CARACTERIZACIÓN FUNCIONAL
DE UNA COLECCIÓN DE GERMOPLASMA DE
OLIVO SILVESTRE
PARA LA MEJORA DEL OLIVAR

TESIS DOCTORAL

PABLO DÍAZ RUEDA

TESIS DOCTORAL

Caracterización funcional de una colección de germoplasma de olivo silvestre para la mejora del olivar

Doctorando:

Pablo Díaz Rueda

Directores:

José Manuel Colmenero Flores

María Nieves Capote Maínez

Tutora:

María Rosario Álvarez Morales

Departamento de Biotecnología vegetal, Grupo de regulación iónica e hídrica en plantas (RIH). Consejo Superior de Investigaciones Científicas (CSIC), Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS).

Memoria de Tesis presentada para la obtención del Título de Doctor por la Universidad de Sevilla.

Sevilla, 2022

Publicaciones / Contribución Científica.

Los resultados obtenidos durante el desarrollo de esta tesis han sido publicados en revistas indexadas (SCI, Science Citation Index) *de primer cuartil (Q1) y primer decil (D1):*

- **Díaz-Rueda, P**., Cantos-Barragán, M., Colmenero-Flores, J.M. (2021). Growth Quality and Development of Olive Plants Cultured *In-Vitro* under Different Illumination Regimes. *Plants*, 10*,* 2214. https://doi.org/10.3390/plants10102214. (*Q1)*

- **Díaz-Rueda, P**., Aguado, A., Romero-Cuadrado, L., Capote, N., Colmenero-Flores, J.M. (2021). Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating *Verticillium dahliae*. *Frontiers in Plant Science* 12, 662060. https://doi.org/10.3389/fpls.2021.662060. (*D1)*

- **Díaz-Rueda, P**., Franco-Navarro, J., Messora, R., Espartero, J., Rivero-Núñez, C., Aleza, P., Capote, N., Cantos, M., García-Fernández, J., De Cires, A., Belaj, A., León, L., Besnard, G., and Colmenero-Flores, J.M. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Frontiers in Plant Science* 11, 629. https://doi.org/10.3389/fpls.2020.00629. (*D1)*

- León, L., **Díaz-Rueda, P**., Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. *Scientia Horticulturae*. 264, 109–157. https://doi.org/10.1016/j.scienta.2019.109157. (*D1)*

- Hernández-Santana, V., **Díaz-Rueda, P**., Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., Pérez-Martín A., Colmenero-Flores J.M. and Rodríguez-Domínguez C.M. (2019). Hydraulic traits emerge as relevant determinants of growth patterns in wild olive genotypes under water stress. *Frontiers in Plant Science* 10, 291. https://doi.org/10.3389/fpls.2019.00291. (*D1)*

- **Díaz-Rueda, P**., Peinado-Torrubia P., Durán-Gutiérrez F.J., Alcántara-Romano P., Aguado A., Capote N., Colmenero-Flores J.M. (2022). Grafting olive cv. Picual onto wild rootstocks from the SILVOLIVE collection as a control method of Verticillium wilt. (En revisión)

Otras aportaciones han sido (i) publicaciones en revistas SCI indirectamente relacionadas con la tesis doctoral, (ii) comunicaciones en congresos nacionales e internacionales en formato póster, capítulo de libro o ponencia y (iii) el registro y protección de una nueva variedad de olivo.

Otras publicaciones en revistas SCI

- Franco-Navarro, J.D., **Díaz-Rueda, P**., Rivero-Núñez, C.M., Brumós, J., Rubio-Casal, A.E., de Cires, A., Colmenero-Flores, J.M., Rosales, M.A. (2021). Chloride nutrition improves drought resistance by enhancing water deficit avoidance and tolerance mechanisms. *Journal of Experimental Botany* 72, 5246–5261. https://doi.org/10.1093/jxb/erab143. (*D1)*

- Rosales, M.A., Franco-Navarro, J.D., Peinado-Torrubia, P., **Díaz-Rueda, P**., Álvarez, R., Colmenero-Flores, J.M. (2020). Chloride Improves Nitrate Utilization and NUE in Plants. *Frontiers in Plant Science* 11, 442. https://doi.org/10.3389/fpls.2020.00442. (*D1)*

- Cubero-Font, P., Maierhofer, T., Jaslan, J., Rosales, M.A., Espartero, J., **Díaz-Rueda, P**., Müller, H.M., Hürter, A.-L., AL-Rasheid, K.A.S., Marten, I., Hedrich, R., Colmenero-Flores, J.M., Geiger, D. (2016). Silent S-Type Anion Channel Subunit SLAH1 Gates SLAH3 Open for Chloride Root-to-Shoot Translocation. *Current Biology* 26, 2213–2220. https://doi.org/10.1016/j.cub.2016.06.045. (*D1)*

Comunicaciones a congresos nacionales

- Paloma Cubero-Font, Miguel A. Rosales, **Pablo Díaz-Rueda**, Juan D. Franco-Navarro, Joaquín Espartero, José M. Colmenero-Flores. "*Functional characterization of the root anion channels SLAH1 and SLAH4: involvement in* Cl^- *and* NO_3^- *nutrition and interaction with abiotic stress*". XII Reunión de Biología Molecular de Plantas. Cartagena, Murcia, 11-13 de junio de 2014.

- Juan D. Franco-Navarro, Paloma Cubero-Font, Miguel A. Rosales, Carlos Rivero, **Pablo Díaz-Rueda,** Joaquín Espartero, José M. Colmenero-Flores. "C*hloride as a beneficial macronutrient in plants: biological functions and regulation".* XVI Simposio Hispano-Luso de Nutrición Mineral de las Plantas. San Pedro del Pinatar, Murcia, 25-28 de septiembre de 2016.

- Paloma Cubero-Font, Joaquín Espartero, Tobias Maierhofer, Miguel Ángel Rosales, **Pablo Díaz-Rueda,** Rainer Hedrich, Dietmar Geiger, José M. Colmenero-Flores. *"Characterization of a root anion channel that plays a pivotal role in plant chloride nutrition"*. XVI Simposio Hispano-Luso de Nutrición Mineral de las Plantas. San Pedro del Pinatar, Murcia, 25-28 de septiembre de 2016.

- Paloma Cubero-Font, José M Barrera-Gavira, **Pablo Díaz-Rueda**, José M. Colmenero-Flores. "*Canales aniónicos SLAH2 y SLAH4".* RED DE EXCELENCIA de Transportadores Na^+ y K^+ (KNaTS). Madrid, 30-31 de marzo de 2017.

- Juan D. Franco-Navarro, Paloma Cubero-Font, Miguel A. Rosales, **Pablo Díaz-Rueda**, Joaquín Espartero, Dietmar Geiger, Colmenero-Flores J.M. "*Chloride as multifunctional beneficial ion: biological functions and regulation*". XXII Reunión de la Sociedad Española de Fisiología Vegetal (SEFV) & XV Spanish-Portuguese Congres of Plant Physiology. Barcelona, 26-29 de junio de 2017.

- **Díaz-Rueda, P.** "*Variabilidad y selección de genotipos resistentes a la enfermedad Verticilosis en* Olea europaea". Jornadas doctorales del programa de doctorado en Biología integrada de la Universidad de Sevilla. Facultad de Biología de la Universidad de Sevilla, 15 de febrero de 2018.

- Juan D. Franco-Navarro, Paloma Cubero-Font, Miguel A. Rosales, **Pablo Díaz-Rueda**, Joaquín Espartero, José M. Colmenero-Flores. "*Cl^- homeostasis as preferential macronutrient: proposed function and regulation".* RED DE EXCELENCIA de Transportadores Na^+ y K^+ (KNaTS). Sevilla, 16-17 de mayo de 2018.

- Cubero-Font P, **Díaz-Rueda P**, Rosales MA, Maierhofer T, Lehmann J, Franco-Navarro JD, Rivero C, Geiger D, Colmenero-Flores JM. "*Characterization of AtSLAH4, a root anion cannel involved in net chloride uptake*". XIV Reunión de Biología Molecular de Plantas. Salamanca 04–06 de julio de 2018.

- **Díaz-Rueda, P.** "*Identificación de portainjertos de bajo vigor en una colección de genotipos silvestres de olivo*". Jornadas doctorales del programa de doctorado en Biología integrada de la Universidad de Sevilla. Facultad de Biología de la Universidad de Sevilla, 21 de febrero de 2019.

- Rosales, M.A., Franco-Navarro, J.D., Peinado-Torrubia, P., **Díaz-Rueda, P.,** Moreno-Racero, Francisco J., Colmenero-Flores, J.M. *"Chloride Uptake and its Effect on Plant Development and Root Hydraulics".* Meeting of the Thematic Research Networ on Sodium and Potassium Transporters in Plants (KNaTs). Estación experimental ZAIDIN (EEZ), 16-18 de septiembre de 2019.

Comunicaciones a congresos internacionales

- Franco-Navarro JD, Brumós J, Rosales MA, Vázquez-Rodríguez A, Sañudo BJ, **Díaz-Rueda P**, Rivero C, Talón M, Colmenero-Flores JM. "*Chloride nutrition regulates development, water balance and drought resistance in plants".* Book of Abstracts of the XII Portuguese-Spanish Symposium on Plant Water Relations, pg. 52. Évora (Portugal), September 30 to October 3, 2014.

- León L., **Díaz-Rueda P.**, Belaj A., Escudier J.G., De la Rosa R., Carrascosa C., Colmenero-Flores JM. *"Characterization of early vigour in wild olive germplasm"*. VIII International Olive Symposium. Split, (Croatia), October 10-14, 2016.

- Colmenero-Flores JM., **Díaz-Rueda P.,** Capote N., Aguado A., Romero-Cuadrado L., León L., Carrascosa C. *"SILVOLIVE, a collection of wild olive genotypes as a source of resistance genes and rootstocks for olive grove cultivation".* VI International Conferences on the Olive Tree and Olive Products OLIVEBIOTECH'18. Seville (Spain), October 15-19, 2018.

- **Díaz-Rueda P.,** Capote N., Aguado A., Romero-Cuadrado L., León L. Carrascosa C., Colmenero-Flores J.M. *"Evaluation of the SILVOLIVE wild olive collection for resistance to Verticillium dahliae".* VI International Conferences on the Olive Tree and Olive Products OLIVEBIOTECH'18. Seville (Spain), October 15-19, 2018.

- **Díaz-Rueda P.,** Carrascosa C., León L., Colmenero-Flores J.M. "*Identification of low-vigour rootstocks in a collection of wild olive genotypes".* VI International Conferences on the Olive Tree and Olive Products OLIVEBIOTECH'18. Seville (Spain), October 15-19, 2018

- Hernández-Santana, V., **Díaz-Rueda, P.**, Díaz-Espejo, A., Raya-Sereno, M.D., Gutiérrez-Gordillo, S, Montero, A., Pérez-Martín, A., Colmenero-Flores, J.M., Rodríguez-Domínguez, C.M. *"Coordination between water supply and demand at leaf and plant level helps to explain differential growth patterns in olive genotypes in response to mild water stress".* VI International Conferences on the Olive Tree and Olive Products OLIVEBIOTECH'18. Seville (Spain), October 15-19, 2018

- Rosales MA, Franco-Navarro JD, **Díaz-Rueda P**, Rivero C, Brumós J, Rubio-Casal A, de Cires A, Colmenero-Flores JM. *"Chloride at macronutrient levels increases drought resistance by improving water balance and water-use efficiency"*. SEB Annual Main Meeting, Heat and Drought Tolerance - From the Lab to Impact in the Field. Sevilla, July 2-5, 2019.

- Colmenero-Flores J.M., Franco-Navarro, J.D., Cubero-Font P., Rosales MA, **Díaz-Rueda P.**, Maierhofer T., Geiger D., Hedrich R. and Díaz-Espejo A. *"The role of Chloride homeostasis on plant drought tolerance"*. SEB Annual Main Meeting, Heat and Drought Tolerance - From the Lab to Impact in the Field. Sevilla, July 2-5, 2019.

- Vargas-Pérez, M.A., Franco-Navarro, J.D., **Díaz-Rueda, P**., Peinado-Torrubia, P., Colmenero-Flores, J.M. *"Molecular basis of chloride nutrition during early*

vegetative development in tobacco plants". XV Meeting of Plant Molecular Biology (XV RBMP). Online, November 26-27, 2020.

- Moreno-Racero, Francisco J., Peinado-Torrubia, P., Lucas, M., **Díaz-Rueda, P**., Rivero-Núñez, C.M., Rosales, M.A., Colmenero-Flores, J.M. *"Functional characterization of the anion channel* AtALMT10*"*. XV Meeting of Plant Molecular Biology (XV RBMP). Online, November 26-27, 2020.

- Cano FJ., Gago J., Sánchez-Gómez D., Granado MR., **Díaz-Rueda P.**, Colmenero-Flores JM., García-Verdugo C., Quintanilla LG., Aranda I. *"Clonal variability of diploid and polyploid genotypes of Olea europaea in response to a CO_2-enriched atmosphere"*. XXXIII Argentinian meeting of Plant Physiology (Reunión Argentina de Fisiología Vegetal). Santa Fe, Argentina (online), September 13-17, 2021.

Protección y registro de la variedad de olivo OESP-A8

En el Proyecto Recupera2020 y en colaboración con la empresa adjudicataria (Viveros Sevilla S.A) se consolidó SILVOLIVE como Grupo Operativo (Nº de registro 2016002000629) donde surgió el registro de la variedad de portainjerto de olivo OESP-A8.**"OESP-A8: Portainjerto de origen silvestre para cultivo intensivo del olivar".**

INVENTORES/AS (p.o. de firma): José M. Colmenero Flores, Carlos Carrascosa Ferrándiz, **Pablo Díaz Rueda**.
TÍTULO: OESP-A8, un Portainjerto de origen silvestre para cultivo intensivo del olivar
Nº DE REGISTRO: NRVP 20155223 (Ministerio de Agricultura, Pesca y Alimentación)
Nº DE REF. CSIC: *PG 200 01_F01 ed05 de 15/04/2015*
PAÍS DE PRIORIDAD: España
ENTIDAD TITULAR: CSIC, Viveros Sevilla, S.A.

Recursos identificativos del doctorando:

ORCID ID: https://orcid.org/0000-0002-7263-9722

Researchgate: https://www.researchgate.net/profile/Pablo_Diaz25

Linkedin: https://www.linkedin.com/in/pablo-d%C3%ADaz-rueda-232243156/

Scopus: https://www.scopus.com/authid/detail.uri?authorId=57191984972

Agradecimientos

Hay un final para cada viaje. Después de un largo camino, cerramos esta historia del olivo para poder darle un punto y aparte. Algunos dicen que un final es sólo un inicio disfrazado, pero ¿quién sabe los que nos depara? En estos años, muchas personas se han cruzado en mi camino a las que tengo indudablemente que agradecer, pues sin su ayuda no habría sido lo mismo.

En primer lugar, quiero mostrar mi más sincero agradecimiento a mis directores de Tesis Doctoral. Al Dr. José Manuel Colmenero Flores (Chema), por su constante dedicación en conseguir financiación para este proyecto y ser de guía durante todos estos años, y a la Dra. María Nieves Capote Maínez por su dedicación y apoyo constante en mi formación científica. A mi tutora académica de la Universidad de Sevilla, Dra. María Rosario Álvarez Morales por su disponibilidad y ayuda en todo momento.

A todos mis compañeros del grupo de investigación del IRNAS, que han sido una gran ayuda tanto en lo laboral como en lo personal desde mis comienzos con el TFM en 2012. Empezando por el más loco de todos, Juan de Dios, gracias por sacarme una sonrisa con esas locuras tuyas en el laboratorio, tu increíble ayuda limpiando raíces y los momentos Monsters. A Carlos por sus chistes malos haciendo más ameno el trabajo del laboratorio. A Kino por su buen humor y paciencia para explicarnos las dudas. A Paloma por enseñarnos como organizar y planificar los experimentos y ser un ejemplo a seguir. A Miriam por escuchar mis confesiones y quejas mientras multiplicábamos el germoplasma de olivo. A Fran, gracias por tu amistad todos estos años y por estar siempre dispuesto a ayudar incluso para cribar una tonelada y media de limo (¡Cuidate esa espalda!). Gracias Pilar, por tu ayuda incondicional, tu disponibilidad para ir al campo, propagar y limpiar esos tropecientos tarros del cultivo *in-vitro,* por tus risas en las cervecitas y sobretodo por tu amistad, todos sabemos que sin ti el laboratorio

no sería el mismo. A Mara por tener siempre un momento para mí demostrándome que siempre puedo contar con tu ayuda tanto fuera como dentro del laboratorio. A Pro, gracias por participar en esas arduas cosechas haciéndolo todo más divertido, y por tus sabios consejos durante esos cafés de tertulias cuando más lo necesitaba. A Migue por tu entusiasmo durante todos estos años. A Isidro que nunca dice que no a una cerveza después del trabajo. A Francito por tu toque mágico con el diseño gráfico. A Rita y Antonella, mis compañeras italianas por su amistad en el breve tiempo de su estancia. Al resto del grupo (Marta, Álvaro, David, Sergio, Ana, Javi y Leti) por vuestra agradable compañía en el laboratorio.

Estos agradecimientos estarían incompletos si no nombrase a mis amigos y compañeros del IRNAS. Desde que entre por esas puertas, encontré estas joyas dignas de mención que sin ellas la tesis no tendría el mismo significado. A Marta Velasco, gracias por esos telefonazos (no wasap, ya que siempre te gusta ir retro) para preguntar como estoy y saber leer entrelineas, por el uno al mes no es de menester, y tus cafes en tu labo. Siempre recordaré cuando éramos cuasi-vecinos, e íbamos a la defensa personal ¡que buenos tiempos! A María, alias la tierna, gracias por tu dulzura todos estos años y tu disponibilidad para cualquier plan. A la amante de los burritos, Marta Gil gracias por todas esas risas y momentos inolvidables. A Marina, la mamá bióloga, que siempre se preocupa por nosotros. A Paloma, la chica con más energía que he conocido en mi vida, gracias por esos bailes de salsa y bachata. A Lolichi, gracias por tu sonrisa y abrazos cada vez que nos veíamos, tus ganas de fiesta y tu actitud positiva. Gracias Layla por ser la alegría del IRNAS, toda una personaje, con la que sin duda la palabra aburrimiento no existe en su vocabulario, aunque la chiquilla tiene el defecto de ver el 'Sálvame'. A Josan, alias modorro, por tenerte como amigo y camarada desde que comenzamos la facultad, y disfrutar de todos esos viajes que nos hemos dado, y los que nos esperan. A Kike, por esas risas y frikadas en el

comedor. Y al resto de compañeros del IRNAS (Letizia, Pablo, Desi, Alba, María del Mar, Ellen, Ornella, Otávio y Dani Hanke), quienes me ayudaron en mis malos momentos y celebraron mis éxitos. Muchísimas gracias por ser quienes sois y estar siempre para una taza de café, una cerveza o una noche de chupitos de Jägermeister. Tambien quiere agradecer al laboratorio de Manolo Cantos (Manolo, Jose Luis, Maria del Mar, Álvaro, Belén, Patricia y María Laura) donde comencé con la propagación de las miles de plantas *in-vitro*, gracias por vuestra calida acogida y amabilidad.

Y como podría olvidarme de la gente del IFAPA 'Las Torres'. Muchas horas echadas en ese cortijo de Alcalá de río realizando inoculaciones de olivo con *Verticillium*. Quiero expresar mi agradecimiento en especial a Laura, Ana Pastrana, Ana Aguado, Mari Carmen y Edu por su ayuda en mis inicios en fitopatología.

También quisiera agradecer a todo el personal de la empresa Viveros Sevilla, donde pude comprobar el arduo trabajo que es estar en un vivero, soportando esos desniveles de temperatura. A su gerente, Carlos Carrascosa, por promover la investigación en el olivo facilitando la financiando de esta Tesis Doctoral, con la participación en el proyecto Recupera2020 y demás contratos que hicieron posible completar este trabajo.

Estos agradecimientos estarían incompletos sin mis amigos de toda la vida. A Rosendo, por apoyarme en la investigación desde nuestra etapa universitaria. A Víctor, por ser mi camarada en esos sábados locos de fiesta. A mi hermano Antonio, con quien unas cervecitas de tranqui acaba transformándose en cubatas dentro del Lobby. A Rafa, por tantos años de amistad terciando, esos planes y viajes increíbles que propones y esas locuras al estilo 'Resacon en las vegas' que en nosotros queda.

Además, quiero mencionar y agradecer (si no me matan!) a mis actuales compañeros del IBVF, las cuales me acogieron como uno más y me dieron fuerzas en esta última etapa de escritura y correcciones de Tesis.

Este párrafo aparte se lo dedico a una maravillosa persona, Elena. Cualquier cosa que escriba me sabrá a poco, pues no tengo palabras para expresar todo lo que ella se merece. Desde el primer momento, me has apoyado en todas mis ideas (algunas más locas que otras) y siempre has luchado porque seamos un equipo. Tus logros son mis logros decías, y hacer cumplir tus sueños es cumplir también los míos. Esto no habría sido lo mismo sin ti. Te quiero.

Y, por último, gracias a mi familia, por haberme apoyado en cada uno de mis pasos y enseñarme lo que es la perseverancia, por la motivación constante que permitieron que hoy en día sea la persona que soy y por vuestro amor incondicional. A mi hermana Ana, por tu apoyo y demostrarme lo que es ser un ejemplo de dedicación, y traer a Gonzalo a nuestras vidas. Y a mis padres, a quienes le debo lo que soy actualmente. Gracias, os quiero.

Índice de contenido

«La ciencia, muchacho, está hecha de errores, pero de errores útiles de cometer, pues poco a poco, conducen a la verdad».

Julio Verne

Resumen

En esta tesis doctoral se ha llevado a cabo la obtención y caracterización morfológica, fisiológica y molecular de una colección de germoplasma de variedades silvestres de olivo pertenecientes a las distintas subespecies de *Olea europaea.* Se realizaron prospecciones directas del material vegetal de partida (semillas procedentes de árboles madre) en distintas regiones de la cuenca mediterránea y también se recolectaron semillas en diferentes bancos mundiales de germoplasmas de olivo. A continuación, se puso a punto el proceso de conservación del germoplasma *in-vitro*, incluyedo el establecimiento, multiplicación, crecimiento y conservación de estos genotipos silvestres, así como de diferentes cultivares maduros, generándose así la colección SILVOLIVE. Se llevó a cabo el estudio de las características lumínicas óptimas para el cultivo *in-vitro* del olivo mediante el empleo de luces LEDs (**Capítulo 1**). Una vez establecida la colección y obtenido el número adecuado de individuos de cada genotipo silvestre, los microtallos se enraizaron y aclimataron *ex-vitro*. Una vez crecidas las plantas durante al menos un año, los genotipos silvestres se caracterizaron genotípica y fenotípicamente (**Capítulo 2**). Se constató una alta variabilidad genética y morfológica en la colección. Al comprobar que algunos genotipos eran capaces de transmitir caracteres de vigor reducido a la variedad 'Picual' injertada sobre ellos, se planteó su potencial uso como portainjertos para sistemas de cultivo intensivo y superintensivo. Además, se estudió el consumo hídrico de los distintos genotipos silvestres bajo condiciones de irrigación óptima (capacidad de campo) y bajo riego deficitario o déficit hídrico moderado (60% de capacidad de campo), demostrando la existencia de genotipos diferenciados por el consumo hídrico y la tolerancia al déficit hídrico (**Anexo I**). Estos resultados fueron la base para la caracterización fisiológica de la colección SILVOLIVE llevada a cabo en colaboración con el grupo del Dr. Antonio Díaz Espejo del IRNAS-CSIC y publicada por Hernández-Santana *et al*., (2019). Posteriormente se evaluó el uso potencial de los genotipos silvestres como portainjertos capaces de reducir el vigor de la variedad injertada en condiciones controladas de invernadero y en condiciones de campo en cultivo de alta densidad. Por un lado, se injertaron los cultivares 'Picual' y 'Arbequina' sobre los genotipos silvestres en maceta, y posteriormente se traspasaron a campo. Paralelamente, los genotipos silvestres sin

injertar se plantaron bajo un marco de plantación super-intensivo y se realizó un estudio de vigor entre 2015 y 2018. Ello permitió seleccionar en 2020 los genotipos de bajo vigor que se injertaron con el cultivar 'Arbequina' en campo, en condiciones de alta densidad de plantación, identificándose portainjertos enanizantes capaces de transmitir vigor reducido a los cultivares injertados (**Capítulo 3**). En relación al uso potencial de los genotipos silvestres como portainjertos para el control de la Verticilosis, se evaluó su susceptibilidad al patotipo defoliante de *V. dahliae*, lo que permitió identificar genotipos resistentes al hongo que no presentaban síntomas de la enfermedad, o los síntomas eran reducidos, y limitaban el desarrollo del hongo mediante dos estrategias (resistencia o prevención de la proliferación del hongo frente a tolerancia al hongo; **Capítulo 4**). Por último, el cultivar susceptible 'Picual' se injertó sobre genotipos silvestres clasificados previamente como de susceptibilidad muy reducida a *V. dahliae* para determinar la capacidad de los genotipos de transferir la resistencia al cultivar injertado. Se observó que los portainjertos resistentes, pero no los tolerantes, controlaban la aparición de síntomas de Verticilosis en el cultivar 'Picual' (**Capítulo 5**).

Capítulo 1

Este capítulo consta de dos secciones. En la primera parte se describe la puesta a punto de un protocolo eficiente para el establecimiento, micropropagación y enraizamiento *in-vitro* de los genotipos silvestres de la colección de germoplama de olivo SILVOLIVE. Todos los genotipos silvestres y las variedades maduras de la colección SILVOLIVE fueron capaces de establecerse y micropropagarse *in-vitro* mediante la esterilización de semillas o explantos y su implantación en tubos con medio nutricional Rugini, observándose un crecimiento generalizado de 40.9 mm de altura en plantas que contienen un promedio de 6.1 nudos por individuo. Estas plantas propagadas y crecidas *in-vitro* se pudieron enraizar eficientemente *in-vitro* o durante el proceso de aclimatación *ex-vitro*, dando lugar posteriormente a plantas viables. La segunda parte describe la optimización de las condiciones lumínicas basadas en el empleo de iluminación LED para el cultivo *in-vitro* del olivo. Los diodos emisores de luz (LED) son útiles para la micropropagación *in-vitro* de plantas, pero se dispone de poca información sobre su uso en especies leñosas. Esta sección compara los efectos de la calidad e

intensidad de la luz sobre el crecimiento y desarrollo de olivos micropropagados de dos subespecies diferentes. Se usaron lámparas fluorescentes y LEDs que cubrían diferentes proporciones de iluminación rojo/azul (90/10, 80/20, 70/30, 60/40) o combinaciones de rojo/azul/ blanco, así como diferentes intensidades de luz (30, 34, 40, 52, 56, 84, 98 y 137 µmol m^{-2} s^{-1} de flujos de fotones fotosintéticos, PPF). Las plantas de olivo mostraron una alta sensibilidad a la calidad e intensidad de la luz. Las proporciones más altas de rojo/azul o las intensidades de luz más bajas estimularon el crecimiento de las plantas principalmente como consecuencia de una mayor tasa de elongación internodal, pero sin afectar el número total de nudos ni el número de brotes. En comparación con la iluminación fluorescente, la iluminación LED mejoró el área foliar y la biomasa, que además se correlacionó positivamente con la intensidad de la luz. La frecuencia de estomas correlacionó positivamente, y el contenido de pigmentos negativamente, con la intensidad de la luz, mientras que no se observó una correlación clara con la calidad de la luz. En comparación con las lámparas fluorescentes, la iluminación LED (particularmente la relación 70/30 rojo / azul con 34 µmol m^{-2} s^{-1} PPF de intensidad) permitió un manejo óptimo del cultivo y mejoró la calidad de las plantas de olivo micropropagadas *in-vitro*.

Capítulo 2

Las subespecies silvestres de *Olea europaea* constituyen una fuente de variabilidad genética con un enorme potencial para la mejora del olivo, adecuada para hacer frente a los cambios globales en las regiones de clima mediterráneo. En este capítulo se pretende identificar genotipos silvestres de olivo con óptima adaptabilidad a diferentes condiciones ambientales para que sirvan como fuente de portainjertos y de genes de resistencia para la mejora del olivo. La colección SILVOLIVE incluye 146 genotipos silvestres representativos de las seis subespecies de *O. europaea* y de híbridos de primera generación. Estos genotipos proceden de colecciones de germoplasma de olivo o de prospecciones directas en España, África continental y el archipiélago Macaronésico. La colección fue genotipada con marcadores moleculares plastídicos y nucleares, pudiéndose confirmar el origen de los genotipos y su alta variabilidad genética. Se cuantificaron parámetros morfológicos en 103 genotipos, lo que permitió

la identificación de tres grupos principales de caracteres correlativos, incluidos el vigor, los hábitos de ramificación y la relación entre la parte aérea y la raíz. Se ha demostrado la alta variabilidad fenotípica en estos caracteres dentro de la colección de germoplasma. Los genotipos silvestres de olivo pueden ser utilizados como portainjertos para el cultivo del olivo. Así, como prueba de concepto, se demostró que diferentes genotipos silvestres utilizados como portainjertos regularon los parámetros de vigor del cultivar 'Picual' injertado, lo que podría mejorar la productividad de los cultivos de alta densidad. Por otro lado, se determinó el consumo hídrico de 39 genotipos silvestres de olivo y su capacidad de resistir un estrés hídrico moderado (60% de capacidad de campo), identificándose genotipos capaces de adaptarse a ambientes adversos.

Capítulo 3

El control del vigor es un factor crítico para asegurar una adecuada producción y longevidad del cultivo del olivo de alta densidad. Para hacer frente a este problema, se quiso aprovechar la variabilidad genética presente en la subespecie silvestre de *Olea europaea* para identificar genotipos de vigor reducido con óptima adaptabilidad a diversos suelos y condiciones ambientales adversas. En primer lugar, se caracterizaron rasgos de vigor temprano en diferentes subespecies de olivos silvestres que podrían usarse como portainjertos para controlar el vigor de los cultivares injertados. En segundo lugar, se evaluó el crecimiento temprano de los vástagos de 'Arbequina' injertados sobre portainjertos silvestres de la colección SILVOLIVE. En el primer estudio, se obtuvieron diferencias significativas entre genotipos para todos los rasgos de vigor y se obtuvieron valores altos de heredabilidad en sentido amplio para la mayoría de ellos, lo que indica un fuerte efecto genético y, por lo tanto, buenas posibilidades de selección. En comparación con los cultivares 'Arbequina' y 'Picual', algunos de los genotipos silvestres evaluados mostraron un vigor claramente menor al final del período experimental. En un segundo estudio, estos genotipos fueron seleccionados por su potencial como portainjertos enanizantes. El genotipo ACO15 y el grupo de genotipos AMK consiguieron reducir el vigor de 'Arbequina' injertados sobre ellos. Estos portainjertos enanos redujeron el tamaño del árbol, por tanto, podrían utilizarse como

nuevos portainjertos enanizantes para su adaptación al sistema de cultivo en seto de alta densidad.

Capítulo 4

En este capítulo se evaluó la resistencia al patotipo defoliante de *Verticillium dahliae* en un grupo de 68 genotipos silvestres de olivo pertenecientes a la colección SILVOLIVE. El nivel de resistencia se determinó evaluando la gravedad de los síntomas en una escala de 0 a 4, estimando el área relativa bajo la curva de progreso de la enfermedad (RAUDPC), determinando el porcentaje de plantas muertas (PDP) y midiendo la evolución de los parámetros morfológicos en las plantas inoculadas a lo largo del tiempo. Además, se cuantificó la densidad de inóculo de *V. dahliae* en el tallo de los genotipos enraizados mediante PCR en tiempo real a los 35 y 120 días después de la inoculación (ddi). Quince genotipos (22%) fueron catalogados como resistentes a *V. dahliae* (es decir, los parámetros de la enfermedad no difirieron significativamente de los del cultivar resistente 'Frantoio', o fueron incluso más bajos). Los genotipos resistentes se caracterizaron por presentar menos síntomas y menor cantidad de ADN de *V. dahliae* a los 120 ddi respecto a los 35 ddi, lo que indica su capacidad para controlar la enfermedad y reducir la densidad del patógeno. El resto de los genotipos evaluados mostraron niveles variables de susceptibilidad. El análisis general de todos los genotipos mostró una alta correlación entre la sintomatología y la cantidad de ADN de *V. dahliae* en el tallo de los genotipos inoculados a los 120 ddi, y no a los 35 ddi. Sin embargo, no se observó correlación a 120 ddi cuando se analizó independientemente el conjunto de genotipos resistentes, lo que sugiere que la resistencia al patotipo defoliante de *V. dahliae* en el olivo se basa en la existencia de diferentes mecanismos, como la prevención o la tolerancia. Estos mecanismos son útiles para el diseño de programas de mejora y para la identificación de genes diana y portainjertos resistentes encaminados a mejorar el control de la Verticilosis en el olivar.

Capítulo 5

La identificación de portainjertos de baja susceptibilidad a *Verticillium dahliae* puede convertirse en un valioso procedimiento para conseguir un control eficaz de la marchitez por Verticillium en el olivar. Esto no solo implica la identificación de genotipos adecuados, sino también el estudio de la interacción entre el portainjerto y el vástago injertado. Así, un portainjerto que previene o minimiza la proliferación de *V. dahliae* (estrategia de evitación/resistencia) puede tener efectos muy diferentes en un vástago susceptible en comparación con portainjertos que muestra pocos o ningún síntoma a pesar de estar infectado (estrategia de tolerancia). Recientemente se han identificado mecanismos tanto de resistencia como de tolerancia en genotipos de olivo silvestre con baja susceptibilidad a *V. dahliae*. Cuando se utilizaron como portainjertos de la variedad altamente susceptible 'Picual', encontramos que los genotipos resistentes, incluido el cultivar 'Frantoio', fueron más efectivos que los genotipos tolerantes para controlar la marchitez por Verticillium. Además, los genotipos tolerantes fueron tan ineficaces como los genotipos susceptibles o extremadamente susceptibles en el control del marchitamiento por Verticillium. También identificamos combinaciones portainjerto-vástago con comportamientos no esperados según el grado de susceptibilidad observado previamente en el portainjerto no injertado. Aunque los portainjertos pudieron controlar la marchitez por Verticillium de acuerdo con su grado de susceptibilidad a *V. dahliae*, la capacidad de controlar la infección no se transfirió adecuadamente al vástago injertado. Nuestros resultados confirmaron que: el grado de susceptibilidad a la marchitez por Verticillium de una variedad de olivo no predice su comportamiento como patrón; injertar un genotipo muy poco susceptible con un vástago susceptible aumenta la susceptibilidad de la planta injertada; en cualquier caso, los portainjertos evitadores/resistentes son más efectivos que los portainjertos tolerantes para reducir la susceptibilidad de la planta injertada a *Verticillium dahliae*.

Abstract

This PhD Thesis addresses the creation of a collection of wild olive germplasm belonging to all subspecies of *Olea europaea*, as well as the subsequent genetic, morphological and physiological characterization of the different genotypes, mainly focused on their future use in olive breeding and cultivation. First, we collected the plant material (seeds obtained from mother trees) in different regions of the Mediterranean Basin, as well as seeds from different world germplasm banks. Then, the *in-vitro* culture process was fine-tuned, including the seedling establishment, micropropagation, elongation and rooting of the wild genotypes and olive cultivars, giving rise to the SILVOLIVE collection. Optimization of the illumination procedure using LED lamps and comparison with traditional phluorescent lamps was carried out (**Chapter 1**). Once the collection was established and a suitable number of explants was obtained, plants were rooted and acclimatized *ex-vitro* for further genotypic and phenotypic characterization (**Chapter 2**). The collection of wild olive genotypes exhibited high genetic and morphologic variability. When used as rootstocks of the cultivar 'Picual' some cultivars transmited features such as reduced vigor to the grafted scion, suggesting their potential use to improve high-density olive cultivation. Water consumption of the wild olive genotypes was studied under optimal (field capacity) and deficit (60% of field capacity) irrigation, showing the occurrence of genotypes with lower water consumption and higher resistance to water deficit (**Annex I**). These results were the starting point for a subsequent study that was conducted in collaboration with the Group of Dr. Antonio Díaz-Espejo from IRNAS-CSIC and published by Hernández-Santana *et al*., (2019). Next, the potential use of wild olive genotypes as rootstocks to improve the agronomic quality of grafted cultivars ('Picual' and 'Arbequina') was assessed under controlled (greenhouse) and field (high-density cultivation) conditions. In addition, ungrafted wild genotypes were also grown in the field under a super-intensive plantation system where a vigor study was carried out between 2015 and 2018. As a result, in 2020 we selected low vigor genotypes, which were grafted with 'Arbequina' cultivar in the field under a super-intensive plantation system, identifying dwarfing rootstocks that can transmit low vigor to the grafted cultivars (**Chapter 3**). Regarding the potential use of wild genotypes as rootstocks to control olive Verticillium wilt, the susceptibility to the defoliating

pathotype of *Verticillium dahliae* was evaluated. Genotypes of very low susceptibility to *V. dahliae* were identified which exhibited no symptoms of the disease and reduced the density of the pathogen in the plant by means of two different defense strategies: avoidance/resistance and tolerance (**Chapter 4**). Finally, the susceptible cultivar 'Picual' was grafted onto wild olive geneotypes of very low susceptibility to determine the ability of the rootstock to transfer the resistance to the grafted cultivar. Our results confirmed that avoidant/resistant rootstocks are more effective than tolerant rootstocks in reducing the susceptibility of the grafted plant to *V dahliae*. (**Chapter 5**).

Chapter 1

This chapter is divided into two sections. The first part describes the development of an efficient protocol for the establishment, propagation and *in-vitro* rooting of the wild genotypes belonging to the germplasm collection SILVOLIVE. The wild genotypes and mature cultivars of the SILVOLIVE collection were established and micropropagated *in-vitro* by means of the sterilization of seeds or explants and their implantation in tubes with Rugini nutritional medium. A generalized growth of 40.9 mm in height and a proportion of 6.1 nodes per plant was observed. These *in-vitro* propagated plants were rooted *ex-vitro* and *in-vitro* resulting in highly efficient rooting and *ex-vitro* acclimatization. The second part describes the optimization of the lighting conditions based on light-emitting diodes (LEDs) for the *in-vitro* cultivation of olive. LEDs are useful for the *in-vitro* micropropagation of plants, but little information is available on woody species. This chapter compares the effects of light quality and intensity on the growth and development of micropropagated olive plants from two different subspecies. Illumination was provided with fluorescent and LED lamps covering different red/blue ratios (90/10, 80/20, 70/30, 60/40) or red/blue/white combinations, as well as different light intensities (30, 34, 40, 52, 56, 84, 98 and 137 µmol m^{-2} s^{-1} of photosynthetic photon fluxes, PPF). Olive plants exhibited high sensitivity to light quality and intensity. Higher red/blue ratios or lower light intensities stimulated plant growth and biomass mainly as a consequence of a higher internodal elongation rate, not affecting either the total number of nodes or shoots. In comparison to fluorescent illumination, LED lighting improved leaf area and biomass, which additionally was positively correlated with light

intensity. Stomatal frequency was positively, and pigments content negatively correlated with light intensity, while no clear correlation was observed with light quality. In comparison with fluorescent lamps, LED illumination (particularly the 70/30 red/blue ratio with 34 µmol m^{-2} s^{-1} PPF intensity) allowed optimal manipulation and improved the quality of *in-vitro* micropropagated olive plants.

Chapter 2

Wild subspecies of *Olea europaea* constitute a source of genetic variability with huge potential for olive breeding to face global changes in Mediterranean-climate regions. We intended to identify wild olive genotypes with optimal adaptability to different environmental conditions to serve as a source of rootstocks and resistance genes for olive breeding. The SILVOLIVE collection includes 146 wild genotypes representative of the six *O. europaea* subspecies and early-generations hybrids. These genotypes came either from olive germplasm collections or from direct prospection in Spain, continental Africa and the Macaronesian archipelago. The collection was genotyped with plastid and nuclear markers, confirming the origin of the genotypes and their high genetic variability. Morphological and architectural parameters were quantified in 103 genotypes allowing the identification of three major groups of correlative traits including vigor, branching habits and the belowground-to-aboveground ratio. The occurrence of strong phenotypic variability in these traits within the germplasm collection has been shown. Furthermore, wild olive relatives are of great significance to be used as rootstocks for olive cultivation. Thus, as a proof of concept, different wild genotypes used as rootstocks were shown to regulate vigor parameters of the grafted cultivar 'Picual' scion, which could improve the productivity of high-density hedgerow orchards. On the other hand, the water consumption of 39 wild genotypes and their ability to resist moderate drought stress (60% field capacity) were determined, and genotypes capable of adapting to more adverse environments were identified.

Chapter 3

The control of the olive tree vigor is a critical factor to ensure adequate production and longevity of high-density olive cultivation. To deal with this problem, we intended to take advantage of the genetic variability present in the wild subspecies of *Olea europaea* to identify genotypes of reduced vigor with optimal adaptability to diverse soils and adverse environmental conditions. First, early vigor traits were characterized in different wild olive subspecies that could potentially be used as rootstocks to control the vigor of grafted cultivars. Secondly, the early growth of 'Arbequina' scions grafted on selected wild rootstocks from the SILVOLIVE collection was evaluated. In the first study, significant differences between genotypes were obtained for all vigor traits and high values of broad sense heritability were obtained for most of them, indicating strong genetic effect and therefore good possibilities for selection. Compared to 'Arbequina' and 'Picual' cultivars, some of the wild genotypes evaluated showed a clearly lower vigor at the end of the experimental period. These genotypes were selected in a second study for their potential as dwarfing rootstocks for high-density olive plantations. The ACO15 and the group of AMK genotypes were able to reduce the vigor of grafted 'Arbequina' cultivar. These dwarfing rootstocks reduced the tree size; therefore, these wild genotypes could be used as new dwarf rootstocks for its adaptation to high-density hedgerow system.

Chapter 4

Resistance to the defoliating pathotype of *Verticillium dahliae* has been evaluated in a pool of 68 wild olive genotypes belonging to the SILVOLIVE collection. Resistance was evaluated by assessing symptoms severity using a 0–4 rating scale, estimating the relative area under the disease progress curve (RAUDPC), determining the percentage of dead plants (PDP), and measuring the evolution of morphological parameters in inoculated plants over time. In addition, the density levels of *V. dahliae* in the stem of rooti noculated genotypes have been quantified by means of quantitative real-time PCR at 35 and 120 days after inoculation (dai). Fifteen genotypes (22%) were cataloged as resistant to *V. dahliae* (i.e., disease parameters did not significantly differ from those of

the resistant cultivar 'Frantoio', or were even lower). Resistant genotypes are characterized by presenting fewer symptoms and a lower amount of *V. dahliae* DNA at 120 dai than at 35 dai, indicating their ability to control the disease and reduce the density of the pathogen. The rest of the evaluated genotypes showed variable levels of susceptibility. Overall analysis of all genotypes showed high correlation between symptomatology and the amount of *V. dahliae* DNA in the stem of inoculated genotypes at 120 dai, rather than at 35 dai. However, correlation at 120 dai was not observed in the set of resistant genotypes, suggesting that resistance to defoliating *V. dahliae* in olive is based on the occurrence of different mechanisms such as avoidance or tolerance. These mechanisms are valuable for designing breeding programs and for the identification of target genes and resistant rootstocks to better control Verticillium wilt in the olive grove.

Chapter 5

The identification of rootstocks of low susceptibility to *Verticillium dahliae* can become a valuable procedure to achieve effective control of verticillium wilt in the olive grove. This not only involves the identification of suitable genotypes, but also the study of the interaction between the rootstock and the grafted scion. Thus, a rootstock that prevents or minimizes *V. dahliae* proliferation (avoidance/resistance strategy) can have very different effects on a susceptible scion compared to a rootstock that shows few or no symptoms despite being infected (tolerance strategy). Both resistance and tolerance mechanisms have been recently identified in wild olive genotypes with low susceptibility to *V. dahliae*. When used as rootstocks of the highly susceptible variety 'Picual', we found that resistant genotypes, including the cultivar 'Frantoio', were more effective than tolerant genotypes in controlling verticillium wilt. Furthermore, tolerant genotypes were as ineffective as susceptible or extremely susceptible genotypes in controlling verticillium wilt. We also identified rootstock-scion combinations with behaviors that were not expected according to the degree of susceptibility previously observed in the non-grafted rootstock. Although the rootstocks were able to control Verticillium wilt according to its degree of susceptibility to *V. dahliae*, the ability to control the infection was not adequately transferred to the grafted scion. Our results confirmed that: the

degree of susceptibility to verticillium wilt of an olive variety does not predict its performance as a rootstock; grafting a very low susceptible genotype with a susceptible scion increases the susceptibility of the grafted plant; in any case, avoidant/resistant rootstocks are more effective than tolerant rootstocks in reducing the susceptibility of the grafted plant to *Verticillium dahliae*.

Introducción General

El cultivo del olivo

Descripción general

El olivo (*Olea europaea* L.) es una planta perenne, caracterizada por ser un árbol de tamaño mediano, de unos 4 a 8 metros de altura según la variedad. A nivel mundial, esta especie presenta un hábitat entre las latitudes 30º y 45º, tanto en el hemisferio norte como en el sur (Therios, 2009). El olivo se considera una de las especies mejor adaptadas al clima mediterráneo (Orlandi et al., 2013; Moriondo et al., 2015), con veranos largos, secos y calurosos, e inviernos suaves y húmedos (Kottek et al., 2006; Peel et al., 2007). El olivo es una planta muy dependiente de la luz, por lo que la deficiencia lumínica reduce el número de flores o su viabilidad. Las temperaturas superiores a 30 ºC pueden limitar el rendimiento del cultivo (Koubouris et al., 2009), y su tasa fotosintética se ve reducida cuando se superan los 40 ºC (Mancuso and Azzarello, 2002). Por otro lado, el olivo no suele tolerar temperaturas inferiores a −10 °C durante períodos prolongados (Palliotti and Bongi, 1996), aunque se ha constatado resistencia a temperaturas tan bajas como −18 ºC (Sanzani et al., 2012). El olivo es una especie bien adaptada a las regiones áridas y semiáridas, capaz de sobrevivir a periodos de intenso déficit hídrico, gracias a adaptaciones morfológicas, fisiológicas y bioquímicas que reducen la pérdida de agua al tiempo que mantienen su captación (Connor and Fereres, 2010). Sin embargo, a pesar de su resistencia a la sequía, el olivo necesita un aporte mínimo de precipitaciones anuales de 350 mm para su supervivencia en zonas áridas y de 500 mm para su producción comercial (Barranco et al., 1998; Fernández, 2014; Ponti et al., 2014). Debido a esta capacidad, aproximadamente el 90% de los olivos cultivados en la cuenca mediterránea se encuentran en condiciones de secano (Gómez et al., 2001; Gómez-Rico et al., 2007).

El olivo es un árbol de crecimiento lento, pudiendo permanecer vivo y productivo durante cientos de años. Es la única especie del género *Olea* que produce frutos comestibles (Barranco et al., 1998), convirtiéndose en una de las más antiguas e

importantes especies frutales de la región Mediterránea (Loumou and Giourga, 2003; Vossen, 2007). Además, la madera, el aceite y las aceitunas de mesa, están profundamente arraigados en la historia de las sociedades mediterráneas por su importancia económica y cultural. Este cultivo oleaginoso es el más importante económicamente en áreas templadas, con 11.7 millones de hectáreas plantadas a nivel mundial, y cuya superficie crece cada año unas 150.000 hectáreas, en su mayoría como olivar en superintensivo. Esta extensión supone el 1% de las tierras cultivadas en el planeta, situándose como el cultivo permanente de mayor distribución en el mundo. Según el consejo oleícola internacional (COI), la producción mundial de aceite de oliva actual oscila entre 2,8 y 3,2 millones de toneladas anuales, con tendencia al aumento de producción (**Figura 1**). El mercado del aceite de oliva está muy demandado, registrándose en la según el Sistema de Información de los Mercados Oleícolas (SIMO) se ha registrado en la última campaña 2020/2021 una producción nacional de 1,389 millones de toneladas de aceite frente a una demanda de mercado de 1,634 millones de toneladas de aceite (Sistema de Información de los Mercados Oleícolas SIMO), compensando esta diferencia mediante importaciones.

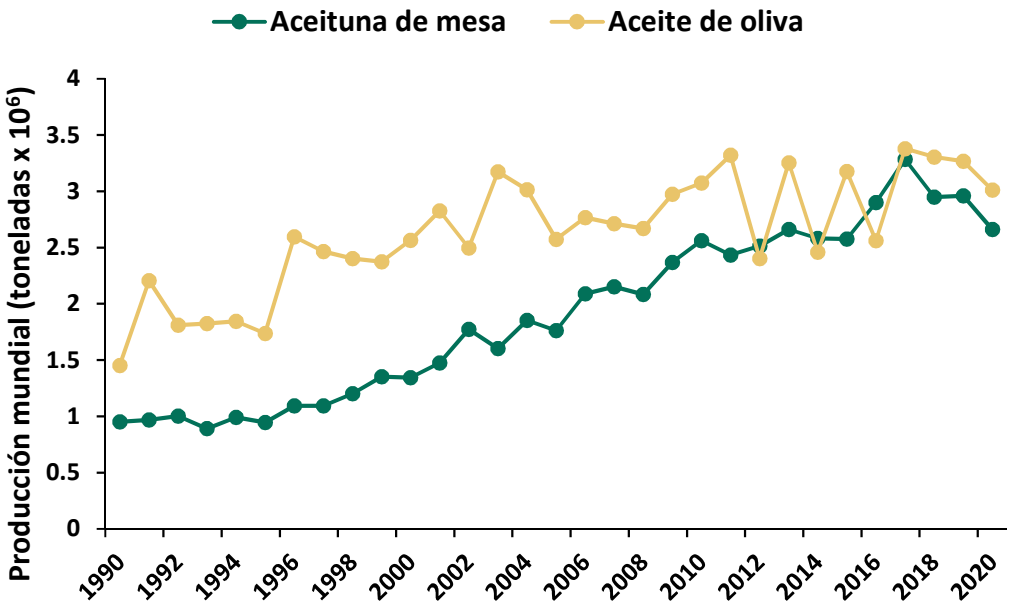


FIGURA 1 | Incremento de la producción mundial anual desde la campaña 1990/91-2020/21 de aceituna de mesa (verde) y aceite de oliva desde (amarillo). Fuente: COI (2021).

España es el principal productor y exportador de aceite de oliva a nivel mundial con una producción de 1.389.000 toneladas de aceite de oliva y 546.600 toneladas de

aceituna de mesa en la campaña 2020/2021 (IOC, 2021). En esta campaña, Grecia se sitúa en segundo puesto con una producción en torno a 275.000 t de aceite de oliva, seguidos de Italia (273.500 t), Turquía (210.000 t) y Marruecos (160.000 t) (**Figura 2**). Recientemente, se ha incorporado el cultivo del olivo en nuevas regiones, como EEUU, Nueva Zelanda, Australia y China (Besnard et al., 2018). En Andalucía se ha incrementado la superficie de olivar en los últimos diez años, con una producción total de aceite de oliva del 80% de la producción nacional y del 46,8% de la producción mundial, situando a Jaén, Córdoba, Granada y Sevilla entre las cuatro regiones más productoras.

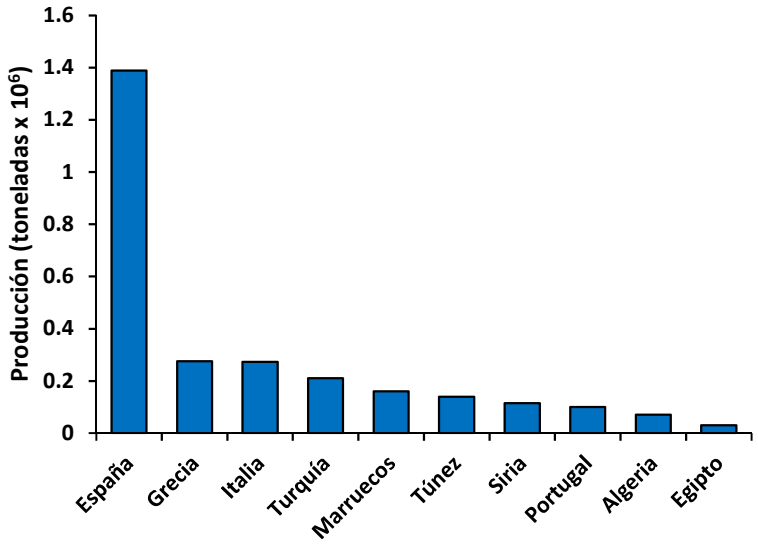


FIGURA 2 | Producción anual de aceite de oliva de los 10 principales países productores en la campaña de 2020/2021. Fuente: COI (2021).

En los últimos años se está produciendo una transición desde un modelo de cultivo tradicional en secano a un sistema de cultivo en regadío y de alta densidad de plantación (Connor et al., 2014). Actualmente, el 28% de la superficie de olivar en España se cultiva en regadío por lo que la mejora en aspectos como la eficiencia en el uso del agua o la resistencia a enfermedades emergentes son factores muy relevantes a mejorar en la olivicultura actual. De esta superficie, unas 725.000 ha se producen bajo el sistema de cultivo intensivo o superintensivo o de seto, por lo que la reducción de vigor es también un factor de gran relevancia a tener en cuenta. Por tanto, se hace esencial la selección de nuevos cultivares y/o portainjertos que añadan valor tanto económico, como fitosanitario y medioambiental al cultivo del olivar.

Medios de propagación y obtención vegetal

Se estima que hay más de 2000 variedades de olivo en todo el mundo (Lavee, 1994). Al igual que en otros países olivareros, el material vegetal de olivo cultivado en España está compuesto por un gran número de variedades, todas ellas muy antiguas y con zonas de difusión restringidas. La fácil propagación vegetativa del olivo ha permitido mantener las características de estas variedades inicialmente seleccionadas que constituyeron los primeros cultivares: mayor productividad, calibre del fruto, producción de aceite y adecuación ambiental (Barranco et al., 1998). La antigüedad de estas variedades de olivo se remonta al inicio del cultivo en España, en el que las variedades actualmente más importantes lo siguen siendo desde el siglo XV (Oliveros and Jordana, 1968). La falta de programas de mejora que hayan sustituido a los cultivares tradicionales, inicialmente seleccionados de poblaciones silvestres, es la causa de esta situación.

El olivo tiene una gran capacidad de regeneración debido a la presencia de yemas latentes que se desarrollan ante estímulos externos. Esa circunstancia junto a la facilidad con la que produce raíces adventicias, le confieren una buena capacidad de multiplicación vegetativa. La propagación sexual, por germinación de semillas, no es adecuada como método de multiplicación porque las plantas resultantes son diferentes entre sí y diferentes a sus parentales, además de tener un largo periodo juvenil (10-15 años). Por tanto, el olivo se ha propagado vegetativamente por medios convencionales (zuecas, estacón, injerto, estaquillado semileñoso bajo nebulización), no siendo estos métodos eficaces debido a limitaciones ambientales o por presentar un éxito limitado para algunos cultivares, como la variedad Gordal. Sin embargo, la mejora de técnicas como el cultivo de tejidos *in-vitro* ha permitido establecer con éxito la micropropagación como uno de los sistemas de multiplicación clonal de plantas de mayor éxito en los últimos tiempos, permitiría la multiplicación adecuada de cultivares de olivo en viveros autorizados. Las ventajas de la micropropagación *in-vitro* son claras, ya que permite: propagar una gran cantidad de material vegetal genéticamente homogéneo en mucho menos tiempo, en un espacio muy reducido y sin depender de las condiciones medioambientales; sanear el material vegetal mediante el cultivo de ápices, eliminando microorganismos, virus y viroides del tejido vegetal, sean o no de naturaleza patógena; propagar el material en condiciones asépticas, evitándose el contacto y la infección por

fitopatógenos durante la multiplicación del material; propagar variedades de interés agronómico que sean poco o nada susceptibles a propagación convencional (variedades estériles, pobres productoras de semilla, así como aquéllas recalcitrantes al enraizamiento de estaquillas); acelerar drásticamente la propagación y, por lo tanto, la entrada en producción de nuevas variedades (el caso, por ejemplo, de plantas recientemente obtenidas de procesos de I+D o recientemente introducidas en un país); y facilitar la diversificación del sistema de producción viverístico por conferirle una enorme versatilidad (ampliando el número de especies que se manejan, acelerando el tiempo de entrada en producción y permitiendo la producción en cualquier época del año) (García-Férriz et al., 2003). La obtención de un banco de germoplasma *in-vitro* con múltiples líneas vegetales de interés permite conservar el material y responder eficazmente a las fluctuaciones en la demanda del sector. Además, estas técnicas proporcionan herramientas eficientes para la propagación y la preservación de germoplasma de plantas amenazadas (Santos et al., 2003; Kozlowski et al., 2012), reduciendo los riesgos de extinción y pérdida de variabilidad genética.

El éxito de la micropropagación *in-vitro* del olivo parece estar influenciada por varios factores tales como la composición mineral del medio, la composición de reguladores de crecimiento vegetal y la fuente de carbono utilizada (Rugini, 1984; Fiorino and Leva, 1986; Leva et al., 1994; Abousalim et al., 2005). Se han realizado estudios comparando diferentes medios de propagación (Rugini, 1984; García-Férriz et al., 2003; Haddad et al., 2018), destacando al medio Rugini como el más prometedor (Rugini, 1984). Asimismo, el uso de diferentes combinaciones y concentraciones de citoquininas favorecen la propagación del tejido vegetal (García-Férriz et al., 2003; Mirzaei et al., 2021). Muchos autores han recomendado el uso de la citoquinina "zeatina" (Chaari et al., 2002; Zuccherelli and Zuccherelli, 2002; Micheli et al., 2010; Mirzaei et al., 2021) y de manitol como fuente de energía debido a su eficacia en el cultivo *in-vitro* del olivo (Leva et al., 1992, 1994; Farahani et al., 2008; Mirzaei et al., 2021). El enraizamiento es la base critica de la micropropagación del olivo, que depende de varios factores como el tipo y concentración de auxina y el genotipo (Rugini, 1984; Leva, 2011; Porfírio et al., 2016).

Se han descrito diversas técnicas para la micropropagación del olivo (Cañas and Benbadis, 1988; Rugini et al., 1990; Benelli and De Carlo, 2018; Mirzaei et al., 2021). Sin embargo, el carácter juvenil del material micropropagado o el bajo rendimiento de los métodos, han perjudicado su uso comercial. En el olivo en particular, por ser un cultivo leñoso con una lenta tasa de crecimiento (Benelli and De Carlo, 2018), es fundamental priorizar su crecimiento y multiplicación, optimizando la temperatura, humedad, iluminación y ventilación. La calidad de la luz posee el papel más importante en la fotosíntesis y organogénesis de las plantas (Hoenecke et al., 1992; Saebo et al., 1995), aunque la iluminación artificial eleva los costes de multiplicación y puede ser una fuente de calor excesiva. Por ello, es necesario optimizar el sistema de iluminación implementando el uso de la iluminación LED (Light Emitting Diode) por su escaso consumo energético y reducida emisión de calor. Poner a punto un sistema de multiplicación eficaz de olivo apto para propagación a gran escala, asegurando la integridad sanitaria y genética (varietal) de las plantas producidas es otro reto de gran importancia en el sector.

Genotipos silvestres de olivo

El olivo (*Olea europaea* L.) es una especie que pertenece a la familia Oleaceae, que comprende alrededor de 30 géneros y más de 600 especies. El género *Olea* tiene unas 35 especies, incluidas *O. europaea* subsp. *europaea* var. *sativa* (olivo cultivado) y *Olea europaea* subsp. *europaea* var. *sylvestris* (Mill.) Lehr (olivo silvestre o acebuche) comunes en la cuenca mediterránea. Además, el olivo silvestre incluye formas asilvestradas que son plántulas de los olivos cultivados o el resultado de hibridaciones entre el acebuche y los cultivares (Angiolillo et al., 1999; Lumaret et al., 2004). Dentro de la biodiversidad del complejo *Olea europea* (L.) se han identificado seis subespecies: (1) *O.e.* subsp. *europaea* (Mill.) Lehr, distribuida por la cuenca mediterránea, (2) *O.e.* subsp. *laperrinei* (Batt. & Trab.) Cif. en los macizos saharianos, (3) *O.e.* subsp. *cuspidata* (Wall. ex G. Don) Cif. distribuidos desde Sudáfrica hasta el sureste de Egipto y desde Oriente Medio hasta India y China, (4) *O.e.* subsp. *guanchica* P. Vargas et al. en las Islas Canarias, (5) *O.e.* subsp. *maroccana* (Greut. and Burd.) P. Vargas et al. en el suroeste de Marruecos y (6) *O.e.* subsp. *cerasiformis* G. Kunkel and Sundig en las Islas Madeira (Médail et al., 2001; Green, 2002) **(Figura 3)**.

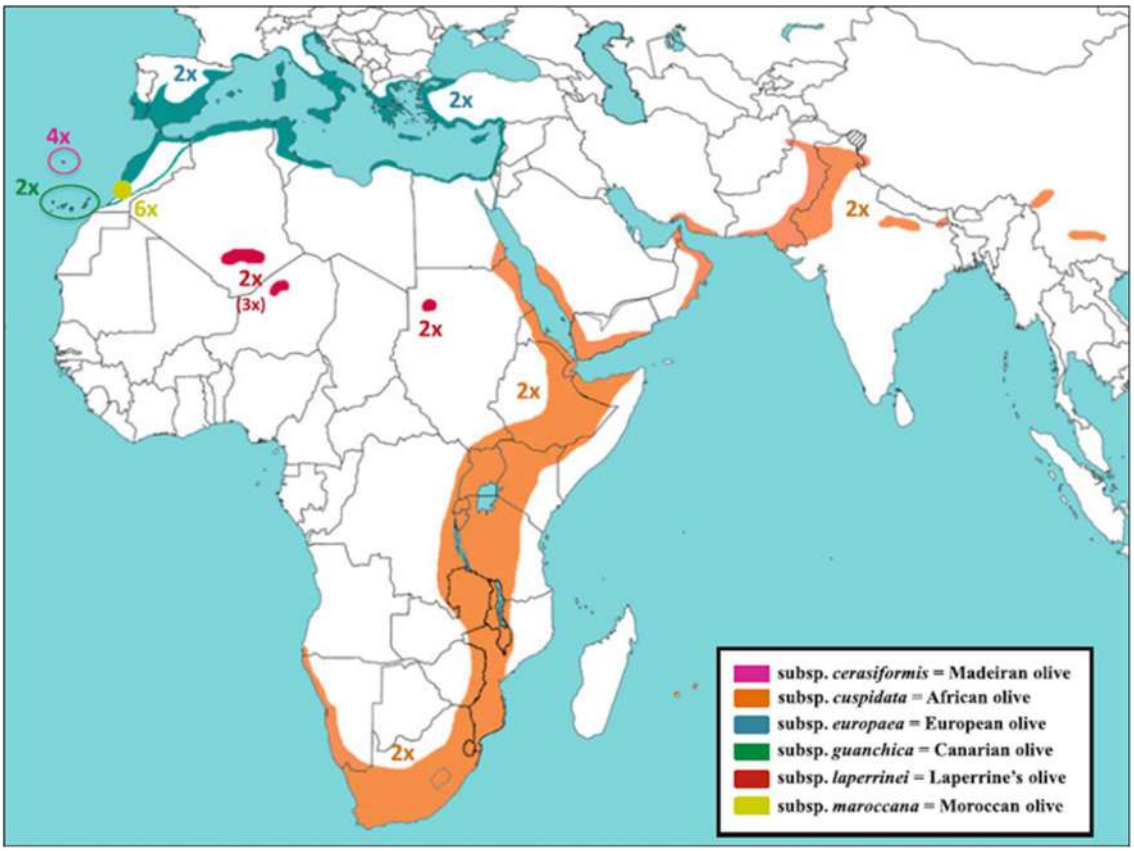


FIGURA 3 | Distribución geográfica del olivo (*Olea europaea* L.; según Rubio de Casas et al., 2006). Actualmente se reconocen seis subespecies en el complejo de olivos (Médail et al., 2001; Green, 2002). El nivel de poliploidía se indica para cada subespecie según Besnard et al. (2008). Se determinó hexaploidía y tetraploidía en las subespecies *maroccana* y *cerasiformis*, respectivamente. También se han detectado algunos triploides (aproximadamente el 3%) en los olivos de la subespecie *lapperine*i (Besnard and Baali-Cherif, 2009).

Esta diversificación de la especie *Olea europaea* L. se remonta al Mioceno tardío o al Plioceno temprano (Besnard et al., 2009), donde la aridificación del Sahara puede haber contribuido a reducir el flujo de genes entre las poblaciones de olivos (Schuster et al., 2006), provocando la diferenciación temprana de la subespecie *cuspidata* (Besnard et al., 2007, 2009). *Olea europaea* subsp. *laperrinei*. (Batt. & Trab.) Cif. es endémica de las cumbres de los macizos del Sahara, presente en tres cadenas montañosas: Aïr (norte de Níger), Hoggar (sur de Argelia) y Jebel Marra (oeste de Darfur, noroeste de Sudán), actuando como refugios aislados para numerosas especies, entre ellas *laperrinei* (Besnard et al., 2012). Debido a este aislamiento, los estudios genéticos muestran que la subsp. *laperrinei* está muy diferenciada de sus parientes mediterráneos, macaronésicos, africanos y asiáticos (Angiolillo et al., 1999). Hay evidencias de que el Sahara ha actuado como un poderoso impulsor de la especiación y de los cambios

evolutivos de especies relictas saharianas (Douady et al., 2003; Genner and Haesler, 2010). Los individuos *laperrinei* se ha caracterizado como diploides, sin embargo, se han detectado algunos casos de triploidía (aproximadamente el 3%) (Besnard and Baali-Cherif, 2009), probablemente causada por fenómenos de especiación. Las subespecies *guanchica*, *cerasiformis* y *maroccana* son endémicas del archipiélago macaronésico. Particularmente, la subespecie *guanchica* endémica de las islas Canarias, distribuyéndose en seis de las siete islas (no se conocen olivos silvestres en Lanzarote) (García-Verdugo et al., 2009; Green, 2002). Esta subespecie muestra un alto grado de diferenciación debido al aislamiento geográfico impuesto por las barreras oceánicas. Este taxón muestra diferencias entre islas, debido al flujo de genes entre las formas silvestres y cultivadas. Sin embargo, se ha determinado mayor parentesco de las poblaciones de *guanchica* de las islas del este (Fuerteventura y Gran Canaria) con el olivo mediterráneo que con las poblaciones de *guanchica* occidental (Tenerife, La Palma, El Hierro, La Gomera) (García-Verdugo et al., 2009). Además, se ha observado un alto grado de diferenciación genética para los marcadores nucleares y cloroplásticos tanto entre diferentes islas como entre subpoblaciones dentro de las islas (García-Verdugo et al., 2010), demostrando la alta variabilidad genética de este taxón. Sin embargo, un estudio mediante marcadores AFLP reveló un bajo nivel de diversidad genética en las poblaciones de *guanchica*, relacionado con los eventos volcánicos de cada isla (García-Verdugo et al., 2009). Las poblaciones jóvenes de olivo de la isla joven de La Palma se ven fuertemente afectadas por los eventos de extinción-recolonización causados por los fenómenos geológicos recientes, que han reducido la varianza genética de la población (García-Verdugo et al., 2009; García-Verdugo et al., 2010).

La subespecie *cerasiformis* es un tetraploide endémico de dos de las tres islas Madeira (Porto Santo y Madeira) que posee una mayor diversidad genética que la subespecie *guanchica* (García-Verdugo et al., 2009). Este taxón es el resultado de un evento de dispersión independiente al taxón de la subsp. *guanchica* (Hess et al., 2000), aunque se planteó la hipótesis de que podría derivar de la hibridación entre los ancestros de las subespecies *guanchica* y *europaea* (Besnard et al., 2008; Kole, 2011).

La subespecie *maroccana* se distribuye en un área muy restringida de unos pocos cientos de hectáreas de la vertiente sur del Alto Atlas Occidental (Médail et al., 2001).

Presenta un origen alopoliploide, lo que probablemente está relacionado con la alta diversidad genética encontrada en este taxón hexaploide (García-Verdugo et al., 2009). Esta subespecie no suele hibridar con las poblaciones concurrentes de *europaea*, probablemente debido a la diferenciación fenológica y a su diferente nivel de ploidía (Kassa et al., 2019).

La subespecie *europaea* var. *sylvestris* se conoce como olivo silvestre mediterráneo o acebuche (Green, 2002), observándose una fuerte diferenciación genética en el ADN nuclear y plastídico entre grupos mediterráneos occidentales y orientales (Besnard et al., 2002; Lumaret et al., 2004; Breton et al., 2006; Besnard et al., 2013b). Dentro de esta subespecie se diferencian tres linajes (conocidos como linajes E1, E2 y E3) basados en el ADN de plástidos (Besnard et al., 2007, 2013b). A causa de fluctuaciones en el clima mediterráneo desde el Pleistoceno, con épocas secas y otras glaciares, las poblaciones de olivo silvestre de la cuenca mediterránea han mostrado diferenciaciones genéticas como resultado de las limitaciones de intercambio génico debido a las distancias geográficas y barreras naturales (desiertos, mares, montañas) durante largos períodos de tiempo (Angiolillo et al., 1999; Besnard et al., 2001, 2007, 2013b, 2013a; Lumaret et al., 2004; Rubio de Casas et al., 2006; Breton et al., 2009; Diez et al., 2015). Debido a la larga historia del cultivo del olivo, específicamente en el Mediterráneo, el origen de los cultivares de olivos locales y su relación genética con los olivos silvestres cercanos (acebuches) es, a menudo, compleja.

Se han realizado estudios filogenéticos y filogeográficos de los olivos silvestres utilizando una gran variedad de marcadores moleculares. Por ejemplo, se utilizaron marcadores de ADN cloroplástico, nuclear y mitocondrial para resolver la relación entre subespecies (Besnard and Bervillé, 2000, 2002; Besnard et al., 2002, 2007; Rubio de Casas et al., 2006; Van de Paer et al., 2018). Los primeros análisis se basaron en polimorfismos de longitud de fragmentos de restricción (RFLP; Besnard and Bervillé, 2000), pero no fueron apropiados para la reconstrucción filogenética. En el análisis filogenético del ADN del cloroplasto se identificaron varios haplotipos dentro del complejo oleícola (Besnard et al., 2002). Un haplotipo o clorotipo se ha definido como el conjunto de polimorfismos que tienden a cosegregar debido a su proximidad. Sin embargo, esta filogenia no fue congruente con los resultados taxonómicos,

morfológicos y moleculares previamente disponibles. La mayoría de los estudios filogenéticos corroboran que la subsp. *cuspidata* está genéticamente más alejada del resto de subespecies. Sin embargo, las relaciones filogenéticas entre los otros taxones permanece indeterminada, y se han resuelto diferentes tipos de relaciones filogenéticas según el tipo de marcadores, orgánulos estudiados o número de muestras analizadas en los diversos estudios (Kassa et al., 2019). Los marcadores relacionados con el ADN cloroplástico y mitocondrial se han utilizado ampliamente en los estudios filogénicos del olivo (Besnard and Bervillé, 2002; Kassa et al., 2019). Estos orgánulos, de herencia materna, son más propensos a la deriva genética que los genes nucleares (Schaal and Olsen, 2000). Además, los polimorfismo del ADN mitocondrial o cloroplástico se diseminan solo por semillas y, por lo tanto, a una distancia más corta que los polimorfismos del ADN nuclear, que también se dispersan por el polen (Ennos, 1994). Por lo tanto, el ADN de estos orgánulos es muy útil para revelar patrones genéticos de fuerte diferenciación y para estudiar procesos filogeográficos.

Variabilidad de los cultivares actuales

El olivo probablemente fue domesticado en el Medio Oriente hace unos 6000 años (Zohary and Spiegel-Roy, 1975; Diez et al., 2015), probablemente a partir de olivos silvestres (*O. europaea* L. subsp. *sylvestris*) (López-Escudero and Mercado-Blanco, 2011). Posteriormente, la difusión de este cultivo se realizó hacia el Oeste a través de la cuenca mediterránea, produciéndose en el proceso relaciones genéticas complejas entre los cultivares (Besnard et al., 2013a) y con las variedades silvestres locales. Así, los agricultores de diversas áreas mediterráneas han seleccionado variedades locales de olivos silvestres o procedentes de cruces entre cultivares introducidos y cultivares locales, dando como resultado una gran cantidad de cultivares cuyo proceso de domesticación aún está en curso (Besnard et al., 2001, 2018; Khadari et al., 2003, 2008; El Bakkali et al., 2013). Estas variedades fueron introducidas y posteriormente propagadas vegetativamente como plántulas, injertos y esquejes, reduciendo fuertemente la diversidad genética de los olivos autóctonos (Baldoni et al., 2006), como los acebuches centro-occidentales (Besnard et al., 2018).

El estudio de la variabilidad genética del olivo cultivado y la identificación de cultivares se ha llevado tradicionalmente a cabo mediante el análisis de la morfología, la composición del aceite y la fenología (Khadari et al., 2003). Sin embargo, actualmente se utilizan marcadores moleculares de ADN para la identificación inequívoca de cultivares (Belaj et al., 2001, 2002, 2003, 2004; Atienza et al., 2013; Trujillo et al., 2014) confirmando la identificación previa realizada por marcadores morfológicos (Barranco, 2000; Barranco and Rallo, 2000). Inicialmente, los cultivares de olivo se nombraron usando criterios genéricos, como sus características morfológicas, localidad de origen, etc (Trujillo et al., 2014). En consecuencia, en los olivos, la existencia de sinonimia (diferentes nombres para el mismo cultivar) y homonimia (mismo nombre para diferentes cultivares) entre y dentro de los países productores de olivos es muy frecuente (Barranco et al., 2000; Corrado et al., 2009; Trujillo et al., 2014). La secuenciación completa del genoma del olivo (Cruz et al., 2016) ha proporcionado las herramientas necesarias para determinar nuevos polimorfismos y correlacionarlos con los rasgos fenotípicos deseados, además de ayudar al estudio de la diversificación y domesticación de la especie. Entre ellos, el empleo de polimorfismos de un solo nucleótido (SNP) se considera más fiable debido a su gran estabilidad, facilidad de puntuación y mejor comprensión de sus tasas de mutación. Recientemente, se han identificado tres clorotipos u haplotipos comunes en los olivos silvestres y pertenecientes al linaje E1 (E1-e.1, E1-e.2 and E1-e.3), presentes en alrededor del 90% de los cultivares (Diez et al., 2015; Besnard et al., 2018). El otro 10% de clorotipos pertenece a los linajes E2 y E3 (es decir, E2.1, E2.2, E3.1 y E3.2) (Besnard et al., 2018). Por tanto, las formas cultivadas provienen de un ancestro mediterráneo precuaternario, pero el largo y continuo proceso de domesticación también provocó la mezcla recurrente con poblaciones silvestres locales, con individuos pre-domesticados y con cultivares introducidos (Besnard et al., 2007, 2013b, 2013a; Diez et al., 2015). Sin embargo, sigue sin documentarse exactamente dónde y cómo se cultivaron por primera vez los cultivares de olivo (Breton et al., 2008). Aunque menos frecuentemente, se han producido contactos entre el olivo cultivado y las subespecies silvestres *cuspidata*, *guanchica* o *laperrinei* (Besnard, 2016). Además, se han observado efectos de hibridación entre las subespecies *laperrinei* y *europaea*, concretamente en la generación del cultivar Dhokar, contribuyendo al proceso de diversificación (Besnard et al., 2013a).

La diversidad genética es aún mayor en el acervo genético silvestre de las diferentes subespecies de olivo (Lumaret et al., 2004; Baldoni et al., 2006, 2009; García-Verdugo et al., 2009; Belaj et al., 2010, 2011; Besnard et al., 2013a, 2013b; Chiappetta et al., 2017; Kassa et al., 2019).

Importancia del uso de genotipos silvestres

La larga historia evolutiva de la especie *Olea europaea,* su adaptación a ambientes muy adversos (Médail et al., 2001; Green, 2002) y la fuerte influencia del cambio climático en la agricultura moderna, hacen que los genotipos silvestres puedan ser considerados como fuente de genes en la mejora del olivo cultivado. Los genotipos silvestres poseen una diversidad genética útil para desarrollar variedades de cultivos más productivas, nutritivas y resilientes (Castañeda-Álvarez et al., 2016). Estos genotipos silvestres están adaptados a las duras condiciones climáticas presentes en regiones áridas y semiáridas, en diferentes altitudes y tipos de suelo, incluidas las expuestas a un severo déficit hídrico, salinidad y bajas temperaturas (Cantos et al., 2002; Baldoni et al., 2006; Klepo et al., 2013; Belaj et al., 2016; Chiappetta et al., 2017). Por lo tanto, la protección de los recursos genéticos del olivo se vuelve fundamental, no solo en formas cultivadas en colecciones *ex-situ*, sino también en los genotipos silvestres en su hábitat natural. Un claro ejemplo son las poblaciones de olivos silvestres de la subsp. *laperrinei,* capaces de sobrevivir en zonas de montaña con precipitaciones anuales a menudo inferiores a 50-100 mm, desarrollando rasgos adaptativos (Besnard et al., 2009), y mostrando un pool genético capaz de adaptarse a ambientes extremos (Besnard et al., 2021). Estas subespecies de olivo silvestre pueden fácilmente cruzarse con los olivos de la cuenca Mediterránea (*Olea europaea* subsp. *europaea*), llegando a ser fuente de variabilidad genética para el olivo cultivado (Zohary, 1994; Hannachi et al., 2009; Besnard et al., 2013a; Klepo et al., 2013; Arias-Calderón et al., 2015c). La formación de híbridos entre grupos genéticos distanciados genéticamente puede resultar en un proceso de heterosis que mejore el rendimiento de la progenie, generando nuevos genotipos superiores con alto potencial agronómico (Biton et al., 2012). Además, el hecho de que los olivos silvestres puedan colonizar hábitats deforestados o suelos marginales puede ser clave en la rehabilitación de sitios devastados en muchas áreas del mundo (Kassa et al., 2019). Los genotipos silvestres también son importantes como recursos genéticos para la

restauración y establecimiento de nuevas combinaciones alélicas, dando nuevas oportunidades para mejorar la adaptación de cultivares a la sequía (Hannachi et al., 2009; Hernández-Santana et al., 2019; Kassout et al., 2021) y para ser utilizados como portainjertos en áreas con problemas de sequía (Baali-Cherif and Besnard, 2005), salinidad (Cantos et al., 2002; Bashir et al., 2021; Tadić et al., 2021), contaminación del suelo (Murillo et al., 2005), o para controlar el vigor (León et al., 2020), el rendimiento y la calidad de los cultivos (Hannachi et al., 2008; Baccouri et al., 2011; León et al., 2018; Espínola et al., 2021), así como una fuente de resistencia a enfermedades como el marchitamiento por *Verticillium* o Verticilosis (Colella et al., 2008; Arias-Calderón et al., 2015c; Trapero et al., 2015; Jiménez-Fernández et al., 2016). Los genotipos silvestres de olivo se han utilizado como parentales en programas de mejora, transmitiendo un período juvenil más corto y con floración más abundante (Klepo et al., 2014), con potencial para mejorar la composición y calidad del aceite (Guerin et al., 2003; Hannachi et al., 2008; León et al., 2018; Elgadi et al., 2021; Espínola et al., 2021) y para superar escenarios futuros de cambio climático en respuesta al estrés abiótico (Mariotti et al., 2020). La identificación de genotipos capaces de hacer frente a los múltiples problemas del cultivo del olivo, permitirá su utilización con fines de mejora, a la vez de preservar la diversidad genética de esta especie de los riesgos de erosión.

Sistemas de plantación para el olivar moderno.

En las últimas décadas se está produciendo una transformación progresiva de las técnicas de cultivo del olivo en respuesta a las condiciones socioeconómicas cambiantes y a los avances tecnológicos. El modelo de cultivo tradicional (70 - 100 árboles por hectárea; **Figura 4A)** en régimen de secano y de recolección manual está transformándose en sistemas de cultivo intensivo (200 - 800 árboles/ha) y superintensivo (1500 - 2000 árboles/ha; **Figura 4B)** sometidos a fertilización y riego (generalmente deficitario), diseñados para la recolección mecanizada (Rallo et al., 2013; Díez et al., 2016; Fernández et al., 2021). Este nuevo sistema superintensivo (HDH, del inglés high-density-hedgerow), también conocido como cultivo de muy alta densidad o cultivo en seto (Rius and Lacarte, 2010; Tous, 2011; Rallo et al., 2013) presenta estructuras geométricas con hileras de árboles espaciados uniformemente creciendo a una altura y anchura predeterminadas y separados a distancias iguales entre sí (Connor

et al., 2014). Estos sistemas de plantación modernos aumentan la rentabilidad del cultivo ya que permiten una fructificación temprana, abundante y consistente, mecanizan totalmente las operaciones de cosecha y, aunque sea parcialmente, de la poda (Barranco et al., 1998; Rallo et al., 2013; Mairech et al., 2020).

FIGURA 4 | Cultivo de olivo tradicional en régimen de secano con amplios marcos de plantación (**A**) y cultivo de olivo en sistema de plantación de alta intensidad con riego por goteo (**B**), ambos cultivos localizados en zonas próxima al municipio de Carmona (Fotos de Pablo Díaz-Rueda).

Los sistemas de producción en seto están bien establecidos en muchos cultivos de árboles frutales templados como el manzano, el melocotonero o el cerezo (Ryugo and Mikuckis, 1969; Heinicke, 1975; DeJong and Doyle, 1985; Robinson et al., 1991), pero para el olivo son relativamente nuevos (Fernández et al., 2021). España es el país con mayor superficie de olivar del mundo, que asciende a 2,65 Mha, el 16% de su suelo cultivado, de ellas, el 71% es olivar tradicional, el 26% olivar intensivo y el 3% olivar superintensivo o en seto (Fernández et al., 2021). Este sistema en seto inició su

plantación en España en 1994 (Rius and Lacarte, 2010), experimentando una rápida difusión en países con o sin tradición olivícola, y cuya implantación no deja de crecer (Rius and Lacarte, 2015). Como en otros árboles frutales, en el cultivo de olivo superintensivo es esencial limitar la altura y el ancho del seto de 4 a 5 años después de su plantación, cuando la altura de los árboles se encuentre dentro de unos 2.5 – 3.5 m (Moutier et al., 2011) para permitir que las cosechadoras cabalgadoras pasen por encima de las hileras de árboles (Connor et al., 2014; **Figura 5**).

FIGURA 5 | Cosechadora de olivo modelo New Holland en vista frontal (**A**) y en funcionamiento (**B**) realizando la recolección mecanizada de aceituna a una hilera de olivos en super-intensivo en la finca experimental Buitrago (Ctra. Mairena del Alcor - Brenes, km 17) (Fotos realizadas por Pablo Díaz-Rueda y JM Colmenero-Flores).

Esta reducción del dosel de los árboles ha provocado un descenso del número de frutos obtenidos por un solo árbol para centrarse en la productividad por superficie de suelo (Jackson and Palmer, 1980). Estos cultivos de alta densidad pensados como inversión a corto plazo, requieren un corto periodo de tiempo (de 3 a 4 años) para alcanzar la plena producción y producir más de 10 t ha^{-1}, en promedio, durante más de 15 años (Rallo et al., 2013). Entre sus ventajas evidentes se encuentra la mecanización integral del cultivo, minimizando los costes por mano de obra, rápida entrada en producción, elevada producción y alta eficiencia de las máquinas de recolección en continuo, generando con ello cultivos más rentables que el modelo tradicional (Connor et al., 2014). Pese al temor inicial de descensos de producción a partir del 6º-7º año de cultivo (debido a la competencia entre árboles y falta de iluminación y aireación en el interior de las copas), se ha comprobado que la producción de fruto y aceite en cultivos superintensivo puede mantener un crecimiento constante durante al menos los

primeros 14 años evaluados (Díez et al., 2016). Además, se ha comprobado que a lo largo de un intervalo de 750 - 2.250 árboles / ha la producción crece proporcionalmente con la densidad de la plantación (Díez et al., 2016; León et al., 2007). Aparte del diseño del cultivo, los factores críticos identificados para garantizar la producción y longevidad del cultivo superintensivo son la selección del cultivar y el control del vigor del árbol (Fernández, 2014). Solo unos pocos cultivares de olivo tradicionales son adecuados para los sistemas de plantación de alta densidad como 'Arbequina', 'Arbosana' o 'Koroneiki' (Rallo et al., 2013; Connor et al., 2014; Díez et al., 2016). La variedad más plantada en cultivo superintensivo es el cultivar 'Arbequina' , que se caracteriza por presentar vigor medio-bajo, productividad alta y estable, producción temprana y un aceite afrutado (Barranco, 2000; Rallo et al., 2013; Rius and Lacarte, 2015). Además, presenta un patrón de desarrollo compacto con alta tasa de ramificación que aumenta la eficiencia del rendimiento en condiciones de HDH en comparación con otros cultivares (Rosati et al., 2013; **Figura 6**).

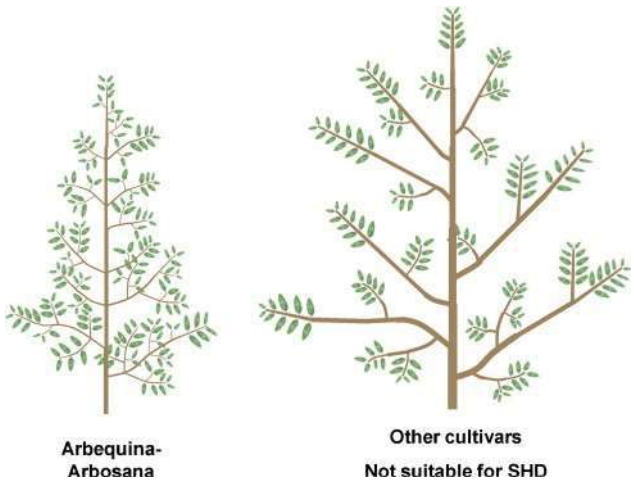


FIGURA 6 | Patrón de ramificación entre cultivares 'Arbequina' y 'Arbosana' adecuados para HDH y otros cultivares no aptos. Estos dos cultivares presentan una mayor ramificación con diámetros más pequeños, proporcionando más sitios de fructificación (brotes) por unidad de volumen del dosel o por unidad de estructuras de soporte. Imagen obtenida de Rosati et al., (2013).

La necesidad de nuevos cultivares específicos para estas nuevas plantaciones ha estimulado el desarrollo de programas de mejora en varios países (De la Rosa and León, 2009) como Turquía (Arsel and Cirik, 1994), Grecia (Pritsa et al., 2003), Italia (Fontanazza et al., 1998; Bellini et al., 2002) e Israel (Lavee et al., 1986). En España, los programas de mejora del olivo comenzaron en 1992 para obtener cultivares adaptados a estos nuevos marcos de plantación (Rallo, 1995). En estos programas se han realizado cruces entre

cultivares para obtener nuevas variedades de vigor reducido, basados en la altura y diámetro del tronco, capaces de adaptarse a los densos marcos de plantación con cosecha mecánica y con cortos periodos juvenil para una rápida entrada en producción (De la Rosa et al., 2006; Hammami et al., 2011; León et al., 2007). Otra característica deseable en estos cultivares es la presencia de un hábito de crecimiento lloroso (Rallo et al., 2008) y un alto número de ramas laterales asociado con diámetros más pequeños de tronco, ramas y brotes (Rosati et al., 2013). Se están llevando a cabo nuevos programas de mejora incorporando la variabilidad genética presente en los olivos silvestres (Klepo et al., 2013, 2014). Recientemente, se han obtenido nuevos cultivares adaptados al cultivo superintensivo: el cultivar 'Sikitita', procedente del cruce entre 'Picual' y 'Arbequina', se caracteriza por su precocidad, alto contenido en aceite, eficiencia de rendimiento y bajo vigor (Rallo et al., 2008); el cultivar 'Fs-17' presenta una entrada en producción precoz, un vigor medio-bajo y buen rendimiento graso (Fontanazza et al., 1998); el cultivar 'Barnea' se caracteriza por un crecimiento erecto y estrecho, precocidad, alta producción y alto rendimiento graso (Lavee et al., 1986); y el recientemente desarrollado cultivar 'Lecciana', un cruce entre 'Arbosana' y 'Leccino', caracterizado por su bajo vigor (Camposeo et al., 2021). El principal inconveniente de las plantaciones de alta densidad es la dificultad de controlar el vigor de los árboles para permitir el paso de la cosechadora. Por ello, las variedades empleadas actualmente ('Arbequina' y 'Arbosana') conllevan un alto coste económico para forzar la reducción del vigor mediante un control estricto de la fertirrigación y la poda (Barranco, 2000). Además, el cultivo en superintensivo excluye la posibilidad de usar cultivares tradicionales como 'Picual' y 'Hojiblanca', comúnmente utilizados por su enorme importancia socioeconómica en la producción de aceite de oliva con alto contenido en ácido oleico y alto contenido fenólico (León et al., 2011), alto rendimiento, elevada capacidad productiva, madurez temprana, desprendimiento fácil y buena adaptación a diferentes condiciones ambientales (Barranco and Rallo, 2000). Tampoco son adecuadas las variedades de aceituna de mesa ('Manzanilla', 'Gordal', etc) debido a su alto vigor, lo que obliga a realizar podas frecuentes (Barranco, 2000). Asimismo, el uso de unas pocas variedades de vigor reducido está produciendo un empobrecimiento progresivo en el uso de cultivares y de la variabilidad genética del cultivo.

Para lograr la introducción de cultivares tradicionales como 'Picual' en el modelo de plantación superintensivo es necesario reducir el tamaño de los árboles. Para ello, se establecen dos estrategias: (1) mediante el fitomejoramiento para obtener y seleccionar portainjertos enanizantes adecuados (Atkinson, 2002) y (2) mediante una gestión cuidadosa del cultivo, analizando las implicaciones positivas del estrés abiótico en la reducción del crecimiento vegetativo, como el déficit de agua y / o nutrientes (Rallo et al., 2013; Connor et al., 2014). Ambas estrategias han permitido obtener plantas cuyos esfuerzos estén dirigidos a la producción de estructuras reproductivas en lugar de estructuras vegetativas (Lo Bianco et al., 2021). Sin embargo, todavía no se han seleccionado portainjertos enanizantes capaces de controlar el vigor del cultivar injertado (Baldoni and Fontanazza, 1990; Troncoso et al., 1990; Nardini et al., 2006; Romero et al., 2014; Rugini et al., 2016). Como todo cambio de modelo, la intensificación del olivar ha traído efectos muy positivos como los mencionados anteriormente, pero también ha desencadenado nuevos problemas que tenían muy poco o ningún impacto en el cultivo tradicional del olivo, como nuevas enfermedades, empobrecimiento genético y mayor sensibilidad a perturbaciones abióticas.

Problemas del cultivo del olivo

Estrés abiótico

El olivo crece íntimamente relacionado con una serie de factores bióticos y abióticos constituyendo un agroecosistema. El olivo es una especie muy bien adaptada al ambiente semiárido de la cuenca mediterránea, y se cultiva tradicionalmente en condiciones de sequía (Fernández, 2014). Sin embargo, según el Panel Intergubernamental sobre Cambio Climático (IPCC), las proyecciones de cambio climático señalan un aumento de las temperaturas y una reducción de los patrones de precipitación (IPCC, 2013). Se espera que los países de la cuenca mediterránea sean los más afectados por las olas de calor y la sequía, y es predecible una reducción en la productividad agrícola (Ponti et al., 2014; Michalopoulos et al., 2020). Además, otros factores abióticos como la radiación solar, la humedad relativa y el viento influyen directamente en la productividad del olivo (Fraga et al., 2020).

El riego del olivar, sobre todo con aguas de mala calidad, ricas en sales, también ha incrementado la incidencia de otra perturbación ambiental propia de la cuenca mediterránea: la salinidad. Debido a la reducción de la disponibilidad de agua por efecto del cambio climático, se prevé una disminución generalizada de su calidad en un futuro próximo y, por tanto, un incremento progresivo de la salinidad. El aumento de la salinidad puede reducir la actividad fotosintética, el crecimiento vegetativo y la producción de fruto y aceite (Ben-Gal, 2011), aunque el grado de tolerancia es específico de la variedad (Ben-Gal, 2011). Este problema se agrava con las malas prácticas de fertilización que suelen producirse en el cultivo forzado del olivo en régimen intensivo y superintensivo. Otra perturbación ambiental propia de la región mediterránea es la presencia de suelos calcáreos y alcalinos inductores de la deficiencia en hierro en los cultivos provocando la clorosis férrica, a la que, por ejemplo, es sensible el cultivar 'Arbequina' (Alcántara et al., 2003). Otro factor asociado a la productividad del olivar cultivado en estas condiciones en regiones semiáridas es la buena adaptación al riego deficitario (Fernández, 2014). Por tanto, el sector oleícola profesional demanda investigación e innovación encaminada a la mejora del material vegetal y de las prácticas del cultivo para un uso más eficiente del agua de riego y una mayor resistencia a diversos estreses ambientales como la sequía, la salinidad y la caliza activa.

Estrés biótico

La intensificación del olivar y el cambio de técnicas de cultivo están provocando la aparición de nuevos problemas o agravando algunos existentes. La lucha contra las plagas del olivo se sigue realizando mediante el uso de insecticidas tradicionales que tienen buena eficacia y bajo coste, pero entrañan efectos secundarios graves, como daño a los insectos auxiliares, residuos en el fruto o contaminación ambiental. En la actualidad, hay que apostar por el desarrollo y uso de medidas alternativas de lucha que disminuyan estos efectos adversos (Barranco et al., 1998).

El estrés biótico es un importante factor determinante de la calidad del aceite de oliva, ejerciendo un efecto perjudicial directo e indirecto en varios parámetros de calidad. Dado que el estrés biótico puede considerarse inevitable en el olivo, es importante desarrollar estrategias adecuadas que minimicen el impacto en el

rendimiento (Corrado et al., 2016). Desde el punto de vista entomológico, el estrés biótico más importante es la mosca del olivo (*Bactrocera oleae* Gmel.), que genera daños directos (disminución de producción, pérdida de peso y/o caída del fruto) e indirectos (pérdida de calidad en el aceite) (Gucci et al., 2012). Otros insectos que afectan negativamente al cultivo del olivo son los Prays o polilla del olivo (*Prays oleae* Bern.), diferentes especies de cochinillas (cochinilla de la tizne: *Saissetia oleae* Bern.; Parlatoria o cochinilla violeta: *Parlatoria oleae* Colvee; Serpeta: *Lepidosaphes ulmi* Linn.), Barrenillo del olivo (*Phloeotribus scarabaeoides* Bern.), Polilla del jazmín o glifode (*Margaronia unionalis* Hubn.), Abichado o euzofera (*Euzophera pinguis* Haw.), Acariosis o sarna (*Aceria oleae* Napela.), Algodón del olivo (Euphyllura olivina Costa.), entre otros (Barranco et al., 1998).

Otro grupo que afecta negativamente al olivo son las enfermedades causadas por bacterias y hongos. Entre las principales enfermedades del olivo se encuentran: el Repilo, causada por el hongo *Spilocea oleaginea* (syn. *Cycloconium oleaginum* Cast.), que provoca defoliación por el debilitamiento del árbol y pérdida de cosecha (Ogawa and English, 1991); la Tuberculosis del olivo, producida por la bacteria *Pseudomonas syringae* pv. *savastanoi,* que causa tumores o agallas afectando al crecimiento de tallos, defoliación y finalmente la muerte del árbol, no conociéndose métodos eficaces de control (Sisto et al., 2004); la Aceituna jabonosa, conocida como antracnosis, lepra o momificado, producida por el hongo *Colletotrichum gloeosporioides,* que causa la podredumbre de las aceitunas, asociada a la pérdida de peso y caída prematura del fruto, originando acidez y muy baja calidad del aceite (Moral et al., 2014); el decaimiento súbito del olivo ocasionado por la bacteria *Xylella fastidiosa* originaria del continente americano, que en los últimos años ha conseguido asentarse en diferentes lugares de la cuenca del Mediterráneo, provocando la desecación y muerte generalizada de los árboles (Morelli et al., 2021). Sin embargo, la enfermedad que actualmente tiene efectos más severos en la productividad del olivar es la Verticilosis o marchitez por *Verticillium*, causada por el hongo *Verticillium dahliae* Kleb. (López-Escudero and Mercado-Blanco, 2011).

Verticillium dahliae

La Verticilosis o marchitez por *Verticillium*, causada por el hongo de suelo *Verticillium dahliae* es actualmente la enfermedad más amenazadora del olivar a nivel mundial (Jiménez-Díaz, et al., 2012; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011). *Verticillium dahliae* tiene un amplio rango de huéspedes, entre los que se encuentran más de 400 especies de plantas leñosas, herbáceas, anuales y perennes, incluidos cultivos de gran importancia económica como el algodón, tomate, patata, coliflor, girasol y olivo (Pegg and Brady, 2002; Fradin and Thomma, 2006). Está ampliamente distribuido, detectándose en casi todas las regiones donde se cultiva el olivo (López-Escudero and Mercado-Blanco, 2011). Las infecciones por *V. dahliae* pueden resultar en pérdidas importantes para el cultivo debido a las altas tasas de mortalidad (Levin et al., 2003), ya que en la actualidad no se dispone de fungicidas eficaces para su control. Este patógeno se caracteriza por la producción de propágulos infecciosos denominados microesclerocios, que son estructuras de resistencia que permiten al hongo persistir en el suelo durante periodos prolongados de tiempo (hasta 14 años) en ausencia de un hospedador (Jiménez-Díaz, et al., 2012; López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Wilhelm, 1955). El ciclo de vida de *V. dahliae* es asexual **(Figura 7)**, siendo la anastomosis de hifas la única forma posible de intercambiar material genético entre sus poblaciones (Montes-Osuna and Mercado-Blanco, 2020). El proceso de infección comienza cuando los propágulos infecciosos germinan en presencia de exudados radiculares y penetran a través de las raíces del huésped (Mol, 1995; Pegg and Brady, 2002). Las pequeñas heridas o aberturas naturales como los pelos radiculares dañados o las células epidérmicas muertas son zonas de penetración del hongo. Posteriormente, el patógeno coloniza sistémicamente el olivo con hifas y conidios a través del sistema vascular antes incluso de que se desarrollen los síntomas (Jiménez-Díaz, et al., 2012).

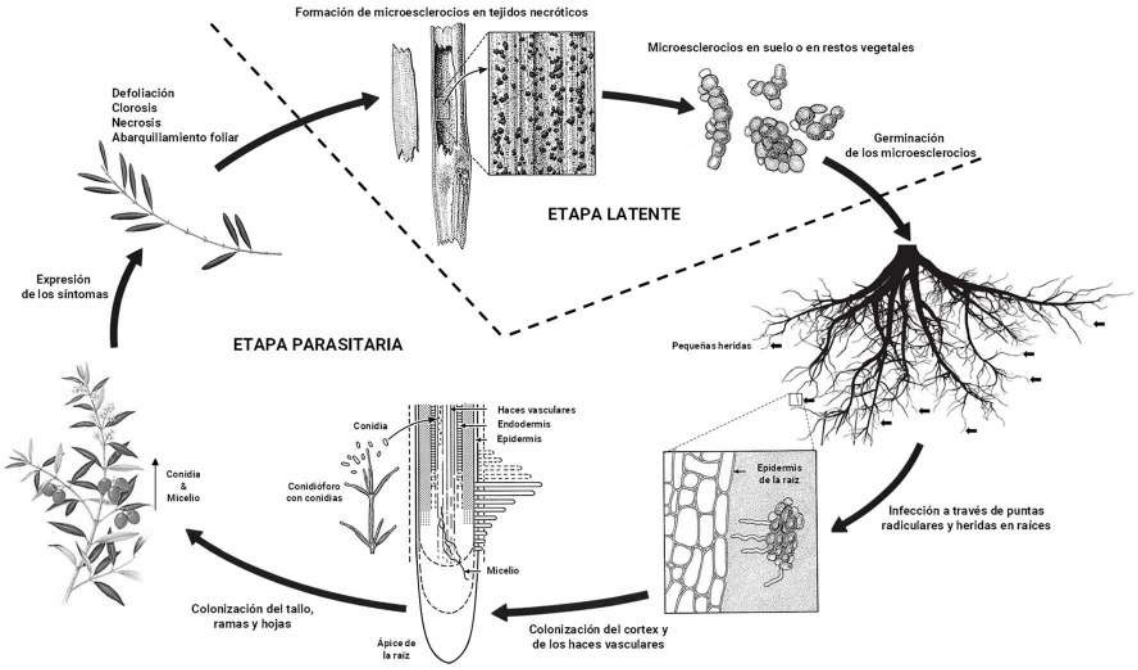


FIGURA 7 | Ciclo de vida de *Verticillium dahliae en olivo. Imagen* adaptada de Keykhasaber et al. (2018).

La acción combinada de la colonización fúngica y de los mecanismos de defensa de la planta infectada provocan la oclusión de los vasos xilemáticos debido a la formación de geles, gomas y tilosas. Esto a su vez genera cavitación o embolia y reduce la conductividad hidráulica de la planta, produciendo su decaimiento (Fradin and Thomma, 2006; Inch and Ploetz, 2012; Yadeta and J. Thomma, 2013; Pouzoulet et al., 2014; Deyett et al., 2019). Además, *V. dahliae* produce micotoxinas, enzimas que degradan la pared celular, elicitores y otros metabolitos que pueden dañar gravemente el metabolismo de la planta y contribuir al marchitamiento de la misma (Fradin and Thomma, 2006; Falcón-Rodríguez et al., 2012; Luo et al., 2014). Este proceso infeccioso desarrolla el síndrome de marchitez por *Verticillium*, con síntomas como clorosis, necrosis, abarquillamiento de las hojas, retraso de crecimiento, defoliación, senescencia precoz y, en algunos casos, la muerte de la planta hospedadora (Fradin and Thomma, 2006; López-Escudero and Mercado-Blanco, 2011; Pegg and Brady, 2002; Trapero et al., 2018; Yadeta and J. Thomma, 2013). Sin embargo, algunos individuos infectados con *V. dahliae* pueden superar la enfermedad, debido a la inactivación o muerte del patógeno en los árboles recuperados (Levin et al., 2003; López-Escudero and Blanco-López, 2005; Bubici and Cirulli, 2014). El diagnóstico de la enfermedad y del grado de susceptibilidad a *V. dahliae* se llevan a cabo mediante visualización de síntomas y aislamiento del hongo

a partir del tejido infectado (López-Escudero et al., 2004; Martos-Moreno et al., 2006; Colella et al., 2008; Markakis et al., 2009; Bubici and Cirulli, 2012; García-Ruíz et al., 2014). Actualmente, el uso de herramientas moleculares basadas en PCR y PCR en tiempo real (qPCR) han permitido detectar y cuantificar de manera específica y altamente sensible al patógeno en plantas infectadas, antes incluso de la aparición de síntomas (Mercado-Blanco et al., 2003; Markakis et al., 2009; Gramaje et al., 2013; Pasche et al., 2013; Jiménez-Fernández et al., 2016).

Se han identificado dos patotipos de *V. dahliae*, clasificados como defoliante (D) y no defoliante (ND) según su capacidad para inducir o no la defoliación de las hojas verdes, respectivamente (Schnathorst and Sibbett, 1971; Bejarano-Alcázar et al., 1996). El patotipo ND causa un síndrome de marchitez moderado y en las plantas infectadas los síntomas pueden desaparecer por completo. Por el contrario, las infecciones por el patotipo D suelen provocar una defoliación grave del árbol, pudiendo ser letales (Keykhasaber et al., 2018; López-Escudero and Mercado-Blanco, 2011).

Debido a que las medidas de control individuales son generalmente ineficaces, se hace necesario implementar una estrategia de manejo integrado para el control efectivo de la enfermedad (Jiménez-Díaz, et al., 2012; López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Tsror, 2011). Esta estrategia debe basarse en la aplicación de medidas de control antes y después de establecer la plantación, dirigidas principalmente a prevenir la penetración del patógeno o a reducir su presencia (López-Escudero and Mercado-Blanco, 2011). Entre las medidas aplicadas antes de la siembra se encuentra la detección preventiva de *V. dahliae* en el suelo mediante el uso de métodos moleculares específicos y sensibles, evitando la siembra en suelos infestados (Pérez-Artés et al., 2005), y la certificación del material vegetal de vivero libre de enfermedad antes de su implantación en el terreno (Morello et al., 2016). La solarización, la aplicación de enmiendas orgánicas al suelo (Varo-Suárez et al., 2018) y el uso de agentes de control biológico son estrategias valiosas de control sostenible (Mercado-Blanco et al., 2004; Prieto et al., 2009; Gómez-Lama Cabanás et al., 2014, 2018; Mulero-Aparicio et al., 2020). Se pueden distinguir dos estrategias diferentes en las plantas para controlar la infección de un patógeno (Robb, 2007). Por un lado, un mecanismo de resistencia que evita o reduce la proliferación del patógeno, impidiendo

o limitando la colonización de la planta. Por otro lado, un mecanismo de tolerancia, que inhibe o reduce los síntomas de la planta a pesar de producirse la proliferación del patógeno. El uso de material vegetal resistente y/o tolerante a *V. dahliae*, es una de las estrategias más prometedoras de control del patógeno, aunque a día de hoy no existen variedades inmunes a la enfermedad (Leyva-Pérez et al., 2018; López-Escudero et al., 2004; Martos-Moreno et al., 2006; Trapero et al., 2013).

Tras el establecimiento de la plantación, la eliminación de la maleza vegetal de los huertos constituye otra medida de control ya que puede reducir la densidad de inóculo en el suelo (Ligoxigakis et al., 2002). Por el mismo motivo, se debe evitar la rotación de cultivos con especies susceptibles (Pegg and Brady, 2002) y eliminar restos de poda que puedan contribuir a una reinfección de las plantas (Morello et al., 2016). Asimismo, se debe realizar un control del riego, ya que la transición de secano a regadío ha propiciado la dispersión del patógeno a través de los sistemas de riego (García-Cabello et al., 2012). También se aplican desinfectantes en el agua de riego para reducir la viabilidad del patógeno (Gómez-Gálvez and Rodríguez-Jurado, 2018; Gómez-Gálvez et al., 2018). El empleo de la solarización y el uso de fungicidas químicos preventivos o curativos reducen la densidad de inóculo, pudiendo controlar parcialmente a la enfermedad (Tjamos and Paplomatas, 1988; Tjamos, 1991; Fradin and Thomma, 2006; Tsror, 2011).

Para determinar el nivel de susceptibilidad a *V. dahliae*, la inoculación por inmersión de raíces desnudas de plántulas jóvenes ha demostrado ser el método más confiable (Trapero et al., 2013). Sin embargo, la vasta mayoría de los cultivares comerciales usados para producción de aceite o aceituna de mesa en España como 'Picual', 'Arbequina', 'Hojiblanca' o 'Manzanilla', son susceptibles o extremadamente susceptibles a *V. dahliae* (García-Ruíz et al., 2014; Hegazi et al., 2012; López-Escudero et al., 2004; López-Escudero and Mercado-Blanco, 2011; Martos-Moreno et al., 2006; Trapero et al., 2015, 2013). Por el contrario, se han identificado buenos niveles de control a *V. dahliae* en algunos cultivares como 'Frantoio', 'Empeltre' o 'Changlot Real', que han mostrando una baja susceptibilidad al patógeno. Esto se traduce en un retraso en el inicio de la enfermedad, capacidad de recuperación y un bajo porcentaje de plantas muertas (López-Escudero et al., 2004; López-Escudero and Mercado-Blanco, 2011; Martos-Moreno et al., 2006). No obstante, esta capacidad de control de la enfermedad

se rompe en suelos con densidad de inóculo de *V. dahliae* moderada y alta (Trapero et al., 2013) o no es duradera a largo plazo en suelos altamente infectados (Valverde et al., 2021). Tampoco se ha establecido si la menor susceptibilidad de estos cultivares se debe a un mayor grado de resistencia o de tolerancia al patógeno. Algunos autores han propuesto que los mecanismos de control a verticilosis se deben a respuestas moleculares (Bubici and Cirulli, 2012; Gómez-Lama Cabanás et al., 2015; Leyva-Pérez et al., 2018), mecanismos estructurales (Báidez et al., 2007; Markakis et al., 2010), bioquímicos y fisiológicos (Gharbi et al., 2016, 2017; Trapero et al., 2018). Sin embargo, el conocimiento de los mecanismos de defensa de la planta tras la infección por *V. dahliae* sigue siendo muy limitado.

Se han realizado programas de mejora para la identificación de variedades de baja susceptibilidad a *Verticillium* (Arias-Calderón et al., 2015a, 2015b, 2015c; Serrano et al., 2021). Pero podrían ser necesarios varios ciclos de mejora para obtener nuevos cultivares resistentes con un rendimiento agronómico adecuado. Se ha mostrado que cultivares de reducida susceptibilidad a *V. dahliae* usados como portainjertos son capaces de reducir la susceptibilidad en la planta injertada (Porras Soriano et al., 2003; Bubici and Cirulli, 2012). Por lo tanto, el uso de portainjertos con resistencia duradera a *V. dahliae* se plantea como una solución eficaz para el control de la Verticilosis en el olivar, lo que podría encontrarse más fácilmente en las poblaciones silvestres de olivo (Arias-Calderón et al., 2015c; Jiménez-Fernández et al., 2016).

Mejora del olivar mediante el uso de portainjertos clonales

El injerto es una antigua práctica agrícola que une el sistema radicular de una planta (portainjerto) con la parte aérea de otra (vástago) mediante un proceso de injerta que hace posible la conexión vascular de ambas partes (Mudge et al., 2009). El portainjerto aporta un adecuado sistema radicular por sus propiedades de arquitectura y función (Lynch, 1995; Kuijken et al., 2015). Debe adaptarse óptimamente a las condiciones edafo-climáticas de su entorno, aportando mayor resistencia a las enfermedades y al estrés ambiental, como el déficit hídrico, la salinidad, la clorosis férrica, etc. También puede alterar beneficiosamente los fenotipos del vástago, como el vigor, la cantidad o

calidad de la producción, o el tiempo de la maduración del fruto (Warschefsky et al., 2016). Este proceso puede ocurrir entre individuos de la misma especie o incluso entre congéneres o plantas de diferentes familias (La Rue, 1934; Jayawickrama et al., 1991). En un contexto de mejora agronómica supone una gran ventaja ya que permite seleccionar por separado las cualidades idóneas antes mencionadas para el portainjerto respecto de las cualidades agronómicas que se le exigen al cultivar injertado.

Mientras que para el resto de especies frutales hace décadas que se adoptó el uso de portainjertos (Gregory et al., 2013; Warschefsky et al., 2016), ésta práctica no se ha implementado aún para el cultivo del olivo, que crece sobre sus propias raíces, limitando claramente la versatilidad del cultivo. Tradicionalmente los portainjertos se han utilizado en el olivo para multiplicar variedades con dificultad para enraizar, como el cultivar 'Gordal'. También se han utilizado para establecer cultivos sobre variedades silvestres ya presentes en un terreno, aprovechando su buena adaptación a éste. Pero estos portainjertos silvestres, procedentes de semillas, constituyen poblaciones genéticamente heterogéneas no aceptables agronómicamente en los sistemas de plantación modernos. Por ello, el uso de un programa de identificación y selección mejora de portainjertos de olivo y su multiplicación clonal es imperativo. Un factor relevante de selección para cualquier portainjerto es su capacidad o compatibilidad para formar el tejido que sirve como unión entre el portainjerto y el vástago: la unión del injerto (Aloni et al., 2010; Goldschmidt, 2014). La curación de la unión del injerto puede tardar entre 360 y 450 días en el caso del olivo (Gascó et al., 2007), pues después de ese periodo la resistencia hidráulica de la unión del injerto representa solo un 3% de la resistencia total de la planta (Nardini et al., 2006).

En el olivo se han realizado algunos estudios para valorar el efecto de los portainjertos en propiedades agronómicas como la precocidad y el rendimiento del cultivo (Fallahi et al., 1989; Troncoso et al., 1990; Tous et al., 1997, 2011; Webster and Lucas, 1997; Lavee and Schachtel, 1999; Pannelli et al., 2002; Romero et al., 2014; Torres-Sánchez et al., 2022), así como en diversos parámetros como la mejora de la resistencia al estrés abiótico y biótico, incluida la susceptibilidad a verticilosis (Bubici and Cirulli, 2012; Porras Soriano et al., 2003), a la escasez de agua y nutrientes (Atkinson, 2002; Ghrab et al., 2017), y a la toxicidad por salinidad o metales pesados (Gallasch and

Dalton, 1989; Savvas et al., 2010). Aunque se ha observado un efecto del portainjerto en la calidad del fruto en muchos cultivos como los cítricos (Morales Alfaro et al., 2021) o la vid (Jin et al., 2016), se ha sugerido que los portainjertos de olivo tienen escasa influencia en la calidad de la fruta (Tous et al., 2011; Romero et al., 2014). Sin embargo, no se han identificado portainjertos idóneos para el cultivo del olivar, ni se ha implementado su multiplicación clonal ni producción de plantas injertadas en viveros autorizados. Dado que en otras especies la compatibilidad del injerto puede ocurrir entre individuos distanciados filogenéticamente, los genotipos silvestres de olivo pueden ser de gran utilidad como portainjertos de cultivares comerciales. Por ello, es necesario mantener colecciones vivas que representen la variabilidad genética no solo de los vástagos, sino también de los posibles portainjertos (Maxted and Kell, 2009).

Portainjertos para controlar el vigor

Se han establecido varios programas de mejora del olivo con el fin de buscar nuevas variedades de alta calidad agronómica y vigor reducido (Rallo, 1995; Fontanazza et al., 1998; León et al., 2007a, 2011, 2015; Rallo et al., 2008; El Riachy et al., 2012; Camposeo et al., 2021). Sin embargo, la regulación del vigor se ha realizado normalmente a través del uso de portainjertos enanizantes y semienanizantes en otras especies frutales (Warschefsky et al., 2016). La reducción del vigor inducida por el portainjerto provoca una disminución en el volumen de la copa, la altura, el diámetro del tronco y la superficie del árbol, lo que permitirá reducir la necesidad de poda en los cultivos de olivo en marcos de plantación intensivo y superintensivo (Koepke and Dhingra, 2013). Las diferencias de vigor del cultivar injertado suelen aparecer en el 4° año después de la brotación (5° año desde su plantación en campo), cuando se producen los altos rendimientos y comienzan a aparecer los primeros problemas de competencia en el cultivo superintensivo (Tous et al., 2011; Romero et al., 2014). La reducción de vigor observada por el uso de portainjertos es dependiente del sistema radicular y no del vástago injertado (Nardini et al., 2006). Así, varios estudios demuestran que el portainjerto tiene una gran influencia en el crecimiento de la planta y en la producción, por lo que portainjertos vigorosos transmiten mayor vigor y altos rendimientos a la variedad injertada, y viceversa (Troncoso et al., 1990; Pannelli et al., 2002; Nardini et al., 2006; Romero et al., 2014). Se han descrito algunos portainjertos enanizantes como Fs-

17 (Baldoni and Fontanazza, 1990), Leccino Dwarf (LD) (Pannelli et al., 2002; Nardini et al., 2006; Rugini et al., 2016) e incluso cultivares usados como portainjertos como 'Arbosana' (Tous et al., 2011). Podría ser particularmente útil el uso de la autoinjerta cuando es necesaria una mínima reducción del vigor de la planta para establecerse en cultivo superintensivo (Rugini et al., 2016). Es por ello que variedades de olivo de reducido vigor se consideran buenos candidatos para reducir el vigor de una plantación injertada. Sin embargo, no siempre existe correlación entre el vigor del patrón y de la planta injertada. Así, el portainjerto enanizante Fs-17 potencia el vigor y el rendimiento del vástago 'Arbequina' (Tous et al., 2011), indicando que la interacción cultivar-portainjerto es también muy relevante en la determinación del vigor resultante de una planta injertada.

Las causas que determinan la reducción del vigor parecen ser el resultado de la interacción compleja de varios factores (Soumelidou, 1994; Cohen and Naor, 2002; Atkinson et al., 2003; Basile et al., 2003; Nardini et al., 2006; Solari et al., 2006; Cohen et al., 2007; Gascó et al., 2007; Trifilò et al., 2007). Se ha propuesto que puede deberse a una perturbación en el transporte del agua en la unión del injerto (Soumelidou, 1994; Atkinson et al., 2003), causado por la unión imperfecta entre el patrón y el vástago. Otros autores lo achacan a descompensaciones de la vástago/portainjerto respecto a conductancia hidráulica de la hoja (Solari et al., 2006) o a la arquitectura hidráulica de la raíz (Nardini et al., 2006; Gascó et al., 2007). Tombesi et al., (2010) observó que una mayor conductancia del xilema del portainjerto se correlaciona con un mayor vigor, determinando que la restricción del xilema en la raíz, el tallo o el vástago puede resultar en un fenotipo enanizante. Por tanto, las relaciones con el agua pueden desempeñar un papel importante en el mecanismo de reducción de vigor inducido por los portainjertos (Beakbane and Thompson, 1939; Basile et al., 2003), y no así la nutrición mineral (Beakbane, 1956).

Portainjertos para el control de la verticilosis

Los portainjertos se seleccionan también por sus rasgos de resistencia a plagas y patógenos transmitidos por el suelo (Louws et al., 2010). Esto permite evitar la aplicación de productos químicos para la gestión o tratamiento de enfermedades. Se ha

demostrado el uso de portainjertos para solventar problemas fitosanitarios. En la lucha contra *V. dahliae* se han seleccionado portainjertos resistentes para diferentes cultivos de especies vegetales como la sandía (Attavar et al., 2020; Devi et al., 2021), el tomate (Papadaki et al., 2017), el algodón (Zhang et al., 2012) y especies leñosas como el aguacate (Haberman et al., 2020) y el pistacho (Epstein et al., 2004). En el olivo se han realizado estudios de susceptibilidad a *Verticillium* usando portainjertos como medida de control de la enfermedad, retrasando la aparición de síntomas y, por ende, el desarrollo de la enfermedad (Hartmann et al., 1971; Porras Soriano et al., 2003; Bubici and Cirulli, 2012). A pesar de los esfuerzos realizados, no se ha seleccionado ningún portainjerto que controle la enfermedad y que impida que el hongo colonice el vástago comercial, generalmente susceptible.

En esta tesis se propone la caracterización de una colección de germoplasma de olivo para su uso en la mejora del olivar. Esta mejora puede llevarse a cabo mediante: i) el trasvase de genes desde genotipos silvestres a cultivares de calidad contrastada mediante cruces sexuales y selección de la descendencia; ii) el uso directo de los genotipos silvestres como portainjertos de cultivares de interés comercial. Nos centraremos principalmente en el fenotipado de los genotipos silvestres de cara a su posible uso como portainjertos para controlar el vigor y la susceptibilidad a verticilosis del cultivar injertado. La mejora simultánea del vigor, resistencia a enfermedades y a las condiciones ambientales adversas debe realizarse a través de la exploración de la máxima variabilidad genética de la especie, que en el caso del olivo incluye necesariamente a las seis subespecies conocidas de *Olea europaea* (Médail et al., 2001; Green, 2002): *europaea, cuspidata, guanchica, cerasiformis, maroccana* y *laperrinei*.

Bibliografía

Abousalim, A., Brhadda, N., and Walali, L. (2005). Essais de prolifération et d'enracinement de matériel issu de rajeunissement par bouturage d'oliviers adultes (*Olea europaea* L.) et de germination *in vitro*: effets de cytokinine et d'auxines. *Biotechnol Agron Soc Env.* 9, 237–240.

Alcántara, E., Cordeiro, A. M., and Barranco, D. (2003). Selection of olive varieties for tolerance to iron chlorosis. *J. Plant Physiol.* 160, 1467–1472. doi: 10.1078/0176-1617-01013.

Aloni, B., Cohen, R., Karni, L., Aktas, H., and Edelstein, M. (2010). Hormonal signaling in rootstock–scion interactions. *Sci. Hortic.* 127, 119–126. doi: 10.1016/j.scienta.2010.09.003.

Angiolillo, A., Mencuccini, M., and Baldoni, L. (1999). Olive genetic diversity assessed using amplified fragment length polymorphisms: *Theor. Appl. Genet.* 98, 411–421. doi: 10.1007/s001220051087.

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and Rodríguez-Jurado, D. (2015a). Resistance to Verticillium wilt in olive progenies from open-pollination. *Sci. Hortic.* 185, 34–42. doi: 10.1016/j.scienta.2015.01.015.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and León, L. (2015b). Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. *Euphytica* 206, 619–629. doi: 10.1007/s10681-015-1463-7.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015c). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi: 10.1016/j.cropro.2015.05.006.

Arsel, H., and Cirik, N. (1994). A general overview of olive breeding in Turkey. *Olivae* 52, 25–27.

Atienza, S. G., de la Rosa, R., Domínguez-García, M. C., Martín, A., Kilian, A., and Belaj, A. (2013). Use of DArT markers as a means of better management of the diversity of olive cultivars. *Food Res. Int.* 54, 2045–2053. doi: 10.1016/j.foodres.2013.08.015.

Atkinson, C. J. (2002). Using Rootstocks to Optimize Fruit Tree Water Use. 35, 7.

Atkinson, C. J., Else, M. A., Taylor, L., and Dover, C. J. (2003). Root and stem hydraulic conductivity as determinants of growth potential in grafted trees of apple (*Malus pumila* Mill.). *J. Exp. Bot.* 54, 1221–1229. doi: 10.1093/jxb/erg132.

Attavar, A., Tymon, L., Perkins-Veazie, P., and Miles, C. A. (2020). Cucurbitaceae Germplasm Resistance to Verticillium Wilt and Grafting Compatibility with Watermelon. *Hortscience* 55 (2), 141–148. doi: 10.21273/hortsci14631-19.

Baali-Cherif, D., and Besnard, G. (2005). High Genetic Diversity and Clonal Growth in Relict Populations of *Olea europaea* subsp. *laperrinei* (Oleaceae) from Hoggar, Algeria. *Ann. Bot.* 96, 823–830. doi: 10.1093/aob/mci232.

Baccouri, B., Guerfel, M., Zarrouk, W., Taamalli, W., Daoud, D., and Zarrouk, M. (2011). Wild olive (*Olea europaea* L.) selection for quality oil production. *J. Food Biochem.* 35, 161–176. doi: 10.1111/j.1745-4514.2010.00373.x.

Báidez, A. G., Gómez, P., Del Río, J. A., and Ortuño, A. (2007). Dysfunctionality of the Xylem in *Olea europaea* L. Plants Associated with the Infection Process by *Verticillium dahliae* Kleb. Role of Phenolic Compounds in Plant Defense Mechanism. *J. Agric. Food Chem.* 55, 3373–3377. doi: 10.1021/jf063166d.

Baldoni, L., Cultrera, N. G., Mariotti, R., Ricciolini, C., Arcioni, S., Vendramin, G. G., et al. (2009). A consensus list of microsatellite markers for olive genotyping. *Mol. Breed.* 24, 213–231. doi: 10.1007/s11032-009-9285-8.

Baldoni, L., and Fontanazza, G. (1990). Preliminary results on olive clonal rootstocks behaviour in the field. *Acta Hortic.*, 37–40. doi: 10.17660/ActaHortic.1990.286.2.

Baldoni, L., Tosti, N., Ricciolini, C., Belaj, A., Arcioni, S., Pannelli, G., et al. (2006). Genetic Structure of Wild and Cultivated Olives in the Central Mediterranean Basin. *Ann. Bot.* 98, 935–942. doi: 10.1093/aob/mcl178.

Barranco, D. (2000). *World Catalogue of Olive Varieties*. International Olive Oil Council Available at: https://books.google.es/books?id=Y7gZywAACAAJ.

Barranco, D., Fernandez-Escobar, R., and Rallo, L. (1998). *El cultivo del olivo*. Sevilla: Junta de Andalucia.

Barranco, D., and Rallo, L. (2000). Olive Cultivars in Spain. *HortTechnology* 10, 107–110. doi: 10.21273/HORTTECH.10.1.107.

Barranco, D., Trujillo, I., and Rallo, P. (2000). Are `Oblonga' and `Frantoio' Olives the Same Cultivar? *HortScience* 35, 1323–1325. doi: 10.21273/HORTSCI.35.7.1323.

Bashir, M. A., Silvestri, C., Coppa, E., Brunori, E., Cristofori, V., Rugini, E., et al. (2021). Response of Olive Shoots to Salinity Stress Suggests the Involvement of Sulfur Metabolism. *Plants* 10, 350. doi: 10.3390/plants10020350.

Basile, B., Marsal, J., and DeJong, T. M. (2003). Daily shoot extension growth of peach trees growing on rootstocks that reduce scion growth is related to daily dynamics of stem water potential. *Tree Physiol.* 23, 695–704. doi: 10.1093/treephys/23.10.695.

Beakbane, A. B. (1956). Possible mechanisms of rootstock effect. *Ann. Appl. Biol.* 44, 517–521. doi: 10.1111/j.1744-7348.1956.tb02147.x.

Beakbane, A. B., and Thompson, E. C. (1939). Anatomical Studies of Stems and Roots of Hardy Fruit Trees II. The Internal Structure of the Roots of Some Vigorous and Some Dwarfing Apple Rootstocks, and the Correlation of Structure with Vigour. *J. Pomol. Hortic. Sci.* 17, 141–149. doi: 10.1080/03683621.1940.11513535.

Bejarano-Alcázar, J., Blanco-López, M. A., Melero-Vara, J. M., and Jiménez-Díaz, R. M. (1996). Etiology, Importance, and Distribution of Verticillium Wilt of Cotton in Southern Spain. *Plant Dis.* 80, 1233–1238. doi: 10.1094/PD-80-1233.

Belaj, A., Gurbuz Veral, M., Sikaoui, H., Moukhli, A., Khadari, B., Mariotti, R., et al. (2016). "Olive Genetic Resources," in *The Olive Tree Genome* Compendium of Plant Genomes., eds. E. Rugini, L. Baldoni, R. Muleo, and L. Sebastiani (Cham: Springer International Publishing), 27–54. doi: 10.1007/978-3-319-48887-5_3.

Belaj, A., León, L., Satovic, Z., and de la Rosa, R. (2011). Variability of wild olives (*Olea europaea* subsp. *europaea* var. *sylvestris*) analyzed by agro-morphological traits and SSR markers. *Sci. Hortic.* 129, 561–569. doi: 10.1016/j.scienta.2011.04.025.

Belaj, A., Muñoz-Diez, C., Baldoni, L., Satovic, Z., and Barranco, D. (2010). Genetic diversity and relationships of wild and cultivated olives at regional level in Spain. *Sci. Hortic.* 124, 323–330. doi: 10.1016/j.scienta.2010.01.010.

Belaj, A., Satovic, Z., Cipriani, G., Baldoni, L., Testolin, R., Rallo, L., et al. (2003). Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. *Theor. Appl. Genet.* 107, 736–744. doi: 10.1007/s00122-003-1301-5.

Belaj, A., Satovic, Z., Rallo, L., and Trujillo, I. (2002). Genetic diversity and relationships in olive (*Olea europaea* L.) germplasm collections as determined by randomly amplified polymorphic DNA. *Theor. Appl. Genet.* 105, 638–644. doi: 10.1007/s00122-002-0981-6.

Belaj, A., Satovic, Z., Trujillo, I., and Rallo, L. (2004). Genetic Relationships of Spanish Olive Cultivars Using RAPD Markers. *HortScience* 39, 948–951. doi: 10.21273/HORTSCI.39.5.948.

Belaj, A., Trujillo, I., de la Rosa, R., Rallo, L., and Giménez, M. J. (2001). Polymorphism and Discrimination Capacity of Randomly Amplified Polymorphic Markers in an Olive Germplasm Bank. *J. Am. Soc. Hortic. Sci.* 126, 64–71. doi: 10.21273/JASHS.126.1.64.

Bellini, E., Giordani, E., Parlati, M. V., and Pandolfi, S. (2002). Olive genetic improvement: thirty years of research. *Acta Hortic.*, 105–108. doi: 10.17660/ActaHortic.2002.586.13.

Benelli, C., and De Carlo, A. (2018). *In vitro* multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™). *3 Biotech* 8, 317. doi: 10.1007/s13205-018-1346-4.

Ben-Gal, A. (2011). Review: Salinity and olive: From physiological responses to orchard management. *Isr. J. Plant Sci.* 59, 15–28. doi: 10.1560/IJPS.59.1.15.

Berlanger, I., and Powelson, M. L. (2000). Verticillium wilt. *Plant Health Instr.* doi: 10.1094/PHI-I-2000-0801-01.

Besnard, G. (2016). "Origin and Domestication," in *The Olive Tree Genome* Compendium of Plant Genomes., eds. E. Rugini, L. Baldoni, R. Muleo, and L. Sebastiani (Cham: Springer International Publishing), 1–12. doi: 10.1007/978-3-319-48887-5_1.

Besnard, G., Anthelme, F., and Baali-Cherif, D. (2012). The Laperrine's olive tree (Oleaceae): a wild genetic resource of the cultivated olive and a model-species for studying the biogeography of the Saharan Mountains. *Acta Bot. Gallica* 159, 319–328. doi: 10.1080/12538078.2012.724281.

Besnard, G., and Baali-Cherif, D. (2009). Coexistence of diploids and triploids in a Saharan relict olive: Evidence from nuclear microsatellite and flow cytometry analyses. *C. R. Biol.* 332, 1115–1120. doi: 10.1016/j.crvi.2009.09.014.

Besnard, G., Baradat, P., Breton, C., Khadari, B., and Bervillé, A. (2001). Olive domestication from structure of oleasters and cultivars using nuclear RAPDs and mitochondrial RFLPs. *Genet. Sel. Evol.* 33, S251. doi: 10.1186/BF03500883.

Besnard, G., and Bervillé, A. (2000). Multiple origins for Mediterranean olive (*Olea europaea* L. ssp. *europaea*) based upon mitochondrial DNA polymorphisms. *Comptes Rendus Académie Sci. - Ser. III - Sci. Vie* 323, 173–181. doi: 10.1016/S0764-4469(00)00118-9.

Besnard, G., and Bervillé, A. (2002). On chloroplast DNA variations in the olive (*Olea europaea* L.) complex: comparison of RFLP and PCR polymorphisms. *Theor. Appl. Genet.* 104, 1157–1163. doi: 10.1007/s00122-001-0834-8.

Besnard, G., El Bakkali, A., Haouane, H., Baali-Cherif, D., Moukhli, A., and Khadari, B. (2013a). Population genetics of Mediterranean and Saharan olives: geographic patterns of differentiation and evidence for early generations of admixture. *Ann. Bot.* 112, 1293–1302. doi: 10.1093/aob/mct196.

Besnard, G., Garcia-Verdugo, C., Rubio De Casas, R., Treier, U. A., Galland, N., and Vargas, P. (2008). Polyploidy in the Olive Complex (*Olea europaea*): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses. *Ann. Bot.* 101, 25–30. doi: 10.1093/aob/mcm275.

Besnard, G., Gorrilliot, O., Raimondeau, P., Génot, B., El Bakkali, A., Anthelme, F., et al. (2021). Contrasting Genetic Footprints among Saharan Olive Populations: Potential Causes and Conservation Implications. *Plants* 10, 1207. doi: 10.3390/plants10061207.

Besnard, G., Khadari, B., Baradat, P., and Bervillé, A. (2002). *Olea europaea* (Oleaceae) phylogeography based on chloroplast DNA polymorphism. *Theor. Appl. Genet.* 104, 1353–1361. doi: 10.1007/s00122-001-0832-x.

Besnard, G., Khadari, B., Navascués, M., Fernández-Mazuecos, M., El Bakkali, A., Arrigo, N., et al. (2013b). The complex history of the olive tree: from Late Quaternary diversification of Mediterranean

lineages to primary domestication in the northern Levant. *Proc. R. Soc. B Biol. Sci.* 280, 20122833. doi: 10.1098/rspb.2012.2833.

Besnard, G., Rubio de Casas, R., Christin, P.-A., and Vargas, P. (2009). Phylogenetics of *Olea* (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: Tertiary climatic shifts and lineage differentiation times. *Ann. Bot.* 104, 143–160. doi: 10.1093/aob/mcp105.

Besnard, G., Rubio de Casas, R., and Vargas, P. (2007). Plastid and nuclear DNA polymorphism reveals historical processes of isolation and reticulation in the olive tree complex (*Olea europaea*). *J. Biogeogr.* 34, 736–752. doi: 10.1111/j.1365-2699.2006.01653.x.

Besnard, G., Terral, J.-F., and Cornille, A. (2018). On the origins and domestication of the olive: a review and perspectives. *Ann. Bot.* 121, 385–403. doi: 10.1093/aob/mcx145.

Biton, I., Shevtsov, S., Ostersetzer, O., Mani, Y., Lavee, S., Avidan, B., et al. (2012). Genetic relationships and hybrid vigour in olive (*Olea europaea* L.) by microsatellites. *Plant Breed.* 131, 767–774. doi: 10.1111/pbr.12001.

Breton, C., Pinatel, C., Médail, F., Bonhomme, F., and Bervillé, A. (2008). Comparison between classical and Bayesian methods to investigate the history of olive cultivars using SSR-polymorphisms. *Plant Sci.* 175, 524–532. doi: 10.1016/j.plantsci.2008.05.025.

Breton, C., Terral, J.-F., Pinatel, C., Médail, F., Bonhomme, F., and Bervillé, A. (2009). The origins of the domestication of the olive tree. *C. R. Biol.* 332, 1059–1064. doi: 10.1016/j.crvi.2009.08.001.

Breton, C., Tersac, M., and Bervillé, A. (2006). Genetic diversity and gene flow between the wild olive (oleaster, *Olea europaea* L.) and the olive: several Plio-Pleistocene refuge zones in the Mediterranean basin suggested by simple sequence repeats analysis. *J. Biogeogr.* 33, 1916–1928. doi: 10.1111/j.1365-2699.2006.01544.x.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9.

Bubici, G., and Cirulli, M. (2014). Natural recovery from Verticillium wilt in olive: can it be exploited in a control strategy? *Plant Soil* 381, 85–94. doi: 10.1007/s11104-014-2112-y.

Camposeo, S., Vivaldi, G. A., Montemurro, C., Fanelli, V., and Cunill Canal, M. (2021). Lecciana, a New Low-Vigour Olive Cultivar Suitable for Super High Density Orchards and for Nutraceutical EVOO Production. *Agronomy* 11, 2154. doi: 10.3390/agronomy11112154.

Cañas, L. A., and Benbadis, A. (1988). *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L). *Plant Sci.* 54, 65–74. doi: 10.1016/0168-9452(88)90056-8.

Cantos, M., Troncoso, J., Liñán, J., Troncoso, A., and Rapaport, H. (2002). Obtaining salt (NaCl) tolerant olive plants: i) some physiological and anatomical characteristics of olive plants growing in harsh saline zones. *Acta Hortic.*, 441–444. doi: 10.17660/ActaHortic.2002.586.90.

Castañeda-Álvarez, N. P., Khoury, C. K., Achicanoy, H. A., Bernau, V., Dempewolf, H., Eastwood, R. J., et al. (2016). Global conservation priorities for crop wild relatives. *Nat. Plants* 2, 16022. doi: 10.1038/nplants.2016.22.

Chaari, A., Chelly Chaabouni, A., Maalej, M., and Drira, N. (2002). Meski olive variety propagated by tissue culture. *Acta Hortic.*, 871–874. doi: 10.17660/ActaHortic.2002.586.189.

Chiappetta, A., Muto, A., Muzzalupo, R., and Muzzalupo, I. (2017). New rapid procedure for genetic characterization of Italian wild olive (*Olea europaea*) and traceability of virgin olive oils by means of SSR markers. *Sci. Hortic.* 226, 42–49. doi: 10.1016/j.scienta.2017.08.022.

Cohen, S., and Naor, A. (2002). The effect of three rootstocks on water use, canopy conductance and hydraulic parameters of apple trees and predicting canopy from hydraulic conductance: *Apple rootstocks influence canopy and hydraulic conductance*. *Plant Cell Environ.* 25, 17–28. doi: 10.1046/j.1365-3040.2002.00795.x.

Cohen, S., Naor, A., Bennink, J., Grava, A., and Tyree, M. (2007). Hydraulic resistance components of mature apple trees on rootstocks of different vigours. *J. Exp. Bot.* 58, 4213–4224. doi: 10.1093/jxb/erm281.

Colella, C., Miacola, C., Amenduni, M., D'Amico, M., Bubici, G., and Cirulli, M. (2008). Sources of verticillium wilt resistance in wild olive germplasm from the Mediterranean region. *Plant Pathol.* 57, 533–539. doi: 10.1111/j.1365-3059.2007.01785.x.

Connor, D. J., and Fereres, E. (2010). "The Physiology of Adaptation and Yield Expression in Olive," in *Horticultural Reviews*, ed. J. Janick (Oxford, UK: John Wiley & Sons, Inc.), 155–229. doi: 10.1002/9780470650882.ch4.

Connor, D. J., Gómez-del-Campo, M., Rousseaux, M. C., and Searles, P. S. (2014). Structure, management and productivity of hedgerow olive orchards: A review. *Sci. Hortic.* 169, 71–93. doi: 10.1016/j.scienta.2014.02.010.

Corrado, G., Garonna, A., Cabanás, C. G.-L., Gregoriou, M., Martelli, G. P., Mathiopoulos, K. D., et al. (2016). "Host Response to Biotic Stresses," in *The Olive Tree Genome* Compendium of Plant Genomes., eds. E. Rugini, L. Baldoni, R. Muleo, and L. Sebastiani (Cham: Springer International Publishing), 75–98. doi: 10.1007/978-3-319-48887-5_6.

Corrado, G., La Mura, M., Ambrosino, O., Pugliano, G., Varricchio, P., and Rao, R. (2009). Relationships of Campanian olive cultivars: comparative analysis of molecular and phenotypic data. *Genome* 52, 692–700. doi: 10.1139/G09-044.

Cruz, F., Julca, I., Gómez-Garrido, J., Loska, D., Marcet-Houben, M., Cano, E., et al. (2016). Genome sequence of the olive tree, *Olea europaea*. *GigaScience* 5, 29. doi: 10.1186/s13742-016-0134-5.

De la Rosa, R., Kiran, A. I., Barranco, D., and León, L. (2006). Seedling vigour as a preselection criterion for short juvenile period in olive breeding. *Aust. J. Agric. Res.* 57, 477. doi: 10.1071/AR05219.

De la Rosa, R., and León, L. (2009). Nuevas variedades y perspectivas futuras de la mejora vegetal del olivo. *Vida Rural*, 41–44.

DeJong, T. M., and Doyle, J. F. (1985). The effect of row orientation on light distribution in hedgerow peach tree canopies. *Acta Hortic.*, 159–166. doi: 10.17660/ActaHortic.1985.173.18.

Devi, P., Tymon, L., Keinath, A., and Miles, C. A. (2021). Progress in grafting watermelon to manage Verticillium wilt. *Plant Pathol.* 70, 767–777.

Deyett, E., Pouzoulet, J., Yang, J. -I., Ashworth, V. E., Castro, C., Roper, M. C., et al. (2019). Assessment of Pierce's disease susceptibility in *Vitis vinifera* cultivars with different pedigrees. *Plant Pathol.* 68, 1079–1087. doi: 10.1111/ppa.13027.

Díez, C. M., Moral, J., Cabello, D., Morello, P., Rallo, L., and Barranco, D. (2016). Cultivar and Tree Density As Key Factors in the Long-Term Performance of Super High-Density Olive Orchards. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01226.

Diez, C. M., Trujillo, I., Martinez-Urdiroz, N., Barranco, D., Rallo, L., Marfil, P., et al. (2015). Olive domestication and diversification in the Mediterranean Basin. *New Phytol.* 206, 436–447. doi: 10.1111/nph.13181.

Douady, C. J., Catzeflis, F., Raman, J., Springer, M. S., and Stanhope, M. J. (2003). The Sahara as a vicariant agent, and the role of Miocene climatic events, in the diversification of the mammalian order Macroscelidea (elephant shrews). *Proc. Natl. Acad. Sci.* 100, 8325–8330. doi: 10.1073/pnas.0832467100.

El Bakkali, A., Haouane, H., Hadiddou, A., Oukabli, A., Santoni, S., Udupa, S. M., et al. (2013). Genetic diversity of on-farm selected olive trees in Moroccan traditional olive orchards. *Plant Genet. Resour.* 11, 97–105. doi: 10.1017/S1479262112000445.

El Riachy, M., Priego-Capote, F., León, L., Luque de Castro, M. D., and Rallo, L. (2012). Virgin olive oil phenolic profile and variability in progenies from olive crosses. *J. Sci. Food Agric.* 92, 2524–2533. doi: 10.1002/jsfa.5662.

Elgadi, S., Ouhammou, A., Zine, H., Maata, N., Ait Babahmad, R., and El Antari, A. (2021). Comparative Oil Composition Study of the Endemic Moroccan Olive (*Olea europaea* subsp. *maroccana*) and Wild Olive (var. *Sylvestris*) in Central West Morocco. *J. Food Qual.* 2021, 1–10. doi: 10.1155/2021/8869060.

Ennos, R. A. (1994). Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* 72, 250–259. doi: 10.1038/hdy.1994.35.

Epstein, L., Beede, R., Kaur, S., and Ferguson, L. (2004). Rootstock Effects on Pistachio Trees Grown in *Verticillium dahliae*-Infested Soil. *Am. Phytopathol. Soc.* 94, 388–395.

Espínola, F., Vidal, A. M., Espínola, J. M., and Moya, M. (2021). Processing Effect and Characterization of Olive Oils from Spanish Wild Olive Trees (*Olea europaea* var. *sylvestris*). *Molecules* 26, 1304. doi: 10.3390/molecules26051304.

Falcón-Rodríguez, A. B., Wégria, G., and Cabrera, J.-C. (2012). "Exploiting Plant Innate Immunity to Protect Crops Against Biotic Stress: Chitosaccharides as Natural and Suitable Candidates for this Purpose," in *New Perspectives in Plant Protection*, ed. A. R. Bandani (InTech). doi: 10.5772/36777.

Fallahi, E., Moon, J. W., and Rodney, D. R. (1989). Yield and quality of redblush grapefruit on 12 rootstocks. *J. Am. Soc. Hortic. Sci.* 114, 187–190.

Farahani, F., Peyvandi, M., and Hosseini-Mazinani, M. (2008). Effect os sucrose and mannitol on *in vitro* regeneration of Iranian olive cv. "rowghani." *Acta Hortic.*, 203–208. doi: 10.17660/ActaHortic.2008.791.28.

Fernández, J. E., Navarro, N., Cuevas, M. V., Hernández–Santana, V., and Alcón, F. (2021). Impacto productivo, ambiental y social del olivar superintensivo frente al tradicional. *Fruticultura* 79.

Fernández, J.-E. (2014). Understanding olive adaptation to abiotic stresses as a tool to increase crop performance. *Environ. Exp. Bot.* 103, 158–179. doi: 10.1016/j.envexpbot.2013.12.003.

Fiorino, P., and Leva, A. (1986). Investigations on the micropropagation of the olive (*Olea europaea* L.) and influence of some mineral elements on the proliferation and rooting of explants. *Olea* 17, 101–104.

Fontanazza, G., Bartolozzi, F., and Vergari, G. (1998). Olivo 'Fs-17'. *Riv Fruttic. Ortofloric.* 60.

Fradin, E. F., and Thomma, B. P. H. J. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Mol. Plant Pathol.* 7, 71–86. doi: 10.1111/j.1364-3703.2006.00323.x.

Fraga, H., Moriondo, M., Leolini, L., and Santos, J. A. (2020). Mediterranean Olive Orchards under Climate Change: A Review of Future Impacts and Adaptation Strategies. *Agronomy* 11, 56. doi: 10.3390/agronomy11010056.

García-Cabello, S., Pérez-Rodríguez, M., Blanco-López, M. A., and López-Escudero, F. J. (2012). Distribution of *Verticillium dahliae* through watering systems in widely irrigated olive growing areas in Andalucia (southern Spain). *Eur. J. Plant Pathol.* 133, 877–885. doi: 10.1007/s10658-012-0011-8.

García-Férriz, L., Ghorbel, R., Ybarra, M., Marí, A., Belaj, A., and Trujillo, I. (2003). Cultivo *in vitro* y comportamiento en campo de olivo adulto. *Horticultura* 172, 64–66.

García-Ruíz, G. M., Trapero, C., Del Río, C., and López-Escudero, F. J. (2014). Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. *Phytoparasitica* 42, 205–212. doi: 10.1007/s12600-013-0353-6.

García-Verdugo, C., Fay, M. F., Granado-Yela, C., De Casas, R. R., Balaguer, L., Besnard, G., et al. (2009). Genetic diversity and differentiation processes in the ploidy series of *Olea europaea* L.: a multiscale approach from subspecies to insular populations. *Mol. Ecol.* 18, 454–467. doi: 10.1111/j.1365-294X.2008.04027.x.

García-Verdugo, C., Forrest, A. D., Fay, M. F., and Vargas, P. (2010). The relevance of gene flow in metapopulation dynamics of an oceanic island endemic, *Olea europaea* subsp. *guanchica*: genetic structure of an *Olea europaea* metapopulation. *Evolution* 64, 3525–3536. doi: 10.1111/j.1558-5646.2010.01091.x.

Gascó, A., Nardini, A., Raimondo, F., Gortan, E., Motisi, A., Lo Gullo, M. A., et al. (2007). Hydraulic kinetics of the graft union in different *Olea europaea* L. scion/rootstock combinations. *Environ. Exp. Bot.* 60, 245–250. doi: 10.1016/j.envexpbot.2006.10.011.

Genner, M. J., and Haesler, M. P. (2010). Pliocene isolation of a north-west Saharan cichlid fish. *J. Fish Biol.* 76, 435–441. doi: 10.1111/j.1095-8649.2009.02549.x.

Gharbi, Y., Barkallah, M., Bouazizi, E., Cheffi, M., Gdoura, R., and Triki, M. A. (2016). Differential fungal colonization and physiological defense responses of new olive cultivars infected by the necrotrophic fungus *Verticillium dahliae*. *Acta Physiol. Plant.* 38, 242. doi: 10.1007/s11738-016-2261-0.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017). Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 97, 30–39. doi: 10.1016/j.pmpp.2016.12.001.

Goldschmidt, E. E. (2014). Plant grafting: new mechanisms, evolutionary implications. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00727.

Gómez, J. A., Giráldez, J. V., and Fereres, E. (2001). Rainfall interception by olive trees in relation to leaf area. *Agric. Water Manag.* 49, 65–76. doi: 10.1016/S0378-3774(00)00116-5.

Gómez-Gálvez, F.-J., and Rodríguez-Jurado, D. (2018). Potential efficacy of soil-applied disinfectant treatments against Verticillium wilt of olive. *Crop Prot.* 106, 190–200. doi: 10.1016/j.cropro.2018.01.002.

Gómez-Gálvez, F.-J., Vargas-Osuna, E., and Rodríguez-Jurado, D. (2018). Suppressive and preventive activity of chemical disinfectants against sclerotia of *Verticillium dahliae* in water. *Crop Prot.* 108, 12–22. doi: 10.1016/j.cropro.2018.02.011.

Gómez-Lama Cabanás, C., Legarda, G., Ruano-Rosa, D., Pizarro-Tobías, P., Valverde-Corredor, A., Niqui, J. L., et al. (2018). Indigenous *Pseudomonas* spp. Strains from the Olive (*Olea europaea* L.) Rhizosphere as Effective Biocontrol Agents against *Verticillium dahliae*: From the Host Roots to the Bacterial Genomes. *Front. Microbiol.* 9, 277. doi: 10.3389/fmicb.2018.00277.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2014). The biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. *Front. Microbiol.* 5. doi: 10.3389/fmicb.2014.00427.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2015). Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00928.

Gómez-Rico, A., Salvador, M. D., Moriana, A., Pérez, D., Olmedilla, N., Ribas, F., et al. (2007). Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. *Food Chem.* 100, 568–578. doi: 10.1016/j.foodchem.2005.09.075.

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A Comparison of Real-Time PCR Protocols for the Quantitative Monitoring of Asymptomatic Olive Infections by *Verticillium dahliae* Pathotypes. *Phytopathology®* 103, 1058–1068. doi: 10.1094/PHYTO-11-12-0312-R.

Green, P. (2002). *A revision of* Olea *L. (Oleaceae). Kew Bull. 57, 91–140.* Kew Bull.

Gregory, P. J., Atkinson, C. J., Bengough, A. G., Else, M. A., Fernández-Fernández, F., Harrison, R. J., et al. (2013). Contributions of roots and rootstocks to sustainable, intensified crop production. *J. Exp. Bot.* 64, 1209–1222. doi: 10.1093/jxb/ers385.

Gucci, R., Caruso, G., Canale, A., Loni, A., Raspi, A., Urbani, S., et al. (2012). Qualitative Changes of Olive Oils Obtained from Fruits Damaged by *Bactrocera oleae* (Rossi). *HortScience* 47, 301–306. doi: 10.21273/HORTSCI.47.2.301.

Guerin, J., Mekuria, G., Burr, M., Collins, G., and Sedgley, M. (2003). Selection of improved olive cultivars. *Acta Hortic.*, 231–234. doi: 10.17660/ActaHortic.2003.622.20.

Haberman, A., Tsror (Lahkim), L., Lazare, S., Hazanovsky, M., Lebiush, S., Zipori, I., et al. (2020). Management of Verticillium Wilt of Avocado Using Tolerant Rootstocks. *Plants* 9, 531. doi: 10.3390/plants9040531.

Haddad, B., Carra, A., Saadi, A., Haddad, N., Mercati, F., Gristina, A. S., et al. (2018). *In vitro* propagation of the relict laperinne's olive (*Olea europaea* L. subsp. *Laperrinei*). *Plant Biosyst. - Int. J. Deal. Asp. Plant Biol.* 152, 621–630. doi: 10.1080/11263504.2017.1306002.

Hammami, S. B. M., León, L., Rapoport, H. F., and De la Rosa, R. (2011). Early growth habit and vigour parameters in olive seedlings. *Sci. Hortic.* 129, 761–768. doi: 10.1016/j.scienta.2011.05.038.

Hannachi, H., Breton, C., Msallem, M., Ben El Hadj, S., El Gazzah, M., and Bervillé, A. (2008). Differences between native and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: A case study in Tunisia. *Sci. Hortic.* 116, 280–290. doi: 10.1016/j.scienta.2008.01.004.

Hannachi, H., Sommerlatte, H., Breton, C., Msallem, M., El Gazzah, M., Ben El Hadj, S., et al. (2009). Oleaster (var. *sylvestris*) and subsp. *cuspidata* are suitable genetic resources for improvement of the olive (*Olea europaea* subsp. *europaea* var. *europaea*). *Genet. Resour. Crop Evol.* 56, 393–403. doi: 10.1007/s10722-008-9374-2.

Hartmann, Schnathorst, W.C, and Whisler, J.E. (1971). Oblonga, a clonal olive rootstock resistant to verticillium wilt. *Calif. Agric.* 25, 12–15.

Hegazi, E. S., Hegazi, A. A., and Allatif, A. M. A. (2012). Resistance of some olive cultivars to verticillium wilt. *J. Appl. Sci. Res.*, 2758–2765.

Heinicke, D. R. (1975). High Density Apple Orchards-Planning, Training and Pruning. *Wash. DC Handb. 4 US Dep. Agric.*

Hernández-Santana, V., Díaz-Rueda, P., Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., et al. (2019). Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress. *Front. Plant Sci.* 10, 291. doi: 10.3389/fpls.2019.00291.

Hess, J., Kadereit, J. W., and Vargas, P. (2000). The colonization history of *Olea europaea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and intersimple sequence repeats (ISSR). *Mol. Ecol.* 9, 857–868. doi: 10.1046/j.1365-294x.2000.00942.x.

Hoenecke, M. E., Bula, R. J., and Tibbitts, T. W. (1992). Importance of "blue" photon levels for lettuce seedlings grown under red-light-emitting diodes. *HortScience Publ. Am. Soc. Hortic. Sci.* 27, 427–430.

Inch, S. A., and Ploetz, R. C. (2012). Impact of laurel wilt, caused by *Raffaelea lauricola*, on xylem function in avocado, *Persea americana*: Impact of laurel wilt on avocado. *For. Pathol.* 42, 239–245. doi: 10.1111/j.1439-0329.2011.00749.x.

IOC (2021). World olive oil and table olive figures. In-ternational Olive Council. https://www.internationaloliveoil.org/what-we-do/economic-affairs-promotion-unit/#figures.

Jackson, J. E., and Palmer, J. W. (1980). A computer model study of light interception by orchards in relation to mechanised harvesting and management. *Sci. Hortic.* 13, 1–7. doi: 10.1016/0304-4238(80)90015-1.

Jayawickrama, K. J. S., Jett, J. B., and McKeand, S. E. (1991). Rootstock effects in grafted conifers: A review. *New For.* 5, 157–173. doi: 10.1007/BF00029306.

Jiménez-Díaz, R., Cirulli, M, Bubici, G, Jiménez-Gasco, MD, Antoniou, PP, and Tjamos, EC (2012). Verticillium Wilt, A Major Threat to Olive Production: Current Status and Future Prospects for its Management. *Plant Dis.* 96, 304–329. doi: 10.1094/PDIS-06-11-0496.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516.

Jin, Z.-X., Sun, T.-Y., Sun, H., Yue, Q.-Y., and Yao, Y.-X. (2016). Modifications of 'Summer Black' grape berry quality as affected by the different rootstocks. *Sci. Hortic.* 210, 130–137. doi: 10.1016/j.scienta.2016.07.023.

Kassa, A., Konrad, H., and Geburek, T. (2019). Molecular diversity and gene flow within and among different subspecies of the wild olive (*Olea europaea* L.): A review. *Flora* 250, 18–26. doi: 10.1016/j.flora.2018.11.014.

Kassout, J., Ater, M., Ivorra, S., Barbara, H., Limier, B., Ros, J., et al. (2021). Resisting Aridification: Adaptation of Sap Conduction Performance in Moroccan Wild Olive Subspecies Distributed Over an Aridity Gradient. *Front. Plant Sci.* 12, 663721. doi: 10.3389/fpls.2021.663721.

Keykhasaber, M., Thomma, B. P. H. J., and Hiemstra, J. A. (2018). Verticillium wilt caused by *Verticillium dahliae* in woody plants with emphasis on olive and shade trees. *Eur. J. Plant Pathol.* 150, 21–37. doi: 10.1007/s10658-017-1273-y.

Khadari, B., Breton, C., Moutier, N., Roger, J., Besnard, G., Bervillé, A., et al. (2003). The use of molecular markers for germplasm management in a French olive collection. *Theor. Appl. Genet.* 106, 521–529. doi: 10.1007/s00122-002-1079-x.

Khadari, B., Charafi, J., Moukhli, A., and Ater, M. (2008). Substantial genetic diversity in cultivated Moroccan olive despite a single major cultivar: a paradoxical situation evidenced by the use of SSR loci. *Tree Genet. Genomes* 4, 213–221. doi: 10.1007/s11295-007-0102-4.

Kirtman, B., Power, S. B., Adedoyin, J. A., Boer, G. J., Bojariu, R., Camilloni, I., et al. (2013). *IPCC, 2013: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)].* Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Klepo, T., De la Rosa, R., Satovic, Z., León, L., and Belaj, A. (2013). Utility of wild germplasm in olive breeding. *Sci. Hortic.* 152, 92–101. doi: 10.1016/j.scienta.2012.12.010.

Klepo, T., Toumi, A., De La Rosa, R., LeÓn, L., and Belaj, A. (2014). Agronomic evaluation of seedlings from crosses between the main Spanish olive cultivar 'Picual' and two wild olive trees. *J. Hortic. Sci. Biotechnol.* 89, 508–512. doi: 10.1080/14620316.2014.11513113.

Koepke, T., and Dhingra, A. (2013). Rootstock scion somatogenetic interactions in perennial composite plants. *Plant Cell Rep.* 32, 1321–1337. doi: 10.1007/s00299-013-1471-9.

Kole, C. ed. (2011). *Wild Crop Relatives: Genomic and Breeding Resources*. Berlin, Heidelberg: Springer Berlin Heidelberg doi: 10.1007/978-3-642-16057-8.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., and Rubel, F. (2006). World Map of the Köppen-Geiger climate classification updated. *Meteorol. Z.* 15, 259–263. doi: 10.1127/0941-2948/2006/0130.

Koubouris, G. C., Metzidakis, I. T., and Vasilakakis, M. D. (2009). Impact of temperature on olive (*Olea europaea* L.) pollen performance in relation to relative humidity and genotype. *Environ. Exp. Bot.* 67, 209–214. doi: 10.1016/j.envexpbot.2009.06.002.

Kozlowski, G., Gibbs, D., Huan, F., Frey, D., and Gratzfeld, J. (2012). Conservation of threatened relict trees through living *ex situ* collections: lessons from the global survey of the genus *Zelkova* (Ulmaceae). *Biodivers. Conserv.* 21, 671–685. doi: 10.1007/s10531-011-0207-9.

Kuijken, R. C. P., van Eeuwijk, Fred. A., Marcelis, L. F. M., and Bouwmeester, H. J. (2015). Root phenotyping: from component trait in the lab to breeding: Table 1. *J. Exp. Bot.* 66, 5389–5401. doi: 10.1093/jxb/erv239.

La Rue, C. D. (1934). Root grafting in trees. *Am. J. Bot.* 21, 121–126. doi: 10.1002/j.1537-2197.1934.tb04952.x.

Lavee, S. (1994). ¿Por qué la necesidad de nuevas variedades de olivos? *Fruticultura* 62, 29–37.

Lavee, S., Haskal, A., and Wodner, M. (1986). 'Barnea', a new olive cultivar from first breeding generation. *Olea* 17, 95–99.

Lavee, S., and Schachtel, J. (1999). Interaction of cultivar rootstock and availability on olive tree performance and fruit production. *Acta Hortic.*, 399–414. doi: 10.17660/ActaHortic.1999.474.83.

León, L., Beltrán, G., Aguilera, M. P., Rallo, L., Barranco, D., and De la Rosa, R. (2011). Oil composition of advanced selections from an olive breeding program. *Eur. J. Lipid Sci. Technol.* 113, 870–875. doi: 10.1002/ejlt.201000535.

León, L., de la Rosa, R., Barranco, D., and Rallo, L. (2007a). Breeding for Early Bearing in Olive. *HortScience* 42, 499–502. doi: 10.21273/HORTSCI.42.3.499.

León, L., De la Rosa, R., Rallo, L., Guerrero, N., and Barranco, D. (2007b). Influence of spacing on the initial production of hedgerow 'Arbequina' olive orchards. *Span. J. Agric. Res.* 5 (4), 554–558.

León, L., de la Rosa, R., Velasco, L., and Belaj, A. (2018). Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. *Front. Plant Sci.* 9, 232. doi: 10.3389/fpls.2018.00232.

León, L., Díaz-Rueda, P., Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. *Sci. Hortic.* 264, 109157. doi: 10.1016/j.scienta.2019.109157.

León, L., Velasco, L., and de la Rosa, R. (2015). Initial selection steps in olive breeding programs. *Euphytica* 201, 453–462. doi: 10.1007/s10681-014-1232-z.

Leva, A. (2011). Innovative protocol for "*ex vitro* rooting" on olive micropropagation. *Open Life Sci.* 6, 352–358. doi: 10.2478/s11535-011-0010-3.

Leva, A. R., Petruccelli, R., and Bartolini, G. (1994). Mannitol *"in vitro"* culture of *Olea europaea* L. (CV. Maurino). *Acta Hortic.*, 43–46. doi: 10.17660/ActaHortic.1994.356.7.

Leva, A. R., Petruccelli, R., Goretti, R., and Panicucci, M. (1992). Ruolo di alcuni microelementi e carboidrati nella proliferazione *in vitro* di cv. di olivo (*Olea europaea* L.). *Atti Congr. Inter "Olive Oil Qual. Firenze*, 333–334.

Levin, A. G., Lavee, S., and Tsror (Lahkim), L. (2003). Epidemiology of *Verticillium dahliae* on olive (cv. Picual) and its effect on yield under saline conditions. *Plant Pathol.* 52, 212–218. doi: 10.1046/j.1365-3059.2003.00809.x.

Leyva-Pérez, M. de la O., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J. B., Luque, F., et al. (2018). Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. *New Phytol.* 217, 671–686. doi: 10.1111/nph.14833.

Ligoxigakis, E. K., Vakalounakis, D. J., and Thanassoulopoulos, C. C. (2002). Weed hosts of *Verticillium dahliae* in crete: Susceptibility, symptomatology and significance. *Phytoparasitica* 30, 511–518. doi: 10.1007/BF02979756.

Lo Bianco, R., Proietti, P., Regni, L., and Caruso, T. (2021). Planting Systems for Modern Olive Growing: Strengths and Weaknesses. *Agriculture* 11, 494. doi: 10.3390/agriculture11060494.

López-Escudero, F. J., and Blanco-López, M. A. (2005). Recovery of Young Olive Trees from *Verticillium dahliae*. *Eur. J. Plant Pathol.* 113, 367–375. doi: 10.1007/s10658-005-3145-0.

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of Olive Cultivars for Resistance to *Verticillium dahliae*. *Eur. J. Plant Pathol.* 110, 79–85. doi: 10.1023/B:EJPP.0000010150.08098.2d.

López-Escudero, F. J., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50. doi: 10.1007/s11104-010-0629-2.

Loumou, A., and Giourga, C. (2003). Olive groves: "The life and identity of the Mediterranean." *Agric. Hum. Values* 20, 87–95. doi: 10.1023/A:1022444005336.

Louws, F. J., Rivard, C. L., and Kubota, C. (2010). Grafting fruiting vegetables to manage soilborne pathogens, foliar pathogens, arthropods and weeds. *Sci. Hortic.* 127, 127–146. doi: 10.1016/j.scienta.2010.09.023.

Lumaret, R., Ouazzani, N., Michaud, H., Vivier, G., Deguilloux, M.-F., and Di Giusto, F. (2004). Allozyme variation of oleaster populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean Basin. *Heredity* 92, 343–351. doi: 10.1038/sj.hdy.6800430.

Luo, X., Xie, C., Dong, J., Yang, X., and Sui, A. (2014). Interactions between *Verticillium dahliae* and its host: vegetative growth, pathogenicity, plant immunity. *Appl. Microbiol. Biotechnol.* 98, 6921–6932. doi: 10.1007/s00253-014-5863-8.

Lynch, J. (1995). Root Architecture and Plant Productivity. *Plant Physiol.* 109, 7–13. doi: 10.1104/pp.109.1.7.

Mairech, H., López-Bernal, Á., Moriondo, M., Dibari, C., Regni, L., Proietti, P., et al. (2020). Is new olive farming sustainable? A spatial comparison of productive and environmental performances between traditional and new olive orchards with the model OliveCan. *Agric. Syst.* 181, 102816. doi: 10.1016/j.agsy.2020.102816.

Mancuso, S., and Azzarello, E. (2002). Heat tolerance in olive. *Adv. Hortic. Sci.* 16 (3–4), 125–130.

Mariotti, R., Belaj, A., De La Rosa, R., Leòn, L., Brizioli, F., Baldoni, L., et al. (2020). EST–SNP Study of *Olea europaea* L. Uncovers Functional Polymorphisms between Cultivated and Wild Olives. *Genes* 11, 916. doi: 10.3390/genes11080916.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Paplomatas, E. J., and Tjamos, E. C. (2009). Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. *Eur. J. Plant Pathol.* 124, 603–611. doi: 10.1007/s10658-009-9447-x.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Roussos, P. A., Paplomatas, E. J., and Tjamos, E. C. (2010). Phenolic Responses of Resistant and Susceptible Olive Cultivars Induced by Defoliating and Nondefoliating *Verticillium dahliae* Pathotypes. *Plant Dis.* 94, 1156–1162. doi: 10.1094/PDIS-94-9-1156.

Martos-Moreno, C., López-Escudero, F. J., and Blanco-López, M. Á. (2006). Resistance of Olive Cultivars to the Defoliating Pathotype of *Verticillium dahliae*. *HortScience* 41, 1313–1316. doi: 10.21273/HORTSCI.41.5.1313.

Maxted, N., and Kell, S. (2009). Establishment of a global network for the *in situ* conservation of crop wild relatives; status and needs. *FAO Comm. Genet. Resour. Food Agric.*, 224.

Médail, F., Quézel, P., Besnard, G., and Khadari, B. (2001). Systematics, ecology and phylogeographic significance of *Olea europaea* L. ssp. *maroccana* (Greuter & Burdet) P. Vargas et al a relictual olive tree in south-west Morocco. *Bot. J. Linn. Soc.* 137, 249–266. doi: 10.1006/bojl.2001.0477.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiol. Mol. Plant Pathol.* 63, 91–105. doi: 10.1016/j.pmpp.2003.10.001.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., and Jiménez-Díaz, R. M. (2004). Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biol. Control* 30, 474–486. doi: 10.1016/j.biocontrol.2004.02.002.

Michalopoulos, G., Kasapi, K. A., Koubouris, G., Psarras, G., Arampatzis, G., Hatzigiannakis, E., et al. (2020). Adaptation of Mediterranean Olive Groves to Climate Change through Sustainable Cultivation Practices. *Climate* 8, 54. doi: 10.3390/cli8040054.

Micheli, M., Standardi, A., El Behi, A. W., Zakhour, D., and Yasin, M. (2010). *In vitro* proliferation of olive ("Dolce agogia" and 'Moraiolo'): effect of different cytokinins. *Acta Hortic.*, 587–590. doi: 10.17660/ActaHortic.2010.884.76.

Mirzaei, L., Yadollahi, A., Kermani, M. J., Naderpour, M., Zeinanloo, A. A., Farsi, M., et al. (2021). Evaluation of genetic stability in olive callus-induced and meristem-induced shoots using flow cytometry and amplified fragment length polymorphism techniques. *Plant Methods* 17, 31. doi: 10.1186/s13007-021-00724-7.

Mol, L. (1995). Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*: II. Quantitative analysis of the luring effect of crops. *Eur. J. Plant Pathol.* 101, 679–685. doi: 10.1007/BF01874872.

Montes-Osuna, N., and Mercado-Blanco, J. (2020). Verticillium Wilt of Olive and Its Control: What Did We Learn during the Last Decade? *Plants* 9, 735. doi: 10.3390/plants9060735.

Moral, J., Xavier, C., Roca, L. F., Romero, J., Moreda, W., and Trapero, A. (2014). Olive Anthracnose and its effect on oil quality. *Grasas Aceites* 65. doi: 10.3989/gya.110913.

Morales Alfaro, J., Bermejo, A., Navarro, P., Quiñones, A., and Salvador, A. (2021). Effect of Rootstock on Citrus Fruit Quality: A Review. *Food Rev. Int.*, 1–19. doi: 10.1080/87559129.2021.1978093.

Morelli, M., García-Madero, J. M., Jos, Á., Saldarelli, P., Dongiovanni, C., Kovacova, M., et al. (2021). *Xylella fastidiosa* in Olive: A Review of Control Attempts and Current Management. *Microorganisms* 9, 1771. doi: 10.3390/microorganisms9081771.

Morello, P., Díez, C. M., Codes, M., Rallo, L., Barranco, D., Trapero, A., et al. (2016). Sanitation of olive plants infected by *Verticillium dahliae* using heat treatments. *Plant Pathol.* 65, 412–421. doi: 10.1111/ppa.12432.

Moriondo, M., Ferrise, R., Trombi, G., Brilli, L., Dibari, C., and Bindi, M. (2015). Modelling olive trees and grapevines in a changing climate. *Environ. Model. Softw.* 72, 387–401. doi: 10.1016/j.envsoft.2014.12.016.

Moutier, N., Ricard, J. M., and Le Verge, S. (2011). Vigour control of the density tree in a high density planting system: two experimental approaches. *Acta Hortic.*, 185–193. doi: 10.17660/ActaHortic.2011.924.23.

Mudge, K., Janick, J., Scofield, S., and Goldschmidt, E. E. (2009). "A History of Grafting," in *Horticultural Reviews*, ed. J. Janick (Hoboken, NJ, USA: John Wiley & Sons, Inc.), 437–493. doi: 10.1002/9780470593776.ch9.

Mulero-Aparicio, A., Varo, A., Agustí-Brisach, C., López-Escudero, F. J., and Trapero, A. (2020). Biological control of Verticillium wilt of olive in the field. *Crop Prot.* 128, 104993. doi: 10.1016/j.cropro.2019.104993.

Murillo, J. M., Madejón, E., Madejón, P., and Cabrera, F. (2005). The response of wild olive to the addition of a fulvic acid-rich amendment to soils polluted by trace elements (SW Spain). *J. Arid Environ.* 63, 284–303. doi: 10.1016/j.jaridenv.2005.03.022.

Nardini, A., Gasco, A., Raimondo, F., Gortan, E., Lo Gullo, M. A., Caruso, T., et al. (2006). Is rootstock-induced dwarfing in olive an effect of reduced plant hydraulic efficiency? *Tree Physiol.* 26, 1137–1144. doi: 10.1093/treephys/26.9.1137.

Ogawa, J. M., and English, H. (1991). Diseases of temperate zone tree fruits and nut crops. *Univ Calif Div. Agr Nat. Resour. Oakl. Calif Publ 3345*.

Oliveros, M. T., and Jordana, J. (1968). *La agricultura de los reinos españoles en tiempo de los Reyes Católicos*. Madrid: Ministerio de Agricultura.

Orlandi, F., Avolio, E., Bonofiglio, T., Federico, S., Romano, B., and Fornaciari, M. (2013). Potential shifts in olive flowering according to climate variations in Southern Italy: Olive flowering in South Italy. *Meteorol. Appl.* 20, 497–503. doi: 10.1002/met.1318.

Palliotti, A., and Bongi, G. (1996). Freezing injury in the olive leaf and effects of mefluidide treatment. *J. Hortic. Sci.* 71, 57–63. doi: 10.1080/14620316.1996.11515382.

Pannelli, G., Rosati, S., and Rugini, E. (2002). The effect of clonal rootstocks on frost tolerance and on some aspects of plant behaviour in Moraiolo and S. Felice olive cultivars. *Acta Hortic.*, 247–250. doi: 10.17660/ActaHortic.2002.586.46.

Papadaki, A. M., Bletsos, F. A., Eleftherohorinos, I. G., Menexes, G., and Lagopodi, A. L. (2017). Effectiveness of seven commercial rootstocks against verticillium wilt and their effects on growth, yield, and fruit quality of tomato. *Crop Prot.* 102, 25–31. doi: 10.1016/j.cropro.2017.08.006.

Pasche, J. S., Mallik, I., Anderson, N. R., and Gudmestad, N. C. (2013). Development and Validation of a Real-Time PCR Assay for the Quantification of *Verticillium dahliae* in Potato. *Plant Dis.* 97, 608–618. doi: 10.1094/PDIS-06-12-0554-RE.

Peel, M. C., Finlayson, B. L., and McMahon, T. A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrol. Earth Syst. Sci.* 11, 1633–1644. doi: 10.5194/hess-11-1633-2007.

Pegg, G. F., and Brady, B. L. eds. (2002). *Verticillium wilts*. Wallingford: CABI doi: 10.1079/9780851995298.0000.

Pérez-Artés, E., Mercado-Blanco, J., Ruz-Carrillo, A. R., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2005). Detection of the defoliating and nondefoliating pathotypes of *Verticillium dahliae* in artificial and natural soils by nested PCR. *Plant Soil* 268, 349–356. doi: 10.1007/s11104-004-0378-1.

Ponti, L., Gutierrez, A. P., Ruti, P. M., and Dell'Aquila, A. (2014). Fine-scale ecological and economic assessment of climate change on olive in the Mediterranean Basin reveals winners and losers. *Proc. Natl. Acad. Sci.* 111, 5598–5603. doi: 10.1073/pnas.1314437111.

Porfírio, S., Gomes da Silva, M. D. R., Cabrita, M. J., Azadi, P., and Peixe, A. (2016). Reviewing current knowledge on olive (*Olea europaea* L.) adventitious root formation. *Sci. Hortic.* 198, 207–226. doi: 10.1016/j.scienta.2015.11.034.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1.

Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., and Rolshausen, P. E. (2014). Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00253.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S. G., Dobinson, K. F., and Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7: Olive- *Pseudomonas-Verticillium* in planta interaction. *Microb. Biotechnol.* 2, 499–511. doi: 10.1111/j.1751-7915.2009.00105.x.

Pritsa, T. S., Voyiatzis, D. G., Voyiatzi, C. J., and Sotiriou, M. S. (2003). Evaluation of vegetative growth traits and their relation to time to first flowering of olive seedlings. *Aust. J. Agric. Res.* 54, 371. doi: 10.1071/AR02131.

Rallo, L. (1995). Selection and breeding of olive in Spain. *Olivae* 59, 46–53.

Rallo, L., Barranco, D., Castro-García, S., Connor, D. J., Gómez del Campo, M., and Rallo, P. (2013). "High-Density Olive Plantations," in *Horticultural Reviews Volume 41*, ed. J. Janick (Hoboken, New Jersey: John Wiley & Sons, Inc.), 303–382. doi: 10.1002/9781118707418.ch07.

Rallo, L., Barranco, D., de la Rosa, R., and León, L. (2008). 'Chiquitita' Olive. *HortScience* 43, 529–531. doi: 10.21273/HORTSCI.43.2.529.

Rius, X., and Lacarte, J. (2015). *La Revolución del Olivar: El Cultivo en Seto.* Barcelona: Agromillora.

Rius, X., and Lacarte, J. M. (2010). *La revolución del olivar: el cultivo en seto*. Subirats: Agromillora.

Robb, J. (2007). *Verticillium* tolerance: resistance, susceptibility, or mutualism? *Can. J. Bot.* 85, 903–910. doi: 10.1139/B07-093.

Robinson, T. L., Lakso, A. N., and Ren, Z. (1991). Modifying Apple Tree Canopies for Improved Production Efficiency. *HortScience* 26, 1005–1012. doi: 10.21273/HORTSCI.26.8.1005.

Romero, A., Hermoso, J. F., and Tous, J. (2014). Olive rootstocks to control' Arbequina IRTAI18' clone vigour results from a second one comparative trial. *Acta Hortic.*, 577–584. doi: 10.17660/ActaHortic.2014.1057.74.

Rosati, A., Paoletti, A., Caporali, S., and Perri, E. (2013). The role of tree architecture in super high density olive orchards. *Sci. Hortic.* 161, 24–29. doi: 10.1016/j.scienta.2013.06.044.

Rubio de Casas, R., Besnard, G., Schönswetter, P., Balaguer, L., and Vargas, P. (2006). Extensive gene flow blurs phylogeographic but not phylogenetic signal in *Olea europaea* L. *Theor. Appl. Genet.* 113, 575–583. doi: 10.1007/s00122-006-0306-2.

Rugini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.* 24, 123–134. doi: 10.1016/0304-4238(84)90143-2.

Rugini, E., Politi, V., Bignami, C., De Agazio, M., and Grego, S. (1990). Effect of polyamine treatments on rooting cutting of three olive cultivars. *Acta Hortic.*, 97–100. doi: 10.17660/ActaHortic.1990.286.17.

Rugini, E., Silvestri, C., Ceccarelli, M., Muleo, R., and Cristofori, V. (2016). Mutagenesis and Biotechnology Techniques as Tools for Selecting New Stable Diploid and Tetraploid Olive Genotypes and Their Dwarfing Agronomical Characterization. *HortScience* 51, 799–804. doi: 10.21273/HORTSCI.51.7.799.

Ryugo, K., and Mikuckis, F. (1969). Sweet cherry. Hedgerow Planting. *Calif Agric* 23, 14–15.

Saebo, A., Krekling, T., and Appelgren, M. (1995). Light quality affects photosynthesis and leaf anatomy of birch plantlets *in vitro*. *Plant Cell Tissue Organ Cult.* 41, 177–185. doi: 10.1007/BF00051588.

Santos, C. V., Brito, G., Pinto, G., and Fonseca, H. M. A. C. (2003). *In vitro* plantlet regeneration of *Olea europaea* ssp. *maderensis*. *Sci. Hortic.* 97, 83–87. doi: 10.1016/S0304-4238(02)00148-6.

Sanzani, S. M., Schena, L., Nigro, F., Sergeeva, V., Ippolito, A., and Salerno, M. G. (2012). Abiotic diseases of olive. *J. Plant Pathol.* 94 (3), 469–491.

Schaal, B. A., and Olsen, K. M. (2000). Gene genealogies and population variation in plants. *Proc. Natl. Acad. Sci.* 97, 7024–7029. doi: 10.1073/pnas.97.13.7024.

Schnathorst, W. C., and Sibbett, G. S. (1971). relation of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. *Plant Dis. Report.* Available at: https://scholar.google.com/scholar_lookup?title=relation+of+strains+of+Verticillium+alboatrum+to+severity+of+Verticillium+wilt+in+Gossypium+hirsutum+and+Olea+europaea+in+California&author=Schnathorst%2C+W.C.&publication_year=1971 [Accessed December 9, 2021].

Schuster, M., Duringer, P., Ghienne, J.-F., Vignaud, P., Mackaye, H. T., Likius, A., et al. (2006). The Age of the Sahara Desert. *Science* 311, 821–821. doi: 10.1126/science.1120161.

Serrano, A., Rodríguez-Jurado, D., Román, B., Bejarano-Alcázar, J., De la Rosa, R., and León, L. (2021). Verticillium Wilt Evaluation of Olive Breeding Selections Under Semi-Controlled Conditions. *Am. Phytopathol. Soc.* doi: 10.1094/PDIS-08- 20-1829-RE.

Sisto, A., Cipriani, M. G., and Morea, M. (2004). Knot Formation Caused by *Pseudomonas syringae* subsp. *savastanoi* on Olive Plants Is *hrp* -Dependent. *Phytopathology®* 94, 484–489. doi: 10.1094/PHYTO.2004.94.5.484.

Solari, L. I., Johnson, S., and DeJong, T. M. (2006). Hydraulic conductance characteristics of peach (*Prunus persica*) trees on different rootstocks are related to biomass production and distribution. *Tree Physiol.* 26, 1343–1350. doi: 10.1093/treephys/26.10.1343.

Soumelidou, K. (1994). The Anatomy of the Developing Bud Union and its Relationship to Dwarfing in Apple. *Ann. Bot.* 74, 605–611. doi: 10.1006/anbo.1994.1161.

Tadić, J., Dumičić, G., Veršić Bratinčević, M., Vitko, S., and Radić Brkanac, S. (2021). Physiological and Biochemical Response of Wild Olive (*Olea europaea* subsp. *europaea* var. *sylvestris*) to Salinity. *Front. Plant Sci.* 12, 712005. doi: 10.3389/fpls.2021.712005.

Therios, I. (2009). Olives. *Crop Prod. Sci. Hortic. Wallingford UK CABI*.

Tjamos, E. C. (1991). Recovery of Olive Trees with Verticillium Wilt After Individual Application of Soil Solarization in Established Olive Orchards. *Plant Dis.* 75, 557. doi: 10.1094/PD-75-0557.

Tjamos, E. C., and Paplomatas, E. J. (1988). Long-term effect of soil solarization in controlling verticillium wilt of globe artichokes in Greece. *Plant Pathol.* 37, 507–515. doi: 10.1111/j.1365-3059.1988.tb02108.x.

Tombesi, S., Johnson, R. S., Day, K. R., and DeJong, T. M. (2010). Relationships between xylem vessel characteristics, calculated axial hydraulic conductance and size-controlling capacity of peach rootstocks. *Ann. Bot.* 105, 327–331. doi: 10.1093/aob/mcp281.

Torres-Sánchez, J., de la Rosa, R., León, L., Jiménez-Brenes, F. M., Kharrat, A., and López-Granados, F. (2022). Quantification of dwarfing effect of different rootstocks in 'Picual' olive cultivar using UAV-photogrammetry. *Precis. Agric.* 23, 178–193. doi: 10.1007/s11119-021-09832-9.

Tous, J. (2011). Olive production systems and mechanization. *Acta Hortic* 924, 169–184.

Tous, J., Romero, A., Hermoso, J. F., and Ninot, A. (2011). Influence of diferent olive rootstocks on growth and yield of the "Arbequina IRTA-I 18®" clone. *Acta Hortic.*, 315–320. doi: 10.17660/ActaHortic.2011.924.39.

Tous, J., Romero, A., Plana, J., Rovira, M., and Vargas, F. J. (1997). Performance of "Negret" hazelnut cultivar on several rootstocks. *Acta Hortic.*, 433–440. doi: 10.17660/ActaHortic.1997.445.56.

Trapero, C., Alcántara, E., Jiménez, J., Amaro-Ventura, M. C., Romero, J., Koopmann, B., et al. (2018). Starch Hydrolysis and Vessel Occlusion Related to Wilt Symptoms in Olive Stems of Susceptible Cultivars Infected by *Verticillium dahliae*. *Front. Plant Sci.* 9, 72. doi: 10.3389/fpls.2018.00072.

Trapero, C., Díez, C. M., Rallo, L., Barranco, D., and López-Escudero, F. J. (2013a). Effective inoculation methods to screen for resistance to Verticillium wilt in olive. *Sci. Hortic.* 162, 252–259. doi: 10.1016/j.scienta.2013.08.036.

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. (2015). Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathol.* 64, 890–900. doi: 10.1111/ppa.12330.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., and López-Escudero, F. J. (2013b). Field Resistance to Verticillium Wilt in Selected Olive Cultivars Grown in Two Naturally Infested Soils. *Plant Dis.* 97, 668–674. doi: 10.1094/PDIS-07-12-0654-RE.

Trifilò, P., Lo Gullo, M. A., Nardini, A., Pernice, F., and Salleo, S. (2007). Rootstock effects on xylem conduit dimensions and vulnerability to cavitation of *Olea europaea* L. *Trees* 21, 549–556. doi: 10.1007/s00468-007-0148-9.

Troncoso, A., Liñán, J., Prieto, J., and Cantos, M. (1990). Influence of diferent olive rootstocks on growth and production of 'Gordal sevillana.' *Acta Hortic.*, 133–136. doi: 10.17660/ActaHortic.1990.286.26.

Trujillo, I., Ojeda, M. A., Urdiroz, N. M., Potter, D., Barranco, D., Rallo, L., et al. (2014). Identification of the Worldwide Olive Germplasm Bank of Córdoba (Spain) using SSR and morphological markers. *Tree Genet. Genomes* 10, 141–155. doi: 10.1007/s11295-013-0671-3.

Tsror (2011). Review: Epidemiology and control of Verticillium wilt on olive. *Isr. J. Plant Sci.* 59, 59–69. doi: 10.1560/IJPS.59.1.59.

Valverde, P., Trapero, C., Arquero, O., Serrano, N., Barranco, D., Muñoz Díez, C., et al. (2021). Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. *Plant Pathol.* 70, 144–153. doi: 10.1111/ppa.13264.

Van de Paer, C., Bouchez, O., and Besnard, G. (2018). Prospects on the evolutionary mitogenomics of plants: A case study on the olive family (Oleaceae). *Mol. Ecol. Resour.* 18, 407–423. doi: 10.1111/1755-0998.12742.

Varo-Suárez, A., Raya-Ortega, M. C., Agustí-Brisach, C., García-Ortiz-Civantos, C., Fernández-Hernández, A., Mulero-Aparicio, A., et al. (2018). Evaluation of organic amendments from agro-industry waste for the control of verticillium wilt of olive. *Plant Pathol.* 67, 860–870. doi: 10.1111/ppa.12798.

Vossen, P. (2007). Olive Oil: History, Production, and Characteristics of the World's Classic Oils. *HortScience* 42, 1093–1100. doi: 10.21273/HORTSCI.42.5.1093.

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., von Wettberg, E. J. B., et al. (2016). Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008.

Webster, A. D., and Lucas, A. (1997). Sweet cherry rootstock studies: Comparisons of *Prunus cerasus* L. and *Prunus* hybrid clones as rootstocks for Van, Merton Glory and Merpet scions. *J. Hortic. Sci.* 72, 469–481. doi: 10.1080/14620316.1997.11515535.

Wilhelm, S (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. *Phytopatthology* 45, 180–181.

Yadeta, K. A., and J. Thomma, B. P. H. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Front. Plant Sci.* 4. doi: 10.3389/fpls.2013.00097.

Zhang, J., Sanogo, S., Flynn, R., Baral, J. B., Bajaj, S., Hughs, S. E., et al. (2012). Germplasm evaluation and transfer of Verticillium wilt resistance from Pima (*Gossypium barbadense*) to Upland cotton (*G. hirsutum*). *Euphytica* 187, 147–160. doi: 10.1007/s10681-011-0549-0.

Zohary, D. (1994). The wild genetic resources of the cultivated olive. *Acta Hortic* 356, 62–65.

Zohary, D., and Spiegel-Roy, P. (1975). Beginnings of Fruit Growing in the Old World. *Science* 187, 319–327. doi: 10.1126/science.187.4174.319.

Zuccherelli, G., and Zuccherelli, S. (2002). *In vitro* propagation of fifty olive cultivars. *Acta Hortic.*, 931–934. doi: 10.17660/ActaHortic.2002.586.204.

Objetivos

El principal objetivo de esta Tesis ha sido la obtención y caracterización de una colección de germoplasma de genotipos silvestres de olivo de alta variabilidad genética (colección SILVOLIVE), para abordar soluciones a problemas a los que se enfrenta el cultivo del olivar. Este objetivo general se ha desarrollado a través de los siguientes objetivos específicos:

1. Establecimiento *in-vitro* de la colección de genotipos silvestres y estudio de la influencia de la calidad e intensidad de las luces LEDs para establecer un sistema de propagación clonal de olivos (**Capítulo 1**).

2. Caracterización genética y morfológica de la colección de olivos silvestres en condiciones de invernadero durante el desarrollo temprano de las plantas y prueba de concepto para su uso como portainjertos (**Capítulo 2**).

3. Caracterización del consumo hídrico y respuesta al riego deficitario de los genotipos de la colección (**Anexo 1**).

4. Caracterización del vigor temprano de los genotipos silvestres y su uso como portainjerto para regular el vigor del cultivar 'Arbequina' (**Capítulo 3**).

5. Evaluación del grado de susceptibilidad de los genotipos silvestres al patotipo defoliante del hongo *V. dahliae*, monitorización de los síntomas y cuantificación de la cantidad de ADN del hongo en las plantas inoculadas mediante qPCR (**Capítulo 4**).

6. Evaluación del grado de susceptibilidad a la verticilosis en el cultivar sensible 'Picual' injertado sobre genotipos silvestres de baja susceptibilidad y comparación de los mecanismos de resistencia *vs* tolerancia a *V. dahliae* en el control de la enfermedad (**Capítulo 5**).

Chapter 1. *In-vitro* establishment of the wild genotypes collection SILVOLIVE and study of the influence of LED lights quality and intensity on olive clonal micropropagation

This chapter is divided into two sections:

Section 1.1. ***In-vitro* establishment, multiplication, rooting and growth of wild olive genotypes and mature cultivars.**

Section 1.2. **Growth Quality and Development of Olive Plants Cultured *In-Vitro* under Different Illumination Regimes.** Published paper: **Díaz-Rueda, P.**, Cantos-Barragán, M., Colmenero-Flores, J.M. (2021). Growth Quality and Development of Olive Plants Cultured In-Vitro under Different Illumination Regimes. Plants, 10, 2214. https://doi.org/10.3390/plants10102214

Section 1.1. *In-vitro* establishment, multiplication, rooting and growth of wild genotypes and mature olive cultivars

Introduction

In-vitro plant tissue culture has been widely studied since its beginning in 1902 (Haberlandt, 1902). Since then, this type of culture has become an essential tool for plant research. Plant cell and tissue culture uses nutritive culture media and controlled aseptic conditions for the growth of plant cells, tissues and organs. *In-vitro* culture techniques are now indispensable for the production of disease-free plants, rapid multiplication of rare or new plant genotypes, plant genome transformation, and production of plant-derived metabolites of important commercial value (Debnath et al., 2006; Altpeter et al., 2016). In addition, it is a fundamental tool for plant biotechnology and plant physiology disciplines. This is possible thanks to a quality of most plant cells, the cell totipotency, defined as the ability of the nucleated plant cell to regenerate part of the plant (root, stem, leaves, organs) or the whole plant. Nevertheless, *in-vitro* tissue culture has some disadvantages, such as the difficulty of keeping the genetic stability of the plants or the high cost of this technique, particularly the expenses associated with culture media (mainly the carbon source, gelling agent and growth regulators), electricity and labor (Espinosa-Leal et al., 2018).

Olive is one of the most important fruit trees cultivated in the Mediterranean basin, where this crop has a great impact on the economy, history, culture, and environment (Barranco et al., 1998). The large expansion area and long life of the olive tree explain the vast number of current cultivars, over 2600 (Therios, 2009). At this time, the increased demand for oil and table olives has led to an expansion of olive plantations, being this demand reflected in the increase of the cultivated area in the last years (FAOSTAT). New olive orchards are being planted in and outside the Mediterranean basin, including non-traditional countries such as Argentina, Australia, Chile, China, Japan and the United States (Besnard et al., 2018; Yadav et al., 2021).

Therefore, the olive sector is demanding a huge volume of plant material from different varieties to carry out the establishment of new plantations.

The olive tree has been propagated vegetatively for centuries by conventional techniques based on both the rooting of stem cuttings, ovuli and suckers and also on grafting (Fabbri et al., 2004). These propagation methods are not always effective, having limited success for some varieties. *In-vitro* propagation is an effective alternative for the clonal multiplication of the olive tree, not only for difficult-to-root varieties, but in general for all cultivars of agronomic interest. The *in-vitro* multiplication of olive has been successfully developed by classic micropropagation (Rugini, 1984) and by less conventional techniques, such as somatic embryogenesis (Rugini, 1988; Leva et al., 1995; Rugini and Caricato, 1995), callus cultures (Martino et al., 1999), protoplast technology and haploid culture (Perri et al., 1994; Solís et al., 2008).

Multiplication by seed is essential in traditional breeding programs, enabling higher genetic variability and production of new genotypes. However, the germination of olive seeds is very inneficient due to the inhibitory effect of woody endocarp, seed integument, and endosperm leading it to occur at a low rate and over a long time (Scaramuzzi, 1958; Crisosto and Sutter, 1985). In order to reduce timing by breaking mechanical dormancy, new protocols for *in-vitro* embryo culture were developed (Acebedo et al., 1997; García et al., 2002). *In-vitro* germination is a process of morphogenesis defined as the development of zygotic embryos separated from the seed and properly cultured in a controlled way to obtain a viable plant. This technique strongly decreases the time and increases the germination rate of olive seeds.

In olive, as occurred in other tree species, somatic embryogenesis is the regeneration method most frequently used (Gupta et al., 1993). The embryogenic cultures can be also maintained *in-vitro* and clonally propagated or used to perform tests for the selection of interesting genotypes. Rugini (1988) was the first who reported embryogenic cultures from immature zygotic embryos of several olive cultivars. Currently, available somatic embryogenesis protocols allow obtaining an acceptable number of plants from embryogenic cultures of juvenile origin (Bradaï et al., 2016; Sánchez-Romero, 2018). Micropropagation by stimulation of axillary buds minimizes the

risk of somaclonal variation, also contributing to 1) the rapid propagation of new genotypes, 2) pathogens elimination from the mother plants or offspring, 3) germplasm preservation, 4) plant regeneration from cell tissues, and 5) synthetic seed production (Rugini et al., 2016).

The success of the *in-vitro* propagation of the olive tree seems to be influenced by several factors, such as the mineral composition of the medium, plant growth regulators and the carbon source used (Rugini, 1984; Fiorino and Leva, 1986; Leva et al., 1994; Abousalim et al., 2005). Several studies have been carried out comparing different propagation media (Rugini, 1984; García-Berenguer and Durán González, 1990; García-Férriz et al., 2003; Haddad et al., 2018), the most promising being the Rugini medium, and the use of different combinations and concentrations of cytokinins (García-Férriz et al., 2003; Mirzaei et al., 2021). Many authors have recommended the use of the cytokinin 'zeatin' (Chaari et al., 2002; Zuccherelli and Zuccherelli, 2002; Micheli et al., 2010; Mirzaei et al., 2021) and mannitol as energy source (Leva et al., 1992, 1994; Farahani et al., 2008; Mirzaei et al., 2021) for their efficacy in olive culture. Rooting is the critical step to obtain viable olive plants, which depends on several factors, such as the type and /or the concentration of auxin and the olive genotype (Rugini, 1984; Leva, 2011; Porfírio et al., 2016). To date, several techniques for olive micropropagation have been described (Cañas and Benbadis, 1988; Rugini et al., 1990; Benelli and De Carlo, 2018; Mirzaei et al., 2021). However, olive micropropagation is highly cultivar-dependent and it is necessary to develop different micropropagation procedures for each cultivar (Bayraktar et al., 2020).

The economic viability of *in-vitro* clonal multiplication of olive trees at an industrial level in nurseries requires the optimization of processes such as temperature, humidity, light, ventilation, rooting and *ex-vitro* acclimatization. It is necessary to develop a commercially efficient multiplication system suitable for large-scale propagation of olive trees, ensuring the sanitary and genetic quality of the plants produced. The aim of the present study was to develop and optimize protocols for the efficient *in-vitro* establishment, propagation and rooting of juvenile wild olive genotypes of the SILVOLIVE collection, as well as mature cultivars. Efficient protocols have been obtained.

Material and Methods

Plant material

Prospections in different geographical areas were carried out to obtain a wild olive collection that includes the six currently recognized subspecies of *Olea europaea* L. (Médail et al., 2001; Green, 2002). The wild olive germplasm collection, called SILVOLIVE, described by Díaz-Rueda et al. (2020) (Chapter 2), includes 146 genotypes coming from seeds of mother trees obtained i) by *in situ* collection of the fruits directly from the mother tree ii) from the world *ex-vitro* germplasm banks of Córdoba and Marrakech; iii) from Dr. Guilleume Besnard's collection (CNRS-UPS-ENFA, Toulouse, France); and iv) from material selected in our Institution (IRNAS-CSIC, Seville, Spain). Collected fruits were stored at 4ºC for further processing.

In-vitro establishment of zygotic embryos of wild olive genotypes

The wild olive genotypes were established *in-vitro* and germinated from zygotic seed embryos of the six currently recognized subspecies of *Olea europaea* L. (Green, 2002), including first generation hybrids (see Chapter 2, Table 1 for details). Seeds were extracted from the inside of the olive pits (**Figure 1A**) by breaking them with a tube-cutter. The seeds (**Figure 1B**) were sterilized by dipping in ethyl alcohol for 3 min, then in a NaClO:HCl (30:1) solution for 10 min adding a drop of Tween-20 to break the surface tension of the water, followed by 5-6 washes with sterile distilled water. These sterilized seeds were kept in sterile water for 48 hours for full tissue hydration (**Figure 1C**), which enables the embryo release. To avoid dormancy induced by the seed coat, this organ, together with the endosperm was removed from the embryos (Lagarda et al., 1983). Then, they were transplanted into glass tubes containing 10 mL of hormone-free medium (Rugini, 1984), embedding the root primordium in the nutrient medium and leaving the cotyledons outside the medium (**Figure 1D**). Finally, the zygotic embryos were incubated in a growth chamber with 16 h light photoperiod (34 µmol $m^{-2}s^{-1}$ intensity with 70% red: 30% blue light-emitting diodes, LEDs), at 25 ± 2 ºC.

FIGURE 1 | *In-vitro* establishment of zygotic embryos of wild olive genotypes. Olive bones (**A**); olive seeds (**B**); sterilized seeds in water (**C**); and zygotic embryos placed in Rugini medium (**D**).

Embryos were maintained in the hormone-free Rugini medium (Rugini, 1984) for approximately 60 days, requiring the renewal of the nutrient medium (**Figure 2**).

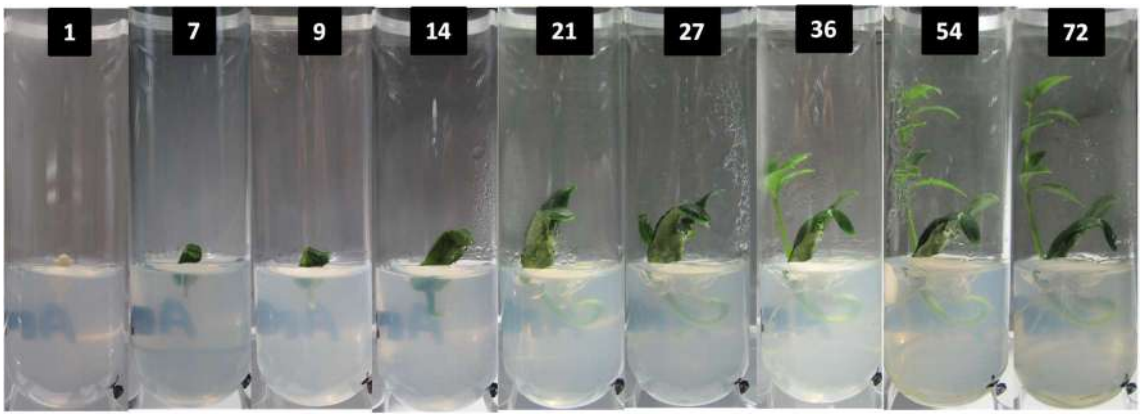


FIGURE 2 | Time-lapse of ANA27 genotype for 72 days.

In-vitro establishment of mature olive varieties by mini-grafting

Mature olive cultivars including 'Picual', 'Arbequina', 'Hojiblanca' and 'Frantoio' were required in this project for different purposes. *In-vitro* establishment of mature olive material was performed through mini-grafting according to Troncoso et al., (2000). On the one hand, 2-years old rooted cuttings were pre-treated by spraying 3 g/L Mancozeb fungicide in the nursery (Viveros Sevilla S.A.). Seven days later, the new shoots were cut, and the apical nodal segment and the leaves were discarded. In the lab, three-to-four nodes explants were selected and disinfected by dipping in 0.1% Tween-20 solution for 5 min, ethanol 70% for 1 min, 200 mg/L $HgCl_2$ plus 0.1% Tween-20 solution for 3 min, and six washes with autoclaved distilled water (**Figure 3A**). On the other hand, *in-vitro* seedlings established from zygotic embryos (see above) were used as rootstocks to perform the mini-graft. The seedling was decapitated, keeping the root part (**Figure 3B**). A central incision was made in the top cut stem while the sterilized explants were cut

into wedge-shaped uninodal segments at the base (**Figure 3C**). The joint was held with a clamp and the grafted plant was carefully reintroduced into a tube containing Rugini medium (**Figure 3D and 3E**). If the plant was grafted correctly the growth of the scion was observed (**Figure 3F**). This newly grown material was further subcultured *in-vitro* to be considered completely established.

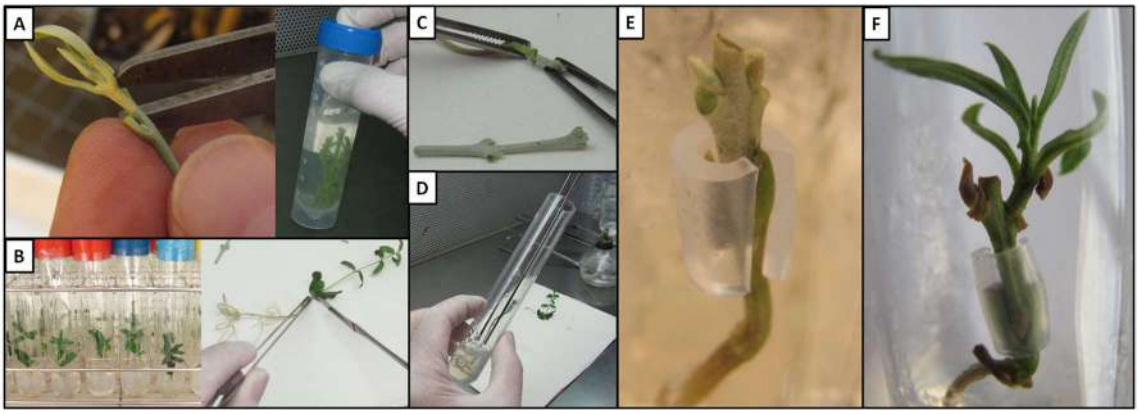


FIGURE 3 | Procedure for the disinfection of the scion explant and olive minigraft for the *in-vitro* establishment of mature cultivars. Explants of mature variety to be disinfected (**A**); *in-vitro* seedlings established from zygotic embryos (**B**); mini-graft technique (**C**); introduction of the grafted plantlet (scion / rootstock combination) in a tube containing Rugini medium (**D**); mini-graft recently performed (**E**); 40 days after mini-grafting *in-vitro* (**F**).

In-vitro micropropagation and rooting of wild olive genotypes and commercial olive varieties.

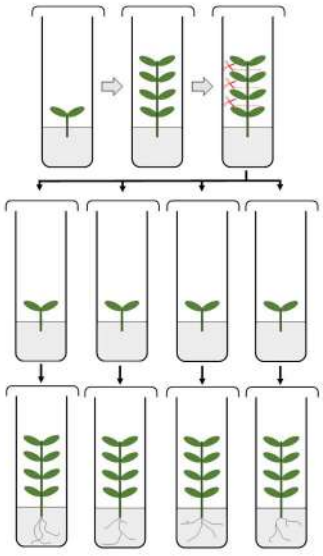


FIGURE 4 | Scheme of *in-vitro* micropropation process.

To maintain maximum genetic estability during *in-vitro* micropropagation of olive explants was based on the growth of axillary buds. Wild olive genotypes and *in-vitro* established commercial cultivars were cut into uninodal segments and inserted in Rugini medium (Rugini, 1984) supplemented with 1 mg/L zeatin under the growing conditions described before (**Figure 4**). For whole plant regeneration, two methods were used: *in-vitro* rooting (A), by which grown shoots were transferred to rooting medium (50% Rugini medium) supplemented with α-naphthalacetic acid (0.8 mg/L) for 21 days, followed by *ex-vitro* acclimatization for 3

weeks under greenhouse conditions; *ex-vitro* rooting (B), by which grown shoots were simultaneously rooted and acclimatized to *ex-vitro* conditions applying α-naphthalacetic acid (NAA) and indole-3-butyric acid (IBA) at the base of the stem for 15 seconds to promote the formation of roots. Three different hormonal combinations were assayed: 0.02% NAA; 0.02% NAA + 0.02% IBA; and 0.04% NAA + 0.04% IBA (**Figure 5**).

FIGURE 5 | *Ex-vitro* rooting process. Fresh shoots from *in-vitro* culture lacking roots (**A**); during shoots dipping in auxin solution (**B**); *ex-vitro* acclimatization of microplants (**C**); and final rooted plant (**D**).

Quantification of the in-vitro growth of the SILVOLIVE collection genotypes.

In-vitro growth of the different genotypes of the SILVOLIVE collection was quantified during 55 days. Uninodal explants of 1 cm in length were implanted into tubes with 10 mL of 1x Rugini medium (Rugini, 1984) supplemented with 1 mg / L zeatin to promote elongation of the aerial part (**Figure 5**). Four to six explants per genotype were grown randomly in a growth chamber at 25 ± 2 ºC, with 16 h light photoperiod (34 µmol $m^{-2}s^{-1}$ intensity with 70% red: 30% blue light-emitting diodes, LEDs). Growth was characterized by quantification of the number of stems generated, the height of the main stem, the total stem length and the number of nodes generated per plant for each genotype.

FIGURE 6 | Micropropagated, grown and rooted olive plants *in-vitro*.

Results and Discussion

The starting plant material for the *in-vitro* establishment of the wild olive genotypes that make up the SILVOLIVE collection was the fruit seed. The olive fruit includes the epicarp or skin, mesocarp or pulp, and the endocarp or stone, with the latter including the seed (Bianchi, 2003). Exceptionally, the Dhokar variety (*Olea europaea* subsp. *laperrinei*) frequently contained two or three seeds per fruit (**Figure 7**). Different seeds from a single fruit were identified with letters and studied as independent genotypes (e.g. DHO10A, DHO10B and DHO6A, DHO6B and DHO6C), showing different genetic profiles (Chapter 2, Supplementary Table S6), displaying different vigor in pot and field conditions (Chapter 2 and 3) and exhibiting different levels of susceptibility to *Verticillium dahliae* (Chapter 4, Table 1).

FIGURE 7 | Olive stone with two seeds inside.

The *in-vitro* establishment and multiplication procedure described in the 'Materials and Methods' section was the same throughout the whole study. However, rooting, a crucial step to obtain viable plants (Rugini, 1992), is a limiting factor in the process of clonal olive micropropagation to produce the large number of plants required for this study, or for scaling up the process to an industrial level (e.g. for nursery production). Therefore, different rooting methods were tested. The first method used an *in-vitro* rooting medium consisting of 50% Rugini medium supplemented with α-naphthalacetic acid (0.8 mg/L) for 21 days to obtain whole plants. The *in-vitro* rooting protocol has several advantages: 1) a high efficiency (efficiency approx 90%); 2) non-rooted explants can be re-used for *in-vitro* propagation, *in-vitro* or *ex-vitro* rooting, avoiding the loss of non-rooted plants.

The second rooting method was the simultaneous *ex-vitro* acclimatization and rooting procedure. Three different hormonal combinations were tested. Althought non statistically significant differences were observed between the three auxin combinations in the three genotypes tested, CUS15, AMK34 and GUA7, the 0.02% NAA + 0.02% IBA treatment resulted in the higher efficiencies, which reached 100% rooting in two out of the three genotypes (**Figure 8**). The *ex-vitro* rooting is much cheaper and requires about four weeks less *in-vitro* residence time of the plant material, so it must be the method adopted for large-scale production. However, non-rotted plants cannot be reused, and the plant material is normally lost. According to Rugini et al. (1990), the rooting efficiency is genotype-dependent. This could be confirmed in our assay, where CUS15 showed around 50% of rooting efficiency, whereas AMK34 and GUA7 presented efficiencies around 90% (**Figure 8**).

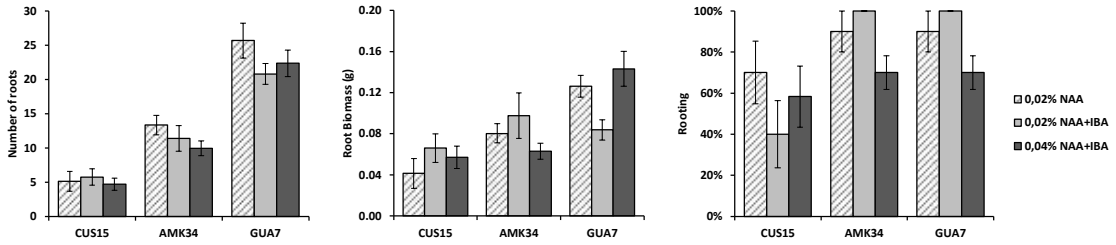


FIGURE 8 | *Ex-vitro* rooting of three genotypes belonging to three different subspecies. Number of roots, root biomass and rooting percentage were tested with three different auxin treatments. Results are means and standard error of 10 plants per genotype.

In-vitro growth of the different genotypes of the SILVOLIVE collection was quantified as number of stems produced per plant, plant height, total stem length and total number of nodes. Results are presented in **Table 1**. The GUA8 genotype showed the highest of number of stems or branching of all germplasm (2.3 stems per plant). The plant height varied 5.6 times between 95.8 mm (AMS15 genotype) and 17.5 mm (GUA9 genotype). However, the longest stem height accumulation was observed in DHO10B and AMS15 plants (98.8 and 95.8 mm respectively). In addition, the highest number of nodes was represented in MAR3 plants (11.0 nodes), which was 6 times higher than that of AOU8 (1.8 nodes per plant). These results showed differences of growth and propagation ratio in the genotypes of the SILVOLIVE collection (**Figure 6**).

TABLE 1 | Growth parameters of the SILVOLIVE genotypes established *in-vitro.*

Genotype	Number of stems	Height	Total Stem lenght	Number of nodes
GUA1	2.0 ± *0.0*	36.0 ± *5.7*	48.0 ± *2.5*	6.7 ± *0.3*
GUA2	2.0 ± *0.0*	21.0 ± *1.9*	23.8 ± *1.9*	7.3 ± *0.9*
GUA3	2.0 ± *0.0*	28.0 ± *3.7*	36.8 ± *6.5*	7.3 ± *1.1*
GUA4	1.5 ± 0.3	40.0 ± 2.7	42.0 ± 2.2	6.0 ± 0.4
GUA6	1.5 ± *0.3*	22.5 ± *3.6*	23.5 ± *3.4*	5.5 ± *1.0*
GUA7	2.0 ± *0.0*	38.3 ± *2.1*	44.5 ± *2.3*	8.5 ± *0.3*
GUA8	2.3 ± *0.5*	24.5 ± *2.7*	29.0 ± *4.5*	8.0 ± *1.1*
GUA9	1.3 ± *0.3*	17.5 ± *0.3*	18.3 ± *0.9*	3.7 ± *0.3*
CUS3	1.5 ± 0.3	40.3 ± 2.6	42.8 ± 2.1	6.3 ± 0.6
CUS4	1.0 ± 0.0	44.0 ± 7.1	44.0 ± 7.1	5.5 ± 1.0
CUS11	2.0 ± 0.0	35.0 ± 2.1	35.0 ± 7.4	5.5 ± 1.5
CUS12	2.0 ± *0.0*	36.3 ± *2.1*	43.3 ± *1.4*	6.8 ± *0.6*
CUS13	2.0 ± *0.0*	27.5 ± *2.1*	33.0 ± *3.4*	5.5 ± *0.3*
CUS14	1.8 ± *0.3*	32.5 ± *2.4*	36.5 ± *4.3*	5.0 ± *0.4*
CUS15	1.3 ± *0.3*	30.3 ± *1.9*	30.5 ± *2.0*	4.5 ± *0.5*
CEH6	1.8 ± *0.3*	40.3 ± *4.4*	43.0 ± *4.9*	5.5 ± *0.5*
CEH7	1.8 ± *0.3*	36.5 ± *4.6*	38.5 ± *3.9*	5.0 ± *0.0*
CEH8	2.0 ± *0.0*	25.3 ± *2.0*	28.5 ± *1.3*	5.3 ± *0.3*
CEH9	1.5 ± *0.3*	40.8 ± *4.5*	45.0 ± *4.4*	5.8 ± *0.6*
CEH17	2.0 ± *0.0*	40.3 ± *3.3*	45.0 ± *2.0*	6.3 ± *0.3*
CEH19	1.5 ± *0.3*	32.5 ± *2.1*	34.3 ± *2.9*	4.5 ± *0.6*
CEH20	1.3 ± *0.3*	34.3 ± *2.6*	35.0 ± *2.9*	4.8 ± *0.3*
CEH21	1.8 ± *0.3*	26.5 ± *2.6*	28.0 ± *2.2*	4.5 ± *0.3*
CEH23	1.8 ± *0.3*	24.8 ± *2.7*	26.3 ± *2.1*	5.3 ± *0.3*
CEH24	2.0 ± *0.0*	36.3 ± *1.8*	41.7 ± *1.5*	6.7 ± *0.3*
CEH25	1.8 ± *0.3*	31.8 ± *2.7*	38.3 ± *3.0*	7.3 ± *0.8*
CEH26	1.5 ± *0.3*	40.3 ± *3.2*	43.0 ± *2.0*	6.5 ± *0.9*
CEH30	2.0 ± *0.0*	33.5 ± *1.7*	34.5 ± *1.7*	6.0 ± *0.0*
CER1	1.8 ± *0.3*	60.0 ± *3.4*	68.3 ± *4.2*	7.8 ± *0.5*
CER3	2.0 ± *0.0*	35.0 ± *2.5*	40.0 ± *0.8*	5.8 ± *0.3*
AMK5	2.0 ± *0.0*	41.0 ± *1.1*	47.5 ± *4.9*	6.5 ± *0.5*
AMK6	1.5 ± *0.3*	38.3 ± *2.7*	44.3 ± *4.0*	7.0 ± *0.9*
AMK14	1.8 ± *0.3*	32.0 ± *1.9*	35.8 ± *3.7*	5.8 ± *0.6*
AMK16	2.0 ± *0.0*	34.5 ± *2.6*	37.5 ± *2.7*	7.5 ± *0.3*
AMK21	2.0 ± *0.0*	25.3 ± *0.5*	31.5 ± *1.5*	7.3 ± *0.8*
AMK26	1.5 ± *0.5*	21.0 ± *4.0*	21.8 ± *4.5*	2.0 ± *0.7*
AMK34	1.0 ± *0.0*	52.0 ± *2.5*	52.0 ± *2.5*	5.8 ± *0.3*
AMK9	2.0 ± *0.0*	32.3 ± *2.5*	42.3 ± *4.5*	8.0 ± *1.1*
AMK12	1.3 ± *0.3*	30.0 ± *6.0*	30.8 ± *5.7*	6.3 ± *1.8*
AMK25	2.0 ± *0.0*	85.3 ± *10.7*	95.3 ± *22.7*	10.0 ± *2.5*
AMK27	2.0 ± *0.0*	34.5 ± *2.5*	38.8 ± *2.5*	6.3 ± *0.6*
ACO1	1.7 ± 0.3	55.3 ± 3.4	56.7 ± 2.7	7.7 ± 0.3
ACO5	1.3 ± *0.3*	72.3 ± *13.4*	75.5 ± *15.7*	6.0 ± *1.1*

ACO14	2.0 ± *0.0*	33.0 ± *1.5*	35.0 ± *1.1*	6.8 ± *0.3*
ACO15	1.3 ± *0.3*	36.3 ± *1.7*	38.3 ± *3.4*	5.5 ± *0.5*
ACO18	1.3 ± *0.3*	28.5 ± *1.7*	32.0 ± *4.0*	6.0 ± *1.7*
AJA1	1.3 ± *0.3*	78.3 ± *10.5*	83.5 ± *8.9*	7.8 ± *0.9*
AJA4	2.0 ± *0.0*	52.5 ± *4.1*	68.0 ± *2.7*	9.0 ± *0.0*
AJA6	1.5 ± *0.3*	69.8 ± *12.8*	80.5 ± *11.7*	7.0 ± *0.8*
AJA12	1.7 ± *0.3*	33.0 ± *5.6*	36.0 ± *6.6*	5.3 ± *1.6*
AJA17	1.7 ± 0.3	42.7 ± 3.4	47.0 ± 0.6	7.0 ± 0.6
ACZ1	2.0 ± *0.0*	43.5 ± *2.3*	46.0 ± *2.5*	8.5 ± *0.5*
ACZ3	2.0 ± *0.0*	37.8 ± *0.6*	39.5 ± *0.6*	7.0 ± *0.4*
ACZ4	2.0 ± *0.0*	50.3 ± *2.0*	53.5 ± *2.3*	7.5 ± *0.3*
ACZ5	1.3 ± *0.3*	40.0 ± *5.4*	40.3 ± *5.4*	5.3 ± *0.5*
ACZ7	1.5 ± *0.3*	42.5 ± *3.4*	44.8 ± *4.4*	6.8 ± *0.5*
ACZ8	2.0 ± *0.0*	35.5 ± *2.3*	41.5 ± *3.3*	7.8 ± *0.3*
ACZ9	1.0 ± *0.0*	25.3 ± *2.7*	25.3 ± *2.7*	4.0 ± *0.0*
ACZ10	2.0 ± *0.0*	31.3 ± *2.9*	32.6 ± *4.9*	6.0 ± *1.6*
ACZ12	1.8 ± *0.3*	28.0 ± *0.9*	29.3 ± *0.6*	6.0 ± *0.4*
ARC1	2.0 ± *0.0*	32.5 ± *4.2*	37.0 ± *4.1*	6.3 ± *0.3*
APR1	1.3 ± *0.3*	23.3 ± *3.3*	25.5 ± *5.5*	5.8 ± *1.8*
FRA1	1.8 ± 0.3	31.8 ± 2.3	32.5 ± 2.1	5.8 ± 0.3
FRA2	1.7 ± *0.3*	28.3 ± *4.2*	30.0 ± *3.0*	3.8 ± *0.9*
FRA3	1.8 ± *0.3*	32.5 ± *5.4*	36.0 ± *4.3*	5.3 ± *0.3*
FRA4	2.0 ± *0.0*	32.0 ± *3.5*	36.0 ± *5.2*	5.8 ± *0.5*
DHO1	1.5 ± *0.3*	39.3 ± *3.8*	41.5 ± *3.4*	5.0 ± *0.4*
DHO2	1.3 ± *0.3*	40.5 ± *4.3*	41.5 ± *4.7*	5.0 ± *0.6*
DHO4	2.0 ± *0.0*	39.0 ± *4.8*	56.0 ± *5.9*	8.3 ± *0.6*
DHO6A	1.8 ± *0.3*	38.0 ± *3.5*	45.8 ± *3.8*	5.3 ± *0.5*
DHO6B	1.3 ± *0.3*	52.8 ± *6.7*	53.8 ± *7.7*	4.8 ± *0.8*
DHO6C	2.0 ± *0.0*	51.8 ± *1.8*	53.5 ± *1.8*	7.0 ± *0.4*
DHO8A	1.8 ± *0.3*	37.0 ± *3.9*	43.0 ± *4.5*	7.3 ± *0.9*
DHO10A	1.8 ± *0.3*	37.5 ± *3.7*	39.3 ± *3.1*	5.5 ± *0.3*
DHO10B	1.8 ± *0.3*	84.0 ± *7.5*	98.8 ± *7.7*	8.0 ± *0.7*
DHO11A	1.3 ± *0.3*	46.0 ± *1.2*	46.8 ± *1.9*	5.3 ± *0.3*
DHO12A	1.5 ± *0.3*	51.8 ± *5.8*	54.3 ± *4.0*	6.0 ± *0.4*
DHO13A	1.5 ± *0.3*	48.3 ± *1.7*	50.8 ± *2.3*	6.0 ± *0.0*
MAR1	2.0 ± *0.0*	39.3 ± *6.2*	45.5 ± *5.2*	9.8 ± *0.5*
MAR2	2.0 ± 0.0	18.5 ± 0.5	17.8 ± 1.7	3.0 ± 1.2
MAR3	2.0 ± *0.0*	61.3 ± *6.6*	73.7 ± *4.4*	11.0 ± *0.6*
AMS7	2.0 ± *0.0*	79.8 ± *2.1*	94.5 ± *9.2*	8.0 ± *0.6*
AMS8	2.0 ± *0.0*	57.0 ± *0.0*	58.0 ± *3.8*	7.0 ± *1.8*
AMS11	1.8 ± *0.3*	65.3 ± *4.9*	66.0 ± *4.9*	6.0 ± *0.4*
AMS12	1.3 ± *0.3*	61.5 ± *6.3*	61.8 ± *6.3*	6.5 ± *0.3*
AMS15	1.0 ± *0.0*	95.8 ± *6.6*	95.8 ± *6.6*	6.5 ± *0.3*
AMS16	1.8 ± *0.3*	41.0 ± *4.0*	44.8 ± *4.0*	7.5 ± *0.3*
AMS17	1.3 ± *0.3*	72.8 ± *9.8*	74.0 ± *10.4*	5.0 ± *0.6*
AMS18	1.5 ± *0.3*	75.0 ± *7.6*	75.5 ± *7.8*	5.8 ± *0.9*
AMS19	2.0 ± *0.0*	42.0 ± *2.9*	45.3 ± *2.9*	8.8 ± *0.5*

AOU1	1.8 ± *0.3*	33.0 ± *7.4*	33.8 ± *7.1*	5.3 ± *0.3*
AOU2	2.0 ± *0.0*	51.3 ± *1.8*	52.3 ± *1.8*	7.0 ± *0.0*
AOU3	2.0 ± *0.0*	45.0 ± *2.0*	46.8 ± *1.9*	5.5 ± *0.3*
AOU4	1.3 ± *0.3*	45.0 ± *19.3*	45.3 ± *19.3*	4.8 ± *0.8*
AOU5	1.5 ± *0.3*	39.0 ± *13.0*	43.0 ± *16.7*	6.0 ± *1.2*
AOU7	1.0 ± *0.0*	44.8 ± *4.5*	44.8 ± *4.5*	5.3 ± *0.3*
AOU8	1.0 ± *0.0*	23.5 ± *1.5*	23.5 ± *1.5*	1.8 ± *0.5*
AOU9	1.8 ± *0.3*	31.8 ± *10.9*	37.3 ± *14.0*	4.3 ± *1.3*
AOU10	1.3 ± *0.3*	76.3 ± *15.2*	76.7 ± *15.2*	8.0 ± *0.7*
AOU11	2.0 ± *0.0*	63.0 ± *6.8*	73.3 ± *2.3*	8.0 ± *0.7*
AOU12	2.0 ± *0.0*	81.0 ± *12.8*	83.5 ± *12.3*	8.3 ± *0.5*
AOU13	2.0 ± *0.0*	44.8 ± *6.9*	52.0 ± *5.9*	6.0 ± *0.6*
TAM3	1.0 ± *0.0*	48.5 ± *2.2*	48.5 ± *2.2*	5.0 ± *0.0*
TAM4	1.5 ± *0.3*	37.5 ± *3.8*	38.5 ± *4.1*	6.3 ± *0.5*
TAM9	1.0 ± *0.0*	59.3 ± *7.6*	59.3 ± *7.6*	6.5 ± *0.6*
TAM12	1.5 ± *0.3*	36.3 ± *8.0*	37.0 ± *7.7*	6.5 ± *0.3*
ANA1	2.0 ± *0.0*	30.3 ± *1.8*	42.0 ± *2.9*	9.0 ± *0.4*
ANA6	1.8 ± *0.3*	34.5 ± *3.0*	38.8 ± *1.4*	7.8 ± *0.5*
ANA8	1.8 ± *0.3*	41.8 ± *4.4*	47.5 ± *7.1*	7.3 ± *1.1*
ANA12	2.0 ± 1.0	21.5 ± 0.5	23.5 ± 3.8	4.0 ± 1.7
ANA13	1.8 ± *0.3*	56.3 ± *5.9*	61.5 ± *5.1*	7.0 ± *0.7*
ANA19	1.5 ± *0.3*	35.0 ± *4.1*	37.5 ± *3.5*	7.3 ± *0.8*
ANA20	1.3 ± *0.3*	38.3 ± *5.3*	40.5 ± *4.8*	6.0 ± *0.4*
ANA23	1.5 ± *0.3*	36.8 ± *5.4*	44.8 ± *5.9*	6.0 ± *0.9*
ANA24	1.5 ± 0.3	23.3 ± 1.2	25.8 ± 1.1	4.5 ± 0.6
ANA25	1.8 ± *0.3*	56.5 ± *5.2*	70.3 ± *6.3*	6.5 ± *0.6*
ANA26	2.0 ± *0.0*	34.5 ± *2.9*	46.8 ± *4.9*	9.0 ± *0.6*
ANA27	1.3 ± *0.3*	36.0 ± *7.0*	36.8 ± *7.8*	5.0 ± *1.1*
ANA28	1.8 ± *0.3*	51.8 ± *1.8*	56.3 ± *3.3*	6.8 ± *0.8*
ANA29	1.3 ± *0.3*	33.7 ± *3.0*	34.0 ± *3.1*	6.3 ± *1.4*
ANA30	1.3 ± *0.3*	26.5 ± *1.9*	28.8 ± *3.1*	3.8 ± *0.5*
ANA31	2.0 ± *0.0*	62.0 ± *2.9*	71.8 ± *1.7*	9.3 ± *0.5*
ANA32	1.3 ± *0.3*	36.5 ± *4.3*	36.8 ± *4.3*	4.8 ± *0.3*
ANA35	1.5 ± *0.3*	33.5 ± *3.5*	35.0 ± *3.2*	5.5 ± *1.1*
HER1	1.8 ± *0.3*	46.8 ± *3.9*	47.8 ± *3.9*	5.8 ± *0.5*
HER2	1.5 ± *0.3*	37.8 ± *1.4*	38.3 ± *1.7*	5.8 ± *0.3*
HER4	2.0 ± 0.0	38.0 ± 9.0	52.7 ± 7.8	4.6 ± 1.6
HER5	1.8 ± *0.3*	42.5 ± *1.9*	47.0 ± *3.4*	7.8 ± *0.8*
HER6	1.0 ± *0.0*	31.7 ± *0.3*	31.7 ± *0.3*	4.7 ± *0.3*
HER8	2.0 ± *0.0*	28.7 ± *1.8*	36.0 ± *5.4*	5.8 ± *1.6*
BAR2	1.8 ± *0.3*	33.5 ± *2.2*	34.8 ± *2.6*	7.3 ± *0.5*
BAR3	1.8 ± *0.3*	31.5 ± *1.0*	34.5 ± *1.9*	6.5 ± *0.3*
BAR4	1.0 ± *0.0*	38.7 ± *4.4*	38.7 ± *4.4*	5.0 ± *1.0*
BAR5	1.8 ± *0.3*	19.5 ± *1.3*	20.3 ± *1.5*	4.0 ± *0.7*
BAR6	1.3 ± *0.3*	25.5 ± *3.4*	25.8 ± *3.4*	4.5 ± *0.6*
BAR8	2.0 ± *0.0*	31.3 ± *1.4*	40.8 ± *4.1*	7.8 ± *0.9*
BAR9	1.8 ± *0.3*	47.5 ± *4.8*	48.3 ± *4.9*	7.5 ± *0.3*
BAR11	1.3 ± *0.3*	28.5 ± *2.2*	28.8 ± *2.3*	5.5 ± *0.6*

BAR12	1.8 ± *0.3*	41.5 ± *5.0*	43.3 ± *4.9*	6.5 ± *0.3*
PICUAL	1.0 ± *0.0*	43.7 ± *1.9*	43.7 ± *1.9*	5.3 ± *0.5*
HOJIBLANCA	1.8 ± *0.3*	41.5 ± *6.1*	44.5 ± *4.4*	5.5 ± *1.0*
ARBEQUINA	1.3 ± *0.3*	35.0 ± *4.0*	35.3 ± *4.3*	5.0 ± *0.7*
FRANTOIO	1.3 ± *0.3*	34.3 ± *2.5*	35.5 ± *2.3*	5.3 ± *0.5*
MEAN	1.6	40.9	43.5	6.1
MIN	1.0	17.5	17.8	1.8
MAX	2.3	95.8	98.8	11.0
CV	0.3	14.9	16.0	1.5

The *in-vitro* establishment of mature cultivars following the protocol described by Troncoso et al. (2000) had 100% success for all varieties used, the the hyperhydricity phenomenon was not observed. Although the olive micropropagation efficiency strongly relies on the cultivar used (Bayraktar et al., 2020), growth of the *in-vitro* propagated explants was observed for all tested genotypes (**Figure 6**). Finally, all genotypes could be successfully acclimatized *ex-vitro* under greenhouse conditions. In short, the resulting micropropagation cycle included: two months for the growth of micro-cuttings *in-vitro*, with an average height of 41 mm containing an average number of 6 nodes; three weeks for *in-vitro* rooting; three weeks for *ex-vitro* acclimatization. In summary, three and a half months are necessary to obtain a batch of viable plants of *in-vitro* origin, and about 3-4 weeks less if plants are rooted *ex-vitro*.

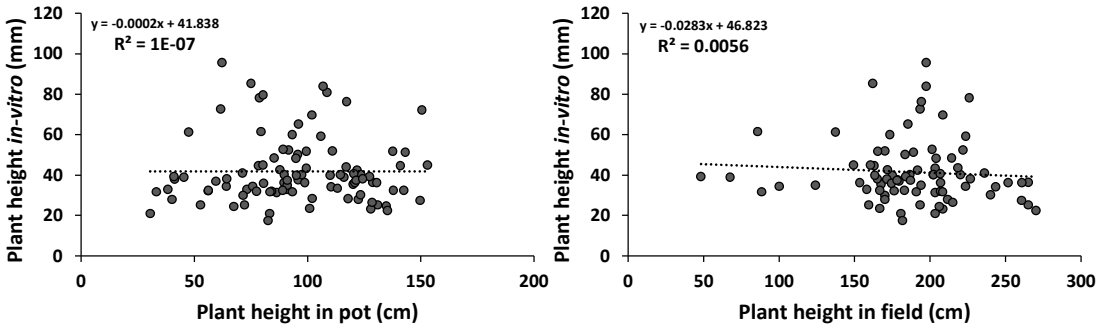


FIGURE 9 | Correlation between *in-vitro* plant height data and potted plant height data (from chapter 2) and plant height in the field (from chapter 3).

The *in-vitro* plantlet height of the different genotypes did not correlate with either the *ex-vitro* height measured in potted-plants (data obtained from chapter 2) or the plant height measured in the field (data obtained from chapter 3; **Figure 9**). Therefore, the *in-vitro* growth of the plantlets is not an indicative of their *ex-vitro* vigor. For example, AMS12 and DHO10A exhibited high *in-vitro* growth but reduced size in pot and

field conditions. However, a number of low-vigor genotypes such as AMK21, GUA9 and MAR1, which showed reduced growth under pot and field conditions, also exhibited lower growth under *in-vitro* conditions. These low-vigor genotypes, which could be of potential interest for the olive sector, have the disadvantage of low *in-vitro* multiplication rate, meaning longer time necessary to obtain complete viable plants in the nursery. In any case, once the plants of interest for the olive industry have been identified, it is necessary to optimize the *in-vitro* multiplication rooting procedures of each genotype for nursery production (Benelli and De Carlo, 2018; Mirzaei et al., 2021).

To optimize the *in-vitro* micropropagation process, the influence of lighting through the use of LEDs lights on the *in-vitro* development and growth of two wild olive genotypes has been addressed in section 1.2 (Díaz-Rueda et al., 2021).

Conclusion

The use of *in-vitro* tissue culture is a feasible strategy for the production of olive plants. We have optimized protocols for the *in-vitro* establishment and micropropagation of wild olive genotypes and mature cultivars. More importantly, we have optimized a procedure for simultaneous rooting and acclimatization to *ex-vitro* conditions that could be of interest for the potential production of grafted olive plants in certified nurseries. These procedures allowed also the production of thousands of viable plants that were later used in the different trials carried out in this PhD project.

Section 1.2. Growth Quality and Development of Olive Plants Cultured *In-Vitro* under Different Illumination Regimes

This section has been published as: **Díaz-Rueda, P.**, Cantos-Barragán, M., Colmenero-Flores, J.M. (2021). Growth Quality and Development of Olive Plants Cultured *In-Vitro* under Different Illumination Regimes. *Plants*, *10*, 2214. https://doi.org/10.3390/plants10102214

plants

MDPI

Article

Growth Quality and Development of Olive Plants Cultured In-Vitro under Different Illumination Regimes

Pablo Díaz-Rueda *, Manuel Cantos-Barragán and José Manuel Colmenero-Flores *

Instituto de Recursos Naturales y Agrobiología, Spanish National Research Council (CSIC), Av. Reina Mercedes 10, 41012 Sevilla, Spain; cantos@irnase.csic.es

* Correspondence: pdiaz@irnas.csic.es (P.D.-R.); chemacf@irnase.csic.es (J.M.C.-F.)

Abstract: Light-emitting diodes (LEDs) are useful for the in-vitro micropropagation of plants, but little information is available on woody species. This work compares the effects of light quality and intensity on the growth and development of micropropagated olive plants from two different subspecies. Illumination was provided with fluorescent and LED lamps covering different red/blue ratios (90/10, 80/20, 70/30, 60/40) or red/blue/white combinations, as well as different light intensities (30, 34, 40, 52, 56, 84, 98 and 137 µmol m^{-2} s^{-1} of photosynthetic photon fluxes, PPF). Olive plants exhibited high sensitivity to light quality and intensity. Higher red/blue ratios or lower light intensities stimulated plant growth and biomass mainly as a consequence of a higher internodal elongation rate, not affecting either the total number of nodes or shoots. In comparison to fluorescent illumination, LED lighting improved leaf area and biomass, which additionally was positively correlated with light intensity. Stomatal frequency was positively, and pigments content negatively, correlated with light intensity, while no clear correlation was observed with light quality. In comparison with fluorescent lamps, LED illumination (particularly the 70/30 red/blue ratio with 34 µmol m^{-2} s^{-1} PPF intensity) allowed optimal manipulation and improved the quality of in-vitro micropropagated olive plants.

Keywords: LED illumination; fluorescent illumination; *Olea europaea*; micropropagation; internodal elongation

check for updates

Citation: Díaz-Rueda, P.; Cantos-Barragán, M.; Colmenero-Flores, J.M. Growth Quality and Development of Olive Plants Cultured In-Vitro under Different Illumination Regimes. *Plants* **2021**, *10*, 2214. https://doi.org/10.3390/plants10102214

Academic Editor: Francisco M. del Amor Saavedra

Received: 4 October 2021
Accepted: 13 October 2021
Published: 18 October 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Olive (*Olea europaea*, L.) is a woody crop belonging to the *Oleaceae* family. It is a medium-sized tree of about 4 to 8 m high, depending on the variety. The *Olea europaea* species is found in the Mediterranean area [1] and is the only one of the genus that produces edible fruit [2]. Olive is a crop that is well adapted to Mediterranean drylands, with very acceptable productions and is capable of surviving periods of intense water deficit. It does not tolerate temperatures below −10 °C for prolonged periods of time. It is a plant highly dependent on light, so that light deficiency reduces the number of flowers or their viability. Despite its good climatic adaptation, it is a slow-growing tree, requiring optimal multiplication procedures. Micropropagation has arisen as a successful technique for plant propagation through in-vitro tissue culture technology. An increase of in-vitro culture efficiency for olive requires the optimization of temperature, lighting and ventilation parameters. Of them, light quality and quantity play essential roles in plant photosynthesis, development and organogenesis [3–6].

Plants respond to light quantity and quality through photoreceptors and photomorphogenic responses [7,8], regulating different aspects of plant development, including seed germination, seedling elongation, vegetative growth and architecture, flowering and senescence [9]. Among photoreceptors, phytochromes absorb light wavelengths from the whole spectrum, but most importantly in the red and far-red regions [10,11]. Phytochromes affect the regulation of phytohormones such as auxin [12] salicylic and jasmonic acid [13,14].

Introduction

Olive (*Olea europaea*, L.) is a woody crop belonging to the *Oleaceae* family. It is a medium-sized tree of about 4 to 8 m high, depending on the variety. The *Olea europaea* species is found in the Mediterranean area (Zohary et al., 2012) and is the only one of the genus that produces edible fruit (Barranco et al., 1998). Olive is a crop that is well adapted to Mediterranean drylands, with very acceptable productions and is capable of surviving periods of intense water deficit. It does not tolerate temperatures below −10 °C for prolonged periods of time. It is a plant highly dependent on light, so that light deficiency reduces the number of flowers or their viability. Despite its good climatic adaptation, it is a slow-growing tree, requiring optimal multiplication procedures. Micropropagation has arisen as a successful technique for plant propagation through *in-vitro* tissue culture technology. An increase of *in-vitro* culture efficiency for olive requires the optimization of temperature, lighting and ventilation parameters. Of them, light quality and quantity play essential roles in plant photosynthesis, development and organogenesis (Hoenecke et al., 1992; Saebo et al., 1995; Li and Kubota, 2009; Vieira et al., 2015).

Plants respond to light quantity and quality through photoreceptors and photomorphogenic responses (Beattie et al., 2018; Paradiso and Proietti, 2021), regulating different aspects of plant development, including seed germination, seedling elongation, vegetative growth and architecture, flowering and senescence (Quail, 2010). Among photoreceptors, phytochromes absorb light wavelengths from the whole spectrum, but most importantly in the red and far-red regions (Shinomura et al., 1996; Davis and Burns, 2016). Phytochromes affect the regulation of phytohormones such as auxin (Marks and Simpson, 1999) salicylic and jasmonic acid (Pieterse et al., 2012; Lazzarin et al., 2021). Other plant photoreceptors include cryptochromes and phototropins, which respond to blue and ultraviolet (UV) light. Cryptochromes perceive blue and UV-A light (370–450 nm), regulating many physiological and developmental processes, such as chlorophyll biosynthesis, response to high-irradiance stress and photomorphogenesis (Zheng et al., 2019). Phototropins mainly mediate phototropic

responses triggered by blue and UV-A light in the 320–500 nm wavelength range (Wang et al., 2013).

Fluorescent lamps, traditionally used for *in-vitro* culture, increase the temperature of the plant culture chamber, making necessary more expensive cooling systems and therefore higher energy consumption. In contrast, light-emitting diodes (LED) lighting has clear benefits for optimizing the performance of plant growth chambers, including smaller size, longer life, lower temperature and the option to regulate single wavelength emission (Bula et al., 1991; Brown et al., 1995; Li et al., 2010). It is well known that the peak spectral output of red and blue LED chips coincide closely with the main absorption peaks of chlorophyll and the reported wavelengths for maximum photosynthetic efficiency (Benedict et al., 1972; Moon et al., 2006). Therefore, the use of LED illumination has become considerably more attractive for plant *in-vitro* culture and, particularly, for micropropagation. Thus, several plant species have been grown successfully *in-vitro* under LED illumination (Gupta and Jatothu, 2013). They include plantlets of banana (Vieira et al., 2015), cotton (Li et al., 2010), *Lippia filifolia* (Chaves et al., 2020) and rapeseed (Li et al., 2013), cultured under different light qualities including fluorescent lamps and different combinations of blue vs. red LEDs. In addition, the use of LED illumination for the *in-vitro* culture of some woody plant species, such as *Eucalyptus urophylla* (Miranda et al., 2020), *Cedrela fissilis* (Oliveira et al., 2020), *Pinus sylvestris* and *Abeis borisii-regis* (Smirnakou et al., 2017), has been reported. These results confirmed positive effects of LED illumination on physiological and morphological parameters of *in-vitro* grown plants. However, responses vary according to plant species and it is necessary to empirically determine the effects of light quality and intensity resulting from different spectral combinations of LED lighting.

LED illumination facilitates the use of different light spectra to induce different developmental responses, for example, shoot multiplication, internode elongation, rooting, hardening, or even flowering in different plant species (McNellis and Deng, 1995; Chory et al., 1996; Chory, 1997; Chang et al., 2003; Kim et al., 2004; Ausin et al., 2005; Folta and Maruhnich, 2007; Kurepin and Pharis, 2014; Luan et al., 2015). Many authors have reported different techniques for *in-vitro* olive culture, such as somatic embryogenesis (Leva et al., 1995; Trabelsi et al., 2011; Mazri et al., 2020), zygotic

embryogenesis (García et al., 2002), callus culture (Gentile and Uccella, 2014; Mohammad et al., 2019) and micropropagation (Zacchini and Agazio, 2004). Micropropagation for the conservation of olive genetic diversity has been widely applied, and many olive cultivars have been micropropagated successfully. This process is influenced by many factors, mainly, the medium composition, the plant genetic background, the physiological and sanitary state of the mother plant and physical factors, among which light and temperature of the growth chamber are particularly relevant. Related to light influence, scarce information is available on *in-vitro* culture of olive, particularly light studies have been reported for the improvement of *ex-vitro* rooting from microplantlets (Leva, 2011); callus culture (Mohammad et al., 2019; Mirzaei et al., 2021) or the reduction of vitrification (Grigoriadou et al., 2007). However, no information is available on the effect of light quality and quantity on the *in-vitro* growth of micropropagated *Olea europaea* plants, nor on the specific benefits of LED lighting. The aim of this study was to evaluate to what extent light quality and intensity regulate developmental, morphological, anatomical, physiological and photosynthetic parameters of micropropagated olive plants. For this purpose, two wild olive genotypes belonging to two different subspecies of *Olea europaea* were micropropagated under different illumination regimes supplied by both fluorescent and LED lighting, and the following parameters were measured: total stem length, internodal length, number of nodes, number of shoots, shoot and callus biomass, total leaf area, number of leaves, stomatal size, stomatal frequency and content of photosynthetic pigments.

Material and Methods

Plant Material

Plantlets of two wild varieties belonging to different subspecies of *Olea europaea* were obtained by *in-vitro* germination: *Olea europaea* subsp. *europaea* genotype AMK34 and *Olea europaea* subsp. *guanchica* genotype GUA7 (**Supplementary Figure S5**; (Díaz-Rueda et al., 2020)). Uninodal explants (1 cm length) with one axillar bud were replicated. Apical explants were avoided in order to assure homogeneous responses. Explants were incubated in sterile 200 mL SIGMA jars (Merck V0633; Sigma-Aldrich,

Darmstadt, Germany) containing 30 mL of olive culture medium (Rugini, 1984), supplemented with zeatin (1 mgL^{-1}), 20 gL^{-1} mannitol and 6 gL^{-1} agar. The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C for 20 min. Six explants were incubated per jar under sterile conditions, avoiding direct contact of axillary buds with the agar culture medium. Jar lids were sealed with breathable sealing film (Parafilm, Pechiney Plastic Packaging, Menasha, WI, USA) and incubated in the growth chamber for 69 days. Growing conditions were 23 ± 2 °C and a 16 h light photoperiod, under the lighting conditions described in Table 1. Twelve explants were evaluated per treatment and genotype.

TABLE 1 | Lighting conditions assayed in this study.

Red/Blue Ratio (%)	Photon Flux (µmol m^{-2} s^{-1})	Nomenclature
LED 90/10	56	R9B1-56
LED 80/20	30	R8B2-30
LED 70/30	34	R7B3-34
LED 70/30	52	R7B3-52
LED 60/40	40	R6B4-40
LED 70/30 + white	84	R7B3W-84
LED 60/40 + white	98	R6B4W-98
Fluorescent	137	TFL-137

Illumination Treatments

Light treatments, which included seven types of LED quality/intensity combinations and one fluorescent light, are summarized in Table 1. Blue/Red illumination was obtained with intercalated red and blue 14.5 W/m LED strips. The blue LED has a peak emission at 455 nm and the red LED at 630 nm. Red/Blue treatment combinations included: 90%/10% with a photosynthetic photon flux (PPF) of 56 µmol $m^{-2}s^{-1}$ or µE (R9B1-56); 80%/20% (R8B2-30) with 30 µmol $m^{-2}s^{-1}$ PPF; 70%/30% with 34 µmol $m^{-2}s^{-1}$ PPF (R7B3-34); 70%/30% with 52 µmol $m^{-2}s^{-1}$ PPF (R7B3-52); and 60%/40% with 40 µmol $m^{-2}s^{-1}$ PPF (R6B4-40). Red/Blue/White illumination was obtained with intercalated red, blue and white 14.5 W/m LED strips: Red/Blue 70%/30% plus white LED with 84 µmol $m^{-2}s^{-1}$ PPF (R7B3W-84); and Red/Blue 60%/40% plus white LED with 98 µmol $m^{-2}s^{-1}$ PPF (R6B4W-98). When white LED lighting was supplemented to the R/B lighting, a 50% white vs. 50% R/B was used. The R/B ratio was also adjusted within the white LEDs lighting in order to equal it to that of the R/B lighting in the corresponding treatment

(R7B3 in the R7B3W-84 treatment and R6B4 in the R6B4W-98 treatment). The fluorescent lighting consisted of daylight (6500 °K) tubular fluorescent lamps with 137 µmol $m^{-2}s^{-1}$ PPF (TFL-137). The resulting spectral patterns are given in Supplementary Figure S6.

For a given number of LED chips, changes in the light quality (R/B ratio) inherently lead to changes in the light intensity (photon flux). To overcome the problem that two variables are assigned to a single treatment, different approaches have been addressed. On the one hand, in order to compare the effect of different light intensities without modifying light quality, we have compared plant responses to the following treatments: (i) R7B3-34 vs. R7B3-52 vs. R7B3-84; (ii) R6B4-40 vs. R6B4-98. The R7B3-34 vs. R7B3-52 comparison was possible by increasing the number of chips without modifying the R/B ratio. The additional treatment R7B3-84 was applied through supplementation with white LEDs. The same approach was used with the R6B4-98 treatment to make possible the comparison of the R6B4-98 vs. the R6B4-40 treatments. On the other hand, in order to compare the effect of light quality (different R/B ratios) without modifying the intensity (so that a single variable is assigned to a single treatment) we have compared the plant responses to the following treatments: (i) R9B1-56 vs. R7B3-52, given that 56 vs. 52 µmol/m^2s^{-1} light intensities are similar enough not to involve significant differences in plant responses; (ii) R8B2-30 vs. R7B3-34, given that 30 vs. 34 µmol/m^2s^{-1} light intensities are similar enough not to involve significant differences in plant responses; iii) R6B4-98 vs. R7B3-84, given that 98 vs. 84 µmol/m^2s^{-1} light intensities are similar enough not to involve significant differences in plant responses.

Determination of Growth and Developmental Parameters

For the quantification of vegetative development, the following parameters were quantified during the course of explants growth: plant height, stem length, number of shoots, number of nodes, and number of leaves. After 69 days of growth, plantlets were harvested and fresh weight (FW) was obtained. Dry weight (DW) and water content (WC) was calculated after drying the explants at 70 °C for 72 h. WC was determined in leaves obtained from six plants, using all leaves per plant according to the following equation:

$$WC\ (\%) = [(100\ x\ (FW - DW)]\ (FW)^{-1.} \quad (1)$$

After removing plantlets from the jars and before oven incubation, the leaves were excised and scanned in an Epson Stylus DX4000 multifunction printer (Seiko Epson Corp., Owa, Japan) to obtain total leaf area through pixel quantification with the Software 'Medición de Hojas v1.0' (Developed at the Department of Ecology, University of Seville, Spain; Taguas and Rivero, 1989).

Determination of Photosynthetic Pigments

Photosynthetic pigments were extracted from fully expanded leaves using 0.1 g of fresh plant material in 10 mL of 80% aqueous acetone (n = 12). After filtering, 1 mL of the suspension was mixed with 2 mL acetone, and chlorophyll-a (Chl a), chlorophyll-b (Chl b) and carotenoid (Cx + c) content (µg g^{-1} FW) was determined using a spectrophotometer (U-2001; Hitachi Ltd., Tokyo, Japan) measuring the absorbance at 663 nm, 645 nm and 470 nm, respectively, according to Arnon (1949).

$$Chlorophyll\ A\ (Chl_a) = 0.0127 \cdot D_{663} - 0.00269 \cdot D_{645} \quad (2)$$

$$Chlorophyll\ B\ (Chl_b) = 0.0229 \cdot D_{645} - 0.00468 \cdot D_{663} \quad (3)$$

$$Chlorophyll\ (Chl) = Chl_a + Chl_b \quad (4)$$

$$Carotenoids = ((1000 \cdot D_{470}) - (1{,}82 \cdot Chl_a) - (85{,}02 \cdot Chl_b)) / 198. \quad (5)$$

Number and Size of Leaf Stomata and Trichomes

Ten samples from three fully expanded leaves, each leaf belonging to a different plant in a total of three plants per treatment, were used. Analysis of abaxial leaf cells was carried out in epidermal impressions, performed as described in Gitz and Baker (2009). Number and size of leaf stomata and trichomes was quantified on a Zeiss Axioskop microscope equipped with Nomarski optics, AxioCam MRc5, and the Zeiss AxioVision software (Freeware 'Carl Zeiss AxioVision Rel.4.9.1.0' available at the Zeiss Homepage http://www.zeiss.com/ (accessed on 20 September 2021), Carl Zeiss Microscopy GmbH, Jena, Germany) as described in Franco-Navarro et al. (2016).

Statistical Analyses

Unless otherwise specified, the data analysis was carried out from 12 single-plantlet replications within each treatment. The data from all experiments were subject to analysis of variance (ANOVA) and levels of significance are indicated in the figures by asterisks: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Non-significant (ns) differences were indicated when $p > 0.05$. Multiple comparisons of means were analyzed by Tukey's HSD (honestly significant difference). A multiple range test was calculated using the Statistical Analysis System (STATGRAPHICS Centurion XVI software; http://www.statgraphics.com (accessed on 20 September 2021); StatPoint Technologies, Warrenton, VA, USA). In the graphics where the correlation between the photosynthetic photon flux and different growth/developmental parameters were plotted, the regression lines and the coefficient of determination (R^2) were obtained using the corresponding function in Excel (Microsoft Office 2016 for Windows; Microsoft Corporation, Redmond, Washington DC, USA).

Results

Effect of Light Quality and Intensity on Stem Growth and Development

A clear variation of plant growth was observed in micropropagated olive plants treated with different light regimes (**Figure 1**).

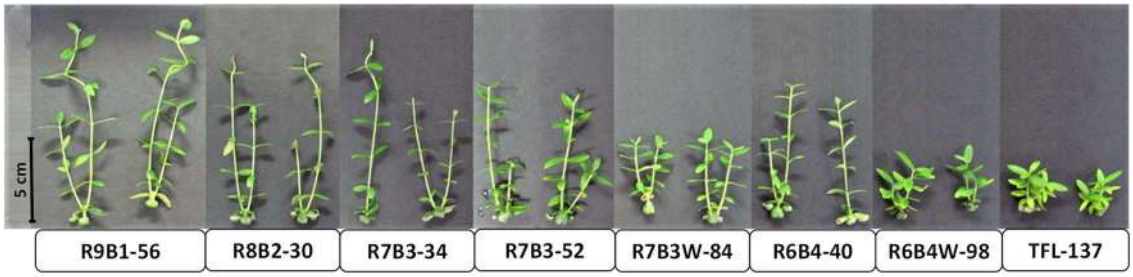


FIGURE 1 | Effect of illumination quality and quantity on micropropagated olive plantlets. Different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) were used for *in-vitro* clonal propagation of the AMK34 genotype of *Olea europaea* subsp. *europaea*. Shoot explants were cultivated for 69 days.

Negative and statistically significant correlations ($p \leq 0.001$) were observed when light intensity was compared with the total stem length (**Figure 2A**) and the internodal

length (**Figure 2C**) of AMK34 and GUA7 plants. A higher stem length correlated not only with a lower light intensity, but also with a higher red:blue ratio (**Figure 2B and 2D**) in both wild olive subspecies. Thus, plants subjected to greater light intensities or lower red:blue ratio exhibited shorter stems (**Figure 2A and 2B**). The effect of light quality and intensity on shoot height was clearly a consequence of the internode length in both genotypes (**Figure 2C and 2D**), not affecting either the total number of shoots or nodes per explant (**Supplementary Figure S1**).

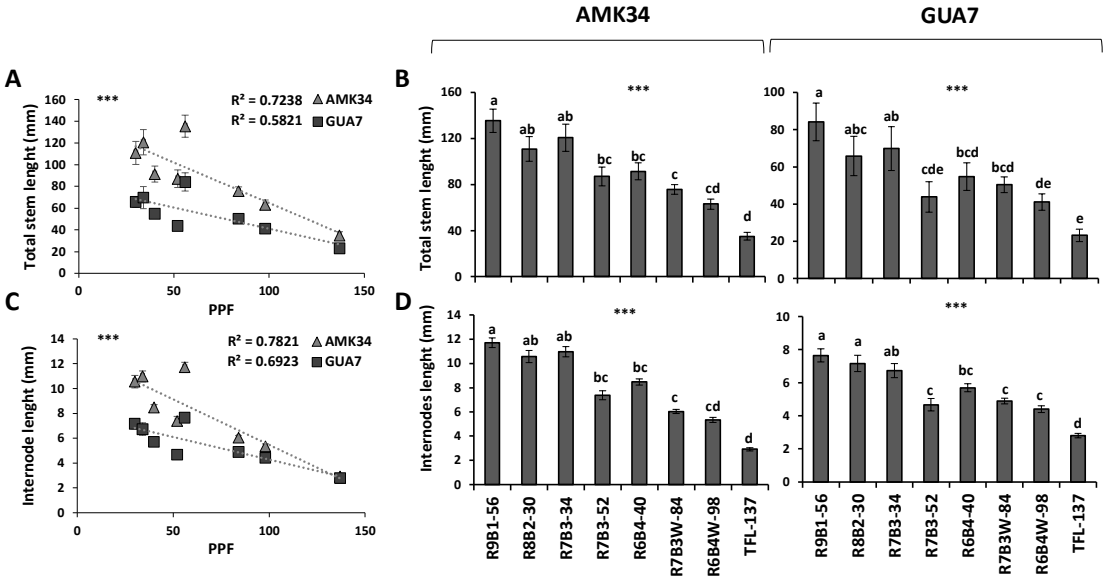


FIGURE 2 | Effect of light quality and quantity on stem length and internodal elongation of micropropagated olive plantlets. Effect of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on total stem length (**A**, **B**) and average internodal length (**C**, **D**) of different *Olea europaea* subspecies: subsp. *europaea* (genotype AMK34); and subsp. *guanchica* (genotype GUA7). PPF, photosynthetic photon flux. Level of significance: *** $p \leq 0.001$.

Therefore, light quality and light intensity determined independent photomorphogenic responses that could be distinguished from each other, comparing the different combinations of lighting regimes applied. On the one hand, for the same light quality (R:B, 70%:30%), the higher light intensity in the R7B3-52 treatment (52 µmol $m^{-2}s^{-1}$ PPF) induced significantly shorter stems (**Figure 2B**) and shorter internodes (**Figure 2D**) than the 34 µmol $m^{-2}s^{-1}$ PPF light intensity (R7B3-34 treatment) in both olive genotypes. A similar response was observed when the effect of the R6B4W-98 treatment on total stem length (**Figure 2B**) and internode length (**Figure 2D**) was compared with that of the R6B4-40 treatment. On the other hand, when similar light intensities (56 and 52 µmol $m^{-2}s^{-1}$ PPF) were compared in the R9B1-56 and R7B3-52

treatments, respectively, the 90% red light present in the R9B1-56 treatment determined significantly longer stems (**Figure 2B**) and internodes (**Figure 2D**) than the 70% red light content of the R7B3-52 treatment.

Plants subjected to daylight white fluorescent illumination (137 µmol $m^{-2}s^{-1}$ PPF) resulted in the shortest internodes and the lowest stem height (**Figures 1 and 2**) throughout the whole period of plant growth (**Supplementary Figure S2**). The strong stem shortening caused by fluorescent lights is a general response in the olive species as could be observed not only in AMK34 and GUA7, but also in other wild olive varieties from the *europaea* subspecies (**Supplementary Figure S3**).

This response determined significant differences in the shoot biomass of AMK34 (**Figure 3A and 3B**) and GUA7 (**Figure 3D and 3E**) genotypes. Thus, a negative correlation ($p \leq 0.001$) between the light intensity (PPF) and the dry biomass was observed in *in-vitro* grown plants (**Figure 3A and 3D**). Interestingly, no differences were observed in the callus biomass (**Figure 3C and 3F**).

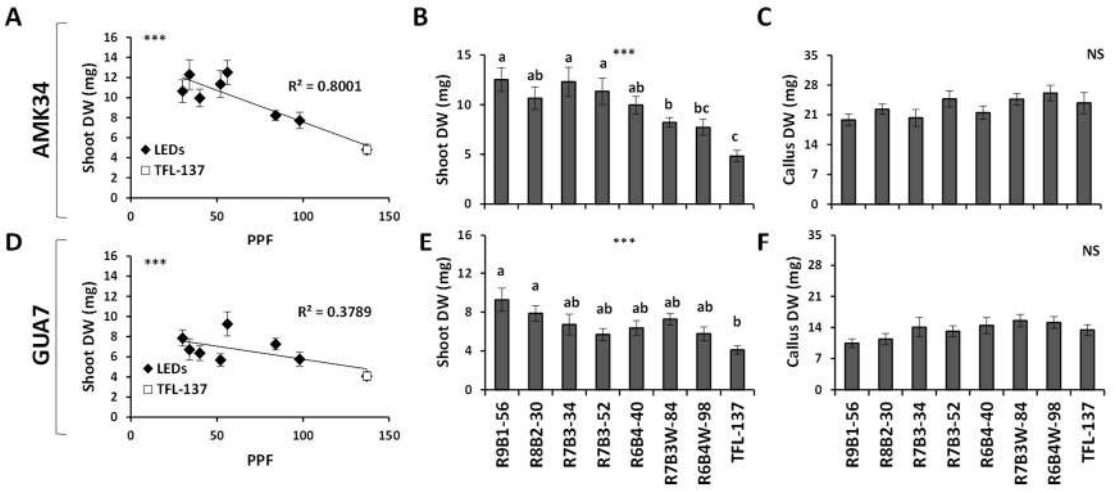


FIGURE 3 | Effect of illumination quality and quantity on plant biomass. Correlation of light intensity with shoot dry weight (DW) in the olive genotypes AMK34 (**A**) and GUA7 (**D**). Effects of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on shoot DW (**B**, **E**) and callus DW (**C**, **F**) of the olive genotypes AMK34 and GUA7, respectively. PPF, photosynthetic photon flux. Levels of significance: *** $p \leq 0.001$; and non-significant (NS).

Therefore, it can be concluded that micropropagated olive plants are highly sensitive to light quality and intensity, which regulates the internodal elongation rate, but not the total number of nodes, affecting plant growth and biomass.

Effect of Light Quality on Leaf Growth, Development and Pigment Composition

LED light intensity showed significant positive correlation ($p \leq 0.001$) with total leaf area (**Figure 4A**) and leaf fresh weight (**Figure 4B**) in both AMK34 and GUA7 genotypes. However, the fluorescent light treatment did not fit this correlation, showing abnormally low values of total leaf area and biomass that did not correlate with the equivalent light intensity in the group of plants treated with LED illumination (**Figure 4A and 4B**).

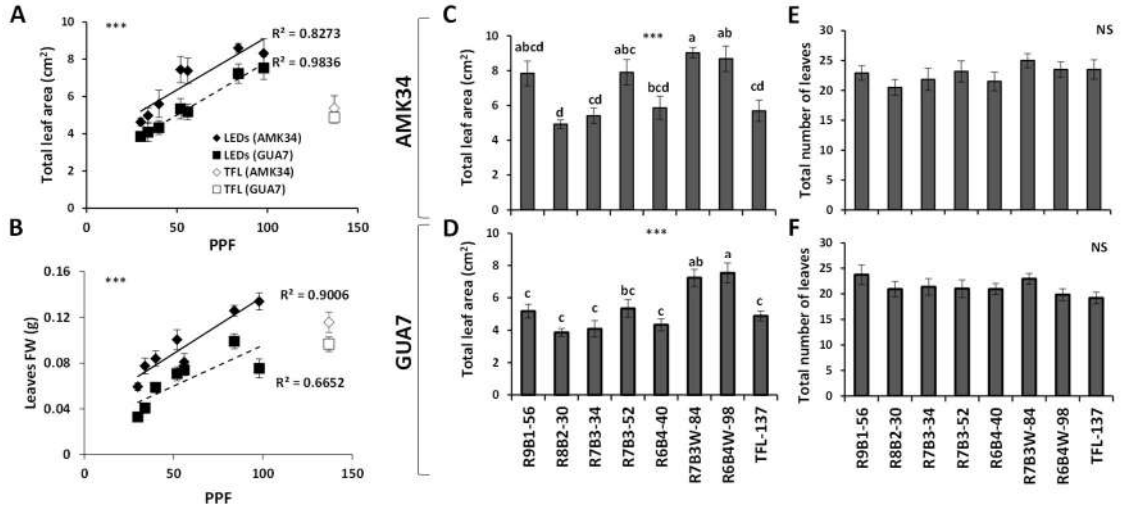


FIGURE 4 | Effect of illumination quality and quantity on leaf area and leaf fresh weight. Correlation of light intensity with the leaf area in the AMK34 and GUA7 olive genotypes (**A**). Correlation of light intensity with the leaf fresh weight in the AMK34 and GUA7 olive genotypes (**B**). Effect of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on the total leaf area of AMK34 (**C**) and GUA7 (**D**) plants. Effects of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on the total number of leaves in AMK34 (**E**) and GUA7 (**F**) plants. PPF, photosynthetic photon flux. Levels of significance: *** $p \leq 0.001$; and non-significant (NS).

Contrary to what was observed for the elongation of internodes and stem, light quality did not clearly affect either the total leaf area (**Figure 4C and 4D**) or the total number of leaves (**Figure 4E and 4F**) in any olive genotype. Thus, different red vs. blue light ratios in R9B1-56 and R7B3-52 treatments having similar light intensities (56 and 52 µmol $m^{-2}s^{-1}$ PPF respectively), did not determine differences in either leaf area (**Figure 4C and 4D**) or total number of leaves (**Figure 4E and 4F**).

Besides total leaf area, stomatal size and density can affect water relations in plants, which is important when considering the *ex-vitro* acclimatization success of micropropagated plants. Slight but significant correlations between light intensity and

stomatal development could be observed: a negative correlation with the stomatal size (**Figure 5A**); and a positive correlation with the stomatal frequency (**Figure 5B**). Thus, a higher light intensity determined a higher density of smaller stomata. Interestingly, the fluorescent light fitted this correlation together with other LED lights, in contrast to the leaf area and biomass responses (**Figure 4**). Light quality did not clearly affect either the stomatal size or stomatal frequency of the olive variety AMK34 (**Figure 5C and 5D**).

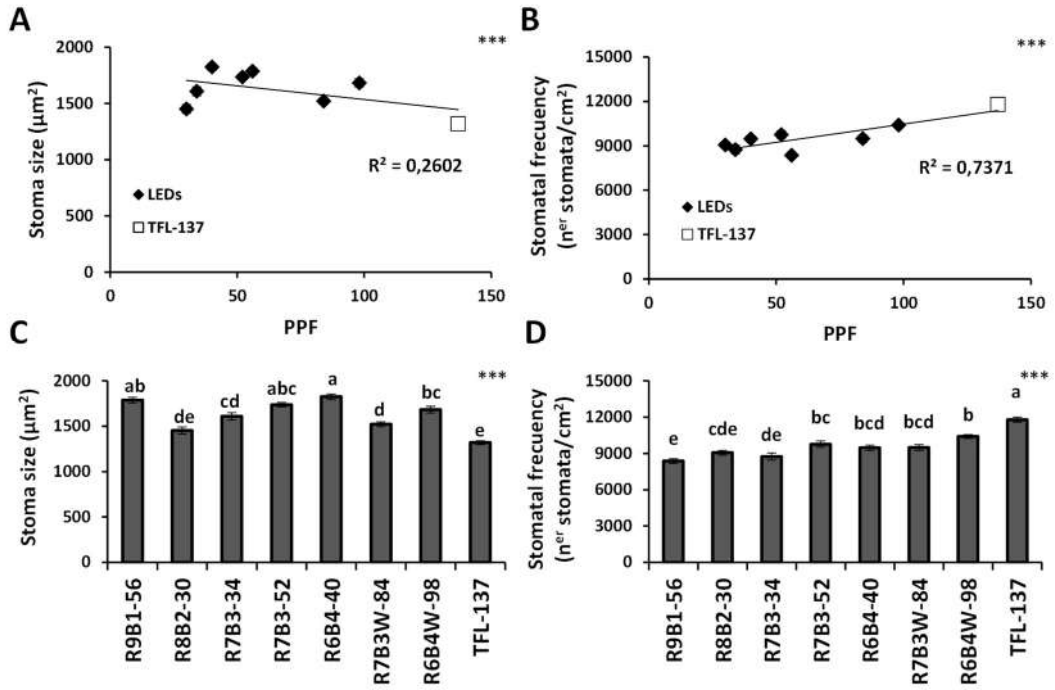


FIGURE 5 | Effect of illumination quality and quantity on stomatal size and frequency. Correlation of light intensity with the stomatal size (A) and the stomatal frequency (B) in *in-vitro* grown AMK34 plants. Effect of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on the stomatal size (C) and the stomatal frequency (D) in *in-vitro* grown AMK34 plants. PPF, photosynthetic photon flux. Levels of significance: *** $p \leq 0.001$.

Finally, light intensity showed negative correlations with the content of the photosynthetic pigments chlorophyll (**Figure 6A**) and carotenoids (**Figure 6B**) in the AMK34 olive genotype. Light quality affected pigment content since different red:blue ratios in the treatments R9B1-56 and R7B3-52, having similar light intensities, determined significant differences in chlorophyll-A (**Figure 6E**). Thus, a lower red:blue ratio determined a reduction of chlorophyll-A. Similar trends were observed in chlorophyll-B (**Figure 6F**) and carotenoids (**Figure 6D**), although differences were not statistically significant. Thus, lower light intensity and/or a higher red:blue ratio determined a higher content of photosynthetic pigments.

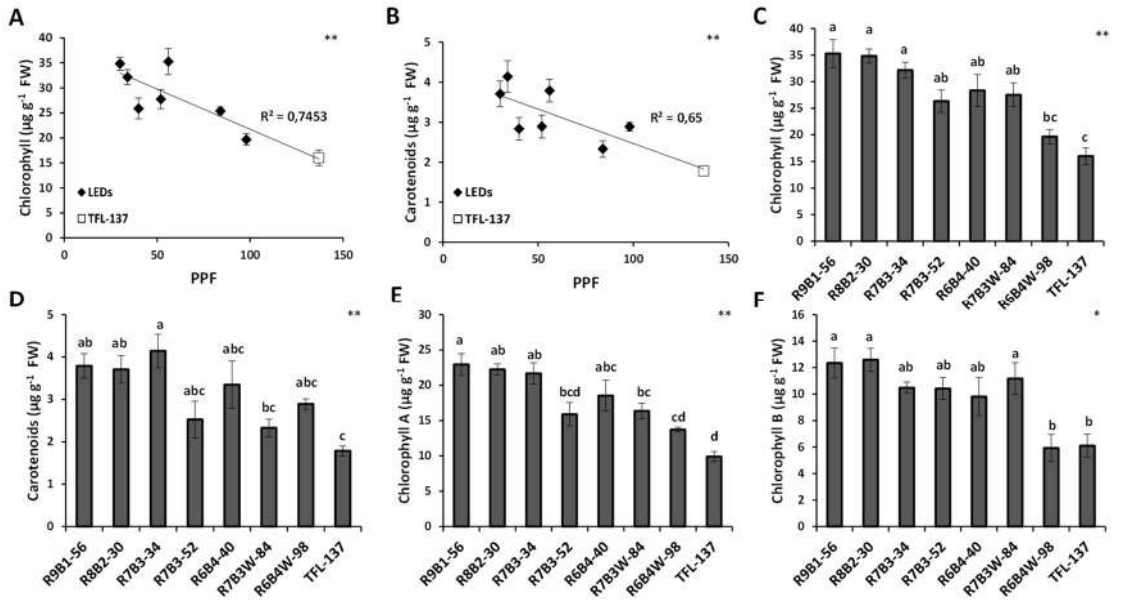


FIGURE 6 | Effect of illumination quality and quantity on pigment composition. Correlation between light intensity and total chlorophylls (**A**) or carotenoid pigments (**B**) of micropropagated AMK34 plant leaves. Effects of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on: total chlorophylls (**C**) and carotenoid pigments (**D**) in micropropagated shoots of AMK34 plants. Effects of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on: chlorophyll-A (**E**) and chlorophyll-B (**F**). PPF, photosynthetic photon flux. Levels of significance: * $p \leq 0.05$; ** $p \leq 0.01$.

In conclusion, light quality and quantity affected important morphological, anatomical and biochemical parameters, allowing optimal regulation of plant quality during *in-vitro* olive micropropagation.

Discussion

Olea europaea is widely used worldwide for the production of oil and table olives, so that improving plant propagation procedures is of great interest for the olive industry. Lighting is one of the most important environmental factors determining optimal plant propagation. Knowledge about plant photomorphogenesis has increased drastically due to the growing use of LED illumination (Paradiso and Proietti, 2021). In this work, we show that LED illumination improved the *in-vitro* growth of olive explants in comparison with fluorescent lamps (**Figures 1–3**). Among different LED lighting regimes, the 70:30 red:blue ratio with 34 µmol $m^{-2}s^{-1}$ PPF combined low energy consumption with adequate development in terms of plant growth (**Figures 1 and 2**), internodal length (**Figure 2**), biomass (**Figure 3**) and accumulation of photosynthetic pigments (**Figure 6**).

Adequate internodal length allows optimal manipulation of *in-vitro* olive explants and therefore higher micropropagation rates. In addition, the R7B3-34 lighting regime reduced the leaf area and biomass (**Figure 4**), as well as the stomatal density (**Figure 5**), which is expected to reduce water loss during the sensitive *ex-vitro* acclimatization process.

The internodal elongation rate of micropropagated olive plants, but not the total number of nodes, was highly sensitive to both light quality and intensity (**Figures 1 and 2**). Thus, low illumination intensities (e.g., 30–50 µmol $m^{-2}s^{-1}$) and/or high red:blue ratios (e.g., 90:10–70:30) enhanced internodal elongation, facilitating the propagation of uninodal explants. Different problems have been associated with the propagation of olive shoots containing short internodal segments: the easy chance to damage axillar meristems, the contact of leaves with the medium and the difficulty to adequately embed the explant into the agar medium. These problems frequently cause necrosis and give rise to undifferentiated structures in micropropagated olive explants. This undesirable growth is induced by high light intensity or low B:R ratios (e.g., R6B4W-98 and, especially, the fluorescent TFL-137 lighting). A negative effect of fluorescent lighting on shoot growth in comparison with LED illumination has also been reported in other plant species (Akoyunoglou and Anni, 1984; Moon et al., 2006; Saito et al., 2010; Li et al., 2013; Vieira et al., 2015; Oliveira et al., 2020). We show in this work the reduction of shoot biomass (**Figure 3A–D**) and lower pigments content (**Figure 6**) in response to higher light intensity, indicating that PPF values higher than 50 µmol $m^{-2}s^{-1}$ impairs *in-vitro* culture of olive plants. Previous reports also observed a decrease of dry weight in plants grown *in-vitro* at high light intensity (Silva et al., 2017; de Hsie et al., 2019). This phenomenon is probably the consequence of a suboptimal cell elongation rate under high light intensity, affecting different photosynthetic organs: the internodes, producing abnormally short stems (**Figure 2C and 2D**); and the leaves, reducing leaf area (**Figure 4A**) and biomass (**Figure 4B**). This ultimately impairs the correct *in-vitro* growth and the development of olive plants. *In-vitro* culture of plant tissues is usually carried out at a relative low PPF (37–70 µmol $m^{-2}s^{-1}$ PPF) (Pierik, 1990) since it is assumed that photosynthesis is limited by the low CO_2 concentration in the vessel, while this limitation is counteracted by sucrose supply in the medium (Serret et al., 2001).

The stimulation of stem elongation in response to a higher red/blue ratio has also been described in other micropropagated species. Conversely, blue light is known to inhibit stem elongation (Mortensen and Strømme, 1987; da Silva and Debergh, 1997; Schuerger, 1997; Kim et al., 2004; Poudel et al., 2008; Simlat et al., 2016; Zheng et al., 2019). These responses are associated with differences in the relative contribution of blue-sensitive photoreceptors (cryptochromes and phototropins) and phytochromes (Paradiso and Proietti, 2021). Blue light has an important role in chlorophyll formation and chloroplast development (Senger, 1982; Kim et al., 2004; Islam et al., 2012). At the molecular level, blue light upregulates genes involved in the synthesis of enzymes involved in chlorophyll biosynthesis, promoting chlorophyll accumulation (Lobiuc et al., 2017). However, we have observed a reduction of leaf chlorophyll content when the red/blue ratio was decreased from 9:1 to 7:3 (**Figure 6A and 6C**). There are also reports showing that monochromatic blue light reduces chlorophyll content in some species, while no effect was found in other ones (Abidi et al., 2013; Wang et al., 2015). In any case, there is consensus that a minimal blue light proportion threshold, quantified as 7%, is required to avoid dysfunctions associated to very high red/blue ratios (Hogewoning et al., 2010). Another component of light quality affecting plant morphological responses is the red/far-red ratio, which regulates stem elongation, branching, leaf expansion, and reproduction (Zheng et al., 2019). Since no far-red light has been used in the LED illumination regimes assayed in this work, we can conclude that regulation of adequate red/blue ratios is sufficient to optimize the shoot elongation rate and the internodal length of micropropagated olive plants.

Light quality/intensity can be used to regulate leaf growth *in-vitro*. We observed a positive correlation between light intensity and both leaf area and leaf fresh weight (Figure 4A,B). This phenomenon, previously observed in other micropropagated plants (Batista et al., 2018), is probably a consequence of an increased net leaf photosynthesis (Zhang et al., 2019). Interestingly, this correlation was not observed in other tissues like the callus (Figure 3). Other authors, such as Morini et al. (2000)**,** reported that callus production was not influenced by light quality in *in-vitro* propagated quince, although far-red plus blue light reduced callus growth. However, it was reported that red light

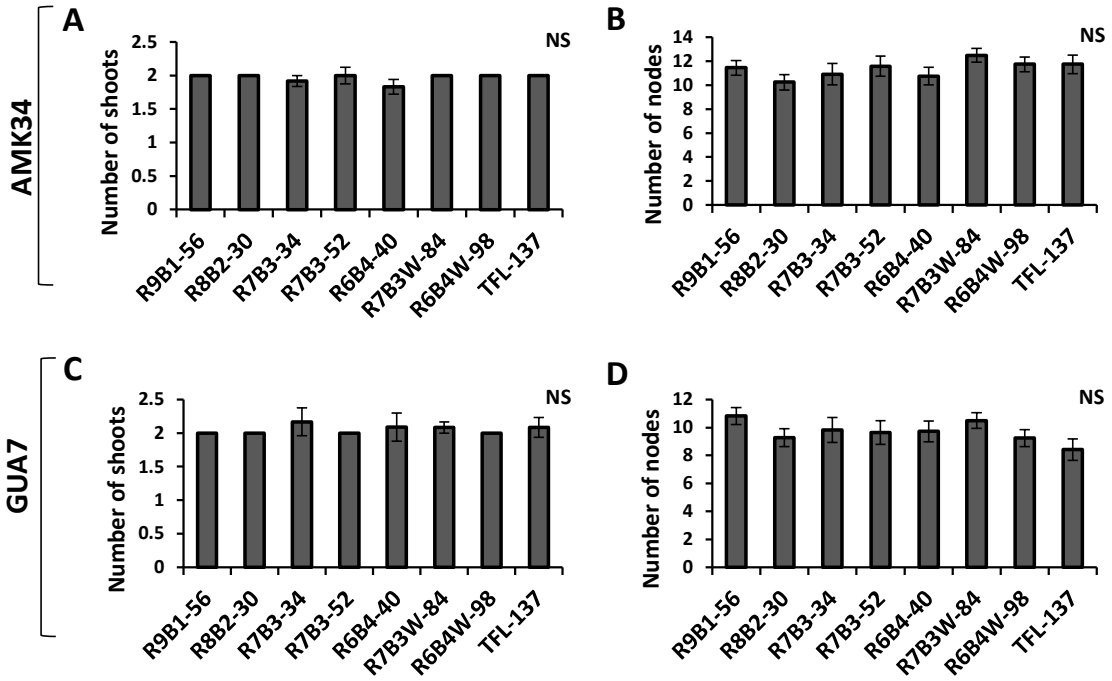
induced greater callus formation in explants of poinsettia cultivars, while the blue light exerted the opposite effect (D'Onofrio and Morini, 2001).

The stomatal distribution and development are highly variable between species, depending mainly on lighting conditions and CO_2 concentration. Our results show that stomatal frequency in micropropagated olive is induced by higher light intensity (Figure 5B) and/or a higher proportion of blue light (Figure 5D). Similar results were reported in several species (Wetzstein and Sommer, 1983; Paek and Hahn, 2000; Kim et al., 2004; Poudel et al., 2008; Pillitteri and Torii, 2012; Zhang et al., 2019). Stomatal frequency impacts water loss through transpiration and water use efficiency (Franco-Navarro et al., 2019). Thus, lower light intensity resulting in lower stomatal frequency is expected to reduce water loss through leaf transpiration as previously observed (Franco-Navarro et al., 2019), possibly improving plant *ex-vitro* acclimatization.

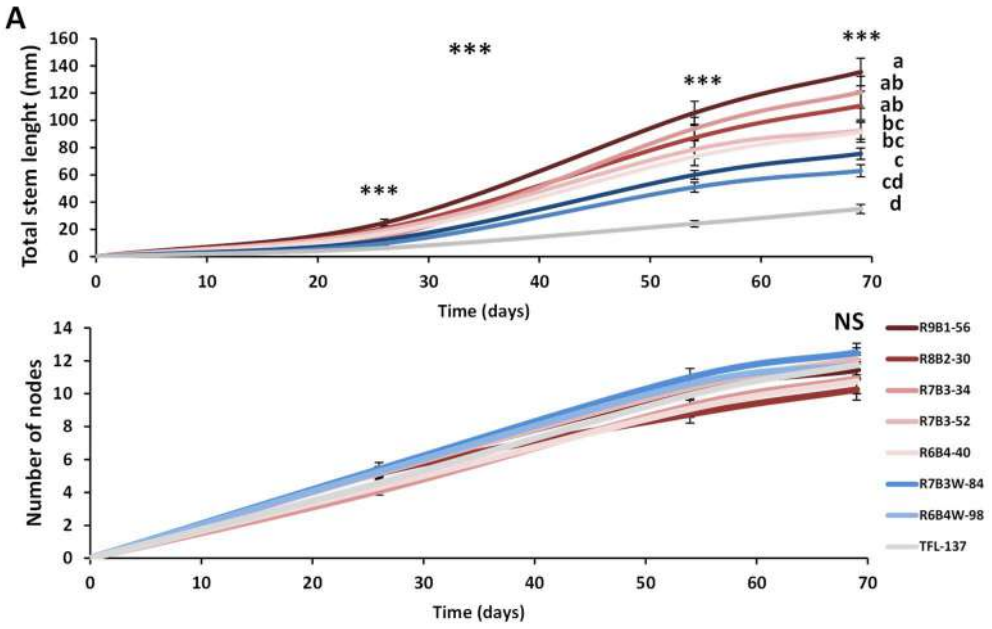
Conclusion

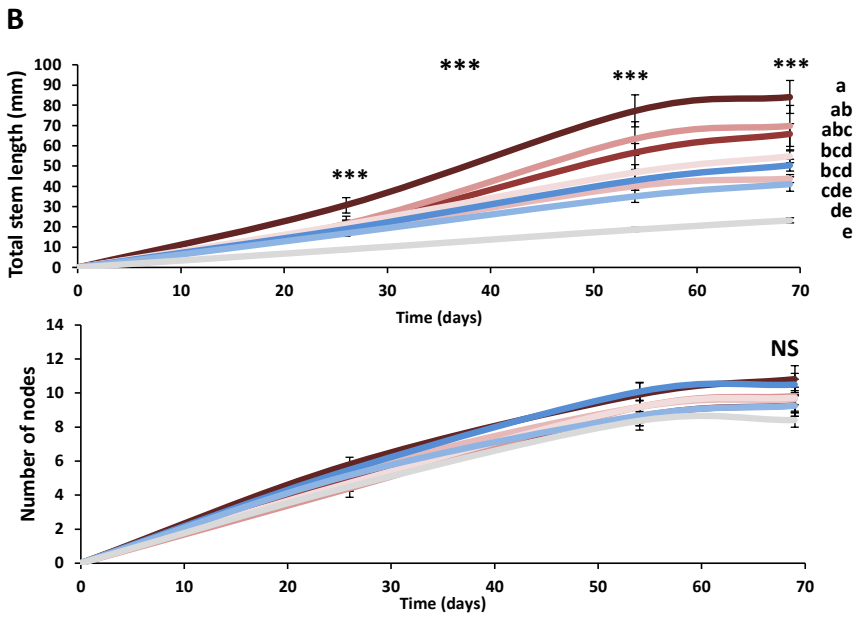
The aim of this study was to evaluate the effect of light quality and intensity on olive micropropagation, also comparing specific responses to LED vs. conventional fluorescent illumination. We concluded that two different subspecies of *Olea europaea* showed better growth and development under LED illumination in comparison to fluorescent lamps. Seventy percent red and thirty percent blue LEDs with a PPF of 34 µmol $m^{-2}s^{-1}$ of light irradiance was the optimal lighting treatment for olive micropropagation in terms of lower energy consumption, higher height and biomass, accumulation of photosynthetic pigments, optimal manipulation of *in-vitro* explants, lower leaf area and minimal stomatal density.

Supplementary Materials

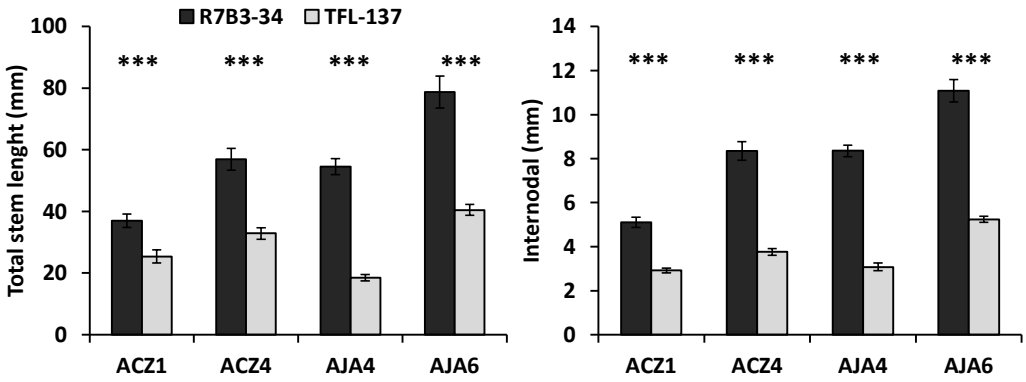


SUPPLEMENTARY FIGURE S1 | Effect of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on: number of shoots (**A, C**) and number of nodes (**B, D**) in *Olea europaea* subsp. *europaea* genotype AMK34 (**A, B**) and *Olea europaea* subsp. *guanchica* genotype GUA7 (**C, D**). Levels of significance: non-significant (NS).

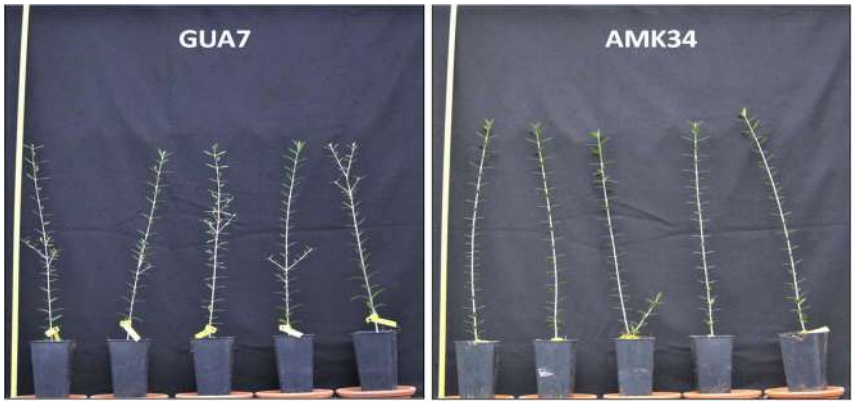




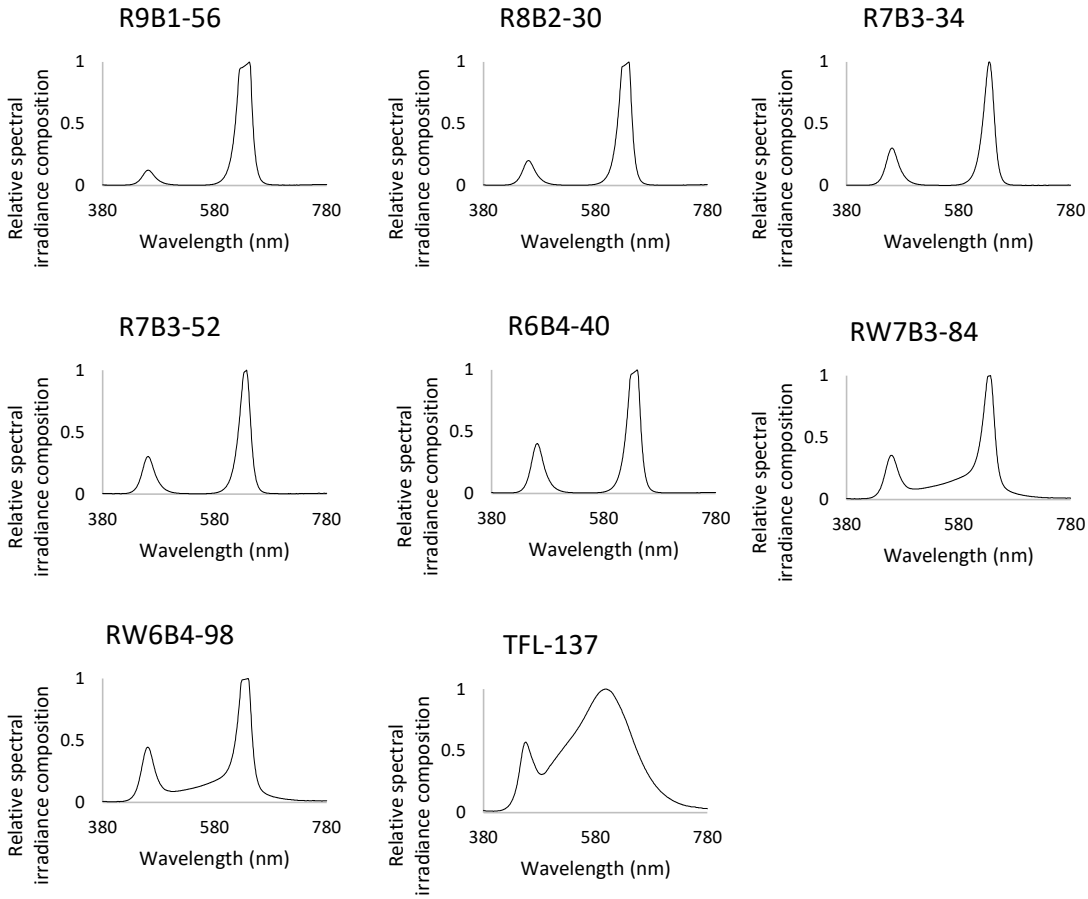
SUPPLEMENTARY FIGURE S2 | Effects of different illumination regimes from light-emitting diodes (LEDs) and fluorescent lighting (TFL-137) on the evolution of stem length and number of nodes in AMK34 (**A**) and GUA7 (**B**) along 69 days of *in-vitro* culture in the different light treatments assayed. Levels of significance: ***P≤0.001; Non-significant (NS).



SUPPLEMENTARY FIGURE S3 | Total stem and internodal length in four different genotypes of *Olea europaea* subsp. *europaea* (Díaz-Rueda et al., 2020) along 69 days of *in-vitro* culture under the LED illumination treatment R7B3-34 and the fluorescent illumination treatment TFL-137. Levels of significance: ***P≤0.001; Non-significant (NS).



SUPPLEMENTARY FIGURE S4 | Images of GUA7 and AMK34 genotypes one year after *ex-vitro* acclimatization.



SUPPLEMENTARY FIGURE S5 | Spectral patterns of the different illumination treatments used in this work (see Table 1), obtained with a spectrometer MK350N PLUS (UPRtek, Zhunan Township, Miaoli County, Taiwan).

References

Abidi, F., Girault, T., Douillet, O., Guillemain, G., Sintes, G., Laffaire, M., et al. (2013). Blue light effects on rose photosynthesis and photomorphogenesis: Blue light and rose development. *Plant Biol.* 15, 67–74. doi: 10.1111/j.1438-8677.2012.00603.x.

Abousalim, A., Brhadda, N., and Walali, L. (2005). Essais de prolifération et d'enracinement de matériel issu de rajeunissement par bouturage d'oliviers adultes (*Olea europaea* L.) et de germination *in vitro*: effets de cytokinine et d'auxines. *Biotechnol Agron Soc Env.* 9, 237–240.

Akoyunoglou, G., and Anni, H. (1984). "Blue light effect on chloroplast development in higher plants," in *Blue Light Effects in Biological Systems* Proceedings in Life Sciences., ed. H. Senger (Berlin, Heidelberg: Springer Berlin Heidelberg), 397–406. doi: 10.1007/978-3-642-69767-8_44.

Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A., Brutnell, T. P., Citovsky, V., et al. (2016). Advancing Crop Transformation in the Era of Genome Editing. *Plant Cell*, tpc.00196.2016. doi: 10.1105/tpc.16.00196.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1.

Ausin, I., Alonso-Blanco, C., and Martinez-Zapater, J.-M. (2005). Environmental regulation of flowering. *Int. J. Dev. Biol.* 49, 689–705. doi: 10.1387/ijdb.052022ia.

Barranco, D., Fernandez-Escobar, R., and Rallo, L. (1998). *El cultivo del olivo*. Sevilla: Junta de Andalucia.

Batista, D. S., Felipe, S. H. S., Silva, T. D., de Castro, K. M., Mamedes-Rodrigues, T. C., Miranda, N. A., et al. (2018). Light quality in plant tissue culture: does it matter? *Vitro Cell. Dev. Biol. - Plant* 54, 195–215. doi: 10.1007/s11627-018-9902-5.

Bayraktar, M., Hayta-Smedley, S., Unal, S., Varol, N., and Gurel, A. (2020). Micropropagation and prevention of hyperhydricity in olive (*Olea europaea* L.) cultivar 'Gemlik.' *South Afr. J. Bot.* 128, 264–273. doi: 10.1016/j.sajb.2019.11.022.

Beattie, G. A., Hatfield, B. M., Dong, H., and McGrane, R. S. (2018). Seeing the light: The roles of red- and blue-light sensing in plant microbes. *Annu. Rev. Phytopathol.* 56, 41–66. doi: 10.1146/annurev-phyto-080417-045931.

Benedict, C. R., McCree, K. J., and Kohel, R. J. (1972). High photosynthetic rate of a chlorophyll mutant of cotton. *Plant Physiol.* 49, 968–971. doi: 10.1104/pp.49.6.968.

Benelli, C., and De Carlo, A. (2018). *In vitro* multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™). *3 Biotech* 8, 317. doi: 10.1007/s13205-018-1346-4.

Besnard, G., Terral, J.-F., and Cornille, A. (2018). On the origins and domestication of the olive: a review and perspectives. *Ann. Bot.* 121, 385–403. doi: 10.1093/aob/mcx145.

Bianchi, G. (2003). Lipids and phenols in table olives. *Eur. J. Lipid Sci. Technol.* 105, 229–242. doi: 10.1002/ejlt.200390046.

Bradaï, F., Pliego-Alfaro, F., and Sánchez-Romero, C. (2016). Long-term somatic embryogenesis in olive (*Olea europaea* L.): Influence on regeneration capability and quality of regenerated plants. *Sci. Hortic.* 199, 23–31. doi: 10.1016/j.scienta.2015.12.010.

Brown, C. S., Schuerger, A. C., and Sager, J. C. (1995). Growth and photomorphogenesis of pepper plants under red light-emitting diodes with supplemental blue or far-red lighting. *J. Am. Soc. Hortic. Sci. Am. Soc. Hortic. Sci.* 120, 808–813.

Bula, R. J., Morrow, R. C., Tibbitts, T. W., Barta, D. J., Ignatius, R. W., and Martin, T. S. (1991). Light-emitting diodes as a radiation source for plants. *HortScience* 26, 203–205. doi: 10.21273/HORTSCI.26.2.203.

Cañas, L. A., and Benbadis, A. (1988). *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L). *Plant Sci.* 54, 65–74. doi: 10.1016/0168-9452(88)90056-8.

Chaari, A., Chelly Chaabouni, A., Maalej, M., and Drira, N. (2002). Meski olive variety propagated by tissue culture. *Acta Hortic.*, 871–874. doi: 10.17660/ActaHortic.2002.586.189.

Chang, H. S., Charkabarty, D., Hahn, E. J., and Paek, K. Y. (2003). Micropropagation of calla lily (*Zantedeschia albomaculata*) via *in vitro* shoot tip proliferation. *Vitro Cell. Dev. Biol. - Plant* 39, 129–134. doi: 10.1079/IVP2002362.

Chaves, M. C., Freitas, J. C. E., Nery, F. C., Paiva, R., Prudente, D. de O., Costa, B. G. P., et al. (2020). Influence of colorful light-emitting diodes on growth, biochemistry, and production of volatile organic compounds in vitro of *Lippia filifolia* (Verbenaceae). *J. Photochem. Photobiol. B* 212, 112040. doi: 10.1016/j.jphotobiol.2020.112040.

Chory, J. (1997). Light modulation of vegetative development. *Plant Cell* 9, 1225–1234. doi: 10.1105/tpc.9.7.1225.

Chory, J., Chatterjee, M., Cook, R. K., Elich, T., Fankhauser, C., Li, J., et al. (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proc. Natl. Acad. Sci.* 93, 12066–12071. doi: 10.1073/pnas.93.22.12066.

da Silva, M. H. M., and Debergh, P. C. (1997). The effect of light quality on the morphogenesis of *in vitro* cultures of *Azorina vidalii* (Wats.) Feer. *Plant Cell Tissue Organ Cult.* 51, 187–193. doi: 10.1023/A:1005988621036.

Davis, P. A., and Burns, C. (2016). Photobiology in protected horticulture. *Food Energy Secur.* 5, 223–238. doi: 10.1002/fes3.97.

de Hsie, B. S., Bueno, A. I. S., Bertolucci, S. K. V., de Carvalho, A. A., da Cunha, S. H. B., Martins, E. R., et al. (2019). Study of the influence of wavelengths and intensities of LEDs on the growth, photosynthetic pigment, and volatile compounds production of Lippia rotundifolia Cham </i>in vitro</i>. *J. Photochem. Photobiol. B* 198, 111577. doi: 10.1016/j.jphotobiol.2019.111577.

Debnath, M., Malik, C., and Bisen, P. (2006). Micropropagation: A Tool for the Production of High Quality Plant-based Medicines. *Curr. Pharm. Biotechnol.* 7, 33–49. doi: 10.2174/138920106775789638.

Díaz-Rueda, P., Cantos-Barragán, M., and Colmenero-Flores, J. M. (2021). Growth Quality and Development of Olive Plants Cultured *In-Vitro* under Different Illumination Regimes. *Plants* 10, 2214. doi: 10.3390/plants10102214.

Díaz-Rueda, P., Franco-Navarro, J. D., Messora, R., Espartero, J., Rivero-Núñez, C. M., Aleza, P., et al. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Front. Plant Sci.* 11, 629. doi: 10.3389/fpls.2020.00629.

D'Onofrio, C., and Morini, S. (2001). Effect of light quality on *in vitro* production of callus in explants of three Poinsettia cultivars. *Acta Hortic.*, 449–452. doi: 10.17660/ActaHortic.2001.560.88.

Espinosa-Leal, C. A., Puente-Garza, C. A., and García-Lara, S. (2018). *In vitro* plant tissue culture: means for production of biological active compounds. *Planta* 248, 1–18. doi: 10.1007/s00425-018-2910-1.

Fabbri, A., Bartolini, G., Lambardi, M., and Kailis, S. (2004). *Olive propagation manual*. CSIRO Publishing, Collingwood.

FAOSTAT. Disponible en línea: http://faostat.fao.org/ (consultado el 24 de febrero de 2021).

Farahani, F., Peyvandi, M., and Hosseini-Mazinani, M. (2008). Effect os sucrose and mannitol on *in vitro* regeneration of Iranian olive cv. "rowghani." *Acta Hortic.*, 203–208. doi: 10.17660/ActaHortic.2008.791.28.

Fiorino, P., and Leva, A. (1986). Investigations on the micropropagation of the olive (*Olea europaea* L.) and influence of some mineral elements on the proliferation and rooting of explants. *Olea* 17, 101–104.

Folta, K. M., and Maruhnich, S. A. (2007). Green light: a signal to slow down or stop. *J. Exp. Bot.* 58, 3099–3111. doi: 10.1093/jxb/erm130.

Franco-Navarro, J. D., Brumós, J., Rosales, M. A., Cubero-Font, P., Talón, M., and Colmenero-Flores, J. M. (2016). Chloride regulates leaf cell size and water relations in tobacco plants. *J. Exp. Bot.* 67, 873–891. doi: 10.1093/jxb/erv502.

Franco-Navarro, J. D., Rosales, M. A., Cubero-Font, P., Calvo, P., Álvarez, R., Díaz-Espejo, A., et al. (2019). Chloride as macronutrient increases water use efficiency by anatomically-driven reduced stomatal conductance and increased mesophyll diffusion to CO_2. *Plant J.*, tpj.14423. doi: 10.1111/tpj.14423.

García, J. L., Troncoso, J., Sarmiento, R., and Troncoso, A. (2002). Influence of carbon source and concentration on the *in vitro* development of olive (*Olea europea* L.) zygotic embryos and explants raised from them. *Plant Cell Tissue Organ Cult.* 69, 95–100. doi: 10.1023/A:1015086104389.

García-Berenguer, A., and Durán González, R. (1990). Mineral media for *in vitro* propagation of juvenile "Picual" microcuttings. *Acta Hortic.*, 61–64. doi: 10.17660/ActaHortic.1990.286.8.

García-Férriz, L., Ghorbel, R., Ybarra, M., Marí, A., Belaj, A., and Trujillo, I. (2003). Cultivo *in vitro* y comportamiento en campo de olivo adulto. *Horticultura* 172, 64–66.

Gentile, L., and Uccella, N. A. (2014). Selected bioactives from callus cultures of olives (*Olea europaea* L. Var. Coratina) by LC-MS. *Food Res. Int.* 55, 128–136. doi: 10.1016/j.foodres.2013.10.046.

Gitz, D. C., and Baker, J. T. (2009). Methods for creating stomatal impressions directly onto archivable slides. *Agron. J.* 101, 232–236. doi: 10.2134/agronj2008.0143N.

Green, P. (2002). *A revision of* Olea *L. (Oleaceae). Kew Bull. 57, 91–140.* Kew Bull.

Grigoriadou, K., Eleftheriou, E. P., and Vasilakakis, M. (2007). Hidden hyperhydricity may be responsible for abnormal development and acclimatization problems of micropropagated olive plantlets: an anatomical leaf study. *Acta Hortic.*, 103–106. doi: 10.17660/ActaHortic.2007.748.10.

Gupta, P. K., Pullman, G., Timmis, R., Kreitinger, M., Carlson, W. C., Grob, J., et al. (1993). Forestry in the 21st Century. *Nat. Biotechnol.* 11, 454–459. doi: 10.1038/nbt0493-454.

Gupta, S. D., and Jatothu, B. (2013). Fundamentals and applications of light-emitting diodes (LEDs) in *in vitro* plant growth and morphogenesis. *Plant Biotechnol. Rep.* 7, 211–220. doi: 10.1007/s11816-013-0277-0.

Haberlandt, G. (1902). Kulturversuche mit isolierten Flanzellen. *Sitzungsber Akad Wiss Wien Math Nat* 111, 69–91.

Haddad, B., Carra, A., Saadi, A., Haddad, N., Mercati, F., Gristina, A. S., et al. (2018). *In vitro* propagation of the relict laperinne's olive (*Olea europaea* L. subsp. *Laperrinei*). *Plant Biosyst. - Int. J. Deal. Asp. Plant Biol.* 152, 621–630. doi: 10.1080/11263504.2017.1306002.

Hoenecke, M. E., Bula, R. J., and Tibbitts, T. W. (1992). Importance of "blue" photon levels for lettuce seedlings grown under red-light-emitting diodes. *HortScience Publ. Am. Soc. Hortic. Sci.* 27, 427–430.

Hogewoning, S. W., Trouwborst, G., Maljaars, H., Poorter, H., van Ieperen, W., and Harbinson, J. (2010). Blue light dose-responses of leaf photosynthesis, morphology, and chemical composition of *Cucumis sativus* grown under different combinations of red and blue light. *J. Exp. Bot.* 61, 3107–3117. doi: 10.1093/jxb/erq132.

Islam, M. A., Kuwar, G., Clarke, J. L., Blystad, D.-R., Gislerød, H. R., Olsen, J. E., et al. (2012). Artificial light from light emitting diodes (LEDs) with a high portion of blue light results in shorter poinsettias compared to high pressure sodium (HPS) lamps. *Sci. Hortic.* 147, 136–143. doi: 10.1016/j.scienta.2012.08.034.

Kim, S.-J., Hahn, E.-J., Heo, J.-W., and Paek, K.-Y. (2004). Effects of LEDs on net photosynthetic rate, growth and leaf stomata of chrysanthemum plantlets *in vitro*. *Sci. Hortic.* 101, 143–151. doi: 10.1016/j.scienta.2003.10.003.

Kurepin, L. V., and Pharis, R. P. (2014). Light signaling and the phytohormonal regulation of shoot growth. *Plant Sci.* 229, 280–289. doi: 10.1016/j.plantsci.2014.10.006.

Lagarda, A., Martin, G. C., and Polite, V. S. (1983). Anatomical and morphological development of "Manzanillo" olive seed in relation to germination. *J Am Soc Hortic Sci* 108, 741–743.

Lazzarin, M., Meisenburg, M., Meijer, D., van Ieperen, W., Marcelis, L. F. M., Kappers, I. F., et al. (2021). LEDs make it resilient: Effects on plant growth and defense. *Trends Plant Sci.* 26, 496–508. doi: 10.1016/j.tplants.2020.11.013.

Leva, A. (2011). Innovative protocol for "*ex vitro* rooting" on olive micropropagation. *Open Life Sci.* 6, 352–358. doi: 10.2478/s11535-011-0010-3.

Leva, A., Muleo, R., and Petruccelli, R. (1995). Long-term somatic embryogenesis from inmature olive cotyledons. *J. Hortic. Sci.* 70, 417–421. doi: 10.1080/14620316.1995.11515311.

Leva, A. R., Petruccelli, R., and Bartolini, G. (1994). Mannitol *"in vitro"* culture of *Olea europaea* L. (CV. Maurino). *Acta Hortic.*, 43–46. doi: 10.17660/ActaHortic.1994.356.7.

Leva, A. R., Petruccelli, R., Goretti, R., and Panicucci, M. (1992). Ruolo di alcuni microelementi e carboidrati nella proliferazione *in vitro* di cv. di olivo (*Olea europaea* L.). *Atti Congr. Inter "Olive Oil Qual. Firenze*, 333–334.

Li, H., Tang, C., and Xu, Z. (2013). The effects of different light qualities on rapeseed (*Brassica napus* L.) plantlet growth and morphogenesis *in vitro*. *Sci. Hortic.* 150, 117–124. doi: 10.1016/j.scienta.2012.10.009.

Li, H., Xu, Z., and Tang, C. (2010). Effect of light-emitting diodes on growth and morphogenesis of upland cotton (*Gossypium hirsutum* L.) plantlets *in vitro*. *Plant Cell Tissue Organ Cult. PCTOC* 103, 155–163. doi: 10.1007/s11240-010-9763-z.

Li, Q., and Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environ. Exp. Bot.* 67, 59–64. doi: 10.1016/j.envexpbot.2009.06.011.

Lobiuc, A., Vasilache, V., Oroian, M., Stoleru, T., Burducea, M., Pintilie, O., et al. (2017). Blue and red LED illumination improves growth and bioactive compounds contents in acyanic and cyanic *Ocimum basilicum* L. microgreens. *Molecules* 22, 2111. doi: 10.3390/molecules22122111.

Luan, V. Q., Huy, N. P., Nam, N. B., Huong, T. T., Hien, V. T., Hien, N. T. T., et al. (2015). *Ex vitro* and *in vitro Paphiopedilum delenatii* Guillaumin stem elongation under light-emitting diodes and shoot regeneration via stem node culture. *Acta Physiol. Plant.* 37, 136. doi: 10.1007/s11738-015-1886-8.

Marks, T. R., and Simpson, S. E. (1999). Effect of irradiance on shoot development *in vitro*. *Plant Growth Regul.* 28, 133–142.

Martino, L., Cuozzo, L., and Brunori, A. (1999). Establishment of meristem tip culture from field-grown olive (*Olea europaea* L.) cv. Moraiolo. *Agr Med* 129, 193–198.

Mazri, M. A., Naciri, R., and Belkoura, I. (2020). Maturation and Conversion of Somatic Embryos Derived from Seeds of Olive (*Olea europaea* L.) cv. Dahbia: Occurrence of Secondary Embryogenesis and Adventitious Bud Formation. *Plants* 9, 1489. doi: 10.3390/plants9111489.

McNellis, T. W., and Deng, X. W. (1995). Light control of seedling morphogenetic pattern. *Plant Cell* 7, 1749–1761. doi: 10.1105/tpc.7.11.1749.

Médail, F., Quézel, P., Besnard, G., and Khadari, B. (2001). Systematics, ecology and phylogeographic significance of *Olea europaea* L. ssp. *maroccana* (Greuter & Burdet) P. Vargas et al a relictual olive tree in south-west Morocco. *Bot. J. Linn. Soc.* 137, 249–266. doi: 10.1006/bojl.2001.0477.

Micheli, M., Standardi, A., El Behi, A. W., Zakhour, D., and Yasin, M. (2010). *In vitro* proliferation of olive ("Dolce agogia" and 'Moraiolo'): effect of different cytokinins. *Acta Hortic.*, 587–590. doi: 10.17660/ActaHortic.2010.884.76.

Miranda, N. A., Xavier, A., Otoni, W. C., Gallo, R., Gatti, K. C., de Moura, L. C., et al. (2020). Quality and intensity of light in the *in vitro* development of microstumps of *Eucalyptus urophylla* in a photoautotrophic system. *For. Sci.* 66, 754–760. doi: 10.1093/forsci/fxaa027.

Mirzaei, L., Yadollahi, A., Kermani, M. J., Naderpour, M., Zeinanloo, A. A., Farsi, M., et al. (2021). Evaluation of genetic stability in olive callus-induced and meristem-induced shoots using flow cytometry and amplified fragment length polymorphism techniques. *Plant Methods* 17, 31. doi: 10.1186/s13007-021-00724-7.

Mohammad, S., Khan, M. A., Ali, A., Khan, L., Khan, M. S., and Mashwani, Z.-R. (2019). Feasible production of biomass and natural antioxidants through callus cultures in response to varying light intensities in olive (*Olea europaea* L.) cult. Arbosana. *J. Photochem. Photobiol. B* 193, 140–147. doi: 10.1016/j.jphotobiol.2019.03.001.

Moon, H. K., Park, S.-Y., Kim, Y. W., and Kim, C. S. (2006). Growth of Tsuru-rindo (*Tripterospermum japonicum*) cultured *in vitro* under various sources of light-emitting diode (LED) irradiation. *J. Plant Biol.* 49, 174–179. doi: 10.1007/BF03031014.

Morini, S., D'Onofrio, C., Bellocchi, G., and Fisichella, M. (2000). Effect of 2,4-DD and light quality on callus production and differentiation from in vitro cultured quince leaves. *Plant Cell Tissue Organ Cult.* 63, 47–55. doi: 10.1023/A:1006456919590.

Mortensen, L. M., and Strømme, E. (1987). Effects of light quality on some greenhouse crops. *Sci. Hortic.* 33, 27–36. doi: 10.1016/0304-4238(87)90029-X.

Oliveira, T. dos R., Aragão, V. P. M., Moharana, K. C., Fedosejevs, E., do Amaral, F. P., Sousa, K. R., et al. (2020). Light spectra affect the *in vitro* shoot development of *Cedrela fissilis* Vell. (*Meliaceae*) by changing the protein profile and polyamine contents. *Biochim. Biophys. Acta BBA - Proteins Proteomics* 1868, 140529. doi: 10.1016/j.bbapap.2020.140529.

Paek, K.-Y., and Hahn, E. (2000). Cytokinins, auxins and activated charcoal affect organogenesis and anatomical characteristics of shoot-tip cultures of lisianthus [*Eustoma grandiflorum* (RAF.) Shinn]. *Vitro Cell. Dev. Biol. - Plant* 36, 128–132. doi: 10.1007/s11627-000-0026-2.

Paradiso, R., and Proietti, S. (2021). Light-quality manipulation to control plant growth and photomorphogenesis in greenhouse horticulture: The state of the art and the opportunities of modern LED systems. *J. Plant Growth Regul.* doi: 10.1007/s00344-021-10337-y.

Perri, E., Parlati, M. V., and Rugini, E. (1994). Isolation and culture of olive (*Olea europaea* L.) cultivar protoplasts. *Acta Hortic.*, 51–53. doi: 10.17660/ActaHortic.1994.356.9.

Pierik, R. L. M. (1990). "Rejuvenation and micropropagation," in *Progress in Plant Cellular and Molecular Biology* Current Plant Science and Biotechnology in Agriculture., eds. H. J. J. Nijkamp, L. H. W. Van Der Plas, and J. Van Aartrijk (Dordrecht: Springer Netherlands), 91–101. doi: 10.1007/978-94-009-2103-0_13.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055.

Pillitteri, L. J., and Torii, K. U. (2012). Mechanisms of Stomatal Development. *Annu. Rev. Plant Biol.* 63, 591–614. doi: 10.1146/annurev-arplant-042811-105451.

Porfírio, S., Gomes da Silva, M. D. R., Cabrita, M. J., Azadi, P., and Peixe, A. (2016). Reviewing current knowledge on olive (*Olea europaea* L.) adventitious root formation. *Sci. Hortic.* 198, 207–226. doi: 10.1016/j.scienta.2015.11.034.

Poudel, P. R., Kataoka, I., and Mochioka, R. (2008). Effect of red- and blue-light-emitting diodes on growth and morphogenesis of grapes. *Plant Cell Tissue Organ Cult.* 92, 147–153. doi: 10.1007/s11240-007-9317-1.

Quail, P. H. (2010). Phytochromes. *Curr. Biol.* 20, 504–507.

Rugini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.* 24, 123–134. doi: 10.1016/0304-4238(84)90143-2.

Rugini, E. (1988). Somatic embryogenesis and plant regeneration in olive (*Olea europaea* L.). *Plant Cell Tissue Organ Cult.* 14, 207–214. doi: 10.1007/BF00043411.

Rugini, E. (1992). Involvement of Polyamides in Auxin and *Agrobacterium rhizogenes* - induced Rooting of Fruit Trees *in Vitro*. *J. Am. Soc. Hortic. Sci.* 117, 532–536. doi: 10.21273/JASHS.117.3.532.

Rugini, E., and Caricato, G. (1995). Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) ?canino? and ?moraiolo? *Plant Cell Rep.* 14. doi: 10.1007/BF00233645.

Rugini, E., Cristofori, V., and Silvestri, C. (2016). Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods. *Biotechnol. Adv.* 34, 687–696. doi: 10.1016/j.biotechadv.2016.03.004.

Rugini, E., Politi, V., Bignami, C., De Agazio, M., and Grego, S. (1990). Effect of polyamine treatments on rooting cutting of three olive cultivars. *Acta Hortic.*, 97–100. doi: 10.17660/ActaHortic.1990.286.17.

Saebo, A., Krekling, T., and Appelgren, M. (1995). Light quality affects photosynthesis and leaf anatomy of birch plantlets *in vitro*. *Plant Cell Tissue Organ Cult.* 41, 177–185. doi: 10.1007/BF00051588.

Saito, Y., Shimizu, H., Nakashima, H., Miyasaka, J., and Ohdoi, K. (2010). The effect of light quality on growth of lettuce. *IFAC Proc. Vol.* 43, 294–298. doi: 10.3182/20101206-3-JP-3009.00052.

Sánchez-Romero, C. (2018). "Olive *Olea europaea* L.," in *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants* Forestry Sciences., eds. S. M. Jain and P. Gupta (Cham: Springer International Publishing), 25–38. doi: 10.1007/978-3-319-79087-9_2.

Schuerger, A. (1997). Anatomical features of pepper plants (*Capsicum annuum* L.) grown under red light-emitting diodes supplemented with blue or far-red light. *Ann. Bot.* 79, 273–282. doi: 10.1006/anbo.1996.0341.

Senger, H. (1982). The effect of blue light on plants and microorganisms. *Photochem. Photobiol.* 35, 911–920. doi: 10.1111/j.1751-1097.1982.tb02668.x.

Serret, M. D., Trillas, M. I., and Araus, J. L. (2001). The effect of *in vitro* culture conditions on the pattern of photoinhibition during acclimation of gardenia plantlets to *ex vitro* conditions. *Photosynthetica* 39, 67–73. doi: 10.1023/A:1012443919022.

Shinomura, T., Nagatani, A., Hanzawa, H., Kubota, M., Watanabe, M., and Furuya, M. (1996). Action spectra for phytochrome A- and B-specific photoinduction of seed germination in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.* 93, 8129–8133. doi: 10.1073/pnas.93.15.8129.

Silva, S. T., Bertolucci, S. K. V., da Cunha, S. H. B., Lazzarini, L. E. S., Tavares, M. C., and Pinto, J. E. B. P. (2017). Effect of light and natural ventilation systems on the growth parameters and carvacrol content in the *in vitro* cultures of *Plectranthus amboinicus* (Lour.) Spreng. *Plant Cell Tissue Organ Cult. PCTOC* 129, 501–510. doi: 10.1007/s11240-017-1195-6.

Simlat, M., Ślęzak, P., Moś, M., Warchoł, M., Skrzypek, E., and Ptak, A. (2016). The effect of light quality on seed germination, seedling growth and selected biochemical properties of *Stevia rebaudiana* Bertoni. *Sci. Hortic.* 211, 295–304. doi: 10.1016/j.scienta.2016.09.009.

Smirnakou, S., Ouzounis, T., and Radoglou, K. (2017). Effects of continuous spectrum LEDs used in indoor cultivation of two coniferous species *Pinus sylvestris* L. and *Abies borisii-regis* Mattf. *Scand. J. For. Res.* 32, 115–122. doi: 10.1080/02827581.2016.1227470.

Solís, M.-T., Pintos, B., Prado, M.-J., Bueno, M.-Á., Raska, I., Risueño, M.-C., et al. (2008). Early markers of *in vitro* microspore reprogramming to embryogenesis in olive (*Olea europaea* L.). *Plant Sci.* 174, 597–605. doi: 10.1016/j.plantsci.2008.03.014.

Therios, I. (2009). Olives. *Crop Prod. Sci. Hortic. Wallingford UK CABI*.

Trabelsi, E. B., Naija, S., Elloumi, N., Belfeleh, Z., Msellem, M., Ghezel, R., et al. (2011). Somatic embryogenesis in cell suspension cultures of olive *Olea europaea* (L.) 'Chetoui.' *Acta Physiol. Plant.* 33, 319–324. doi: 10.1007/s11738-010-0550-6.

Troncoso, A., Liñán, J., Cantos, M., and Acebedo, M. M. (2000). Injerto *in vitro* del olivo. *Mercacei* 23, 171–173.

Vieira, L. do N., de Freitas Fraga, H. P., dos Anjos, K. G., Puttkammer, C. C., Scherer, R. F., da Silva, D. A., et al. (2015). Light-emitting diodes (LED) increase the stomata formation and chlorophyll content in *Musa acuminata* (AAA) 'Nanicão Corupá' *in vitro* plantlets. *Theor. Exp. Plant Physiol.* 27, 91–98. doi: 10.1007/s40626-015-0035-5.

Wang, X. Y., Xu, X. M., and Cui, J. (2015). The importance of blue light for leaf area expansion, development of photosynthetic apparatus, and chloroplast ultrastructure of *Cucumis sativus* grown under weak light. *Photosynthetica* 53, 213–222. doi: 10.1007/s11099-015-0083-8.

Wang, Y., Maruhnich, S. A., Mageroy, M. H., Justice, J. R., and Folta, K. M. (2013). Phototropin 1 and cryptochrome action in response to green light in combination with other wavelengths. *Planta* 237, 225–237. doi: 10.1007/s00425-012-1767-y.

Wetzstein, H., and Sommer, H. (1983). Scanning electron microscopy of *in vitro*-cultured liquidambar styraciflua plantlets during acclimatization. *J AM SOC HORTIC SCI* 108, 475–480.

Yadav, S., Carvalho, J., Trujillo, I., and Prado, M. (2021). Microsatellite Markers in Olives (*Olea europaea* L.): Utility in the Cataloging of Germplasm, Food Authenticity and Traceability Studies. *Foods* 10, 1907. doi: 10.3390/foods10081907.

Zacchini, M., and Agazio, M. (2004). Micropropagation of a Local Olive Cultivar for Germplasm Preservation. *Biol. Plant.* 48, 589–592. doi: 10.1023/B:BIOP.0000047156.57328.27.

Zhang, Y., Kaiser, E., Zhang, Y., Yang, Q., and Li, T. (2019). Red/blue light ratio strongly affects steady-state photosynthesis, but hardly affects photosynthetic induction in tomato (*Solanum lycopersicum*). *Physiol. Plant.* 167, 144–158. doi: 10.1111/ppl.12876.

Zheng, L., He, H., and Song, W. (2019). Application of light-emitting diodes and the effect of light quality on horticultural crops: A review. *HortScience* 54, 1656–1661. doi: 10.21273/HORTSCI14109-19.

Zohary, D., Hopf, M., and Weiss, E. (2012). *Domestication of plants in the Old World: the origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin*. 4th ed. Oxford: Oxford University Press.

Zuccherelli, G., and Zuccherelli, S. (2002). *In vitro* propagation of fifty olive cultivars. *Acta Hortic.*, 931–934. doi: 10.17660/ActaHortic.2002.586.204.

Chapter 2. SILVOLIVE, a germplasm collection of wild subspecies with high genetic variability as a source of rootstocks and resistance genes for olive breeding

This chapter has been published as**: Díaz-Rueda, P**., Franco-Navarro, J., Messora, R., Espartero, J., Rivero-Núñez, C., Aleza, P., Capote, N., Cantos, M., García-Fernández, J., De Cires, A., Belaj, A., León, L., Besnard, G., and Colmenero-Flores, J. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. Frontier Plant Science 11:629. https://doi.org/10.3389/fpls.2020.00629

This chapter contains an annex whose data is published as: Hernández-Santana, V., **Díaz-Rueda, P.**, Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., Montero A, Perez-Martin A, Colmenero-Flores JM and Rodriguez-Dominguez CM. (2019). Hydraulic traits emerge as relevant determinants of growth patterns in wild olive genotypes under wáter stress. Frontier Plant Science 10:291. https://doi.org/10.3389/fpls.2019.00291

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 May 2020
doi: 10.3389/fpls.2020.00629

SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding

Pablo Díaz-Rueda[1], Juan D. Franco-Navarro[1], Rita Messora[2], Joaquín Espartero[1], Carlos M. Rivero-Núñez[1], Pablo Aleza[3], Nieves Capote[4], Manuel Cantos[1], Jose L. García-Fernández[1], Alfonso de Cires[5], Angjelina Belaj[6], Lorenzo León[6], Guillaume Besnard[7] and Jose M. Colmenero-Flores[1]**

[1] Instituto de Recursos Naturales y Agrobiología, Spanish National Research Council (CSIC), Seville, Spain, [2] Plant Physiology Laboratory, Dipartimento Sci Vita, Univ Modena & Reggio Emilia, Modena, Italy, [3] Centro de Citricultura y Producción Vegetal, Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain, [4] Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA) Centro Las Torres, Seville, Spain, [5] Departamento de Biología Vegetal y Ecología, Fac Biología, Univ de Sevilla, Seville, Spain, [6] Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA) Centro Alameda del Obispo, Córdoba, Spain, [7] CNRS-UPS-IRD, EDB, UMR 5174, Université Paul Sabatier, Toulouse, France

OPEN ACCESS

Edited by:
Jaime Prohens,
Universitat Politècnica de València, Spain

Reviewed by:
Francesco Paolo Marra,
University of Palermo, Italy
Innocenzo Muzzalupo,
Research Center for Citrus Fruit and Mediterranean Crops, Council for Agricultural Research and Economics, Italy

***Correspondence:**
Pablo Díaz-Rueda
pdiaz@irnas.csic.es
Jose M. Colmenero-Flores
chemacf@irnase.csic.es

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 06 March 2020
Accepted: 23 April 2020
Published: 28 May 2020

Citation:
Díaz-Rueda P, Franco-Navarro JD, Messora R, Espartero J, Rivero-Núñez CM, Aleza P, Capote N, Cantos M, García-Fernández JL, de Cires A, Belaj A, León L, Besnard G and Colmenero-Flores JM (2020) SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. Front. Plant Sci. 11:629. doi: 10.3389/fpls.2020.00629

Wild subspecies of *Olea europaea* constitute a source of genetic variability with huge potential for olive breeding to face global changes in Mediterranean-climate regions. We intend to identify wild olive genotypes with optimal adaptability to different environmental conditions to serve as a source of rootstocks and resistance genes for olive breeding. The SILVOLIVE collection includes 146 wild genotypes representative of the six *O. europaea* subspecies and early-generations hybrids. These genotypes came either from olive germplasm collections or from direct prospection in Spain, continental Africa and the Macaronesian archipelago. The collection was genotyped with plastid and nuclear markers, confirming the origin of the genotypes and their high genetic variability. Morphological and architectural parameters were quantified in 103 genotypes allowing the identification of three major groups of correlative traits including vigor, branching habits and the belowground-to-aboveground ratio. The occurrence of strong phenotypic variability in these traits within the germplasm collection has been shown. Furthermore, wild olive relatives are of great significance to be used as rootstocks for olive cultivation. Thus, as a proof of concept, different wild genotypes used as rootstocks were shown to regulate vigor parameters of the grafted cultivar "Picual" scion, which could improve the productivity of high-density hedgerow orchards.

Keywords: *Olea europaea*, wild germplasm, molecular markers, genetic variability, vigor, branching, rootstock, grafting

INTRODUCTION

The wild relatives of domesticated crops possess genetic diversity useful for developing more productive, nutritious and resilient crop varieties (Castaneda-Alvarez et al., 2016), and for preserving global food security against the serious threat of climate change (Vincent et al., 2013). Wild relatives of the domesticated olive tree (*Olea europaea* L.) are evergreen, drought tolerant,

Introduction

The wild relatives of domesticated crops possess genetic diversity useful for developing more productive, nutritious and resilient crop varieties (Castaneda-Alvarez *et al*., 2016), and for preserving global food security against the serious threat of climate change (Vincent *et al*., 2013). Wild relatives of the domesticated olive tree (*Olea europaea* L.) are evergreen, drought tolerant, usually multi-stemmed small trees or large shrubs with very good adaptability to different environmental conditions (Médail *et al*., 2001; Green, 2002; Kassa *et al*., 2019). Wild olives grow in arid and semiarid regions at different altitudes and soil types, including those exposed to severe water deficit, salinity and low temperatures (Cantos *et al*., 2002; Baldoni *et al*., 2006; Klepo *et al*., 2013; Belaj *et al*., 2016; Chiappetta *et al*., 2017). This adaptability to adverse environmental conditions makes wild olive trees suitable to grow in marginal soils (e.g., at risk of desertification), to colonize deforested habitats or to rehabilitate devastated regions (Bekele, 2005; Kassa *et al*., 2019). Six olive subspecies have been recognized that occur in different natural distribution ranges in Europe, Africa, and Asia (Green, 2002): (1) *O. europaea* subsp. *europaea*, which includes wild types or oleasters [var. *sylvestris* (Mill.) Lehr] and the domesticated olive (var. *europaea*) that are common in the whole Mediterranean basin; (2) *O. e.* subsp. *cuspidata* (Wall. ex G. Don) Cif. distributed from South Africa to south-eastern Egypt and from the Middle East to India and China; (3) *O. e.* subsp. *laperrinei* (Batt. & Trab.) Cif. in the central Saharan mountains; (4) *O. e*. subsp. *maroccana* (Greut. & Burd.) P. Vargas et al. in southwestern Morocco; (5) *O. e.* subsp. *cerasiformis* Kunk. & Sund. In Madeira; and (6) and *O. e.* subsp. *guanchica* P. Vargas *et al*. in the Canary Islands.

Besnard *et al*. (2007) showed through nuclear and plastid DNA data that the main wild progenitor of the cultivated olive (*O. e.* subsp. *europaea* var. *europaea*) is the wild Mediterranean olive, also known as oleaster (*O. e.* subsp. *europaea* var. *sylvestris*). Olive domestication from wild oleaster populations has involved the selection of a small number of desirable genotypes with bigger fruits, which were asexually propagated through cuttings. Such selection and propagation practices may contribute to reduce genetic diversity of the cultivated genepool (Rugini *et al*., 2011), but continuous

hybridization events with local wild populations have, however, occurred during the long and ongoing domestication process (Besnard *et al*., 2013b, 2007). A higher genetic diversity is still observed in the wild genepool (Lumaret *et al*., 2004; Baldoni *et al*., 2009; Belaj *et al*., 2010; Besnard *et al*., 2013a; Chiappetta *et al*., 2017; Kassa *et al*., 2019). Wild olives therefore represent an important source of genes for crop improvement of resistance to abiotic stresses [e.g., salinity (Cantos *et al*., 2002), water deficit (Hernández-Santana *et al*., 2019), soil pollution (Murillo *et a*l., 2005)], vigor (León *et al*., 2020), crop yield and quality (Hannachi *et al*., 2008; Baccouri *et al*., 2011; León *et al*., 2018), as well as for resistance to biotic factors such as the Verticillium wilt (Colella *et al*., 2008; Arias-Calderon *et al*., 2015b; Trapero *et al.,* 2015; Jimenez- Fernandez *et al.,* 2016). Wild olive genotypes have been tested in limited breeding studies, showing potential to shorten the juvenile period or to increase flower production (Klepo *et al*., 2014), to improve oil composition (Hannachi *et al*., 2008; León *et al*., 2018) and to improve resistance to soil-borne diseases (Arias-Calderon *et al.,* 2015a).

An alternative and direct approach to take advantage of the gene-pool of wild germplasm is the use of selected wild genotypes as rootstocks, which greatly increases the efficiency of perennial crops. Rootstocks are commonly chosen for rooting capacity, abiotic and biotic stress resistance, and their ability to beneficially alter scion phenotypes such as precocity (early bearing), production, and fruit quality (Warschefsky *et al*., 2016). It is interesting to note that wild olive rootstocks were widely used in ancient cultivation systems (Barazani *et al*., 2014), while modern olive crops, unlike other perennial woody crops, use self-rooted cultivars. Reduction of vigor through the use of dwarf rootstocks is of particular interest in the cultivation of woody fruit trees. The main drawback of super-intensive olive orchards, also known as high-density hedgerow (HDH) system, is the difficulty to control the tree size to allow the movement of the harvesting machines (Tous *et al.,* 2010). Cultivars used for HDH exhibit greater branching associated with smaller vigor parameters (Rosati *et al*., 2013). These features, which are difficult to gather in the same variety, determine that only a few traditional olive cultivars meet partially the low vigor requirement for HDH system, mostly ‘Arbequina’, ‘Arbosana’, and ‘Koroneiki’ (Diez *et al*., 2016). Even these cultivars require tree size control by means of strict pruning and fertirrigation practices (Fernandez *et al*.,

2013), which are expensive procedures. In addition, the HDH system excludes the possibility of using traditional cultivars of higher vigor, but of outstanding socioeconomic importance. Some studies indicate that certain olive cultivars used as rootstocks can regulate vigor traits like the canopy volume, stem section and production of the grafted scion (Baldoni and Fontanazza, 1990; Pannelli *et al.*, 2002; Del Río and Caballero, 2006; Tous *et al*., 2012; Romero *et al*., 2014; Rugini *et al*., 2016). The use of wild genotypes to control the vigor of the grafted cultivar is also a matter of great interest (León *et al*., 2020), but no rootstocks of proven quality are currently available at either commercial or experimental levels.

It would therefore be desirable to have a catalog of wild genotypes representing most of the variability of the *O. europaea* species characterized for agronomical or eco-physiological traits of greatest interest. In the present study, we have characterized a germplasm collection of 146 olive genotypes representative of the six *Olea europaea* subspecies including hybrids. The collection has been genotyped and phenotyped for a number of morphological and developmental traits of interest. As a proof of concept, the ability of a number of wild genotypes to modify vigor features of the olive cultivar 'Picual' has been addressed.

Materials and Methods

Plant Material and Culture Conditions

The wild olive germplasm collection, called SILVOLIVE, includes 146 genotypes obtained from seeds of mother trees prospected in their natural habitats or maintained in different Olive Germplasm Banks (WOGB-IFAPA Córdoba, WOGB-INRA Marrakech, and CEFE Montpellier; **Table 1**). The genotypes were *in-vitro* germinated from zygotic embryos of seeds from olive tres belonging to all subspecies of *Olea europaea* L. including hybrids (see **Table 1** for detail): *O. e*. subsp. *guanchica* (GUA, ANA, HER, and BAR); *O. e*. subsp. *cerasiformis* (CER); *O. e*. subsp. *maroccana* (MAR); *O. e.* subsp. *cuspidata* (CUS, CEH); *O. e.* subsp. *europaea* (ACO, ACZ, AJA, AMK, AMS, AOU, APR, ARC, FRA, and TAM); and *O. e.* subsp. *laperrinei* (DHO) consisting of zygotic embryos of "Dhokar," a Maghreb cultivated hybrid between *laperrinei* and *europaea* (Besnard *et al*.,

2013a). The 'Frantoio' (FRA) cultivar was the only elite olive variety exceptionally used as mother tree because of its potential interest in transmitting resistance to Verticillium wilt (Lopez-Escudero *et al*., 2004). The genotypes APR1 and ARC1 were previously obtained as seeds from salt-resistant wild olive trees present in Puerto Real (Cádiz, Spain) and Odiel (Huelva, Spain) salt marshes, respectively (Cantos *et al*., 2002) (**Image 1**).

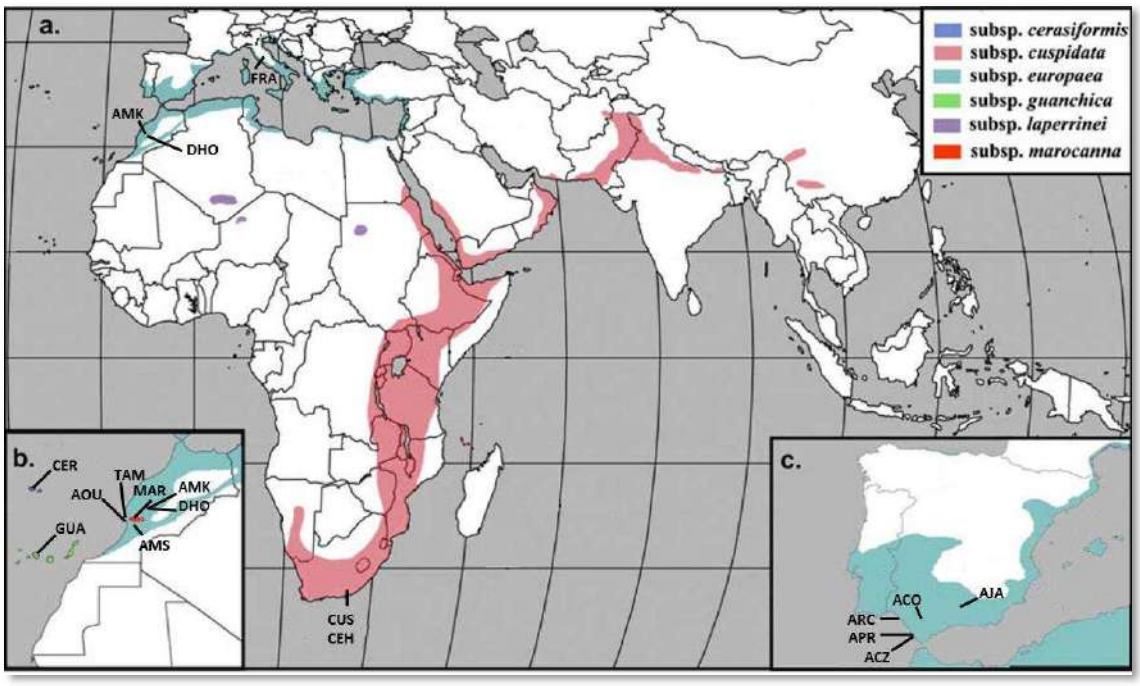


IMAGE 1 | Geographical distribution of the six currently recognised subspecies of *Olea europaea* L. and locations of the genotypes prospected described in Table 1. a) Distribution of genotypes from subsp. *europaea*, *laperrinei* and *cuspidata*; b) Distribution of genotypes from subsp. *cerasiformis*, *guanchica*, *maroccana* and *europaea* (partial); c) Distribution of genotypes from *O.e.* subsp. *europaea* in the Iberian Peninsula. *Modified from Rubio de Casas et al., 2006.*

Seeds were surface-sterilized and germinated *in-vitro* in a hormone-free medium (Rugini, 1984) incubated in a growth chamber with 16 h light photoperiod (34 µmol m^{-2} s^{-1} intensity with 70% red: 30% blue light-emitting diodes, LEDs) at 25 ± 2 ºC. Seedlings were cut into uninodal segments and micropropagated in the same Rugini medium supplemented with 1 mg/L zeatin in the same growth chamber described before. For whole plant regeneration, grown shoots were transferred to rooting medium (50% strength Rugini medium) supplemented with a-naphthalacetic acid (0.8 mg/L). Rooted seedlings were *ex-vitro* acclimatized for 3 weeks, transplanted to 2.5 L pots and then grown under greenhouse conditions.

Morphological and Architectural Traits

Different morphological and architectural traits were evaluated on *ex-vitro* potted plants at different growing stages. In potted plants, 13 months after transplanting *ex-vitro* acclimatized seedlings, we recorded: primary shoot height; number of secondary stems; number of tertiary stems; number of total nodes; total number of leaves; basal stem diameter (measured at 5 cm above ground with a vernier caliper); and fresh weight (leaf, shoot, and root). The morphological parameters were calculated according to the following equations:

Internode length = Height / Number of nodes on the main stem

R/A = Root fresh weight / Shoot fresh weight

Branching = Number of secondary stems

Branching frequency = Number of secondary stems / Number of nodes

(Rosati *et al*., 2013)

Branching efficiency = Number of secondary stems/ Basal Diameter 5 cm above ground

(Rosati *et al*., 2013)

Morphological traits of 103 wild genotypes were measured in three independent experiments, using 7–10 plants per genotype (**Supplementary Table S4**). In order to compare the results obtained from the different assays, the GUA1 variety was grown in the three different assays to normalize the data. Ratios obtained from two parameters measured in the same plant were calculated from absolute (non-normalized) values. Correlations between vigor parameters measured in grafted plants represent the average value of 8–12 plants per grafted genotype ± standard errors. Correlation graphics and the respective R^2-values were calculated with the Excel software.

Ploidy Level

Polyploids have been described within the *O. europaea* complex as a consequence of recent neopolyploidization events in Macaronesia (i.e., hexaploid *maroccana* and tetraploid *cerasiformis*; Besnard *et al*., 2008) and in the Hoggar mountains (i.e., presence of a few triploids in subsp. *laperrinei*; Besnard *et al*., 2008). It was thus necessary to first

determine the ploidy level of each individual of the SILVOLIVE collection. This was determined by flow cytometry according to the methodology described by Aleza *et al*. (2009). Samples consisted of small pieces of leaves (~ 0.5 mm^2) collected from each genotype, which were directly compared to a well-known diploid cultivar (Córdoba WOGB, acc. number W45) as a control. Samples were chopped together using a razor blade in the presence of a nuclei isolation solution (High Resolution DNA Kit Type P, solution A; Partec®, Münster, Germany). Nuclei were filtered through a 30 mm nylon filter and stained with a DAPI solution (4,6-diamine-2-phenylindol; High Resolution DNA Kit Type P, solution B; Partec®). Following a 5 min incubation period, stained samples were run in a CyFlowR Ploidy Analyzer (Partec®) flow cytometer equipped with optical parameters for the detection of DAPI fluorescence at 365 nm. Histograms were analyzed using the CyView software (Partec®), which determines peak position, co-efficient of variation (CV), arithmetic mean and median of the samples.

Chloroplastic DNA Polymorphism

Genomic DNA was extracted from leaf disks using the Sigma kit REDExtract-N-AmPlant PCR. We then used plastid markers to discriminate between the different wild olive provenances in our collection [Note that three plastid lineages have been described in the Mediterranean olive (Besnard *et al*., 2011): lineages E1 from the eastern Mediterranean basin, and lineages E2 and E3, both from the western Mediterranean region (hereafter referred to E1, E2, and E3, respectively)]. Ten chloroplastic DNA (cpDNA) loci previously reported (Weising and Gardner, 1999; Besnard *et al*., 2003, 2011, 2013a; Baali-Cherif and Besnard, 2005; Besnard, 2008; Garcia-Verdugo *et al*., 2010) were analyzed in the present study (**Supplementary Tables S1, S2**).

Primers for PCR-amplification of the cpDNA markers are listed in **Supplementary Table S2**. Polymerase chain reactions (PCR) were performed at a final volume of 20 mL with 10 ng of template DNA, 0.5 mM primer concentration, and 2 units of MyTaqTM Red DNA Polymerase (BIOLINE) through conventional PCR procedures using a BIO-RAD T100 thermal cycler. After amplification, 2 mL of the PCR product was run on a 2% agarose gel to verify amplification product size. PCR products were sequenced and chromatograms were visualized using the "Chromas" software to identify SNPs and

indels. For each genotype, a final sequence was obtained through concatenation of the loci following this order: ccmp5, OeR16Qa, matK2-3, QR-1, QR-2, QR-3, trnTD-2, trnTL-1, SSR-31, and SSR-45. For each subspecies and Mediterranean lineage, we also added as a reference the same concatenated sequences extracted from full plastomes available in the NCBI database. A full chloroplastic sequence was, however, not available for subspecies *cerasiformis*. All sequences were then aligned and analyzed with the "MEGA6" software (Tamura *et al*., 2013). A phylogenetic analysis was performed by maximum likelihood based on the Tamura 3- parameter model (Tamura, 1992).

Nuclear Microsatellite (SSR) Markers

Leaf samples from *in-vitro* grown seedlings were used to purify genomic DNA with the Sigma kit RED-Extract-N-AmPlant PCR. Five polymorphic nuclear SSR markers (Sefc *et al*., 2000) were then used to establish a genetic profile for every individual. The description of the SSR markers, including primer sequences, repetitive motif, allele size, and references are described in **Supplementary Table S3**. To get reference genotypes, DNA was also obtained from wild and cultivated olives maintained in different germplasm collections: subsp. *cerasiformis* (CEFE Montpellier, accession Cer3), subsp. *guanchica* (WOGB Córdoba, accession W49), subsp. *europaea* E1 (WOGB Córdoba, accession W45), subsp. *europaea* E3 (WOGB Córdoba, accession W69), as well as the cultivars "Dhokar" (WOGB Marrakech, accession Oct413) and "Frantoio" (WOGB Córdoba, accession 80). PCR reactions were performed as previously explained. SSR fragment analysis was performed with the "Peak Scanner" program (Applied Biosystems). A genotype matrix was built (**Supplementary Table S3**) and analyzed in R as explained below.

Grafting

To determine grafting compatibility, the olive 'Picual' and 'Hojiblanca' cultivars were grafted onto 43 wild genotypes using 10 potted plants per genotype. As a control, plants from both cultivars were also grafted onto their own roots. Leaves were removed from semi-hardwood wild genotypes grown for 18 months under greenhouse conditions after *ex-vitro* acclimatization. Rootstock plantlets were cut 20 cm from the ground level. 'Picual' scions with similar stem diameter, or slightly thinner than those of the rootstocks, were cut into sections containing 3–4 nodes and their leaves removed from

the base. To avoid tearing the bark, two slanted downward notches were rapidly made in the basal node of the scion (characteristic tip shape). Then, a 2-cm lengthwise incision was made in the cut tip of the rootstock stem using a sharp knife to quickly insert the scion, making sure that the two sets of cambial tissue coincide. A biodegradable synthetic tape was used to seal the graft unión in order to stop the entry of microorganisms and to prevent the rootstock and scion tissue cells from drying out. The grafted plant was grown for a year under greenhouse conditions before performing measurements of morphological scion features.

R Functions and Statistical Analyses

The software R was used for different genotyping and phenotyping analyses (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria)[1]. For the analyses of the morphological traits, a principal component analysis (PCA) based on the correlation data matrix was performed using the package "mice" to interpolate missing values, and "factoextra" to calculate the principal components. For the analysis of nuclear markers, a genotype matrix was built considering allele sizes (**Supplementary Table S3**) and analyzed with the "POLYSAT" package (Clark and Jasieniuk, 2011). Then, a matrix of genetic distances was created according to Bruvo *et al*. (2004). The principal coordinate analysis was plotted using the "POLYSAT" package. Morphological parameters of grafted plants were represented as the average values of 8–10 plants ± standard errors. Asterisks indicate significant differences with the selfgrafted 'Picual' plants value. The data were subjected to analysis of variance (ANOVA) and multiple comparisons of means were analyzed by Tukey's HSD (honestly significant difference). The multiple range test was calculated using the Statistical Analysis System (STATGRAPHICS Centurion XVI software; http://www. statgraphics.com; StatPoint Technologies, Warrenton, VA, United States).

Results

The wild olive germplasm collection SILVOLIVE includes 146 genotypes obtained from seeds of up to 120 mother trees from 19 different locations, prospected in their natural habitats or from Olive Germplasm Banks (**Table 1**). After *in-vitro* germination, the

genotypes were micropropagated to ensure the availability of clonal plant material required for subsequent genotyping and phenotyping assays.

Genotyping and Ploidy Level Determination of the SILVOLIVE Collection

To determine the origin and the degree of genetic variability of the SILVOLIVE genotypes, chloroplastic and nuclear markers have been used. Chloroplastic polymorphisms have been widely used to analyze the phylogeographic history of the olive complex (Besnard *et al*., 2003, 2007, 2018). According to the cpDNA markers obtained (**Supplementary Tables S1, S4**), the main maternal lineages of African and Mediterranean wild olives are represented in the SILVOLIVE collection (**Figure 1**), confirming the wide genetic variability represented in the germplasm collection. The three chloroplastic lineages previously identified in the subsp. *europaea* – as E1, E2, and E3 (Besnard *et al*., 2007) – were also sampled. We could also identify differences in the cpDNA of *cuspidata* genotypes. The previously fully sequenced *cuspidata* haplotype (NCBI accession number FN650747) includes the genotypes CUS12, CUS13, CUS14, and CUS15. The *cuspidata* genotypes CUS3, CUS4, CUS6, and CUS11 showed a polymorphism (C to T change) in the trnT-trnL spacer (Besnard *et al*., 2003). This attests that CUS trees (**Table 1**) were issued from two mothers (Gr3 and Gr5), not sharing the same chloroplastic haplotype.

TABLE 1 | Origin and code of the 146 olive genotypes of the SILVOLIVE collection.

Subspecies	Lineage	Mother tree: Variety	Mother tree: Natural Localization	Prospection	Acronym	Number of Genotypes
europaea	*E1*	'Frantoio' Ω	Tuscany, Italy	WOGB, Córdoba, Spain Acc. Number 80	FRA	4
		'Acebuche de Puerto Real' oleaster Ω	Puerto Real saline marshes, Cádiz, Spain	*In-vitro* germplasm collection M. Cantos (IRNAS, CSIC)	APR	1
		Unnamed oleaster Ω	Cádiz Mountains, Spain	WOGB, Córdoba, Spain Acc. Number W45	ACZ	10
		Unnamed oleaster×	Coria del Río, Seville, Spain	Coria del Río, Sevilla, Spain	ACO	5
		Unnamed oleaster×	Marrakech Mountains, Morocco	Marrakech, Morocco	AMK	11
		Unnamed oleaster×	Amskroud, Morocco	Amskroud, Morocco	AMS	9
	E2	'Raboconejo' oleaster Ω	Saltés Island in Odiel saline marshes, Huelva, Spain	*In-vitro* germplasm collection M. Cantos (IRNAS, CSIC)	ARC	1
		Unnamed oleaster×	Tamri, Morocco	Tamri, Morocco	TAM	5
		Unnamed oleaster×	Aourir, Morocco	Aourir, Morocco	AOU	12
	E3	Unnamed oleaster Ω	Sierra de Jaén, Spain	WOGB, Córdoba, Spain Acc. Number W69	AJA	6
laperrinei x *europaea*	*E1-/1*	'Dhokar'×	Tataouin zone, Tunisia	WOGB, Marrakech, Morocco Acc. Number Oct413	DHO	12
guanchica	*M-g1*	*guanchica* Ω	Tenerife, Canary Islands, Spain	WOGB, Córdoba, Spain Acc. Number W49	GUA	9
	M-g1	*guanchica*×	Tenerife, Canary Islands, Spain	Anaga, Tenerife, Spain	ANA	18
	M-g1	*guanchica*×	La Gomera, Canary Islands, Spain	Hermigua, La Gomera, Spain	HER	6
	M-g2	*guanchica*×	Gran Canaria, Canary Islands, Spain	Cañón del Cernícalo, Gran Canarias, Spain	BAR	9
cerasiformis	*M-c*	*cerasiformis* Ω	Madeira Islands, Portugal	CEFE Montpellier, France Acc. Number Cer3	CER	2
maroccana	*M-m*	*maroccana*×	Imouzzer, Morocco	Imouzzer, Morocco	MAR	3
cuspidata x *europaea*	*A*	*cuspidata*×	Grahamstown, South Africa	CEFE Montpellier Acc. Number Gr3 & Gr5	CUS	8
	A	*cuspidata*×	Kirstenbosch, South Africa	CEFE Montpellier, France Acc. Kirstenbosch	CEH	15
Total number of genotypes						146

× Seeds obtained from different mother trees.
Ω Seeds obtained from the same mother tree.

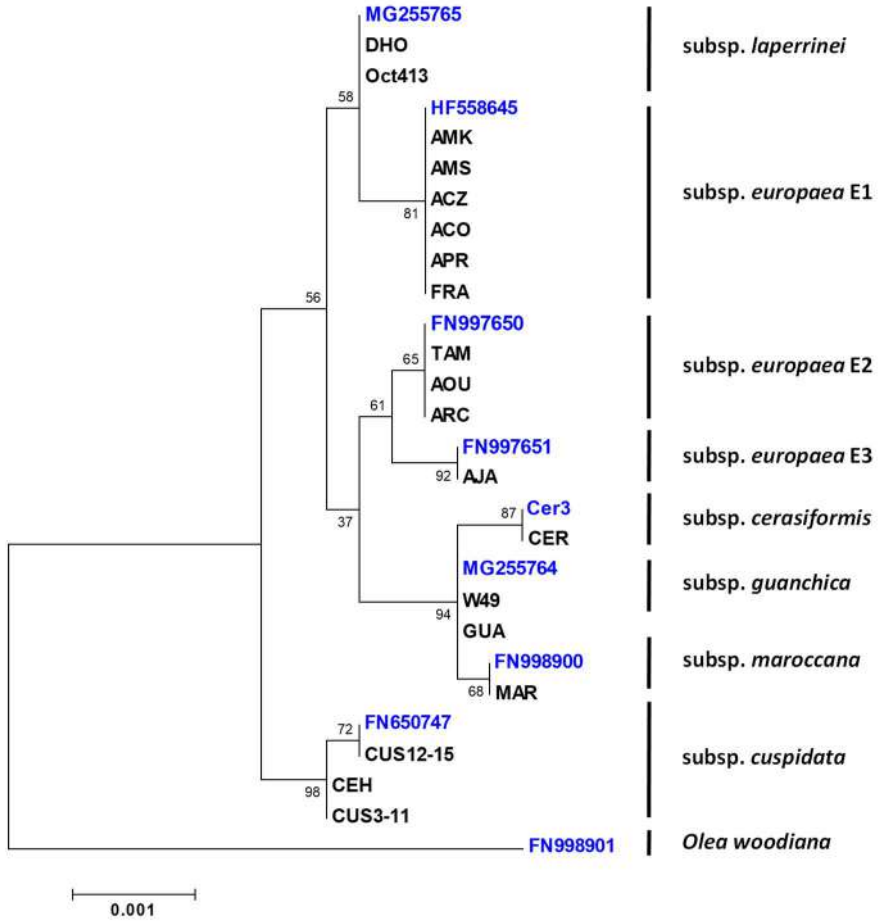


FIGURE 1 | Phylogenetic relationships among wild olive genotypes of the SILVOLIVE collection according to plastid markers. Sequences of the chloroplast markers were used to determine the phylogenetic relationships through the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Phylogenetic analyses were conducted with MEGA6 (Tamura *et al*., 2013). The tree with the highest log likelihood (–5551.0632) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.05)]. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The total number of positions in the final dataset was 3,818. Support of nodes was estimated with 1,000 bootstraps. NCBI Accession numbers of the plastomes, used here as reference genomes, are the following: *Olea europaea* subsp. *europaea* lineage E1 (HF558645); *O. e*. subsp. *europaea* lineage E2 (FN997650); *Olea europaea* subsp. *europaea* lineage E3 (FN997651); *O. e*. subsp. *laperrinei* (MG255765); *O. e.* subsp. *maroccana* (FN998900); *O. e.* subsp. *guanchica* (MG255764); *O. e*. subsp. *cuspidata* (FN650747); *O. woodiana* (FN998901). For *cerasiformis*, with no available full plastome sequence, the mother tree from the CEFE Montpellier collection (Cer3) was used to sequence the chloroplast markers. Other mother trees were also verified: W49 = *Olea europaea* subsp. guanchica from the WOGB Córdoba collection; Oct413 = *Olea europaea* subsp. *laperrinei* variety Dhokar from the WOGB Marrakech collection. *Olea woodiana* (FN998901) served as the outgroup species to root the tree.

To assess the possible genetic admixture imposed by the open pollination of the trees that gave rise to the genotypes of the SILVOLIVE collection, nuclear microsatellite markers, which are co-dominantly inherited, were analyzed. Five SSR markers were

sufficient to distinguish all individuals of the SILVOLIVE collection (**Supplementary Table S5**). While the cpDNA markers allowed distinguishing genotypes according to their maternal origin (following to some extent the taxonomy; Besnard *et al*., 2018) our SSR dataset did not allow a clear distinction of taxa (**Figure 2**), with many SSR alleles shared between subspecies (**Supplementary Table S5**). Thus, individuals belonging to the same chloroplastic lineage (e.g., *europaea*-E1) exhibited a relatively large nuclear diversity (**Figure 2**). This may result from the admixture produced by sexual crossings of wild mother trees with pollen of genetically diverse trees present in the prospection sites, mainly in WOGBs.

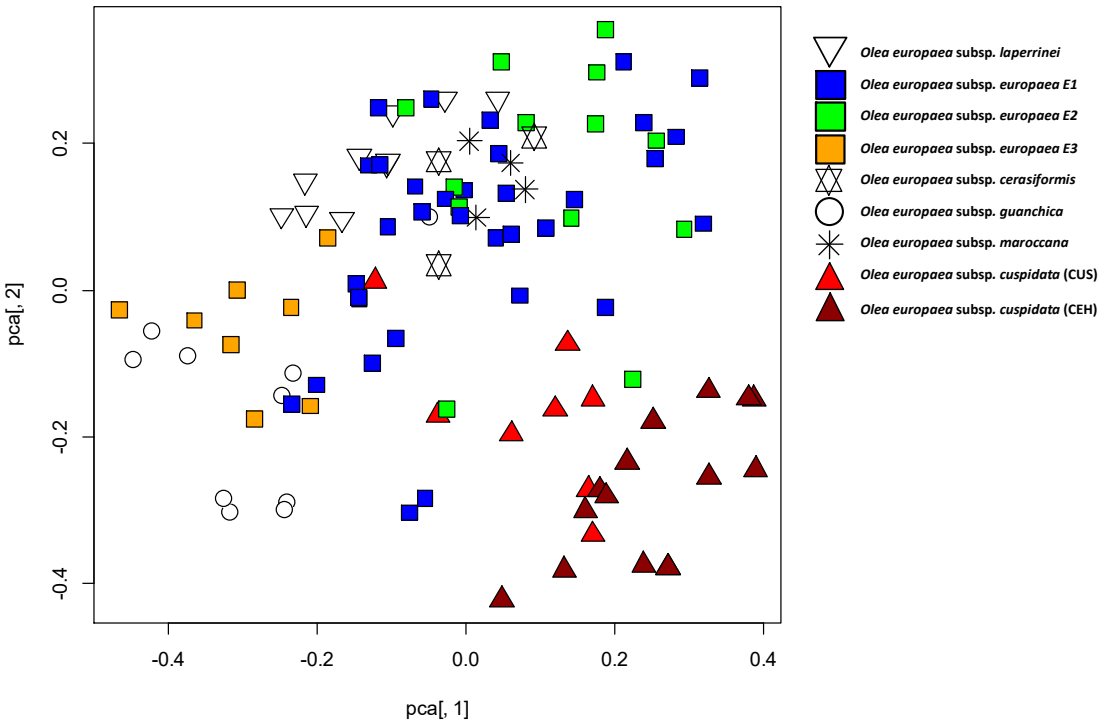


FIGURE 2 | Genetic dispersion of 105 genotypes of the SILVOLIVE collection according to nuclear SSR markers. Genetic distance matrices were calculated according to Bruvo *et al*. (2004). To represent the distribution of the genotypes according to their genetic distances, Principal Coordinate Analysis and plotting were subsequently performed with the package "POLYSAT" in R (Clark and Jasieniuk, 2011). Plotted symbols represent different subspecies or lineages of Olea europaea, and different colors identified the genotypes according to the classification obtained by chloroplastic markers. To compute allele copy number, POLYSAT uses the combinatorics utilities in R (the "combn" and "permn" functions from the COMBINAT package) to match all possible combinations of alleles and find the smallest sum of geometrically transformed distances between alleles (Equation 2) of Bruvo *et al*. (2004).

Multiple polyploidy levels were revealed in the SILVOLIVE collection through flow cytometry (**Supplementary Figure S1**) and nuclear microsatellite analyses (**Supplementary Table S5**). The results showed the presence of triploids harboring the cpDNA of subsp. *europaea*-E2 (AOU10), *laperrinei* (DHO10A, DHO11A) and *cerasiformis*

(CER1 and CER3); and hexaploid genotypes harboring the cpDNA of all genotypes of subsp. *maroccana*. All genotypes of *cuspidata*, *guanchica*, *europaea*-E1 and *europaea*-E3, as well as most *europaea*-E2 genotypes and *laperrinei* x *europaea* hybrids were confirmed as diploids. The presence of triploid genotypes suggests the occurrence of hybridization between diploid and polyploid genotypes in the prospecting zones, or spontaneous events of polyploidization.

Morphological Traits of the SILVOLIVE Collection

Growth habits and vigor traits can be quantified in olive seedlings a minimum of 9 months after germination (Hammami *et al*., 2011). Different morphological features of root and shoot parts have thus been measured to define vigor and branching habits in 103 wild olive genotypes 13 months after *ex-vitro* acclimatization. In **Supplementary Table S6**, genotypes have been primarily classified according to their height because it has been described as a good trait to predict the vigor of olive plants grown in pots (De la Rosa *et al.*, 2006). A strong phenotypic variability was observed in the height of the wild genotypes, which displayed differences of up to five times between the máximum and minimum values (**Supplementary Figure S1**). Among the genotypes analyzed, two hexaploids were included in the group of very reduced vigor; four triploid genotypes were distributed along the low, medium and high vigor groups; and no multiploid genotypes were present in the group of very high vigor genotypes (**Supplementary Table S6**). Vigor parameters, represented by the plant height showed high and positive correlations with the basal stem diameter, the shoot biomass, the total leaf biomass and the total plant biomass (**Supplementary Table S7**). Other group of correlated parameters comprises features characteristic of branching habits, including the branching efficiency, the branching frequency and the number of tertiary stems. The total number of nodes (and leaves) and the number of secondary stems correlated with both vigor and branching parameters. Finally, the root-to-shoot ratio showed negative correlations with both vigor and branching parameters. We observed that the high R/S ratio and the low shoot branching are traits highly represented in genotypes of very low and low vigor (**Supplementary Table S8**). High branching is overrepresented in the group of intermediate vigor genotypes. Finally, the low R/S ratio is typical of genotypes with high or very high vigor (**Supplementary Table S8**).

According to a PCA analysis that explains 66.1% of total variability of the morphological traits measured, different groups of genotypes could be distinguished (**Figure 3**). ARC, AOU, CUS, and CEH genotypes are mainly characterized by high values of vigor traits: plant height, stem basal diameter, plant biomass, shoot biomass, root biomass, total leaf biomass and internode length; APR, ACZ, CER, and CEH are mainly characterized by high values of branching traits: branching efficiency branching frequency, number of secondary and tertiary stems, total number of nodes and total number of leaves; AMK, GUA, AJA and DHO genotypes are mainly characterized by low vigor traits; MAR and DHO genotypes are mainly characterized by low branching habits and high root-to-shoot ratio. The high variability of genotypes was not only observed at the level of the whole collection, but also within the same subspecies, and even within the offspring of the same tree, as observed for example in ACZ, CUS, AOU, DHO, or CEH genotypes (**Figure 3**).

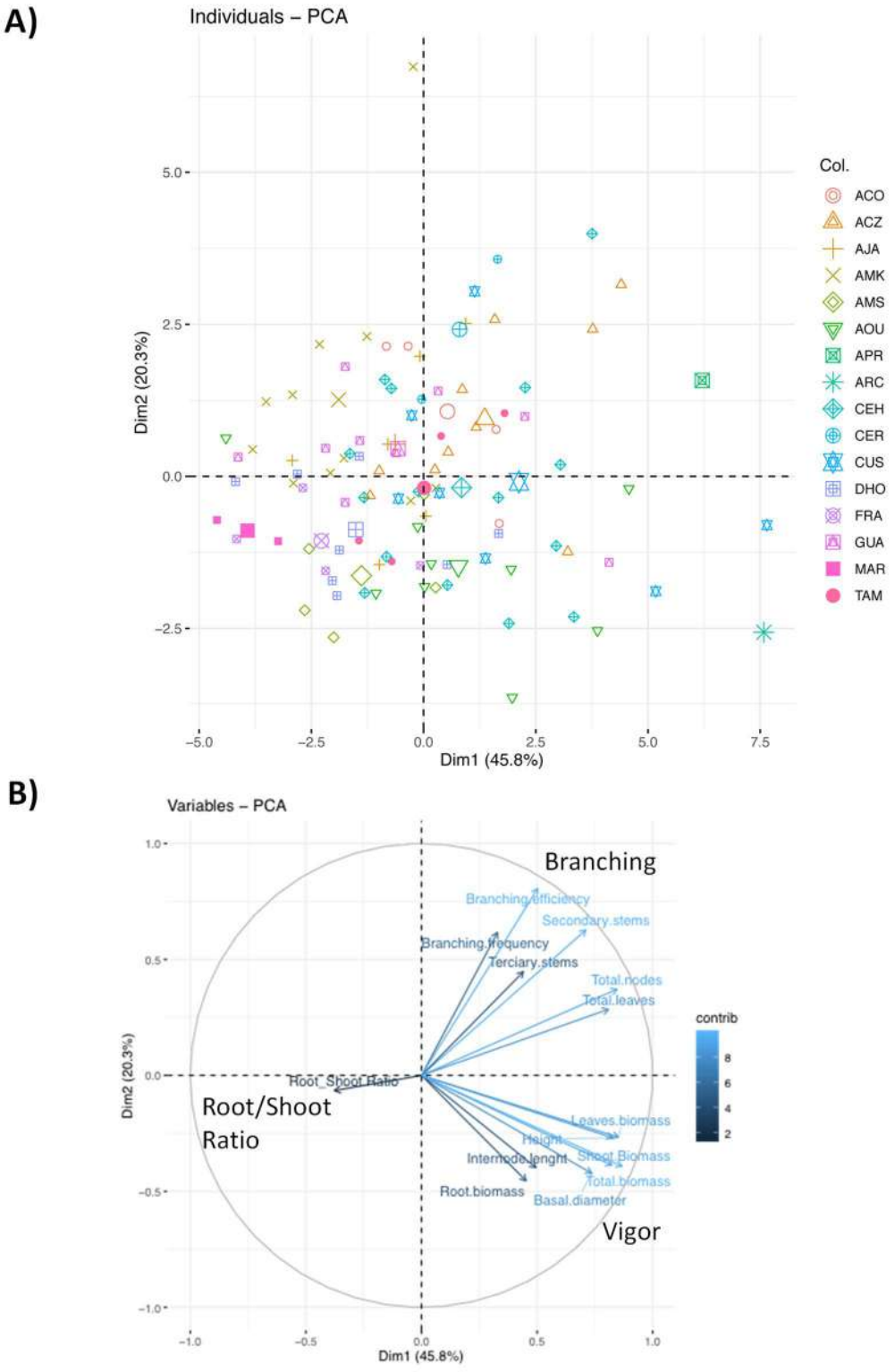


FIGURE 3 | Principal Component Analysis (PCA) of morphological parameters of wild olive subspecies. Data concerning morphological parameters were converted into a matrix of numerical values. The missing data were interpolated using the "Mice" package as a preliminary step to the calculation of the main components using "Prcomp." In (A) the two main components of variability, explaining here 66.5% of the total variability, were identified using the "Fviz_eig (res.pca)" algorithm and the results were plotted in a graphic. In (**B**) the contribution of each variable is depicted in the two main components previously represented in (**A**).

Regulation of Scion Features by Wild Genotypes Used as Rootstocks

Tree grafting on clonal rootstocks is an important practice for morphological uniformity, improvement of environmental adaptability and crop quality of plants. However, it is not a widespread procedure in olive nursery production. In a first attempt to confirm the grafting compatibility of the wild genotypes with commonly used cultivars, the two high-vigor Spanish varieties 'Picual' and 'Hojiblanca' were grafted on 43 genotypes belonging to subspecies *guanchica*, *cerasiformis*, *laperrinei*, *cuspidata*, and *europaea* (lineages E1, E2, and E3) or their hybrids. All the accessions assayed, including the most genetically distant subsp. *cuspidata* showed grafting compatibility with the cultivated olive varieties used (**Supplementary Table S9**). Low efficient grafting compatibility was observed only for subsp. *europaea* lineage E2, for which a single genotype was tested.

As a proof of concept, morphological traits were examined in the "Picual" scion grafted on 20 different wild olive genotypes. When used as rootstocks, many wild genotypes modified vigor parameters of the "Picual" scion (**Figure 4A**). As expected, most of the genotypes classified as very-low to intermediate vigor (**Supplementary Table S6**; DHO10B, ACO15, AMK14, GUA8, GUA2, FRA4, AJA17, AMK6, and FRA3) significantly reduced vigor properties of the grafted scion (**Figures 5A–E**). Conversely, high vigor genotypes such as ACZ9, CUS13 and CUS15 increased the vigor of the grafted scion (**Figures 5A–E**). However, some contradictory relationships were also observed. Thus, AMK5, AMK21, and GUA9, classified as very low- and low-vigor genotypes (**Supplementary Table S6**), significantly increased vigor parameters in the grafted "Picual" variety (**Figure 5E**). This indicates that not only the rootstock but also some other unknown effects (e.g., the rootstock x scion interaction) determined the scion properties in the grafted plant. As a result, when vigor traits such as basal diameter and height were compared within the same grafted plant, a positively significant correlation was observed (**Figure 4A**). However, when the vigor trait either height (**Figure 4B**) or basal diameter (**Figure 4C**) was compared between the grafted and the non-grafted plant, no clear correlation was observed.

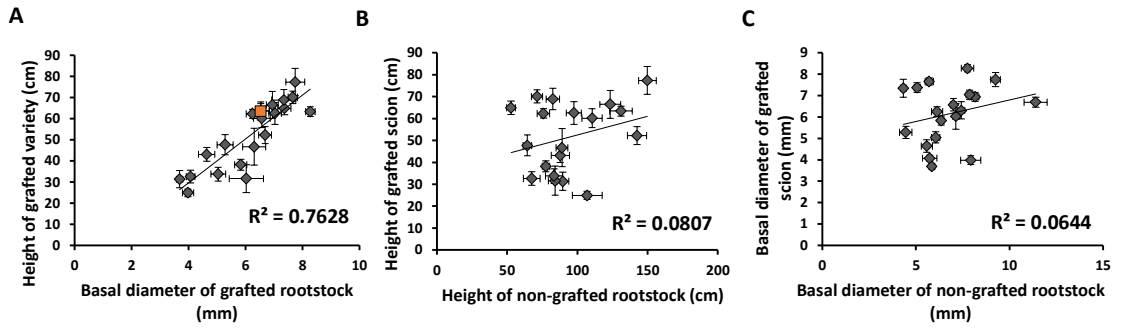


FIGURE 4 | Vigor regulation of the "Picual" scion by wild rootstock genotypes. Wild genotypes grown in pots for 1 year after *ex-vitro* acclimatization were grafted with the cv. "Picual". Morphological features were measured 1 year after grafting. (A) Correlation between the rootstock basal diameter and the scion height. Values correspond to the average value of different individuals (N = 8–12), with self-grafted "Picual" labeled in red color. (B) Correlation between height of the non-grafted rootstock and height of the grafted scion. Values correspond to the average value of different individuals (N = 8–12) of each genotype. (C) Correlation between the basal diameter of the non-grafted rootstock and the height of the grafted scion. Values correspond to the average value of different individuals (N = 8–12) of each genotype. Error bars in 2 dimensions (in A–C).

While many of the tested genotypes reduced to some extent vigor parameters of the 'Picual' scion (9 out of 20 genotypes showed statistically significant reductions of most vigor traits assayed; **Figures 5A–F**), modification of branching traits was less evident (**Figures 5G, H**). Most of the genotypes that reduced vigor also significantly reduced branching efficiency (**Figure 5G**), although no significant differences in branching frequency were observed (**Figure 5F**). However, FRA3, which showed significant reductions of some vigor traits, maintained similar branching efficiency, and higher (although not statistically significant) branching frequency, than the self-grafted 'Picual'.

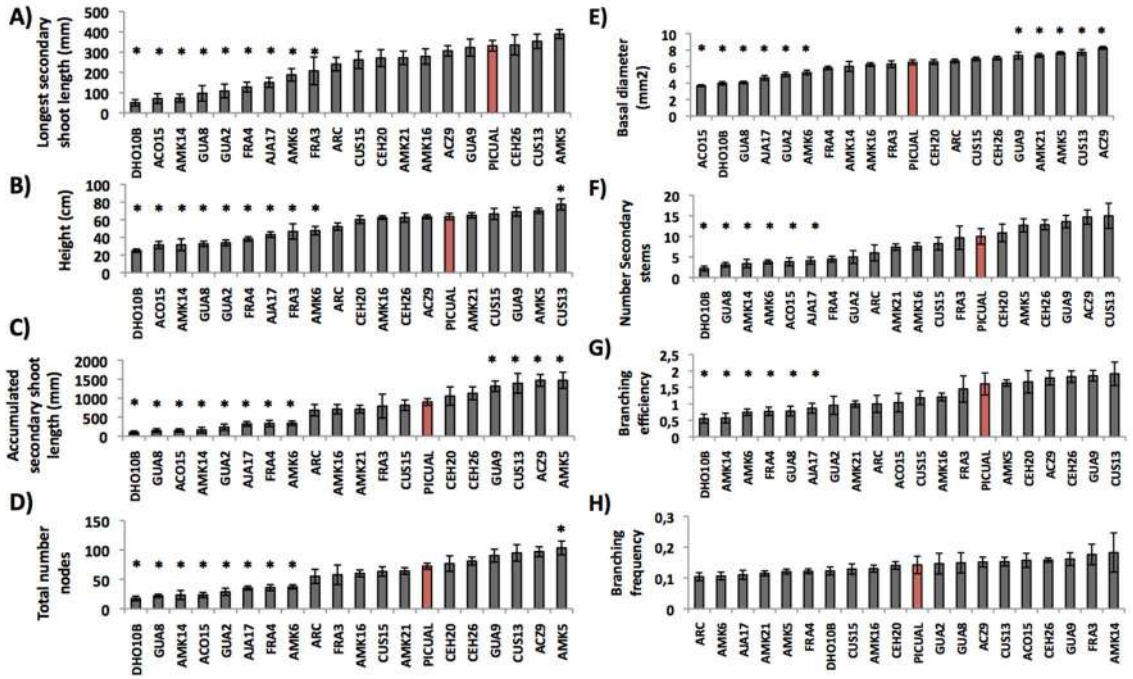


FIGURE 5 | Comparison of morphological parameters measured in the 'Picual' scion grafted on different genotypes of the SILVOLIVE collection. Potted plants grown under greenhouse conditions were used 1 year after grafting. The parameters measured were: The longest secondary shoot length (A); the plant height (B); the accumulated secondary shoot length calculated as the sum of all secondary shoots length (C); the total number of nodes (D); the basal stem diameter at 5 cm from ground (E); the total number of secondary stems (F); the branching efficiency (G); and the branching frequency (H). Bars are the average of 7–10 plants. Asterisks indicate significant differences with self-grafted 'Picual' plants labeled as red bar. The data were subject to analysis of variance (ANOVA) and multiple comparisons of means were analyzed by Tukey's HSD (honestly significant difference). Multiple range test was calculated using the Statistical Analysis System (STATGRAPHICS Centurion XVI software; http://www.statgraphics.com; StatPoint Technologies, Warrenton, VA, United States).

Discussion

Ecological and socio-economic issues regarding the future of olive cultivation are essential in the light of present global changes, including agronomic, climatic, economic societal, or political changes (Besnard *et al*., 2018). Genetic erosion has dangerously shrunk the genetic pool of crop species. In the olive cultivation, the use of a small number of cultivars for "modern" olive orchards (e.g., in the HDH system) may lead to genetic erosion in the near future, which may increase the susceptibility of the crop to abrupt climate changes, and to the emergence of new diseases and pests (Esquinas-Alcázar, 2005). Therefore, identification of unexploited adaptive traits in wild olive genotypes and their subsequent utilization is expected to be a major goal of olive crop breeding and rootstock programmes in the close future.

Genetic Diversity of the SILVOLIVE Collection

Germplasm characterization is a key starting point of the pre-breeding process, and molecular markers are a valuable tool for identifying and characterizing olive genotypes (do Val *et al*., 2012). The gain of genetic diversity in the SILVOLIVE collection was achieved at two levels: (i) firstly, at the whole collection level, it includes genotypes related to all known subspecies of *O. europaea*, that were here characterized with both plastid and nuclear markers. Besides the genetically distant subspecies *cuspidata* (**Figure 1**), genotypes of subspecies *europaea*, *laperrinei*, *cerasiformis*, *guanchica*, and *maroccana* belong to a monophyletic lineage from North Africa and the Mediterranean area, as previously reported (Médail *et al*., 2001; Rubio de Casas *et al*., 2006; Besnard *et al*., 2018); (ii) secondly, at the level of individuals, many accessions of the collection resulted from admixture between genetically distant parents. It is expected that hybrid offspring displays phenotypic performance superior to their parents due to a higher heterozygosity (Shull, 1948). The greatest manifestation of heterosis in the SILVOLIVE collection is expected to occur in genotypes resulting from the cross between *cuspidata* and *europaea* (CUS and CEH genotypes) since both parental subspecies present the maximum genetic distance (**Figure 1**). The admixture produced by sexual crossings of wild mother trees with pollen of diverse origin in collections or natural conditions (**Figure 2**), has further increased the genetic diversity and heterosis of the SILVOLIVE collection. Different ploidy levels are also represented in the collection, first with the hexaploid *maroccana*, and second with triploids of different origins. The triploid genotypes CER1 and CER3 were obtained from a verified 4x *cerasiformis* mother tree (CEFE Montpellier, accession Cer3) and may thus result from a cross with a diploid father tree of the CEFE collection. The triploid AOU10, harboring the *europaea*-E2 chloroplast lineage, was prospected in the Southwest Morocco, where the hexaploid *maroccana* subspecies is endemic. AOU10 could be the product of a sexual crossing between a diploid female parent (*europaea*) with a hexaploid male parent (*maroccana*), although a tetraploid genotype should be rather expected. The possibility that AOU10 just resulted from a spontaneous triploidization between two oleasters cannot be ruled out (Besnard and Baali-Cherif, 2009). Similarly, the two triploid progenies issued from “Dhokar” from the WOGB Marrakech are very likely issued from a spontaneous events

of polyploidization. A high level of unreduced gametes is indeed expected in "Dhokar" due to its hybrid status (Besnard *et al*., 2013a) that may lead to abnormalities during the formation of gametes (Mason and Pires, 2015).

Phenotypic Diversity of the SILVOLIVE Collection

The diversity of morphological features in olive trees (shoot growth, root development, root-to-shoot biomass ratio, branching habits, total leaf surface, etc.) can be enormously relevant in terms of plant-soil interaction, plant hydraulic properties, water and nutrient uptake abilities, photosynthetic capacity, abiotic stress resistance, etc., as previously shown for a number of SILVOLIVE genotypes (Hernández-Santana *et al*., 2019). Illustrating the huge phenotypic variability of the collection, genotypes like DHO6A have a root biomass that approximates that of the aerial part (**Supplementary Figure S2**), whereas other genotypes like AMK34, AJA7 or CUS14 have a root biomass five times lower than that of their corresponding aerial biomass. Root anatomy can mediate responses to a range of abiotic and biotic stressors. In many soils, a deep-rooting, waterconserving root phenotype is likely to have several advantages (Warschefsky *et al*., 2016). Besides the root development, the morphological traits measured have been organized in two groups of correlative traits, which identifies two sets of vigor and branching parameters respectively (**Figure 3** and **Supplementary Table S7**) Particularly plant vigor traits show great variability (e.g., up to 5 times differences in height; **Supplementary Figure S2**). Highly vigorous phenotypes with high root biomass could be of interest to rehabilitate deforested habitats or at risk of desertification (Bekele, 2005; Kassa *et al*., 2019), while low-vigor genotypes could be particularly interesting for different reasons. On the one hand, they could reasonably be better suited to reduce vigor of grafted scions, which should be of great relevance for HDH olive plantations. On the other hand, 45% of very low- and low-vigor genotypes present high root-to-shoot ratio (**Supplementary Table S8**), which may determine a more favorable plant-soil interaction in term of stress resistance, nutrient- and water-use efficiencies. These traits may favor the reduction of irrigation and fertilizers, promoting a more sustainable agriculture. This is particularly true for very low- and low-vigor genotypes like DHO6A, AMK21, DHO12A, AMK9, GUA8, AMK12, CEH2, FRA4, DHO10B, and AMS7 (**Supplementary Table S6** and **Supplementary Figure S1**). Most AMK genotypes (10 out

of 11) are classified as low or very low vigor genotypes (**Supplementary Figure S2**), being a possible source of low-vigor genes. However, genotypes with low vigor are frequently discarded in olive breeding programs because of their long juvenile period (De la Rosa *et al*., 2006). This wide variability of vigor features was also observed in wild olive genotypes from the SILVOLIVE collection grown in the field for a longer time (León *et al*., 2020). Thus, similarly to the phenotype observed in potted plants, AMK genotypes showed low vigor when grown in the field, supporting the fact that morphological features observed in young plants grown in pots, like vigor, are still observed in field-grown plants. The possibility that the low-vigor AMK genotypes are inbred individuals should be bear in mind, because they could be more susceptible to adverse situations of biotic or abiotic origin. Around 3.3 times variability was observed in the basal stem diameter of the wild olives collection. In general, genotypes with greater heights have thicker trunks, showing a positive correlation in potted plants (R^2 = 0.7581), as reported also in SILVOLIVE genotypes grown in the field (León *et al*., 2020).

However, some varieties such as FRA1, AMS15, AMK26 and DHO6A, showed thicker primary stems despite having low vigor traits according to other morphological features. The number of secondary stems also showed high variability (**Supplementary Figure S1** and **Supplementary Table S6**), with CEH3, ACZ7, and ACZ8 exhibiting the highest number of lateral branches. The AMK26 genotype is remarkable, with the smallest size and the highest branching frequency (**Supplementary Figure S1**). Cultivars used for HDH orchards, such as "'Arbequina' " and "Arbosana," exhibit greater branching frequency associated with smaller diameters of trunk, branches and shoots. This means low-vigor plants producing a greater number of smaller secondary stems and shoots that reduce permanent structures for a given canopy volume (Rosati *et al*., 2013). In this study, genotypes with low basal diameter and high branching like AMK26, CUS14, AMK27, AMK5, AJA17, AOU13, AMS12, GUA9, ACO15, ACO14, CER3, GUA4, ACZ4, and CUS3, have been identified. The branching habit is one of the main factors affecting carbon partitioning between wood and leaves. Low-vigor and highbranching genotypes of the SILVOLIVE collection have a higher number of nodes and leaves (R^2 = 0.7), which means increased canopy density and may determine higher number of potential fruiting sites, becoming an important trait to be used to produce and export more assimilates

toward fruits (Rosati *et al*., 2018). Otherwise, the hexaploid MAR1 and MAR3, together with most FRA and AMS genotypes show high apical growth with a low number of secondary stems.

SILVOLIVE, a Germplasm Collection for the Identification of Rootstocks That Improve Olive Cultivation

Traditional olive plantations are characterized by low tree density and rain fed orchards with low yields. Progressive intensification of olive cultivation, with higher densities, irrigated, and mechanically harvested orchards has significantly increased crop productivity [e.g., higher production at lower costs; Rallo *et al*. (2013)]. But intensive cultivation has strongly reduced the diversity of cultivars in olive orchards, increased the demand of inputs and the risk of environment unbalances (Rallo *et al*., 2016) dealing, for example, to higher incidence of soil-borne diseases (Lopez-Escudero and Blanco-Lopez, 2005; Lopez-Escudero and Mercado-Blanco, 2011; Perez-Rodríguez *et al*., 2015). Given that grafting compatibility can occur across broad phylogenetic distances, crop wild relatives are of great significance to grafted perennial crops. Olive cultivation, specially intensive crops, could benefit from the use of clonal rootstocks, which have been proposed to potentially improve a number of agronomic traits such as: resistance to Verticillium wilt (Porras-Soriano *et al*., 2003; Bubici and Cirulli, 2012); tolerance to frost injury (Pannelli *et al*., 2002) and iron chlorosis (Alcantara *et al*., 2003); and early maturation (Malik and Bradford, 2004). But the main drawback of HDH orchards is the difficulty to control the tree size to allow the movement of the harvesting machines (Tous *et al*., 2010). Only a few traditional olive cultivars meet partially the low vigor requirement for HDH plantations, mostly ‘Arbequina’, ‘Arbosana’, and ‘Koroneiki’ (Diez *et al*., 2016). Even these cultivars require tree size control by means of strict pruning and fertirrigation practices (Fernandez *et al*., 2013), which are expensive procedures. In addition, the HDH system excludes the possibility of using traditional cultivars of higher vigor, but outstanding socioeconomic importance. Rootstock-induced reduction in scion vigor, or ‘dwarfing’ causes a decrease in tree size, reducing the need for pruning in commercial orchards. But to our knowledge, no rootstocks of proven quality are presently available at either commercial or experimental levels. The question arises

whether the phenotypic features previously described in genotypes of the SILVOLIVE collection can be somehow transmitted to the grafted scion. Plant shoot vigor is affected by numerous root-depending factors including root hydraulic pressure, water uptake efficiency, hormone production, nutrient uptake, and stomatal conductance. Convincing evidence has been provided that these traits are genetically encoded by the root portion of the grafted plant, playing the rootstock genotype essential roles in shaping variation of these traits in the scion (Warschefsky *et al*., 2016). In addition, rootstocks can also affect the branching pattern of the scion (Costes *et al*., 2010) and promote early and more abundant bearing in young olive trees (Rosati *et al*., 2017, 2018). On the one hand, we have verified that all subspecies can be grafted with commercial olive varieties, including *cuspidata*, which has the maximum genetic distance with *europaea* (**Supplementary Table S9**). On the other hand, we have also confirmed that wild genotypes used as rootstocks modify the morphological properties of the grafted scion (**Figures 4A**, **5**). In general, a correspondence is observed between the vigor of the genotype and the vigor transmitted to the grafted scion. However, graft combinations have also been observed in which this correlation does not occur, with low-vigor genotypes increasing the vigor of the grafted scion (e.g., AMK5, AMK21, and GUA9; **Figure 5E**), strongly suggesting that other factors (e.g., the rootstock x scion interaction) are also relevant to determine the properties of the grafted tree. It is noteworthy that in a first trial with 20 genotypes of variable vigor, 9 genotypes significantly reduced vigor parameters of the grafted 'Picual' scion (**Figure 5**). This represents 45% of the genotypes tested, which indicates that the ability to reduce vigor is a common trait in the collection of wild genotypes. Most of the rootstocks that reduce vigor of the 'Picual' scion also significantly reduced the branching efficiency as a consequence of the reduction in the number of secondary stems (**Supplementary Figure S2**), given the strong correlation between both parameters (R^2 = 0.93). However, no significant differences were observed in the branching frequency or average number of secondary stems per bud.

Conclusion

This work has been carried out on plants grown in pots 1 year after grafting. It is therefore necessary to extend this study to field conditions for a greater number of years

to obtain more conclusive data. For example, determining the rootstock effect on the branching frequency might require greater root development in the soil and a larger canopy volume. With this aim, field trials are currently being developed under HDH conditions with 35 selected genotypes used as rootstocks of the 'Picual' and 'Arbequina' scions. In addition, some of the genotypes tested have been proven resistant or extremely resistant to Verticillium wilt (to be published), one of the most threatening disease for olive cultivation due to the severity of damage caused and its rapid extension (Inderbitzin and Subbarao, 2014). The identification of one or several Verticillium-resistant dwarfing rootstocks in the collection of wild genotypes could give an important boost to the high density cultivation of olive grove, opening the possibility of: increasing the sustainability of the crop (e.g., reducing pruning and the use of fungicides); and introducing new oil or table olive cultivars (of higher vigor) into the HDH system. Therefore, the SILVOLIVE collection, which represents an important part of the genetic variability of the species, has been genotyped and phenotyped. Great variability has been found in the wild genotypes at both genotypic and phenotypic levels. When used as rootstocks, wild genotypes modify growth parameters of the grafted scion. According to their specific characteristics, genotypes of the SILVOLIVE collection have great potential interest to: (i) restore vegetation on degraded soils or at risk of desertification; (ii) provide relevant traits or genes in breeding programs; (iii) be used as rootstocks for olive cultivation, mostly for high-density hedgerow (HDH) orchards.

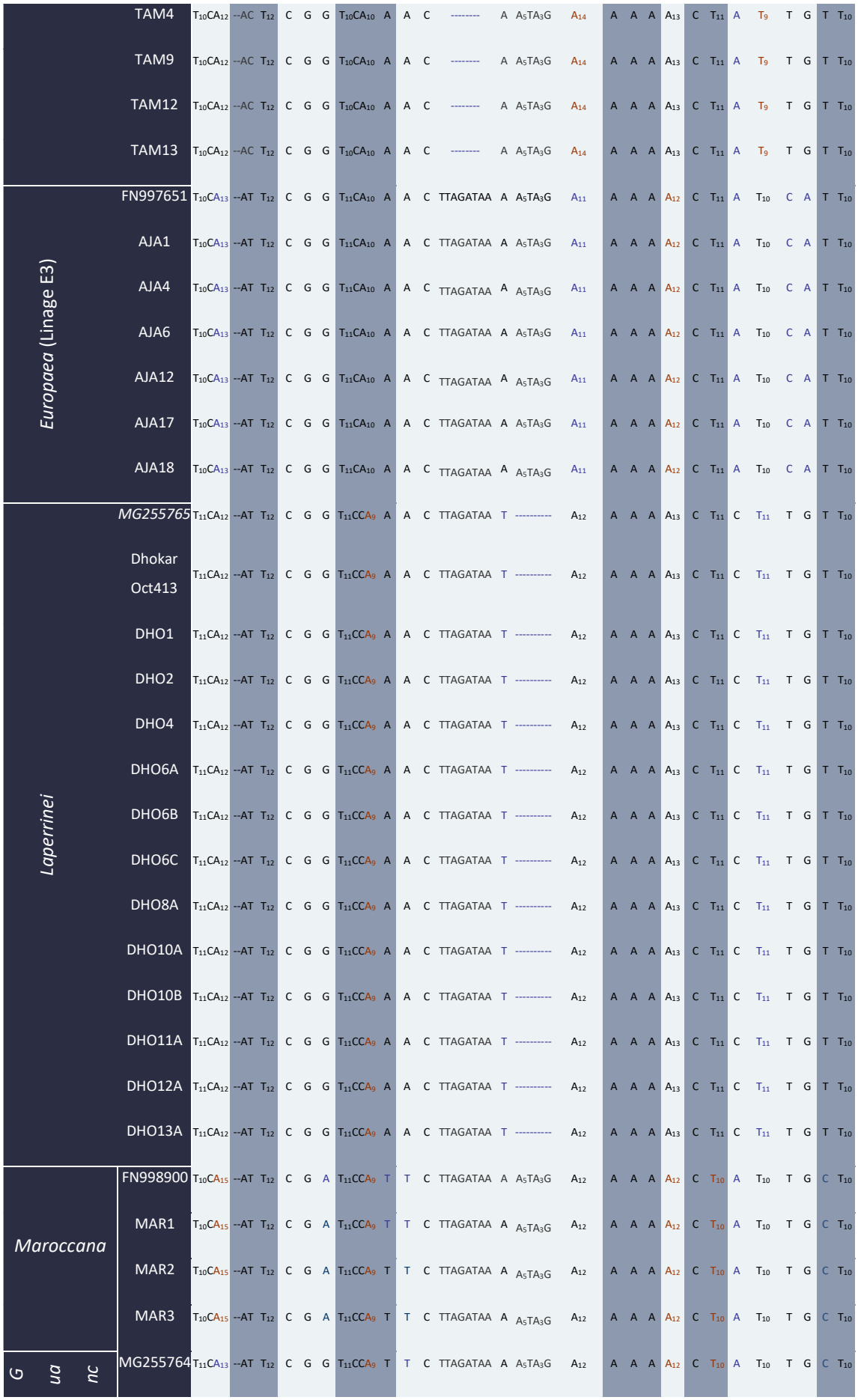
Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00629/

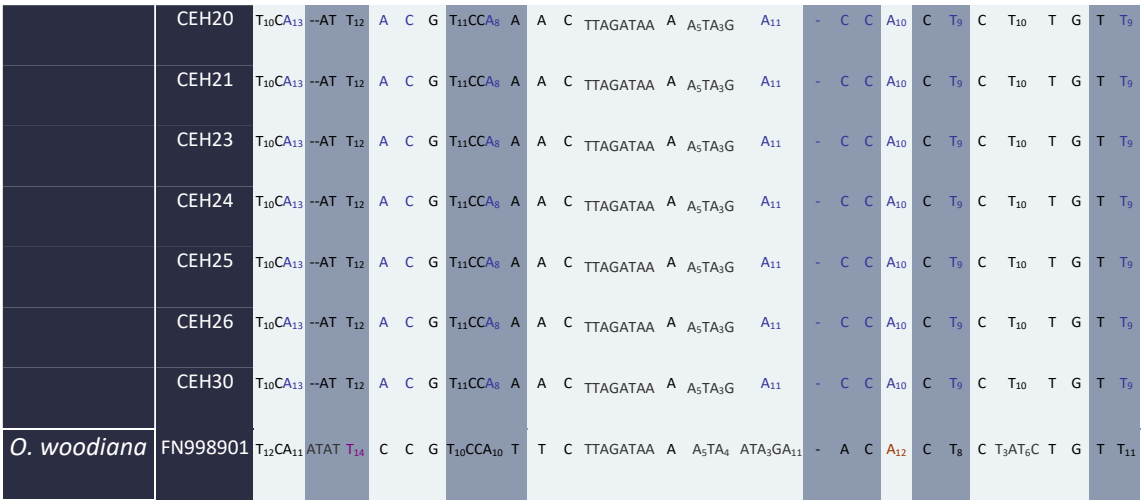
SUPPLEMENTARY TABLE S1 | Chloroplast markers.

Subspecies	Varieties	cpDNA MARKERS										
		CCMP5R	OeR16Qa-F	matK2-3F	QR-1RF	QR-2F	QR-3R	TrnTD-2F	TrnTL1F	SSR31F	SSR45F
Europaea (Linage E1)	HF558645	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACO1	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACO5	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACO14	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACO15	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACO18	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ1	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ2	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ3	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ4	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ5	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ7	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ8	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ9	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ10	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	ACZ12	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK5	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK6	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK14	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK16	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK21	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK26	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK34	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK9	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK12	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}
	AMK25	$T_{11}CA_{12}$	--AT T_{12}	C G G	$T_{11}CA_{10}$ A	A T TTAGATAA T ---------- A_{12}	A A A	A_{13}	C T_{11}	C T_{10} T G	T T_{10}

	AMK27	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS7	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS8	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS11	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS12	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS15	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS16	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS17	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS18	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	AMS19	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	FRA1	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	FRA2	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	FRA3	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	FRA4	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
	APR	$T_{11}CA_{12}$	--AT	T_{12}	C	G	G	$T_{11}CA_{10}$	A	A	T	TTAGATAA	T	---------	A_{12}	A	A	A	A_{13}	C	T_{11}	C	T_{10}	T	G	T	T_{10}
Europaea (Linage E2)	FN997650	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	ARC	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU1	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU2	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU3	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU4	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU5	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU7	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU8	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU9	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU10	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU11	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU12	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	AOU13	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}
	TAM3	$T_{10}CA_{12}$	--AC	T_{12}	C	G	G	$T_{10}CA_{10}$	A	A	C	--------	A	A_5TA_3G	A_{14}	A	A	A	A_{13}	C	T_{11}	A	T_9	T	G	T	T_{10}


TAM4
TAM9
TAM12
TAM13
FN997651
AJA1
AJA4
AJA6
AJA12
AJA17
AJA18
Europaea (Linage E3)
MG255765
Dhokar Oct413
DHO1
DHO2
DHO4
DHO6A
DHO6B
DHO6C
DHO8A
DHO10A
DHO10B
DHO11A
DHO12A
DHO13A
Laperrinei
FN998900
MAR1
MAR2
MAR3
MG255764
Maroccana
Gua nc

	W49	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA1	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA2	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA3	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA4	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA5	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA6	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA7	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA8	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	GUA9	$T_{11}CA_{13}$	--AT	T_{12}	C	G	G	$T_{11}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
Cerasiformis	Cer3 (EDB)	$T_{10}CA_{13}$	----	T_{12}	C	G	G	$T_{12}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	CER1	$T_{10}CA_{13}$	----	T_{12}	C	G	G	$T_{12}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
	CER3	$T_{10}CA_{13}$	----	T_{12}	C	G	G	$T_{12}CCA_9$	T	T	C	TTAGATAA	A	A_5TA_3G	A_{12}	A	A	A	A_{12}	C	T_{10}	A	T_{10}	T	G	C	T_{10}
Cuspidata	FN650747	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	T	T_9	C	T_{10}	T	G	T	T_9
	CUS3	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CUS4	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CUS6	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CUS11	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CUS12	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	T	T_9	C	T_{10}	T	G	T	T_9
	CUS13	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	T	T_9	C	T_{10}	T	G	T	T_9
	CUS14	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	T	T_9	C	T_{10}	T	G	T	T_9
	CUS15	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	T	T_9	C	T_{10}	T	G	T	T_9
	CEH2	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH3	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH6	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH7	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH8	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH9	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH17	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9
	CEH19	$T_{10}CA_{13}$	--AT	T_{12}	A	C	G	$T_{11}CCA_8$	A	A	C	TTAGATAA	A	A_5TA_3G	A_{11}	-	C	C	A_{10}	C	T_9	C	T_{10}	T	G	T	T_9



The ‘ccmp5’ marker is a microsatellite region present in the atpB-rbcL spacer (Weising and Gardner, 1999) that identifies subsp. *maroccana*. The ‘OeR16Qa’ marker is an indel in the rps16-trnQ spacer (Garcia-Verdugo *et al*., 2010) that identifies subsp. *cerasiformis*. The ‘MatK2-3’ marker in the matK region (Baali-Cherif and Besnard, 2005), includes a G to A transversion in subsp. *maroccana*, and the C to A transversion and G to C transition in subsp. *cuspidata*. The ‘QR-1’ marker in the psbK-trnS spacer (Besnard *et al*., 2003), contains an A to T transition in subsp. *maroccana*, *guanchica* and *cerasiformis*, and length allelic variants at a microsatellite locus allowing distinction of several taxa: “$T_{11}CCA_8$” in subsp. *cuspidata*, “$T_{11}CCA_9$” in subsp. *cerasiformis*, *maroccana* and *laperrinei*, “$T_{11}CA_{10}$” in subsp. *europaea* E1 and E3, and “$T_{10}CA_{10}$” in subsp. *europaea* E2. The ‘QR-2’ marker in the psbK-trnS spacer (Besnard *et al*., 2003) includes two different indels, one specific to subsp. *europaea* E2, and the other to subsp. *europaea* E1 and *laperrinei*. Other markers present in the QR-2 region include a C to T transversion specific to subsp. *europaea* E1; an A to T transition specific to subsp. *maroccana*, *guanchica* and *cerasiformis*; and length allelic variants at a poly-A stretch specific to subsp. *cuspidata* and *europaea* E3 (“poly-A11”), subsp. *cerasiformis*, *guanchica*, *laperrinei*, *maroccana*, and *europaea* E1 (“poly-A12”), and subsp. *europaea* E2 (“poly-A14”). The ‘QR-3’ marker in the trnS-trnG spacer (Besnard *et al*., 2003), includes an indel (loss of an A) and two A to C transversions specific to subsp. *cuspidata*. The ‘TrnTD-2’ marker in the trnD-trnT intergenic spacer (Besnard, 2008), includes a length allelic variant at a poly-T specific to both subsp. *laperrinei* and *europaea* E1 and E2. The ‘TrnTL-1’ marker in the trnT-trnL spacer (Besnard *et al*., 2003), includes a length allelic variant at a poly-T specific to subsp. *cuspidata*. The ‘SSR31’ marker includes a length allelic variant at a poly-T (Besnard *et al*., 2013a) specific to both subsp. *laperrinei*, and *europaea* E2; two specific transitions (T to C, and G to A) in *europaea* E3; and an A to C transversion specific to subsp. *laperrinei*, *cuspidata* and *europaea* E1. The ‘SSR45’ marker (Besnard *et al*., 2011) includes a T to C transition specific to subsp. *cerasiformis* and a length allelic variant at a poly-T specific to subsp. *cuspidata*.

SUPPLEMENTARY TABLE S2 | Primer sequences and PCR products used as polimorphic cpDNA markers.

Polymorphism		Primer sequences (5'–>3')	Length	TM	Size (pb)	Reference
Ccmp5	F	CAAAATGAACTCCTGCCTCC	20	58.4	262-266	*This Work*
	R	ATTCGAGGGGGAAATGAGAG	20	58.4		
OeR16Qa	F	TTGGAACCGGTATGGAATTG	20	56.4	329-335	*García-Verdugo et al., 2010*
	R	TTTGAAATCAGTGAAGGGTCAA	22	56.4		
MatK2-3	F	CCTTGTTTTGACTGTATCGCAC	22	60.1	494	*This Work*
	R	GGATGGGGTGGGGTATTAG	19	59.5		
QR-1	F	GGGTCTCCCACAATATCGAA	20	58.4	445-447	*This Work*
	R	GTGGACTAAAGCGTCGGATTG	21	61.2		
QR-2	F	CCTGGACGTGAAGAATAAAATCC	23	60.9	383-398	*This Work*
	R	CAGGCCATCAGAATAAGAAGG	21	59.5		
QR-3	F	GGGTCTTTCGGTCTTATTCATATTC	25	62.5	466-467	*This Work*
	R	CACCTATGTCAGCTTTTTGTCTG	23	60.9		
TrnTD-2	F	TTTACAGTCCGTCCCCATTAAC	22	60.1	409-430	*This Work*
	R	CCCTGAACAAATCCTATCCCTC	22	62.1		
TrnTL1	F	AACATTCCTCCGCTTTCATTC	21	57.5	231-234	*This Work*
	R	CTGGAACCCTTGAATTCATTAG	22	58.4		
SSR-31	F	GTTGACCCCTTGGCTTTTC	19	57.5	445-447	*Besnard et al., 2013*
	R	AGTGCCTTCCCCCTTTTC	18	56.3		
SSR-45	F	GTACGGGCATTATCTTTTGTGC	22	60.1	293-295	*Besnard et al., 2011*
	R	GAGTCCAGTTCAGCGTCACA	20	60.5		

SUPPLEMENTARY TABLE S3 | SSR markers used in this study.

Name		Primer sequences (5' to 3')	LABEL	TM	Repeat motif	PCR Product size	Reference
DCA3	F	CCCAAGCGGAGGTGTATATTGTTAC	5'FAM	65.8	$(GA)_{19}$	227-253	*Sefc et al., 2000*
	R	TGCTTTTGTCGTGTTTGAGATGTTG		62.5			
DCA5	F	AACAAATCCCATACGAACTGCC	5'FAM	60.3	$(GA)_{15}$	196-240	*Sefc et al., 2000*
	R	CGTGTTGCTGTGAAGAAAATCG		60.3			
DCA16	F	TTAGGTGGGATTCTGTAGATGGTTG	5'FAM	64.2	$(GT)_{13}(GA)_{29}$	122-210	*Sefc et al., 2000*
	R	TTTTAGGTGAGTTCATAGAATTAGC		59.2			
DCA9	F	AATCAAAGTCTTCCTTCTCATTTCG	5'HEX	60.9	$(GA)_{23}$	164-204	*Sefc et al., 2000*
	R	GATCCTTCCAAAAGTATAACCTCTC		62.5			
DCA18	F	AAGAAAGAAAAAGGCAGAATTAAGC	5'HEX	59.2	$(CA)_4CT(CA)_3(GA)_{19}$	158-182	*Sefc et al., 2000*
	R	GTTTTCGTCTCTCTACATAAGTGAC		62.5			

SUPPLEMENTARY TABLE S4 | Amplicons size of cpDNA markers.

Subspecies	cpDNA Markers									
	CCMP5	OeR16Qa-F	matK2-3F	QR-1RF	QR-2F	QR-3R	TrnTD-2F	TrnTL1F	SSR31F	SSR45F
Europaea-E1	263	331	494	446	383	467	412	234	446	294
Europaea-E2	263	331	494	445	387	467	412	234	445	294
Europaea-E3	264	331	494	446	392	467	411	234	446	294
Laperrine	263	331	494	446	383	467	412	234	447	294
Guanchica	264	331	494	446	393	467	411	233	446	294
Cerasiformis	264	329	494	447	393	467	411	233	446	294
Maroccana	266	331	494	446	393	467	411	233	446	294
Cuspidata	264	331	494	445	392	466	409	232	446	293
Cuspidata x Europaea	264	331	494	445	392	466	409	232	446	293
Olea Woodiana	262	335	494	446	398	466	430	231	447	295
Size range	262-266	329-335	494	445-447	383-398	466-467	409-430	231-234	445-447	293-295

Amplicons were truncated avoiding blunt ends to optimize MEGA alignment.

SUPPLEMENTARY TABLE S5 | Fragment analysis of SSR markers.

Subspecies	Genotypes	Ploidy	SSR Markers				
			DCA3	DCA5	DCA16	DCA9	DCA18
Guanchica	**W49**	**2X**	**230/251**	**207/203**	**173/173**	**161/194**	**168/178**
Guanchica	**GUA1**	**2X**	**230/251**	**207/203**	**145/173**	**161/183**	**168/166**
Guanchica	**GUA2**	**2X**	**241/251**	**203/203**	**121/173**	**183/194**	**166/178**
Guanchica	**GUA3**	**2X**	**230/241**	**207/199**	**145/173**	**194/194**	**176/178**
Guanchica	**GUA4**	**2X**	**230/230**	**203/203**	**145/173**	**161/161**	**168/176**
Guanchica	**GUA5**	**2X**	**241/251**	**207/203**	**145/173**	**161/183**	**166/178**
Guanchica	**GUA6**	**2X**	**237/251**	**196/203**	**149/173**	**185/194**	**174/178**
Guanchica	**GUA7**	**2X**	**230/251**	**199/203**	**121/173**	**161/194**	**168/176**
Guanchica	**GUA8**	**2X**	**230/237**	**207/203**	**145/173**	**161/183**	**166/178**
Guanchica	**GUA9**	**2X**	**241/251**	**207/199**	**121/173**	**161/183**	**168/166**
Cuspidata	**CUS3**	**2X**	**237/230**	**197/197**	**141/141**	**171/165**	**166/178**
Cuspidata	**CUS4**	**2X**	**230/249**	**197/197**	**141/138**	**173/165**	**162/178**
Cuspidata	**CUS6**	**2X**	**230/249**	**197/199**	**141/147**	**173/171**	**162/178**
Cuspidata	**CUS11**	**2X**	**230/230**	**197/197**	**138/154**	**173/173**	**166/178**
Cuspidata	**CUS12**	**2X**	**237/230**	**199/203**	**141/121**	**173/181**	**174/186**
Cuspidata	**CUS13**	**2X**	**237/247**	**199/203**	**138/173**	**173/183**	**178/178**
Cuspidata	**CUS14**	**2X**	**230/247**	**197/203**	**141/124**	**173/183**	**174/174**
Cuspidata	**CUS15**	**2X**	**230/230**	**197/196**	**145/130**	**173/183**	**166/178**
Cuspidata	**CEH2**	**2X**	**230/237**	**197/203**	**124/154**	**183/165**	**168/184**
Cuspidata	**CEH5**	**2X**	**230/237**	**197/203**	**124/154**	**191/165**	**174/184**
Cuspidata	**CEH6**	**2X**	**230/237**	**197/203**	**170/154**	**183/165**	**174/184**
Cuspidata	**CEH7**	**2X**	**230/251**	**197/203**	**154/154**	**161/165**	**170/184**
Cuspidata	**CEH8**	**2X**	**230/247**	**197/203**	**170/154**	**191/165**	**158/168**
Cuspidata	**CEH9**	**2X**	**230/251**	**197/203**	**173/170**	**191/165**	**158/170**

Cuspidata	CEH17	2X	230/251	197/203	121/170	161/165	174/184
Cuspidata	CEH19	2X	230/247	197/203	154/154	191/165	174/184
Cuspidata	CEH20	2X	230/237	197/203	173/170	191/165	170/184
Cuspidata	CEH21	2X	230/237	197/203	154/154	191/165	158/174
Cuspidata	CEH23	2X	230/247	197/203	124/170	183/165	158/174
Cuspidata	CEH24	2X	230/237	197/203	124/170	191/165	158/174
Cuspidata	CEH25	2X	230/237	197/203	124/170	183/165	158/168
Cuspidata	CEH26	2X	230/247	197/203	170/154	191/165	158/168
Cuspidata	CEH30	2X	230/251	197/203	121/154	161/165	174/184
Cerasiformis	CER0	4X	237/249	199/192	134/121	181/191/175	170/176/184 /166
Cerasiformis	CER1	3X	251/237	199/196/205	134/121	175/181	178/194/182
Cerasiformis	CER3	3X	251/265	199/196/202	134/121	175/187	176/194/182
Europaea	AMK5	2X	230/247	199/203	136/173	193/165	178/178
Europaea	AMK6	2X	230/247	203/203	136/145	193/165	168/178
Europaea	AMK14	2X	251/247	203/203	136/154	180/196	170/168
Europaea	AMK16	2X	251/247	209/203	136/136	196/196	170/170
Europaea	AMK21	2X	251/247	199/203	124/173	193/193	168/178
Europaea	AMK26	2X	237/247	203/203	136/154	193/161	170/184
Europaea	AMK34	2X	237/237	209/203	136/136	193/165	170/178
Europaea	ACO1	2X	237/253	192/207	160/173	183/161	174/174
Europaea	ACO5	2X	237/251	192/203	173/173	183/161	170/184
Europaea	ACO14	2X	237/241	199/196	136/173	183/175	178/174
Europaea	ACO15	2X	237/243	192/203	143/165	171/161	170/184
Europaea	ACO18	2X	237/241	199/196	143/173	183/161	172/174
Europaea	W69	2X	251/253	196/203	145/173	204/204	172/178
Europaea	AJA1	2X	253/241	199/203	121/173	183/204	172/166
Europaea	AJA4	2X	253/230	199/203	121/173	183/204	172/176
Europaea	AJA6	2X	251/241	199/196	145/145	161/204	172/168
Europaea	AJA12	2X	251/241	199/203	121/173	183/204	176/178
Europaea	AJA17	2X	251/237	203/203	145/145	204/204	184/178
Europaea	AJA10	2X	251/230	199/203	145/121	183/204	166/178
Europaea	AJA18	2X	251/241	203/203	145/173	183/204	166/178
Europaea	W45	2X	237/237	199/196	154/154	183/177	184/174
Europaea	ACZ1	2X	237/230	199/203	154/145	183/177	166/174
Europaea	ACZ2	2X	237/241	199/203	154/145	183/177	166/174
Europaea	ACZ3	2X	237/247	199/198	154/154	183/183	170/184
Europaea	ACZ4	2X	237/230	196/203	154/121	177/177	166/184
Europaea	ACZ5	2X	237/241	196/203	154/145	183/177	166/184
Europaea	ACZ7	2X	237/237	196/196	154/154	183/204	172/174
Europaea	ACZ8	2X	237/247	196/203	154/124	171/177	184/174

Europaea	ACZ9	2X	237/241	199/196	154/154	183/183	184/174
Europaea	ACZ10	2X	237/247	196/196	154/145	183/189	166/174
Europaea	ACZ12	2X	237/247	199/211	154/154	171/177	164/184
Europaea	ARC1	2X	237/243	199/211	145/145	161/175	166/174
Europaea	APR1	2X	234/239	199/203	173/157	183/191	166/166
Europaea	FRANTOIO NR80	2X	235/241	196/203	149/149	183/183	174/174
Europaea	FRA1	2X	237/241	199/196	121/149	183/175	172/174
Europaea	FRA2	2X	237/241	203/203	154/149	183/191	174/174
Europaea	FRA3	2X	235/237	203/203	124/149	183/161	174/174
Europaea	FRA4	2X	247/241	203/203	121/149	183/183	168/174
Europaea	AMS7	2X	237/237	203/203	149/154	183/204	178/166
Europaea	AMS11	2X	237/237	203/203	173/154	183/193	178/166
Europaea	AMS12	2X	237/251	203/203	149/154	204/204	166/182
Europaea	AMS16	2X	237/237	203/203	149/154	193/204	178/178
Europaea	AOU1	2X	237/239	213/207	200/183	181/181	196/172
Europaea	AOU2	2X	230/239	213/198	154/183	181/191	196/180
Europaea	AOU3	2X	237/230	196/203	173/143	165/204	164/178
Europaea	AOU5	2X	237/237	213/198	157/183	181/179	196/182
Europaea	AOU10	3X	237/237	213/203	154/173	183/204	178/182/196
Europaea	AOU13	2X	239/251	213/203	154/154	181/193	170/170
Europaea	TAM3	2X	237/237	199/198	183/183	193/193	182/186
Europaea	TAM4	2X	237/237	196/202	154/183	193/191	176/166
Europaea	TAM9	2X	237/237	198/198	159/170	193/175	176/184
Europaea	TAM12	2X	237/237	196/202	164/170	193/165	174/180
Europaea	TAM13	2X	237/230	199/202	164/170	193/165	186/180
Europaea	TAM11	2X	237/237	198/198	161/170	193/204	166/172
Europaea	ARBEQUINA	2X	230/241	199/203	121/145	183/204	176/166
Laperrine	Dhokar (Oct413)	2X	237/237	213/203	149/149	171/193	178/170
Laperrine	DHO1	2X	237/237	213/203	154/149	193/204	178/170
Laperrine	DHO2	2X	237/251	213/203	154/149	193/204	178/170
Laperrine	DHO4	2X	237/251	213/203	154/149	171/204	178/178
Laperrine	DHO6A	2X	237/251	213/203	173/149	171/193	178/170
Laperrine	DHO6B	2X	237/251	203/203	173/149	171/204	170/170
Laperrine	DHO8A	2X	237/251	203/203	154/149	171/161	178/166
Laperrine	DHO10A	3X	237/237	203/203	173/149/121	193/161	178/166
Laperrine	DHO11A	3X	237/237	213/203/199	154/149	171/193/183	178/166/170
Laperrine	DHO12A	2X	237/237	213/203	173/149	193/191	178/166
Maroccana	MAR0	6X	241/237	196/203/199/201	149/146	177/175/185	176/174/172 /180/178
Maroccana	MAR1	6X	247/237/243/ 230	213/203/199	154/149/183/121 /132	171/185	172/176/184
Maroccana	MAR2	6X	247/241/243	202/203/199	156/145	171/185	172/184/182

Maroccana	MAR3	6X	247/237/243	202/203/199	154/126	171/204	170/176/186

Supplementary Table S6 | Morphological features of wild genotypes from the SILVOLIVE collection.

Category	Variety	Ploidy	Height	Basal diameter	Shoot biomass	Leaves biomass	Total biomass	Total number nodes	Root biomass	Number Secondary stems	Branching efficiency	Branching frequency	Root / Shoot Ratio
Very Low Vigor	AMK26	2X	--	--	+/-	--	--	+	--	++	++	++	+
	FRA1	2X	--	--	--	-	--	--	-	--	--	--	+
	AOU1	2X	--	--	--	--	--	--	--	--	--	--	--
	GUA3	2X	--	--	--	--	--	--	--	--	--	-	-
	MAR1	6X	--	--	--	--	--	--	--	--	--	--	--
	DHO6A	2X	--	-	--	--	-	--	+	-	-	++	++
	DHO4	2X	--	--				--		--	--	--	
	MAR3	6X	--	--	-	--	-	--	+/-	--	--	--	+/-
	AMK21	2X	--	--	--	--	--	-	-	-	+/-	+/-	++
	DHO12A	2X	--	--	--	--	--	--	--	--	--	--	++
	AMK9	2X	--	--	--	--	--	--	-	--	-	+/-	++
	CUS14	2X	--	-	+	+	+	-	+/-	+	+	++	--
	DHO8A	2X	--	-	-	-	+/-	-	+	+/-	+/-	+	+/-
	AMS17	2X	--	+/-	-	--	-	--	+/-	--	--	--	+
	AMS15	2X	--	+	--	--	--	--	-	--	--	-	+/-
	AMK27	2X	--	--	--	--	--	--	--	+/-	+	++	-
	AMK6	2X	--	--	--	--	--	-	--	-	+	+/-	+
	GUA8	2X	--	--	--	-	--	-	-	+/-	+	+/-	++
	AMK5	2X	--	--	--	--	--	+/-	-	+	++	++	+
	AMK12	2X	--	+/-	--	--	-	-	+	+/-	+/-	+	++
	CEH8	2X	--	+/-	--	-	-	+	+/-	+	+	+	+/-
Low Vigor	AJA12	2X	-	--	--	--	--	-	--	-	-	-	+
	DHO1	2X	-	+/-	-	-	-	-	+/-	--	--	--	-
	AMK25	2X	-	-	--	--	--	-	-	-	-	-	+/-
	AMK16	2X	-	-	--	--	--	+/-	-	-	-	--	--

	CEH2	2X	-	+/-	+/-	+/-	-	+/-	-	+/-	+/-	+/-	++
	DHO13A	2X	-	+/-				-		--	--	--	
	FRA4	2X	-	-	-	-	--	--	--	-	-	-	++
	DHO10B	2X	-	-	+/-	+/-	+	--	++	--	--	--	++
	AOU13	2X	-	-	++	+	++	--	++	-	+/-	++	+/-
	AJA1	2X	-	-	+/-	+	+/-	+/-	+/-	+/-	+	-	-
	AMS12	2X	-	--	+	+	+	--	++	+/-	+	++	+/-
	AOU3	2X	-	-	+	+/-	+	--	++	--	--	-	+/-
	AMS7	2X	-	+/-	+/-	-	+/-	--	++	--	--	--	++
	GUA1	2X	-	--	--	-	-	+/-	-	+/-	+	-	-
	GUA9	2X	-	--	--	--	--	-	--	+/-	+	++	-
	GUA2	2X	-	-	-	+/-	-	-	+	-	+/-	-	+
	CEH25	2X	-	+/-	-	-	--	+	--	+	+	+	+/-
	AMK14	2X	-	+/-	+/-	-	+/-	+	+	-	-	-	-
	DHO10A	**3X**	-	++	+	++	++	+/-	++	+/-	-	+/-	+
	TAM3	2X	-	+	-	--	-	-	+/-	--	--	-	+
	ACZ10	2X	-	+	+/-	+	+/-	++	+/-	++	++	+	--
Intermediate Vigor	**AJA17**	2X	+/-	--	+/-	+/-	-	+	--	+	++	+	--
	FRA3	2X	+/-	+	-	+/-	-	--	--	--	--	--	+/-
	DHO6B	2X	+/-	-	-	-	+/-	--	++	--	--	--	++
	AJA18	2X	+/-	+/-	-	-	-	+	-	++	++	++	+
	ACO15	2X	+/-	-	-	-	-	+	-	++	++	+	+/-
	CEH17	2X	+/-	+	-	+	+	-	++	--	--	--	++
	CER3	**3X**	+/-	-	+/-	+/-	+/-	+/-	+/-	+	+	++	+/-
	ACO14	2X	+/-	--	--	--	--	+/-	--	+	+	++	+/-
	TAM4	2X	+/-	+	+	+	+	++	+	+	+	+/-	-
	AJA4	2X	+/-	+/-	-	+	+/-	+/-	+/-	--	--	--	--
	GUA5	2X	+/-	-	-	+/-	+/-	+/-	+/-	+/-	+	+	+
	CER1	**3X**	+/-	+/-	+/-	+/-	-	++	--	++	++	++	--
	AOU9	2X	+/-	+/-	+	+/-	+	-	+	+/-	+/-	+	-
	GUA4	2X	+/-	-	-	+/-	+/-	+	+	++	++	++	+
	ACZ4	2X	+/-	-	+/-	+/-	+/-	+	+/-	++	++	+	-
	ACZ3	2X	+/-	+/-	+/-	-	-	+/-	--	+/-	+/-	+/-	+/-

	AMS11	2X	+/-	+/-	+	+/-	++	-	++	-	-	+	+/-
	CUS3	2X	+/-	--	-	+	+/-	+	+/-	+	++	++	--
	CEH26	2X	+/-	+	+/-	-	-	+/-	--	-	-	-	-
	TAM12	2X	+/-	+	-	-	+/-	++	+	+	+	-	+
	TAM13	2X	+/-	+				+/-		-	-	-	
High Vigor	**ACZ1**	2X	+	+	-	+	+/-	+	+/-	+	+	+/-	-
	AOU8	2X	+	+/-	++	+/-	+	--	+	-	-	+/-	--
	CEH3	2X	+	+/-	+/-	++	+	++	+/-	++	++	+	-
	AJA6	2X	+	-	+/-	+/-	+	++	+	-	-	--	--
	ACO18	2X	+	++	+	+/-	+	+	+	++	+	++	+
	ACZ2	2X	+	+	+/-	-	-	-	--	+/-	-	+/-	+
	TAM9	2X	+	+/-	+/-	-	+/-	-	++	-	-	-	+
	AOU12	2X	+	+	++	+	++	+/-	+	+/-	+/-	+/-	--
	ACZ5	2X	+	+	-	+/-	+/-	+	+/-	+/-	+/-	+/-	-
	CEH20	2X	+	+/-	+	+	+/-	+/-	-	+/-	+/-	+/-	+
	AMK34	2X	+	-	+	+/-	+/-	+	-	-	+/-	--	--
	CEH30	2X	+	+	+/-	+	-	-	-	--	--	-	+/-
	AOU5	2X	+	+/-				++		++	++	++	
	AOU10	**3X**	+	++	++	++	++	-	++	-	--	-	--
	FRA2	2X	+	++	++	+/-	+	-	-	-	-	-	-
	ACZ8	2X	+	+/-	+	++	++	++	+/-	++	++	+/-	--
	CEH9	2X	+	+	++	++	++	++	++	-	-	--	-
	CEH24	2X	+	-	+	++	+	++	+	++	++	+	-
	ACZ7	2X	+	++	+	+	+	++	+/-	++	++	++	--
	ACZ12	2X	+	+	+	+	+/-	+	--	+	+/-	+/-	+/-
	CUS15	2X	+	+	+/-	-	--	+/-	--	+/-	-	-	--
	CEH6	2X	+	+	+	+	+	+	+	+	+	+	+/-
Very High Vigor	**GUA7**	2X	++	-	+	+	+	++	+	++	++	+	+
	CUS4	2X	++	++	++	++	++	++	+	+	+/-	--	-
	APR	2X	++	++	++	++	++	++	++	++	+	+	-
	CEH21	2X	++	+	+	++	++	+	+	++	++	+	--
	CEH7	2X	++	++	+	++	++	+/-	++	+/-	-	-	+/-
	CUS12	2X	++	++	+/-	-	-	+	--	+	+/-	+/-	+

	ACZ9	2X	++	+	+	++	++	+	++	+	+	+	++
	CEH23	2X	++	++	+	+	+	+/-	+/-	-	-	-	+
	GUA6	2X	++	+	++	++	++	++	++	+	+	--	--
	DHO6C	2X	++	+	++	+	+	+	-	+	+/-	+/-	--
	CEH19	2X	++	++	++	++	++	+	+	+	+/-	+	+
	AOU7	2X	++	++				+/-		+/-	+/-	+/-	
	ARC	2X	++	++	++	++	++	++	++	+	+/-	+	-
	AOU2	2X	++	++	++	++	+	++	-	++	+	++	-
	CUS13	2X	++	++	++	+/-	+/-	+/-	-	+/-	-	+/-	+/-
	ACO5	2X	++	++	++	+	+	+	-	+	+/-	+	+
	AOU4	2X	++	++	++	++	++	+/-	+/-	+	+/-	+	--
	CUS6	2X	++	++	++	++	++	++	+	++	++	+/-	+/-
MEAN			94.54	7.04	8.70	7.63	25.11	125.61	9.30	12.94	1.87	0.23	0.41
MIN			30.50	3.49	1.65	1.26	3.42	24.22	0.60	0.00	0.00	0.00	0.18
MAX			183.77	11.40	32.92	28.99	78.34	353.21	26.17	45.15	6.90	0.54	0.78
CV			0.32	0.21	0.67	0.61	0.51	0.59	0.55	0.70	0.70	0.42	0.31

Early vigor parameters measured in 103 genotypes of the SILVOLIVE collection 13 months after *ex-vitro* acclimatization. The genotypes are sorted from lower to higher vigor according to the plant height and classified in five categories: very low (- -); low (-); medium (+/-); high (+); and very high (++) vigor.

Supplementary Table S7 | Correlations between morphological parameters.

	VIGOUR PARAMETERS								BRANCHING PARAMETERS				ROOT / SHOOT
	Height	Basal diameter	Shoot biomass	Leaves biomass	Total biomass	Internode lenght	Root biomass	Total number nodes	Total leaves	Secondary stems	Branching efficiency	Terciary stems	Branching frequency	Root/Shoot Ratio
Height	1.00	0.76	0.75	0.73	0.73	0.41	0.30	0.60	0.58	0.43	0.21	0.17	0.10	-0.29
Basal diameter	0.76	1.00	0.79	0.72	0.73	0.24	0.34	0.47	0.44	0.28	-0.04	0.19	0.00	-0.17
Shoot biomass	0.75	0.79	1.00	0.82	0.90	0.50	0.45	0.50	0.48	0.31	0.06	0.29	0.11	-0.32
Leaves biomass	0.73	0.72	0.82	1.00	0.86	0.49	0.46	0.61	0.58	0.43	0.20	0.24	0.11	-0.31
Total biomass	0.73	0.73	0.90	0.86	1.00	0.49	0.73	0.57	0.58	0.35	0.13	0.27	0.08	-0.24
Internode lenght	0.41	0.24	0.50	0.49	0.49	1.00	0.10	0.02	0.02	0.04	0.00	0.00	0.02	0.07
Root biomass	0.30	0.34	0.45	0.46	0.73	0.10	1.00	0.18	0.24	0.05	-0.05	0.05	0.01	0.11
Total number nodes	0.60	0.47	0.50	0.61	0.57	0.02	0.18	1.00	0.96	0.83	0.69	0.50	0.27	-0.35
Total leaves	0.58	0.44	0.48	0.58	0.58	0.02	0.24	0.96	1.00	0.73	0.60	0.39	0.16	-0.37
Secondary stems	0.43	0.28	0.31	0.43	0.35	0.04	0.05	0.83	0.73	1.00	0.93	0.43	0.62	-0.23
Branching efficiency	0.21	-0.04	0.06	0.20	0.13	0.00	-0.05	0.69	0.60	0.93	1.00	0.44	0.71	-0.19
Terciary stems	0.17	0.19	0.29	0.24	0.27	0.00	0.05	0.50	0.39	0.43	0.44	1.00	0.46	-0.11
Branching frequency	0.10	0.00	0.11	0.11	0.08	0.02	0.01	0.27	0.16	0.62	0.71	0.46	1.00	0.00
Root/Shoot Ratio	-0.29	-0.17	-0.32	-0.31	-0.24	0.07	0.11	-0.35	-0.37	-0.23	-0.19	-0.11	0.00	1.00

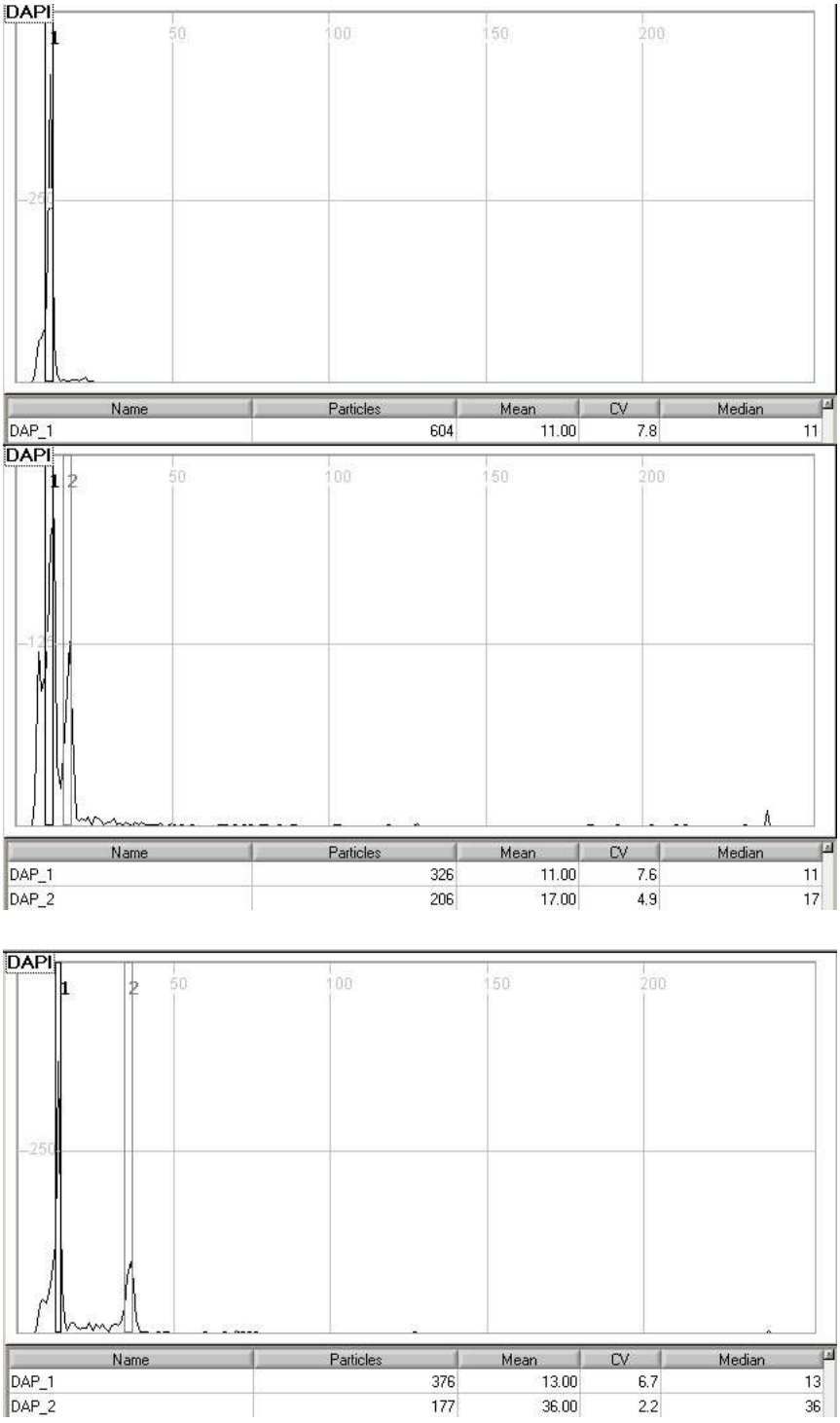
Supplementary Table S8 | Distribution of root-to-shoot ratio and branching patterns according to plant vigor.

	Number of Genotypes analyzed	High R/S Ratio (%)	Low R/S Ratio (%)	High Branching (%)	Low Branching (%)
Very low- and low-vigor Genotypes	42	45%	28%	21%	36%
Intermediate vigor Genotypes	21	28%	38%	47%	19%
High- and very high-vigor Genotypes	42	28%	52%	24%	9%

Values calculated from the data present in the Supplementary Table S4. High Root/Shoot ratio genotypes include those with ++ or + values. Low Root/Shoot ratio genotypes include those with -- or - values. High branching genotypes include those with ++ or + values. Low branching genotypes include those with -- or - values.

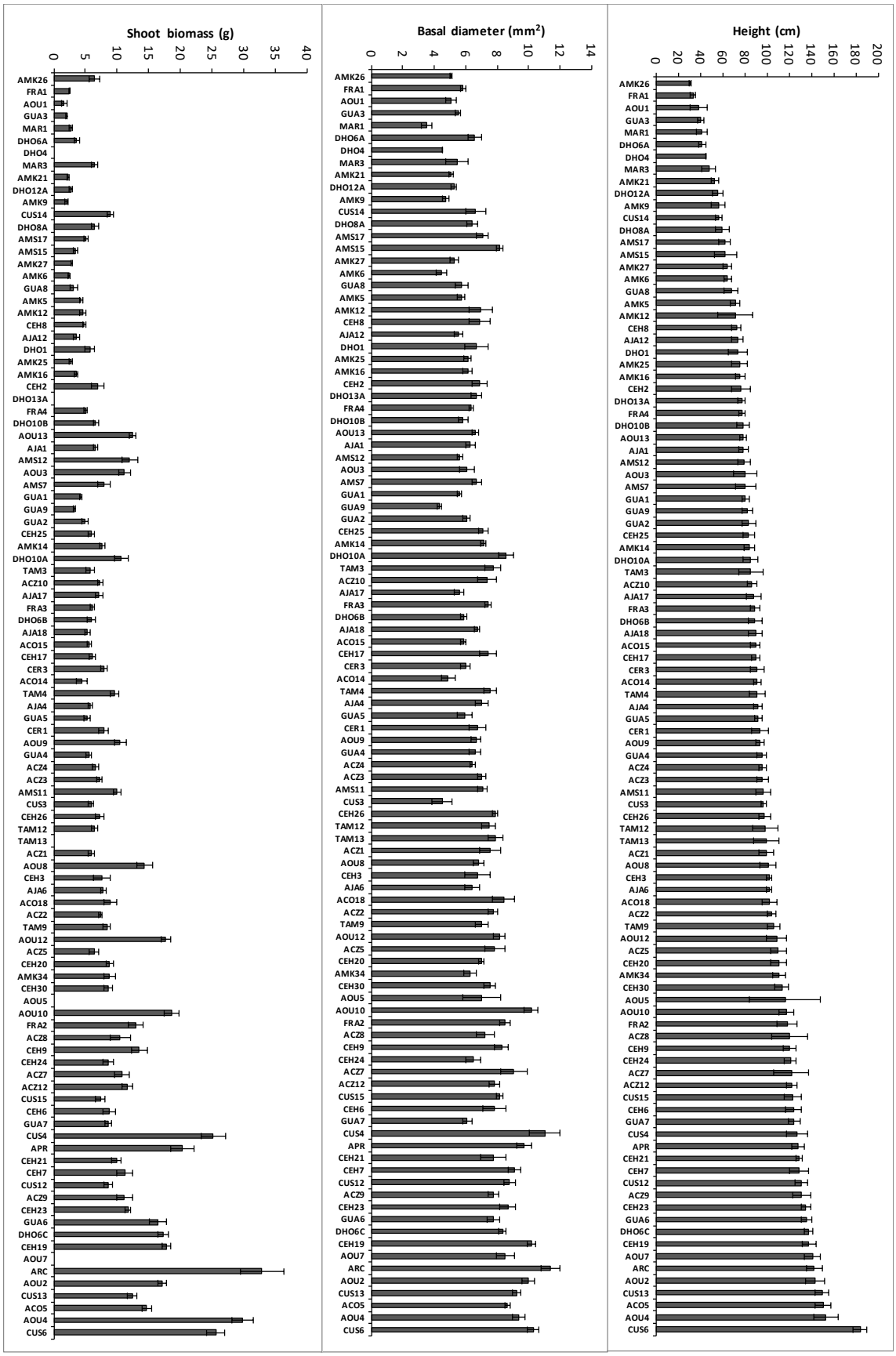
Supplementary Table S9 | Grafting compatibility.

		Grafting efficiency (%)	
		var. Picual	**var. Hojiblanca**
Subsp.*europaea E1*	***AMK5-6-14-16-21-; ACO5-15-18; ACZ3-5-8-9; APR; FRA2-3-4***	**65.9**	**83.3**
Subsp.*europaea E2*	***ARC***	**61.5**	**0**
Subsp.*europaea E3*	***AJA6-12-17-18***	**55.8**	**100**
Subsp. *laperrinei*	**DHO10B**	**55.6**	**-**
Subsp. *cerasiformis*	**CER3**	**20.0**	**-**
Subsp.*maroccana*	**-**	**-**	**-**
Subsp. *guanchica*	***GUA1-2-3-7-8-9***	**61.6**	**40**
Subsp. *cuspidata*	***CUS4-6-11-13-15;CEH6-7-8-17-19-20-21-24-26***	**78.6**	**97.0**
Picual self-grafted		**67.7**	**-**
Hojiblanca selft-grafted		**-**	**83.5**
TOTAL		**57.0**	**80.4**

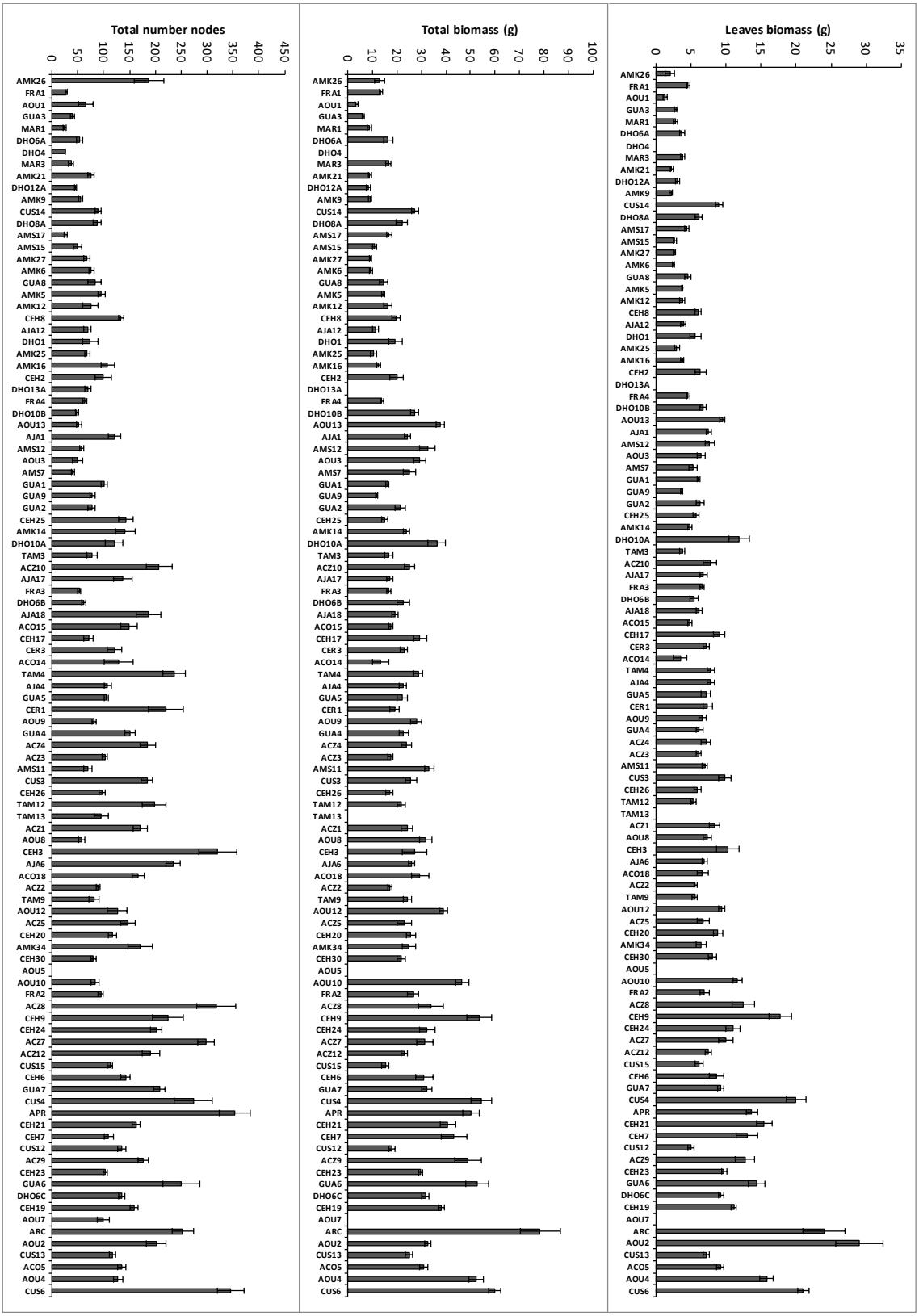


SUPPLEMENTARY FIGURE S1. Flow cytometric measurements of DAPI-stained leaves. (A) Histogram of the control diploid plant GUA1 (peak 1). (B) Histogram of the diploid control plant GUA1 (peak 1) and triploid plant AOU10 (peak 2) with index peak ratio 17/11=1.54. (C) Histogram of the diploid control plant GUA1 (peak 1) and hexaploid plant MAR2 (peak 2) with index peak ratio 36/13=2.8.

SUPPLEMENTARY FIGURE S2 | Quantification of morphological parameters of wild olive genotypes sorted by plant height, represented in the top graph.

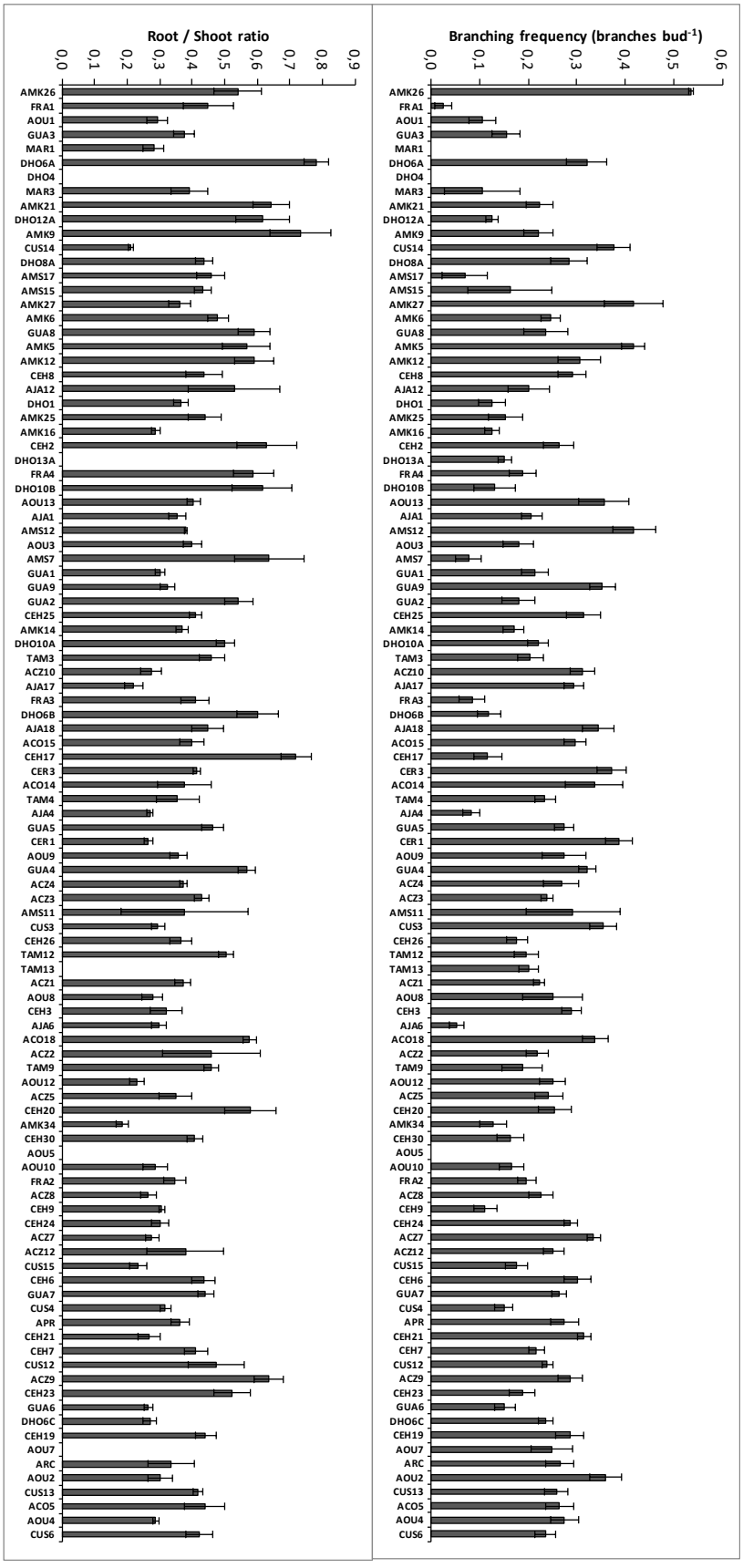


SUPPLEMENTARY FIGURE S2 | Quantification of morphological parameters of wild olive genotypes sorted by plant height, represented in the top graph (continued).



SUPPLEMENTARY FIGURE S2 | Quantification of morphological parameters of wild olive genotypes sorted by plant height, represented in the top graph (continued).

SUPPLEMENTARY FIGURE S2 | Quantification of morphological parameters of wild olive genotypes sorted by plant height, represented in the top graph (continued).



Annex 1. Water consumption of wild olive genotypes

Results obtained in this annex have been published in a collaborative research with Dr. Antonio Díaz-Espejo's lab, published as: Hernández-Santana, V., **Díaz-Rueda, P.**, Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., Montero A, Pérez-Martín A, Colmenero-Flores JM and Rodríguez-Domínguez CM. (2019). Hydraulic traits emerge as relevant determinants of growth patterns in wild olive genotypes under water stress. Frontiers in Plant Science 10:291. https://doi.org/10.3389/fpls.2019.00291

In this annex, only those results entirely obtained by Pablo Díaz Rueda and his research group were described.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 March 2019
doi: 10.3389/fpls.2019.00291

Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress

Virginia Hernandez-Santana[1], Pablo Diaz-Rueda[1], Antonio Diaz-Espejo[1], María D. Raya-Sereno[1,2], Saray Gutiérrez-Gordillo[1,3], Antonio Montero[1], Alfonso Perez-Martin[1], Jose M. Colmenero-Flores[1] and Celia M. Rodriguez-Dominguez[1,4]*

[1] Irrigation and Crop Ecophysiology Group, Instituto de Recursos Naturales y Agrobiología de Sevilla, Consejo Superior de Investigaciones Científicas, Seville, Spain, [2] School of Agricultural Engineering, CEIGRAM, Universidad Politécnica de Madrid, Madrid, Spain, [3] Centro "Las Torres-Tomejil", Instituto Andaluz de Investigación y Formación Agraria y Pesquera, Seville, Spain, [4] School of Biological Sciences, University of Tasmania, Hobart, TAS, Australia

OPEN ACCESS

Edited by:
Johannes Kromdijk, University of Cambridge, United Kingdom

Reviewed by:
Meisha Holloway-Phillips, Australian National University, Australia
Brunella Morandi, University of Bologna, Italy

***Correspondence:**
Virginia Hernandez-Santana
virginiahsa@gmail.com

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 15 November 2018
Accepted: 22 February 2019
Published: 13 March 2019

Citation:
Hernandez-Santana V, Diaz-Rueda P, Diaz-Espejo A, Raya-Sereno MD, Gutiérrez-Gordillo S, Montero A, Perez-Martin A, Colmenero-Flores JM and Rodriguez-Dominguez CM (2019) Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress. Front. Plant Sci. 10:291. doi: 10.3389/fpls.2019.00291

The hydraulic traits of plants, or the efficiency of water transport throughout the plant hydraulic system, could help to anticipate the impact of climate change and improve crop productivity. However, the mechanisms explaining the role of hydraulic traits on plant photosynthesis and thus, plant growth and yield, are just beginning to emerge. We conducted an experiment to identify differences in growth patterns at leaf, root and whole plant level among four wild olive genotypes and to determine whether hydraulic traits may help to explain such differences through their effect on photosynthesis. We estimated the relative growth rate (RGR), and its components, leaf gas exchange and hydraulic traits both at the leaf and whole-plant level in the olive genotypes over a full year. Photosynthetic capacity parameters were also measured. We observed different responses to water stress in the RGRs of the genotypes studied being best explained by changes in the net CO_2 assimilation rate (NAR). Further, net photosynthesis, closely related to NAR, was mainly determined by hydraulic traits, both at leaf and whole-plant levels. This was mediated through the effects of hydraulic traits on stomatal conductance. We observed a decrease in leaf area: sapwood area and leaf area: root area ratios in water-stressed plants, which was more evident in the olive genotype *Olea europaea* subsp. *guanchica* (GUA8), whose RGR was less affected by water deficit than the other olive genotypes. In addition, at the leaf level, GUA8 water-stressed plants presented a better photosynthetic capacity due to a higher mesophyll conductance to CO_2 and a higher foliar N. We conclude that hydraulic allometry adjustments of whole plant and leaf physiological response were well coordinated, buffering the water stress experienced by GUA8 plants. In turn, this explained their higher relative growth rates compared to the rest of the genotypes under water-stress conditions.

Keywords: hydraulic allometry, leaf hydraulic conductance, leaf:sapwood area ratio, leaf:root area ratio, net photosynthesis rate, stomatal conductance

Introduction

Olea europaea L. is considered one of the species best adapted to the semi-arid environment of the Mediterranean Basin, growing under conditions in which seasonal, but prolonged, situations of water deficit occur (Gimenez et al., 1997; Fernández, 2014). The olive tree resists drought stress as a result of morphological, physiological, and biochemical adaptations that reduce water loss while maintaining water uptake (Connor and Fereres, 2010). Therefore, it is able to grow and produce acceptable yields under harsh environmental conditions (Fernández, 2014). Due to this capacity, approximately 90% of the olive trees cultivated in the Mediterranean basin are mainly found in rainfed conditions (Gómez et al., 2001; Gómez-Rico et al., 2007). However, despite their tolerance to drought, olives need a minimum of 350 mm rainfall $year^{-1}$ for their survival in arid areas and a minimum of 500 mm rainfall $year^{-1}$ to guarantee the productivity of commercial cultivars (Ponti et al., 2014). In addition, water deficit can be quite damaging in young plantations (Sanzani et al., 2012).

Extensive rainfed olive cultivation, with 70–80 trees ha^{-1} densities, is being transformed into more profitable intensive (200-600 trees ha^{-1}) and super-intensive (HDH) systems (1500-2000 trees ha^{-1}) (Connor, 2006; Rallo et al., 2013). The increase of yield and production of olive orchards under intensive cultivation requires fertigation (Rallo et al., 2013; Díez et al., 2016). However, water is a scarce resource in mediterranean regions and its availability is increasingly limited. Regulated deficit irrigation (RDI) optimizes the use of water in intensive and HDH olive orchards (García et al., 2017) and can also improve the olive grove yield and quality (Gómez-Rico et al., 2007), even when the water supply is very low (Barranco et al., 1998). Therefore, the identification of genotypes well adapted to RDI conditions can be of great relevance if used as rootstocks that improve the performance of the intensive and HDH olive grove.

In recent years, climate change has widely affected different ecosystem conditions across different regions of the world. Climate models predict increased temperatures for the Mediterranean Basin in response to increasing greenhouse-gases and a negative trend in precipitation (Dell'Aquila et al., 2012; IPCC, 2013) (**Figure 1**).

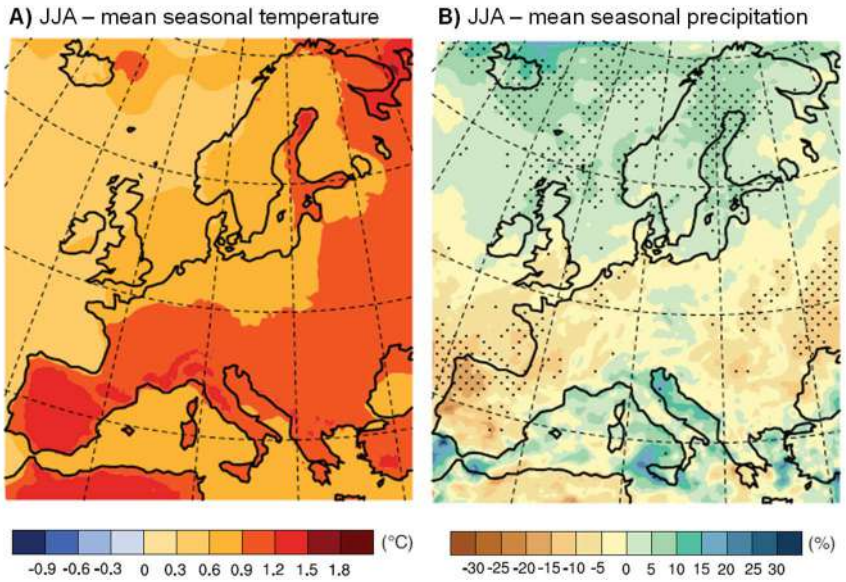


FIGURE 1 | **Map obtained from IPPC 2013, Figure 11.18.** European-scale projections from the ENSEMBLES regional climate modelling project for 2016–2035 relative to 1986–2005, applicable to June, July and August (JJA). For temperature, projected changes (°C) are displayed as changes of mean seasonal surface temperature (A). For precipitation, projected changes (%) sre displayed as changes of mean seasonal precipitation (B).

The Mediterranean ecosystem is very sensitive to climate change because a shift towards less precipitation and higher temperature can increase aridity, being this region a transition zone between the super arid systems of Morocco and the temperate ecosystems of Europe (**Figure 2**). The effect of climate change in the Mediterranean region, such as higher incidence of water deficit and desertification, are disturbing crop yields (United Nations, 2015). While the increase of food production is an essential objective of the Agenda 2030 of the European Union (ODS 2 and 15) (United Nations, 2015), 12 million hectares of arable land are lost each year around the world due to environmental disturbances. In this scenario of limited water availability, crop breeders are taking advantage of the genetic variability present in wild relatives of plant crop species, better adapted to environmental limitations (Nevo et al., 2012; Burnett et al., 2016), such as water deficit (Ruane et al., 2008; Reddy et al., 2017; Trentacoste et al., 2018).

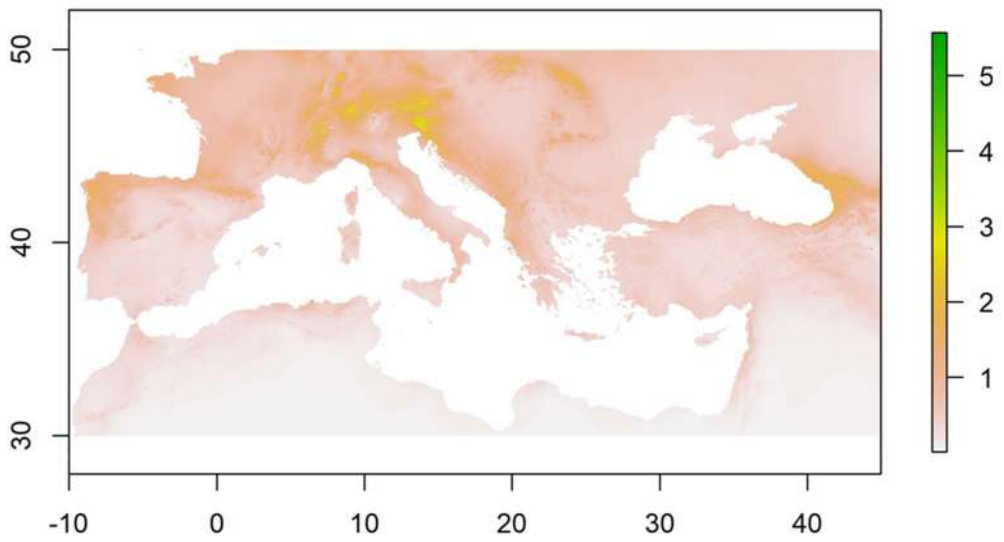


FIGURE 2 | Map obtained from the CGIAR-CSI geoportal (Trabucco and Zomer, 2018) using the "raster" package (Hijmans, 2021) in software R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria), for the period 1970-2000 at a spatial resolution of 30 arc-seconds (~ 1km^2 in Ecuador). The variable represented is the aridity index for the Mediterranean Basin (lower values correspond to more arid conditions and higher values to more humid conditions). The axes show the coordinates (latitude and longitude).

Several studies have been conducted to determine water consumption of olive orchards (Palomo et al., 2002; Ezzahar et al., 2009), although the response to water stress of the olive tree has not been thoroughly investigated (Sanzani et al., 2012). Different drought-resistance mechanisms have been proposed (Chartzoulakis et al., 1999; Bacelar et al., 2004; Fernández, 2014; Díaz-Espejo et al., 2018), and differences among *Olea europaea* subsp. *europaea* cultivars have been observed (Chartzoulakis et al., 1999; Hernández-Santana et al., 2019; Edziri et al., 2021). If a higher genetic diversity is explored, other subspecies can be found like: the subsp. *laperrine*, which is adapted to an extremely arid climate and can survive in mountain areas within the Sahara desert with annual rainfall often less than 50-100 mm (Besnard et al., 2009); or the subsp. *maroccana* from Morocco, an extraordinary hotspot for plant diversity and endemism (Lala et al., 2018), developed in an arid region (Médail et al., 2001). Therefore, these wild genotypes show a genetic pool capable of adapting to extreme environments (Besnard et al., 2021) in the new climate change scenario. From the point of view of the climate change forecast, the use of wild olive genotypes in the olive orchard can be used: (i) as a source of resistance genes in breeding programs of new cultivars or rootstocks; (ii) as rootstocks themselves to improve water-use efficiency or drought tolerance in the

modern olive orchard; iii) to restore deforested regions in arid zones, avoiding soil loss and desertification.

The aims of this study were: (i) to evaluate the water consumption of 39 wild olive genotypes representative of the different subspecies of the SILVOLIVE collection under conditions of full (100% field capacity) and deficit (60% field capacity) irrigation; (ii) to identify genotypes with contrasting behaviors in terms of water use and plant, shoot and root fresh weight as starting plant material for a collaborative work on the effect of hydraulic traits on plant growth patterns under water stress.

Material and Methods

Plant material and culture conditions

A total of 39 genotypes from the SILVOLIVE collection (Díaz-Rueda et al., 2020**; Table 1**) were analyzed in five experiments to compare plant biometric parameters. Plants were propagated and rooted *in-vitro* following the procedure previously described (Chapter 1; Díaz-Rueda et al., (2020)). After *ex-vitro* acclimatization, plants were grown under greenhouse conditions for one year in 1 L pots. Healthy and homogenous plants were selected and transplanted into 10 L pots containing a vermiculite:peat:perlite substrate (40:40:20) and were acclimatized outdoor for 2 additional months. Plants of all genotypes were the same batch (13-18 months of age) when the experiment started, using GUA1 genotype in the five experiments as normalizing control.

TABLE 1 | Origin and code of the 39 wild olive genotypes of the SILVOLIVE collection used in water use efficiency.

		Mother tree				
Subspecies	**Lineage**	**Variety**	**Natural Localization**	**Prospection**	**Acronym**	**Number of Genotypes**
europaea	*E1*	'Acebuche de Puerto Real' oleaster Ω	Puerto Real saline marshes, Cádiz, Spain	*In-vitro* germplasm collection M. Cantos (IRNAS, CSIC)	APR	1
		Unnamed oleaster Ω	Cádiz Mountains, Spain	WOGB, Córdoba, Spain Acc. Number W45	ACZ	7
		Unnamed oleaster×	Coria del Río, Seville, Spain	Coria del Río, Sevilla, Spain	ACO	3
		Unnamed oleaster×	Marrakech Mountains, Morocco	Marrakech, Morocco	AMK	6
	E2	'Raboconejo' oleaster Ω	Saltés Island in Odiel saline marshes, Huelva, Spain	*In-vitro* germplasm collection M. Cantos (IRNAS, CSIC)	ARC	1
	E3	Unnamed oleaster Ω	Sierra de Jaén, Spain	WOGB, Córdoba, Spain Acc. Number W69	AJA	4
guanchica	*M-g1*	*guanchica* Ω	Tenerife, Canary Islands, Spain	WOGB, Córdoba, Spain Acc. Number W49	GUA	8
cuspidata x *europaea*	*A*	*cuspidata*×	Grahamstown, South Africa	CEFE Montpellier Acc. Number Gr3 & Gr5	CUS	1
	A	*cuspidata*×	Kirstenbosch, South Africa	CEFE Montpellier, France Acc. Kirstenbosch	CEH	8
Total number of genotypes						39

× Seeds obtained from different mother trees.

Ω Seeds obtained from the same mother tree.

Experimental design

The 39 wild olive genotypes were evaluated during 2015 in La Hampa CSIC experimental orchard, near Seville (Spain) (37º 17´N, 6º 30´W, altitude 30 m) (**Figure 3**). For each of these 39 genotypes, six well-irrigated plants (100% field capacity; WW) and six water-stressed plants (60% field capacity; WS) were distributed randomly in rows of 16 plants at 1 x 1.5 m, alternating well-watered (WW) rows and water-stressed rows (WS). This distribution was sufficient to avoid shading by neighboring plants (based on *in situ* observations). Pots were kept on a concrete block to avoid root contact with the soil. In addition, a plastic was placed on top of the pot to avoid the loss of water by evaporation. Plants were harvested at the end of the trial (four weeks in each experiment), and the fresh and dry weights of aerial organs (leaves and stems) and the root were recorded.

FIGURE 3 | Quantification of water use in representative genotypes of the SILVOLIVE collection. Assays were performed in the experimental station "La Hampa" in 2015.

Water Use, Growth and Stress Parameters

Water loss was quantified comparing the weight of the pot immediately after watering with that obtained 3-4 days after watering. After the measurement, plants were re-watered up to their corresponding water status. Plants were harvested at the end of the assay, and the fresh and dry weights of shoots (leaves and stems) and roots were recorded.

As a stress marker, the integrity of photosystem II was determined through measurement of chlorophyll fluorescence using a portable PAM fluorometer (FluorPen FP-100; Photon System Instruments, Brno, Czech Republic) that enables quick and precise quantification of quantum yield (Qy) in light-adapted plants following the protocol described by Franco-Navarro et al. (2016). For each treatment and genotype, six fully expanded and photosynthetically active leaves from three plants were used. Qy measurements were conducted between 10:00 – 12:00 am, and plants were then harvested.

Data Processing and Statistical Analysis

Statistical analyses were performed to assess the effect of the irrigation treatment and genotype on leaf, root and the whole-plant weight values. One-way ANOVA was used in

cases where more than two levels were compared, while the multiple comparisons of means were analyzed by Tukey's HSD (honestly significant difference) for comparisons between two levels. The multiple range test was calculated using the Statistical Analysis System (STATGRAPHICS Centurion XVI software; http://www.statgraphics.com; StatPoint Technologies, Warrenton, VA, United States).

Results and Discussion

Deficit irrigation determined a reduction in water consumption with respect to well-irrigated plants, although this reduction varies between different genotypes (**Figure 4**). While a wide variability in water consumption was observed in the set of 39 wild olive genotypes, those from the same origin showed similar water consumption habits (**Figure 4**). The value range showed a variance of 5.5 times from ACZ8 (0.41 ± 0.036) to AMK6 (2.30 ± 0.110) in WW plants. Thus, AMK genotypes showed higher, while ACZ and CEH genotypes lower water consumption than the average. Some ACZ genotypes showed greater differences between water consumption under WW and WS conditions.

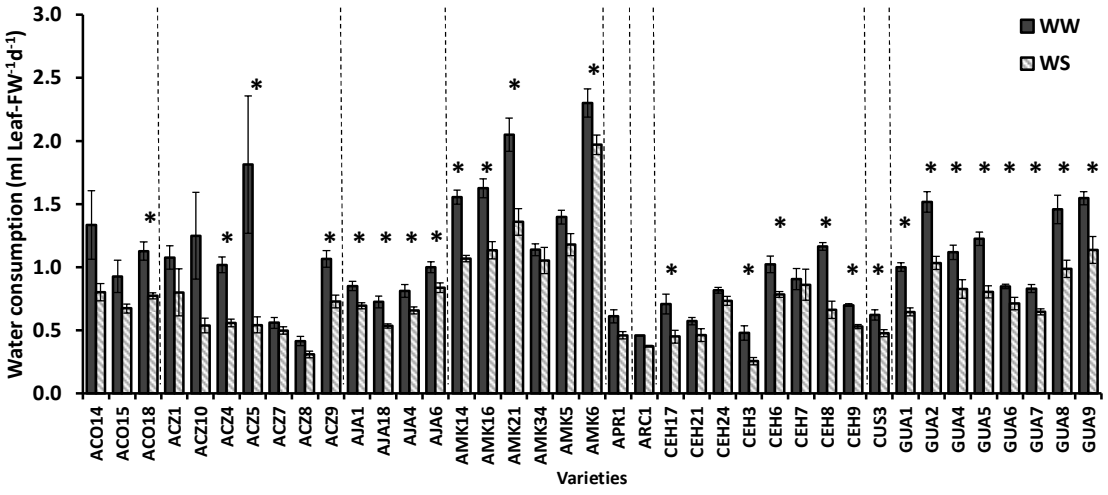


FIGURE 4 | Water use habits of wild olive genotypes. Water consumption relative to leaf fresh weight (FW) was quantified in potted seedlings from selected genotypes of the subspecies *europaea* (ACZ8, ARC1, ACZ27, APR1, AJA18, AJA4, AJA1, ACO15, AJA6, ACZ4, ACZ9, ACZ1, ACO18, AMK34, ACZ10, ACO14, AMK5, AMK14, AMK16, ACZ5, AMK21, and AMK6), *guanchica* (GUA7, GUA6, GUA1, GUA4, GUA5, GUA8, GUA2 and GUA9), and *cuspidata* (CEH3, CEH9, CEH24, CEH6, CEH8). Bars are the average of six plants ± SE. Asterisks indicate significant differences between well-watered (WW) and water-stressed (WS) plants for each genotype ($p < 0.05$).

Because water consumption must be normalized with respect to the total leaf area or biomass, both parameters showed inverse correlations (**Figure 5**). Plants with lower aerial biomass (e.g. AMK16, AMK21 and AMK6) showed higher consumption per unit of leaf biomass, which probably means greater water availability in plants with lower transpiration surface rather than greater water use.

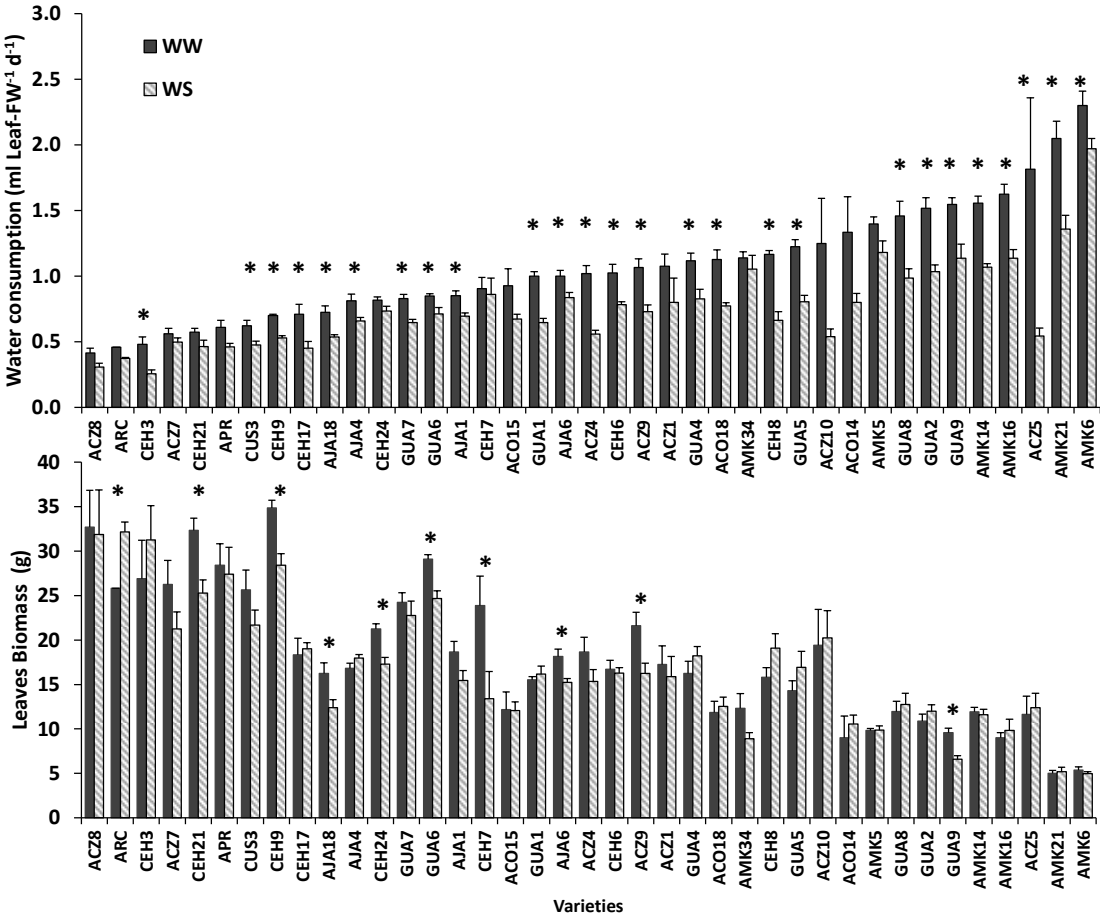


FIGURE 5 | Comparison of water consumption and total plant biomass in selected genotypes from the subspecies *europaea* (ACZ8, ARC1, ACZ27, APR1, AJA18, AJA4, AJA1, ACO15, AJA6, ACZ4, ACZ9, ACZ1, ACO18, AMK34, ACZ10, ACO14, AMK5, AMK14, AMK16, ACZ5, AMK21, and AMK6), *guanchica* (GUA7, GUA6, GUA1, GUA4, GUA5, GUA8, GUA2 and GUA9), and *cuspidata* (CEH3, CEH9, CEH24, CEH6, CEH8). Bars are the average of six plants ± standard error. Asterisks indicate significant differences between well-watered (WW) and water-stressed (WS) plants for each genotype ($p < 0.05$).

Water deficit frequently impairs the development of the aerial part of the plants, although a stimulating effect on the root growth was also observed in many genotypes (**Figure 6**).

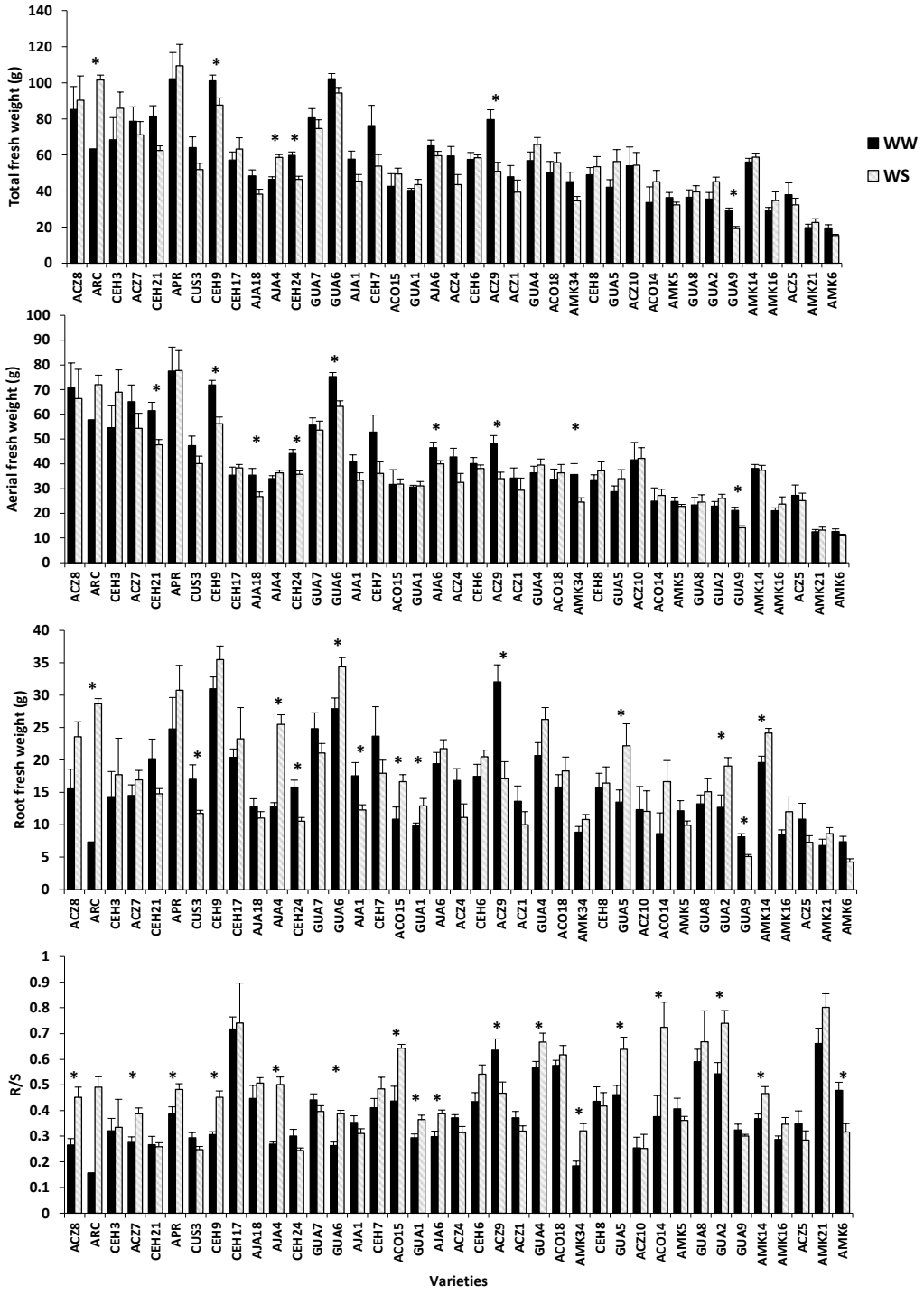


FIGURE 6 | Growth of wild olive genotypes. Total FW, shoot FW, root FW, and the root vs. shoot (R/S) biomass ratio was quantified in potted seedlings during the 2015 screening in selected genotypes from the subspecies *europaea* (ACZ8, ARC1, ACZ27, APR1, AJA18, AJA4, AJA1, ACO15, AJA6, ACZ4, ACZ9, ACZ1, ACO18, AMK34, ACZ10, ACO14, AMK5, AMK14, AMK16, ACZ5, AMK21, and AMK6), *guanchica* (GUA7, GUA6, GUA1, GUA4, GUA5, GUA8, GUA2 and GUA9), and *cuspidata* (CEH3, CEH9, CEH24, CEH6, CEH8). Bars are the average of six plants ± standard error. Asterisks indicate significant differences between well-watered (WW) and water-stressed (WS) plants for each genotype ($p < 0.05$).

As a first approach to know the degree of water deficit resistance of the wild genotypes studied, growth inhibition of the plant aerial part relative to that of the corresponding well-irrigated control treatments was quantified (**Figure 7**). The aerial growth of most genotypes was inhibited by water deficit (61.5%). However, fifteen genotypes (38.5%) were identified as water deficit resistant since deficit irrigation did not impair the growth of the aerial plant part, or it was even stimulated (**Figure 7**). In all resistant genotypes, except in one case (93.3%), plants stimulated root growth in response to water deficit, while this percentage was reduced to 29.1% in the rest of the genotypes. Therefore, the resistant genotypes where those that most importantly stimulated root growth in response to deficit irrigation (20-100% stimulation). The case of ARC was especially noteworthy, given that its root biomass quadrupled due to the effect of deficit irrigation. Thus, GUA2, GUA8, GUA5 and GUA4 from the subsp. *guanchica*, as well as ARC and AJA4 from the subsp. *europaea*, were identified as the most resistant to water deficit (**Figure 7**). The three genotypes whose growth was most negatively affected by water deficit were ACZ5, ACZ1 and ACZ9.

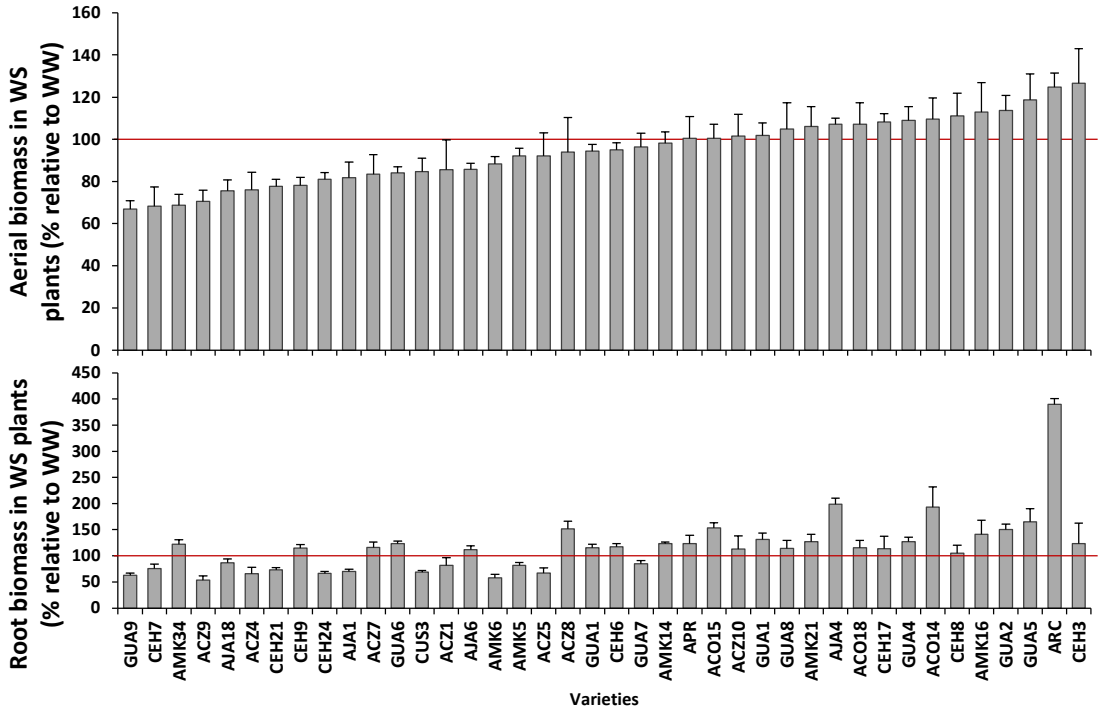


FIGURE 7 | Growth Effect of water deficit on the growth of aboveground and belowground plant biomass in the wild genotypes. Plants were grown under optimal (field capacity, FC) or deficit (60% FC) irrigation. The results represent the biomass of water-stressed plants relative (%) to their well-watered controls.

We also used the integrity of Photosystem-II (PS-II) as a stress marker (**Figure 7**). In agreement with the growth-inhibition results, the photosystem-II was less damaged in the *guanchica* resistant genotypes, while sensitive ACZ genotypes were much more affected. We can see, for example, that the variety that shows the greatest loss of integrity of the PS-II under deficit irrigation conditions is ACZ5 genotype, which coincides with being the one identified as the most sensitive to water deficit due to loss of aerial biomass (**Figure 6**).

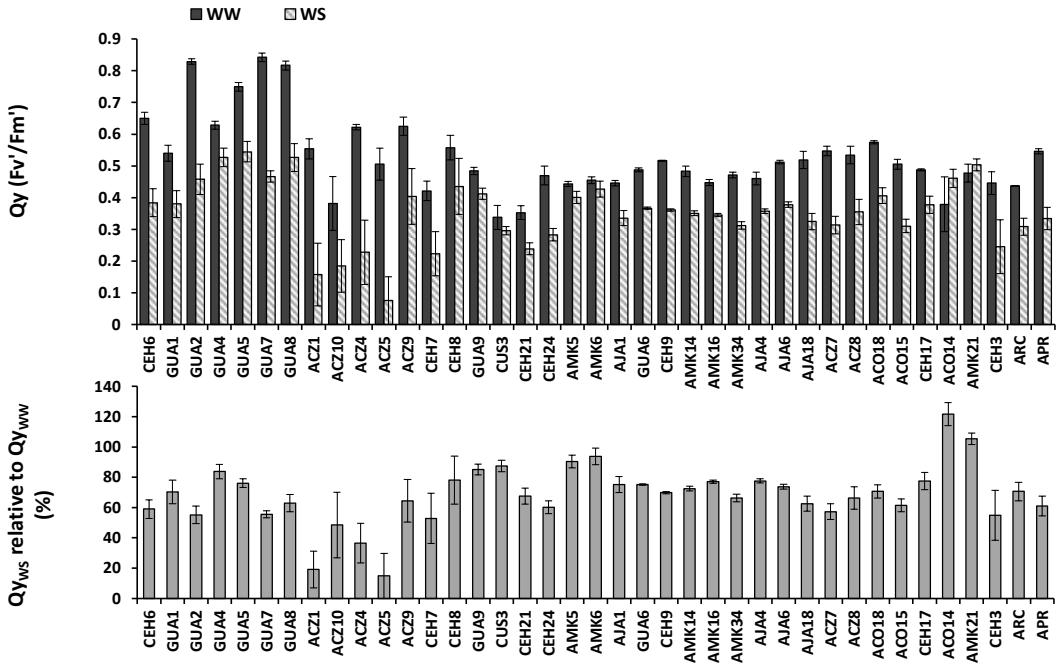


FIGURE 8 | Effect of the water deficit on the integrity of Photosystem-II in the different deficit irrigation tests. Plants were grown under optimal (field capacity, WW) or deficit (WS, 60% WW) irrigation. The results represent the Quantum yield (Qy) of water-stressed plants relative (%) to their well-watered controls.

We have made a first approach on the water use and deficit irrigation responses in 39 wild olive genotypes of the SILVOLIVE collection (Table 1) belonging to three out of the six subspecies currently recognized within *Olea europaea* (Médail et al., 2001; Green, 2002a). Among the six most resistant genotypes, two different developmental and/or physiological strategies were identified (**Figure 5**): 1) ARC and CEH3 present high vigor and a very low rate of water consumption per unit of leaf biomass; 2) GUA5, GUA2, AMK16 and ACO14 present lower vigor and a high rate of water consumption per unit of leaf biomass. Therefore, we propose that the stimulation of root growth in response to water deficit is the adaptive mechanism that is best correlated with the plant

resistance to water deficit in the olive genotypes. ARC and APR were previously described as salt-tolerant genotypes (Cantos et al., 2002). Both genotypes are characterized by being very vigorous and by increasing the root/aerial-part ratio in response to deficit irrigation (**Figure 6**). Therefore, we propose that both genotypes increase the plant's ability to explore a greater soil volume and depth, allowing higher water uptake. These genotypes can be used as a plant cover to restore deforested regions and degraded soils at risk of desertification, as well as rootstocks in olive production for arid or super-arid areas.

Sensitive genotypes such as ACZ5, ACZ1, ACZ9, ACZ4 and ACZ10 showed a strong reduction in water consumption during the period of water stress (**Figure 4**), and their photosynthetic system was more damaged (**Figure 8**), resulting highly affected by water deficit. ACZ genotypes are also characterized by high inhibition of root growth in response to water deficit, which is consistent with the fact that this adaptive response is important for resistance to water deficit.

From these results four genotypes were selected due to their contrasting behaviour to water deficit in terms of water use and plant, shoot and root fresh weight (Hernández-Santana et al., 2019) (see published manuscript in Apendix 1).

References

Alcántara, E., Cordeiro, A. M., and Barranco, D. (2003). Selection of olive varieties for tolerance to iron chlorosis. *J. Plant Physiol.* 160, 1467–1472. doi: 10.1078/0176-1617-01013.

Aleza, P., Juárez, J., Hernández, M., Pina, J. A., Ollitrault, P., and Navarro, L. (2009). Recovery and characterization of a Citrus clementinaHort. ex Tan. "Clemenules" haploid plant selected to establish the reference whole Citrus genome sequence. *BMC Plant Biol.* 9, 110. doi: 10.1186/1471-2229-9-110.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and León, L. (2015a). Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. *Euphytica* 206, 619–629. doi: 10.1007/s10681-015-1463-7.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015b). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi: 10.1016/j.cropro.2015.05.006.

Baali-Cherif, D., and Besnard, G. (2005). High Genetic Diversity and Clonal Growth in Relict Populations of *Olea europaea* subsp. *laperrinei* (Oleaceae) from Hoggar, Algeria. *Ann. Bot.* 96, 823–830. doi: 10.1093/aob/mci232.

Baccouri, B., Guerfel, M., Zarrouk, W., Taamalli, W., Daoud, D., and Zarrouk, M. (2011). Wild olive (*Olea europaea* L.) selection for quality oil production. *J. Food Biochem.* 35, 161–176. doi: 10.1111/j.1745-4514.2010.00373.x.

Bacelar, E. A., Correia, C. M., Moutinho-Pereira, J. M., Goncalves, B. C., Lopes, J. I., and Torres-Pereira, J. M. G. (2004). Sclerophylly and leaf anatomical traits of five field-grown olive cultivars growing under drought conditions. *Tree Physiol.* 24, 233–239. doi: 10.1093/treephys/24.2.233.

Baldoni, L., Cultrera, N. G., Mariotti, R., Ricciolini, C., Arcioni, S., Vendramin, G. G., et al. (2009). A consensus list of microsatellite markers for olive genotyping. *Mol. Breed.* 24, 213–231. doi: 10.1007/s11032-009-9285-8.

Baldoni, L., and Fontanazza, G. (1990). Preliminary results on olive clonal rootstocks behaviour in the field. *Acta Hortic.*, 37–40. doi: 10.17660/ActaHortic.1990.286.2.

Baldoni, L., Tosti, N., Ricciolini, C., Belaj, A., Arcioni, S., Pannelli, G., et al. (2006). Genetic Structure of Wild and Cultivated Olives in the Central Mediterranean Basin. *Ann. Bot.* 98, 935–942. doi: 10.1093/aob/mcl178.

Barazani, O., Westberg, E., Hanin, N., Dag, A., Kerem, Z., Tugendhaft, Y., et al. (2014). A comparative analysis of genetic variation in rootstocks and scions of old olive trees – a window into the history of olive cultivation practices and past genetic variation. *BMC Plant Biol.* 14, 146. doi: 10.1186/1471-2229-14-146.

Barranco, D., Fernandez-Escobar, R., and Rallo, L. (1998). *El cultivo del olivo*. Sevilla: Junta de Andalucia.

Bekele, T. (2005). Recruitment, survival and growth of *Olea europaea* subsp *cuspidata* seedlings and juveniles in dry Afromontane forests of northern Ethiopia. *Trop Ecol* 46, 113–126.

Belaj, A., Gurbuz Veral, M., Sikaoui, H., Moukhli, A., Khadari, B., Mariotti, R., et al. (2016). "Olive Genetic Resources," in *The Olive Tree Genome* Compendium of Plant Genomes., eds. E. Rugini, L. Baldoni, R. Muleo, and L. Sebastiani (Cham: Springer International Publishing), 27–54. doi: 10.1007/978-3-319-48887-5_3.

Belaj, A., Muñoz-Diez, C., Baldoni, L., Satovic, Z., and Barranco, D. (2010). Genetic diversity and relationships of wild and cultivated olives at regional level in Spain. *Sci. Hortic.* 124, 323–330. doi: 10.1016/j.scienta.2010.01.010.

Besnard, G. (2008). Chloroplast DNA variations in Mediterranean olive. *J. Hortic. Sci. Biotechnol.* 83, 51–54. doi: 10.1080/14620316.2008.11512346.

Besnard, G., and Baali-Cherif, D. (2009). Coexistence of diploids and triploids in a Saharan relict olive: Evidence from nuclear microsatellite and flow cytometry analyses. *C. R. Biol.* 332, 1115–1120. doi: 10.1016/j.crvi.2009.09.014.

Besnard, G., De Casas, R. R., and Vargas, P. (2003). A set of primers for length and nucleotide-substitution polymorphism in chloroplastic DNA of *Olea europaea* L. (Oleaceae). *Mol. Ecol. Notes* 3, 651–653. doi: 10.1046/j.1471-8286.2003.00547.x.

Besnard, G., El Bakkali, A., Haouane, H., Baali-Cherif, D., Moukhli, A., and Khadari, B. (2013a). Population genetics of Mediterranean and Saharan olives: geographic patterns of differentiation and evidence for early generations of admixture. *Ann. Bot.* 112, 1293–1302. doi: 10.1093/aob/mct196.

Besnard, G., Garcia-Verdugo, C., Rubio De Casas, R., Treier, U. A., Galland, N., and Vargas, P. (2008). Polyploidy in the Olive Complex (*Olea europaea*): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses. *Ann. Bot.* 101, 25–30. doi: 10.1093/aob/mcm275.

Besnard, G., Gorrilliot, O., Raimondeau, P., Génot, B., El Bakkali, A., Anthelme, F., et al. (2021). Contrasting Genetic Footprints among Saharan Olive Populations: Potential Causes and Conservation Implications. *Plants* 10, 1207. doi: 10.3390/plants10061207.

Besnard, G., Hernández, P., Khadari, B., Dorado, G., and Savolainen, V. (2011). Genomic profiling of plastid DNA variation in the Mediterranean olive tree. *BMC Plant Biol.* 11, 80. doi: 10.1186/1471-2229-11-80.

Besnard, G., Khadari, B., Navascués, M., Fernández-Mazuecos, M., El Bakkali, A., Arrigo, N., et al. (2013b). The complex history of the olive tree: from Late Quaternary diversification of Mediterranean lineages to primary domestication in the northern Levant. *Proc. R. Soc. B Biol. Sci.* 280, 20122833. doi: 10.1098/rspb.2012.2833.

Besnard, G., Rubio de Casas, R., Christin, P.-A., and Vargas, P. (2009). Phylogenetics of *Olea* (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: Tertiary climatic shifts and lineage differentiation times. *Ann. Bot.* 104, 143–160. doi: 10.1093/aob/mcp105.

Besnard, G., Rubio de Casas, R., and Vargas, P. (2007). Plastid and nuclear DNA polymorphism reveals historical processes of isolation and reticulation in the olive tree complex (*Olea europaea*). *J. Biogeogr.* 34, 736–752. doi: 10.1111/j.1365-2699.2006.01653.x.

Besnard, G., Terral, J.-F., and Cornille, A. (2018). On the origins and domestication of the olive: a review and perspectives. *Ann. Bot.* 121, 385–403. doi: 10.1093/aob/mcx145.

Bruvo, R., Michiels, N. K., D'Souza, T. G., and Schulenburg, H. (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level: MICROSATELLITE DISTANCES IN POLYPLOIDS. *Mol. Ecol.* 13, 2101–2106. doi: 10.1111/j.1365-294X.2004.02209.x.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9.

Burnett, A. C., Rogers, A., Rees, M., and Osborne, C. P. (2016). Carbon source-sink limitations differ between two species with contrasting growth strategies: Source-sink limitations vary with growth strategy. *Plant Cell Environ.* 39, 2460–2472. doi: 10.1111/pce.12801.

Cantos, M., Troncoso, J., Liñán, J., Troncoso, A., and Rapaport, H. (2002). Obtaining salt (NaCl) tolerant olive plants: i) some physiological and anatomical characteristics of olive plants growing in harsh saline zones. *Acta Hortic.*, 441–444. doi: 10.17660/ActaHortic.2002.586.90.

Castañeda-Álvarez, N. P., Khoury, C. K., Achicanoy, H. A., Bernau, V., Dempewolf, H., Eastwood, R. J., et al. (2016). Global conservation priorities for crop wild relatives. *Nat. Plants* 2, 16022. doi: 10.1038/nplants.2016.22.

Chartzoulakis, K., Patakas, A., and Bosabalidis, A. M. (1999). Changes in water relations, photosynthesis and leaf anatomy induced by intermittent drought in two olive cultivars. *Environ. Exp. Bot.* 42, 113–120. doi: 10.1016/S0098-8472(99)00024-6.

Chiappetta, A., Muto, A., Muzzalupo, R., and Muzzalupo, I. (2017). New rapid procedure for genetic characterization of Italian wild olive (*Olea europaea*) and traceability of virgin olive oils by means of SSR markers. *Sci. Hortic.* 226, 42–49. doi: 10.1016/j.scienta.2017.08.022.

Clark, L. V., and Jasieniuk, M. (2011). POLYSAT : an R package for polyploid microsatellite analysis. *Mol. Ecol. Resour.* 11, 562–566. doi: 10.1111/j.1755-0998.2011.02985.x.

Colella, C., Miacola, C., Amenduni, M., D'Amico, M., Bubici, G., and Cirulli, M. (2008). Sources of verticillium wilt resistance in wild olive germplasm from the Mediterranean region. *Plant Pathol.* 57, 533–539. doi: 10.1111/j.1365-3059.2007.01785.x.

Connor, D. J. (2006). Towards optimal designs for hedgerow olive orchards. *Aust. J. Agric. Res.* 57, 1067. doi: 10.1071/AR05448.

Connor, D. J., and Fereres, E. (2010). "The Physiology of Adaptation and Yield Expression in Olive," in *Horticultural Reviews*, ed. J. Janick (Oxford, UK: John Wiley & Sons, Inc.), 155–229. doi: 10.1002/9780470650882.ch4.

Costes, E., Lauri, P. É., and Regnard, J. L. (2010). "Analyzing Fruit Tree Architecture: Implications for Tree Management and Fruit Production," in *Horticultural Reviews*, ed. J. Janick (Oxford, UK: John Wiley & Sons, Inc.), 1–61. doi: 10.1002/9780470767986.ch1.

De la Rosa, R., Kiran, A. I., Barranco, D., and León, L. (2006). Seedling vigour as a preselection criterion for short juvenile period in olive breeding. *Aust. J. Agric. Res.* 57, 477. doi: 10.1071/AR05219.

Del Río, C., and Caballero, J. M. (2006). Resultados preliminares sobre el empleo de patrones para modificar el vigor y/o la producción de la variedad de olivo 'Picual.' *Actas Hortic. SECH* 45, 179–180.

Dell'Aquila, A., Calmanti, S., Ruti, P., Struglia, M., Pisacane, G., Carillo, A., et al. (2012). Effects of seasonal cycle fluctuations in an A1B scenario over the Euro-Mediterranean region. *Clim. Res.* 52, 135–157. doi: 10.3354/cr01037.

Díaz-Espejo, A., Fernández, J. E., Torres-Ruíz, J. M., Rodríguez-Domínguez, C. M., Pérez-Martín, A., and Hernández-Santana, V. (2018). "The Olive Tree Under Water Stress," in *Water Scarcity and Sustainable Agriculture in Semiarid Environment* (Elsevier), 439–479. doi: 10.1016/B978-0-12-813164-0.00018-1.

Díaz-Rueda, P., Franco-Navarro, J. D., Messora, R., Espartero, J., Rivero-Núñez, C. M., Aleza, P., et al. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Front. Plant Sci.* 11, 629. doi: 10.3389/fpls.2020.00629.

Díez, C. M., Moral, J., Cabello, D., Morello, P., Rallo, L., and Barranco, D. (2016). Cultivar and Tree Density As Key Factors in the Long-Term Performance of Super High-Density Olive Orchards. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01226.

do Val, A. D. B., Ferreira, J. L., Vieira Neto, J., Pasqual, M., de Oliveira, A. F., Borém, A., et al. (2012). Genetic diversity of Brazilian and introduced olive germplasms based on microsatellite markers. *Genet. Mol. Res.* 11, 556–571. doi: 10.4238/2012.March.8.4.

Edziri, H., Chehab, H., Aissaoui, F., Boujnah, D., and Mastouri, M. (2021). Photosynthetic, anatomical and biochemical responses of olive tree (*Olea europaea*) cultivars under water stress. *Plant Biosyst. - Int. J. Deal. Asp. Plant Biol.* 155, 740–746. doi: 10.1080/11263504.2020.1779844.

Esquinas-Alcázar, J. (2005). Protecting crop genetic diversity for food security: political, ethical and technical challenges. *Nat. Rev. Genet.* 6, 946–953. doi: 10.1038/nrg1729.

Ezzahar, J., Chehbouni, A., Er-Raki, S., Khabba, S., and Hanich, L. (2009). Estimating and monitoring seasonal water consumption of olive orchards in a semi-arid region (Morocco) using the Eddy-covariance system. *Options Méditerranéennes A* 88.

Fernández, J. E., Perez-Martin, A., Torres-Ruiz, J. M., Cuevas, M. V., Rodriguez-Dominguez, C. M., Elsayed-Farag, S., et al. (2013). A regulated deficit irrigation strategy for hedgerow olive orchards with high plant density. *Plant Soil* 372, 279–295. doi: 10.1007/s11104-013-1704-2.

Fernández, J.-E. (2014). Understanding olive adaptation to abiotic stresses as a tool to increase crop performance. *Environ. Exp. Bot.* 103, 158–179. doi: 10.1016/j.envexpbot.2013.12.003.

Franco-Navarro, J. D., Brumós, J., Rosales, M. A., Cubero-Font, P., Talón, M., and Colmenero-Flores, J. M. (2016). Chloride regulates leaf cell size and water relations in tobacco plants. *J. Exp. Bot.* 67, 873–891. doi: 10.1093/jxb/erv502.

García, J. M., Morales-Sillero, A., Pérez-Rubio, A. G., Díaz-Espejo, A., Montero, A., and Fernández, J. E. (2017). Virgin olive oil quality of hedgerow 'Arbequina' olive trees under deficit irrigation: Oil quality from deficit irrigated olive hedgerow. *J. Sci. Food Agric.* 97, 1018–1026. doi: 10.1002/jsfa.7828.

García-Verdugo, C., Forrest, A. D., Fay, M. F., and Vargas, P. (2010). The relevance of gene flow in metapopulation dynamics of an oceanic island endemic, *Olea europaea* subsp. *guanchica*: genetic structure of an *Olea europaea* metapopulation. *Evolution* 64, 3525–3536. doi: 10.1111/j.1558-5646.2010.01091.x.

Gimenez, C., Fereres, E., Ruz, C., and Orgaz, F. (1997). Water relations and gas exchange of olive trees: diurnal and seasonal patterns of leaf water potential, photosynthesis and stomatal conductance. *Acta Hortic.*, 411–416. doi: 10.17660/ActaHortic.1997.449.57.

Gómez, J. A., Giráldez, J. V., and Fereres, E. (2001). Rainfall interception by olive trees in relation to leaf area. *Agric. Water Manag.* 49, 65–76. doi: 10.1016/S0378-3774(00)00116-5.

Gómez-Rico, A., Salvador, M. D., Moriana, A., Pérez, D., Olmedilla, N., Ribas, F., et al. (2007). Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. *Food Chem.* 100, 568–578. doi: 10.1016/j.foodchem.2005.09.075.

Green, P. (2002a). *A revision of* Olea *L. (Oleaceae). Kew Bull. 57, 91–140.* Kew Bull.

Green, P. S. (2002b). A Revision of *Olea* L. (Oleaceae). *Kew Bull.* 57, 91. doi: 10.2307/4110824.

Hammami, S. B. M., León, L., Rapoport, H. F., and De la Rosa, R. (2011). Early growth habit and vigour parameters in olive seedlings. *Sci. Hortic.* 129, 761–768. doi: 10.1016/j.scienta.2011.05.038.

Hannachi, H., Breton, C., Msallem, M., Ben El Hadj, S., El Gazzah, M., and Bervillé, A. (2008). Differences between native and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: A case study in Tunisia. *Sci. Hortic.* 116, 280–290. doi: 10.1016/j.scienta.2008.01.004.

Hernández-Santana, V., Díaz-Rueda, P., Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., et al. (2019). Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress. *Front. Plant Sci.* 10, 291. doi: 10.3389/fpls.2019.00291.

Hijmans, R. J. (2021). Raster: Geographic Data Analysis and Modeling. R package version 3.4-13. https://CRAN.R-project.org/package=raster. Data downloaded on 16 september 2021.

Inderbitzin, P., and Subbarao, K. V. (2014). *Verticillium* Systematics and Evolution: How Confusion Impedes Verticillium Wilt Management and How to Resolve It. *Phytopathology®* 104, 564–574. doi: 10.1094/PHYTO-11-13-0315-IA.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516.

Kassa, A., Konrad, H., and Geburek, T. (2019). Molecular diversity and gene flow within and among different subspecies of the wild olive (*Olea europaea* L.): A review. *Flora* 250, 18–26. doi: 10.1016/j.flora.2018.11.014.

Kirtman, B., Power, S. B., Adedoyin, J. A., Boer, G. J., Bojariu, R., Camilloni, I., et al. (2013). *IPCC, 2013: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)].* Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Klepo, T., De la Rosa, R., Satovic, Z., León, L., and Belaj, A. (2013). Utility of wild germplasm in olive breeding. *Sci. Hortic.* 152, 92–101. doi: 10.1016/j.scienta.2012.12.010.

Klepo, T., Toumi, A., De La Rosa, R., LeÓn, L., and Belaj, A. (2014). Agronomic evaluation of seedlings from crosses between the main Spanish olive cultivar 'Picual' and two wild olive trees. *J. Hortic. Sci. Biotechnol.* 89, 508–512. doi: 10.1080/14620316.2014.11513113.

Lala, S., Amri, A., and Maxted, N. (2018). Towards the conservation of crop wild relative diversity in North Africa: checklist, prioritisation and inventory. *Genet. Resour. Crop Evol.* 65, 113–124. doi: 10.1007/s10722-017-0513-5.

León, L., de la Rosa, R., Velasco, L., and Belaj, A. (2018). Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. *Front. Plant Sci.* 9, 232. doi: 10.3389/fpls.2018.00232.

León, L., Díaz-Rueda, P., Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. *Sci. Hortic.* 264, 109157. doi: 10.1016/j.scienta.2019.109157.

López-Escudero, F. J., and Blanco-López, M. A. (2005). Recovery of Young Olive Trees from *Verticillium dahliae*. *Eur. J. Plant Pathol.* 113, 367–375. doi: 10.1007/s10658-005-3145-0.

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of Olive Cultivars for Resistance to *Verticillium dahliae*. *Eur. J. Plant Pathol.* 110, 79–85. doi: 10.1023/B:EJPP.0000010150.08098.2d.

López-Escudero, F. J., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50. doi: 10.1007/s11104-010-0629-2.

Lumaret, R., Ouazzani, N., Michaud, H., Vivier, G., Deguilloux, M.-F., and Di Giusto, F. (2004). Allozyme variation of oleaster populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean Basin. *Heredity* 92, 343–351. doi: 10.1038/sj.hdy.6800430.

Malik, N., and Bradford, J. (2004). Reciprocal grafting between early maturing and normal maturing olive varieties: Preliminary effects on the nature of juvenility and flowering. *J. Food Agric. Environ.* 2, 197–200.

Mason, A. S., and Pires, J. C. (2015). Unreduced gametes: meiotic mishap or evolutionary mechanism? *Trends Genet.* 31, 5–10. doi: 10.1016/j.tig.2014.09.011.

Médail, F., Quézel, P., Besnard, G., and Khadari, B. (2001). Systematics, ecology and phylogeographic significance of *Olea europaea* L. ssp. *maroccana* (Greuter & Burdet) P. Vargas et al a relictual olive tree in south-west Morocco. *Bot. J. Linn. Soc.* 137, 249–266. doi: 10.1006/bojl.2001.0477.

Murillo, J. M., Madejón, E., Madejón, P., and Cabrera, F. (2005). The response of wild olive to the addition of a fulvic acid-rich amendment to soils polluted by trace elements (SW Spain). *J. Arid Environ.* 63, 284–303. doi: 10.1016/j.jaridenv.2005.03.022.

Nevo, E., Fu, Y.-B., Pavlicek, T., Khalifa, S., Tavasi, M., and Beiles, A. (2012). Evolution of wild cereals during 28 years of global warming in Israel. *Proc. Natl. Acad. Sci.* 109, 3412–3415. doi: 10.1073/pnas.1121411109.

Palomo, M. J., Moreno, F., Fernández, J. E., Díaz-Espejo, A., and Girón, I. F. (2002). Determining water consumption in olive orchards using the water balance approach. *Agric. Water Manag.* 55, 15–35. doi: 10.1016/S0378-3774(01)00182-2.

Pannelli, G., Rosati, S., and Rugini, E. (2002). The effect of clonal rootstocks on frost tolerance and on some aspects of plant behaviour in Moraiolo and S. Felice olive cultivars. *Acta Hortic.*, 247–250. doi: 10.17660/ActaHortic.2002.586.46.

Pérez-Rodríguez, M., Alcántara, E., Amaro, M., Serrano, N., Lorite, I. J., Arquero, O., et al. (2015). The Influence of Irrigation Frequency on the Onset and Development of Verticillium Wilt of Olive. *Plant Dis.* 99, 488–495. doi: 10.1094/PDIS-06-14-0599-RE.

Ponti, L., Gutierrez, A. P., Ruti, P. M., and Dell'Aquila, A. (2014). Fine-scale ecological and economic assessment of climate change on olive in the Mediterranean Basin reveals winners and losers. *Proc. Natl. Acad. Sci.* 111, 5598–5603. doi: 10.1073/pnas.1314437111.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1.

Rallo, L., Barranco, D., Castro-García, S., Connor, D. J., Gómez del Campo, M., and Rallo, P. (2013). "High-Density Olive Plantations," in *Horticultural Reviews Volume 41*, ed. J. Janick (Hoboken, New Jersey: John Wiley & Sons, Inc.), 303–382. doi: 10.1002/9781118707418.ch07.

Rallo, L., Caruso, T., Díez, C. M., and Campisi, G. (2016). "Olive Growing in a Time of Change: From Empiricism to Genomics," in *The Olive Tree Genome* Compendium of Plant Genomes., eds. E. Rugini, L. Baldoni, R. Muleo, and L. Sebastiani (Cham: Springer International Publishing), 55–64. doi: 10.1007/978-3-319-48887-5_4.

Reddy, K. R., Brand, D., Wijewardana, C., and Gao, W. (2017). Temperature Effects on Cotton Seedling Emergence, Growth, and Development. *Agron. J.* 109, 1379–1387. doi: 10.2134/agronj2016.07.0439.

Romero, A., Hermoso, J. F., and Tous, J. (2014). Olive rootstocks to control' Arbequina IRTAI18' clone vigour results from a second one comparative trial. *Acta Hortic.*, 577–584. doi: 10.17660/ActaHortic.2014.1057.74.

Rosati, A., Paoletti, A., Al Hariri, R., Morelli, A., and Famiani, F. (2018). Partitioning of Dry Matter into Fruit Explains Cultivar Differences in Vigor in Young Olive (*Olea europaea* L.) Trees. *HortScience* 53, 491–495. doi: 10.21273/HORTSCI12739-17.

Rosati, A., Paoletti, A., Caporali, S., and Perri, E. (2013). The role of tree architecture in super high density olive orchards. *Sci. Hortic.* 161, 24–29. doi: 10.1016/j.scienta.2013.06.044.

Rosati, A., Paoletti, A., Pannelli, G., and Famiani, F. (2017). Growth Is Inversely Correlated with Yield Efficiency across Cultivars in Young Olive (*Olea europaea* L.) Trees. *HortScience* 52, 1525–1529. doi: 10.21273/HORTSCI12321-17.

Ruane, J., Sonnino, A., Steduto, P., and Deane, C. (2008). Coping With Water Scarcity: What Role for Biotechnologies? Land and Water Discussion Paper.

Rubio de Casas, R., Besnard, G., Schönswetter, P., Balaguer, L., and Vargas, P. (2006). Extensive gene flow blurs phylogeographic but not phylogenetic signal in *Olea europaea* L. *Theor. Appl. Genet.* 113, 575–583. doi: 10.1007/s00122-006-0306-2.

Rugini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.* 24, 123–134. doi: 10.1016/0304-4238(84)90143-2.

Rugini, E., De Pace, C., Gutiérrez-Pesce, P., and Muleo, R. (2011). "Olea," in *Wild Crop Relatives: Genomic and Breeding Resources*, ed. C. Kole (Berlin, Heidelberg: Springer Berlin Heidelberg), 79–117. doi: 10.1007/978-3-642-16057-8_5.

Rugini, E., Silvestri, C., Ceccarelli, M., Muleo, R., and Cristofori, V. (2016). Mutagenesis and Biotechnology Techniques as Tools for Selecting New Stable Diploid and Tetraploid Olive Genotypes and Their Dwarfing Agronomical Characterization. *HortScience* 51, 799–804. doi: 10.21273/HORTSCI.51.7.799.

Sanzani, S. M., Schena, L., Nigro, F., Sergeeva, V., Ippolito, A., and Salerno, M. G. (2012). Abiotic diseases of olive. *J. Plant Pathol.* 94 (3), 469–491.

Sefc, K. M., Lopes, M. S., Mendonça, D., Santos, M. R. D., Machado, M. L. D. C., and Machado, A. D. C. (2000). Identification of microsatellite loci in olive (*Olea europaea*) and their characterization in Italian and Iberian olive trees. *Mol. Ecol.* 9, 1171–1173. doi: 10.1046/j.1365-294x.2000.00954.x.

Shull, G. H. (1948). What Is "Heterosis"? *Genetics* 33 (5), 439–446.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol.* 9, 678–687. doi: 10.1093/oxfordjournals.molbev.a040752.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197.

Tous, J., Romero, A., and Hermoso, J. F. (2010). New trends in olive orchard design for continuous mechanical harvesting. *Adv. Hortic. Sci.* 24, 43–52.

Tous, J., Romero, A., Hermoso, J. F., and Ninot, A. (2011). Influence of diferent olive rootstocks on growth and yield of the "Arbequina IRTA-I 18®" clone. *Acta Hortic.*, 315–320. doi: 10.17660/ActaHortic.2011.924.39.

Trabucco, A., and Zomer, R. J. (2018). Global Aridity Index and Potential Evapo-Transpiration (ET0) Climate Database v2. CGIAR Consortium for Spatial Information (CGIAR-CSI). Published online, available from the CGIAR-CSI GeoPortal at https://cgiarcsi.community. Data downloaded on 13 september 2021.

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. (2015). Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathol.* 64, 890–900. doi: 10.1111/ppa.12330.

Trentacoste, E. R., Contreras-Zanessi, O., Beyá-Marshall, V., and Puertas, C. M. (2018). Genotypic variation of physiological and morphological traits of seven olive cultivars under sustained and cyclic drought in Mendoza, Argentina. *Agric. Water Manag.* 196, 48–56. doi: 10.1016/j.agwat.2017.10.018.

United Nations (2015). Transforming Our World: The 2030 Agenda for Sustainable Development. New York, NY: United Nations.

Vincent, H., Wiersema, J., Kell, S., Fielder, H., Dobbie, S., Castañeda-Álvarez, N. P., et al. (2013). A prioritized crop wild relative inventory to help underpin global food security. *Biol. Conserv.* 167, 265–275. doi: 10.1016/j.biocon.2013.08.011.

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., von Wettberg, E. J. B., et al. (2016). Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008.

Weising, K., and Gardner, R. C. (1999). A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome* 42, 9–19. doi: 10.1139/g98-10

Chapter 3. Evaluation of early vigor traits of wild olive germplasm in the field and potential use as dwarfing rootstocks: a proof of concept

Some of the results from this chapter have been used in the following publication:

Evaluation of early vigor traits in wild olive germplasm. Published as: León, L., **Díaz-Rueda, P.,** Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. Sci. Horticult. 264, 109–157. https://doi.org/10.1016/j.scienta. 2019.109157

This publication is not part of the compendium of articles of this thesis since the doctoral student does not appear as the main author. This publication is attached to the Thesis as Annex-1 document.

Introduction

Crop wild relatives could represent interesting sources of variability for agronomic traits difficult to find in cultivated materials, such as resistance to pest and diseases and abiotic stresses, yield and quality traits. Thus, in the last years, the use of crop wild relatives germplasm has been extensively promoted in breeding programs of several fruit trees and nut crops (Aradhya et al., 2015). *Olea europaea* subsp. *europaea* var. *europaea* and var. *sylvestris* constitute the botanical varieties of cultivated and wild olives respectively, both of them widely spread throughout the whole Mediterranean basin. Besides the subsp. *europaea*, five other subspecies are included within the *Olea europaea* species, based on morphology and geographical distribution, including subsp. *cuspidata* and laperrinei spread in tropical and subtropical regions, subsp. *guanchica*, only recognized in the Canary Islands as well as the only two polyploid subsp. *maroccana* (6n) and *cerasiformis* (4n) present in south-western Morocco; and Madeira archipelago, respectively (Besnard et al., 2013; Green, 2002).

The potential use of olive wild genetic resources also known as oleasters as a source of genetic variability for important agronomic traits has been suggested, particularly regarding resistance to specific adverse biotic and abiotic environmental conditions (Hernández-Santana et al., 2019; Lavee and Zohary, 2011). Agronomic evaluation of progenies involving wild parents showed higher vigor, shorter juvenile period and more abundant flowering than progenies from cultivated materials (Klepo et al., 2013). Improved oil composition for some important quality components has been also reported (Hannachi et al., 2009; León et al., 2018). However, wild olives also transmit undesirable fruit traits to its descendants (particularly lower fruit size and oil content), which indicates the need of additional back-cross generations and, therefore, slow down the selection process (Klepo et al., 2014). The need for a longer selection period compared with the cultivated germplasm has limited the potential use of wild relatives as parents in olive breeding programs up to now.

Rootstock selection allows a straightforward way of valorizing wild genetic resources (Warschefsky et al., 2016). In fact, grafting on wild materials was extensively

used in the antiquity as a propagation technique in olive. Thus, the analysis of ancient olive trees in several countries reveals that grafting by using seedling, pre-selected clonal rootstocks or oleasters has been a practice widely used in the Middle East (Barazani et al., 2014) and at a lower extent in central (Lazović et al., 2016) and western part (Ninot et al., 2018) of the Mediterranean Basin. However, it should be noted that olive trees are currently grown on their own roots and rootstocks are exceptionally used in olive compared to other fruit tree species. The use of rootstocks has been recommended to cope with adverse biotic factors such as soilborne disease (Bubici and Cirulli, 2012; Porras Soriano et al., 2003) and abiotic environmental conditions such as preventing frost injury (Pannelli et al., 2002) or iron chlorosis caused by Fe deficiency (Alcántara et al., 2003). Moreover, the use of wild genetic resources as rootstocks may represent a new source of variability for important agronomic traits. In olive, it has been only studied as a way to improve resistance to Verticillium wilt of susceptible cultivars (Arias-Calderón et al., 2015; Jiménez-Fernández et al., 2016). However, to the best of our knowledge, a comprehensive evaluation of vigor traits in olive wild materials and the potential use of wild genetic resources as rootstocks for vigor control has not been attempted so far in olive. This acquires great relevance if we take into account the spreading of high density hedgerow olive orchards in recent years and the need for increasing the number of cultivars suitable for this new growing system (Rallo et al., 2013).

Traditional olive orchards are designed for densities of 70-80 trees ha^{-1} whereas the super high-density (SHD) system with narrow hedgerows of 1500-2000 trees ha^{-1} are gaining popularity worldwide. These modern super intensitive systems have several advantages including rapid mechanized harvesting and pruning, high crop levels, and early bearing (Díez et al., 2016b). However, only a few traditional olive cultivars are suitable for SDH olive hedgerow orchads such as ‘Arbequina’, ‘Arbosana’ or ‘Koroneiki’ cultivars (De la Rosa et al., 2007; Díez et al., 2016b; Rallo et al., 2013). The use of medium-low vigor cultivars allows less expensive mechanical harvesting and pruning and avoids shadowing problems that could compromise the long-term health of SHD orchards (Rallo et al., 2013; Rosati et al., 2013; Tous et al., 1999).

'Arbequina' is the dominant cultivar used in SDH orchards given its medium-low vigor, very early bearing, high and stable productivity, and fruity oil (Barranco and Rallo, 2000; De la Rosa et al., 2007; Rallo et al., 2013; Rius and Lacarte, 2015). Better vigor control of this variety cultivated under the SHD regime will increase the profitability of the crop. In addition, traditional cultivars widely used in oil production ('Picual', 'Hojiblanca', 'Cornicabra', etc.) or table olives ('Manzanilla', 'Gordal', etc.), have greater vigor, which is incompatible with SHD cultivation. Breeding programs have been undertaken to obtain olive varieties of high agronomic quality, reduced vigor and short juvenile period (JP) (De la Rosa et al., 2006; Hammami et al., 2021, 2011; León et al., 2007). Thus, low vigour cultivars, such as 'Sikitita', have been obtained which are adapted to this cropping system (Rallo et al., 2008). A reduction in the tree vigor was also observed in comercial cultivars by constriction of the trunk to obstruct the flow of sap in the xylem and phloem, although a reduction of yield was also observed (Tombesi and Farinelli, 2016). Likewise, water management through deficit irrigation can be an efficient horticultural practice to control vigor (Di Vaio et al., 2012). But as is done with almost all fruit trees, the use of dwarfing rootstocks seems to be the most reasonable system to reduce the vigor of a crop. The use of rootstocks to control the vigor of the grafted olive cultivar has been tested in several works (Baldoni and Fontanazza, 1990; Pannelli et al., 2002; Romero et al., 2014; Rugini et al., 2016), but currently there are no commercial dwarfing olive rootstocks have been identified that control the vigor of modern plantations.

The aim of this work was: i) the characterization of early vigor traits in different wild olive subspecies representing a wide range of genetic variability of the species *Olea europaea* in order to study the variability and heritability for these traits and the relationships between them; ii) to identify genotypes that could be potentially used as rootstocks to optimize intensive olive cultivation; iii) to use, as a proof of concept, genotypes identified as having low or very low vigor as rootstocks of the 'Arbequina' cultivar to assess their ability to further reduce the tree vigor.

Material and Methods

Plant material

For vigor traits measurements of field-grown wild olive genotypes, 88 genotypes belonging to 6 different subspecies (**Table 1**) were propagated as previously described in chapters 1 and 2 (Díaz-Rueda et al., 2020). 'Arbequina' and 'Picual' plants obtained by standard vegetative propagation of semi-hardwood stem cuttings were included for comparison. Homogeneous plants of each wild genotype were initially obtained from *in-vitro* seedlings germinated in Rugini medium (Rugini, 1984) without hormones. Uninodal segments were further micropropagated in 100 % strength Rugini medium (Rugini, 1984) supplemented with 1 mg/l zeatin. *In-vitro* explants were incubated in a room chamber under 16 h light photoperiod (34 µmol $m^{-2}s^{-1}$ intensity with light emitting diode (LED) 70 % red plus 30 % blue) at 25 ± 2 °C. Explants were grown up to 7−8 cm height and transferred for 21 days to rooting medium with auxins, consisting on 50 % strength Rugini medium supplemented with α-naphthalacetic acid (0.8 mg / l). Rooted seedlings were acclimatized *ex-vitro* for 3 weeks, transplanted to 1 L pots and grown under greenhouse conditions. Plants with 1 year in pot were transplanted into the field. Standard cultural practices were followed, with no pruning to allow free development of plants and irrigation by in-line drip to avoid water stress of plants. Vigor traits were recorded in two independent assays performed in different locations.

The first batch consisted of 43 genotypes, which were transplanted to the field at the experimental farm of IFAPA Centre "Alameda del Obispo", Cordoba (Spain) in May 2015 (**Table 1**). Plants were established at 4 × 2m spacing in a randomized design with 6 replicates per genotype. The second batch consisted of 49 genotypes that were set in the experimental farm Buitrago, Agrasur S.A. (37º 28´ 55.2´´ N, 5º 50´ 31.196´´ O; Ctra. Mairena del Alcor - Brenes, km 17. Brenes, Spain) in November 2019 (**Table 1**) at 4 × 2 m spacing in a randomized design with 6 to 8 replicates per genotype. Four genotypes were common to both assays to allow the possibility of normalization and comparisons between the two assays.

TABLE 1 | Origin and code of the wild olive genotypes of the SILVOLIVE collection used in this chapter.

		Mother tree				Number of Genotypes	
Subspecies	**Lineage**	**Variety**	**Natural Localization**	**Prospection**	**Acronym**	**First batch**	**Second batch**
europaea	*E1*	'Frantoio'	Tuscany, Italy	WOGB, Córdoba, Spain Acc. Number 80	FRA		1
		Unnamed oleaster	Cádiz Mountains, Spain	WOGB, Córdoba, Spain Acc. Number W45	ACZ	11	1
		Unnamed oleaster	Coria del Río, Seville, Spain	Coria del Río, Sevilla, Spain	ACO		1
		Unnamed oleaster	Marrakech Mountains, Morocco	Marrakech, Morocco	AMK	7	6
		Unnamed oleaster	Amskroud, Morocco	Amskroud, Morocco	AMS		5
	E2	Unnamed oleaster	Tamri, Morocco	Tamri, Morocco	TAM		5
		Unnamed oleaster	Aourir, Morocco	Aourir, Morocco	AOU		9
	E3	Unnamed oleaster	Sierra de Jaén, Spain	WOGB, Córdoba, Spain Acc. Number W69	AJA	6	
laperrinei x *europaea*	*E1-/1*	'Dhokar'	Tataouin zone, Tunisia	WOGB, Marrakech, Morocco Acc. Number Oct413	DHO		9
guanchica	*M-g1*	*guanchica*	Tenerife, Canary Islands, Spain	WOGB, Córdoba, Spain Acc. Number W49	GUA	9	1
cerasiformis	*M-c*	*cerasiformis*	Madeira Islands, Portugal	CEFE Montpellier, France Acc. Number Cer3	CER		2
maroccana	*M-m*	*maroccana*	Imouzzer, Morocco	Imouzzer, Morocco	MAR		2
cuspidata x *europaea*	*A*	*cuspidata*	Grahamstown, South Africa	CEFE Montpellier Acc. Number Gr3 & Gr5	CUS	1	4
	A	*cuspidata*	Kirstenbosch, South Africa	CEFE Montpellier, France Acc. Kirstenbosch	CEH	9	3
Total number of genotypes						43	49

To characterize vigor regulation by wild genotypes used as rootstocks of the cv. 'Arbequina', fourteen genotypes with different growth and vigor habits were selected (**Table 2**).

TABLE 2 | Vigor measurements of 1 year-old seedlings in pots. Data from Díaz-Rueda et al., 2020 (Table S6).

Category (Díaz-Rueda et al., 2020) Table S6	Variety	Height	Basal diameter	Shoot biomass	Leaves biomass	Total biomass	Total number nodes	Root biomass	Number Secondary	Root / Shoot Ratio
Very low vigor	**AMK21**	--	--	--	--	--	-	-	-	++
Very low vigor	**AMK6**	--	--	--	--	--	-	--	-	+
Very low vigor	**AMK5**	--	--	--	--	--	+/-	-	+	+
Very low vigor	**GUA8**	--	--	--	-	--	-	-	+/-	++
Low vigor	**AMK16**	-	-	--	--	--	+/-	-	-	--
Low vigor	**GUA9**	-	--	--	--	--	-	--	+/-	-
Low vigor	**AMK14**	-	+/-	+/-	-	+/-	+	+	-	-
Intermediate vigor	**AJA17**	+/-	--	+/-	+/-	-	+	--	+	--
Intermediate vigor	**ACO15**	+/-	-	-	-	-	+	-	++	+/-
Intermediate vigor	**CEH26**	+/-	+	+/-	-	-	+/-	--	-	-
High Vigour	**CEH20**	+	+/-	+	+	+/-	+/-	-	+/-	+
High Vigour	**CUS15**	+	+	+/-	-	--	+/-	--	+/-	--
Very High Vigour	**ARC**	++	++	++	++	++	++	++	+	-
Very High Vigour	**CUS13**	++	++	++	+/-	+/-	+/-	-	+/-	+/-

Self-grafted (ARB/ARB) and self-rooted (non-grafted 'Arbequina') plants were obtained by the company 'Viveros Sevilla S.A.' and included for comparisons. Wild rootstocks were obtained by *in-vitro* micropropagation and acclimatized *ex-vitro* in November 2016 as previously described Díaz-Rueda et al. (2020). Plants grown under greenhouse conditions for 1.5 years were grafted using the 'spike' technique. Grafted plants were kept in 3.5 L pots for one year and vigor measurements were initially made in the pot (Díaz-Rueda et al., 2020). Standard cultural practices were followed, including fertigation and pruning to allow optimal development of grafted plants. Plants were further established in the field at the experimental farm 'Buitrago' in November 2019 at 4 × 1.5 m spacing (**Figure 1**). The plantation was established in a randomized design with 6 to 10 plants per genotype. After 1 year growing in the field, different vigor parameters defined below were quantified.

FIGURE 1 | Plantation of olive trees in Buitrago farm consisting of 'Arbequina' grafted onto wild olive genotypes **(A)**. Detail of the graft section **(B)**.

Measurements

In the first batch of field-grown genotypes (Córdoba), early vigor parameters were recorded in Summer (July) and Winter (January) from 2015 to 2018. In Summer 2015 (S15) the following traits were measured: plant height (PH), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), maximum internode length (IM), total number of nodes (NN) and number of branches (NB), and length of the longest branch (BL). Plant height, diameter of the main axis at different heights and length of the longest branch measurements were repeated in Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18). Finally, canopy volume (CV) was also calculated in Winter 2018 from measurements of canopy height and width for final categorization of plant vigor.

In the second batch (Sevilla), a single measurement was done 1 year after transplanting, including tree height, canopy volume (CV) and trunk diameter at 5 cm (DG), 50 cm (DL) and 100 cm (DC) from ground. The canopy height (H) and width along two perpendicular axes (D1 and D2) were measured. The canopy volume (CV, formula 1) and the productive surface of the canopy (PS, formula 2) were calculated resembling its shape to that of a spherical cap:

$$\mathbf{CV} = 2/3\pi r^2 H = 2/3\pi D^2/4H = \pi D^2 H/6 \quad (1)$$

$$\mathbf{PS} = 2\pi rH = \pi DH \quad (2)$$

where D is the mean of the D1 and D2 values. The measurement of the trunk cross section was made with a caliper at 5 cm above the grafting point.

Statistical Analyses

Statistical analyses were performed using R statistical software version 4.0.2 (R Core Team 2021). Data was explored with histograms and boxplots. Broad sense heritability was estimated as $h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_E)$, where σ^2_G is the variance between genotypes and σ^2_E is the residual variance. Spearman's rank correlation between characters was calculated from average values per genotype. Differences between experimental groups were assessed by one-way analysis of variance (ANOVA). Post-hoc pairwise comparisons between all means were made with Tukey´s HSD (95 % confidence interval).

Results

Evaluation of early vigor traits in wild olive genotypes grown in the field

Overall, a wide variability was observed for all vigor traits measured at different time points (**Figure 2**).

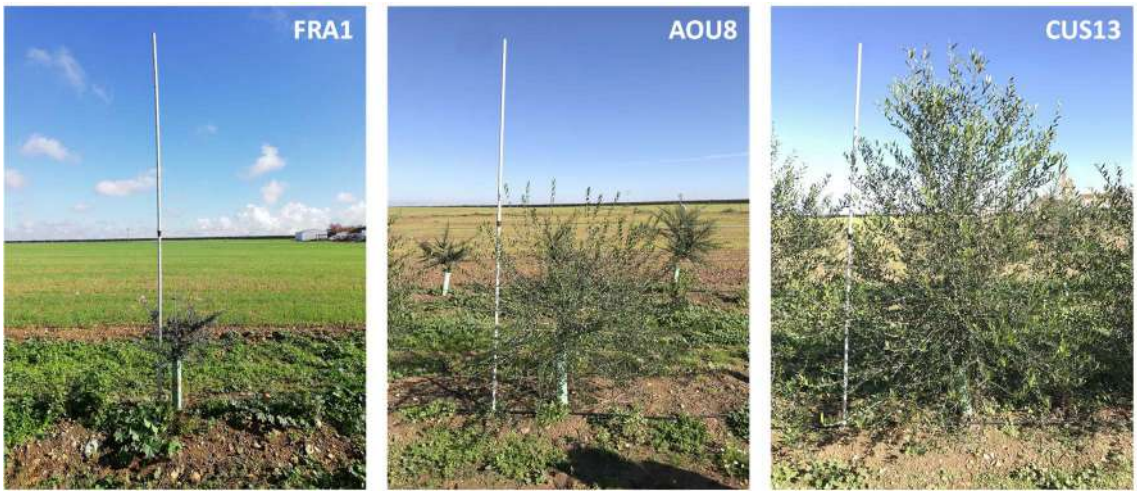


FIGURE 2 | Representation of genotypes with different contrasting vigor habits are shown: low vigor (FRA1 genotype); medium vigor (AOU8 genotype); and high vigor (CUS13 genotype). A 3-meter surveying rod is used as a length reference. Images correspond to the trial carried out at the "Finca Buitrago" experimental farm, Sevilla.

This variability was maintained throughout the evaluation period for some of them such as PH, or gradually increased for others such as DG (**Figure 3**). Average PH increased from 94 cm PH-S15 to 199 cm PH-W18, while DG increased from 6mm DG-S15 to 39mm TDG-W18.

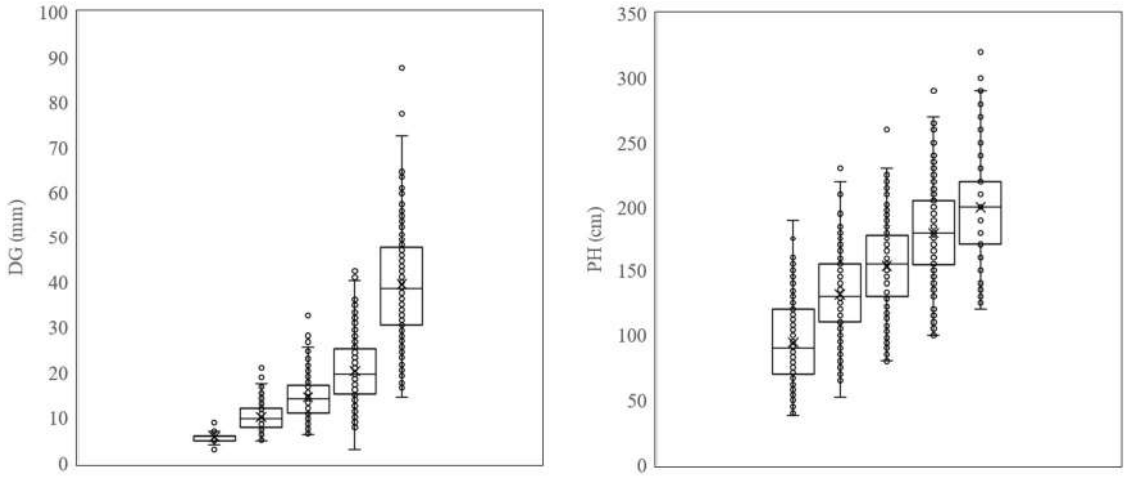


FIGURE 3 | Box and whisker plots for diameter of the main axis at ground level (DG) and plant height (PH) measured sequentially during the evaluation period (from left to right: Summer 2015, Winter 2016, Summer 2016, Winter 2017 and Winter 2018). Results obtained from the trial carried out at the 'Alameda del Obispo' experimental farm, IFAPA, Córdoba.

Significant differences between genotypes were obtained for all vigor traits, being the variance component between genotypes higher than the error variance for most of them (**Table 3**). As a consequence, high values of broad sense heritability were obtained for most of the evaluated vigor traits. It should be noted that the highest heritability values were obtained at the beginning of the evaluation period just after planting in the field, but heritability values decreased throughout the evaluation period for those traits measured sequentially due to a higher proportional increase of error variance as the plants grew older. For instance, heritability values for PH decreased from 0.93 in S15 to a half 0.47 in W18.

Average data per genotype indicated also a high variability for vigor traits among genotypes and a general reduction on the differences between genotypes as the plants grew older (**Table 4**). As an example, coefficient of variation values for PH decreased from 34.2 % in S15 to 15.1 % in W18. Only for diameter of the main axis measured at ground level (DG) variability among genotypes remained similar during the evaluation period, with coefficients of variation from 21.3 to 26.5 %. In any case, a general wide

variability and, therefore, possibilities for selection according to vigor characterization could be inferred for all the evaluated traits and periods.

TABLE 3 | Variance components and heritability of vigor traits measured during the evaluation period.

	PH-S15	**PH-W16**	**PH-S16**	**PH-W17**	**PH-W18**
Genotype	1017,05	697,88	649,20	764,21	762,25
Error	72,39	255,47	404,22	571,94	846,73
h2	0,93	0,73	0,62	0,57	0,47
	BL-S15	**BL-W16**	**BL-S16**	**BL-W17**	
Genotype	58,73	214,85	278,26	356,11	
Error	38,35	204,11	333,89	469,62	
h2	0,60	0,51	0,45	0,43	
	DG-S15	**DG-W16**	**DG-S16**	**DG-W17**	**DG-W18**
Genotype	1,45	5,77	13,39	25,07	80,98
Error	0,47	3,50	8,70	23,84	73,65
h2	0,75	0,62	0,61	0,51	0,52
		DL-W16	**DL-S16**	**DL-W17**	**DL-W18**
Genotype		4,30	7,72	16,92	44,58
Error		2,26	5,26	15,20	44,18
h2		0,66	0,59	0,53	0,50
			DC-S16	**DC-W17**	**DC-W18**
Genotype			7,56	13,70	43,04
Error			4,59	11,92	46,48
h2			0,62	0,53	0,48
	IM-S15				
Genotype	76,90				
Error	36,34				
h2	0,68				
	NN-S15				
Genotype	113,04				
Error	20,46				
h2	0,85				
	NB-S15				
Genotype	99,88				
Error	40,85				
h2	0,71				

Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB), Summer 2015 (S15). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18).

TABLE 4 | Mean, minimum, maximum and coefficient of variation for vigor traits (average values per genotype) measured during the evaluation period on 43 wild genotypes.

	PH-S15	PH-W16	PH-S16	PH-W17	PH-W18
Mean	93,8	130,9	153,1	179,3	199,5
Min	41,8	79,2	97,5	115,8	150,8
Max	163,7	197,5	219,5	253,8	270,0
CV (%)	34,2	20,7	17,5	16,3	15,1
	BL-S15	**BL-W16**	**BL-S16**	**BL-W17**	
Mean	16,9	40,9	57,0	82,2	
Min	4,5	13,8	19,7	28,6	
Max	43,2	88,2	103,8	117,8	
CV (%)	47,6	38,8	32,6	25,5	
	DG-S15	**DG-W16**	**DG-S16**	**DG-W17**	**DG-W18**
Mean	5,8	10,2	14,5	20,3	39,4
Min	3,5	5,9	8,1	9,9	18,7
Max	9,2	16,6	25,9	37,4	67,6
CV (%)	21,3	24,8	26,5	26,5	24,5
		DL-W16	**DL-S16**	**DL-W17**	**DL-W18**
Mean		7,3	10,4	15,1	30,1
Min		3,4	4,6	7,1	15,5
Max		11,5	16,6	26,5	44,6
CV (%)		29,7	28,3	29,2	23,8
			DC-S16	**DC-W17**	**DC-W18**
Mean			6,4	9,5	22,6
Min			1,3	2,3	8,4
Max			12,8	20,3	41,5
CV (%)			47,3	42,4	31,3

	IM-S15
Mean	37,9
Min	27,0
Max	63,8
CV (%)	23,9
	NN-S15
Mean	39,0
Min	20,2
Max	68,3
CV (%)	27,7
	NB-S15
Mean	23,7
Min	3,8
Max	48,7
CV (%)	43,9

Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB), Summer 2015 (S15). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18).

Spearman's rank analysis showed significant correlations among the different vigor parameters (**Figure 4**). In general, correlation values between different vigor traits measured at the same timepoint were higher than comparisons made at different timepoints. Similarly, for any given vigor trait correlation values were higher between consecutive timepoints than between more distanced measurements. However, some traits such as NB-S15 and PH-W18 showed always slight correlations with the other evaluated vigor parameters (r<0.60). Canopy volumen (CV), calculated in Winter 2018 showed significant correlation with all the evaluated vigor traits, the highest values (> 0.80) been observed with traits measured from Summer 2016, i.e. one year after planting in field. Among them, CV showed the highest correlation with diameter of the main axis measured at 50 cm from ground in Winter 2018 (DL-W18, r=0.901).

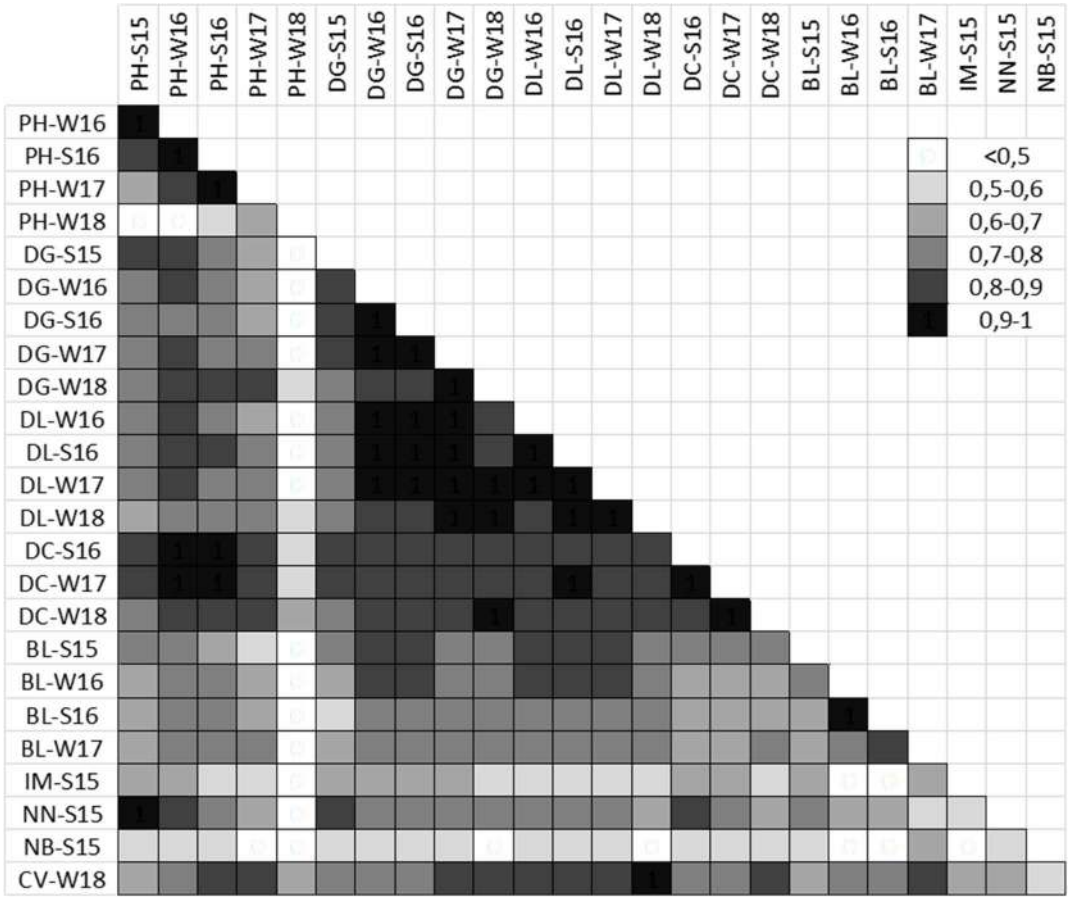


FIGURE 4 | Spearman's rank correlations between average vigor traits per genotype measured during the evaluation period. Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (TDG, TDL, TDC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB), Summer 2015 (S15). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18). Results obtained from the trial carried out at the 'Alameda del Obispo' experimental farm, IFAPA, Córdoba.

A classification of genotypes based on DL vs CV Scatterplots obtained from the trials carried out at 'Alameda del Obispo' Córdoba (**Figure 5**) and 'Finca Buitrago', Sevilla (**Figure 6**), showed some clear differences regarding the origin of the plant materials.

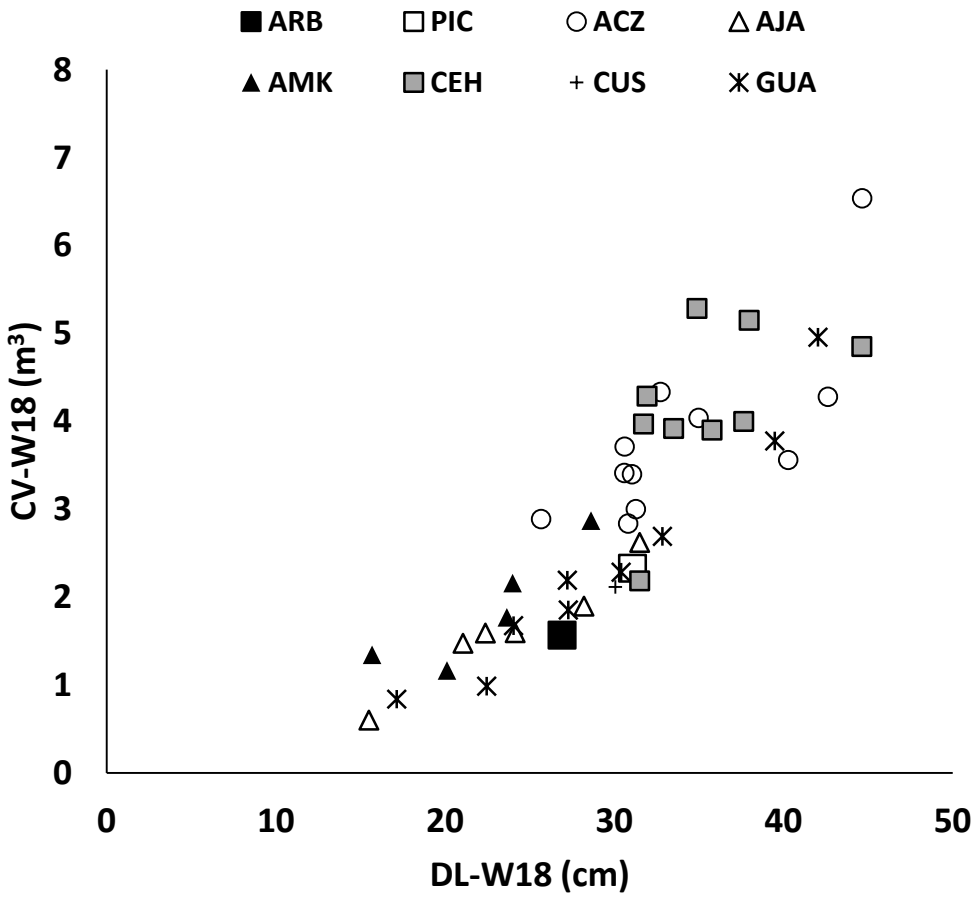


FIGURE 5 | Scatter plot of average data per genotype for diameter of the main axis at 50 cm from ground and canopy volume measured at the end of the experimental period in Winter 2018, TDL-W18 and CV-W18, respectively. Data are grouped according to the origin of the genotypes, including 'Arbequina' and 'Picual' as controls for comparison. Data were normalized by the mean and SD for each trait. Results obtained from the trial carried out at the "Alameda del Obispo" experimental farm, IFAPA, Córdoba.

Thus, genotypes from Marrakech (Morocco; AMK), Jaen (Spain; AJA) and Tuscany (Italy; FRA) provenances showed very low vigor (**Supplementary Figure S1**). The highest vigor was obtained in wild genotypes from Cadiz (Spain; ACZ) and genotypes from crosses *cuspidata* (from Grahamstown and Kirstenbosch, South Africa; CEH) x *europaea*. However, a wide variability was obtained in most cases, particularly in Guanchica (Canary Islands, Spain; GUA), Aourir (Morocco) and Tamri (Morocco) genotypes (**Figure 5 and 6**). Compared to 'Arbequina' and 'Picual' cultivars, included as controls for

comparison, some of the wild genotypes evaluated showed a clearly lower vigor at the end of the experimental period.

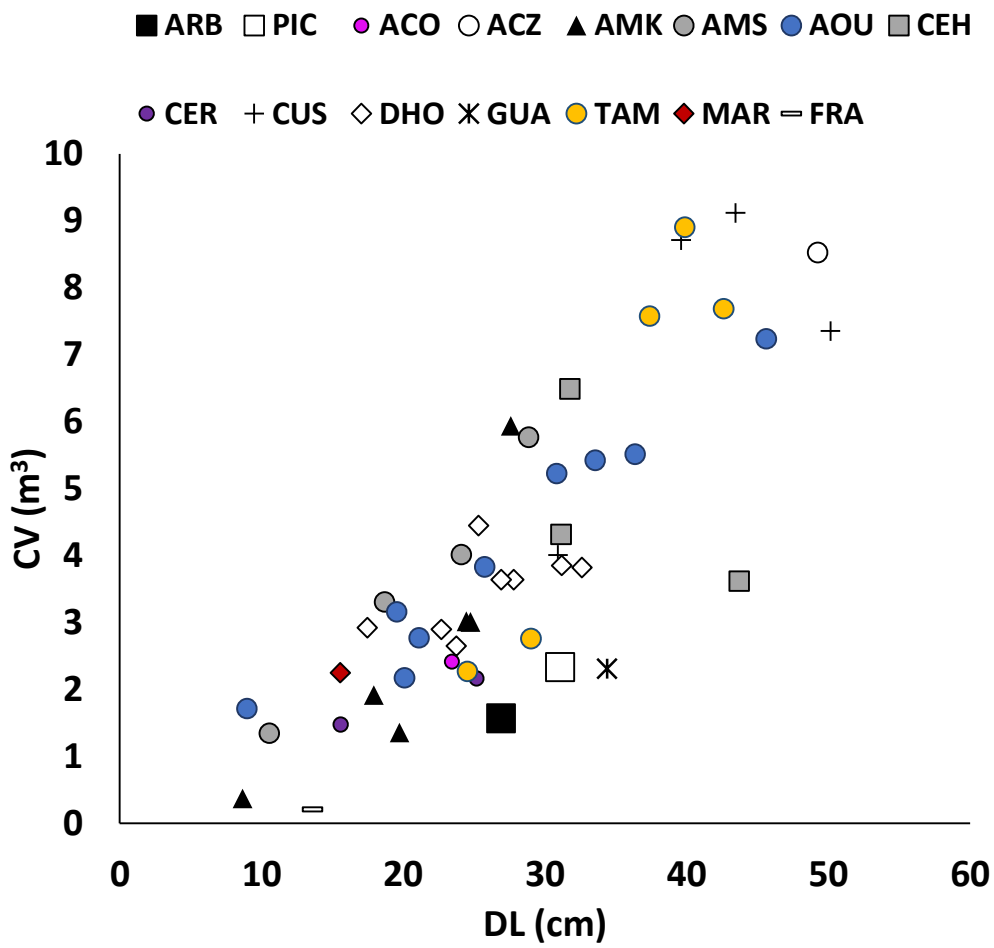


FIGURE 6 | Scatter plot of average data per genotype for diameter of the main axis at 50 cm from ground and canopy volume measured one year after transplanting. Data are grouped according to the origin of the genotypes, including 'Arbequina' and 'Picual' as controls for comparison. Data were normalized by the mean and SD for each trait. Results obtained from the trial carried out at the "Finca Buitrago" experimental farm, Seville.

Compared to 'Arbequina' and 'Picual' cultivars, included as controls for comparison, some of the wild genotypes evaluated showed a clearly lower vigor at the end of the experimental period (**Table 5**). These genotypes are good candidates as dwarfing rootstocks.

TABLE 5 | Significant differences between wild genotypes and cultivars 'Picual' and 'Arbequina' about vigor traits. Significant differences are marked with an asterik (p-value < 0.05).

	Height		Volume canopy		DS		DL		DC	
Genotypes	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual
MAR1	*	*				*				
DHO4	*	*								
AMS12	*	*								
FRA1	*	*		*	*	*	*	*		
AMK27	*	*			*	*	*	*		
CER3	*	*						*		*
AMS7		*				*	*	*		
MAR3		*								
AOU4		*						*		
TAM12		*								
AJA12										
AMK16		*								
AOU3								*		
AMK21						*		*		
AOU12										
AOU13										
GUA4		*								
AMK6								*		
DHO12A										
AOU8										
ARC										
ACZ8										
AJA6										
AMK34										
GUA3										
DHO6A										
AJA17						*		*		
CER1										
GUA1		*								
ACZ5										

AMK9								*		
CEH3										
GUA5										
TAM4										
ACZ3										
DHO8A										
AMK25										
GUA9								*		
CUS14										
ACZ4			*							
DHO1										
AMS11										
AOU7										
CEH2										
CUS3										
DHO10A								*		
AOU2										
AOU9			*							
ACZ7			*							
CEH8			*							
AMS17								*		
ARBEQUINA										
AOU10			*	*						
AMS15										
DHO10B										
DHO6B										
CEH17			*		*	*				
CEH26										
ACZ1										
ACZ10			*		*		*		*	*
GUA2										
DHO13A										
GUA8										
CEH9			*							
ACO15										
AMK12										

CEH25			*							
AJA4										
APR			*							
ACZ12			*							
TAM3			*	*						
CEH21			*							
TAM13			*							
PICUAL										
CEH6			*							
AJA18										
AMK14										
TAM9			*	*						
ACZ2			*	*						
AJA1										
GUA7			*		*				*	*
AMK5										
CUS15	*		*	*						
CEH20	*		*	*						
CEH24	*		*	*						
CUS13	*		*	*						
CUS12	*		*	*			*			
ACZ9	*		*	*	*	*	*		*	*
CEH7	*		*	*	*	*	*	*	*	*
GUA6	*		*	*	*	*	*		*	*

Genotypes showing lower values of vigor parateters in comparioson to 'Picual' and/or 'Arbequina' cultivars. Asterisks indicate statistically significant differences. DS = Diameter measured at ground level, DL = Diameter measured at 50 cm from ground, DC = Diameter measured at 100 cm from ground.

The canopy volume of the tested genotypes showed a significant positive correlation with the plant height (R^2 = 0.6124). ACZ9, which was previously defined as the the most vigorous genotype according to pot assays (chapter 2), had the highest canopy volume (6.53 m^3). Conversely, MAR1, previously catalogued as a genotype with very low vigor (chapter 2), showed the lowest canopy volume (0.0017 m^3). These results confirmed that vigor of olive plants can be successfully evaluated in potted seedlings.

The trunk diameter measured at three heights from the ground (DG, DL and DC) showed a positive correlation with the plant height (R^2 = 0.5191, R^2 = 0.5605, R^2 = 0.5309, respectively).

Vigor regulation through the use of wild olive rootstocks

A wide variability of plant vigor was observed in 'Arbequina' cultivars grafted onto wild olive rootstocks from different origins **(Figure 7).**

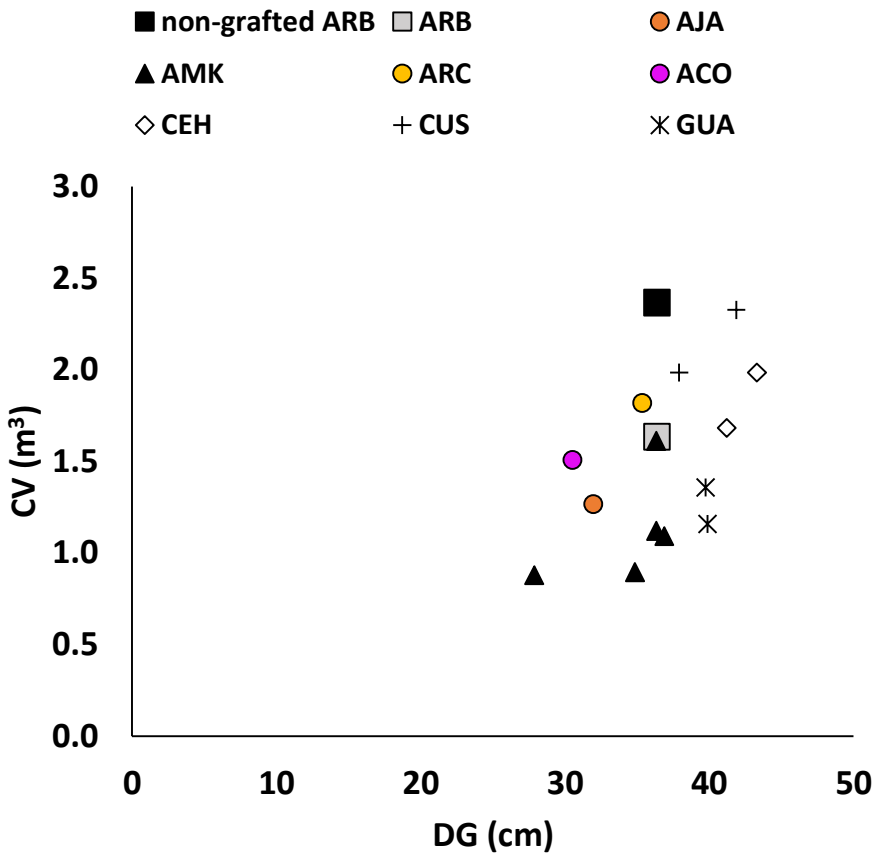


FIGURE 7 | Scatter plot of average data per grafted genotype for diameter of the main axis at 50 cm from ground and canopy volume measured one year after transplanting. Data are grouped according to the origin of the genotypes, including 'Arbequina' and 'Picual' as controls for comparison. Data were normalized by the mean and SD for each trait. Results obtained from the trial carried out at the 'Finca Buitrago' experimental farm, Seville.

Early vigor measurements of the 'Arbequina' scion grafted onto wild rootstocks are detailed in **Table 6**. When used as rootstocks, very low-vigor genotypes such as AMK6, AMK21 and GUA8, and low-vigor genotypes such as AMK14, AMK16 and GUA9 (**Table 2**), reduced the vigor of the 'Arbequina' scion (**Table 6**). Significant reductions were observed for different vigor parameters in comparison to the self-grafted

'Arbequina' (ARB/ARB). The very high-vigor genotypes CUS13 and ARC, as well as the high-vigor genotypes CEH20 and CUS15 increased the vigor of the self-grafted 'Arbequina' (ARB/ARB; **Table 6**). It must be noted that all rootstocks, with the exception of CEH20, reduced the vigor of the 'Arbequina' scion when compared to the self-rooted 'Arbequina' tree. However, only 7 out of 14 rootstocks reduced the tree vigour in comparison to the self-grafted 'Arbequina' (ARB/ARB), indicating that the grafting process itself reduces the vigor of the grafted scion.

TABLE 6 | Vigor parameters of 'Arbequina' plants grafted onto wild olive rootstocks in the field, 2.5 years after grafting.

Rootstock / Scion combination*	Height (m)	Canopy volume (m^3)	Productive surface (m^2)	Longest secondary shoot length (cm)	Suckers (Nº / plant)	Diameter at 5 cm above the graft (mm)
AMK14 / ARB	1.45 ± *0.00* *	0.88 ± *0.00* *	4.90 ± *0.00* *	55.0 ± *0.00* *	0.00 ± *0.00*	27.86 ± *0.00*
AMK6 / ARB	1.50 ± *0.06* *	1.09 ± *0.14* *	5.49 ± *0.46* *	60.5 ± *3.47* *	3.70 ± *1.31* *	36.85 ± *2.86*
ACO15 / ARB	1.51 ± *0.09* *	0.90 ± *0.16* *	4.96 ± *0.63* *	52.5 ± *4.19* *	1.57 ± *0.78* *	30.52 ± *2.56*
AMK21 / ARB	1.53 ± *0.04* *	0.89 ± *0.09* *	5.04 ± *0.31* *	52.2 ± *2.73* *	8.13 ± *0.67* *	34.83 ± *1.56*
GUA8 / ARB	1.53 ± *0.04* *	1.36 ± *0.05* *	6.25 ± *0.20* *	67.5 ± *2.09* *	1.00 ± *0.45* *	39.73 ± *1.25*
AMK16 / ARB	1.53 ± *0.05* *	1.12 ± *0.11* *	5.63 ± *0.39* *	60.3 ± *2.92* *	1.80 ± *0.70* *	36.30 ± *1.98*
GUA9 / ARB	1.54 ± *0.09* *	1.16 ± *0.22* *	5.73 ± *0.73* *	59.6 ± *3.73* *	0.67 ± *0.67*	39.85 ± *2.73*
ARB / ARB	**1.61 ± *0.11*** *	**1.63 ± *0.37*** *	**6.89 ± *1.10*** *	**68.0 ± *7.72***	**0.20 ± *0.20***	**36.36 ± *5.16***
AJA17 / ARB	1.61 ± *0.04* *	1.27 ± *0.13* *	6.19 ± *0.39* *	63.8 ± *2.39* *	0.00 ± *0.00*	31.96 ± *0.99*
ARC / ARB	1.65 ± *0.03* *	1.82 ± *0.09* *	7.52 ± *0.25* *	74.2 ± *2.20*	5.33 ± *1.20* *	35.34 ± *0.27*
AMK5 / ARB	1.69 ± *0.05* *	1.61 ± *0.11* *	7.12 ± *0.34* *	70.3 ± *2.43* *	7.90 ± *1.55* *	36.29 ± *1.49*
CEH26 / ARB	1.70 ± *0.00* *	1.68 ± *0.00* *	7.34 ± *0.00* *	70.0 ± *0.00*	2.00 ± *0.00* *	43.29 ± *0.00*
CUS15 / ARB	1.78 ± *0.08* *	1.98 ± *0.02*	8.15 ± *0.14* *	77.5 ± *5.00*	0.50 ± *0.50*	37.90 ± *3.29*
CUS13 / ARB	1.78 ± *0.13* *	2.33 ± *0.38*	8.81 ± *1.04*	83.8 ± *3.75*	2.50 ± *2.50* *	41.86 ± *0.35*
non-grafted ARB	**2.02 ± *0.03***	**2.37 ± *0.09***	**9.47 ± *0.23***	**78.3 ± *1.45***	**0.10 ± *0.10***	
CEH20 / ARB	2.13 ± *0.13*	2.76 ± *0.07*	10.50 ± *0.45*	83.8 ± *3.75*	2.50 ± *0.50* *	41.21 ± *6.22*

*Abbreviations: non-grafted ARB = self-rooted 'Arbequina' clones; ARB/ARB = self-grafted 'Arbequina' clones; AMK14/ARB = 'Arbequina' scion grafted on AMK14 rootstock; AMK6/ARB = 'Arbequina' scion grafted on AMK6 rootstock; ACO15/ARB = 'Arbequina' scion grafted on ACO15 rootstock; AMK21/ARB = 'Arbequina' scion grafted on AMK21 rootstock; GUA8/ARB = 'Arbequina' scion grafted on GUA8 rootstock; AMK16/ARB = 'Arbequina' scion grafted on AMK16 rootstock; GUA9/ARB = 'Arbequina' scion grafted on GUA9 rootstock; AJA17/ARB = 'Arbequina' scion grafted on AJA17 rootstock; ARC/ARB = 'Arbequina' scion grafted on ARC rootstock; AMK5/ARB = 'Arbequina' scion grafted on AMK5 rootstock; CEH26/ARB = 'Arbequina' scion grafted on CEH26 rootstock; CUS15/ARB = 'Arbequina' scion grafted on CUS15 rootstock; CUS13/ARB = 'Arbequina' scion grafted on CUS13 rootstock; CEH20/ARB = 'Arbequina' scion grafted on CEH20 rootstock. Values are means ± standard errors of 6-10 plants. Asterisks indicate significant differences with the non-grafted 'Arbequina'.

The AMK21 rootstock is the genotype that developed the greatest number of suckers in plants grafted with 'Arbequina' (8.13). The AMK5 and ARC rootstocks also exhibited a high production of suckers in plants grafted with 'Arbequina' (7.9 and 5.3 respectively). On the other hand, AMK14/ARB, AJA17/ARB and DHO10B/PIC were the combinations that produced the lowest number of suckers.

We observed that those rootstocks with greater trunk diameter transmitted greater vigor to the grafted variety (**Figure 8**).

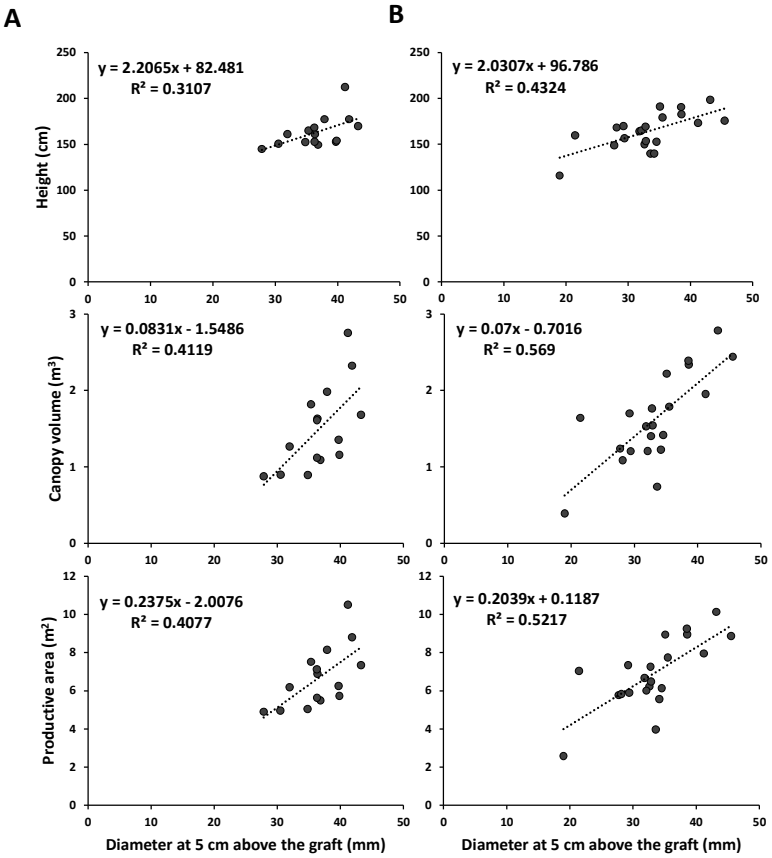


FIGURE 8 | Correlation between the trunk diameter at 5 cm above the graft and the height, the canopy volume and the productive area of 'Arbequina' (**A**) and 'Picual' scions (**B**).

Discussion

Only a few early-bearing, highly productive, low vigor and weeping habit cultivars such as 'Arbequina' and 'Arbosana' are considered suitable for high density olive hedgerow orchards (De la Rosa et al., 2007), which limits the potential expansion of this modern growing system. The use of dwarfing rootstocks has been widely used to control tree

vigor in many fruit species (Gregory et al., 2013), which allows design of specific growing systems and planting densities according to rootstock vigor (Cummins and Aldwinckle, 1983), but this approach has been scarcely studied in olive.

Seedlings of wild olive genotypes from the SILVOLIVE collection were previously characterized in pots, and classified into five categories based on their vigor (Díaz-Rueda et al., 2020). A wide intraspecific variability for vigor traits has been reported from the evaluation in cultivar collections (Del Río et al., 2005; Tous et al., 2005). A quite similar variability has been found in this work in genotypes from different subspecies and areas of origin. Thus, for instance, a coefficient of variation of 24.8 % was obtained from the analysis of trunk diameter ten years after planting in 61 cultivars evaluated at the World Olive Germplasm of IFAPA in Córdoba compared to 24.5 % obtained in 43 olive wild genotypes in this work. A high heritability and strong genetic effect could be also inferred from the evaluation of vigor traits in these cultivar collections. However, significant environmental influence has also been observed for some cultivars, which underlines the need of extensive testing in different environments for adequate selection. In fact, a high correlation has been reported between olive tree vigor and different soil and landscape characteristics such as thickness of the soil root zone, clay and cation exchange capacity and distance to mountain summit (Gálvez et al., 2004).

The whole results of vigor evaluation should be considered in terms of variability, heritability and correlation among them. A high correlation between 17 different vigor parameters was also observed in the characterization of olive progenies from crosses between different cultivars, which allows the selection of a group of them as the best growth descriptors based on their influence by parent genotype (Hammami et al., 2011). Moreover, a high influence of plant age on growth habit traits was also determined and early characterization at the time of planting in the field was suggested as the most appropriate age for evaluating seedling growth parameters (Hammami et al., 2011). Early vigor measurements at the seedling stage before planting in the field was also proposed in olive progenies as a tool for selection for short juvenile period (De la Rosa et al., 2006). In this work however, measurements taken early after planting showed high heritability and variability among genotypes, but lower correlation with final

characterization of the genotypes. The results obtained suggest that measurements taken from one year after planting could be considered more efficient for selection.

Among the wild genotypes evaluated in this work, some of them showed a clear lower vigor than 'Arbequina' at the end of the experimental period and could be considered potentially interesting dwarfing rootstocks. Future works should be carried out to test whether the differential vigor in the evaluated wild materials is extensible to the grafted cultivars of interest. Previous works testing single cultivars grafted on a range of potential interesting rootstocks indicate that the intrinsic vigor of some rootstock is transferred to the grafted cultivar. However, opposite results were also found in some cases, although it should be noted that explicit evaluation of vigor of the rootstock used was not carried out but based on previous literature (Romero et al., 2014; Troncoso et al., 1990). Similarly, different vigor control ability has been obtained for some rootstocks depending on the cultivar grafted, while a general dwarfing effect was observed for some others (Pannelli et al., 2002). These partly contradictory results suggest that further research is still necessary to guarantee adequate choice of rootstocks.

Again, the environmental conditions should be also carefully considered in testing. For instance, water availability showed a marked effect on growth and production parameters that obscure the potential rootstock effect in experiments with reciprocal scion/rootstock combination of three olive cultivars (Hernández-Santana et al., 2019; Lavee and Schachtel, 1999). Moreover, the effect of grafting itself has been showed to control vigor by reducing plant size of self-grafted genotypes (Rugini et al., 2016), although no effect of self-grafting has also been reported for some cultivars (Pannelli et al., 2002). Several mechanisms have been suggested as responsible of the dwarfing potential of olive rootstock, mainly related to the hydraulic architecture of the different scion/rootstock combinations (Nardini et al., 2006), as also reported for other fruit tree species (Cohen et al., 2007). The hydraulic characterization of the wild plant materials showing contrasting dwarf effect in our work could provide valuable information in future.

Theses wild olive genotypes were also used as rootstocks to evaluate the ability to transmit the trait of reduced vigour to the 'Arbequina' and 'Picual' varieties grafted onto them in controlled conditions (Díaz-Rueda et al., 2020). In this work, the ability of the rootstocks to transmit reduced vigour to the variety grafted onto them has been evaluated in the field. Nowadays, several studies have been performed for early selection of olive progenies in breeding programs using quantitative parameter of vigor such as plant height (De la Rosa et al., 2006), branch orientation, architectural types and association of plant height with architectural types (Hammami et al., 2021). Reciently, the unmanned aerial vehicle (UAV) methodology has been confirmed as a suitable method for the measurement of olive tree vigor parameters, which could be exploited to select potential cultivars for different growing systems (Rallo et al., 2020; Gómez-Gálvez et al., 2021; Torres-Sánchez et al., 2021). The identification of new cultivars with early bearing and high early yield are objetives of the breeding programs (De la Rosa et al., 2006; León et al., 2007). Olive breeding programs are based on cultivated olive germplasm (Hassani and Tombesi, 2008; Hammami et al., 2011; Rallo, 2014) and crosses between cultivars and wild genotypes (Klepo et al., 2014; Díaz-Rueda et al., 2020; León et al., 2020). The use of dwarfing rootstocks has been widely used to control tree size in many species of fruit trees (Gregory et al., 2013), allowing a design of growing in high-density planting systems based on rootstock vigor (Cummins and Aldwinckle, 1983), but this approach has been scarcely studied in olive.

In this study, the rootstock/scion combinations differed markedly in its influence on the scion growth. Specifically, rootstocks such as ACO15, AMK21, AMK6, and GUA8, identified as low vigor (Díaz-Rueda et al., 2020) with a dwarf type root system, reduced the growth of the scion, developing canopy volumes and canopy areas significantly smaller than those of plants onto high vigor rootstocks, such as CEH (e.g. CEH20 and CEH26) and CUS (e.g. CUS13 and CUS15) genotypes. In this sense, the vegetative growth of the different rootstock/scion combinations appeared to depend on genetically determined root system characteristics. Therefore, they could be considered as potential dwarfing rootstocks for its use in high-density olive hedgerow orchads. For example, ACO15/ARB plants developed a canopy volume that was three-fold lower than that of CEH20/ARB plants, and almost half than that of self-grafted 'Arbequina' (0.9

versus 2.76 m^3 and 0.9 versus 1.63 m^3, respectively; **Table 1**). Similar results were observed when grafting 'Picual' scions: ACO15/PIC plants developed a canopy volumen six-fold lower than CEH20/PIC plants, and three-fold lower than self-grafted 'Picual' plants (0.4 versus 2.4 m^3 and 0.4 versus 1.4 m^3, respectively; **Table 2**). Therefore, the canopy volume can be used as an intuitive measure to select rootstocks that reduce vigor to the grafted plant in the short theme. Theses rootstocks which reduced vigor can be selected for its use in high density plantation systems. These observations showed the role of roots in determining the dwarfing character observed in low-vigor plants. According to Rugini et al., (2016) and our results, the self-grafting could be particularly useful for reducing the canopy volume in plants, when a minimal plant reduction is necessary to establish high-density olive orchards. However, genotypes such as GUA9 (a low-vigor genotype) produced a high canopy volume in 'Picual' while self-grafted 'Picual' produced a vigor canopy volume similar to that cultivar grown on its own roots. Conversely, when GUA9 rootstook was grafted with 'Arbequina' showed lower canopy volume than that of the self-grafted 'Arbequina' and 'Arbequina' plants. Although the dwarfing effect observed in most genotypes were rootstock-dependent, some genotypes like GUA9 did not exhibit such a very clear effect.

The dwarfing effect might be caused by several mechanisms. Some authors have suggested that rootstock-induced dwarfing might arise from a perturbation of the water transport at the graft union (Soumelidou, 1994; Atkinson et al., 2003), caused by graft-incompatibility between the rootstock and scion union. Nardini et al. (2006) suggested that it is an effect of the hydraulic architecture of the different scion/rootstock combinations, caused by a reduction in root hydraulic conductance. The influence of the age of the plant at the time of growth parameters measurement has been observed (Gascó et al., 2007), who demonstrated that it is necessary at least 250 days after grafting for the hydraulic resistance of the graft union to drop to less than 20% of the hydraulic resistance of the plant. Moreover, the graft union represented only about 3% of the hydraulic resistance of the plant and the root system of olive plants represented between 60–70% of the whole plant one year after grafting (Nardini et al., 2006). In this sense, the continuity of the xylem system across the graft union was completely restored in the plants assayed in this work, which measured 2.5 years after grafting.

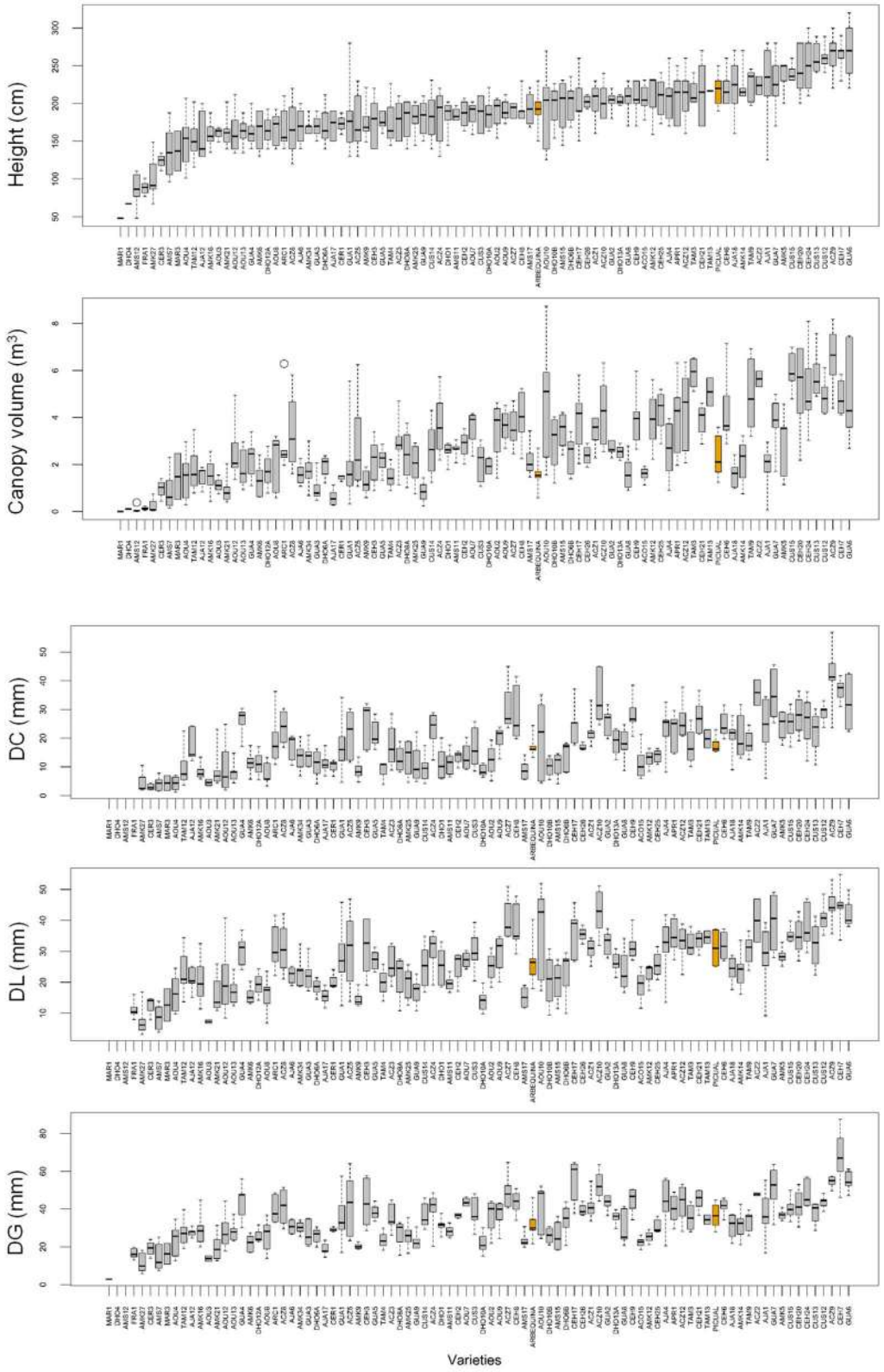
Trifilò et al., (2007) reported that dwarfing rootstocks that reduce the grafted plant size might cause some reduction of vessel diameter and length of vigorous scion. However, a previous study reported that the influence of rootstocks on the vigor of the grafted cultivar usually appears in the 4th year after budding (5th year of planting) (Tous et al., 2011). Our results agree with Nardini et al. (2006) and Lovisolo et al., (2007) for young-grafted olive plants, since dwarfing rootstocks induced smaller canopy volume (CV) when compared with scions grafted onto vigorous genotypes. However, a deeper study would be necessary to understand the mechanisms of dwarfing induction of the SILVOLIVE genotypes. Moreover, due to the possible scion size reduction by self-grafting of the elite cultivars, this approach could be interesting for produce semi-dwarf plants for intensive olive orchards.

A parameter of great interest for nurseries and farmers was the number of suckers produced from the base of the stem or from the roots. Suckers are shoots that rise from the main branches, trunk or roots of the rootstock. The identification of rootstocks with little or no ability to form these vegetative structures is a desirable trait. These vegetative structures spend a lot of energy, which could influenze negatively on the olive tree production. In agriculture, selective pruning is carried out to remove these undesirable shoots, generating a cost for the farmer. Therefore, obtaining a rootstock that does not generate suckers is a desirable trait. The removal of suckers is an operation that is not usual in hedgerow plantations, although differ significantly among plantation systems (Rallo et al., 2013). Some assayed rootstocks did not generate suckers during the experimental period in the field (e.g. AMK14 / ARB, AJA17 / ARB or DHO10B / PIC). However, other rootstocks such as AMK21, AMK5 or ARC showed a high sucker production. The AMK21 rootstock is one of the genotypes that most reduces vigor in the grafted varieties, but at the same time presents a high formation of suckers in both combinations (AMK21 / ‘Arbequina’ and AMK21 / ‘Picual’). In ‘Arbequina’ and ‘Picual’ varieties, an increase of suckers was observed in AMK5 and ARC, both rootstocks confer high vigor to ‘Arbequina’ and ‘Picual’ varieties so they are not good candidates as drafting rootstocks in high-density hedgerow orchards.

Conclusion

A wide variability for early vigor traits has been obtained from the evaluation of wild olive germplasms from different origins. In particular, low vigor and potentially interesting dwarfing effect was obtained in some sylvestris genotypes from Jaen (Spain) and Marrakech (Morocco) provenances and some genotypes from guanchica origin. Future works should be carried out to test whether the differences in growth traits between the studied plant materials are translated into vigor control of grafted materials and, moreover, whether it represents a general effect or it is limited to particular scion/rootstock combinations. Moreover, the potential effect of the rootstock on other important agronomic traits of the grafted cultivar, such as earliness of bearing, productivity, fruit and oil traits, should be also evaluated to guarantee adequate choice of rootstock. The use of rootstocks as a tool to control vigor in olive could open new possibilities for future olive growing, mainly regarding high-density hedgerow olive orchards where only a very limited number of cultivars have been successfully adapted up to now. Wild olive genotypes tested as rootstocks have a different influence on 'Arbequina' cultivar grafted onto them. Genotypes from AMK transmitted a reduced vigor to both varieties, being a source of genetic variability to be exploted as rootstock in HDH olive orchards. The most suitable rootstock to be used as dwarfing rootstock was ACO15, although a long juvenile period is developed in the variety grafted onto these dwarfing rootstocks in contrast to vigorous ones. These are preliminary studies in olive plants one year after planting. New analyses should be done in the future after longer time in the field.

Supplementary Materials



SUPPLEMENTARY FIGURE S1 | Vigor parameters of 90 genotypes from the SILVOLIVE collection in field conditions; Height (cm), canopy volume (m^3), Diameter of trunk at 5 cm (DG), 50 cm (DL) and 100 cm (DC) from ground. Data were grouped according to their height values, including 'Arbequina' and 'Picual' as controls for comparison. Data were normalized by the mean and SD for each trait.

References

Alcántara, E., Cordeiro, A. M., and Barranco, D. (2003). Selection of olive varieties for tolerance to iron chlorosis. *J. Plant Physiol.* 160, 1467–1472. doi: 10.1078/0176-1617-01013.

Aradhya, M. K., Preece, J., and Kluepfel, D. A. (2015). Genetic conservation, characterization and utilization of wild relatives of fruit and nut crops at the USDA germplasm repository in Davis, California. *Acta Hortic.*, 95–104. doi: 10.17660/ActaHortic.2015.1074.14.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi: 10.1016/j.cropro.2015.05.006.

Atkinson, C. J., Else, M. A., Taylor, L., and Dover, C. J. (2003). Root and stem hydraulic conductivity as determinants of growth potential in grafted trees of apple (*Malus pumila* Mill.). *J. Exp. Bot.* 54, 1221–1229. doi: 10.1093/jxb/erg132.

Baldoni, L., and Fontanazza, G. (1990). Preliminary results on olive clonal rootstocks behaviour in the field. *Acta Hortic.*, 37–40. doi: 10.17660/ActaHortic.1990.286.2.

Barazani, O., Westberg, E., Hanin, N., Dag, A., Kerem, Z., Tugendhaft, Y., et al. (2014). A comparative analysis of genetic variation in rootstocks and scions of old olive trees – a window into the history of olive cultivation practices and past genetic variation. *BMC Plant Biol.* 14, 146. doi: 10.1186/1471-2229-14-146.

Barranco, D., and Rallo, L. (2000). Olive Cultivars in Spain. *HortTechnology* 10, 107–110. doi: 10.21273/HORTTECH.10.1.107.

Besnard, G., El Bakkali, A., Haouane, H., Baali-Cherif, D., Moukhli, A., and Khadari, B. (2013). Population genetics of Mediterranean and Saharan olives: geographic patterns of differentiation and evidence for early generations of admixture. *Ann. Bot.* 112, 1293–1302. doi: 10.1093/aob/mct196.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9.

Cohen, S., Naor, A., Bennink, J., Grava, A., and Tyree, M. (2007). Hydraulic resistance components of mature apple trees on rootstocks of different vigours. *J. Exp. Bot.* 58, 4213–4224. doi: 10.1093/jxb/erm281.

Cummins, J. N., and Aldwinckle, H. S. (1983). "Breeding Apple Rootstocks," in *Plant Breeding Reviews*, ed. J. Janick (Boston, MA: Springer US), 294–394. doi: 10.1007/978-1-4684-8896-8_10.

De la Rosa, R., Kiran, A. I., Barranco, D., and León, L. (2006). Seedling vigour as a preselection criterion for short juvenile period in olive breeding. *Aust. J. Agric. Res.* 57, 477. doi: 10.1071/AR05219.

De la Rosa, R., León, L., Guerrero, N., Rallo, L., and Barranco, D. (2007). Preliminary results of an olive cultivar trial at high density. *Aust. J. Agric. Res.* 58, 392. doi: 10.1071/AR06265.

Del Río, C., Caballero, J. M., and García-Fernández, M. D. (2005). Vigor. Banco de Germoplasma de Córdoba. En: Variedades de olivo en España (Libro II: Variabilidad y selec- ción). In: Rallo, L., Barranco, D., Caballero, J.M., Del Río, C., Martín, A., Tous, J., Trujillo, I. (Eds.), Junta de Andalucía. M.A.P.A. y Ediciones Mundi-Prensa, Madrid.

Di Vaio, C., Marra, F. P., Scaglione, G., La Mantia, M., and Caruso, T. (2012). The effect of different vigour olive clones on growth, dry matter partitioning and gas exchange under water deficit. *Sci. Hortic.* 134, 72–78. doi: 10.1016/j.scienta.2011.11.001.

Díaz-Rueda, P., Franco-Navarro, J. D., Messora, R., Espartero, J., Rivero-Núñez, C. M., Aleza, P., et al. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Front. Plant Sci.* 11, 629. doi: 10.3389/fpls.2020.00629.

Díez, C. M., Moral, J., Cabello, D., Morello, P., Rallo, L., and Barranco, D. (2016). Cultivar and Tree Density As Key Factors in the Long-Term Performance of Super High-Density Olive Orchards. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01226.

Gálvez, M., Parra, M. A., and Navarro, C. (2004). Relating tree vigour to the soil and landscape characteristics of an olive orchard in a marly area of southern Spain. *Sci. Hortic.* 101, 291–303. doi: 10.1016/j.scienta.2003.12.001.

Gascó, A., Nardini, A., Raimondo, F., Gortan, E., Motisi, A., Lo Gullo, M. A., et al. (2007). Hydraulic kinetics of the graft union in different *Olea europaea* L. scion/rootstock combinations. *Environ. Exp. Bot.* 60, 245–250. doi: 10.1016/j.envexpbot.2006.10.011.

Gómez-Gálvez, F. J., Pérez-Mohedano, D., de la Rosa-Navarro, R., and Belaj, A. (2021). High-throughput analysis of the canopy traits in the worldwide olive germplasm bank of Córdoba using very high-resolution imagery acquired from unmanned aerial vehicle (UAV). *Sci. Hortic.* 278, 109851. doi: 10.1016/j.scienta.2020.109851.

Green, P. S. (2002). A Revision of *Olea* L. (Oleaceae). *Kew Bull.* 57, 91. doi: 10.2307/4110824.

Gregory, P. J., Atkinson, C. J., Bengough, A. G., Else, M. A., Fernández-Fernández, F., Harrison, R. J., et al. (2013). Contributions of roots and rootstocks to sustainable, intensified crop production. *J. Exp. Bot.* 64, 1209–1222. doi: 10.1093/jxb/ers385.

Hammami, S. B. M., León, L., Rapoport, H. F., and De la Rosa, R. (2011). Early growth habit and vigour parameters in olive seedlings. *Sci. Hortic.* 129, 761–768. doi: 10.1016/j.scienta.2011.05.038.

Hammami, S. B. M., León, L., Rapoport, H. F., and de la Rosa, R. (2021). A new approach for early selection of short juvenile period in olive progenies. *Sci. Hortic.* 281, 109993. doi: 10.1016/j.scienta.2021.109993.

Hannachi, H., Sommerlatte, H., Breton, C., Msallem, M., El Gazzah, M., Ben El Hadj, S., et al. (2009). Oleaster (var. *sylvestris*) and subsp. *cuspidata* are suitable genetic resources for improvement of the olive (*Olea europaea* subsp. *europaea* var. *europaea*). *Genet. Resour. Crop Evol.* 56, 393–403. doi: 10.1007/s10722-008-9374-2.

Hassani, D., and Tombesi, A. (2008). Vegetative growth of olive genotypes from a diallel cross. *Acta Hortic.*, 137–142. doi: 10.17660/ActaHortic.2008.791.17.

Hernández-Santana, V., Díaz-Rueda, P., Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., et al. (2019). Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress. *Front. Plant Sci.* 10, 291. doi: 10.3389/fpls.2019.00291.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516.

Klepo, T., De la Rosa, R., Satovic, Z., León, L., and Belaj, A. (2013). Utility of wild germplasm in olive breeding. *Sci. Hortic.* 152, 92–101. doi: 10.1016/j.scienta.2012.12.010.

Klepo, T., Toumi, A., De La Rosa, R., LeÓn, L., and Belaj, A. (2014). Agronomic evaluation of seedlings from crosses between the main Spanish olive cultivar 'Picual' and two wild olive trees. *J. Hortic. Sci. Biotechnol.* 89, 508–512. doi: 10.1080/14620316.2014.11513113.

Lavee, S., and Schachtel, J. (1999). Interaction of cultivar rootstock and availability on olive tree performance and fruit production. *Acta Hortic.*, 399–414. doi: 10.17660/ActaHortic.1999.474.83.

Lavee, S., and Zohary, D. (2011). The potential of genetic diversity and the effect of geographically isolated resources in olive breeding. *Isr J Plant Sci* 59, 3–13.

Lazović, B., Adakalić, M., Pucci, C., Perović, T., Bandelj, D., Belaj, A., et al. (2016). Characterizing ancient and local olive germplasm from Montenegro. *Sci. Hortic.* 209, 117–123. doi: 10.1016/j.scienta.2016.06.022.

León, L., de la Rosa, R., Barranco, D., and Rallo, L. (2007). Breeding for Early Bearing in Olive. *HortScience* 42, 499–502. doi: 10.21273/HORTSCI.42.3.499.

León, L., de la Rosa, R., Velasco, L., and Belaj, A. (2018). Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. *Front. Plant Sci.* 9, 232. doi: 10.3389/fpls.2018.00232.

León, L., Díaz-Rueda, P., Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. *Sci. Hortic.* 264, 109157. doi: 10.1016/j.scienta.2019.109157.

Nardini, A., Gasco, A., Raimondo, F., Gortan, E., Lo Gullo, M. A., Caruso, T., et al. (2006). Is rootstock-induced dwarfing in olive an effect of reduced plant hydraulic efficiency? *Tree Physiol.* 26, 1137–1144. doi: 10.1093/treephys/26.9.1137.

Ninot, A., Howad, W., Aranzana, M. J., Senar, R., Romero, A., Mariotti, R., et al. (2018). Survey of over 4, 500 monumental olive trees preserved on-farm in the northeast Iberian Peninsula, their genotyping and characterization. *Sci. Hortic.* 231, 253–264. doi: 10.1016/j.scienta.2017.11.025.

Pannelli, G., Rosati, S., and Rugini, E. (2002). The effect of clonal rootstocks on frost tolerance and on some aspects of plant behaviour in Moraiolo and S. Felice olive cultivars. *Acta Hortic.*, 247–250. doi: 10.17660/ActaHortic.2002.586.46.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1.

Rallo, L. (2014). Breeding Oil and Table Olives for Mechanical Harvesting in Spain. *HortTechnology* 24, 295–300. doi: 10.21273/HORTTECH.24.3.295.

Rallo, L., Barranco, D., Castro-García, S., Connor, D. J., Gómez del Campo, M., and Rallo, P. (2013). "High-Density Olive Plantations," in *Horticultural Reviews Volume 41*, ed. J. Janick (Hoboken, New Jersey: John Wiley & Sons, Inc.), 303–382. doi: 10.1002/9781118707418.ch07.

Rallo, L., Barranco, D., de la Rosa, R., and León, L. (2008). 'Chiquitita' Olive. *HortScience* 43, 529–531. doi: 10.21273/HORTSCI.43.2.529.

Rallo, P., de Castro, A. I., López-Granados, F., Morales-Sillero, A., Torres-Sánchez, J., Jiménez, M. R., et al. (2020). Exploring UAV-imagery to support genotype selection in olive breeding programs. *Sci. Hortic.* 273, 109615. doi: 10.1016/j.scienta.2020.109615.

Rius, X., and Lacarte, J. (2015). *La Revolución del Olivar: El Cultivo en Seto.* Barcelona: Agromillora.

Romero, A., Hermoso, J. F., and Tous, J. (2014). Olive rootstocks to control' Arbequina IRTAI18' clone vigour results from a second one comparative trial. *Acta Hortic.*, 577–584. doi: 10.17660/ActaHortic.2014.1057.74.

Rosati, A., Paoletti, A., Caporali, S., and Perri, E. (2013). The role of tree architecture in super high density olive orchards. *Sci. Hortic.* 161, 24–29. doi: 10.1016/j.scienta.2013.06.044.

Rugini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.* 24, 123–134. doi: 10.1016/0304-4238(84)90143-2.

Rugini, E., Silvestri, C., Ceccarelli, M., Muleo, R., and Cristofori, V. (2016). Mutagenesis and Biotechnology Techniques as Tools for Selecting New Stable Diploid and Tetraploid Olive Genotypes and Their Dwarfing Agronomical Characterization. *HortScience* 51, 799–804. doi: 10.21273/HORTSCI.51.7.799.

Soumelidou, K. (1994). The Anatomy of the Developing Bud Union and its Relationship to Dwarfing in Apple. *Ann. Bot.* 74, 605–611. doi: 10.1006/anbo.1994.1161.

Tombesi, S., and Farinelli, D. (2016). Trunk Constriction Effects on Vegetative Vigour and Yield Efficiency in Olive Tree (*Olea europaea* L.). *J Agr Sci Tech* 18, 1667–1680.

Torres-Sánchez, J., de la Rosa, R., León, L., Jiménez-Brenes, F. M., Kharrat, A., and López-Granados, F. (2021). Quantification of dwarfing effect of different rootstocks in 'Picual' olive cultivar using UAV-photogrammetry. *Precis. Agric.* doi: 10.1007/s11119-021-09832-9.

Tous, J., Romero, A., Hermoso, J. F., and Ninot, A. (2011). Influence of diferent olive rootstocks on growth and yield of the "Arbequina IRTA-I 18®" clone. *Acta Hortic.*, 315–320. doi: 10.17660/ActaHortic.2011.924.39.

Tous, J., Romero, A., and Plana, J. (2005). Vigor. Banco de Germoplasma de Cataluña. en: variedades de olivo en españa (libro II: variabilidad y selección). In: Rallo, L., Barranco, D., Caballero, J.M., Del Río, C., Martín, A., Tous, J., Trujillo, I. (Eds.), Junta de Andalucía. M.A.P.A. y Ediciones Mundi-Prensa, Madrid.

Tous, J., Romero, A., Plana, J., and Baiges, F. (1999). Planting density trail with '"Arbequina"' olive cultivar in Catalonia (Spain). *Acta Hort* 474, 177–179.

Trifilò, P., Lo Gullo, M. A., Nardini, A., Pernice, F., and Salleo, S. (2007). Rootstock effects on xylem conduit dimensions and vulnerability to cavitation of *Olea europaea* L. *Trees* 21, 549–556. doi: 10.1007/s00468-007-0148-9.

Troncoso, A., Liñán, J., Prieto, J., and Cantos, M. (1990). Influence of diferent olive rootstocks on growth and production of 'Gordal sevillana.' *Acta Hortic.*, 133–136. doi: 10.17660/ActaHortic.1990.286.26.

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., von Wettberg, E. J. B., et al. (2016). Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008.

Chapter 4. Wild olive genotypes as a valuable source of resistance to defoliating *Verticillium dahliae*

This chapter has been published as: **Díaz-Rueda, P**., Aguado, A., Romero-Cuadrado, L., Capote, N., Colmenero-Flores, J.M., (2021). Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating *Verticillium dahliae*. Frontier Plant Science 12:662060. https://doi.org/10.3389/fpls.2021.662060

frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 July 2021
doi: 10.3389/fpls.2021.662060

Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating *Verticillium dahliae*

Pablo Díaz-Rueda[1], Ana Aguado[2], Laura Romero-Cuadrado[2], Nieves Capote[2] and José M. Colmenero-Flores[1]**

[1] *Instituto de Recursos Naturales y Agrobiología, Spanish National Research Council (CSIC), Seville, Spain,* [2] *Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA) Centro Las Torres, Seville, Spain*

Resistance to the defoliating pathotype of *Verticillium dahliae* has been evaluated in a pool of 68 wild genotypes of olive belonging to the SILVOLIVE collection. Resistance was evaluated by assessing symptom severity using a 0–4 rating scale, estimating the relative area under the disease progress curve (RAUDPC), determining the percentage of dead plants (PDP), and measuring the evolution of morphological parameters in inoculated plants over time. In addition, the density levels of *V. dahliae* in the stem of root-inoculated genotypes have been quantified by means of quantitative real-time PCR at 35 and 120 days after inoculation (dai). Fifteen genotypes (22%) were cataloged as resistant to *V. dahliae* (i.e., disease parameters did not significantly differ from those of the resistant cultivar Frantoio, or were even lower). Resistant genotypes are characterized by presenting fewer symptoms and a lower amount of *V. dahliae* DNA at 120 dai than at 35 dai, indicating their ability to control the disease and reduce the density of the pathogen. The rest of the evaluated genotypes showed variable levels of susceptibility. Overall analysis of all genotypes showed high correlation between symptomatology and the amount of *V. dahliae* DNA in the stem of inoculated genotypes at 120 dai, rather than at 35 dai. However, correlation at 120 dai was not observed in the set of resistant genotypes, suggesting that resistance to defoliating *V. dahliae* in olive is based on the occurrence of different mechanisms such as avoidance or tolerance. These mechanisms are valuable for designing breeding programs and for the identification of target genes and resistant rootstocks to better control Verticillium wilt in the olive grove.

Keywords: SILVOLIVE, Verticillium wilt, resistance, *Olea europaea* subspecies, tolerance, olive crop, qPCR

OPEN ACCESS

Edited by:
Sabrina Sarrocco, University of Pisa, Italy

Reviewed by:
David Ruano-Rosa, Instituto Tecnológico Agrario de Castilla y León, Spain
Olere Francis Emeriewen, Julius Kühn Institute (JKI), Germany

****Correspondence:***
Nieves Capote
marian.capote@juntadeandalucia.es
José M. Colmenero-Flores
chemacf@irnase.csic.es

Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *31 January 2021*
Accepted: *31 May 2021*
Published: *01 July 2021*

Citation:
Díaz-Rueda P, Aguado A, Romero-Cuadrado L, Capote N and Colmenero-Flores JM (2021) Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating Verticillium dahliae. Front. Plant Sci. 12:662060. doi: 10.3389/fpls.2021.662060

INTRODUCTION

Olive (*Olea europaea* L) is one of the most cultivated woody crops in the world, having high socio-economic importance in all olive-producing areas (Blázquez-Martínez, 1996; Civantos, 2004). Verticillium wilt is considered the most important soilborne disease affecting olive (Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011), causing severe losses to growers, nurseries and olive industries (Jiménez-Díaz et al., 2012). The causal agent, *Verticillium dahliae* Kleb., is a soilborne fungus that penetrates the roots of the host plant and colonizes its vascular system. The combined action of the fungal colonization and the defense response of the infected plant, e.g., formation of gels, gums, and tyloses (Fradin and Thomma, 2006;

Introduction

Olive (*Olea europaea* L.) is one of the most cultivated woody crops in the world, having high socioeconomic importance in all olive-producing areas (Blázquez-Martínez, 1996; Civantos, 2004). Verticillium wilt is considered the most important soilborne disease affecting olive (Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011), causing severe losses to growers, nurseries and olive industries (Jiménez-Díaz *et al*., 2012). The causal agent, *Verticillium dahliae* Kleb., is a soilborne fungus that penetrates the roots of the host plant and colonizes its vascular system. The combined action of the fungal colonization and the defense response of the infected plant, e.g., formation of gels, gums, and tyloses (Fradin and Thomma, 2006; Yadeta and Tomma, 2013) provokes the obstruction of xylem vessels, reducing the transport of water and nutrients and producing wilt symptoms, defoliation, dieback of shoots, and the eventual death of the whole plant (Gharbi *et al*., 2016). In addition, *V. dahliae* produces mycotoxins that can seriously damage the metabolism of the plant and contribute to plant wilting (Fradin and Thomma, 2006; Luo *et al*., 2014). Resistance has been evaluated on the basis of external symptoms, vascular browning, and isolation of the fungus from plant tissues (López- Escudero *et al*., 2004; Martos-Moreno *et al*., 2006; Colella *et al*., 2008; Markakis *et al*., 2009; Bubici and Cirulli, 2012; García-Ruiz *et al*., 2014) even in naturally infected soils (Trapero *et al*., 2013). In addition, the qPCR quantification of *V. dahliae* in inoculated plants has been used to determine the resistance of different olive genotypes (Mercado-Blanco *et al*., 2003; Markakis *et al*., 2009; Jiménez-Fernández *et al*., 2016).

Verticillium dahliae isolates from olive can be classified into defoliating (D) and non-defoliating (ND) pathotypes, depending on the virulence and the symptoms caused in the infected plant (Schnathorst and Sibbett, 1971; Bejarano-Alcázar *et al*., 1996). The D pathotype is highly virulent and is able to cause the drop of leaves and the complete death of the tree (Sánchez- Hernández *et al*., 1998). The ND pathotype is mildly virulent and does not cause defoliation of the tree. Although ND isolates can eventually cause wilting and death of susceptible cultivars, remission of symptoms with time has been observed (López-Escudero and Mercado-Blanco, 2011). The D pathotype has

progressively spread and displaced the ND pathotype in most olive cropping areas in southern Spain, severely affecting olive production yields (López-Escudero *et al*., 2010; Jiménez-Díaz *et al*., 2011, 2012; Areal and Riesgo, 2014).

Besides having a wide range of host species, including many common crops such as sunflower and cotton (Hiemstra and Harris, 1998; Pegg and Brady, 2002), *V. dahliae* can also asymptomatically colonize other plant species and persist in organic plant residues (Bhat and Subbarao, 1999; Pegg and Brady, 2002). In addition, the fungus is well adapted to long-term survival in soil because it can form resistant structures named microsclerotia (Wilhelm, 1955). All these properties increase the inoculum density in the soil and the dispersion of the pathogen, making the use of crop rotation an unwise strategy to control Verticillium wilt. In recent years, changes in cropping practices including drip irrigation and high-density plantings have also influenced the worsening of the phytosanitary status of olive crops (López-Escudero and Blanco-López, 2005; Villalobos *et al.,* 2006; Pérez-Rodríguez *et al*., 2015). All these factors, joined to the inefficacy of chemical control, make eradication of *V. dahliae* a very difficult task in olive plantations (López-Escudero *et al*., 2004; Hegazi *et al*., 2012; Trapero *et al.*, 2013; García-Ruiz *et al.,* 2014). Because single control measures are generally ineffective, integrated approaches are frequently implemented (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020). Cultural methods e.g., control of irrigation, avoiding rotation with susceptible crops, and pruning of branches before defoliation, can contribute to the decrease of the density of inoculum in the soil. Soil solarization, the application of organic amendments to the soil (Varo-Suárez *et al*., 2018), and the use of biological control agents are sustainable and promising control strategies (Areal and Riesgo, 2014; Gómez- Lama Cabanas *et al*., 2018; Azabou *et al*., 2020; Boutaj *et al*., 2020; Castro *et al*., 2020; Mulero-Aparicio *et al*., 2020). Preventive or curative chemical fungicides can only partially control the disease (Tjamos, 1993; Fradin and Thomma, 2006; Tsror, 2011; Gómez-Gálvez *et al*., 2020). Biotechnological approaches such as the preventive detection of *V. dahliae* by using specific and highly sensitive molecular methods such as quantitative real-time PCR (qPCR), allows the identification of both healthy plant material and free-pathogen pre-planting soils.

The use of resistant cultivars is probably the most effective and sustainable strategy for the control of Verticillium wilt disease. However, most of the commercial cultivars used in Spain (including the most used cultivars 'Picual', 'Hojiblanca', 'Cornicabra', and 'Arbequina') are susceptible or highly susceptible to *V. dahliae* (López-Escudero *et al*., 2004; Hegazi *et al*., 2012; Trapero *et al*., 2013, 2015; García-Ruiz *et al*., 2014). The few available resistant cultivars such as Frantoio are not used in olive production because they have lower agronomic quality. However, their use as rootstock has demonstrated to confer resistance to *V. dahliae* to the grafted variety (Porras-Soriano *et al*., 2003; Bubici and Cirulli, 2012). However, this resistance is slightly broken in soils with moderate and high inoculum density of *V. dahliae* (Trapero *et al.*, 2013) or is not durable in the long term in highly infected soils (Valverde *et al*., 2021). Therefore, new genotypes with long-lasting resistance to *V. dahliae* are needed as cultivars or rootstocks for adequate control of Verticillium wilt in olive plantations. Breeding programs have been developed with the aim of identifying resistant cultivars (Arias-Calderón *et al*., 2015a,b,c; Serrano *et al*., 2021) although satisfactory planting material with appropriate agronomic traits and longterm resistance are not available yet. Several breeding cycles could be necessary to obtain new cultivars with improved agronomic performance. Finally, the use of resistant cultivars (Porras-Soriano *et al.,* 2003; Bubici and Cirulli, 2012) or wildolive genotypes (Arias-Calderón *et al*., 2015c; Jiménez-Fernández *et al.*, 2016) as rootstocks has emerged as a feasible alternative for getting *V. dahliae* resistance, although a limited number of genotypes have been evaluated.

The SILVOLIVE collection comprises an extensive number of wild genotypes belonging to all the known subspecies of *Olea europaea*, providing a natural source of genetic variability with high potential to be used for breeding and as rootstocks of commercial varieties (Díaz-Rueda *et al.*, 2020). When used as rootstocks, different genotypes have demonstrated to regulate morphological parameters of the grafted scion and could potentially provide the grafted cultivar with resistance to *V. dahliae*. Taking advantage of the genetic and phenotypic variability of the SILVOLIVE collection, our objectives were (i) to evaluate the resistance to *V. dahliae* of 68 artificially inoculated wild olive genotypes and three control reference cultivars, the highly susceptible 'Picual', the moderately susceptible 'Arbequina' and the resistant 'Frantoio'; (ii) to

determine by qPCR technology if quantification of fungal DNA in the basal stem of inoculated plants is a reliable tool to predict resistance to *V. dahliae* and what is the optimal infection time to assess the degree of susceptibility according to DNA quantification (35 or 120 dai); and (iii) to go in depth into the mechanisms of resistance/tolerance based in the quantification of both fungal density and the development of symptoms at different times after inoculation.

Materials and Methods

Plant Material

Plant material consisted in 6 months-old, 20–30 cm high olive plantlets belonging to the SILVOLIVE collection, which comprises genotypes from all known subspecies of *Olea europaea* (*europaea*, *laperrinei*, *cuspidata*, *cerasiformis*, *guanchica*, and *maroccana*) (Díaz-Rueda *et al*., 2020). A total of 71 genotypes were evaluated including 68 wild-olive genotypes, and three commercial varieties ('Frantoio', 'Arbequina', and 'Picual') used as highly resistant, moderately susceptible, and extremely susceptible reference controls, respectively (**Table 1**). Wild-olive genotypes were micropropagated from the *in-vitro* SILVOLIVE germplasm collection (Díaz-Rueda *et al*., 2020). Seedlings were cut into uninodal segments and incubated in Rugini medium (Rugini, 1984) supplemented with 1 mg/L zeatin in a growth chamber with 16 h light photoperiod (34 µmol m^{-2} s^{-1} intensity with 70% red: 30% blue light-emitting diodes, LEDs) at 25 ± 2 ºC. For whole plant regeneration, grown shoots were transferred to rooting medium (50% strength Rugini medium) supplemented with α-naphthalacetic acid (0.8 mg/L). Rooted seedlings were acclimatized *ex-vitro* for 3 weeks, transplanted into 1-L pots and then grown under common greenhouse conditions (Díaz-Rueda *et al*., 2020).

Fungal Isolate and Inoculum Production

The defoliating *V. dahliae* pathotype VD-117, obtained from the culture collection of IFAPA research center, Córdoba (Spain) (Bejarano-Alcázar *et al*., 1996), was used in the inoculation tests for resistance assessment. Conidial suspensions for resistance tests were prepared by transferring five 8 mm-Potato Dextrose Agar (PDA) discs of actively

growing mycelium of VD-117 isolate to flasks containing 100 mL potato dextrose broth (PDB) and incubated at 150 rpm in an orbital shaker at 24 ºC in the dark for 7 days. The conidial suspension was filtered through four layers of sterile cheesecloth and adjusted to 1 x 10^7 conidia/ml with sterile distilled water using a hemocytometer.

Inoculation of Olive Plants

For assessing resistance to *V. dahliae* in the 68 wild-olive genotypes (**Supplementary Figure 2**), four experiments were carried out over four different years: 2016, 2017, 2018, and 2019. The reference cultivars were repeated in the four trials, and a number of wild genotypes have been repeated in, at least, two different assays. Thus, ACZ1 and ACO15 have been repeated in three different assays while APR, ACZ7, ACZ9, ARC, GUA8, GUA9, CUS6, GUA5, AMK16, AMK5, AMK6, and DHO6A have been repeated in two different assays. Repetitions of both reference cultivars and wild-type genotypes in different trials and years have shown consistent results. Twenty-four plants were inoculated for each genotype. Plant roots were washed under abundant tap water to remove the substrate. Secondary roots were cut in 4–5 positions and the whole bare root system was dipped in the *V. dahliae* conidial suspension for 15 min. Control plants were treated by immersion of roots in PDB:sterile distilled water (1:1, v:v). Each plant was individually transplanted into 1 L pots containing sterilized 2:1 silt:peat moss (v:v). A completely randomized blocks design with four blocks and six plants (repetitions) per block for each genotype was used. Plants were maintained at 24/18 ºC and 60/40% relative humidity (day/night) in a greenhouse with a daily 14-h photoperiod supplemented with fluorescent illumination (360 mmol/m^2). Plants were watered as needed, and fertilized weekly with Hoagland's nutrient solution (Hoagland and Arnon, 1950). The most resistant genotypes from each pathogenicity test were re-evaluated in the following experiment, so that susceptible and highly susceptible genotypes were evaluated once, whereas moderately susceptible and resistant genotypes were evaluated twice.

Symptoms Assessment

Symptoms of aerial organs were evaluated on each plant every 2 weeks, starting 35 days after inoculation. Symptoms were registered following a 0–4 rating scale according to the percentage of Maximum Intensity Symptoms (MIS): chlorosis, leaf curl, stunting, leaf

and shoot necrosis or defoliation: 0 = 0% MIS or no symptoms; 1 = 25% MIS; 2 = 50% MIS; 3 = 75% MIS; 4 = 100% MIS or dead plants. At the end of the experiment, the following disease parameters were estimated from these scale values: (i) the relative area under the disease progress curve (RAUDPC), calculated for each cultivar considering its percentage with regard to the maximum possible value that could be reached in the period of assessment, based on the calculation formula according to (Campbell and Madden, 1990):

$$RAUDPC = \left[\sum_{i=1}^{n}\left(\frac{S_i + S_{i-1}}{2}\right)\Delta t\right]\left[\frac{100}{S_{max}T}\right] \quad (1)$$

where Si = mean severity of the experimental unit in the observation i; 4t = the number of days between observations; Smax = maximum disease rating (=4); T = experimental period in days (=120); n = number of observations; (ii) the final mean severity of symptoms (FMS), calculated according to López-Escudero *et al*. (2007); (iii) the percentage of dead plants (PDP) from the total of inoculated plants. Using all these parameters, the resistance level (RL) of each genotype was determined according to (López-Escudero *et al.,* 2007): ES = extremely susceptible; S = susceptible; MS = moderately susceptible; R = resistant. In assay 3, morphological parameters such as the number of nodes, the accumulated length of branches, and the plant height of each plant were also measured over time at 0, 35, 50, 70, 85, 100, and 120 dai. The relative growth rate (RGR) was calculated as the number of new nodes per day.

Quantification of Verticillium dahliae in Plant Tissues by Real-Time PCR

Quantification of *V. dahliae* levels in the inoculated plants was carried out by qPCR at two times, 35 and 120 dai, using half of the inoculated plants for each time of analysis (three inoculated plants/block and four blocks, in a total of 12 plants per analyzed time). Plants were removed from the pots and two thirds of the stem, corresponding to the basal portion, was deprived of leaves, and immediately frozen at -80 ºC until analysis. Stems of plants from the same experimental block were grouped in a composite sample, giving rise to four biological replicates per treatment and harvesting time. Samples were ground in a mortar in presence of liquid nitrogen until getting a fine powder. Total DNA

was extracted using the Isolate II Plant DNA Kit (Bioline, London, United Kingdom) following the manufacturers' instructions. DNA concentration was accurately determined in triplicate measurements by using a fluorescent spectrophotometer (ModulusTM II Microplate Multimode reader, Turner Biosystems, United States). PCR reactions amplifying an intergenic spacer of *V. dahliae* previously characterized (Bilodeau *et al*., 2012) were performed in a 20 mL final volume, in 96-well plates in a CFX Connect thermocycler (Bio-Rad). The reaction cocktail contained 1x SensiMix (SensiMixTM Probe Kit, Bioline), 1 mM each forward and reverse primers (Bilodeau *et al*., 2012), 5 mM TaqMan probe labeled with 6'FAM fluorescein (Bilodeau *et al*., 2012), 0.1 mg/mL BSA, and 3 mL of extracted DNA adjusted to a concentration of 15 ng/mL. Amplifications were carried out at 95 ºC for 10 min, and 55 cycles of 15 s at 95 ºC and 30 s at 62 ºC. For normalization of DNA loading, a conserved region of the plant housekeeping cytochrome oxidase gene (*cox*) was amplified in a parallel qPCR reaction with the same conditions as those used for *V. dahliae* amplification (Garrido *et al*., 2009). The standard curve for *V. dahliae* quantification was obtained through serial dilutions of genomic DNA from *V. dahliae* VD- 117 (10 ng to 1 fg) in a fixed background of plant DNA (20 ng/ml) obtained from healthy olive stems. *V. dahliae* DNA was extracted from 0.1 g VD-117 mycelium grown for 10 days in PDA medium, using the Isolate II Plant DNA Kit, as described above. The standard curve for *cox* quantification was obtained through serial dilutions of DNA from nontreated olive plants (10 ng to 0.1 pg). The relative amounts of *V. dahliae* and *cox* DNA in the inoculated olive plants were obtained by extrapolation of cycle threshold value from the respective standard curves through the CFX Manager software (Bio-Rad). The threshold position of the DNA standard curves generated from different plates was manually fixed at the same position for all treatments and experiments for better comparison (Vaerman *et al*., 2004). The efficiencies of the reactions were calculated from the slope of the standard curves according to the following formula: $E = 10^{(-1/slope)}$. Four biological replicates (one DNA extractions x each block x each time) and three technical replicates (PCR reaction) of standards, samples and DNA template-free controls were used. Results were expressed as means ± standard errors of the *V. dahliae*:*cox* ratios (ng *V. dahliae* DNA · ng plant DNA^{-1}), previously defined as mean normalized quantity (MNQ) in Garrido *et al*. (2009). Quantifications were also calculated as ng of *V. dahliae* DNA per ng of plant DNA, and

similar quantitative results were obtained than when using *cox* normalization (data not shown).

Statistical Analysis and Normalization

Statistical analyses were performed with Statistix 9.0 (Analytical Software, Tallahassee, FL, United States). Data from varieties that were repeated twice were subjected to analysis of variance (ANOVA) using the assays as a factor. Where significant differences were not detected, data were pooled for the calculation of the means. For those varieties repeated with differences in their results, the chosen data was that with the highest severity. Means of FMS, RAUDPC and DNA content at 120 dai were compared between each genotype and the means of 'Frantoio', 'Arbequina', and 'Picual' reference controls, respectively, by the LSD test at $P = 0.05$. Percentage data were previously transformed by arcsine $(Y/100)^{1/2}$. Linear regression analyses were performed for estimating relationships between biometric parameters and FMS or RAUDPC in inoculated plants at 120 dai, respectively.

Results

Development of Symptoms in Inoculated Wild Olive Genotypes

In preliminary assays, not reported here, quantification of *Verticillium* DNA content and symptoms was carried out at times shorter than 120 dai. We noticed that, in order to obtain clear results on the resistance/tolerance mechanisms displayed by olive wild genotypes, both *Verticillium* DNA content and symptoms had to be recorder in 120 dai trials. The inoculated cultivars used as control references developed symptoms of Verticillium wilt according to their previously established RLs (**Table 1**, **Figure 1**, and **Supplementary Figure 1**). Thus, 'Picual' plants presented severe wilting symptoms, showing early defoliation around 21 dai, chlorosis, leaf curl, leaf and shoot necrosis, stunting, and death. 'Picual' plants exhibited a RAUDPC of 78.0% and reached a final mean severity (FMS) index of 4 on the 0–4 symptoms scale. 'Arbequina', considered as moderately susceptible, showed mild symptoms starting around 50 dai, consisting on chlorosis, moderate defoliation, leaf, and shoot necrosis. RAUDPC and FMS values in

'Arbequina' plants were 24.2%, and 1.9, respectively. 'Frantoio' plants behaved as resistant and displayed very slight symptoms showing occasional defoliation or leaf curl, although most of the inoculated plants did not show symptoms. 'Frantoio' plants achieved a RAUDPC value of 2.7%, and an FMS value of 0.5. At the end of the experiment, the PDP was 100% for 'Picual', 23.1% for 'Arbequina' and 0% for 'Frantoio' varieties. RAUDPC, FMS, and PDP values were significantly different ($P < 0.05$) between each of the 'Picual', 'Arbequina' and 'Frantoio' cultivars (**Figure 2**).

A wide variability of the disease progress was observed in wild olive genotypes (**Supplementary Figure 1**). Wild olive genotypes and reference olive cultivars were classified into four groups of resistance (**Table 1**) according to the criteria defined by López-Escudero *et al*. (2007): extremely susceptible (**Figure 1A**); susceptible (**Figure 1B**); moderately susceptible (**Figure 1C**); and resistant (**Figure 1D**) genotypes. Thirty out of 68 wild olive genotypes assayed (44%) were extremely susceptible to the defoliating isolate VD117 of *V. dahliae*. With the exception of DHO12A, AMK34, AOU11, and ACZ8, all extremely susceptible genotypes showed a mortality rate of at least 50% at 120 dai (**Table 1**). A group of 12 genotypes (18%) exhibited RAUDPC values ranging from 18.6 to 38.7%, FMS between 2.6 and 3.0, and PDP from 8.3 to 50%, and were considered as susceptible (**Table 1** and **Figure 1B**). Eleven genotypes (16%) showed disease parameters similar to those of 'Arbequina', i.e., RAUDPC values from 5.0 to 29.2%, FMS between 1.0 and 2.2, and PDP from 0 to 33.3%, and were classified as moderately susceptible (**Table 1** and **Figure 1C**).

Table 1. Mean disease parameters assessed in wild olive genotypes inoculated with the defoliating isolate VD117 of *Verticillium dahliae.* Reference control cultivars 'Picual' (extremely susceptible) 'Arbequina' (moderately susceptible) and 'Frantoio' (resistant) are indicated in bold.

VARIETY[a]	RAUDPC[b]	FMS[c]	PDP[d]	R.L.[e]
GUA4	83,0	4,0	100,0	ES
PICUAL	**78,0**	**4,0**	**100,0**	**ES**
FRA1	76,9	4,0	100,0	ES
AMK25	74,7	3,9	91,7	ES
AJA4	73,3	4,0	100,0	ES
CUS3	69,1	3,8	92,9	ES
MAR1	69,0	4,0	100,0	ES
CER3	68,8	4,0	100,0	ES
GUA1	68,7	4,0	100,0	ES
GUA2	68,3	3,8	87,5	ES
CUS6	64,0	3,9	87,5	ES
AMK16	63,9	4,0	100,0	ES
DHO8A	57,4	4,0	100,0	ES
AJA17	56,7	4,0	100,0	ES
ACZ10	56,2	3,3	61,5	ES
DHO12A	54,0	3,9	33,3	ES
AMK34	52,6	3,4	0,0	ES
GUA5	52,4	4,0	93,8	ES
AMK6	51,5	3,3	66,7	ES
AOU4	50,4	4,0	100,0	ES
AOU11	48,8	3,5	9,1	ES
CUS14	47,5	3,6	88,9	ES
AMS19	44,6	3,3	75,0	ES
FRA2	44,1	3,9	50,0	ES
ACZ8	43,4	3,5	38,5	ES
DHO10B	40,1	3,4	80,0	ES
DHO10A	39,7	3,5	75,0	ES
AMK9	33,9	2,6	60,0	ES
DHO6C	33,7	2,8	75,0	ES
CEH20	21,7	3,9	87,5	ES
GUA8	16,6	3,4	56,3	ES
GUA7	38,7	2,8	16,7	S
AOU10	36,3	2,9	50,0	S
CER1	36,3	2,9	41,7	S
AMK12	35,4	2,8	50,0	S
AJA1	34,2	2,8	16,7	S
GUA9	33,3	2,8	43,8	S
FRA3	33,0	2,8	18,2	S
ACZ9	32,5	2,7	12,5	S
CEH21	31,2	3,0	25,0	S
AMS15	28,7	2,6	14,3	S
TAM4	23,2	3,0	8,3	S
ACZ5	18,6	2,7	25,0	S
ACZ1	29,2	2,2	12,0	MS
AMK21	28,9	1,9	33,3	MS
ARBEQUINA	**24,2**	**1,9**	**23,1**	**MS**
ARC1	19,3	1,6	13,3	MS
GUA6	16,7	2,0	11,1	MS
ACZ7	16,4	1,9	0,0	MS
AJA12	15,7	1,0	16,7	MS
ACO18	14,5	1,3	20,0	MS
TAM3	10,0	1,5	33,3	MS
DHO1	9,3	1,4	16,7	MS
AJA6	9,3	1,8	0,0	MS
APR1	5,0	1,6	0,0	MS
AMK27	10,8	0,9	0,0	R
AMK14	9,6	0,9	0,0	R
CEH23	9,1	1,0	0,0	R
AMS17	5,1	0,7	0,0	R
CEH8	4,3	0,7	0,0	R
AOU3	4,3	0,6	0,0	R
FRANTOIO	**2,7**	**0,5**	**0,0**	**R**
DHO6A	2,2	0,3	0,0	R
ACO1	2,0	0,6	0,0	R
ACO14	1,9	0,3	0,0	R
DHO6B	1,6	0,4	0,0	R
AMK5	1,3	0,1	0,0	R
ACZ3	0,6	0,3	0,0	R
TAM12	0,4	0,1	0,0	R
ACO15	0,4	0,1	0,0	R
GUA3	0,2	0,0	0,0	R

[a]Genotypes from the SILVOLIVE collection (Díaz-Rueda *et al*., 2020).

[b]RAUDPC: Relative area under the disease progress curve estimated as the percentage with regard to the potential maximum value.

[c]FMS: Final mean severity of symptoms.

[d]PDP: Percentage of dead plants.

[e]R.L. Resistance level of each genotype (López-Escudero *et al*., 2007). ES = extremely susceptible; S = susceptible; MS = moderately susceptible; R = resistant

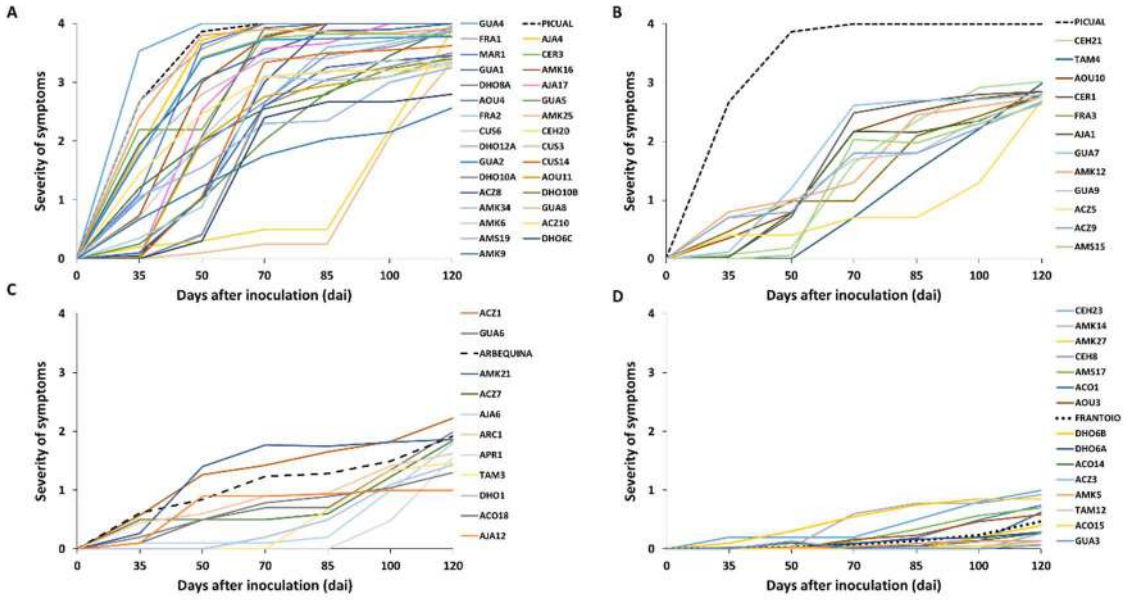


FIGURE 1 | Progress of the severity of symptoms recorded in wild olive genotypes inoculated with the defoliating isolate VD117 of *Verticillium dahliae* (assays 1–4). Values are the means of 24 plants per assay. Severity of symptoms was assessed each time on a 0–4 rating scale according to the percentage of Maximum Intensity Symptoms (MIS): chlorosis, leaf and shoot necrosis or defoliation: 0 = 0% MIS or no symptoms; 1 = 25% MIS; 2 = 50% MIS; 3 = 75% MIS; 4 = 100% MIS or dead plants. (A) extremely susceptible, (B) susceptible, (C) moderately susceptible, and (D) resistant genotypes. The extremely susceptible cultivar 'Picual' was maintained in panel 1B as a reference. Reference control cultivars 'Picual', 'Arbequina', and Frantoio are indicated in dashed lines.

A group of 15 wild olive genotypes (22%) were defined as resistant and presented low RAUDPC values (0.2–10.8%), moderate or no symptoms (FMS from 0.0 to 1.0), and no dead plants (PDP 0%) (**Table 1** and **Figure 1D**). Within this group, genotypes GUA3, ACO15, TAM12, ACZ3, and AMK5 exhibited lower symptoms than 'Frantoio', with significantly lower values in RAUDPC and/or FMS parameters, pointing to greater resistance to Verticillium wilt than the resistant cultivar of reference (**Figure 2**). The resistant genotypes DHO6B, ACO14, ACO1, DHO6A, AOU3, CEH8, AMS17, AMK14, and AMK27 showed non-significant differences with the resistant cultivar 'Frantoio' in RAUDPC and FMS values (**Figure 2**).

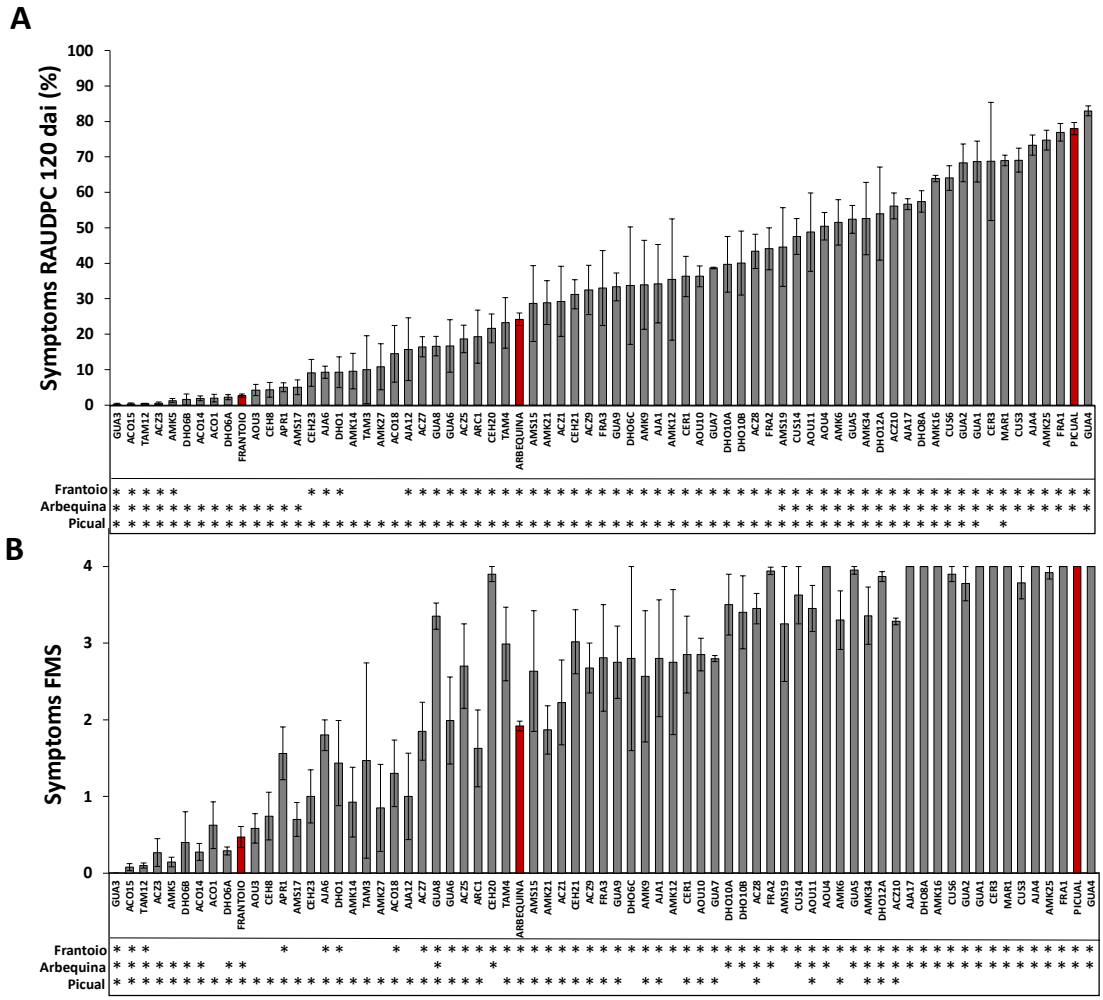


FIGURE 2 | Symptoms of infection of olive genotypes inoculated with the *Verticillium dahliae* defoliating isolate VD117. The Relative Area Under the Disease Progress Curve (RAUDPC) (A) and the Final Mean Severity (FMS) (B) of all wild olive genotypes was evaluated at 120 days after inoculation (dai). Red bars correspond to the reference cultivars: the resistant 'Frantoio' (RAUDPC < 10, FMS < 1.5 and PDP = 0); the moderately susceptible 'Arbequina' (RAUDPC 31–50, FMS 1.5–2.5 and PDP = 0–30); and the extremely susceptible 'Picual' (RAUDPC > 71, FMS > 3.0 and PDP > 51), according to the levels of resistance described by López-Escudero *et al*. (2007). Asterisks indicate significant differences with cvs. 'Frantoio', 'Arbequina' and 'Picual', respectively. Results are the mean of 24 plants and error bars correspond to the standard error. Data were subjected to analysis of variance (ANOVA) and multiple comparisons of means were analyzed by LSD (Least Significant Difference) at P = 0.05. Multiple range test was performed by Statistix 9.0 (Analytical Software, Tallahase, FL, United States).

Evolution of morphological parameters after treatment was measured in all genotypes analyzed in assay 3 (**Figure 3** and **Supplementary Figure 2**). After inoculation, the relative growth rate was strongly inhibited in susceptible, but not in resistant genotypes (**Figure 3A**). Resistant genotypes inoculated with the fungus showed little or no growth inhibition when compared to their non-inoculated controls (**Figure 3B**). In contrast, a remarkable growth inhibition was detected in the inoculated 'Arbequina'

(**Figure 3A**), and full growth inhibition was observed in the inoculated 'Picual' susceptible cultivar (**Figure 3B**). Production of new nodes was similar in inoculated and non-inoculated resistant genotypes compared to the scarce or null production of new nodes observed in susceptible genotypes (**Supplementary Figures 2A, B**, respectively). Accumulated secondary shoot length was more inhibited in susceptible than in resistant genotypes (**Supplementary Figures 2C, D**). This is, at least in part, a result of the reduction of the stem intermodal elongation in inoculated plants (**Supplementary Figures 2E, F**). At the end of the experiment, average growth inhibition by Verticillium wilt (relative to non-inoculated plants), measured as production of new nodes, accumulated secondary shoot length, and plant height, was significantly lower in the group of resistant genotypes than in the moderately or extremely susceptible ones (**Figure 4A**). Significant negative correlations were observed between FMS and the production of new nodes (R^2 = -0.8646); the accumulated secondary shoot length (R^2 = -0.698); and the plant height (R^2 = -0.5723), respectively (**Figure 4B**).

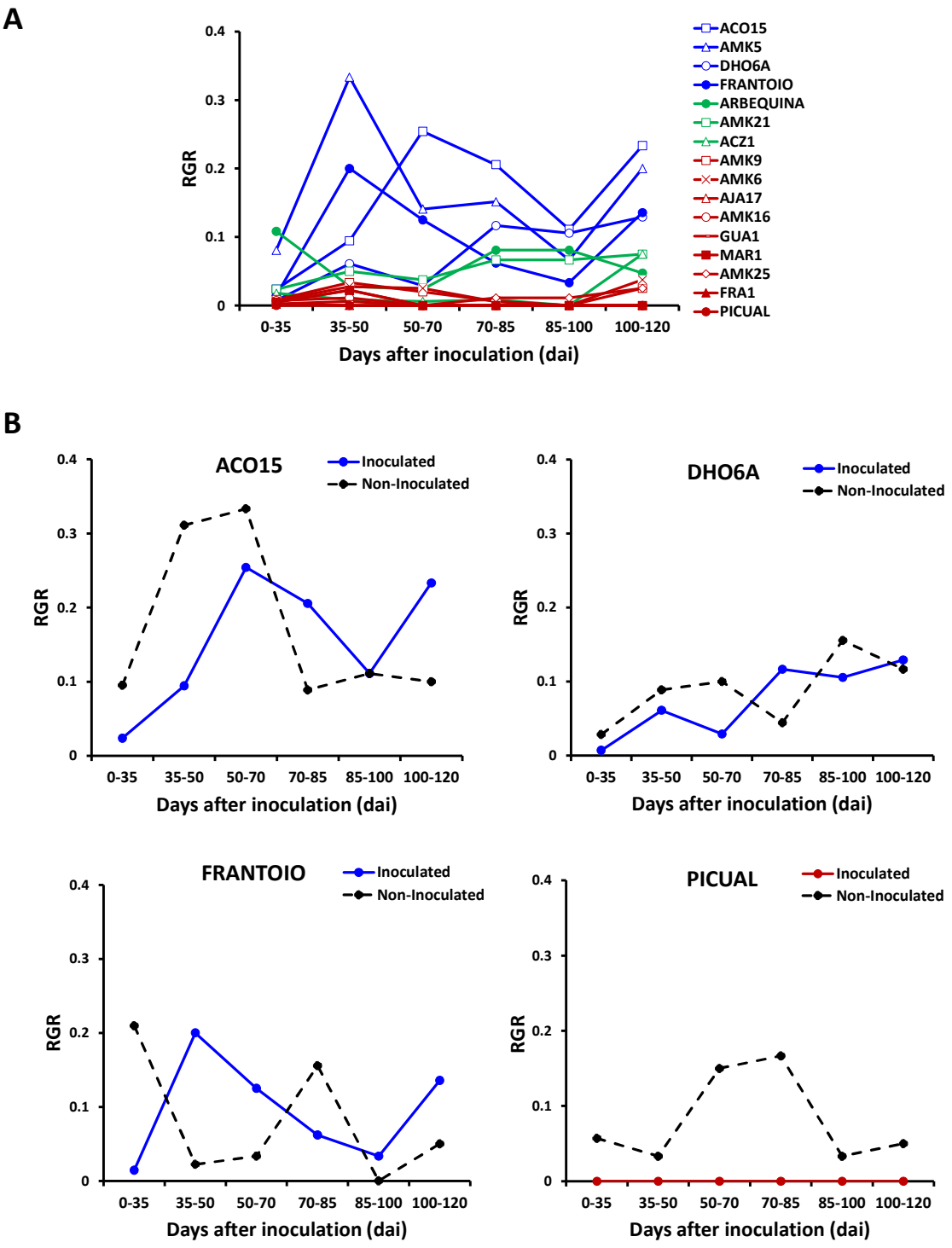


FIGURE 3 | Relative growth rate (RGR) quantified as the production of new nodes per day in wild genotypes and cultivars of assay 3. (A) RGR values of plants inoculated with the defoliating isolate VD117 of *Verticillium dahliae* were compared between resistant (blue), moderately susceptible (green) and susceptible or extremely susceptible (red) genotypes. (B) Comparison of inoculated (plain lines) vs. non-inoculated (dashed black lines) RGR in resistant wild genotypes and in representative cultivars: resistant 'Frantoio' and extremely susceptible 'Picual'.

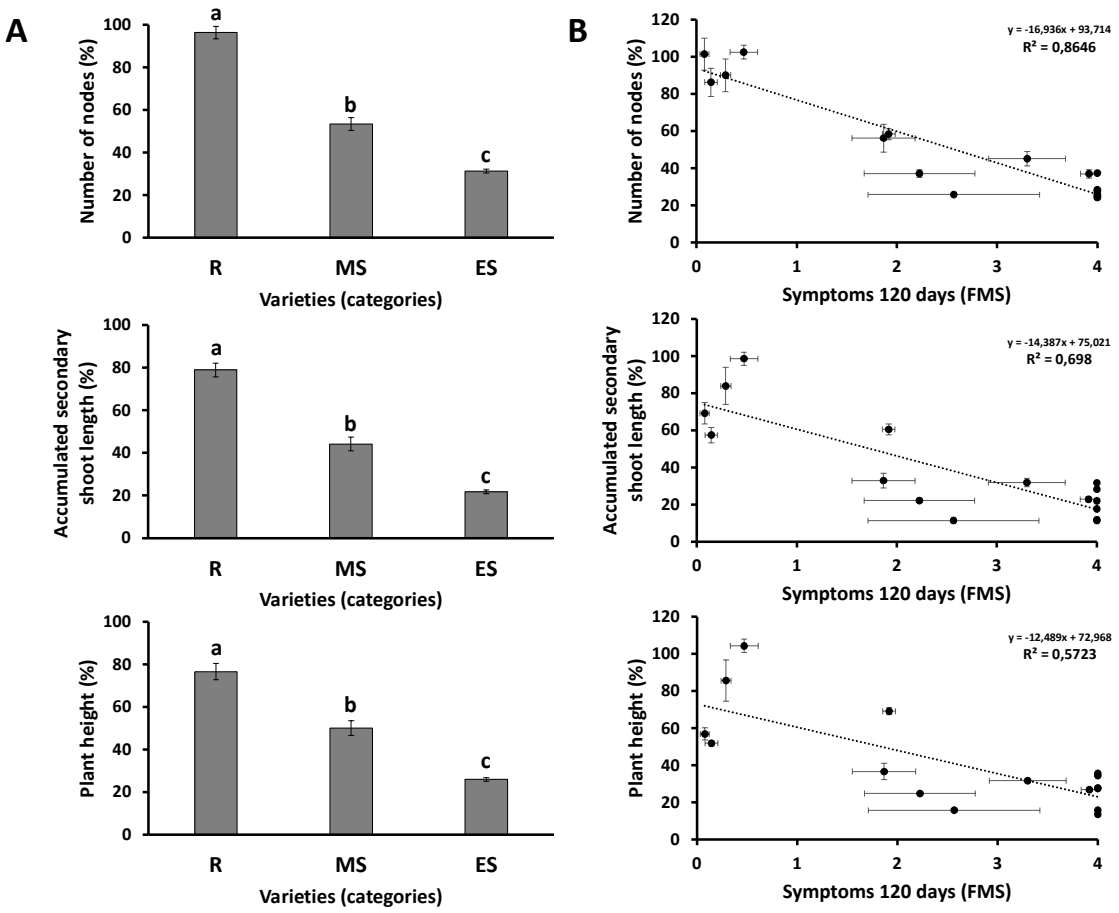


FIGURE 4 | (A) Morphological parameters in different groups of susceptibility at 120 days after inoculation (dai). Results are given as the average value of the different genotypes included in each group of susceptibility: R, resistant genotypes; MS, moderately susceptible genotypes; ES, extremely susceptible genotypes. For each genotype, the morphological value was normalized with the average value of the respective non-inoculated plants. Letters above bars represent significant differences at $P < 0.05$. (B) Correlations between morphological parameters and symptoms, measured as final mean severity of symptoms (FMS) at 120 dai. Scatter plots including the regression line, the regression equation and the coefficient of determination (R^2) are given for each morphological parameter: number of nodes; accumulated secondary shoot length; and plant height. Bars and dots represent the average value of 12 plants normalized with the average value of the respective non-inoculated plants for each genotype. Error bars correspond to standard errors of the mean.

Quantification of Verticillium dahliae in Inoculated Wild Olive Genotypes

The limit of detection of *V. dahliae* in the basal stems of inoculated plants using the TaqMan-based qPCR protocol was 15 fg of fungal DNA. No differences between the efficiency and C_t values of the standard curves were found between experiments, so that the data from experiments 1–4 could be compared. Average values of *V. dahliae* DNA content (MNQ) in the basal stem of the reference cultivars at 120 dai were: 3,130.93 x 10^{-3} in the susceptible 'Picual'; 1,594.41 x 10^{-3} in the moderately susceptible

'Arbequina'; and 0.41 x 10^{-3} in the resistant 'Frantoio' (**Supplementary Table 1** and **Figure 5A**).

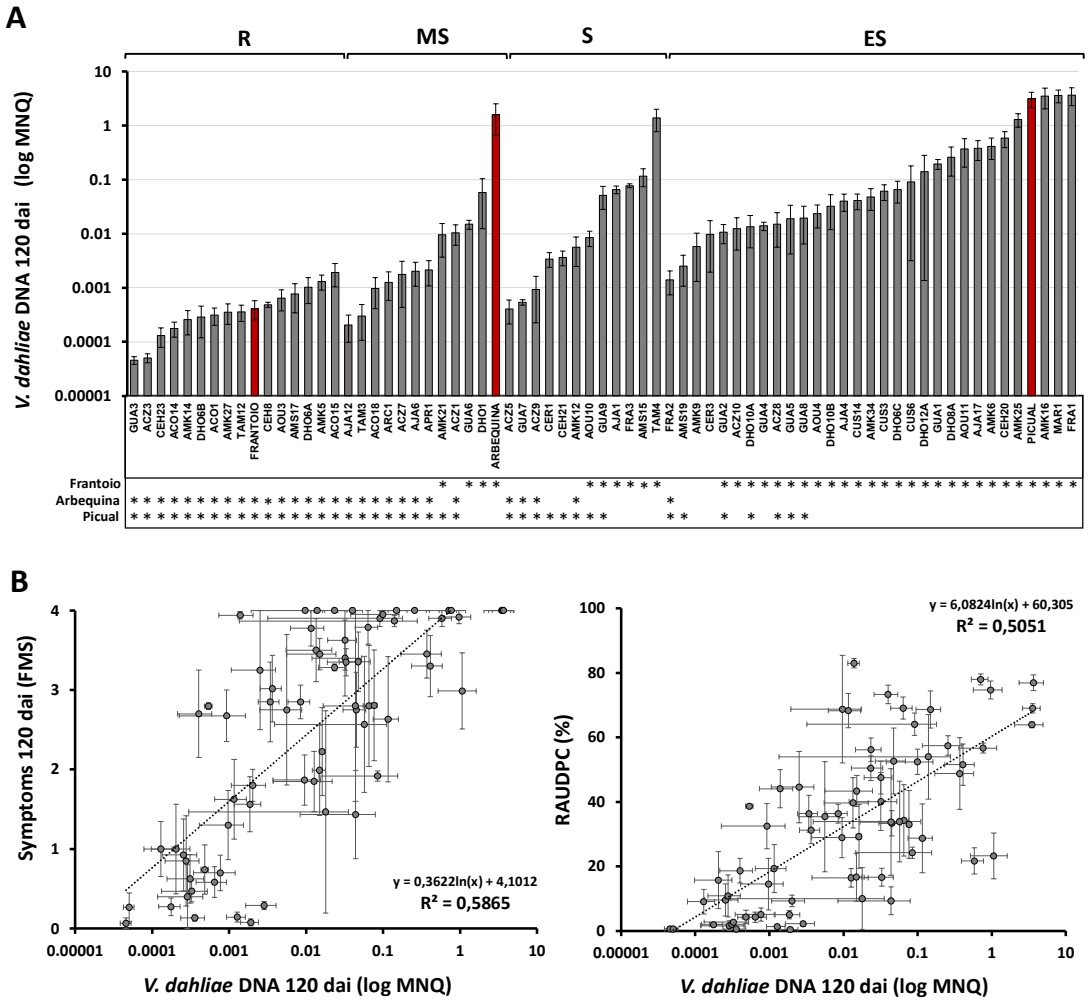


FIGURE 5 | (A) Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA of wild olive genotypes evaluated at 120 days after inoculation (dai) with the *V. dahliae* defoliating isolate VD117. Red bars represent cvs. 'Frantoio', 'Arbequina' and 'Picual' as reference cultivars of resistant, moderately susceptible, and extremely susceptible genotypes, respectively, according to the resistance level described by López-Escudero *et al*. (2007). Asterisks indicate significant differences with cvs. 'Frantoio', 'Arbequina' and 'Picual', respectively. (B) Scatter plots including regression line, regression equation and coefficient of determination (R^2) for the relationships between the Mean Normalized Quantity (MNQ) of *V. dahliae* DNA and the Final Mean Severity (FMS) (left) or the RAUDPC (right) at 120 dai. Results are the mean of 12 plants per assay, or 24 plants for those genotypes repeated in two different assays. Error bars in two dimensions indicate the standard error of the mean.

According to the classification of RLs of genotypes shown in **Table 1**, average MNQ values of *V. dahliae* DNA at 120 dai ranged as follows (**Supplementary Table 1**): from 3,658.79 x 10^{-3} (FRA1) to 1.39 x 10^{-3} (FRA2) in extremely susceptible genotypes; from 1,392.34 x 10^{-3} (TAM4) to 0.40 x 10^{-3} (ACZ5) in susceptible genotypes; from 58.25 x 10^{-3} (DHO1) to 0.20 x 10^{-3} (AJA12) in moderately susceptible genotypes; and from 1.90 x 10^{-}

^{3} (ACO15) to 0.04 x 10^{-3} (GUA3) in resistant genotypes (**Supplementary Table 1**). Therefore, the highest amounts of *V. dahliae* DNA were detected in extremely susceptible genotypes, whereas the lowest DNA amounts of *V. dahliae* DNA were detected in resistant genotypes. In the group of resistant genotypes, GUA3, ACZ3, CEH23, ACO14, AMK14, DHO6B, ACO1, AMK27, and TAM12 showed lower content of *V. dahliae* DNA than the resistant cultivar 'Frantoio' at 120 dai, although differences were not statistically significant. The lowest content of *V. dahliae* DNA was detected in ACZ3 and GUA3 genotypes, about ten times lower than that of the resistant 'Frantoio'.

Correlation Patterns Between Verticillium dahliae DNA Content and Plant Symptoms

In agreement with the results previously shown, a statistically significant positive linear correlation was established between the amount *V. dahliae* in plant shoot tissues and the plant symptoms at 120 dai, measured as FMS (R^2 = 0.5865) or RAUDPC (R^2 = 0.5051) (**Figure 5B**). This positive correlation was observed not only when the genotypes of the different assays were analyzed together (**Figure 5B**), but also when they were analyzed as separated assays (**Supplementary Figure 3**). However, in the subset of resistant genotypes, no correlation could be observed between the *V. dahliae* DNA content and symptoms at 120 dai calculated as RAUDPC (R^2 = 0.1987) or FMS (R^2 = 0.0202) (**Supplementary Figure 4A**). DNA of *V. dahliae* was also quantified at 35 dai (in assays 1, 2, and 3), but a poor correlation between the amount of *V. dahliae* DNA and symptoms was observed at this early period of infection (FMS R^2 = 0.21; **Supplementary Figure 4B**). And no correlation at all was observed between *V. dahliae* DNA content at 35 dai and symptoms at 120 dai (FMS R^2 = 0.0782; **Supplementary Figure 4B**), suggesting that diagnosis of Verticillium wilt based upon DNA quantification cannot be performed during an early stage of infection. We took advantage of having available DNA content values at early and late infection periods in assays 1, 2, and 3, to study the occurrence of different evolution patterns of infection over time, and to compare it with the degree of susceptibility to the disease in the different genotypes (**Figure 6A**). We observed that the lack of correlation between *V. dahliae* DNA content and symptoms in the group of resistant genotypes was even more pronounced at 35 than at 120 dai (**Supplementary**

Figure 5). Thus, genotypes with very low or no symptoms such as ACO15 and DHO6A, showed significantly higher *V. dahliae* DNA content at 35 dai than genotypes such as Frantoio or CEH23, with lower *V. dahliae* DNA and higher symptoms (**Supplementary Figure 5** and **Figure 2**). The data suggest the occurrence of genotypes such as ACO15 and DHO6A that tolerate the presence of relatively high amounts of the fungus. Therefore, different response patterns in the evolution of the fungus in plant tissues after infection can be distinguished (**Figure 6B**). The first one (pattern 1) consisted of a significant decrease in the amount of *V. dahliae* DNA between 35 and 120 dai. All the resistant genotypes, and the moderately susceptible AMK21 genotype, followed this pattern (**Figure 6B**). Pattern 2 showed no significant variation in the quantity of *V. dahliae* DNA between 35 and 120 dai. This pattern included 43% of moderately susceptible, 33% of susceptible, and 15% of extremely susceptible genotypes to *V. dahliae*, but none of the resistant genotypes (**Figure 6B**). Finally, in pattern 3, a significant increase in *V. dahliae* DNA at 120 dai respect to 35 dai was observed, including most of extremely susceptible (85%), 67% susceptible, 43% moderately susceptible genotypes, and none of the resistant genotypes (**Figure 6B**). The results point to the occurrence of different mechanisms of resistance to Verticillium wilt in wild olive genotypes.

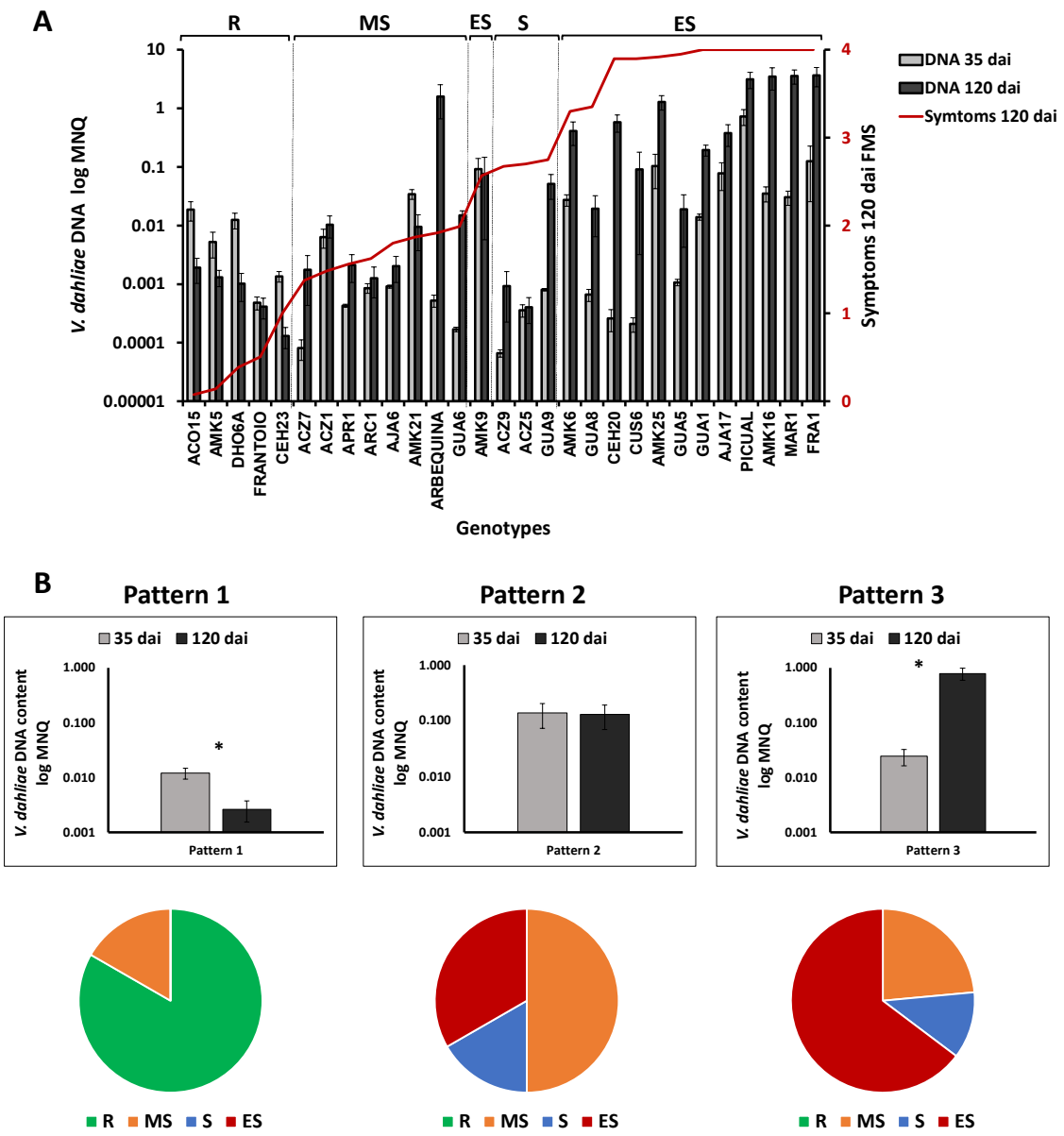


FIGURE 6 | (A) Evolution of *Verticillium dahliae* content after inoculation in genotypes tested in assays 1, 2, and 3 (A) Quantification of *V. dahliae* DNA (Mean Normalized Quantity, MNQ) in wild olive genotypes inoculated with the defoliating isolate VD117 of *V. dahliae* at 35 and 120 days after inoculation (dai). Bars represent the mean of 12 plants per assay, or 24 plants for those genotypes repeated in two different assays. The red line represents the average final mean severity (FMS) at 120 dai for each genotype. (B) Patterns of variation of *V. dahliae* DNA quantity between 35 and 120 dai (upper diagram), and relative content of genotypes according to the resistance level described by López-Escudero *et al.*, 2007; lower diagram) for each pattern. Asterisks indicate significant differences at $P < 0.05$. The data were subjected to analysis of variance (ANOVA) and multiple comparisons of means were analyzed by Tukey's HSD (Honestly significant difference). Error bars represent the standard error of the mean. R, resistant; MS, moderately susceptible; S, susceptible; ES, extremely susceptible.

Discussion

Control of Verticillium wilt in olive is nowadays a challenge that must be addressed under an integrated management strategy (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020), in which the search for sources of resistance should be of highest priority. Most of the commercial olive varieties domesticated for higher fruit and olive oil yields and quality are susceptible to *V. dahliae*. First steps in the search for resistant genotypes based on germplasm collections composed of commercial varieties, wild genotypes or breeding crosses have been addressed (Caballero and Del Río, 2008; Arias-Calderón *et al*., 2015c; Jiménez-Fernández *et al*., 2016), but still with no practical transference of results to olive production. In this work, the resistance to a defoliating isolate of *V. dahliae* has been evaluated for a high number of wild olive genotypes belonging to the SILVOLIVE collection. This collection is composed of individuals representative of all known subspecies of *Olea europaea* (*europaea*, *cuspidata*, *laperrinei*, *cerasiformis*, *guanchica*, and *maroccana*), which confers it a high diversity and genetic variability (Díaz-Rueda *et al*., 2020). Our results provide keys to better understand the resistance mechanisms to Verticillium wilt and a source of genotypes to be used in breeding programs or as rootstocks to improve the control of the disease in the olive grove.

Wild Olive Genotypes: A Valuable Source of Resistance to Verticillium Wilt

A wide spectrum of RLs was found among the SILVOLIVE genotypes tested (**Table 1** and **Figures 1**, **2**). This is in accordance with the variability of RLs previously described in olive cultivars, wild olive genotypes, and the offspring of breeding crosses (Colella *et al.*, 2008; Arias-Calderón *et al*., 2015b; Jiménez-Fernández *et al*., 2016). The percentage of resistant genotypes found in our work was 22% (15 out of 68), similar to another screening of comparable dimensions that reported 23% (13 out of 55) resistant genotypes (Arias-Calderón *et al*., 2015c). The resistant genotypes displayed similar or even better behavior than the resistant cultivar 'Frantoio', i.e., delay of the disease progress, scarce wilt symptoms, lower reduction of growth parameters, and no incidence of dead plants (**Table 1** and **Figures 1**–**3**). The mean content of *V. dahliae* DNA

quantified in the stem of root-inoculated resistant genotypes was 1,647 (ACO15) to 68,043 (GUA3) times lower than that of the susceptible cultivar 'Picual'. Other genotypes previously reported as resistant showed DNA contents that were 249–1,537 times lower than 'Picual' (Jiménez-Fernández *et al*., 2016). The content of *V. dahliae* DNA was 837 (ACO15) to 34,565 (GUA3) times lower than that of the moderately susceptible 'Arbequina', which is the most widely used cultivar in super-intensive olive orchards. Four of the six subspecies of *O. europaea* are represented in the resistant pool of the SILVOLIVE collection (*europaea*, *cuspidata*, *laperrine*, and *guanchica*) with no relationship between resistance and olive subspecies. We have not found resistant genotypes among the subspecies *maroccana* and *cerasiformis*, probably due to the low number of genotypes assayed (MAR1 from subsp. *maroccana* and CER1 and CER3 from subsp. *cerasiformis*). These genotypes were classified as susceptible (CER3) or extremely susceptible (MAR1, CER1) to *V. dahliae* despite being polyploid genotypes, a characteristic previously related to resistance to abiotic and biotic stress in plants (Sattler *et al*., 2016; Ruiz *et al*., 2020; Russo *et al*., 2020).

The presence of genotypes that showed significant higher RLs than 'Frantoio' is remarkable (**Figure 2**). The resistant genotype GUA3 belongs to the subspecies *guanchica* (Canary Islands, Spain). Progenies of this subspecies have been previously reported as resistant to the D pathotype of *V. dahliae* (Arias-Calderón *et al*., 2015b). However, most of the *guanchica* genotypes assayed in our study were classified as susceptible (GUA7 and GUA9) or extremely susceptible (GUA1, GUA2, GUA4, GUA5, GUA7, and GUA8), indicating that *guanchica* subspecies include genotypes with different levels of resistance to the fungus.

Verticillium dahliae inoculation did not significantly inhibit the relative growth rate of resistant genotypes (**Figure 3**). Most susceptible genotypes showed severe reduction of the intermodal length (**Supplementary Figure 2E**), probably as a result of impaired cell elongation. This phenomenon may be due to the loss of hydraulic conductivity in infected plants as a consequence of vascular occlusion by accumulation of defense metabolites such as tyloses and gels (Yadeta *et al*., 2013; Gharbi *et al*., 2017) or by cavitation of xylem vessels (Pouzoulet *et al*., 2014; Trapero *et al*., 2018).

Strategies for the Control of Verticillium Wilt in Resistant Genotypes

Different patterns of *V. dahliae* DNA at 35 and 120 dai vs. different degree of symptoms developed by wild genotypes, point to the occurrence of different mechanisms of resistance to Verticillium wilt. On the one hand, we propose that genotypes with relatively high content of *V. dahliae* at 35 dai and low level at 120 dai (e.g., ACO15, AMK5, and DHO6A) tolerate moderate infection levels at the short term, and control the infection at the medium- and long-term, reducing the amount of fungus in the plant tissues and exhibiting minimal symptoms (**Figures 2**, **3**). On the other hand, genotypes like Frantoio and CEH23 maintained low *V. dahliae* DNA levels at 35 and 120 dai, suggesting a more effective disease avoidance ability, since they prevent the fungus to proliferate in the shorter and longer terms. The occurrence of the two resistance mechanisms, tolerance and avoidance, may be the reason explaining the lack of correlation between the content of *V. dahliae* DNA and symptoms in the pool of resistant genotypes (**Supplementary Figure 5**). Anyway, it is clearly stated here the fact that resistant genotypes limit the spread of *V. dahliae*. Thus, a significant decrease in the amount of fungus DNA at 120 days compared to 35 days is observed in most resistant plants, a phenomenon that did not occur in non-resistant genotypes.

Different physiological, cellular and molecular mechanisms of resistance have been proposed in *V. dahliae* resistant genotypes (Gómez-Lama Cabanas *et al*., 2015; Trapero *et al*., 2018): the reinforcement of the cell wall by deposition of lignin and suberin at the site of infection (Gharbi *et al*., 2017); the production of reactive oxygen species such as H2O2 (Gharbi *et al*., 2017); and the early activation of plant defense mechanisms (Gharbi *et al*., 2016), such as the induction of genes coding for chitinases and b-1,3-glucanase to degrade the pathogen cell wall. Elucidating what type of molecular mechanisms determines the tolerance vs. the avoidance response to Verticillium wilt is of prime interest.

Potential Use of Genotypes as Resistant Rootstocks

Therefore, grafting susceptible cultivars of economic relevance, such as ‘Picual’ and ‘Arbequina’, onto resistant genotypes is a necessary approach to identify the most convenient strategy of resistance. From the resistance mechanisms previously

proposed, tolerance to *V. dahliae* would be optimal to be implemented in cultivars through breeding programs, while the disease avoidance would be optimal for rootstocks. Thus, although exhibiting minimal symptoms, the tolerance strategy of ACO15, AMK5, and DHO6A may have the disadvantage of allowing the fungus to proliferate and reach the grafted scion during the early infection period. From this perspective, the strategy of minimizing the proliferation of the fungus in the rootstock, represented by the resistant 'Frantoio' and CEH23 genotypes, could be more appropriate. We are currently conducting these assays with grafted plants to clarify these points.

These and other resistant genotypes can be used as rootstocks to improve Verticillium wilt resistance in the grafted plant as previously shown (Bubici and Cirulli, 2012; Porras-Soriano *et al.*, 2003). Furthermore, other traits previously characterized in these genotypes make them of special interest for their potential use as rootstocks. Thus, ACO15, AMK5, and DHO6A (*europaea* subspecies, Marrakech, Morocco), ACZ3 (*europaea* subspecies, Cádiz Mountains, Spain) and TAM12 (*europaea* subspecies, Tamri, Morocco) were classified as very low to intermediate vigor and high branching genotypes. Vigor reduction is a desirable trait in genotypes to be used as rootstocks for high and super high-density hedgerow orchards, a trait that can be transmitted to the grafted scion (Díaz-Rueda *et al*., 2020). It may be also the case of high branching, which means increased canopy density and high number of potential fruiting sites.

DNA Quantification of Verticillium dahliae as a Tool for Diagnosis of Verticillium Wilt

In planta quantification of *V. dahliae* DNA through TaqMan qPCR technology allowed specific detection and accurate quantification of the pathogen in this work, as previously shown (Mercado-Blanco *et al*., 2003; Gramaje *et al*., 2013; Jiménez-Fernández *et al*., 2016). DNA of *V. dahliae* was detected in all genotypes assayed, including the highly resistant ones, demonstrating that the fungus penetrated the root and colonized the stem of the plant. The lack of correlation between *V. dahliae* DNA at 35 dai and plant symptoms indicates that a screening of resistant genotypes cannot be performed at early infection times (e.g., 35 dai). For instance, some genotypes behaved as resistant

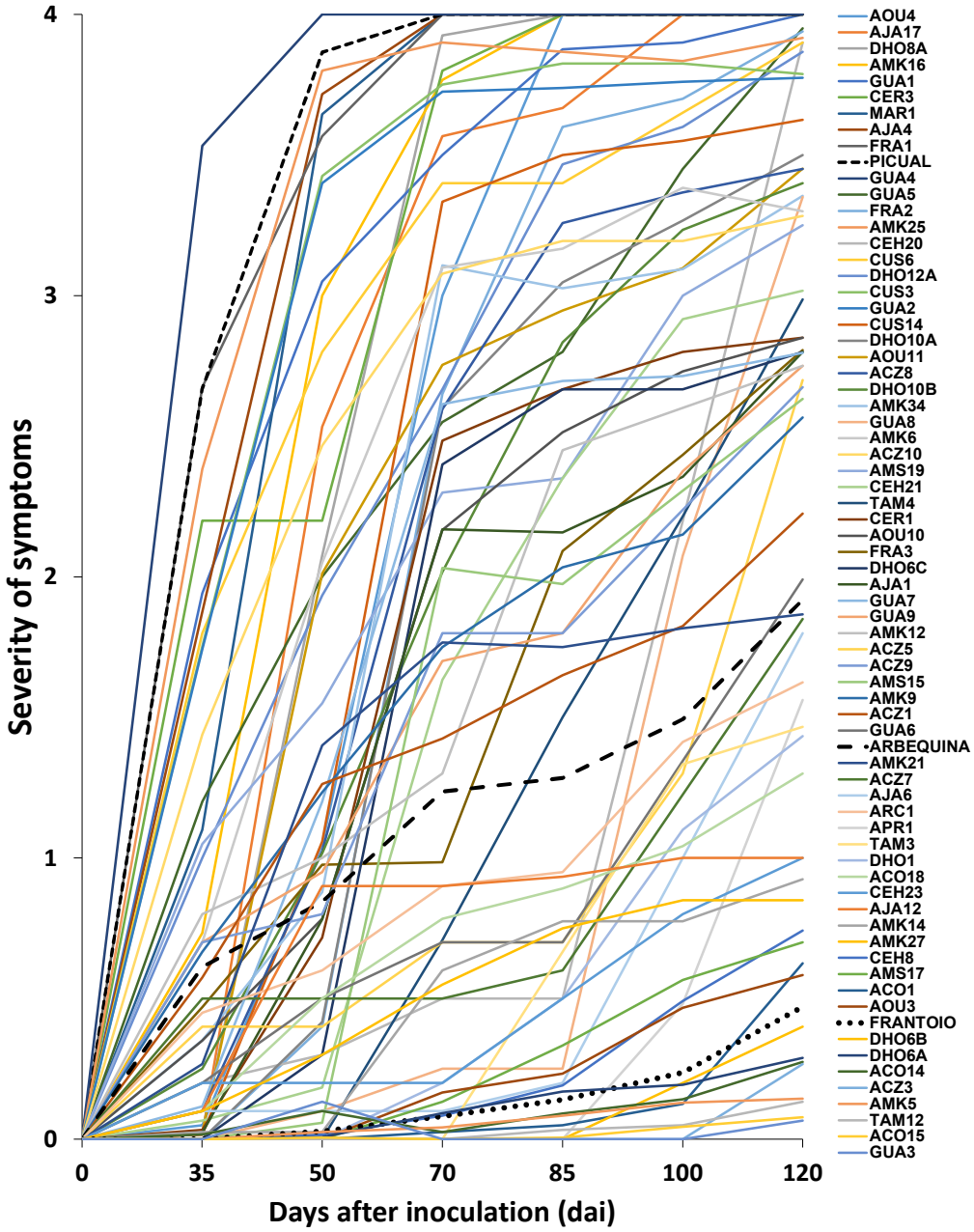
up to at least 85 dai, after which they suffered an abrupt increase in symptoms and *V. dahliae* DNA content, which determined them to be finally classified as extremely susceptible (**Figure 1A**). This indicates that the plants can prevent proliferation of the fungus for a time, after which the barriers of resistance are overcome, and the disease eventually develops. This is in line with results showing that disease symptoms can appear long after planting olive cultivars in naturally infected soils (Trapero *et al*., 2013; Valverde *et al*., 2021). To postulate resistant genotypes as useful for the control of Verticillium wilt, it is necessary to assess the resistance of susceptible cultivars grafted on the resistant wild genotypes identified in this work. In this regard, we are currently evaluating the resistance of commercial cultivars grafted on different wild olive genotypes that have demonstrated resistance to the disease. Field evaluation in naturally infected soils is also needed to test the long-term persistence of the resistance trait.

Conclusion

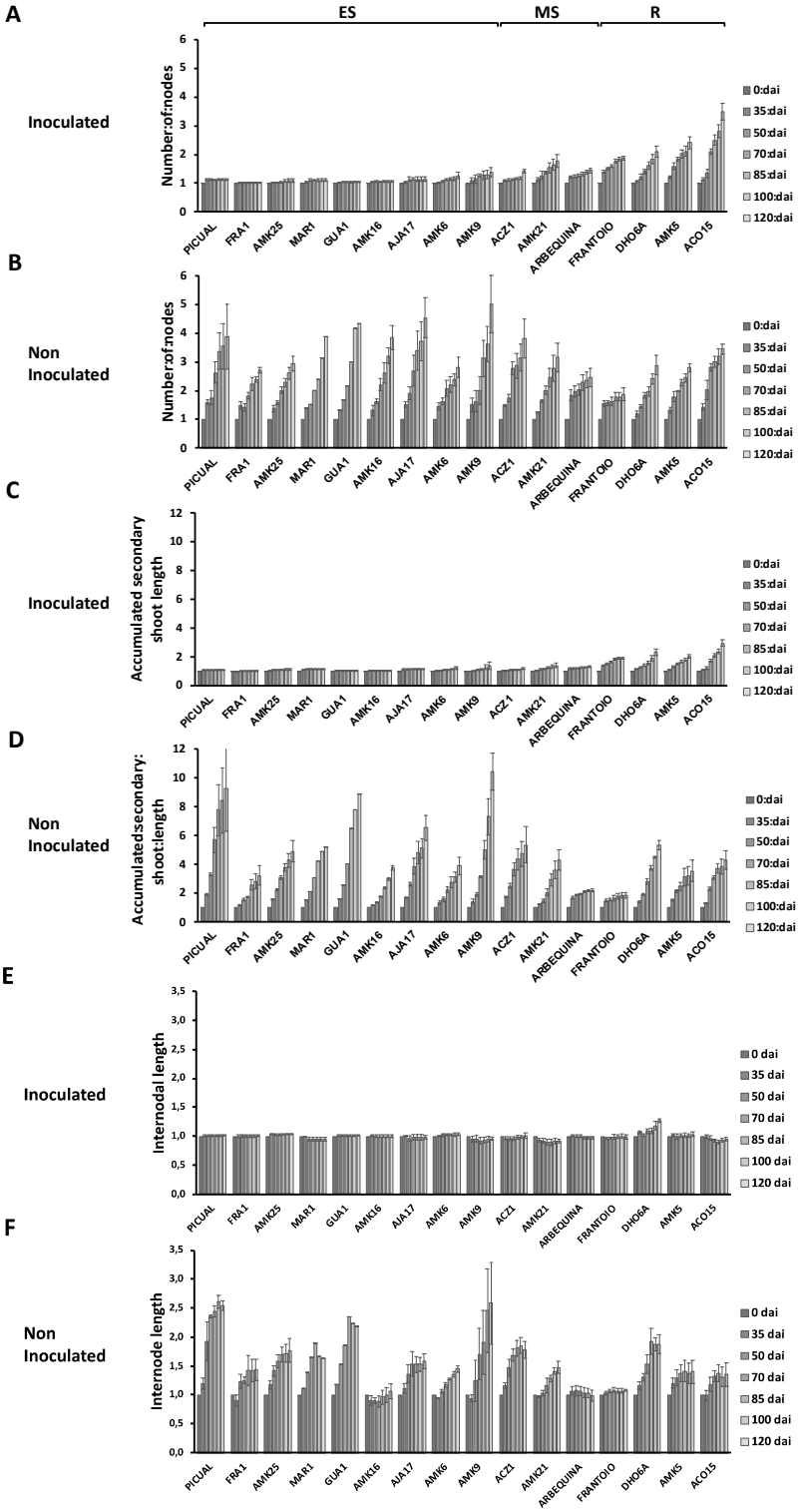
We have identified 15 wild genotypes displaying similar or better resistance to Verticillium wilt than the resistant cultivar Frantoio. Measurement of *Verticillium* DNA content at early and late stages of infection, together with correlations with plant symptoms, made it possible to identify specific patterns of response in wild olive genotypes, pointing to the occurrence of different strategies of resistance to Verticillium wilt, such as avoidance and tolerance mechanisms. Therefore, this work represents a valuable source of resistant genotypes to be used as rootstocks and in breeding programs. Our findings contribute to the improvement of an integrated, effective, and sustainable strategy for optimal control of Verticillium wilt in the olive grove.

Supplementary Materials

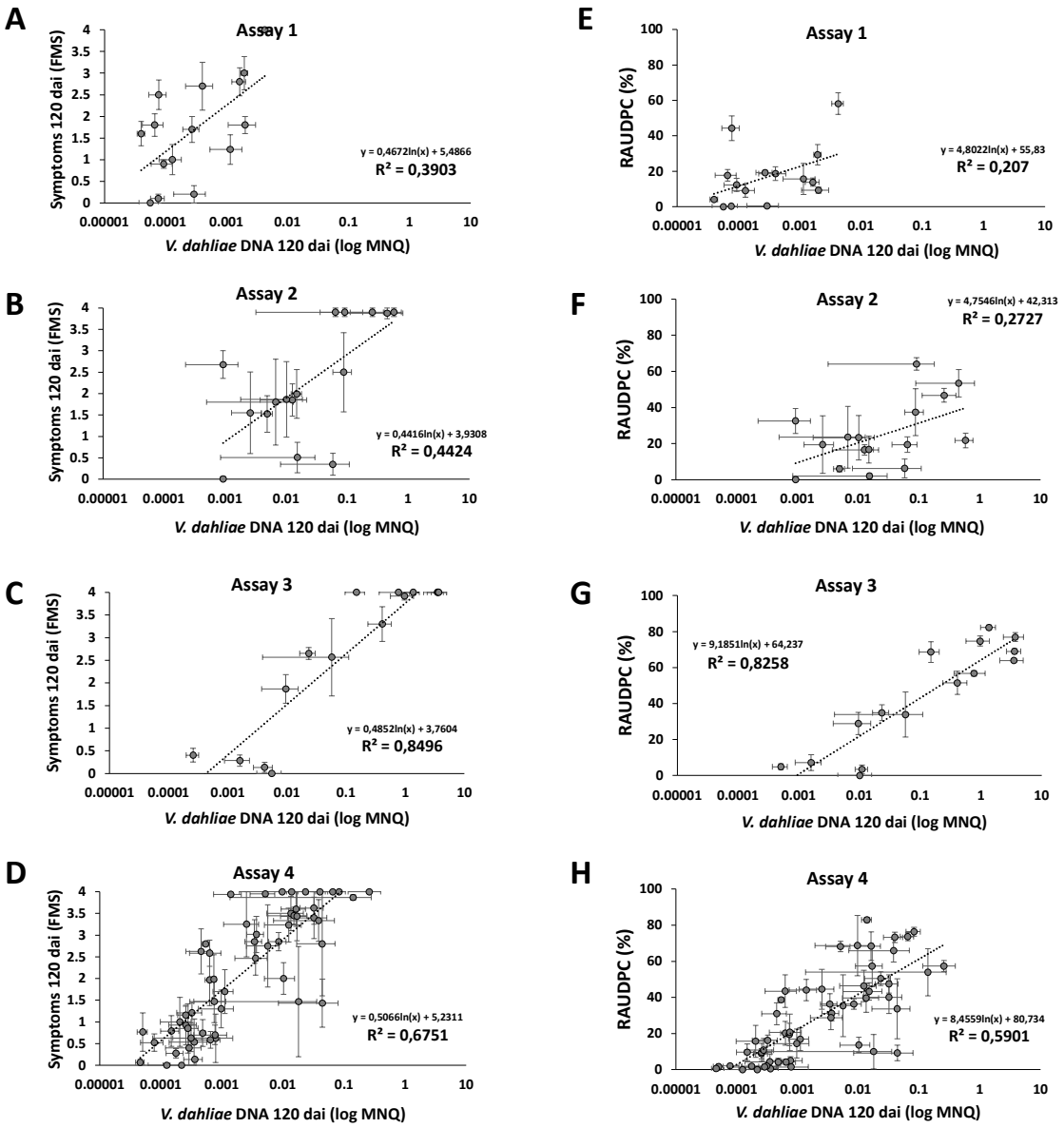
The Supplementary Material for this chapter can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.662060/full#supplementary-material



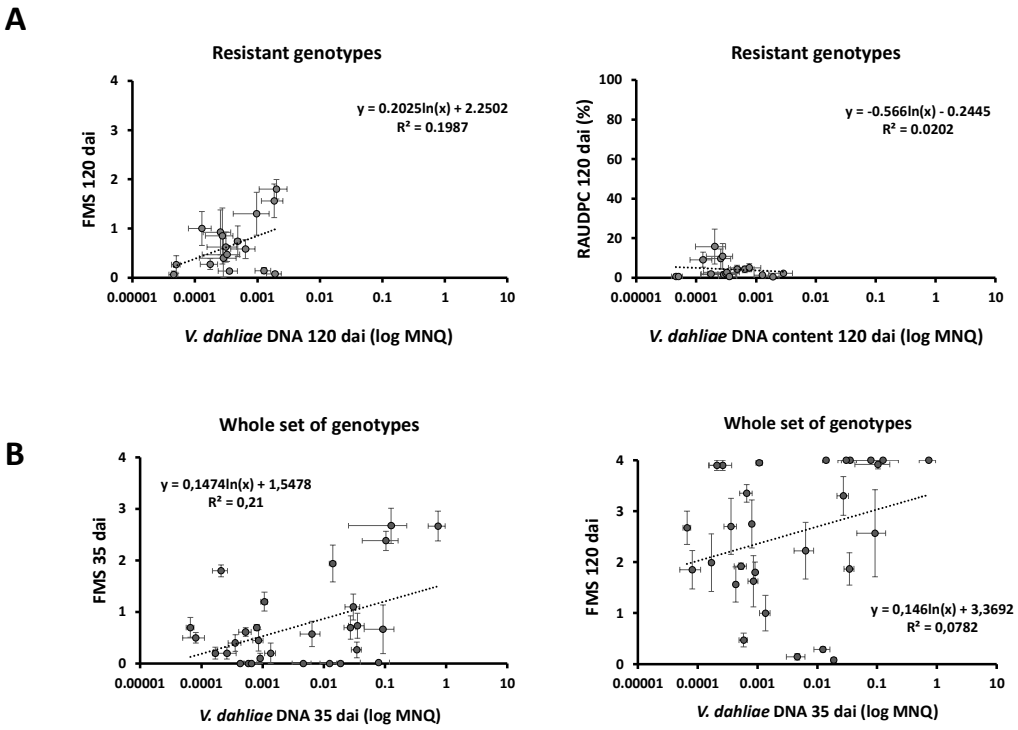
SUPPLEMENTARY FIGURE S1 | Progress of the severity of symptoms recorded in wild olive genotypes inoculated with the defoliating isolate VD117 of *Verticillium dahliae* (sum of assays 1 to 4). Values are the means of 24 plants per assay. Severity of symptoms was assessed each time on a 0-4 rating scale according to the percentage of *M*aximum *I*ntensity *S*ymtoms (*MIS*): chlorosis, leaf and shoot necrosis or defoliation: 0 = 0% *MIS* or no symptoms; 1 = 25% *MIS*; 2 = 50% *MIS*; 3 = 75% *MIS*; 4 = 100% *MIS* or dead plants. Reference control cultivars 'Picual', 'Arbequina' and 'Frantoio' are indicated as dashed lines.



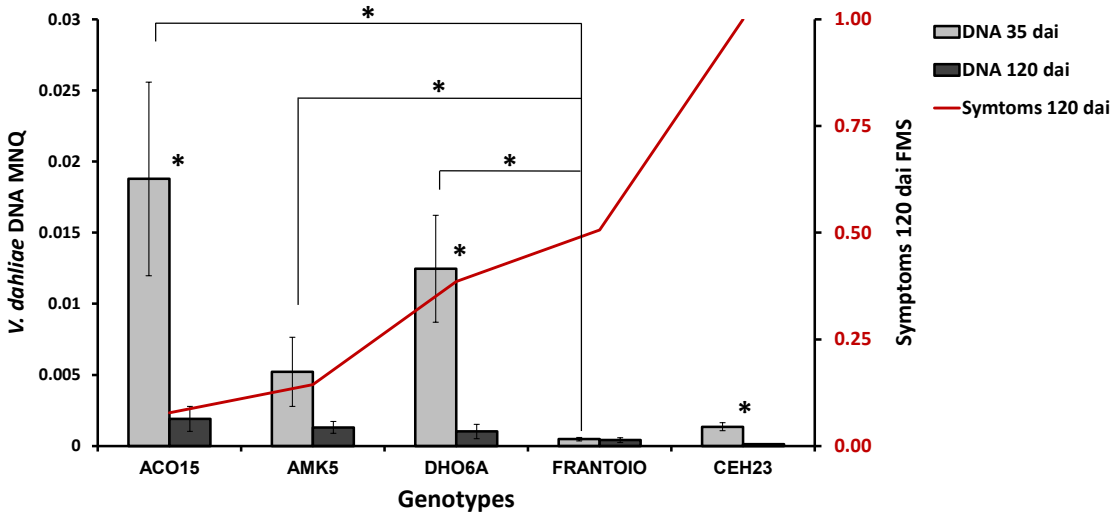
SUPPLEMENTARY FIGURE S2 | Evolution of morphological parameters with time in wild olive genotypes inoculated with *V. dahliae* isolate VD117 (A, C, E) or non-inoculated (B, D, F). Representative cultivars were also included: resistant 'Frantoio', moderately susceptible 'Arbequina' and extremely susceptible 'Picual'. Values of morphological parameters were normalized with the corresponding value at 0 dai. Results represent the average of the relative values measured in 12 plants per genotype and bars correspond to the standard error.



SUPPLEMENTARY FIGURE S3 | Scatter plots including regression line, regression equation and coefficient of determination (R^2) for the relationships between the Mean Normalized Quantity (MNQ) of *V. dahliae* DNA and the Final Mean Severity (FMS) (left graphics) or the RAUDPC (right graphics) at 120 dai. Results are the mean of 12 plants per assay, or 24 plants for those genotypes repeated in two different assays. Error bars in 2 dimensions indicate the standard error of the mean.



SUPPLEMENTARY FIGURE S4 | **(A)** Scatter plots including regression line, regression equation and coefficient of determination (R^2) for the relationships between the Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA and the Final Mean Severity (FMS) (left graphic) or the RAUDPC (right graphic) at 120 days after inoculation (dai) in the group of resistant genotypes from assays 1, 2, 3 and 4. **(B)** Correlations between the Mean Normalized Quantity (MNQ) of *V. dahliae* DNA at 35 dai and the Final Mean Severity (FMS) at both 35 (left graphic) and 12 dai (right graphic) in the whole set of genotypes from assays 1, 2 and 3. Results are the mean of 12 plants per assay, or 24 plants for those genotypes repeated in two different assays. Error bars in two dimensions indicate the standard error of the mean.



SUPPLEMENTARY FIGURE S5 | Quantification of *Verticillium dahliae* DNA (Mean Normalized Quantity, MNQ) in wild olive genotypes inoculated with the defoliating isolate VD117 of *Verticillium dahliae* at 35 and 120 days after inoculation (dai) in assays 1, 2 and 3. Bars represent the average values of 12-24 plants per genotype. Error bars indicate the standard error of the mean. The red line represents the average final mean severity (FMS) at 120 dai for each genotype.

References

Areal, F., and Riesgo, L. (2014). Farmers' views on the future of olive farming in Andalusia, Spain. Land Use Policy 36, 543–553. doi: 10.1016/j.landusepol.2013.10.005

Arias-Calderón, R., Leon, L., Bejarano-Alcazar, J., Belaj, A., de la Rosa, R., and Rodriguez-Jurado, D. (2015a). Resistance to Verticillium wilt in olive progenies from open-pollination. Sci. Hortic. 185, 34–42. doi: 10.1016/j.scienta.2015.01.015

Arias-Calderón, R., Rodriguez-Jurado, D., Bejarano-Alcazar, J., Belaj, A., de la Rosa, R., and Leon, L. (2015b). Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica 206, 619–629. doi: 10.1007/s10681-015-1463-7

Arias-Calderón, R., Rodriguez-Jurado, D., Leon, L., Bejarano-Alcazar, J., de la Rosa, R., and Belaj, A. (2015c). Pre-breeding for resistance to Verticillium wilt in olive: fishing in the wild relative gene pool. Crop Prot. 75, 25–33. doi: 10.1016/j.cropro.2015.05.006

Azabou, M. C., Gharbi, Y., Medhioub, I., Ennouri, K., Barham, H., Tounsi, S., *et al*. (2020). The endophytic strain *Bacillus velezensis* OEE1: an efficient biocontrol agent against Verticillium wilt of olive and a potential plant growth promoting bacteria. Biol. Control 142:104168. doi: 10.1016/j.biocontrol.2019.104168

Bejarano-Alcázar, J., Blanco-Lopez, M. A., Melero-Vara, J. M., and Jiménez-Díaz, R. M. (1996). Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Dis. 80, 1233–1238. doi: 10.1094/pd-80-1233

Bhat, R. G., and Subbarao, K. V. (1999). Host range specificity in *Verticillium dahliae*. Phytopathology 89, 1218–1225. doi: 10.1094/phyto.1999.89.12.1218

Bilodeau, G., Koike, S., Uribe, P., and Martin, F. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology 102, 331–343. doi: 10.1094/PHYTO-05-11-0130

Blázquez-Martínez, J. (1996). Origine e Diffusione Della Coltivazione. Enciclopedia Mondiale dell'olivo. Madrid: COI.

Boutaj, H., Chakhchar, A., Meddich, A., Wahbi, S., El Alaoui-Talibi, Z., Douira, A., *et al*. (2020). Bioprotection of olive tree from Verticillium wilt by autochthonous endomycorrhizal fungi. J. Plant Dis. Prot. 127, 349–357. doi: 10.1007/s41348-020-00323-z

Bubici, G., and Cirulli, M. (2011). "Verticillium wilt of olives," in Olive Diseases and Disorders, eds L. Schena, G. E. Agosteo, and S. O. Cacciola (Kerala: Research Signpost).

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. Plant Soil 352, 363–376. doi: 10.1007/s11104-011-1002-9

Caballero, J. M., and Del Río, C. (2008). "The olive world germplasm bank of Spain," in Proceedings of the 15th International Symposium on Olive Growing, Lovaina, 31–38. doi: 10.17660/actahortic.2008.791.1

Campbell, C., and Madden, L. (1990). Introduction to Plant Disease Epidemiology. New York, NY: JohnWiley & Sons.

Castro, D., Torres, M., Sampedro, I., Martinez-Checa, F., Torres, B., and Bejar, V. (2020). Biological control of Verticillium Wilt on olive trees by the salttolerant strain *bacillus velezensis* XT1. Microorganisms 8:1080. doi: 10.3390/microorganisms8071080

Civantos, L. (2004). "La olivicultura en el mundo y en España," in El Cultivo del Olivo, eds D. Barranco, R. Fernández Escobar, and L. Rallo (Madrid: Ediciones Mundi-Prensa), 19–35.

Colella, C., Miacola, C., Amenduni, M., D'Amico, M., Bubici, G., and Cirulli, M. (2008). Sources of Verticillium wilt resistance in wild olive germplasm from the Mediterranean region. Plant Pathol. 57, 533–539. doi: 10.1111/j.1365-3059.2007.01785.x

Díaz-Rueda, P., Franco-Navarro, J., Messora, R., Espartero, J., Rivero-Núñez, C., Aleza, P., *et al*. (2020). SILVOLIVE, a germplasm collection of wild subspecies with high genetic variability as a source of rootstocks and resistance genes for olive breeding. Front. Plant Sci. 11:629. doi: 10.3389/fpls.2020.00629

Fradin, E. F., and Thomma, B. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. alboatrum*. Mol. Plant Pathol. 7, 71–86. doi: 10.1111/j.1364-3703.2006.00323.x

García-Ruiz, G. M., Trapero, C., Del Rio, C., and Lopez-Escudero, F. J. (2014). Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. Phytoparasitica 42, 205–212. doi: 10.1007/s12600-013-0353-6

Garrido, C., Carbu, M., Fernández-Acero, F. J., Boonham, N., Colyer, A., Cantoral, J. M., and Budge, G. (2009). Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. Plant Pathol. 58, 43–51. doi: 10.1111/j.1365-3059.2008.01933.x

Gharbi, Y., Barkallah, M., Bouazizi, E., Cheffi, M., Gdoura, R., and Triki, M. A. (2016). Differential fungal colonization and physiological defense responses of new olive cultivars infected by the necrotrophic fungus *Verticillium dahliae*. Acta Physiol. Plant 38:242.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017). Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. Physiol. Mol. Plant Pathol. 97, 30–39. doi: 10.1016/j.pmpp.2016.12.001

Gómez-Gálvez, F. J., Vega-Macias, V., Hidalgo-Moya, J. C., Hidalgo-Moya, J. J., and Rodriguez-Jurado, D. (2020). Application to soil of disinfectants through irrigation reduces *Verticillium dahliae* in the soil and Verticillium wilt of olive. Plant Pathol. 69, 272–283. doi: 10.1111/ppa.13114

Gómez-Lama Cabanas, C., Ruano-Rosa, D., Legarda, G., Pizarro-Tobias, P., Valverde-Corredor, A., Carlos Trivino, J., *et al*. (2018). Bacillales members from the olive rhizosphere are effective biological control agents against the defoliating pathotype of *Verticillium dahliae*. Agriculture 8:90. doi: 10.3390/agriculture8070090

Gómez-Lama Cabanas, C., Schiliro, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2015). Systemic responses in a tolerant olive (Olea europaea L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. Front. Microbiol. 6:928. doi: 10.3389/fmicb.2015.00928

Gramaje, D., Perez-Serrano, V., Montes-Borrego, M., Navas-Cortes, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A Comparison of real-time pcr protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. Phytopathology 103, 1058–1068. doi: 10.1094/phyto-11-12-0312-r

Hegazi, E., Hegazi, A., and Allatif, M. (2012). Resistence of olive cultivars to Verticillium wilt. J. Appl. Sci. Res. 8, 2758–2765.

Hiemstra, J., and Harris, D. (1998). A Compendium of Verticillium Wilt in Tree Species. Wageningen: Ponsen & Looije.

Hoagland, D., and Arnon, D. (1950). The Water Culture Method for Growing Plants Without Soils. Berkeley, CA: Agricultura Expermental Station.

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., delMar Jimenez-Gasco, M., Antoniou, P. P., and Tjamos, E. C. (2012). Verticillium Wilt, a major threat to olive production: current status and future prospects for its management. Plant Dis. 96, 304–329. doi: 10.1094/pdis-06-11-0496

Jiménez-Díaz, R. M., Olivares-Garcia, C., Landa, B. B., del Mar Jiménez-Gasco, M., and Navas-Cortes, J. A. (2011). Region-wide analysis of genetic diversity in *Verticillium dahliae* populations infecting olive in southern Spain and agricultural factors influencing the distribution and prevalence of vegetative compatibility groups and pathotypes. Phytopathology 101, 304–315. doi: 10.1094/phyto-07-10-0176

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortes, J. A., Bubici, G., Cirulli, M., *et al*. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. Plant Pathol. 65, 1279–1291.

López-Escudero, F., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1–50. doi: 10.1007/s11104-010-0629-2

López-Escudero, F., Mercado-Blanco, J., Manuel Roca, J., Valverde-Corredor, A., and Angel Blanco-Lopez, M. (2010). Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. Phytopathol. Mediterr. 49, 370–380.

López-Escudero, F. J., and Blanco-López, M. A. (2005). Recovery of young olive trees from *Verticillium dahliae.* Eur. J. Plant Pathol. 113, 367–375. doi: 10.1007/s10658-005-3145-0

López-Escudero, F. J., del Rio, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. Eur. J. Plant Pathol. 110, 79–85. doi: 10.1023/b:ejpp.0000010150.08098.2d

López-Escudero, F. J., Mwanza, C., and Blanco-Lopez, M. A. (2007). Reduction of *Verticillium dahliae* microsclerotia viability in soil by dried plant residues. Crop Prot. 26, 127–133. doi: 10.1016/j.cropro.2006.04.011

Luo, X., Xie, C., Dong, J., Yang, X., and Sui, A. (2014). Interactions between *Verticillium dahliae* and its host: vegetative growth, pathogenicity, plant immunity. Appl. Microbiol. Biotechnol. 98, 6921–6932. doi: 10.1007/s00253-014-5863-8

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Paplomatas, E. J., and Tjamos, E. C. (2009). Symptom development, pathogen isolation and real-time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. Eur. J. of Plant Pathol. 124, 603–611. doi: 10.1007/s10658-009-9447-x

Martos-Moreno, C., Lopez-Escudero, F. J., and Blanco-Lopez, M. A. (2006). Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. Hortscience 41, 1313–1316. doi: 10.21273/hortsci.41.5.1313

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodriguez-Jurado, D., and Jiménez-Díaz, R. M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. Physiol. Mol. Plant Pathol. 63, 91–105. doi: 10.1016/j.pmpp.2003.10.001

Montes-Osuna, N., and Mercado-Blanco, J. (2020). Verticillium wilt of olive and its control: what did we learn during the last decade? Plants 9:735. doi: 10.3390/plants9060735

Mulero-Aparicio, A., Varo, A., Agusti-Brisach, C., Lopez-Escudero, F. J., and Trapero, A. (2020). Biological control of Verticillium wilt of olive in the field. Crop Prot. 128:104993. doi: 10.1016/j.cropro.2019.104993

Pegg, G., and Brady, B. (2002). Verticillium Wilts. Wallingford,EN: CABI International.

Pérez-Rodríguez, M., Alcantara, E., Amaro, M., Serrano, N., Lorite, I. J., Arquero, O., *et al*. (2015). The influence of irrigation frequency on the onset and development of Verticillium wilt of olive. Plant Dis. 99, 488–495. doi: 10.1094/pdis-06-14-0599-re

Porras-Soriano, A., Soriano Martin, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. Crop Protection 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1

Pouzoulet, J., Pivovaroff, A., Santiago, L., and Rolshausen, P. (2014). . Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. Front. Plant Sci. 5:253. doi: 10.3389/fpls.2014.00253

Rugini, E. (1984). *In vitro*-propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24, 123–134. doi: 10.1016/0304-4238(84)90143-2

Ruiz, M., Oustric, J., Santini, J., and Morillon, R. (2020). Synthetic Polyploidy in Grafted Crops. Front. Plant Sci. 11:540894. doi: 10.3389/fpls.2020.540894

Russo, R., Caruso, M., Arlotta, C., Lo Piero, A. R., Nicolosi, E., and Di Silvestro, S. (2020). Identification of field tolerance and resistance to mal secco disease in a citrus germplasm collection in sicily. Agronomy 10:1806. doi: 10.3390/agronomy10111806

Sánchez-Hernández, M., Ruiz-Dávila, A., Pérez de Algaba, A., Blanco-López, M., and Trapero-Casas, A. (1998). Occurrence and etiology of death of young olive trees in southern Spain. Eur. J. Plant Pathol. 104, 347–357.

Sattler, M. C., Carvalho, C. R., and Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. Planta 243, 281–296. doi: 10.1007/s00425-015-2450-x

Schnathorst, W., and Sibbett, G. (1971). Relation of strains of *Verticillium alboatrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. Plant Dis. Rep. 55:780.

Serrano, A., Rodriguez Jurado, M. D., Roman, B., Bejarano-Alcazar, J., de la Rosa, R., and Leon, L. (2021). Verticillium wilt evaluation of olive breeding selections under semi-controlled conditions. Plant Dis. (in press). doi: 10.1094/PDIS-08-20-1829-RE

Tjamos, E. (1993). Prospects and strategies in controlling Verticillium wilt of olive. EPPO Bull. 23, 505–512. doi: 10.1111/j.1365-2338.1993.tb01361.x

Trapero, C., Alcantara, E., Jimenez, J., Amaro-Ventura, M. C., Romero, J., Koopmann, B., *et al*. (2018). Starch hydrolysis and vessel occlusion related to wilt symptoms in olive stems of susceptible cultivars infected by *Verticillium dahliae*. Front. Plant Sci. 9:72. doi: 10.3389/fpls.2018.00072

Trapero, C., Rallo, L., Lopez-Escudero, F. J., Barranco, D., and Diez, C. M. (2015). Variability and selection of Verticillium wilt resistant genotypes in cultivated olive and in the Olea genus. Plant Pathol. 64, 890–900. doi: 10.1111/ppa.12330

Trapero, C., Serrano, N., Arquero, O., Del Rio, C., Trapero, A., and Lopez-Escudero, F. J. (2013). Field resistance to Verticillium Wilt in selected olive cultivars grown in two naturally infested soils. Plant Dis. 97, 668–674. doi:10.1094/pdis-07-12-0654-re

Tsror, L. (2011). Epidemiology and control of Verticillium wilt on olive. Isr. J. Plant Sci. 59, 59–69. doi: 10.1560/ijps.59.1.59 Vaerman, J. L., Saussoy, P., and Ingargiola, I. (2004). Evaluation of real-time PCR data. J.Biol. Regul. Homeost. Agents 18, 212–214.

Valverde, P., Trapero, C., Arquero, O., Serrano, N., Barranco, D., Munoz Diez, C., *et al*. (2021). Highly infested soils undermine the use of resistant olive rootstocks as a control method of Verticillium wilt. Plant Pathol. 70, 144–153. doi: 10.1111/ppa.13264

Varo-Suárez, A., Raya-Ortega, M. C., Agusti-Brisach, C., García-Ortíz-Civantos, C., Fernández-Hernández, A., Mulero-Aparicio, A., *et al*. (2018). Evaluation of organic amendments from agro-industry waste for the control of Verticillium wilt of olive. Plant Pathol. 67, 860–870. doi: 10.1111/ppa.12798

Villalobos, F., Testi, L., Hidalgo, J., Pastor, M., and Orgaz, F. (2006). Modelling potential growth and yield of olive (*Olea europaea* L.) canopies. Eur. J. Agron. 24, 296–303. doi: 10.1016/j.eja.2005.10.008

Wilhelm, S. (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology 45, 180–181.

Yadeta, K., and Tomma, B. (2013). The xylem as battle-ground for plant hosts and vascular wilt pathogens. Front. Plant Sci. 4:97. doi: 10.3389/fpls.2013.00097

Yadeta, K. A., Elmore, J. M., and Coaker, G. (2013). Advancements in the analysis of the arabidopsis plasma membrane proteome. Front. Plant Sci. 4:86. doi:10.3389/fpls.2013.00086

Chapter 5. Avoidant/resistant rather than tolerant olive rootstocks are more effective in controlling Verticillium wilt

Introduction

The olive (*Olea europ*aea subsp. *europaea* L.) crop has enormous economic, cultural and ecological importance within the Mediterranean basin. Spain is the main producer and exporter of olive oil worldwide. In recent years, olive cultivation has undergone a green revolution based on the change from a traditional model to a high-density plantation cultivation system (Connor et al., 2014). In addition, the use of drip irrigation, the application of forced agronomic practices and the use of young and highly productive cultivars have managed to increase production (Gucci et al., 2007) and yield (Lodolini et al., 2016; Marra et al., 2016). This new scenario, with high soil humidity and the proximity of the tree roots, has facilitated, however, the prevalence and spread of soilborne fungi (Rodríguez et al., 2008).

Verticillium wilt of olive is probably the most devastating fungal disease of the olive tree worldwide (López-Escudero and Mercado-Blanco, 2011; Tsror, 2011). In Mediterranean climate areas, this disease is considered the main limiting factor in olive cultivation because it causes high levels of tree mortality and a reduction in productivity and fruit yield (Montes-Osuna and Mercado-Blanco, 2020). The disease is caused by the soilborne hemibiotrophic fungus *Verticillium dahliae* Kleb. This pathogen is characterized by the production of infectious propagules named microsclerotia, which are resistance structures that allow the fungus to persist in the soil for long time (up to 14 years) in the absence of a host (Wilhelm, S, 1955; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz, et al., 2012; Montes-Osuna and Mercado-Blanco, 2020). Thus, long-lasting perennial crops such as olive trees are continuously exposed to the fungal infection (Tjamos and Jiménez-Díaz, 1998).

V. dahliae germinates in response to plant root exudates, penetrates through the roots (Mol, 1995) and colonizes the host's vascular system, progressively decreasing the conductivity of the xylem due to the occlusion of the vessels by tyloses and gels generated by the plant to compartmentalize the infection (Yadeta and J. Thomma, 2013). The loss of hydraulic conductivity has negative effects on the plant, causing cavitation or embolism, and eventually plant wilting and death (Inch and Ploetz, 2012;

Pouzoulet et al., 2014; Deyett et al., 2019). Disease symptoms mainly depends on fungal virulence, and host susceptibility. The defoliating pathotype (D) of *V. dahliae* causes the most severe symptoms, with young olive trees exhibiting more susceptibility than adult ones, where the infection generally spreads more slowly (Sinclair and Hudler, 1998; López-Escudero and Mercado-Blanco, 2011).

The control of Verticillium wilt is not easy. Drip irrigation, the use of infected planting material and inappropriate agronomic practices contribute to the spread of infective propagules (Montes-Osuna and Mercado-Blanco, 2020). The adequate management of the irrigation system (Baroudy et al., 2018) and the control of the phytosanitary status of the plant material are essential to control the incidence and severity of the disease. Crop rotation is not adequate due to the wide host range of *V. dahliae* (McCain and Raabe, 2002; Pegg and Brady, 2002) and the persistence of microesclerotia in the soil (Umaerus et al., 1989). The application of thermal treatments such as soil solarization (Tjamos and Paplomatas, 1988), hot air (Morello et al., 2016) or the use of organic amendments to inhibit or reduce the viability of the microsclerotia (Varo-Suárez et al., 2018) help to manage Verticillium wilt of olive. On the other hand, the use of chemical fumigation is not very effective or is not allowed due to environmental concerns (Tsror, 2011). The use of biological control agents based in bacterial strains and not pathogenic fungi within an integrated management, delays the appearance of symptoms, and reduces the incidence and severity of the disease (Carrero-Carrón et al., 2016; Gómez-Lama Cabanás et al., 2018; Mercado-Blanco et al., 2004; Mulero-Aparicio et al., 2020; Papasotiriou et al., 2013; Prieto et al., 2009; Varo et al., 2016).

Due to the presence of asymptomatic infected plants and the constant presence of latent microsclerotia in the soil, among other epidemiological factors, the development of molecular tools for the accurate and sensitive detection of *V. dahliae* is essential to control the silent spread of the disease. The real-time quantitative polymerase chain reaction (qPCR) for the specific detection and quantification of *V. dahliae* in infected tissues has allowed a better understanding of the dynamics of *V. dahliae* infection in infected olive tissues and its relationship with the degree of susceptibility of different olive cultivars (Mercado-Blanco et al., 2003; Markakis et al.,

2009; Gramaje et al., 2013; Díaz-Rueda et al., 2021). The use of qPCR detection protocols establishes a preventive control method within an integrated management strategy (Montes-Osuna and Mercado-Blanco, 2020), allowing an early diagnosis of Verticillium wilt of olive, even when the symptoms are not evident.

The genetic resistance of the cultivar is the most recommended measure to control Verticillium wilt of olive (Trapero et al., 2015). However, most of the agronomically and economically relevant olive cultivars, such as 'Picual' or 'Arbequina' , are susceptible or extremely susceptible to the defoliant pathotype of *V. dahliae* (López-Escudero and Mercado-Blanco, 2011). On the contrary, a high level of resistance has been found in cultivars such as 'Empeltre', 'Frantoio', 'Oblonga' and 'Changlot Real'. These genotypes have shown a late onset of the disease, ability to recover and a low percentage of dead plants under *V. dahliae* pressure (López-Escudero et al., 2004; López-Escudero and Mercado-Blanco, 2011). However, none of these resistant cultivars are widely used in commercial olive production due to their poor agronomic properties. Studies on resistance to Verticillium wilt of olive have mainly focused on the analysis of biochemical and physiological responses (Gharbi et al., 2016, 2017; Trapero et al., 2018) or on genetic and transcriptomic analyses (Gómez-Lama Cabanás et al., 2015; Leyva-Pérez et al., 2018; Serrano et al., 2020) of olive cultivars that show different levels of susceptibility to *V. dahliae*. However, the genetic origin of resistance in olive trees has not been accurately identified yet.

Grafting susceptible olive cultivars onto resistant rootstocks is an approach that has recently gained interest as a control strategy for Verticillium wilt. The appearance of diseases caused by soil pathogens such as *Verticillium* (Montes-Osuna and Mercado-Blanco, 2020) or *Phytophthora* (González et al., 2019) and the need to obtain dwarfing varieties in super-intensive or hedge cultivation systems (Rallo, 2014) has increased the number of studies on the use of rootstocks in the olive grove. Numerous studies report the use of rootstocks to solve the problem of Verticillium wilt in different herbaceous species such as watermelon (Attavar et al., 2020; Devi et al., 2021), and tomato (Papadaki, A. M. et al., 2017), and in woody plants such as avocado (Haberman et al., 2020) and pistachio (Epstein et al., 2004). In olive, the grafting strategy has been studied under controlled conditions (Porras Soriano et al., 2003; Bubici and Cirulli, 2012) and in

field trials with naturally infested soils (Hartmann et al., 1971; Valverde et al., 2021). The use of resistant rootstocks has been shown to reduce the susceptibility to *V. dahliae*, delaying the onset of symptoms and therefore the development of the disease (Porras Soriano et al., 2003; Bubici and Cirulli, 2012; Yildiz et al., 2020), although this tolerance could be broken over time in soils with high inoculum density (Valverde et al., 2021). However, no resistant rootstocks have been currently identified that fully prevents Verticicillium infection of the grafted variety. For this reason, identification of new resistant olive rootstocks that can control the disease in soils with low and / or high inoculum density is demanded by the olive sector.

Two different strategies can be distinguished in plants to control the infection of pathogens (Robb, 2007). On the one hand, the mechanism that avoids pathogen proliferation, by which plant defenses prevent or limit the colonization of the pathogen is commonly referred to by the generic term of resistance. On the other hand, the tolerance mechanism inhibits or reduce the symptoms despite the occurrence of pathogen proliferation. Interestingly, both strategies have been recently identified as mechanisms of avoidance/resistance or tolerance to Verticillium wilt in different wild olive genotypes of the SILVOLIVE collection (Díaz-Rueda et al., 2021). The SILVOLIVE collection comprises an extensive number of wild genotypes belonging to different subspecies of *Olea europaea*, providing a natural source of genetic variability with high potential to be used for cultivar or rootstock breeding.

The objective of this study was to clarify which one of the two mechanisms, avoidance/resistance or tolerance, is more effective for controlling Verticillium wilt in olive plants through the use of low-susceptibility rootstocks. With this aim, the highly susceptible ‘Picual’ cultivar was grafted onto different wild olive genotypes of very low susceptibility to Verticillium wilt that display the two mechanisms (Díaz-Rueda et al., 2021): On the one hand, ACO15, AMK5, and DHO6A genotypes exhibited the tolerance strategy, allowing moderate levels of *V. dahliae* proliferation, but showing minimal symptoms. On the other hand, the cultivar ‘Frantoio’ and the wild genotypes GUA3, CEH23 and AMK27 exhibited the avoidance/resistance strategy, maintaining minimal *V. dahliae* DNA levels since they prevented the fungus to proliferate, also showing reduced

symptoms (Díaz-Rueda et al., 2021); **Supplementary Figure S1**). Verticillium wilt susceptibility was evaluated by estimating the final mean severity of symptoms (FMS), the relative area under the disease progress curve (RAUDPC), and the percentage of dead plants (PDP) in greenhouse conditions. The content of *V. dahliae* DNA was quantified in the rootstocks and in the grafted 'Picual' scions at 35 and 120 days after inoculation (dai) by qPCR. Growth parameters such as plant biomass, accumulated length of the grafted scion, number of nodes and number of stem branching were also measured for all combinations and compared with their corresponding non-inoculated plants. The results indicate that olive rootstocks that avoid/resist the pathogen, controlling its proliferation, were much more efficient in controlling Verticillium wilt than tolerant rootstocks.

Material and Methods

Plant Material

Plant material consisted in cv. 'Picual' grafted onto micropropagated wild olive genotypes one year after *ex-vitro* acclimatization. The different rootstocks used in this study were selected according to their different levels of resistance to Verticillium wilt, previously characterized in Díaz-Rueda et al., (2021). Six genotypes were identified as low susceptible to Verticillium wilt (GUA3, CEH3, AMK27, ACO15, AMK5 and DHO6A). Among them, GUA3, CEH3, AMK27 as well as the cultivar 'Frantoio' showed the Verticillium wilt avoidance/resistance strategy, with MNQ values of $0.45 \cdot 10^{-4}$ - $3.55 \cdot 10^{-4}$, whereas ACO15, AMK5 and DHO6A showed the Verticillium wilt tolerance strategy, with MNQ values of $1.02 \cdot 10^{-3}$ - $1.91 \cdot 10^{-3}$ (Díaz-Rueda et al., 2021; **Supplementary Figure S1**). AMK21, with a MNQ value of $9.54 \cdot 10^{-3}$ was defined as moderately susceptible to Verticillium wilt, while ACZ10 (MNQ value of $1.23 \cdot 10^{-2}$) and GUA8 (MNQ value of $1.93 \cdot 10^{-2}$) were defined as extremely susceptible. In addition to the resistant 'Frantoio', the cultivar 'Arbequina' (MNQ value of 1.59) and 'Picual' (MNQ value of 3.13) were described as moderately susceptible and extremely susceptible reference controls, respectively (Díaz-Rueda et al., 2021). Cultivars were obtained from a commercial nursery (**Table 1**). Wild-olive genotypes were micropropagated *in-vitro* from the

SILVOLIVE germplasm collection (Díaz-Rueda et al., 2020). Two types of grafting procedures were used: 'spike' graft and 'gusset' or 'T' graft. For the 'spike' graft, the rootstock was decapitated 15 cm from the base of the stem and the 5-6 cm shoot of the scion containing four to six buds was inserted in an incision made in the cut end of the rootstock trunk. For the 'gusset' graft, a T-shaped cut was made at about 20 cm from the base of the rootstock stem, where a rectangular bark slice of the scion containing two buds was introduced. To assure a close contact and the immobility between the rootstock and the scion, the graft area was tied with plastic film.

The newly grafted plants were maintained in 3-L pots in greenhouse conditions for 4 months at 28 ºC and high relative humidity to promote the union and the growth of the graft. During this phase, plants were fertilized, and the lateral buds of the rootstocks were removed, facilitating graft cicatrization and the development of the scions. After this time, inoculation tests for resistance assessment were carried out.

Fungal isolate and inoculum production

The defoliating *V. dahliae* pathotype VD-117, obtained from the collection of the Plant Pathology Laboratory of Department of Agronomy, University of Córdoba (Spain) (Blanco-López et al., 1984), was used in the inoculation tests for resistance assessment. Conidial suspensions were prepared and adjusted to 1 x 10^7 conidia/ml, according to Díaz-Rueda et al., (2021).

Inoculation of olive plants

For resistance tests, twenty-four plants were inoculated for each rootstock × scion combination. Plant roots were washed under tap water to remove the substrate. The bare root system of each plant were cut 4-5 times in the secondary roots and dipped in the *V. dahliae* conidial suspension for 15 min. Roots of non-inoculated control plants were subjected to the same treatment, but immersed in PDB:sterile distilled water (1:1, v:v). Plants were individually transplanted into 3-L pots containing sterilized 2:1 silt:peat moss (v:v). The experimental design was complete randomized blocks with four blocks and six plants per block and 5-7 non-inoculated plants for each combination. Moreover,

non-grafted 'Frantoio', 'Picual' and 'Arbequina' were used as controls following the same experimental design. Plants were maintained at 24/18 °C and 60/40 % relative humidity (day/night) in a greenhouse where natural illumination was supplemented with 360 µmol m^{-2} s^{-1} fluorescent lighting to ensure 14-h photoperiod. Plants were watered three times per week and fertilized weekly with Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Symptoms assessment

Symptoms were evaluated on each plant at 0, 35, 50, 70, 85, 100 and 120 days after inoculation, following a 0-4 rating scale according to the percentage of *M*aximum *I*ntensity *S*ymptoms (*MIS*): total or partial apoplexy, chlorosis, leaf curl, stunting, leaf and shoot necrosis or defoliation: 0 = 0% *MIS* or no symptoms; 1 = 25% *MIS*; 2 = 50% *MIS*; 3 = 75% *MIS*; 4 = 100% *MIS* or dead plants.

At the end of the experiment, the following disease parameters were estimated from these scale values: i) the relative area under the disease progress curve (RAUDPC) was obtained for each plant considering its percentage respect to the maximum value that could be reached in the period of assessment, using the following formula based on Campbell and Madden (1990):

$$RAUDPC = \left[\sum_{i=1}^{n}\left(\frac{S_i + S_{i-1}}{2}\right)\Delta t\right]\left[\frac{100}{S_{max}T}\right] \quad (1)$$

where S_i = severity of the experimental unit in the observation i; Δt = the number of days between observations; S_{max} = maximum disease rating (=4); T = experimental period in days (=120); n = number of observations; ii) the final mean severity of symptoms (FMS), calculated according to (López-Escudero et al., 2007); iii) the percentage of dead plants (PDP) from the total of inoculated plants.

Growth parameters

Accumulated length of the grafted scion (including secondary and tertiary branches), number of nodes, and number of stem branching were measured at 0 and 120 dai. The

increase of the growth for each combination was calculated as the mean ± the standard error of the difference between the value at 120 dai and 0 dai. The relative increase of the growth parameters was calculated as the ratio between the growth increase in inoculated plants and the growth values of non-inoculated plants.

Quantification of Verticillium dahliae in the scion stem and rootstock by real-time PCR

Quantification of the amount of *V. dahliae* DNA in the inoculated plants was carried out at 35 and 120 dai by qPCR using the primers and TaqMan probe designed by Bilodeau et al. (2012) and following the procedure described by Díaz-Rueda et al. (2021). Results were expressed as means ± standard errors of the *V. dahliae*:*cox* ratios (ng *V. dahliae* DNA · ng plant DNA^{-1}), previously defined as mean normalized quantity (MNQ) in Garrido et al. (2009). Eight inoculated plants were used at 35 dai (two inoculated plants /block and four blocks), and 16 inoculated plants at 120 dai (four inoculated plants /block and four blocks). Non-inoculated plants were quantified as negative controls.

Plants were removed from the pots and both the rootstock trunck and the stem of the scion were separated and immediately frozen at - 80 ºC until analysis. For each rootstock x scion combination, grafted scions and rootstocks from plants from the same experimental block were grouped, respectively, in a composite sample, giving rise to four biological replicates per plant tissue, rootstock/scion combination, and harvesting time. Moreover, the emergence of new shoots from the rootstock stems were also recorded and photographed in inoculated plants. Theses new shoots were harvested from 'Frantoio', GUA3, AMK27, AMK21 and DHO6A genotypes at 120 dai and *V. dahliae* DNA was quantified and compared to the amount detected in the corresponding 'Picual' grafted scion.

Samples were ground by a blender (Taurus JB1501, Lleida, Spain) and by a mortar in presence of liquid nitrogen until getting a fine powder. Total DNA was extracted using the Isolate II Plant ADN Kit (Bioline, London UK) following the manufacturers' instructions. DNA concentration was accurately determined in duplicate measurements by using a fluorescent spectrophotometer (Modulus™ II Microplate Multimode reader,

Turner Biosystems, USA). PCR reactions and DNA normalization were carried out according to Díaz-Rueda et al. (2021).

Statistical analysis

Statistical analyses were performed with the software R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Data were subjected to the analysis of variance (ANOVA). The means of FMS, RAUDPC, and DNA content of the rootstock and the scion at 35 and 120 dai were compared between each genotype and the means of the grafted reference controls FRA/PIC, ARB/PIC and PIC/PIC by LSD test at P = 0.05. The percentage data were previously transformed by arcsine $(Y/100)^{1/2.}$ Linear regression analyses were performed to estimate relationships between symptomatology parameters (RAUDPC) and the DNA content of the rootstock and stem at 35 and 120 dai, respectively.

Results

Development of symptoms and growth parameters in inoculated plants

No symptoms were developed in non-inoculated, grafted and non-grafted, plants. The inoculated non-grafted 'Picual', 'Arbequina' and 'Frantoio' cultivars used as reference controls, developed symptoms of Verticillium wilt according to their previously established susceptibility level, except for 'Arbequina' that behaved as an extremely susceptible variety in this work (**Table 1, Figures 1 and 2**). Thus, 120 dai 'Picual' plants presented severe wilting symptoms, showing apoplexy and defoliation in all inoculated plants, which exhibited RAUDPC values of 66.97% and FMS values of 4 on the 0-4 symptoms scale (**Table 1**). Although commonly behaving as a moderately susceptible cultivar, 'Arbequina' showed the most severe symptoms, including chlorosis, severe defoliation, and leaf and shoot necrosis, with RAUDPC and FMS values of 81.61%, and 4, respectively (**Table 1**). The low-susceptibility cultivar 'Frantoio' displayed very slight symptoms, showing occasional defoliation or leaf curl, although most of the inoculated plants did not show symptoms. 'Frantoio' plants reached RAUDPC and FMS values of 7.48% and 0.48, respectively (**Table 1**). At the end of the experiment, the percentage of

dead plants (PDP) was 100% for 'Picual' and 'Arbequina', and 9.1% for 'Frantoio' cultivars (**Table 1**).

TABLE 1 | Mean disease parameters assessed in cultivar 'Picual' grafted onto wild olive rootstocks inoculated with the defoliating isolate VD117 of Verticillium dahliae. 'Picual' grafted onto the reference cultivars 'Picual' PIC/PIC (extremely susceptible), 'Arbequina' ARB/PIC (moderately susceptible) and 'Frantoio' FRA/PIC (resistant) are indicated in blue. Non-grafted 'Picual', 'Arbequina' and 'Frantoio' reference controls are indicated in bold.

Varieties[a]	RL[b]	RAUDPC[c]	FMS[d]	PDP[e]
Arbequina	MS	81.61	4.00	100.0
ARB/PIC	MS	68.10	4.00	100.0
Picual	ES	66.97	4.00	100.0
PIC/PIC	ES	64.06	4.00	100.0
ACZ10/PIC	S	63.94	4.00	100.0
GUA8/PIC	ES	62.04	4.00	100.0
ACO15/PIC	R	58.82	4.00	100.0
CEH23/PIC	R	58.66	4.00	100.0
AMK5/PIC	R	54.56	4.00	100.0
DHO6A/PIC	R	54.20	3.75	93.8
AMK21/PIC	MS	46.41	4.00	100.0
GUA3/PIC	R	40.18	2.88	62.5
AMK27/PIC	R	36.20	3.46	81.3
FRA/PIC	R	18.94	1.56	28.6
Frantoio	R	7.48	0.48	9.1

[a] 'Picual' grafted onto genotypes from the SILVOLIVE collection (Díaz-Rueda et al., 2020).
[b]RL: Resistance level of each rootstock genotype according to Díaz-Rueda et al. (2021). ES = extremely susceptible; S = susceptible; MS = moderately susceptible; R = resistant.
[c]RAUDPC: Relative area under the disease progress curve estimated as the percentage with regard to the potential maximum value.
[d]FMS: Final mean severity of symptoms.
[e]PDP: Percentage of dead plants.

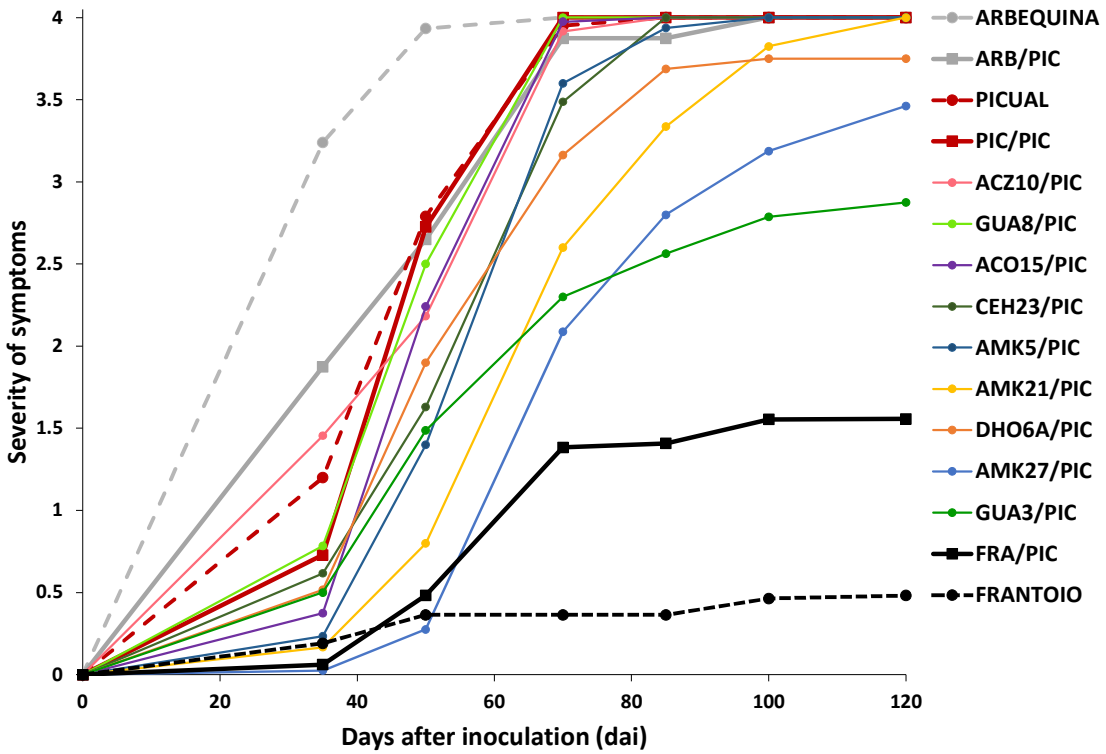


FIGURE 1 | Progress of the severity of symptoms recorded in cultivar 'Picual' grafted onto wild olive rootstocks inoculated with the defolianting isolate VD117 of *Verticillium dahliae*. Values are the means of 24 plants. Severity of symptoms was assessed each time on a 0-4 rating scale, according to the percentage of *M*aximum *I*ntensity *S*ynthoms (*MIS*): apoplexy, chlorosis, leaf and shoot necrosis or defoliation: 0 = 0% *MIS* or no symptoms; 1 = 25% *MIS*; 2 = 50% *MIS*; 3 = 75% *MIS*; 4 = 100% *MIS* or dead plants. Non-grafted 'Picual', 'Arbequina' and 'Frantoio' reference cultivars are indicated in dashed lines.

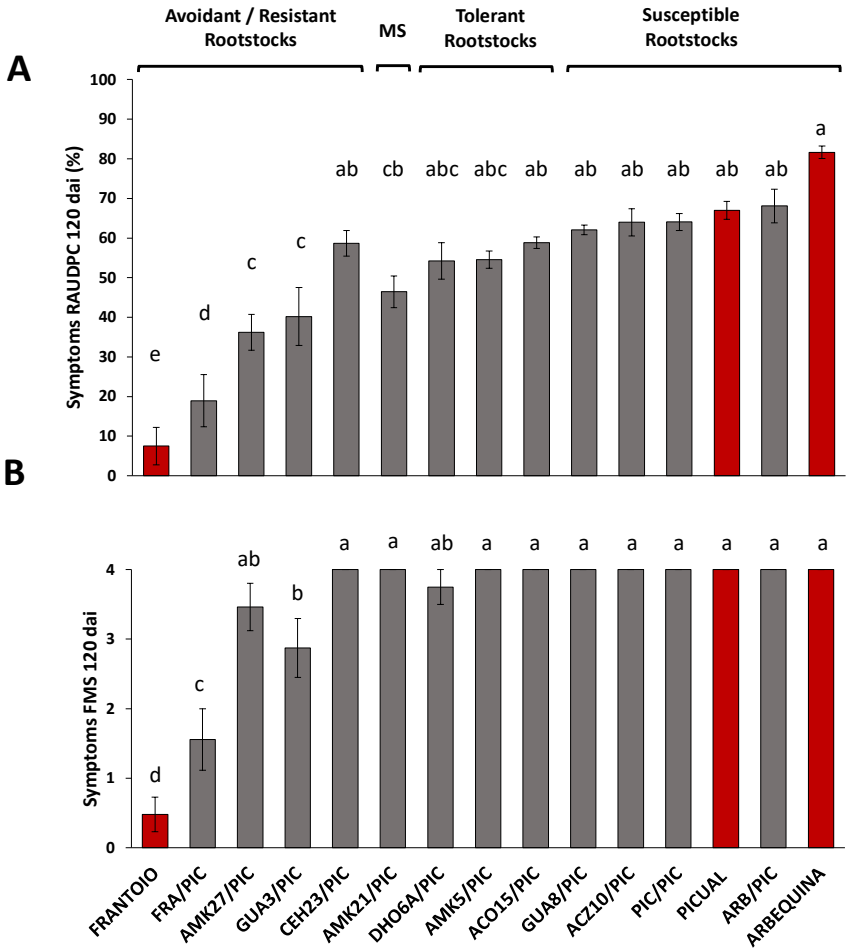


FIGURE 2 | Relative Area Under the Disease Progress Curve (RAUDPC) **(A)** and Final Mean Severity (FMS) **(B)** of cultivar 'Picual' grafted onto different olive rootstocks inoculated with the *Verticillium dahliae* defoliating isolate VD117, at 120 days after inoculation (dai). Red bars correspond to non-grafted reference cultivars: the resistant 'Frantoio', the moderately susceptible 'Arbequina', and the extremely susceptible 'Picual', according to the levels of resistance described by López-Escudero et al. (2007). Results are the mean of 16 plants and error bars correspond to the standard error.

The reference cultivars and the wild genotypes were used as rootstocks of the susceptible scion 'Picual'. It should be noted that the type of grafting procedure did not affect the symptomatology of the inoculated plants, showing not significant differences in the development of the disease (**Supplementary Figure S2**). In the short term (35 dai),'Picual' grafted on 'Picual' (PIC/PIC), 'Picual' grafted on 'Arbequina' (ARB/PIC) and 'Picual' grafted on 'Frantoio' (FRA/PIC) showed lower susceptibility symptoms than the corresponding non-grafted rootstocks (**Supplementary Figure S3A and B**), although this trend changed at 120 dai (**Supplementary Figures S3C and D**). At the end of the assay (120 dai) RAUDPC and FMS values were, respectively: 64.06% and 4 in PIC/PIC; 68.1% and 4 in ARB/PIC; and 18.94% and 1.56 in FRA/PIC (**Table 1**). The non-grafted 'Frantoio'

displayed the lowest susceptibility at 120 dai, wth RAUDPC and FMS values of, 7.5% and 0.5, respectively (**Table 1**; **Supplementary Figures S3C and D**). No PIC/PIC or ARB/PIC plants survived at the end of experiment (120 dai), while the FRA/PIC combination and the ungrafted Frantoio kept 71.4% and 91.9% of the plants alive at the end of experiment. These results confirmed the lower susceptibility to Verticillium wilt of the cultivar 'Frantoio' as previously reported, and confirmed its ability to significantly reduce symptoms when used as a rootstock of a susceptible cultivar (**Figure 1**).

The susceptible scion 'Picual' was also grafted onto the wild olive genotypes. When grafted onto the extremely susceptible genotypes ACZ10 and GUA8, plants showed the highest RAUDPC values (63.94% and 62.04%, respectively) of the whole set of wild olive genotypes tested (**Table 1, Figures 1 and 2**). These susceptible genotypes showed FMS values of 4, and 100% of PDP, not differing significantly from the behaviour exhibited by either the PIC/PIC and ARB/PIC combinations or by the non-grafted 'Picual' and 'Arbequina' cultivars. Interestingly, Picual scions grafted onto the tolerant wild genotypes AMK5, DHO6A and ACO15, also showed high susceptibility to *V. dahliae*, with RAUDPC values between 58.82% and 54.20% and FMS values of 4 (with the exception of DHO6A, with one surviving, symptomless plant 120 dai; **Table 1**). The susceptibility of plants grafted onto the tolerant genotypes was even higher than that of plants grafted onto the moderately susceptible AMK21 (AMK21/PIC), which showed a moderate RAUDPC value of 46.41%, a FMS value of 4, and 100% of PDP.

Except for CEH23, the avoidant/resistant genotypes were the most effective in controlling Verticillium wilt when used as rootstocks of the cultivar 'Picual' (**Table 1, Figures 1 and 2**). GUA3, AMK27 and, especially, the cultivar 'Frantoio' showed lower symptoms than plants grafted onto the tolerant genotypes. Thus, GUA3/PIC, AMK27/PIC and FRA/PIC showed a delay in the development of the disease exhibiting low RAUDPC values (40.18%, 36.20% and 18.94), moderate symptoms (FMS 2.88, 3.46 and 1.56), and PDP of 62.5% and 81.3% and 28.6%, respectively. GUA3/PIC, AMK27/PIC and FRA/PIC plants that remained alive presented no symptoms at the end of experiment. The lowest susceptibility was achieved by the non-grafted 'Frantoio' cultivar. Despite being very efficient in preventing the proliferation of *Verticillium dahliae*, the non-grafted CEH23 genotype presented more symptoms than the other low susceptibility genotypes

(**Supplementary Figure S1**). Moreover, when it was used as a rootstock, the CEH3/PIC combination behaved as susceptible (**Table 1, Figure 1**). Taken together, the results show that avoidant/resistant genotypes are more effective than tolerant genotypes in controlling Verticillium wilt (**Figure 2**).

Inoculated grafted plants exhibited lower growth than non-inoculated grafted plants at 120 dai (**Supplementary Figure S4A and S4B**), but plant growth was less inhibited when the 'Picual' scion was grafted onto the avoidant/resistant genotypes (**Figure 3; Supplementary Figure S4C and S4D)**. Particularly, the avoidant/resistant rootstocks Frantoio, and GUA3 showed the highest increase of scion length and number of nodes relative to non-inoculted plants.

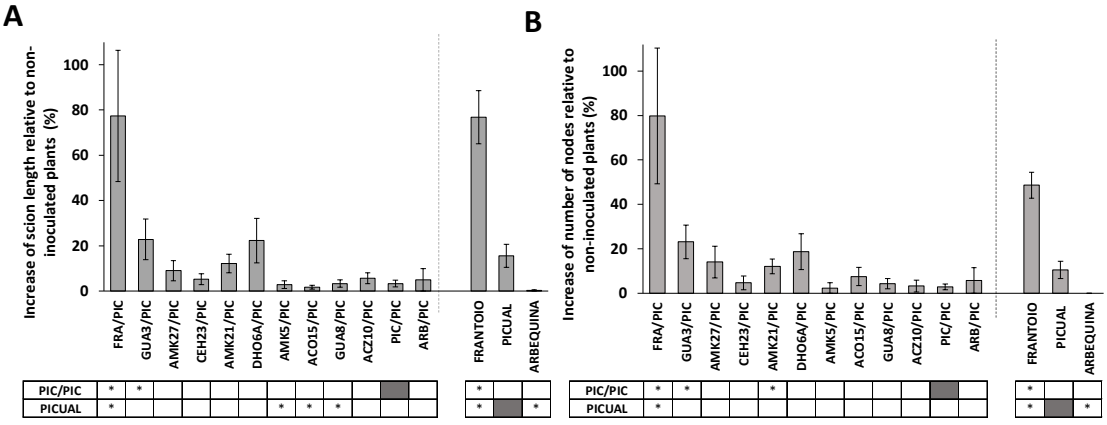


FIGURE 3 | Percentage of increase of scion length **(A)** and number of nodes **(B)** of *Verticillium dahliae*-inoculated 'Picual' x rootstock combinations at 120 days after inoculation (dai), relative to non-inoculated plants. Asterisks indicate significant differences with the inoculated self-grafted 'Picual' (PIC/PIC) and non-grafted 'Picual' plants, respectively. Results are the mean of 12-16 inoculated plants and 3-6 non-inoculated plants. Error bars correspond to the standard error.

Quantification of Verticillium dahliae DNA by real-time PCR

After root inoculation with *V. dahliae*, the degree of infection (fungal proliferation) was measured through direct quantification of *V. dahliae* DNA content in aerial plant tissues. The fungal DNA was quantified in the stem of both the rootstock and the 'Picual' scion, below and above the graft site, respectively, at 35 and 120 dai (**Figure 4**).

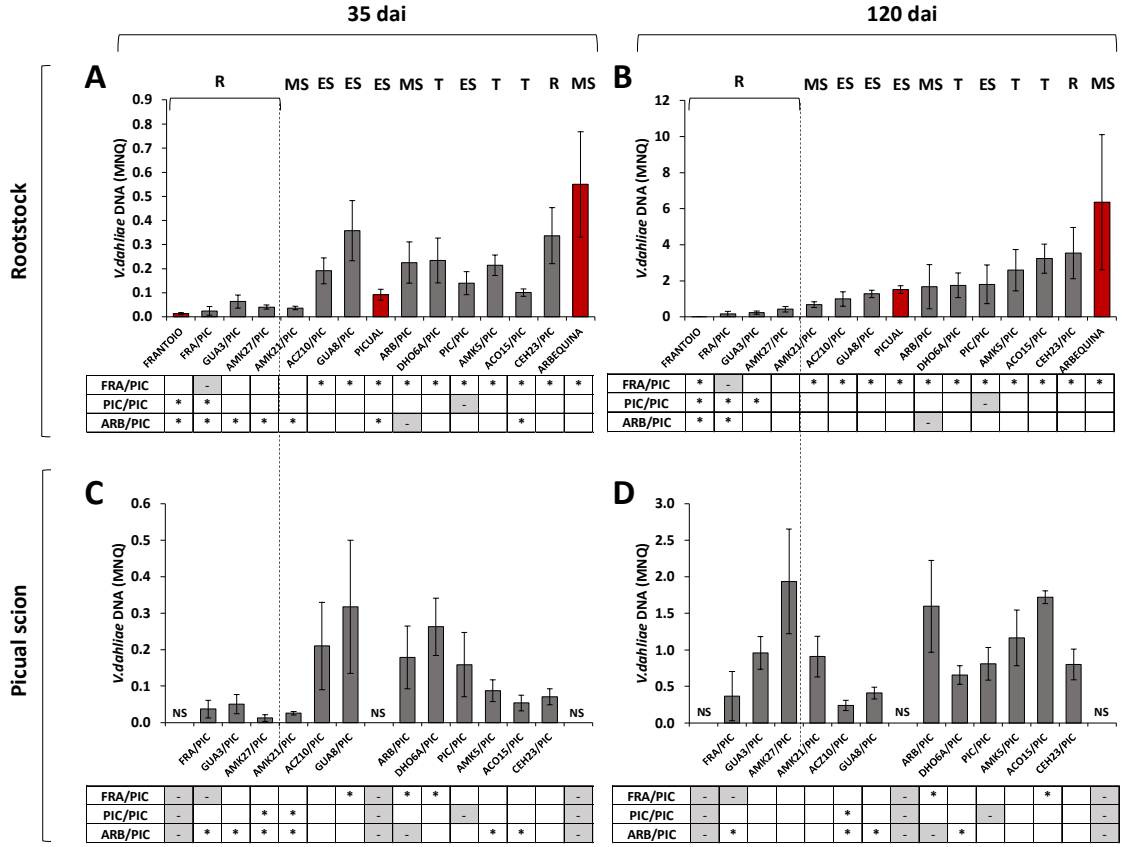


FIGURE 4 | Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA in the stem of grafted olive rootstocks evaluated at 35 days after inoculation (dai) with the defoliating isolate VD117 **(A)** and at 120 dai **(B)**. MNQ of *V. dahliae* DNA in the 'Picual' grafted scion evaluated at 35 dai **(C)** and at 120 dai **(D)**. Red bars represent non-grafted 'Frantoio', 'Arbequina', and 'Picual' plants as reference cultivars of resistant, moderately susceptible, and extremely susceptible genotypes, respectively. Asterisks indicate significant differences with the DNA values of 'Picual' grafted onto 'Frantoio' (FRA/PIC), 'Arbequina' (ARB/PIC), and 'Picual' (PIC/PIC) plants, respectively. NS means No Scion in non-grafted plants. Results are the mean of 16 plants per genotype and error bars correspond to the standard error.

The ungrafted cultivar 'Frantoio' showed the lowest content of *V. dahliae* DNA at both 35 and 120 dai (**Figure 4A and 4B**). In grafted plants, the avoidant/resistant rootstocks (FRA/PIC, AMK27/PIC and GUA3/PIC) had lower amounts of *V. dahliae* DNA than torerant and susceptible rootstocks at 35 and 120 dai (**Figure 4A and 4B**), accordingly to the symptoms previously observed. Tolerant grafted rootstocks (DHO6A/PIC, AMK5/PIC, ACO15/PIC) and susceptible grafted rootstocks (GUA8/PIC, ACZ10/PIC, PIC/PIC) showed statistically significant higher levels of *V. dahliae* DNA in the stem than FRA/PIC at 120 dai (**Figure 4B**). Two wild genotypes showed anomalous performance as rootstocks relative to to their behavior as ungrafted plants (Díaz-Rueda et al., 2021), in close correlation with the symptoms exhibited (**Table 1**). On the one hand, the moderately susceptible genotype AMK21 behaved like a resistant rootstock,

showing low *V. dahliae* DNA levels in the rootstock stem of the grafted AMK21/PIC plant at 35 dai and 120 dai (**Figure 4A and 4B**). On the other hand, the resistant genotype CEH23 behaved like a susceptible rootstock, showing high *V. dahliae* DNA levels in the rootstock stem of the grafted CEH23/PIC plant at 35 dai and 120 dai (**Figure 4A and 4B**).

At 35 dai, Picual scions grafted onto the avoidant/resistant genotypes FRA/PIC, AMK27/PIC and GUA3/PIC and also on the moderately susceptible genotype AMK21/PIC had lower *V. dahliae* DNA levels than those grafted on tolerant and susceptible genotypes (**Figure 4C**). These differences were statistically significant in comparison to the susceptible ARB/PIC plants, but only AMK27/PIC and AMK21/PIC showed significantly lower *V. dahliae* DNA levels in comparison to PIC/PIC. However, at 120 dai, a significant increase in fungal DNA was quantified in the stem of the susceptible scion Picual, even in plants grafted onto resistant genotypes (**Figure 4D**). Thus, the DNA content detected in 'Picual' scions grafted onto the resistant and tolerant wild genotypes did not significantly differ from that quantified in PIC/PIC plants at 120 dai.

The content of *V. dahliae* DNA in the stem of the Picual scion grafted onto the avoidant/resistant genotypes at 120 dai (around 0.3 - 1.9 MNQ) was anormally high if compared to the values previously quantified in the stem of the corresponding ungrafted genotypes (around $0.1 \cdot 10^{-3}$ - $0.5 \cdot 10^{-3}$; **Supplementary Figure S1**). To ascertain which correlation exists between *V. dahliae* DNA content in the rootstock or the scion and the plant symptoms, scatter plots between the RAUDPC and MNQ values were plotted (**Figure 5**). Results indicated that the rootstock is able to control Verticillium wilt according to its degree of susceptibility to *V. dahliae*, as shown by the high correlation between the amount of *V. dahliae* DNA in the rootstock and the plant symptoms (RAUDPC) at both 35 dai (R^2 = 0.7676; **Figure 5A**) and 120 dai (R^2 = 0.7889; **Figure 5B**). However, the ability to control the infection is not adequately transferred to the grafted scion, which shows a worse correlation at 35 dai (R^2 = 0.5034; **Figure 5C**) and a complete loss of correlation at 120 dai (R^2 = 0.0022; **Figure 5D**).

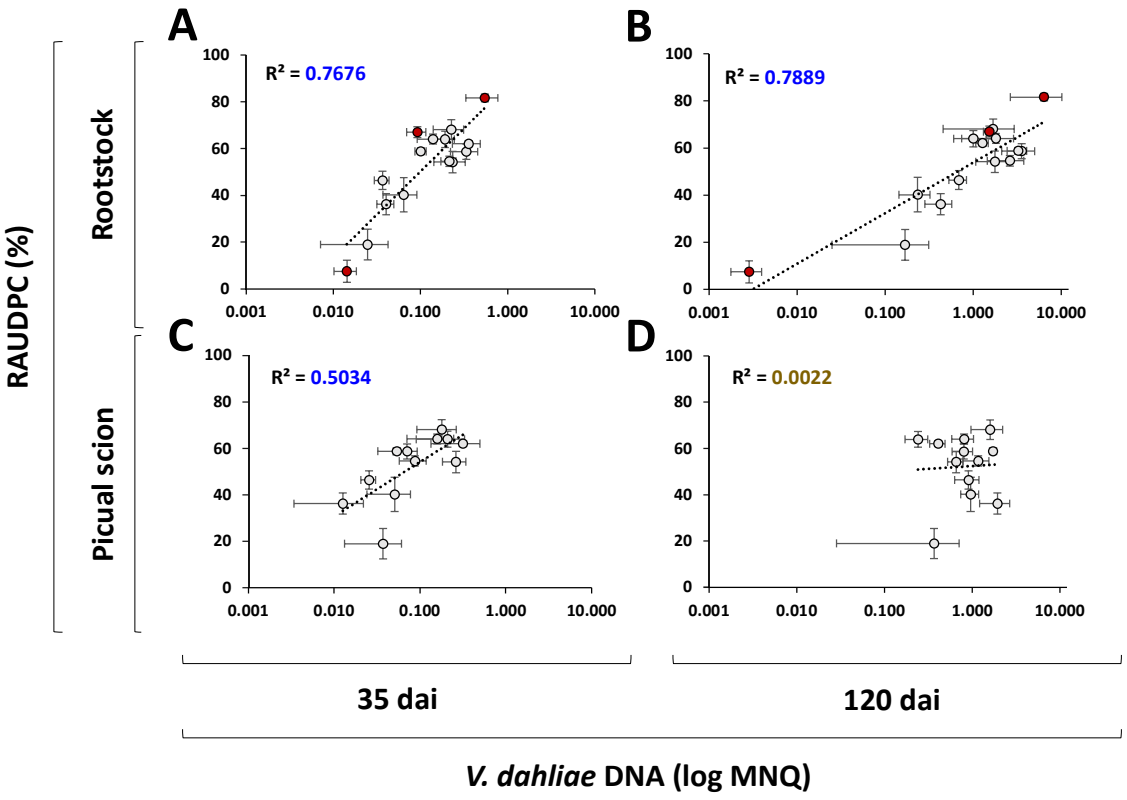


FIGURE 5 | Scatter plots including regression line and coefficient of determination (R^2) for the relationships between the RAUDPC and the Mean Normalized Quantity (log MNQ) of *Verticillium dahliae* DNA in rootstock trunks at 35 dai (**A**) and 120 dai (**B**), and in the Picual scions at 35 dai (**C**) and 120 dai (**D**). Results are the mean of 16 plants. Red points indicate non-grafted 'Frantoio', 'Arbequina' and 'Picual' plants. Error bars in two dimensions indicate the standard error of the mean.

The loss of capacity to control the infection by a low-susceptibility genotype when grafted with a susceptible scion becomes evident when the evolution of the infection over time is analyzed (**Figure 6**). Genotypes that present low susceptibility to *V. dahliae* are characterized by controlling the fungus content, so that at a late infection time (120 dai), the content of the fungus is lower than that at an early infection time (35 dai) as we previously reported in Díaz-Rueda et al., (2021). However, this ability was lost when genotypes of low susceptibility were grafted with the highly susceptible 'Picual' cultivar. Thus, we could observe that the ungrafted 'Frantoio' resistant cultivar reduced the content of *V. dahliae* in the stem at 120 dai, but not when it was used as a rootstock of the grafted 'Frantoio' cultivar (**Figure 6A**). All the genotypes used as rootstocks in this work, including those with low or high susceptibility to *V. dahliae*, showed an increase in fungal DNA at 120 dai compared to 35 dai in both the rootstock (**Figure 6B**) and the scion (**Figure 6C**). Despite the deleterious effect of the scion on the susceptibility to *V.*

dahliae in grafted plants, avoidant/resistant rootstocks still had the lowest fungal DNA levels at both 35 and 120 dai (**Figure 6B**).

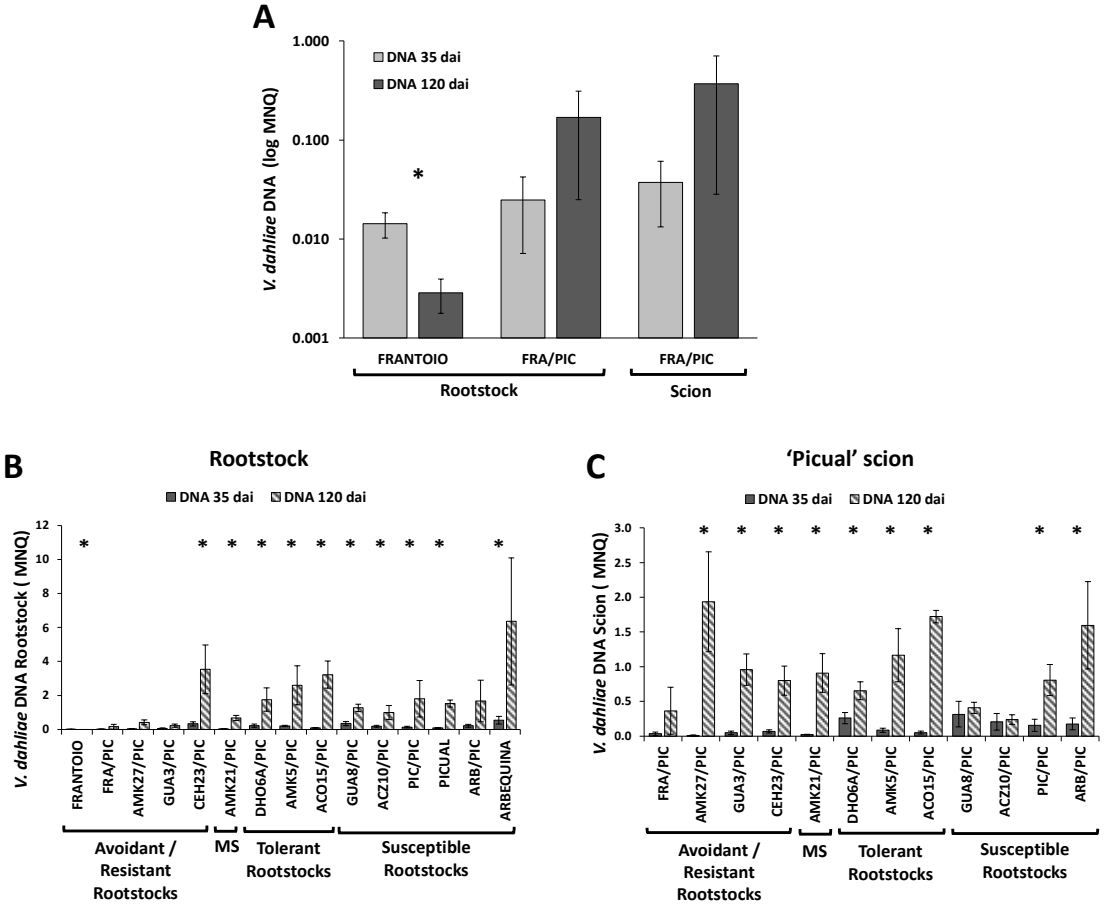


FIGURE 6 | Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA in the rootstock trunks of non-grafted 'Frantoio' and 'Frantoio' grafted with 'Picual' (FRA/PIC) and in the corresponding grafted 'Picual' scion **(A)**, and in the rootstock trunks (**B**) and 'Picual' scions (**C**) of wild olive genotypes grafted with 'Picual' at 35 and 120 days after inoculation (dai) with the *V. dahliae* defoliating isolate VD117. Asterisks indicate significant differences between 35 and 120 dai. Results are the mean of 8 plants at 35 dai and 16 plants at 120 dai, and error bars correspond to the standard error.

As expected, *V. dahliae* DNA levels were lower in the avoidant/resistant rootstocks compared to its corresponding grafted scion after 120 days of infection (**Figure 7**). However, the opposite trend was observed in plants grafted with tolerant and susceptible rootstocks, which contained more *V. dahliae* DNA in the rootstock stem than in the Picual scion stem at 120 dai (**Figure 7**). Especially remarkable, the stem of the tolerant rootstocks CEH23 and ACO15 had higher content of fungal DNA than the rootstock of the tolerant genotypes (**Figure 6B and 7**).

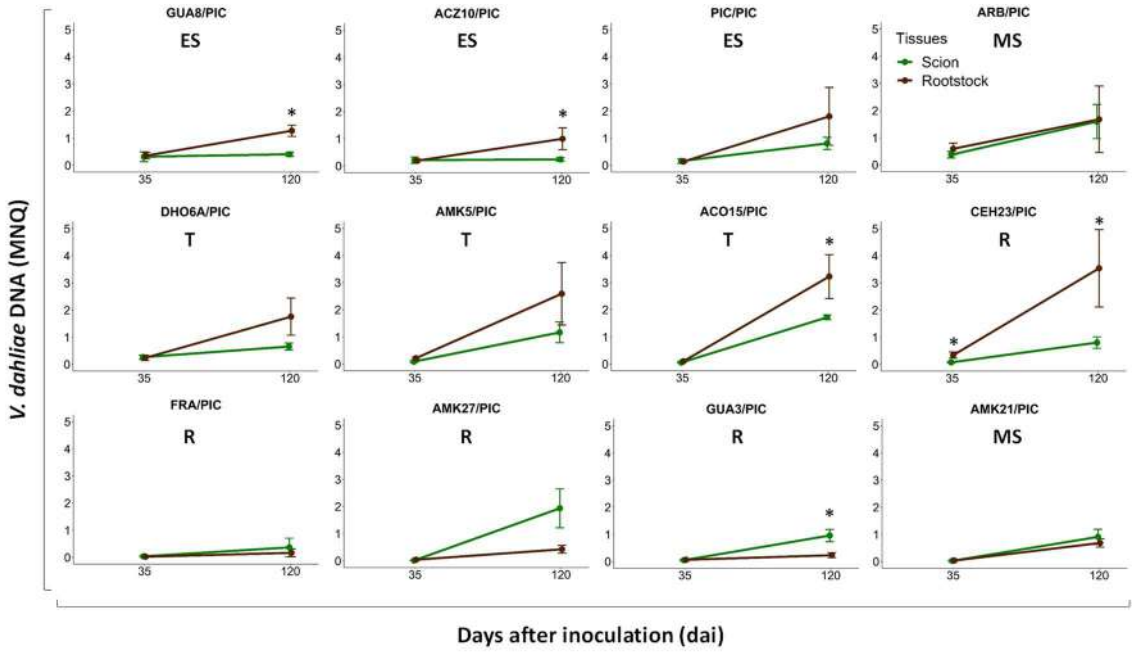


FIGURE 7 | Evolution of *Verticillium dahliae* DNA content in 'Picual' scions (green line) and rootstock trunks (brown line) at 35 and 120 days after inoculation (dai) with the defoliating isolate VD117. Points represent the mean of 4 values (one value per block) for each genotype. Error bars represent the standard error of the mean. Asterisks indicate significant differences between different tissues at the same time.

The ability of avoidant/resistant rootstocks to control *V. dahliae* proliferation resulted in the reduction of wilting in the rootstock stem, with frequent sprouting of new shoots that showed no symptoms. Interestingly, new sprouts contained around 10^2 to 10^5 lower *V. dahliae* levels than the main rootstock stem, regardless of the symptoms observed in the grafted scion (**Supplementary Figure S5**). Conversely, sprouting was much less frequent in susceptible rootstocks, and new sprouts turned necrotic within a few days.

Discussion

The use of resistant rootstocks is one of the most promising and environmentally friendly strategies to control Verticillium wilt of olive. The resistance of the rootstock could be transmitted to the grafted variety, either by avoiding, limiting or delaying the fungus spreading, thus impeding the vascular dissemination during the systemic colonization phase, which is the basis of the plant resistance against *V. dahliae* (Pegg and Brady, 2002; Markakis et al., 2009; Gramaje et al., 2013). The use of resistant rootstocks might provide an improvement in the integrated management of Verticillium

wilt by grafting onto them susceptible cultivars that are agronomically adapted and commercially desirable (Jiménez-Fernández et al., 2016). Therefore, and taking advantage of the characterization of the wild olive SILVOLIVE germplasm collection in terms of *V. dahliae* susceptibility, the potential of grafting susceptible cv. 'Picual' onto resistant rootstocks was evaluated for the control of Verticillium wilt. Two control mechanisms were previously identified among the SILVOLIVE wild genotypes: avoidance/resistance (that limits the proliferantion of the fungus) and tolerance (that allows the fungal proliferation although exhibiting low symptoms) (Díaz-Rueda et al., 2021; **Supplementary Figure S1**). In this point, SILVOLIVE wild olive genotypes showing different degrees of susceptibility were used as rootstocks of the susceptible cv. 'Picual' to discern which of the two mechanisms is more effective for controlling Verticillium wilt of olive plants.

The wild genotypes of *Olea europaea* evaluated in this study present a wide genetic variability (Díaz-Rueda et al., 2020), and belong to the subspecies *guanchica* (GUA3 and GUA8), *laperrinei* (DHO6A), *cuspidata* (CEH23) and *europaea* (ACO15, AMK5, AMK21, AMK27 and ACZ10). Other studies have evaluated the susceptibility to *V. dahliae* in wild genotypes of the subspecies *guanchica*, indicating to be a source of resistance genes (Arias-Calderón et al., 2015c), subsp. *cuspidata*, considered as a powerful transmitter of resistance (Trapero et al., 2015), and subsp. *europaea* (Jiménez-Fernández et al., 2016). However, this is the first study evaluating the use of wild genotypes of the subspecies *laperrinei, guanchica* and *cuspidata* as rootstock for the control of Verticillium wilt of olive. All the wild genotypes showed compatibility with the graft of the 'Picual' variety, even when using genotypes phylogenetically distant to *europaea* subspecies. No significant differences were observed between the two types of grafting tested within the same rootstock / scion combination, in terms of final symptoms or development of the disease (RAUDPC) (**Supplementary Figure S2**). Consequently, the type of graft did not significantly influence the susceptibility of the grafted variety. Grafted plants exhibited adequate growth in terms of branch length and number of nodes, demonstrating that the vascular connections were successfully functional.

Non-grafted 'Frantoio' and 'Picual' reference cultivars used as controls, behaved as resistant and highly susceptible to *V. dahliae*, respectively, as previously reported in controlled conditions (Arias-Calderón et al., 2015a, 2015b; Jiménez-Fernández et al., 2016; Sanei and Razavi, 2017; Anguita-Maeso et al., 2021; Díaz-Rueda et al., 2021). However, non-grafted 'Arbequina' plants showed high susceptibility to *V. dahliae* in this study (**Figures 1 and 2**). This variety has been previously reported as susceptible (López-Escudero and Mercado-Blanco, 2011; Arias-Calderón et al., 2015a), moderately susceptible (Pérez-Rodríguez et al., 2015; Díaz-Rueda et al., 2021) and even as resistant (Serrano et al., 2021) to the defolianting pathotype of *V. dahliae* in controlled conditions.

Picual plants grafted onto the reference cultivars (FRA/PIC, ARB/PIC and PIC/PIC) presented a delay in the appearance of symptoms with respect to the non-grafted reference plants at short times (35 dai). This delay in the onset of symptoms has been previously reported in field trials in which the non-grafted 'Picual' exhibited disease symptoms earlier than the self-grafted 'Picual' (Valverde et al., 2021). The stress caused in the plants by the grafting process has been reported as one of the causes of the vascular occlusion, which would prevent the spread of the pathogen, and could explain the delay in the appearance of the disease at short times (Bubici and Cirulli, 2011). This initial resistance is not maintained at 120 dai in ARB/PIC and PIC/PIC plants which presented similar symptoms than non-grafted inoculated plants. However, 'Picual' grafted onto 'Frantoio' (FRA/PIC) and onto two of the the avoidant/resistant genotypes (GUA3/PIC and AMK27/PIC) presented a disease progression and final symptoms significantly lower than 'Picual' and self-grafted Picual (PIC/PIC) plants at 120 dai (**Figures 1 and 2**). These avoidant/resistant wild genotypes probably display the same control mechanism proposed for 'Frantoio', consisting in a high vascular plugging that limit or delay the colonization of *V. dahliae,* thus providing a partial disease control (Bubici and Cirulli, 2011). The ability to control Verticilliun wilt exhibited by 'Frantoio' and other resistant cultivars can be, however, overcome under moderate and high fungal inoculum density under long-term field conditions, both when used on their own roots (Trapero et al., 2013) as well as when used as rootstocks (Valverde et al., 2021), even under different irrigation systems (Pérez-Rodríguez et al., 2015).

Some genotypes that showed a resistance degree when growth under their own roots did not display the same behabiour when were grafted with a susceptible cultivar. This is the case of the previously identified tolerant genotypes DHO6A, AMK5, ACO15 and the avoidant/resistant genotype CEH23 which were not able to transmit the resistance to the grafted 'Picual' at long times (**Figures 2A and 2B**). Previous susceptibility studies of these non-grafted tolerant genotypes showed reduced symptomatology and low amount of *V. dahliae* DNA in the plant stems (Díaz-Rueda et al., 2021). However, once the fungus overcome the resistance offered by the rootstock, it could spread to the susceptible variety developing wilt symptoms. The opposite scenario occurs when the moderately susceptible genotype AMK21 is used as rootstock, providing better control than the tolerant genotypes. Therefore, the degree of susceptibility to Verticillium wilt of an olive variety does not always predict its performance as a rootstock. In this way, and looking for a way to predict the degree of susceptibility of an olive genotype, some studies have revealed a relationship between functional traists of olive cultivars and the tolerance to *V. dahliae* (Cardoni et al., 2021), being the root system architecture the most relevant difference between tolerant and susceptible cultivars. In addition, a different gene expression pattern have been detected in roots of highly resistant and extremely susceptible cultivars: Susceptible cultivars have higher expression of genes related to root growth and development, while resistant cultivars show a higher expression of genes related to the defense against pathogens and protein turnover (Ramirez-Tejero et al., 2021). Despite these morphological and genetics markers related to tolerance, we would like to highlight the importance of conducting resistance trials on grafted plants to evaluate the ability of new olive genotypes to control the disease.

The inoculated plants that transmitted the partial resistance to the grafted scion FRA/PIC, GUA3/PIC, AMK27/PIC and the moderately susceptible AMK21/PIC were also the ones that showed higher growth parameters than the reference self-grafted 'Picual' (PIC/PIC), although only FRA/PIC plants showed higher growth parameters than those of non-grafted Picual (**Figure 3**).

In addition, these four combinations presented lower amount of *V. dahliae* DNA in the rootstock than in the susceptible grafted 'Picual' stem at 120 dai (**Figures 4 and 7**), conversely to the scenario occurred in the rest of the combinations evaluated. This suggests that the avoidance/resistance mechanism against *V. dahliae* could be carried out in the rootstock which could slow down the growth of the fungus. In addition, the low *V. dahliae* DNA content of the 'Picual' scions relative to the tolerant and susceptible rootstocks could be due to the high percentage of dead plants occurred in these combinations, in which a reduction of the fungal concentration in dead plant tissues could have take place.

GUA8 and ACZ10 rootstocks, previously considered as susceptible genotypes to Verticillium wilt in controlled susceptibility trials (Díaz-Rueda et al., 2021), showed a disease progression and final symptoms similar to those of self-grafted 'Picual', high incidence of the disease, scarce growth after infection, and high amount of *V. dahliae* DNA in the rootstock and scion stems at 35 dai. However, the amount of *V. dahliae* DNA in the 'Picual' scion at 120 dai was the lowest of all the wild genotypes/scion combinations tested, with values very similar to that of the resistant FRA/PIC combination (**Figure 6**). The low amount of *V. dahliae* DNA present in the grafted scion could be due to the development of a hypersensitive response to the fungus in these plants. This hypersensitive response is a form of cell death at the site of pathogen infection that leads to a dysfunction of the vascular bundles of the olive tree, preventing the transport of water and nutrients (100% of the plants were dead 85 days after inoculation) and hence the spread of the fungus towards the grafted variety (Heath, 1998). This type of resistance is activated only against biotrophic or hemi-biotrophic pathogens like *Verticillium* (Chakraborty et al., 2017). Although, this sophisticated defense mechanisms against *V. dahliae* was not efficient to GUA8 and ACZ10 plants.

Adventitious shoots emerged from resistant rootstocks ('Frantoio', AMK27, GUA3 and AMK21) showed an asymptomatic behavior with little or no presence of the pathogen compared to that registered in the rootstock trunk and in the grafted stem (**Supplementary Figure S4**). It has been recently reported that the size of the xylem vessels is related to the resistance of plants to pathogens (Pouzoulet et al., 2017). Plants with wider vessels can loss hydraulic function due to occlusion or embolism triggered by

pathogen infection, resulting in a greater loss of hydraulic conductance compared to narrower vessels (Newbanks et al., 1983; Solla and Gil, 2002; Pérez-Donoso et al., 2007; Pouzoulet et al., 2014). Consequently, hosts with wider vessels could be exposed to higher risks of hydraulic failure upon infection. Therefore, these new emerging shoots probably present narrower vessels could be less exposed to the risks of hydraulic failure after infection, and to a restricted spread of the fungal propagules.

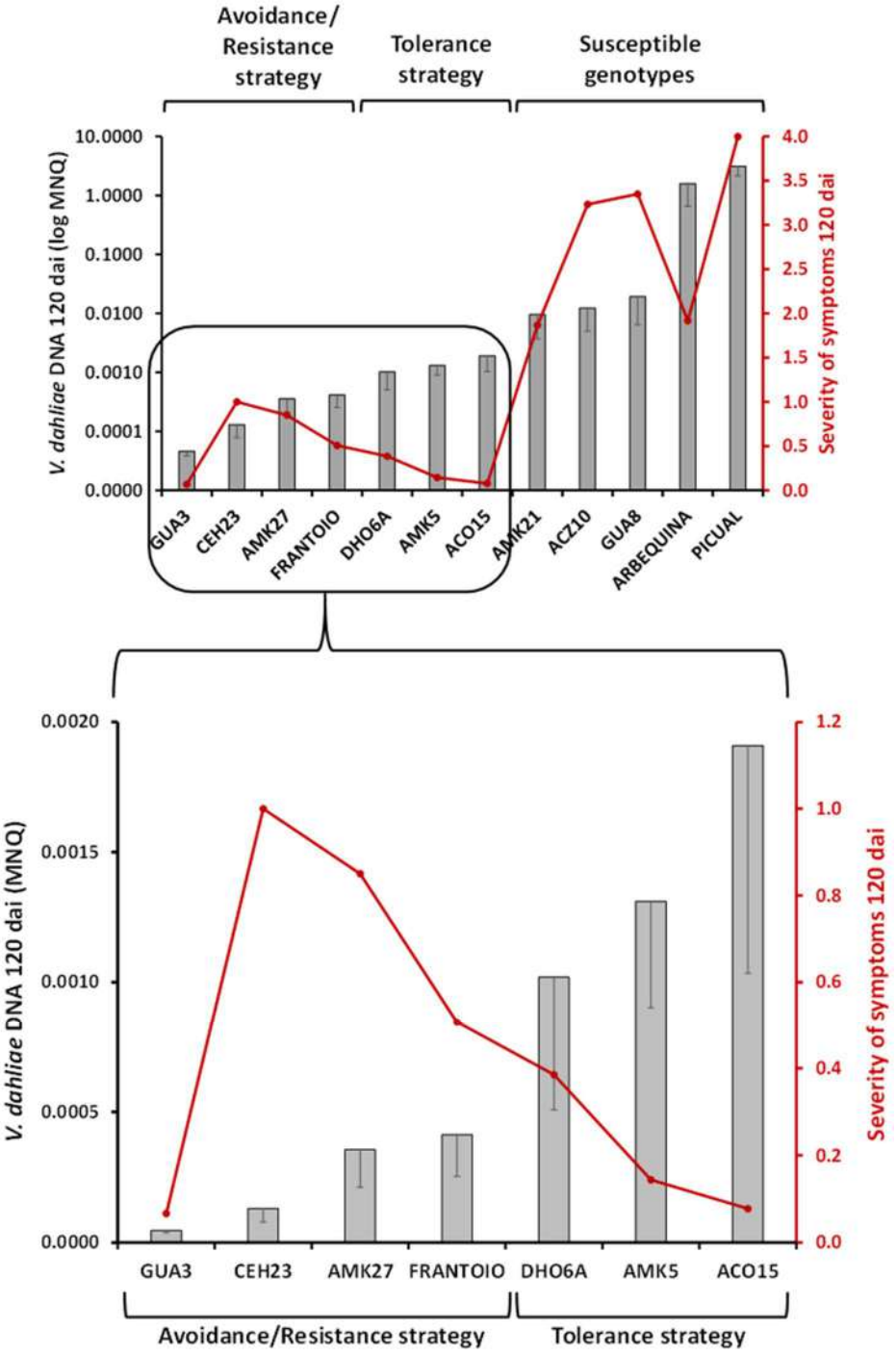
Despite the partial resistance shown by some avoidant/resistant genotypes used as rootstocks, the fungus was able to colonize the grafted 'Picual' variety, even detecting larger amounts of DNA in the stem scion than in the rootstock. Despite this, the disease symptoms of these plants were less severe, and the plants showed greater growth parameters than the self-grafted or non-grafted 'Picual' plants. Some aspects should be considered in this study that could explain the high disease incidence observed and the presence of *V. dahliae* in the 'Picual' scion even when grafted onto resistant roostocks: i) the high virulence of the defoliating isolate V117, which made even 'Arbequina' plants behaved as extremely susceptible to *V. dahliae;* ii) the high inoculum density used in the inoculation tests (10^7 microsclerotia / ml), far from the inoculum densities found in a real scenario: Susceptibility tests carried out under field conditions, determined that the concentration of *V. dahliae* was about 21 microsclerotia / g in a highly infested plot, and 5 microsclerotia / g in a moderately one (Trapero et al., 2015; Valverde et al., 2021); iii) the young plant material used in the study: Young grafted olive plants are more sensitive to environmental stresses, which even implies slight reductions in xylem efficiency, such as xylem cavitation and embolism. An adaptation time is necessary so that the graft and the plant have the same hydraulic resistance (Nardini et al., 2006; Gascó et al., 2007); iv) *in-vitro* micropropagation as a method of obtaining the plant material: The plant material used as rootstocks in this study consisted of micropropagated plants rooted *in-vitro*. It has been shown that *in-vitro* micropropagated plants show an alteration in the composition of the xylem microbiome, with olive trees from aseptic propagation being more susceptible to the defoliant pathotype of *V. dahliae* than those obtained by conventional nursery multiplication (Anguita-Maeso et al., 2021); v) the hydraulic resistances of the graft union in the new vascular connections of the grafted plants. All these characteristics make the plants more susceptible to the pathogen. From this point

of view, avoidant/resistant genotypes used as rootstocks restricts the spread of the fungus inside the rootstock could be more effective control measure of Verticillium wilt of olive in soils under conditions of low or moderate inoculum density (López-Escudero and Mercado-Blanco, 2011). Moreover, the use of other control measures of Verticillium wilt such as irrigation control or the use of several biological control agents (BCAs) such as *Pseudomonas Fluorescens* (Maldonado-González et al., 2015) or *Trichoderma harzianum* (Carrero-Carrón et al., 2018) could complete the integrated management strategy of *V. dahliae* in olive cultivation (Montes-Osuna and Mercado-Blanco, 2020; Mulero-Aparicio et al., 2020). Resistant rootstocks have been successfully applied to control *V. dahliae* in different herbaceous species as watermelon (Devi et al., 2021), eggplant (Attavar and Miles, 2021) and tomato (Papadaki, A. M. et al., 2017), and also in different woody species such as avocado (Haberman et al., 2020) and pistachio (Epstein et al., 2004). The evaluation of new olive material from the SILVOLIVE collection with high level of resistance (Díaz-Rueda et al., 2021) or from other collections (Arias-Calderón et al., 2015b; Trapero et al., 2015), could provide rootstocks capable of effectively controlling Verticillium wilt of olive.

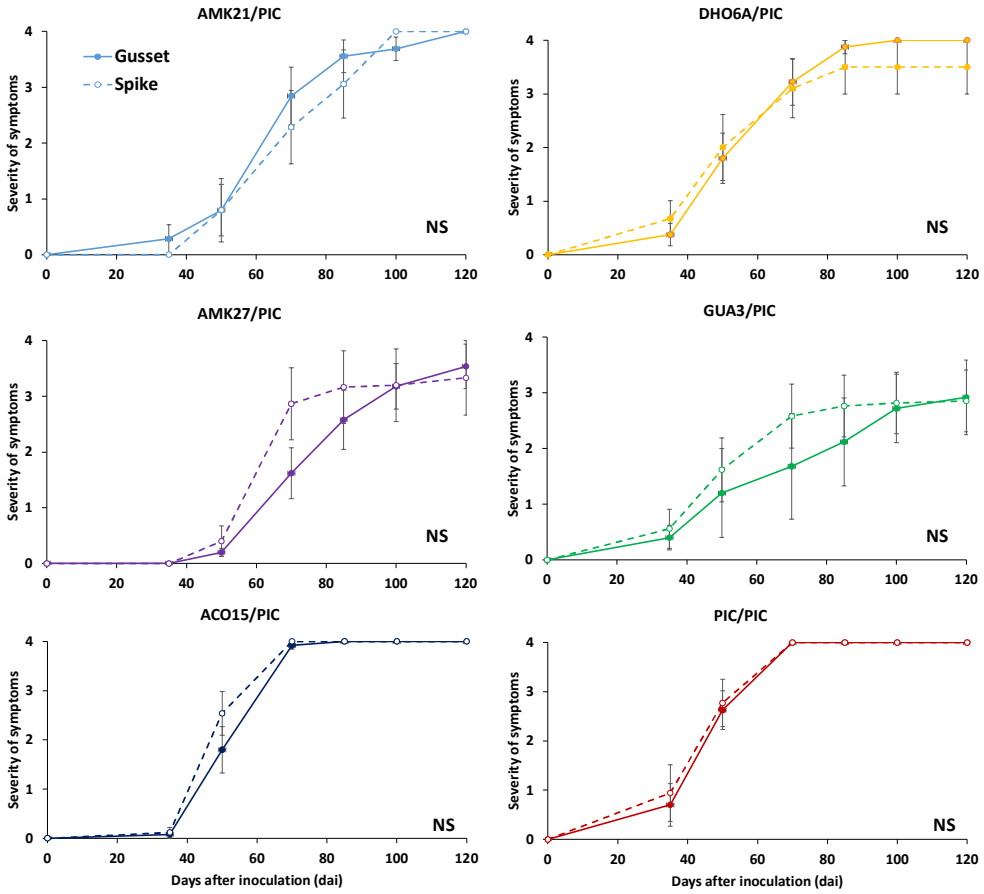
Conclusion

The degree of susceptibility to Verticillium wilt of an olive variety does not always predict its performance as a rootstock, so that grafting experiments are advised. Our results reveal that, in general, avoidant/resistant rootstocks are more effective than tolerant rootstocks in reducing the susceptibility of the grafted plant to V. dahliae. However long-term field experiments under moderate fungal pressure and the evaluation of new olive material from the SILVOLIVE or other collections are necessary, to provide rootstocks capable of effectively controlling Verticillium wilt of olive.

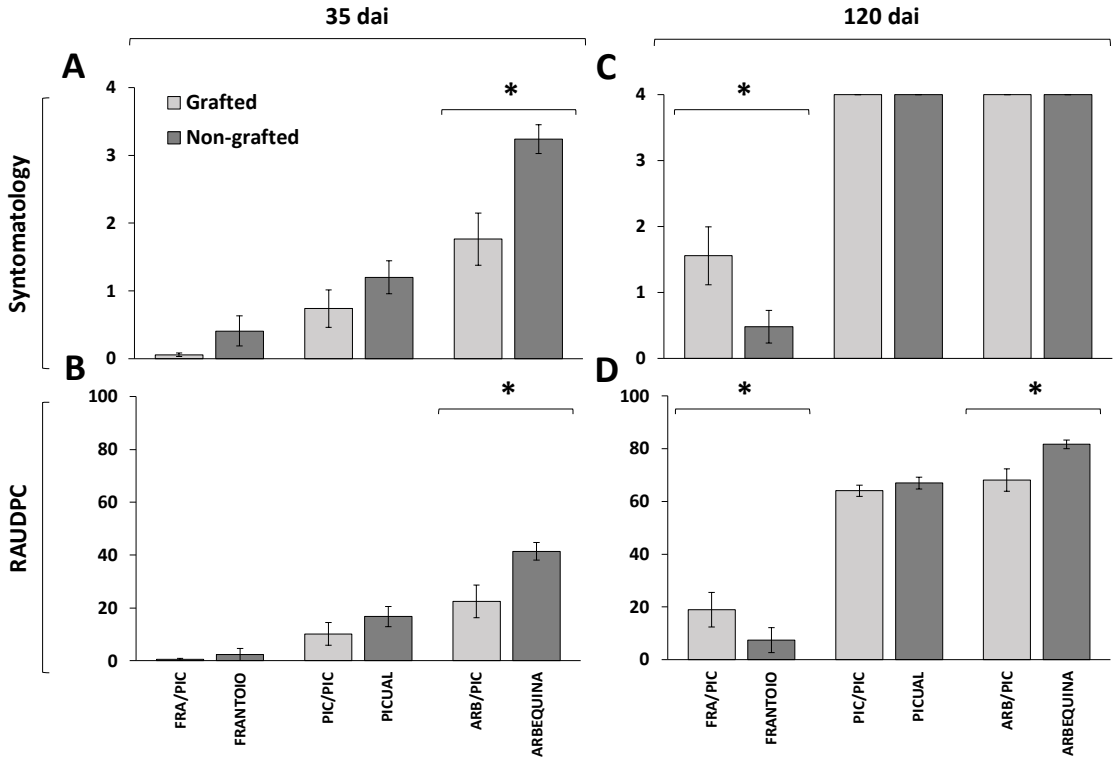
Supplementary Materials



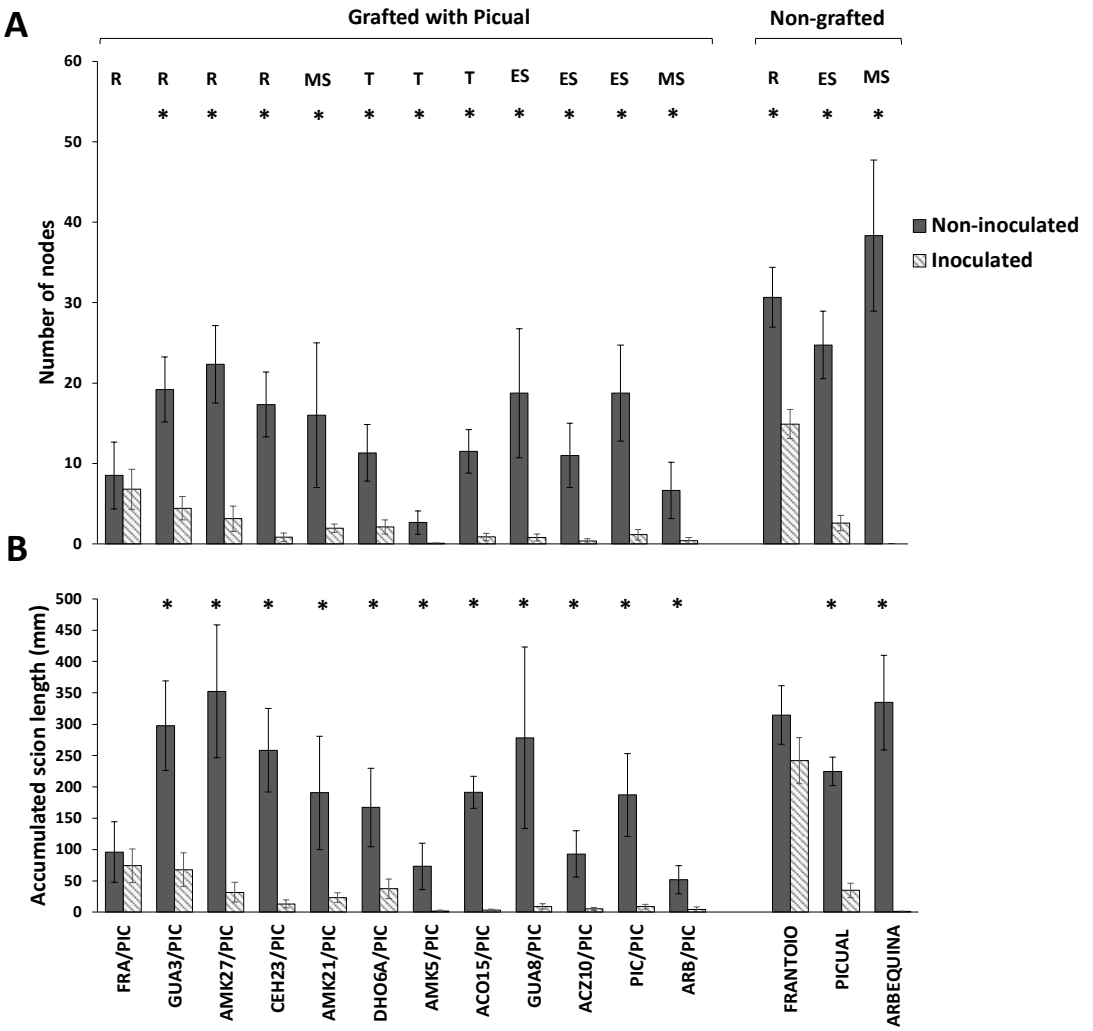
SUPPLEMENTARY FIGURE S1 | Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA and severity of symptoms in non-grafted wild olive genotypes and reference cultivars 'Frantoio', 'Picual' and 'Arbequina' at 120 days after inoculation (dai), as described by Díaz-Rueda et al. (2021).



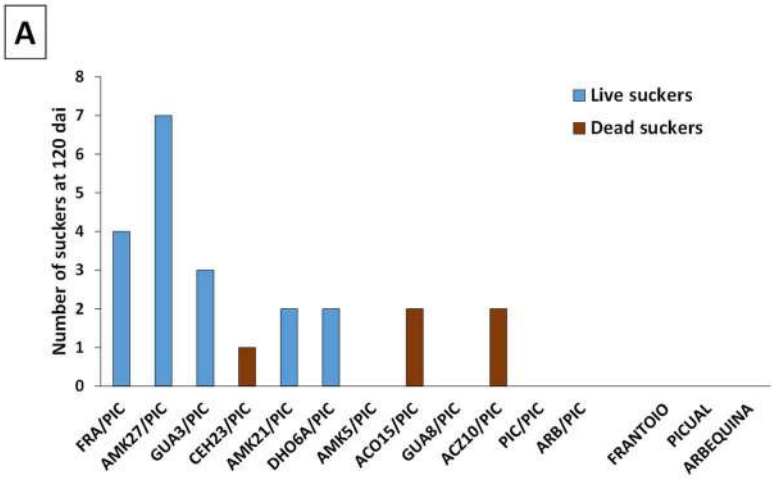
SUPPLEMENTARY FIGURE S2 | Progress of the severity of symptoms (in a 0-4 scale) of cv. 'Picual' onto wild olive rootstocks inoculated with the defolianting isolate VD117 of *Verticillium dahliae* grafted by two different methods: 'skipe' (dashed line) and 'gusset' (line). Values are the means of 8 plants for each grafting method and error bars represent the standard error.



SUPPLEMENTARY FIGURE S3 | Symptoms of olive reference cultivars 'Frantoio, 'Picual' and 'Arbequina' non-grafted and grafted with 'Picual' scions, inoculated with the *Verticillium dahliae* defoliating isolate VD117. (A) Final Mean Severity (FMS) and (B) Relative area under the disease progress curve (RAUDPC) at 35 days after inoculation (dai); (C) FMS and (D) RAUDPC at 120 dai. Asterisks indicate significant differences between grafted and non-grafted plants.

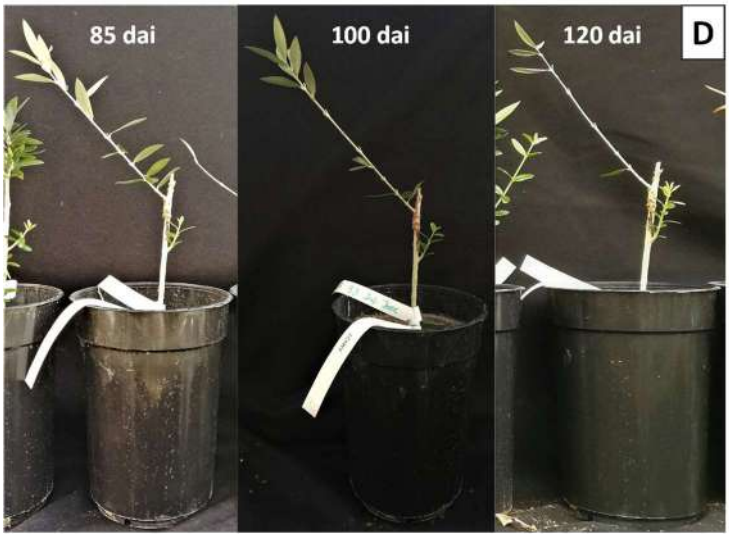


SUPPLEMENTARY FIGURE S4 | (A) Number of nodes and (B) accumulated lenght of secondary and tertiary shoots branches of 'Picual' scions grafted onto different rootstocks inoculated with *Verticillium dahliae* (striped bars) and non-inoculated (dark bars) at 120 days after inoculation (dai). Non-grafted 'Frantoio', 'Picual' and 'Arbequina' olives were used as controls (right). Asterisks indicate significant differences between inoculated and non-inoculated plants. Results are the mean of 12-16 inoculated plants and 3-6 non-inoculated plants. Error bars correspond to the standard error.



C. Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA in shoots emitted from rootstock olives

Combination (Rootstock/Scion)	Block	Time	DNA Shoot	DNA Scion	DNA Rootstock	FMS	Scion *vs* Shoot	Rootstock *vs* Shoot
FRA/PIC	1	120	8.18782E-05	0.020	0.022	0.4	248	264
AMK27/PIC	1	120	5.3428E-05	0.432	0.026	3.4	8078	489
AMK27/PIC	1	120	2.36196E-07	0.432	0.026	4	1827279	110573
AMK27/PIC	1	120	8.47574E-06	0.432	0.026	0	50921	3081
AMK27/PIC	2	120	9.28049E-07	1.490	0.611	4	1605859	658182
AMK27/PIC	4	120	6.87641E-05	3.867	0.399	4	56236	5808
AMK27/PIC	4	120	7.80638E-05	3.867	0.399	4	49537	5116
AMK21/PIC	2	120	0.000322394	1.729	0.756	4	5363	2346
AMK21/PIC	2	120	0.00334434	1.729	0.756	4	517	226
GUA3/PIC	1	120	5.67065E-06	0.974	0.032	1.6	171716	5619
GUA3/PIC	3	120	6.16139E-05	0.042	0.570	1	678	9257
GUA3/PIC	3	120	0.000763514	0.002	0.031	0	2	41
DHO6A/PIC	1	120	5.26809E-07	0.320	0.354	0	608126	672034



SUPPLEMENTARY FIGURE S5 | (A) Number of rootstock suckers for each genotype at 120 days after inoculation (dai) (Blue bars correspond to alive suckers and brown bars correspond to dead suckers). (B) Example of an inoculated AMK27 plant at 120 dai showing dead 'Picual' scion and asymptomatic suckers grown from the rootstock. (C) Mean Normalized Quantity (MNQ) of *Verticillium dahliae* DNA in rootstock suckers at 120 dai with the *V. dahliae* defoliating isolate VD117. (D) Examples of inoculated AMK27/PIC plants at 85, 100 and 120 dai, showing severity of symptoms (defoliation and necrosis) on 'Picual' scion and asymptomatic rootstock suckers.

References

Anguita-Maeso, M., Trapero-Casas, J. L., Olivares-García, C., Ruano-Rosa, D., Palomo-Ríos, E., Jiménez-Díaz, R. M., et al. (2021). *Verticillium dahliae* Inoculation and in vitro Propagation Modify the Xylem Microbiome and Disease Reaction to Verticillium Wilt in a Wild Olive Genotype. *Front. Plant Sci.* 12, 632689. doi: 10.3389/fpls.2021.632689.

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and Rodríguez-Jurado, D. (2015a). Resistance to Verticillium wilt in olive progenies from open-pollination. *Sci. Hortic.* 185, 34–42. doi: 10.1016/j.scienta.2015.01.015.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and León, L. (2015b). Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. *Euphytica* 206, 619–629. doi: 10.1007/s10681-015-1463-7.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015c). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi: 10.1016/j.cropro.2015.05.006.

Attavar, A. S., and Miles, C. A. (2021). Screening World Vegetable Center eggplant and pepper rootstocks for resistance to Verticillium wilt. *Acta Hortic.*, 155–162. doi: 10.17660/ActaHortic.2021.1302.21.

Attavar, A., Tymon, L., Perkins-Veazie, P., and Miles, C. A. (2020). Cucurbitaceae Germplasm Resistance to Verticillium Wilt and Grafting Compatibility with Watermelon. *Hortscience* 55 (2), 141–148. doi: 10.21273/hortsci14631-19.

Baroudy, F., Habib, W., Tanos, G., Gerges, E., Saab, C., Choueiri, E., et al. (2018). Long-Distance Spread of Verticillium dahliae Through Rivers and Irrigation Systems. *Plant Dis.* 102, 1559–1565. doi: 10.1094/PDIS-08-17-1189-RE.

Bilodeau, G. J., Koike, S. T., Uribe, P., and Martin, F. N. (2012). Development of an Assay for Rapid Detection and Quantification of *Verticillium dahliae* in Soil. *PHYTOPATHOLOGY* 102, 331–343. doi: 10.1094/PHYTO-05-11-0130.

Blanco-López, M. A., Jiménez-Díaz, R. M., and Caballero, J. M. (1984). Symptomatology, incidence and distribution of Verticillium wilt of olive tree in Andalucía. *Phytopathol. Mediterr.* 23, 1–8.

Bubici, G., and Cirulli, M. (2011). *"Verticillium wilt of olives," in Olive Diseases and Disorders, eds L. Schena, G. E. Agosteo, and S. O. Cacciola (Kerala: Research Signpost).*

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9.

Campbell, C. L., and Madden, L. V. (1990). *Introduction to plant disease epidemiology*. New York: Wiley.

Cardoni, M., Mercado-Blanco, J., and Villar, R. (2021). Functional Traits of Olive Varieties and Their Relationship with the Tolerance Level towards Verticillium Wilt. *Plants* 10, 1079. doi: 10.3390/plants10061079.

Carrero-Carrón, I., Rubio, M. B., Niño-Sánchez, J., Navas-Cortés, J. A., Jiménez-Díaz, R. M., Monte, E., et al. (2018). Interactions between *Trichoderma harzianum* and defoliating *Verticillium dahliae* in resistant and susceptible wild olive clones. *Plant Pathol.* 67, 1758–1767. doi: 10.1111/ppa.12879.

Carrero-Carrón, I., Trapero-Casas, J. L., Olivares-García, C., Monte, E., Hermosa, R., and Jiménez-Díaz, R. M. (2016). *Trichoderma asperellum* is effective for biocontrol of Verticillium wilt in olive caused

by the defoliating pathotype of *Verticillium dahliae*. *Crop Prot.* 88, 45–52. doi: 10.1016/j.cropro.2016.05.009.

Chakraborty, S., Moeder, W., and Yoshioka, K. (2017). "Plant Immunity," in *Reference Module in Life Sciences* (Elsevier), B9780128096338120000. doi: 10.1016/B978-0-12-809633-8.12154-5.

Connor, D. J., Gómez-del-Campo, M., Rousseaux, M. C., and Searles, P. S. (2014). Structure, management and productivity of hedgerow olive orchards: A review. *Sci. Hortic.* 169, 71–93. doi: 10.1016/j.scienta.2014.02.010.

Devi, P., Tymon, L., Keinath, A., and Miles, C. A. (2021). Progress in grafting watermelon to manage Verticillium wilt. *Plant Pathol.* 70, 767–777.

Deyett, E., Pouzoulet, J., Yang, J. -I., Ashworth, V. E., Castro, C., Roper, M. C., et al. (2019). Assessment of Pierce's disease susceptibility in *Vitis vinifera* cultivars with different pedigrees. *Plant Pathol.* 68, 1079–1087. doi: 10.1111/ppa.13027.

Díaz-Rueda, P., Aguado, A., Romero-Cuadrado, L., Capote, N., and Colmenero-Flores, J. M. (2021). Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating *Verticillium dahliae*. *Front. Plant Sci.* 12, 662060. doi: 10.3389/fpls.2021.662060.

Díaz-Rueda, P., Franco-Navarro, J. D., Messora, R., Espartero, J., Rivero-Núñez, C. M., Aleza, P., et al. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Front. Plant Sci.* 11, 629. doi: 10.3389/fpls.2020.00629.

Epstein, L., Beede, R., Kaur, S., and Ferguson, L. (2004). Rootstock Effects on Pistachio Trees Grown in *Verticillium dahliae*-Infested Soil. *Am. Phytopathol. Soc.* 94, 388–395.

Garrido, C., Carbu, M., Fernandez-Acero, F. J., Boonham, N., Colyer, A., Cantoral, J. M., et al. (2009). Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. *Plant Pathol.* 58, 43–51. doi: 10.1111/j.1365-3059.2008.01933.x.

Gascó, A., Nardini, A., Raimondo, F., Gortan, E., Motisi, A., Lo Gullo, M. A., et al. (2007). Hydraulic kinetics of the graft union in different *Olea europaea* L. scion/rootstock combinations. *Environ. Exp. Bot.* 60, 245–250. doi: 10.1016/j.envexpbot.2006.10.011.

Gharbi, Y., Barkallah, M., Bouazizi, E., Cheffi, M., Gdoura, R., and Triki, M. A. (2016). Differential fungal colonization and physiological defense responses of new olive cultivars infected by the necrotrophic fungus *Verticillium dahliae*. *Acta Physiol. Plant.* 38, 242. doi: 10.1007/s11738-016-2261-0.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017). Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 97, 30–39. doi: 10.1016/j.pmpp.2016.12.001.

Gómez-Lama Cabanás, C., Ruano-Rosa, D., Legarda, G., Pizarro-Tobías, P., Valverde-Corredor, A., Triviño, J., et al. (2018). Bacillales Members from the Olive Rhizosphere Are Effective Biological Control Agents against the Defoliating Pathotype of *Verticillium dahliae*. *Agriculture* 8, 90. doi: 10.3390/agriculture8070090.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2015). Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00928.

González, M., Pérez-Sierra, A., and Sánchez, M. E. (2019). *Phytophthora oleae*, a new root pathogen of wild olives. *Plant Pathol.* 68, 901–907. doi: 10.1111/ppa.13024.

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A Comparison of Real-Time PCR Protocols for the Quantitative Monitoring of Asymptomatic Olive Infections by *Verticillium dahliae* Pathotypes. *Phytopathology®* 103, 1058–1068. doi: 10.1094/PHYTO-11-12-0312-R.

Gucci, R., Lodolini, E., and Rapoport, H. F. (2007). Productivity of olive trees with different water status and crop load. *J. Hortic. Sci. Biotechnol.* 82, 648–656. doi: 10.1080/14620316.2007.11512286.

Haberman, A., Tsror (Lahkim), L., Lazare, S., Hazanovsky, M., Lebiush, S., Zipori, I., et al. (2020). Management of Verticillium Wilt of Avocado Using Tolerant Rootstocks. *Plants* 9, 531. doi: 10.3390/plants9040531.

Hartmann, Schnathorst, W.C, and Whisler, J.E. (1971). Oblonga, a clonal olive rootstock resistant to verticillium wilt. *Calif. Agric.* 25, 12–15.

Heath, M. C. (1998). Apoptosis, programmed cell death and the hypersensitive response. *Eur. J. Plant Pathol.* 104, 117–124. doi: 10.1023/A:1008645520976.

Hoagland, D., and Arnon, D. (1950). The water culture method for growing plants without soils. *Berkeley Calif. Agric. Expermental Stn.* 32.

Inch, S. A., and Ploetz, R. C. (2012). Impact of laurel wilt, caused by *Raffaelea lauricola*, on xylem function in avocado, *Persea americana*: Impact of laurel wilt on avocado. *For. Pathol.* 42, 239–245. doi: 10.1111/j.1439-0329.2011.00749.x.

Jiménez-Díaz, R., Cirulli, M, Bubici, G, Jiménez-Gasco, MD, Antoniou, PP, and Tjamos, EC (2012). Verticillium Wilt, A Major Threat to Olive Production: Current Status and Future Prospects for its Management. *Plant Dis.* 96, 304–329. doi: 10.1094/PDIS-06-11-0496.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516.

Leyva-Pérez, M. de la O., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J. B., Luque, F., et al. (2018). Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. *New Phytol.* 217, 671–686. doi: 10.1111/nph.14833.

Lodolini, E. M., Polverigiani, S., Ali, S., Mutawea, M., Qutub, M., Pierini, F., et al. (2016). Effect of complementary irrigation on yield components and alternate bearing of a traditional olive orchard in semi-arid conditions. *Span. J. Agric. Res.* 14, e1203. doi: 10.5424/sjar/2016142-8834.

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of Olive Cultivars for Resistance to *Verticillium dahliae*. *Eur. J. Plant Pathol.* 110, 79–85. doi: 10.1023/B:EJPP.0000010150.08098.2d.

López-Escudero, F. J., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50. doi: 10.1007/s11104-010-0629-2.

López-Escudero, F. J., Mwanza, C., and Blanco-López, M. A. (2007). Reduction of *Verticillium dahliae* microsclerotia viability in soil by dried plant residues. *Crop Prot.* 26, 127–133. doi: 10.1016/j.cropro.2006.04.011.

Maldonado-González, M. M., Bakker, P. A. H. M., Prieto, P., and Mercado-Blanco, J. (2015). Arabidopsis thaliana as a tool to identify traits involved in Verticillium dahliae biocontrol by the olive root endophyte Pseudomonas fluorescens PICF7. *Front. Microbiol.* 06. doi: 10.3389/fmicb.2015.00266.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Paplomatas, E. J., and Tjamos, E. C. (2009). Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. *Eur. J. Plant Pathol.* 124, 603–611. doi: 10.1007/s10658-009-9447-x.

Marra, F. P., Marino, G., Marchese, A., and Caruso, T. (2016). Effects of different irrigation regimes on a super-high-density olive grove cv. "Arbequina": vegetative growth, productivity and polyphenol content of the oil. *Irrig. Sci.* 34, 313–325. doi: 10.1007/s00271-016-0505-9.

McCain, A. H., and Raabe, R. D. (2002). Plants Resistant or Susceptible to Verticillium Wilt. *Univ. Calif. Div. Agric. Nat. Resour.* Available at: http://anrcatalog.ucdavis.edu.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiol. Mol. Plant Pathol.* 63, 91–105. doi: 10.1016/j.pmpp.2003.10.001.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., and Jiménez-Díaz, R. M. (2004). Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biol. Control* 30, 474–486. doi: 10.1016/j.biocontrol.2004.02.002.

Mol, L. (1995). Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*: II. Quantitative analysis of the luring effect of crops. *Eur. J. Plant Pathol.* 101, 679–685. doi: 10.1007/BF01874872.

Montes-Osuna, N., and Mercado-Blanco, J. (2020). Verticillium Wilt of Olive and Its Control: What Did We Learn during the Last Decade? *Plants* 9, 735. doi: 10.3390/plants9060735.

Morello, P., Díez, C. M., Codes, M., Rallo, L., Barranco, D., Trapero, A., et al. (2016). Sanitation of olive plants infected by *Verticillium dahliae* using heat treatments. *Plant Pathol.* 65, 412–421. doi: 10.1111/ppa.12432.

Mulero-Aparicio, A., Varo, A., Agustí-Brisach, C., López-Escudero, F. J., and Trapero, A. (2020). Biological control of Verticillium wilt of olive in the field. *Crop Prot.* 128, 104993. doi: 10.1016/j.cropro.2019.104993.

Nardini, A., Gasco, A., Raimondo, F., Gortan, E., Lo Gullo, M. A., Caruso, T., et al. (2006). Is rootstock-induced dwarfing in olive an effect of reduced plant hydraulic efficiency? *Tree Physiol.* 26, 1137–1144. doi: 10.1093/treephys/26.9.1137.

Newbanks, D., Bosch, A., and Zimmermann, M. (1983). Evidence for Xylem Dysfunction by Embolization in Dutch Elm Disease. *Phytopathology* 73, 1060. doi: 10.1094/Phyto-73-1060.

Papadaki, A. M., Bletsos, F. A., Eleftherohorinos, I. G., Menexes, G., and Lagopodi, A. L. (2017). Effectiveness of seven commercial rootstocks against verticillium wilt and their effects on growth, yield, and fruit quality of tomato. *Crop Prot.* 102, 25–31. doi: 10.1016/j.cropro.2017.08.006.

Papasotiriou, F. G., Varypatakis, K. G., Christofi, N., Tjamos, S. E., and Paplomatas, E. J. (2013). Olive mill wastes: A source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. *Biol. Control* 67, 51–60. doi: 10.1016/j.biocontrol.2013.07.008.

Pegg, G. F., and Brady, B. L. eds. (2002). *Verticillium wilts*. Wallingford: CABI doi: 10.1079/9780851995298.0000.

Pérez-Donoso, A. G., Greve, L. C., Walton, J. H., Shackel, K. A., and Labavitch, J. M. (2007). *Xylella fastidiosa* Infection and Ethylene Exposure Result in Xylem and Water Movement Disruption in Grapevine Shoots. *Plant Physiol.* 143, 1024–1036. doi: 10.1104/pp.106.087023.

Pérez-Rodríguez, M., Alcántara, E., Amaro, M., Serrano, N., Lorite, I. J., Arquero, O., et al. (2015). The Influence of Irrigation Frequency on the Onset and Development of Verticillium Wilt of Olive. *Plant Dis.* 99, 488–495. doi: 10.1094/PDIS-06-14-0599-RE.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1.

Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., and Rolshausen, P. E. (2014). Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Front. Plant Sci.* 5. doi: 10.3389/fpls.2014.00253.

Pouzoulet, J., Scudiero, E., Schiavon, M., and Rolshausen, P. E. (2017). Xylem Vessel Diameter Affects the Compartmentalization of the Vascular Pathogen *Phaeomoniella chlamydospora* in Grapevine. *Front. Plant Sci.* 8, 1442. doi: 10.3389/fpls.2017.01442.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S. G., Dobinson, K. F., and Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7: Olive- *Pseudomonas-Verticillium* in planta interaction. *Microb. Biotechnol.* 2, 499–511. doi: 10.1111/j.1751-7915.2009.00105.x.

Rallo, L. (2014). Breeding Oil and Table Olives for Mechanical Harvesting in Spain. *HortTechnology* 24, 295–300. doi: 10.21273/HORTTECH.24.3.295.

Ramírez-Tejero, J. A., Jiménez-Ruiz, J., Serrano, A., Belaj, A., León, L., de la Rosa, R., et al. (2021). Verticillium wilt resistant and susceptible olive cultivars express a very different basal set of genes in roots. *BMC Genomics* 22, 229. doi: 10.1186/s12864-021-07545-x.

Robb, J. (2007). *Verticillium* tolerance: resistance, susceptibility, or mutualism? *Can. J. Bot.* 85, 903–910. doi: 10.1139/B07-093.

Rodríguez, E., García-garrido, J. M., García, P. A., and Campos, M. (2008). Agricultural factors affecting Verticillium wilt in olive orchards in Spain. *Eur. J. Plant Pathol.* 122, 287–295. doi: 10.1007/s10658-008-9287-0.

Sanei, seyed J., and Razavi, S. E. (2017). Resistance and vegetative growth analysis of some olive cultivars in response to a defoliating pathotype of *Verticillium dahliae* Kleb. *Int. J. Hortic. Sci. Technol.* 4. doi: 10.22059/ijhst.2018.241208.206.

Serrano, A., León, L., Belaj, A., and Román, B. (2020). Nucleotide diversity analysis of candidate genes for Verticillium wilt resistance in olive. *Sci. Hortic.* 274, 109653. doi: 10.1016/j.scienta.2020.109653.

Serrano, A., Rodríguez-Jurado, D., Román, B., Bejarano-Alcázar, J., De la Rosa, R., and León, L. (2021). Verticillium Wilt Evaluation of Olive Breeding Selections Under Semi-Controlled Conditions. *Am. Phytopathol. Soc.* doi: 10.1094/PDIS-08- 20-1829-RE.

Sinclair, W. A., and Hudler, G. W. (1998). Verticillium wilt. *Cornell Coop. Ext.-Suffolk Cty.*

Solla, A., and Gil, L. (2002). Xylem vessel diameter as a factor in resistance of Ulmus minor to Ophiostoma novo-ulmi. *For. Pathol.* 32, 123–134. doi: 10.1046/j.1439-0329.2002.00274.x.

Tjamos, E. C., and Paplomatas, E. J. (1988). Long-term effect of soil solarization in controlling verticillium wilt of globe artichokes in Greece. *Plant Pathol.* 37, 507–515. doi: 10.1111/j.1365-3059.1988.tb02108.x.

Tjamos, E., and Jiménez-Díaz, R. (1998). Management of disease. In: Hiemstra J and Harris D (eds) Compendium of Verticillium Wilt in Tree Species. *Ponsen Looijen Wagening. Neth.*, 55–57.

Trapero, C., Alcántara, E., Jiménez, J., Amaro-Ventura, M. C., Romero, J., Koopmann, B., et al. (2018). Starch Hydrolysis and Vessel Occlusion Related to Wilt Symptoms in Olive Stems of Susceptible Cultivars Infected by *Verticillium dahliae*. *Front. Plant Sci.* 9, 72. doi: 10.3389/fpls.2018.00072.

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. (2015). Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathol.* 64, 890–900. doi: 10.1111/ppa.12330.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., and López-Escudero, F. J. (2013). Field Resistance to Verticillium Wilt in Selected Olive Cultivars Grown in Two Naturally Infested Soils. *Plant Dis.* 97, 668–674. doi: 10.1094/PDIS-07-12-0654-RE.

Tsror (2011). Review: Epidemiology and control of Verticillium wilt on olive. *Isr. J. Plant Sci.* 59, 59–69. doi: 10.1560/IJPS.59.1.59.

Umaerus, V. R., Scholte, K., and Turkensteen, L. J. (1989). "Crop Rotation and the Occurrence of Fungal Diseases in Potatoes," in *Effects of Crop Rotation on Potato Production in the Temperate Zones*, eds. J. Vos, C. D. Van Loon, and G. J. Bollen (Dordrecht: Springer Netherlands), 171–189. doi: 10.1007/978-94-009-2474-1_15.

Valverde, P., Trapero, C., Arquero, O., Serrano, N., Barranco, D., Muñoz Díez, C., et al. (2021). Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. *Plant Pathol.* 70, 144–153. doi: 10.1111/ppa.13264.

Varo, A., Raya-Ortega, M. C., and Trapero, A. (2016). Selection and evaluation of micro-organisms for biocontrol of *Verticillium dahliae* in olive. *J. Appl. Microbiol.* 121, 767–777. doi: 10.1111/jam.13199.

Varo-Suárez, A., Raya-Ortega, M. C., Agustí-Brisach, C., García-Ortiz-Civantos, C., Fernández-Hernández, A., Mulero-Aparicio, A., et al. (2018). Evaluation of organic amendments from agro-industry waste for the control of verticillium wilt of olive. *Plant Pathol.* 67, 860–870. doi: 10.1111/ppa.12798.

Wilhelm, S (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. *Phytopatthology* 45, 180–181.

Yadeta, K. A., and J. Thomma, B. P. H. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Front. Plant Sci.* 4. doi: 10.3389/fpls.2013.00097.

Yildiz, M., Yildiz, F., and Erten, L. (2020). Research on Control of Verticillium Wilt of Olive by Grafting Susceptible Cultivars onto Resistant Rootstocks and Varieties. *J Turk Phytopath* 49, 19–24.

Resumen de Resultados

Capítulo 1. Métodos para el establecimiento, multiplicación y crecimiento *in-vitro* de genotipos silvestres y cultivares de olivo maduros.

En este trabajo se estableció un germoplasma *in-vitro* de olivos silvestres procedentes de prospecciones *in situ* y de varios bancos de germoplasma de olivo, conocido como colección SILVOLIVE. Se pusieron a punto los protocolos de establecimiento, micropropagación y enraizamiento *in-vitro* haciendo hincapié en el empleo de luces LEDs durante la fase de propagación. Todos los genotipos de la colección SILVOLIVE fueron susceptibles de ser micropropagados *in-vitro.* Se caracterizó el crecimiento *in-vitro* de todos los genotipos silvestres de olivo de la colección, que presentaron un crecimiento promedio de 40.9 mm de altura y 6.1 nudos por planta tras un período de 55 días. La altura de las plántulas micropropagadas *in-vitro* no se correlacionó con su crecimiento temprano en maceta ni en campo. Por otro lado, el uso de distintos tipos y concentraciones de auxinas no afectó al éxito del enraizamiento, que fue más dependiente de la variedad de olivo.

Se optimizó el uso de luces LEDs para la multiplicación *in-vitro* de explantos de genotipos silvestres de olivo, estableciéndose un 70% rojo + 30% azul con 34 µmol de m^{-2} s^{-1} de intensidad como la condición más favorable para la propagación *in-vitro* de los dos genotipos estudiados. La calidad e intensidad de las luces LEDs tuvo un efecto en la longitud internodal, sin afectar al número de nudos por planta. Las plantas crecidas bajo iluminación fluorescente blanca mostraron los entrenudos más cortos y menor altura que las crecidas bajo luces LED, siendo ésta una respuesta general en el olivo. Este efecto repercutió en la biomasa de las plantas, mostrándose una correlación negativa entre la biomasa del tallo y la intensidad lumínica. La intensidad lumínica mostró una correlación positiva con el área foliar y el peso fresco de las hojas, al contrario que la calidad de la luz. El aumento de la intensidad de luz determinó una mayor densidad de estomas más pequeños, mientras que la calidad de la luz no afectó al tamaño ni a la frecuencia de los estomas. Además, una menor intensidad de luz y / o una mayor proporción de rojo: azul determinaron un mayor contenido de pigmentos fotosintéticos.

Por tanto, la calidad e intensidad de la luz son factores muy influyentes en el proceso de multiplicación *in-vitro*. La cantidad y la calidad de la luz afectan a importantes parámetros morfológicos, anatómicos y bioquímicos, permitiendo una regulación óptima de la micropropagación en el olivo.

Capítulo 2. SILVOLIVE, una colección de germoplasma de subespecies silvestres con alta variabilidad genética como fuente de portainjertos y genes de resistencia para la mejora del olivo.

En este trabajo se determinó el origen y la variabilidad genética de la colección de germoplasma SILVOLIVE que incluye las seis subespecies de *Olea europaea*. Se secuenciaron 10 marcadores cloroplásticos para asegurar el origen filogenético de los genotipos y demostrar, junto con el estudio de análisis de fragmentos de 5 marcadores nucleares (SSR), la alta variabilidad genética impuesta por la polinización abierta de la colección. Al contrario que los cpDNA, los marcadores SSR no realizaron una distinción clara en taxones, por lo tanto, los individuos pertenecientes al mismo linaje cloroplástico exhibieron una alta diversidad nuclear. Además, mediante citometría de flujo se determinó el nivel de ploidía de los genotipos, identificándose variedades diploides en su gran mayoría, además de variedades triploides en genotipos de las subspecies *europaea*-E2 (AOU10), *laperrinei* (DHO10A y DHO11A) y *cerasiformis* (CER1 y CER3), y variedades hexaploides en la subsp. *maroccana* (MAR1, MAR2 y MAR3).

Se caracterizó el crecimiento de 103 genotipos silvestres de olivo en maceta en condiciones de invernadero después de 13 meses desde su aclimatación *ex-vitro*, y se determinó el vigor tanto aéreo como radicular. Se establecieron tres grupos de parámetros con altos niveles de correlación positiva dentro de cada grupo: i) parámetros de vigor (altura, diámetro basal del tallo, biomasa de las ramas, biomasa total de las hojas y biomasa total de la planta); ii) parámetros de ramificación (eficiencia de ramificación, frecuencia de ramificación, número de tallos secundarios y terciarios, número de nudos y de hojas); y iii) relación de biomasa raíz / tallo, que a su vez mostró correlaciones negativas con los parámetros de vigor y ramificación. Los datos fueron sometidos a un análisis de PCA explicando el 66.1% de la variabilidad total. Se distinguieron genotipos de alto vigor (ARC, AOU, CUS y CEH); de bajo vigor (AMK, GUA,

AJA y DHO); con altos niveles de ramificación (APR, ACZ, CER y CEH); y con bajos valores de ramificación y alta relación raíz / parte aérea, como MAR y DHO. Además, aquellos genotipos de bajo vigor y alta ramificación mostraron una elevada relación biomasa raíz / biomasa tallo.

Posteriormente, se seleccionaron genotipos para ser usados como portainjertos del cultivar 'Picual' en maceta. Se demostró la compatibilidad de la injerta y la modificación de los parámetros de vigor del vástago 'Picual', aunque la injerta no afectó sustancialmente a los hábitos de ramificación. Los genotipos de bajo vigor DHO10B, ACO15, AMK14, GUA8, GUA2, FRA4, AJA17, AMK6 y FRA3 redujeron el tamaño del vástago 'Picual', mientras que genotipos de alto vigor ACZ9, CUS13 y CUS15 incrementaron el tamaño del cultivar. Sin embargo, genotipos de vigor reducido como AMK5, AMK21 y GUA9 aumentaron el tamaño del 'Picual' injertado, por lo que otros factores deben influir en las propiedades del vástago. En las plantas injertadas se observó una alta correlación entre parámetros de vigor del portainjerto (diámetro basal) y el vástago (altura de la planta). Sin embargo, no se observó correlación entre los parámetros de la planta injertada y la no injertada.

Por otro lado, se identificaron genotipos con bajo consumo hídrico como ACZ8 (0.41 ± 0.036 ml agua diarios/biomasa foliar fresca), valores 5.5 veces menores que el genotipo con el mayor consumo, AMK6 (2.30 ± 0.110). Este consumo hídrico parece relacionarse con su origen, pues genotipos con el mismo origen materno mostraron un consumo hídrico similar cuando las plantas fueron bien irrigadas (WW). Sin embargo, genotipos con caracteres deseables de bajo vigor exhibieron mayor consumo hídrico por unidad foliar. Además, se identificaron genotipos resistentes al déficit hídrico de las subspecies *guanchica* (GUA2, GUA8, GUA5 y GUA4) y *europaea* (ARC y AJA4) que mostraron una estimulación del 20-100% en el desarrollo de la raíz tras el tratamiento moderado de déficit hídrico (WS).

Capítulo 3. Evaluación del vigor temprano en campo de cultivares de olivo injertados sobre genotipos silvestres de olivo.

En este trabajo se estudió el vigor de los genotipos silvestres de olivo en condiciones de campo. En primer lugar, 43 genotipos fueron evaluados bajo régimen superintensivo, exhibiendo una amplia variabilidad en los parámetros de vigor, siendo la varianza entre genotipos mayor que la varianza de error para la mayoría de ellos. Como consecuencia, se obtuvieron valores altos de heredabilidad en los parámetros de vigor, aunque disminuyeron durante el período de evaluación. Se observaron correlaciones significativas entre los diferentes parámetros de vigor, cuyos valores de correlación fueron más altos para los rasgos medidos en el mismo punto del tiempo. El volumen de copa (CV) demostró ser un buen indicador de vigor mostrando correlaciones significativas con todos los rasgos de vigor evaluados, siendo la medida de 2016 la de valores más altos. Este parámetro (CV) medido en 2018 mostró una buena correlación con el diámetro del eje principal a 50 cm del suelo DL-W18 (r=0.901). Debido a esta buena correlación se realizó una clasificación final determinando diferencias claras en el origen de los genotipos: los genotipos silvestres de Jaén (AJA) y de Marrakech (AMK) se clasificaron como genotipos de bajo vigor; los genotipos silvestres de Cádiz (ACZ) y los híbridos *cuspidata* x *europaea* (CEH) resultaron ser de alto vigor; los genotipos de origen guanchica (GUA) presentaron una alta varibilidad, por lo que solo se seleccionaron aquellos genotipos enanizantes y no toda la línea hereditaria.

En un segundo estudio se evaluaron otros 45 genotipos diferentes: los genotipos silvestres de Cádiz (ACZ) y los híbridos *cuspidata* x *europaea* (CEH) exhibieron alto vigor tanto en altura como en volumen de copa. Genotipos de las subsp. *maroccana* (MAR), genotipos silvestres de Marrakech (AMK) y genotipos silvestres de Aourir (AOU) mostraron características enanizantes. Los parámetros de vigor mostraron correlaciones positivas entre ellos, tomando de referencia la altura como parámetro principal (R^2 = 0.6124, R^2 = 0.5191, R^2 = 0.5605, R^2 = 0.5309 con CV, DG, DL y DC, respectivamente).

Se llevó a cabo un tercer estudio para evaluar el potencial de estos genotipos como portainjertos capaces de reducir el vigor al cultivar 'Arbequina'. Dentro de la amplia variabilidad observada, los portainjertos que exhibieron mayor vigor (CEH y CUS) produjeron un incremento en el tamaño de las plantas injertadas respecto a los propios cultivares autoinjertados y sin injertar. En cambio, genotipos de vigor reducido como AMK, algunos GUA (GUA8 y GUA9) y ACO15 transmitieron reducido vigor al cultivar 'Arbequina' injertado. Sin embargo, algunos genotipos identificados como de bajo vigor (AMK5) produjeron un incremento en el tamaño del cultivar injertado. Además, se determinó que los genotipos AMK21, AMK5 y ARC produjeron un mayor número de chupones, mostrando una mayor tendencia al rebrote del patrón.

Capítulo 4. Genotipos silvestres de olivo como fuente de resistencia a la Verticilosis del olivo.

En este trabajo se evaluó el nivel de susceptibilidad de los genotipos silvestres de la colección SILVOLIVE al patotipo defoliante del hongo *Verticillium dahliae*. Los 68 genotipos silvestres evaluados se clasificaron según los síntomas finales observados (FMS), el desarrollo del progreso de la enfermedad (RAUDPC) y el porcentaje de plantas muertas (PDP) en 4 categorías: Extremadamente susceptible (ES, 30 genotipos, representando el 44.1%); Susceptible (S, 12 genotipos, representando el 17.6%); Moderadamente susceptible (MS, 11 genotipos, representando el 16.2%); y Resistentes (R, 15 genotipos, representando el 22.1%). Dentro del grupo de resistentes, los genotipos GUA3, ACO15, TAM12, ACZ3 y AMK5 presentaron síntomas menos severos que los del cultivar resistente de referencia 'Frantoio'. Después de la inoculación, la tasa de crecimiento relativa se inhibió fuertemente en los genotipos susceptibles, pero no en los resistentes, que mostraron poca o ninguna inhibición de la longitud acumulada de las ramas, altura y número de nudos de las plantas.

El nivel de infección por *V. dahliae* de cada genotipo se cuantificó en el tallo de las plantas inoculadas mediante qPCR a 35 y 120 días después de la inoculación (ddi). Los genotipos extremadamente susceptibles presentaron los mayores niveles de ADN de *V. dahliae*, mientras que las cantidades más bajas de ADN de *V. dahliae* se detectaron en genotipos resistentes. Los genotipos resistentes GUA3, ACZ3, CEH23, ACO14, AMK14,

DHO6B, ACO1, AMK27 y TAM12 mostraron menor contenido de ADN de *V. dahliae* que el cultivar resistente 'Frantoio' a los 120 ddi. A partir de estos resultados, se observó una correlación positiva entre la cantidad de ADN de *V. dahliae* en el tallo y los síntomas de la planta a los 120 ddi, medidos como FMS (R^2 = 0.5865) o RAUDPC (R^2 = 0.5051). Sin embargo, no se observó ninguna correlación entre el contenido de ADN de *V. dahliae* a los 35 ddi y los síntomas a los 120 ddi, lo que sugiere que el diagnóstico no se debe realizar durante una etapa temprana de infección. Se identificaron genotipos, como ACO15 y DHO6A, que toleraban la presencia de cantidades relativamente altas del hongo a pesar de mostrar síntomas muy leves o nulos. Se distinguieron, por tanto, diferentes patrones de respuesta en función de la evolución del hongo en los tejidos vegetales. El patrón 1 experimenta una disminución significativa de la cantidad de ADN de *V. dahliae* entre 35 y 120 ddi. En este patrón se agrupan todos los genotipos resistentes y el genotipo AMK21. El patrón 2, sin variación significativa en la cantidad de ADN de *V. dahliae* entre 35 y 120 ddi, incluye a la mayoría de los genotipos moderadamente susceptibles. El patrón 3, con un aumento significativo de ADN de *V. dahliae* a 120 ddi con respecto a 35 ddi, incluye a la mayoría de los genotipos extremadamente susceptibles (85%), 67% de los susceptibles y 43% de los moderadamente susceptibles, pero a ninguno de los genotipos resistentes.

Capítulo 5. Injerto del cultivar Picual sobre portainjertos silvestres de la colección SILVOLIVE como método de control de la Verticilosis del olivo.

En este trabajo se evaluó la capacidad de transferir la resistencia al patotipo defoliante de *V. dahliae* de los genotipos resistentes de olivo al cultivar 'Picual' injertado sobre ellos. Se analizó la susceptibilidad de 'Picual' injertado sobre 6 genotipos resistentes (AMK27, GUA3, DHO6A, ACO15, AMK5 y CEH23), 1 moderadamente susceptible (AMK21) y 2 susceptibles (ACZ10 y GUA8). Los portainjertos ACZ10 y GUA8, extremadamente susceptibles, mostraron los síntomas más severos, los valores más altos de RAUDPC, y el 100% de plantas muertas, y no se distinguieron significativamente de 'Picual' y 'Arbequina' sin injertar ni autoinjertados. Los portainjertos silvestres resistentes, ACO15, CEH23, AMK5 y DHO6A, mostraron alta susceptibilidad a *V. dahliae*, no mostrando diferencias significativas con los controles 'Picual' y 'Arbequina'. El portainjerto moderadamente susceptible AMK21 presentó un valor de RAUDPC

moderado, síntomas severos y 100% de plantas muestas. Los genotipos resistentes GUA3 y AMK27 fueron los más tolerantes, presentando valores bajos de RAUDPC (40,18% y 36,20%), síntomas moderados (FMS 2,88 y 3,46), y 62,5% y 81,3% de plantas muertas, respectivamente. La tolerancia más alta la presentó 'Frantoio', tanto sin injertar como injertado con 'Picual'. Se utilizaron dos tipos de injerta (púa y escudete), siendo ambas igualmente eficientes. A tiempos cortos (35 ddi) el proceso de la injerta retrasó la aparición de síntomas en 'Frantoio', 'Picual' y 'Arbequina', no así a tiempos largos de 120 ddi. Las plantas inoculadas presentaron menor crecimiento de los órganos aéreos que las plantas no inoculadas. Los portainjertos AMK27, GUA3, AMK21 y DHO6A mostraron un mayor crecimiento que el resto de portainjertos silvestres. Las plantas de 'Frantoio' exhibieron los mayores valores de crecimiento sin mostrar diferencias significativas entre las plantas inoculadas y no inoculadas.

Las plantas que utilizaron como portainjerto 'Frantoio', AMK27, GUA3 y AMK21 presentaron los valores más bajos de ADN de *V. dahliae* a 35 ddi, tanto en el tallo del portainjerto como en el vástago 'Picual'. También presentaron bajos niveles de hongo a 120 ddi en el tallo del portainjerto, pero no así en el vástago 'Picual'. Se detectó una alta correlación entre los síntomas de la planta (medidos como RAUDPC) y el contenido de ADN de *V. dahliae* en los portainjertos a los 35 (R^2 = 0,7676) y 120 ddi (R^2 = 0,7889). Sin embargo, en el vástago 'Picual' la correlación entre los síntomas y la cantidad de ADN se observó a los 35 ddi (R^2 = 0.5034), pero no a los 120 ddi (R^2 = 0.0022). Los brotes adventicios producidos a partir de los portainjertos resistentes fueron asintomáticos y la cantidad de ADN de *V. dahliae* a 120 ddi fue aproximadamente 10000 veces menor que la detectada en el correspondiente 'Picual' injertado, independientemente de los síntomas observados en el vástago injertado.

Discusión General

El cultivo del olivo, de enorme importancia socioeconómica, se enfrenta a antiguos y nuevos retos, entre los que destacan su integración al nuevo sistema de producción de alta densidad, la adaptación al cambio climático y los problemas fitosanitarios, con especial atención a la Verticilosis del olivo. La falta de variedades adaptadas a los nuevos marcos de plantación, el enorme coste del mantenimiento del cultivo (fertirrigación, poda, etc), y la necesidad de aumentar la producción del aceite de oliva y la aceituna de mesa hacen que la revolución del olivar sea inevitable. El desarrollo de variedades con propiedades organolépticas deseables que cumplan los criterios para ser cultivadas en superintensivo o en seto, y el uso de portainjertos capaces de mejorar las características productivas de la variedad injertada, adicionando los beneficios del sistema radicular del portainjerto (que le permita un consumo más eficiente del agua y resistencia a *Verticillium dahliae*), se encuentran entre las tareas puestas en marcha por diversos grupos de investigación. Además, debido a los cambios climatológicos causados por el calentamiento global, se ha acentuado la necesidad de optimizar el manejo de los recursos hídricos y la adaptación del cultivo a condiciones de suelo menos favorables. Por ello, en esta Tesis doctoral hemos establecido y caracterizado un nuevo germoplasma de olivo silvestre para dar solución a los múltiples problemas mencionados. El **Capítulo 1** de esta Tesis pone de manifiesto la utilidad de la micropropagación *in-vitro* del cultivo del olivo como método factible para la obtención de plantas viables a nivel viverístico. La optimización del empleo de luces LEDs ha supuesto una mejora en la obtención de explantos viables, debido al aumento de la longitud internodal sin afectar al número de segmentos nodales, favoreciendo la micropropagación del olivo. Esta tecnología permite responder de manera rápida y eficaz a la creciente demanda por parte del sector de material libre de enfermedades, homogéneo y con unas características de vigor, productividad, calidad de la fruta y resistencia a estreses bióticos y abióticos. Además, es aplicable para multiplicar cultivares o portainjertos que sean recalcitrantes al estaquillado o produzcan pocas semillas. Asimismo, permite multiplicar plántulas de manera rápida y fácilmente, en un tiempo y espacio limitados durante todo el año en comparación con los métodos tradicionales. Debido a las condiciones asépticas de la técnica, ajenas a las condiciones

medioambientales, proporciona herramientas eficientes para la propagación y la preservación de germoplasma de plantas amenazadas (Kozlowski et al., 2012; Santos et al., 2003), reduciendo los riesgos de extinción y pérdida de biodiversidad. Por tanto, este protocolo de multiplicación *in-vitro* es adecuado como una herramienta integral para propagar y preservar los genotipos seleccionados en los programas de mejora I+D o los de reciente introducción en un país. La colección de germoplasma de olivo silvestre, colección SILVOLIVE, ha permitido, además, el establecimiento *in-vitro* de cultivares comerciales de olivo como 'Picual', 'Frantoio' o 'Arbequina' mediante mini-injerta (Troncoso et al., 2000). Esta técnica permite desinfectar e introducir material foráneo maduro para su propagación y conservación *in-vitro,* permitiendo (i) la multiplicación clonal de los cultivares del mismo modo que los genotipos silvestres de la colección, generando un material vegetal homogéneo de partida; y (ii) conservar un germoplasma de cultivares en condiciones de esterilidad para responder de forma eficiente a las necesidades del sector y evitar el riesgo de erosión genética.

La caracterización molecular del germoplasma SILVOLIVE mediante marcadores moleculares es el principio del proceso de pre-mejoramiento, ayudando a identificar y caracterizar los genotipos de olivo (Belaj et al., 2001; do Val et al., 2012; Trujillo et al., 2014). La variabilidad del olivo representa un recurso importante que se puede utilizar en los programas de mejora (Bartolini et al., 1998). La alta diversidad genética de la colección se consiguió al incluir genotipos de todas las subespecies conocidas de *O. europaea*, que se caracterizaron mediante el uso de marcadores plastídicos y nucleares (**Capítulo 2**). Esta alta diversidad ha permitido seleccionar entre los 146 genotipos ensayados: i) genotipos de tamaño reducido con un vigor bajo y un alto número de ramas laterales (branching), potencialmente útiles para el cultivo de alta densidad (HDH) que ha experimentado un auge sustancial en la última década; ii) genotipos de escaso consumo hídrico y resistentes al déficit hídrico, potencialmente útiles para ser usados como portainjertos capaces de afrontar los problemas de falta de agua a causa del cambio climático y útiles en la recuperación de zonas áridas: y iii) genotipos tolerantes a la Verticilosis, uno de las mayores problemas fitosanitarios del olivo. La colección SILVOLIVE es también una fuente de diversidad para explorar otros caracteres deseables en el olivo, como por ejemplo, resistencia a la bacteria *Xylella fastidiosa*, una de las

mayores amenazas del cultivo del olivo en Europa (Montilon et al., 2021), o resistencia a otros factores bióticos como *Mycocentrospora cladosporioides*, *Bactrocera oleae*, *Prays oleae* y *Pseudomonas syringae* pv. *savastanoi* (Fontanazza and Baldoni, 1990; Rugini and Gutiérrez-Pesce, 2006). Del mismo modo, puede ser fuente de variabilidad para búsqueda de resistencia a condiciones ambientales desfavorables como la temperatura, la salinidad, la clorosis férrica o la sequía. Además, estos genotipos silvestres de olivo pueden usarse como portainjertos aportando otras características agronómicas de interés, como acortamiento del período juvenil de las variedades comerciales para una rápida entrada en producción; obtención de un aceite y aceituna de mayor calidad (contenido de aceite, composición de ácidos grasos, peso de la fruta, etc.), y una buena adaptación a la recolección mecánica. Recientemente, los programas de mejora del olivo están desarrollando nuevos métodos de detección para seleccionar de forma rápida y eficiente caracteres tempranos de vigor (Rallo et al., 2020; Torres-Sánchez et al., 2021), y búsqueda de genes diana como marcadores de resistencia a *Verticillium* (Serrano et al., 2020), agilizando el proceso de selección.

En la colección SILVOLIVE están representados diferentes niveles de ploidía (6x, 3x, 2x). La poliploidía es una fuerza importante en la evolución y especiación de las plantas silvestres y cultivadas, siendo una característica relacionada con la resistencia al estrés abiótico y biótico en plantas (Sattler et al., 2016; Ruiz et al., 2020; Russo et al., 2020). Estos genotipos poliploides suelen presentar un mayor vigor a causa del aumento del tamaño celular, aunque no siempre se produce, ya que a menudo se reduce el número de divisiones celulares, con tasas de crecimiento más bajas generándose plántulas de reducido vigor (Sattler et al., 2016). Además, afecta positivamente a la tolerancia de deficiencias en nutrientes, la sequía, el déficit de agua, la temperatura, las plagas y los patógenos. Estas características ventajosas del genoma poliploide ha llevado a los fitomejoradores a la obtención de genotipos poliploides artificiales como herramienta para la mejora de los cultivos (Rugini et al., 2016).

Para el mejorador es interesante la obtención de variedades donde se combinen caracteres agronómicos de interés, caracteres de resistencia a enfermedades y a otros estreses ambientales. Desde esta perspectiva se ha llevado a cabo en esta Tesis Doctoral la evaluación de la resistencia a la Verticilosis de la colección SILVOLIVE priorizando

aquellos genotipos con características deseables de bajo vigor (**Capítulo 4**). El control de la Verticilosis no es tarea fácil y es necesario abordarla mediante una estrategia de gestión integrada de medidas preventivas y/o paliativas (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020). Entre estas estrategias se ha destacado la resistencia genética como la medida más recomendada (Trapero et al., 2015), siendo respetuosa con el medio ambiente y con la posibilidad de combinarse con otras medidas de control. En el **Capítulo 4** de Tesis se evalúa el grado de susceptibilidad al patotipo defoliante V117 de *V. dahliae* de 68 genotipos (46.5 % de la colección) mediante la inoculación artificial en condiciones de invernadero, de los cuales el 22% (15 genotipos) han mostrado niveles de resistencia a la enfermedad. Similar proporción de resistentes se detectó en otro cribado de dimensiones comparables (Arias-Calderón et al., 2015b). Según Niks et al. (1993) la resistencia es la capacidad de la planta para reducir el crecimiento y desarrollo del patógeno después del contacto entre el huésped y el patógeno, o después de iniciado su desarrollo o cuando esté establecido. Este concepto se asemeja a lo observado en las evaluaciones con el cultivar 'Frantoio' y otros genotipos silvestres resistentes que mostraron una recuperación de la enfermedad junto a una disminución del contenido de hongo entre los 35 y 120 ddi. Sin embargo, aunque prevalece el concepto de 'resistencia', los diferentes contenidos de ADN de *V. dahliae* y los diferentes grados de síntomas desarrollados por los genotipos resistentes, apuntan a la aparición de diversos mecanismos de resistencia. Según Robb (2007), las plantas pueden usar dos estrategias para contrarrestar la infección por patógenos que causan enfermedades: (i) la 'resistencia' que inhibe o limita la colonización de la planta por patógenos y, por lo tanto, reducen el desarrollo de enfermedades, y (ii) la 'tolerancia' que inhiben o limitan la expresión de los síntomas a pesar de la proliferación agresiva de los patógenos. Este término de tolerancia ha sido utilizado por otros autores como Gómez-Lama Cabanás et al. (2015) para referirse a aquellos genotipos capaces de hacer frente a las infecciones por *V. dahliae* sin desarrollar síntomas graves de la enfermedad. En este sentido, genotipos como ACO15, AMK5 y DHO6A pueden ser considerados tolerantes, ya que albergan niveles elevados de hongo, pero manifiestan síntomas leves, tolerando niveles moderados de infección a corto plazo y controlando la infección a medio y largo plazo. Por otro lado, genotipos como 'Frantoio', GUA3 y AMK27 mantuvieron niveles bajos de ADN de *V. dahliae* en 120 dai, lo que sugiere una

capacidad de respuesta de resistencia. Estos mecanismos son difíciles de discernir mediante síntomas visuales de la enfermedad, siendo necesario el uso de estudios moleculares para su interpretación.

Comprender los mecanismos desencadenados en la planta huésped por la presencia del patógeno *V. dahliae* es fundamental para diseñar estrategias de control de la enfermedad. La mecanismos de resistencia en estos portainjertos pueden ser fisiológicos, celulares y moleculares (Gómez-Lama Cabanás et al., 2015; Trapero et al., 2018), incluyendo i) el refuerzo de la pared celular por deposición de lignina y suberina en el sitio de infección; ii) la producción de especies reactivas de oxígeno como H_2O_2, responsable de impedir el crecimiento fúngico en raíces de cultivares de olivo resistentes y moderadamente susceptibles (Gharbi et al., 2016, 2017), además de actuar como molécula señalizadora, provocando así un despliegue más rápido de efectos estructurales (gomas, lignina, depósito de tilosa en los vasos infectados) y/o bioquímicos (producción de fitoalexinas y otras sustancias antifúngicas) (López-Escudero and Blanco-López, 2005); y iii) la activación temprana de mecanismos de defensa de las plantas, como la inducción de genes que codifican para quitinasas y β-1,3-glucanasa capaces de degradar la pared celular del patógeno (Gharbi et al., 2016), o la activación de la vías del ácido jasmónico, etileno y ácido salicílico, necesarias para la defensa contra los patógenos hemibiotróficos (Gharbi et al., 2017). Recientemente se publicó el mecanismo molecular de la senescencia foliar prematura causada por *V. dahliae.* Bajo la infección por el hongo, la proteína efectora PevD1 (Proteína elicitora de *V. dahliae* 1, un miembro de la familia de proteínas Alt A 1) se secreta en la célula vegetal y genera una cascada de señalización (PevD1-ORE1-ACS6-etileno), promoviendo la biosíntesis de etileno, lo que conduce a la senescencia de las hojas (Zhang et al., 2021). Estos hallazgos hacen más comprensible el modo de actuación del hongo con el huésped ayudando a encontrar solución al problema desencadenado.

En general, el fitomejorador busca aquel cultivar o portainjerto que no presente síntomas de la enfermedad ni colonización de la planta por el hongo, lo que se conoce como resistencia completa (Niks et al., 1993). Sin embargo, ningún genotipo evaluado en este trabajo y en otros previos mostró un comportamiento asintomático y ausencia total del patógeno en el tejido del huésped tras el periodo experimental. Actualmente,

el cultivar 'Arbequina', usado como referencia debido a su uso extendido en el olivar superintensivo, muestra valores elevados de susceptibilidad al hongo (Díaz-Rueda et al., 2021). Por lo tanto, nuevos genotipos con niveles de resistencia superiores al cv. 'Arbequina' son potencialmente útiles para su uso en la mejora del olivar.

La característica de resistencia del cultivar 'Frantoio' ha sido explotada en diversos programas de mejora para obtener progenies mediante polinización abierta o mediante cruces controlados con otros cultivares, observándose una alta heredabilidad del carácter de resistencia a *V. dahliae* al actuar como progenitor femenino o masculino (Arias-Calderón et al., 2015a; Trapero et al., 2015). Por otro lado, esta resistencia lo hace idóneo para su uso como portainjerto para controlar la Verticilosis (Porras Soriano et al., 2003; Bubici and Cirulli, 2012). Desde esta perspectiva, 'Frantoio' y otros genotipos silvestres seleccionados por su nivel de resistencia al hongo fueron evaluados como portainjertos para controlar la enfermedad. Los genotipos silvestres GUA3 y AMK27 usados como portainjertos del cultivar susceptible 'Picual' retrasaron el desarrollo de la enfermedad (**Capítulo 5**). Estos genotipos resistentes pueden ser utilizados como portainjertos para mejorar la resistencia al marchitamiento por *Verticillium* como demostraron otros portainjertos anteriormente (Porras Soriano et al., 2003; Bubici and Cirulli, 2012). Además, estos portainjertos son idóneos para cultivos HDH: i) por su bajo vigor (tanto en maceta como en campo) (Díaz-Rueda et al., 2020; León et al., 2020), lo que disminuye el coste de poda y facilita su recolección mecánica. Aunque no tenemos resultados de vigor de variedades injertadas sobre estos portainjertos, la mayoría de genotipos AMKs y GUAs transmitieron vigor reducido a los cultivares injertados 'Picual' y 'Arbequina' y, por ende, estos genotipos pueden tener un comportamiento similar a sus genotipos hermanos y disminuir el tamaño de los vástagos injertados; ii) por su rápida entrada en producción al menos el portainjerto GUA3 injertado con 'Picual', y iii) por su potencial de restaurar y/o mejorar la diversidad, tanto por encima como por debajo del suelo. Todo ello aumentará no solo la productividad a corto plazo sino también la viabilidad a largo plazo de las plantaciones de olivo en un escenario de cambio climático (Gómez-González et al., 2022).

Bibliografía de la Discusión

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., and Rodríguez-Jurado, D. (2015a). Resistance to Verticillium wilt in olive progenies from open-pollination. *Sci. Hortic.* 185, 34–42. doi:10.1016/j.scienta.2015.01.015.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015b). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi:10.1016/j.cropro.2015.05.006.

Bartolini, G., Prevost, G., Messeri, C., and Carignani, G. (1998). Olive Germplasm: Cultivars and World-Wide Collections. *FAO Roma Italy*.

Belaj, A., Trujillo, I., de la Rosa, R., Rallo, L., and Giménez, M. J. (2001). Polymorphism and Discrimination Capacity of Randomly Amplified Polymorphic Markers in an Olive Germplasm Bank. *J. Am. Soc. Hortic. Sci.* 126, 64–71. doi:10.21273/JASHS.126.1.64.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi:10.1007/s11104-011-1002-9.

Díaz-Rueda, P., Aguado, A., Romero-Cuadrado, L., Capote, N., and Colmenero-Flores, J. M. (2021). Wild Olive Genotypes as a Valuable Source of Resistance to Defoliating *Verticillium dahliae*. *Front. Plant Sci.* 12, 662060. doi:10.3389/fpls.2021.662060.

Díaz-Rueda, P., Franco-Navarro, J. D., Messora, R., Espartero, J., Rivero-Núñez, C. M., Aleza, P., et al. (2020). SILVOLIVE, a Germplasm Collection of Wild Subspecies With High Genetic Variability as a Source of Rootstocks and Resistance Genes for Olive Breeding. *Front. Plant Sci.* 11, 629. doi:10.3389/fpls.2020.00629.

do Val, A. D. B., Ferreira, J. L., Vieira Neto, J., Pasqual, M., de Oliveira, A. F., Borém, A., et al. (2012). Genetic diversity of Brazilian and introduced olive germplasms based on microsatellite markers. *Genet. Mol. Res.* 11, 556–571. doi:10.4238/2012.March.8.4.

Fontanazza, G., and Baldoni, L. (1990). Proposed programme for the genetic improvement of the olive. *Olivæ* 34, 32–40.

Gharbi, Y., Barkallah, M., Bouazizi, E., Cheffi, M., Gdoura, R., and Triki, M. A. (2016). Differential fungal colonization and physiological defense responses of new olive cultivars infected by the necrotrophic fungus *Verticillium dahliae*. *Acta Physiol. Plant.* 38, 242. doi:10.1007/s11738-016-2261-0.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017). Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 97, 30–39. doi:10.1016/j.pmpp.2016.12.001.

Gómez-González, S., Paniw, M., Blanco-Pastor, J. L., García-Cervigón, A. I., Godoy, O., Herrera, J. M., et al. (2022). Moving towards the ecological intensification of tree plantations. *Trends Plant Sci.*, S1360138521003526. doi:10.1016/j.tplants.2021.12.009.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2015). Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00928.

León, L., Díaz-Rueda, P., Belaj, A., De la Rosa, R., Carrascosa, C., and Colmenero-Flores, J. M. (2020). Evaluation of early vigor traits in wild olive germplasm. *Sci. Hortic.* 264, 109157. doi:10.1016/j.scienta.2019.109157.

López-Escudero, F. J., and Blanco-López, M. A. (2005). Recovery of Young Olive Trees from *Verticillium dahliae*. *Eur. J. Plant Pathol.* 113, 367–375. doi:10.1007/s10658-005-3145-0.

López-Escudero, F. J., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50. doi:10.1007/s11104-010-0629-2.

Montes-Osuna, N., and Mercado-Blanco, J. (2020). Verticillium Wilt of Olive and Its Control: What Did We Learn during the Last Decade? *Plants* 9, 735. doi:10.3390/plants9060735.

Montilon, V., Susca, L., Potere, O., Roseti, V., Campanale, A., Saponari, A., et al. (2021). Embryo culture, *in vitro* propagation, and molecular identification for advanced olive breeding programs. *Horticulturae* 8, 36. doi:10.3390/horticulturae8010036.

Niks, R. E., Ellis, P. R., and Parlevliet, J. E. (1993). "Resistance to parasites," in *Plant Breeding*, eds. M. D. Hayward, N. O. Bosemark, I. Romagosa, and M. Cerezo (Dordrecht: Springer Netherlands), 422–447. doi:10.1007/978-94-011-1524-7_26.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi:10.1016/S0261-2194(02)00180-1.

Rallo, P., de Castro, A. I., López-Granados, F., Morales-Sillero, A., Torres-Sánchez, J., Jiménez, M. R., et al. (2020). Exploring UAV-imagery to support genotype selection in olive breeding programs. *Sci. Hortic.* 273, 109615. doi:10.1016/j.scienta.2020.109615.

Robb, J. (2007). *Verticillium* tolerance: resistance, susceptibility, or mutualism? *Can. J. Bot.* 85, 903–910. doi:10.1139/B07-093.

Rugini, E., and Gutiérrez-Pesce, P. (2006). Genetic improvement of olive. *Pomol Croata* 12, 43–72.

Rugini, E., Silvestri, C., Ceccarelli, M., Muleo, R., and Cristofori, V. (2016). Mutagenesis and Biotechnology Techniques as Tools for Selecting New Stable Diploid and Tetraploid Olive Genotypes and Their Dwarfing Agronomical Characterization. *HortScience* 51, 799–804. doi:10.21273/HORTSCI.51.7.799.

Ruiz, M., Oustric, J., Santini, J., and Morillon, R. (2020). Synthetic Polyploidy in Grafted Crops. *Front. Plant Sci.* 11, 540894. doi:10.3389/fpls.2020.540894.

Russo, R., Caruso, M., Arlotta, C., Lo Piero, A. R., Nicolosi, E., and Di Silvestro, S. (2020). Identification of Field Tolerance and Resistance to Mal Secco Disease in a Citrus Germplasm Collection in Sicily. *Agronomy* 10, 1806. doi:10.3390/agronomy10111806.

Sattler, M. C., Carvalho, C. R., and Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. *Planta* 243, 281–296. doi:10.1007/s00425-015-2450-x.

Serrano, A., León, L., Belaj, A., and Román, B. (2020). Nucleotide diversity analysis of candidate genes for Verticillium wilt resistance in olive. *Sci. Hortic.* 274, 109653. doi:10.1016/j.scienta.2020.109653.

Torres-Sánchez, J., de la Rosa, R., León, L., Jiménez-Brenes, F. M., Kharrat, A., and López-Granados, F. (2021). Quantification of dwarfing effect of different rootstocks in 'Picual' olive cultivar using UAV-photogrammetry. *Precis. Agric.* doi:10.1007/s11119-021-09832-9.

Trapero, C., Alcántara, E., Jiménez, J., Amaro-Ventura, M. C., Romero, J., Koopmann, B., et al. (2018). Starch Hydrolysis and Vessel Occlusion Related to Wilt Symptoms in Olive Stems of Susceptible Cultivars Infected by *Verticillium dahliae*. *Front. Plant Sci.* 9, 72. doi:10.3389/fpls.2018.00072.

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. (2015). Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathol.* 64, 890–900. doi:10.1111/ppa.12330.

Troncoso, A., Liñán, J., Cantos, M., and Acebedo, M. M. (2000). Injerto in vitro del olivo. *Mercacei* 23, 171–173.

Trujillo, I., Ojeda, M. A., Urdiroz, N. M., Potter, D., Barranco, D., Rallo, L., et al. (2014). Identification of the Worldwide Olive Germplasm Bank of Córdoba (Spain) using SSR and morphological markers. *Tree Genet. Genomes* 10, 141–155. doi:10.1007/s11295-013-0671-3.

Zhang, Y., Gao, Y., Wang, H.-L., Kan, C., Li, Z., Yang, X., et al. (2021). *Verticillium dahliae* secretory effector PevD1 induces leaf senescence by promoting ORE1-mediated ethylene biosynthesis. *Mol. Plant* 14, 1901–1917. doi:10.1016/j.molp.2021.07.014.

Conclusiones Generales

- Se optimizó el proceso de micropropagación *in-vitro* de olivos silvestres mediante el estudio de la calidad e intensidad del empleo de luces LEDs frente a la iluminación fluorescente convencional. Concluimos que dos subespecies diferentes de *Olea europaea* mostraron un mejor crecimiento y desarrollo bajo iluminación LED en comparación con las lámparas fluorescentes.

- Setenta por ciento de LED rojos y treinta por ciento azules con un PPF de 34 µmol m^{-2} s^{-1} de irradiancia de luz fue el tratamiento de iluminación óptimo para la micropropagación del olivo en términos de menor consumo de energía, mayor altura y biomasa, acumulación de pigmentos fotosintéticos, manipulación óptima de explantes *in-vitro*, menor área foliar y mínima densidad estomática.

- Se estableció el germoplasma silvestre de olivo 'SILVOLIVE', que representa una parte importante de la variabilidad genética de la especie tanto a nivel genotípico como fenotípico.

- Entre los 146 genotipos silvestres de la colección, diferenciados en 19 zonas de recolección, los genotipos AMK, MAR, GUA presentan características deseables por sus parámetros de vigor reducido en maceta y en campo, rasgos interesantes para su uso como portainjertos en un sistema de cultivo super intensivo o de alta densidad (HDH).

- Los genotipos silvestres de olivo usados como portainjertos modifican los parámetros de crecimiento del vástago injertado. De acuerdo a sus características específicas, los genotipos de la colección SILVOLIVE tienen un gran interés potencial para: (i) restaurar la vegetación en suelos degradados o en riesgo de desertificación; (ii) proporcionar rasgos o genes relevantes en los programas de mejoramiento; (iii) ser utilizados como portainjertos para el cultivo del olivo, principalmente para sistema de cultivo HDH.

- Los portainjertos AMK21, AMK6 y ACO15 injertados con los cultivares 'Arbequina' y 'Picual' en condiciones de campo demostraron ser candidatos aptos para reducir vigor y hacerlos asequibles para sistemas de plantación de alta densidad. Además, genotipos injertados con los cultivares 'Hojiblanca' y 'Picual' mostraron una rápida entrada en producción, destacando los portainjertos vigorizantes y algunos genotipos enanizantes como GUA3 con valores medio/altos de fructificación.

- De 68 genotipos de la colección SILVOLIVE (50% de la colección) evaluados frente al patotipo defoliante V117 de *Verticillium dahliae*, se han identificado 15 genotipos resistentes al hongo en condiciones de invernadero, como posibles candidatos para controlar la Verticilosis del olivo.

- La medición del contenido de ADN de *Verticillium* en etapas tempranas y tardías de la infección, junto con las correlaciones con los síntomas de las plantas, permitió identificar patrones específicos de respuesta en los genotipos silvestres de olivo, apuntando a la aparición de diferentes estrategias de resistencia a la Verticilosis, como mecanismos de evitación y tolerancia.

- Los genotipos resistentes reducen el inóculo del hongo en sus tejidos y retrasan la aparición de los síntomas de la enfermedad, representando una valiosa fuente para ser utilizados como portainjertos y en programas de mejora en el marco de un control integrado de la enfermedad.

- Genotipos seleccionados como portainjertos por su resistencia a *V. dahliae* retrasan el desarrollo de la enfermedad, destacándose los portainjertos AMK27 y GUA3. Sin embargo, ningún genotipo silvestre evaluado como portainjerto mejoró la resistencia proporcionada por el cultivar resistente 'Frantoio' en las condiciones ensayadas.

- Los portainjertos enanizantes y resistentes a *V. dahliae* de la colección silvestre de olivo (AMK27 y GUA3) son buenos candidatos para la mejora del olivar. Estos genotipos presentan varias ventajas para el cultivo: (1) mayor resistencia transmitida a los cultivares 'Picual' y 'Arbequina' frente al patógeno *V. dahliae*; (2)

menor tamaño de árbol que los cv. ‘Arbequina’ y ‘Picual’ autoenraizado y autoinjertado para su introducción en sistemas HDH, (3) rápida entrada en producción, (4) aumento de la variabilidad genética de los olivares para hacer frente a diferentes condiciones edafoclimáticas y desafíos fenológicos y, por lo tanto, (5) ser potenciales recursos genéticos de interés en los programas de mejora.

Perspectivas de Futuro

1. Debido a la importancia de adaptar el cultivo del olivo al sistema de alta densidad de plantación para disminuir los costes de poda y recolección mediante la mecanización, estamos llevando a cabo un experimento a gran escala en la finca Buitrago para evaluar el uso potencial de 28 genotipos seleccionados como portainjertos enanizantes injertando sobre ellos cultivares de gran importancia económica ('Arbequina', 'Manzanilla de Sevilla' y 'Picual'). El crecimiento de las plantas injertadas será evaluado a lo largo de varios años sucesivos, así como su producción dentro de la finca.

2. A causa de la necesidad de encontrar portainjertos resistentes que puedan ayudar al control integrado de la Verticilosis, se evaluará la capacidad de los restantes genotipos resistentes de la colección de transmitir resistencia a *V. dahliae* al cultivar injertado 'Picual'. Además, se evaluará su comportamiento en campos infestados con el patógeno a densidades de inóculo conocidas.

3. Por otro lado, la reforestación a escala mundial es una de las soluciones para reducir el creciente CO_2 atmosférico antropogénico y reducir sus graves efectos en el clima. Las plantas absorben este CO_2 y lo fijan en sus tejidos, ayudando a que se almacene en el suelo. Las numerosas iniciativas de reforestación actuales no incluyen actuaciones en zonas áridas, aunque la aridez esté aumentando rápidamente por el cambio climático y afecte ya a una gran parte del planeta. Se propone una solución que permita aumentar la capacidad de absorción y almacenamiento de CO_2 mediante el uso de plantas leñosas idóneas en regiones áridas. Las condiciones de estas regiones hacen que las especies vegetales que se utilicen deban presentar unas características peculiares. Una especie que reúne rasgos clave para responder eficazmente a la sequía es el olivo, que además puede tener un valor añadido por su producción. Nuestro objetivo específico es identificar genotipos silvestres de olivo que produzcan una cantidad elevada de biomasa subterránea, con mayor capacidad de acumulación de polímeros estables en la raíz (suberina y lignina) y que respondan de manera

efectiva a la sequía. Básicamente, se trata de encontrar una “superplanta” para la recuperación de regiones áridas, con capacidad para almacenar grandes cantidades de CO_2 en estructuras vegetales difíciles de degradar, como la suberina y lignina en la raíz, y por tanto este carbono permanezca inmovilizado en el subsuelo largo tiempo. Esta “superplanta”, además, necesita hacer todo lo anterior, utilizando la menor cantidad posible de agua. Publicaciones recientes han constatado la presencia de características relevantes para este objetivo en diversos cultivares y genotipos silvestres de olivo, por lo que se empezará la búsqueda en la colección SILVOLIVE. Además, se identificarán rasgos fisiológicos clave que permiten definir las características idóneas para identificar “superplantas” en otras especies.

Ápendice 1

Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress

Virginia Hernández-Santana, **Pablo Díaz-Rueda**, Antonio Díaz-Espejo, María D. Raya-Sereno, Saray Gutiérrez-Gordillo, Antonio Montero, Alfonso Pérez-Martín, Jose M. Colmenero-Flores and Celia M. Rodríguez-Domínguez

Front. Plant Sci., 13 March 2019 | https://doi.org/10.3389/fpls.2019.00291

Abstract

The hydraulic traits of plants, or the efficiency of water transport throughout the plant hydraulic system, could help to anticipate the impact of climate change and improve crop productivity. However, the mechanisms explaining the role of hydraulic traits on plant photosynthesis and thus, plant growth and yield, are just beginning to emerge. We conducted an experiment to identify differences in growth patterns at leaf, root and whole plant level among four wild olive genotypes and to determine whether hydraulic traits may help to explain such differences through their effect on photosynthesis. We estimated the relative growth rate (RGR), and its components, leaf gas exchange and hydraulic traits both at the leaf and whole-plant level in the olive genotypes over a full year. Photosynthetic capacity parameters were also measured. We observed different responses to water stress in the RGRs of the genotypes studied being best explained by changes in the net CO_2 assimilation rate (NAR). Further, net photosynthesis, closely related to NAR, was mainly determined by hydraulic traits, both at leaf and whole-plant levels. This was mediated through the effects of hydraulic traits on stomatal conductance. We observed a decrease in leaf area: sapwood area and leaf area: root area ratios in water-stressed plants, which was more evident in the olive genotype *Olea europaea* subsp. *guanchica* (GUA8), whose RGR was less affected by water deficit than the other olive genotypes. In addition, at the leaf level, GUA8 water-stressed plants

presented a better photosynthetic capacity due to a higher mesophyll conductance to CO_2 and a higher foliar N. We conclude that hydraulic allometry adjustments of whole plant and leaf physiological response were well coordinated, buffering the water stress experienced by GUA8 plants. In turn, this explained their higher relative growth rates compared to the rest of the genotypes under water-stress conditions.

Introduction

One of the main challenges facing the world today is to achieve food security, a problem that is aggravated by climate change, natural resource depletion and adverse impacts of environmental degradation (desertification, drought, freshwater scarcity, etc.) (United Nations, 2015). Promising approaches for ensuring the stability of food production under limited water availability involve breeding practices that take advantage of the genetic variability of wild related species and different cultivars that have better adapted to environmental constraints (Nevo et al., 2012; Burnett et al., 2016), such as water-deficit conditions (Ruane et al., 2008; Reddy et al., 2017; Trentacoste et al., 2018). Crop breeders seek to identify and select traits or mechanisms that enable high biological or reproductive yields to be achieved under water-limited conditions (Turner, 2017). As demonstrated in recent studies, some morphological leaf traits (López-Sampson et al., 2017) or processes such as osmotic adjustment (Blum, 2017) explain a large proportion of a tree species' growth, which indicates the potential value of focusing on certain traits to help in the selection of the most productive species or varieties.

Specific knowledge of how hydraulic traits of plants (i.e., the efficiency of water transport throughout the plant) limit plant performance could help to anticipate the impact of climate change (Anderegg et al., 2016) and to improve the security and sustainability of our food supply. Nevertheless, the mechanisms explaining the role of hydraulic traits on growth are complex and only just beginning to be elucidated (Sack et al., 2016). Stomatal control of transpiration is directly or indirectly regulated by changes in plant water status, produced by changes in the soil-to-leaf water transport properties (Buckley, 2005). Under water deficit conditions, stomata close to avoid leaf desiccation but in doing so, carbon dioxide uptake is restricted, and in turn, assimilation rate. Thus,

growth can be limited by both carbon supply and turgor pressure. Above-ground hydraulic resistances to water flow mainly lie in leaves (Nardini and Salleo, 2000; Nardini et al., 2001, Brodribb and Holbrook, 2003; Sack et al., 2003), creating a positive link between leaf hydraulics and leaf gas exchange (Brodribb and Holbrook, 2004, 2006; Brodribb et al., 2005, 2007; Brodribb and Jordan, 2008; Scoffoni et al., 2016; Reddy et al., 2017; Xiong et al., 2018). In that sense, leaf hydraulics have been suggested to be important to both water and carbon (C) fluxes (Reich, 2014).

These studies highlight the coordination of maximum values of leaf gas exchange and leaf hydraulic conductance, i.e., under steady-state, non-stress conditions. The potential relevance of this coordination to plant performance under water-deficit conditions has also been investigated (Brodribb and Holbrook, 2004; Lo Gullo et al., 2005; Gortan et al., 2009; Chen et al., 2010; Hernández-Santana et al., 2016). Besides these short-term mechanisms of stomatal control through leaf hydraulics, plants also respond to water stress through processes influencing equilibria and steady-state behaviors across the entire plant system, adjusting their root/shoot functional balance accordingly (Mencuccini, 2014), i.e., changing the hydraulic allometry of the plant. Nevertheless, in response to water stress, more research is needed to quantify responses in relation to plant anatomy, allocation, architecture and physiology (Addington et al., 2006; Martínez-Vilalta et al., 2009; Zhou et al., 2016; Martin-StPaul et al., 2017) to better understand how development is coordinated in different environments based on the underlying mechanisms (Sterck and Zweifel, 2016).

In relation to olive genotypes, very little is known about how hydraulic traits and photosynthetic assimilation rates in response to water stress influence growth. We know that olive species rely on a range of physiological traits and mechanisms to cope with water deficit (Fernández, 2014; Díaz-Espejo et al., 2018). However, to progress breeding efforts, knowledge of genotypic variation for water-use traits and how they influence plant performance under water stress is required. As such, we conducted an experiment that employed both well-irrigated and water-stress conditions to identify differences in growth patterns among different wild olive genotypes, and to determine whether hydraulic traits may help to explain such differences through their effect on stomatal conductance and photosynthesis rate.

Our objectives were: (i) to evaluate whether different relative growth rate (RGR) patterns representing different physiological strategies arise in wild olive genotypes at leaf, root and plant level and to determine the effects of water availability on these growth patterns and (ii) to determine the role of hydraulic traits (mediated by their effect on leaf gas exchange) at the leaf and whole-plant levels to explain differences in RGR patterns at plant scale in two contrasting olive genotypes.

Materials and Methods

Experimental Overview

We conducted our experiment using four genotypes (AMK6, ACZ9, GUA6, and GUA8) selected from a first screening of 39 wild genotypes representing three different subspecies of *Olea europaea* (*europaea* var. *sylvestris*, *guanchica* and *cuspidata*). We assessed the effect of long-term deficit irrigation on growth patterns of these four genotypes, and afterward, we focused on two of them that presented the most contrasting trends in growth (GUA6 and GUA8) to explore the physiological and morphological traits that explained these differences in growth performance. The specific measurements performed during each period of the experiment are provided in **Table 1**.

TABLE 1 | Period, frequency, and number of replicates per genotype and irrigation treatment for the variables measured along the experiment and the genotypes where they were measured.

Measurement period	Dates	Genotypes	Variables	Measurement frequency	Replicates
Harvest 1–Harvest 2	From 06-04-2016 to 05-04-2017	ACZ9 AMK6 GUA6 GUA8	Relative growth rate (RGR, g g^{-1} day^{-1})	Once	Four
			Leaf mass fraction (LMF, g g^{-1})	Once	Four
			Root mass fraction (RMF, g g^{-1})	Once	Four
			Specific leaf area (SLA, m^2 g^{-1})	Once	Four
			Specific root length (SRL, m g^{-1})	Once	Four
			Net assimilation rate (NAR, g m^{-2} day^{-1})	Once	Four
			Maximum stomatal conductance ($g_{s,max}$, mol m^{-2} s^{-1})	Fortnightly-Monthly	Two (2016) and three (2017)
			Maximum net photosynthesis rate ($A_{N,max}$, µmol m^{-2} s^{-1})	Fortnightly-Monthly	Two (2016) and three (2017)
After Harvest 2	From 06-04-2017 to 29-08-2017	GUA6 GUA8	Maximum stomatal conductance ($g_{s,max}$, mol m^{-2} s^{-1})	Twice	Four
			Maximum net photosynthesis rate ($A_{N,max}$, µmol m^{-2} s^{-1})	Twice	Four
			Maximum velocity of carboxylation (V_{cmax}, µmol m^{-2} s^{-1})	Once	Four
			Mesophyll conductance (g_m, mol m^{-2} s^{-1})	Once	Four
			Leaf water potential (Ψ_{leaf}, MPa)	Twice	
			Foliar N (gN m^{-2})	Once	Four
			Osmotic pressure at full turgor (Π_0, MPa)	Once	Four
			Turgor loss point (TLP, MPa)		
			Vulnerability curve of leaf hydraulic conductance (K_{leaf}, mmol m^{-2} s^{-1} MPa^{-1})	Once	–
			Leaf:sapwood area (cm^2 mm^{-2})	Once	Four
			Leaf:root area (m^2 m^{-2})	Once	Four

Screening of 39 Wild Olive Genotypes Before Harvest 1

The seeds for this first screening were obtained from trees located in the World Olive Germplasm Collection of Córdoba (Spain) and Grahamstown (South Africa). The plants were propagated and rooted in vitro from zygotic embryos obtained from the prospected seeds during 2014. Seeds were obtained by breaking olive pits with a tube cutter and surface sterilized with hypochlorite. Sterile embryos were obtained from the seeds and placed in test tubes with hormone-free olive medium (Rugini, 1984). After in vitro germination, the genotypes were multiplied through propagation of nodal segments in Rugini medium supplemented with 1 mg L^{-1} zeatin (Rugini, 1984). The explants were kept in an in vitro culture chamber at 25°C and subjected to a photoperiod of 16 h light/8 h darkness, using LED illumination 70% red plus 30% blue (70/30) with 34 µmol m^{-2} s^{-1} of photosynthetic photon flux. Plants were rooted in 1/2x Rugini medium supplemented with 0.8 mg/L Naphthaleneacetic acid for 3 weeks. After ex vitro acclimatization, plants were grown under greenhouse conditions for 9 months in 1 L pots. Healthy and homogenous plants were selected and transplanted into 10 L pots containing vermiculite:peat:perlite substrate (40:40:20) and acclimatized for a further 2 months. The 39 genotypes were evaluated during 2015 to assess their water use and fresh weight below and above-ground components as well as the whole plant. For each of these 39 genotypes, six well-irrigated plants (100% field capacity) and six water-stressed plants (60% field capacity) were maintained. Every 2–3 days water loss was quantified and plants were re-watered up to their corresponding water status. Plants were harvested at the end of the trial, and the fresh and dry weights of shoots (leaves and stems) and roots were recorded.

Experimental Management During the Measurements Performed in AMK6, ACZ9, GUA6, and GUA8 From Harvest 1 to Harvest 2

We selected these four genotypes because they presented contrasting behaviors to water deficit in terms of water use and plant, shoot and root fresh weight (**Supplementary Figure S1**). Plants from these four genotypes were grown outdoors in 25 L pots in La Hampa CSIC experimental orchard, near Seville (Spain) (37°17'N, 6°3'W, altitude 30 m), filled with soil (sandy loam) from this orchard. The size of the pot was

not limiting for plant growth. This was based on the observation that roots did not grow enough to fill the entire volume of the pots and some parts of the soil were not explored by them at the end of the experiment. The pots were distributed randomly in rows of 20 plants at 1 × 1.5 m, alternating well-watered (WW) rows and water-stressed rows (WS). This distribution was sufficient to avoid shading by neighboring plants (based on in situ observations). Initial sizes of the plants are shown in **Table 2** and although sizes were different among the groups, we calculated growth using RGR, which uses initial and final sizes, to minimize size dependent effects (Hunt et al., 2002). The experiments lasted for 19 months (from February 2016 to August 2017) including the measurements specifically performed only in GUA6 and GUA8 (see next section).

TABLE 2 | Average and standard error of the leaf area, basal diameter and maximum height of the plants used in the beginning of the experiment (H1).

		Leaf area (cm^2)	Basal diameter (mm)	Maximum height (cm)
WW	ACZ9	370.45 ± 27.87	6.16 ± 0.35	92.92 ± 2.47
	AMK6	89.81 ± 15.45	4.74 ± 0.39	51.82 ± 7.07
	GUA6	178.49 ± 18.58	4.51 ± 0.29	72.27 ± 9.49
	GUA8	59.09 ± 15.92	3.47 ± 0.14	20.84 ± 6.08
WS	ACZ9	518.15 ± 28.42	6.37 ± 0.23	99.92 ± 10.33
	AMK6	99.59 ± 10.78	4.59 ± 0.22	53.72 ± 4.11
	GUA6	257.13 ± 13.95	5.68 ± 0.20	83.02 ± 5.64
	GUA8	47.75 ± 12.11	2.99 ± 0.23	17.70 ± 3.26

The plants of all genotypes were the same age (16 months) when the experiment started. Plants were well-irrigated from February 18 to April 26, 2016. After this date they were irrigated differently until the end of the experiment: WW plants, in which plants were irrigated daily to non-limiting soil water conditions to achieve the highest possible stomatal conductance ($g_{s,max}$); and WS, in which plants were irrigated to a level representing 40% of the $g_{s,max}$ measured in WW plants throughout the experiment to achieve a moderate water-stress status. To achieve these values of $g_{s,max}$, we conducted regular gas exchange measurements and modified the irrigation schedule accordingly (**Figure 1**), i.e., reducing or increasing the frequency and time of irrigation to change the total amount of water depending on WS $g_{s,max}$ values compared to WW $g_{s,}s_{max}$. Reference evapotranspiration (ETo) was collected from a nearby standard weather station (37°13'N, 6°8'W) belonging to the Agroclimatic Information Network of the local government (Junta de Andalucía, Spain). Two harvests were conducted: on April 6, 2016 after a period when all the plants were well irrigated (harvest 1, H1) and on the April 5,

2017, to assess the effect of the long-term deficit irrigation treatment on the olive plants (harvest 2, H2).

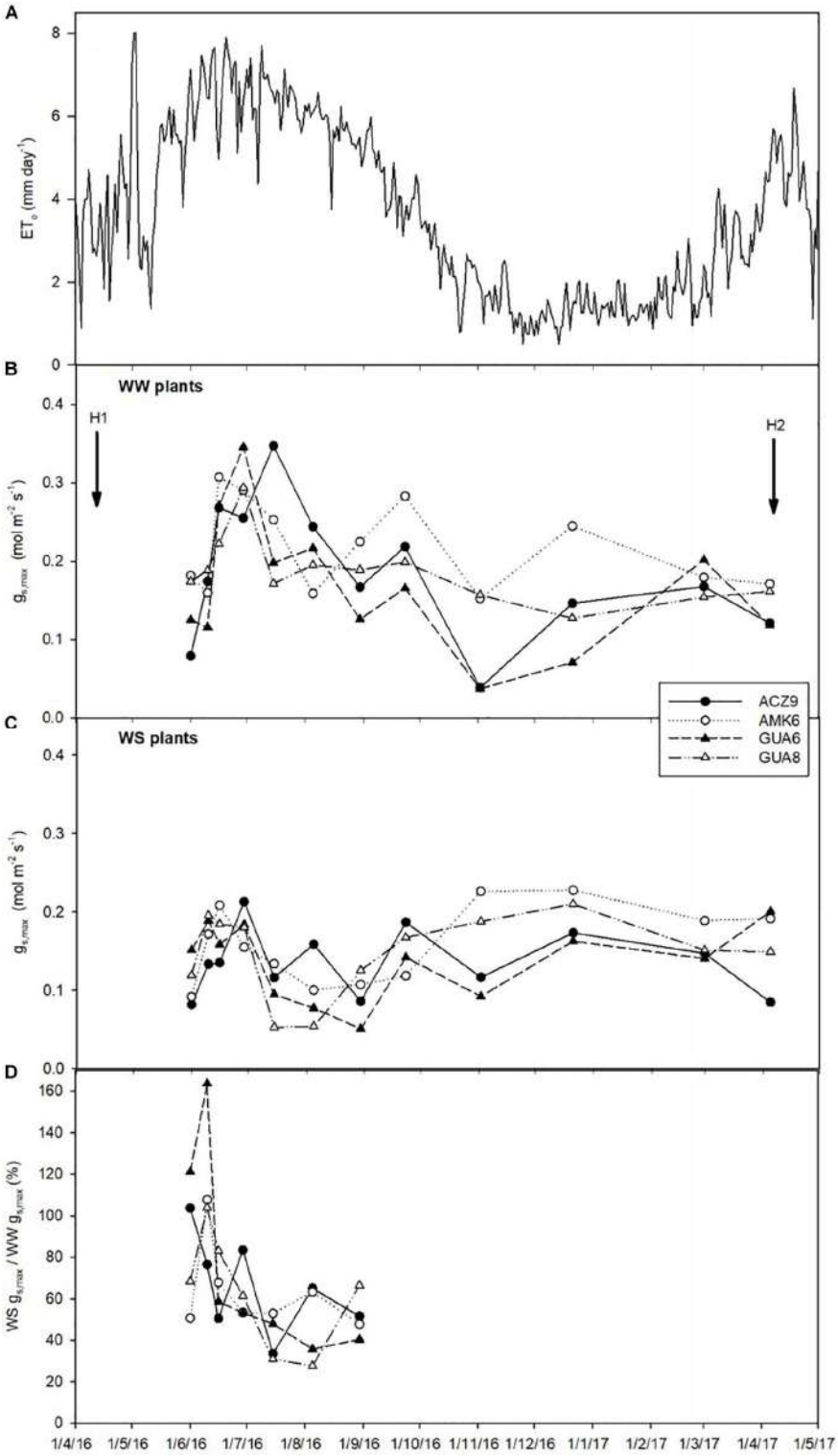


FIGURE 1 | Temporal dynamics of reference evapotranspiration, ETo (A), maximum stomatal conductance ($g_{s,max}$) for the different genotypes for well-watered plants (WW) (B), water-stressed plants (WS) (C), and percent of WS compared to WW $g_{s,max}$ (D) (only June–August data shown for clarity purposes). Each data-point in 2016 represents the average of two plants, while from January to April 2017 the average of three plants is used. H1 and H2 indicate when harvests 1 and 2 took place.

Experimental Management During the Measurements Performed in GUA6 and GUA8 After Harvest 2

After H2, 20 pots of GUA6 and GUA8 were kept under the described irrigation treatments (WS and WW) at the same field experimental site prior to conducting leaf hydraulic conductance measurements, pressure–volume curves and photosynthetic response curves, together with additional gas exchange, plant water status and morphological measurements (**Table 1**).

Growth Parameters (AMK6, ACZ9, GUA6, GUA8, From Harvest 1 to Harvest 2)

Plant growth was determined by harvesting four plants per genotype and irrigation treatment (n = 4) at H1 and H2. Before harvesting, basal stem diameter was measured to estimate sapwood area (m^2). After harvesting, total plant leaf area (m^2) was determined using a Li-Cor 3000-A area meter (equipped with a LI-3050C Transparent Belt Conveyor; Li-Cor, Lincoln, NE, United States). To calculate the biomass (g) of roots, stems and leaves, each component was separated and oven-dried at 60°C for at least 2 days. The following plant traits were calculated for each harvest based on the material obtained: leaf mass fraction (LMF, $g\ g^{-1}$), root mass fraction (RMF, $g\ g^{-1}$), specific leaf area (SLA, $m^2\ g^{-1}$) and specific root length (SRL, $m\ g^{-1}$). The data from each genotype and irrigation treatment for the two consecutive harvests were used to compute the net assimilation rate (NAR; $g\ m^{-2}\ day^{-1}$) and the RGR ($g\ g^{-1}\ day^{-1}$) for the plant (RGR_{plant}), roots (RGR_{root}) and leaves (RGR_{leaf}) according to Hunt et al. (2002):

$$RGR = NAR \times SLA \times LMF \quad (1)$$

Each component was calculated as follows:

$$(1W/)(dWdt/) = (1LA/)(dWdt/) \times LALW/\times LWW/ \quad (2)$$

where t is time between harvest 1 and 2, W is total dry weight per plant, LA is total leaf area per plant and LW is total leaf dry weight per plant.

Root Length and Area (AMK6, ACZ9, GUA6, GUA8, From Harvest 1 to Harvest 2)

The root samples were separated into two groups: fine roots or roots thinner than 2 mm and roots thicker than 2 mm. From the first group of roots (thinner than 2 mm), roots were randomly subsampled, scanning 10% of total biomass using the WinRHIZO system (Regent Instruments, Québec, Canada). The roots thicker than 2 mm were not considered in this analysis as fine roots constitute the primary exchange surface between plants and soil (Jackson et al., 1997). The scanning enabled us to directly obtain the root length (cm) and root area (cm^2) through the WinRHIZO software.

Field Gas Exchange Measurements (AMK6, ACZ9, GUA6, GUA8, From Harvest 1 to Harvest 2)

To verify our irrigation treatments, maximum stomatal conductance ($g_{s,max}$ mol m^{-2} s^{-1}) and net photosynthesis rate ($A_{N,max}$, µmol m^{-2} s^{-1}) were measured at ∼10.30–11.30 GMT from May 2016 to April 2017 (H2) with a portable gas analyzer (Li-6400; Li-Cor, Lincoln, NE, United States) using a 2 × 3 cm standard clear-top chamber under ambient light, vapor pressure deficit and CO_2 conditions in healthy, sunny leaves. Preliminary measurements demonstrated that $g_{s,max}$ occurred at this time of the day. During this period, gas exchange was measured fortnightly during the summer months, and once every month during the rest of the year. In 2016 we measured gas exchange in one leaf from each of two plants of every genotype and for the two irrigation treatments (n = 2). From January 2017 to April 2017 we increased the number of sampled plants to three (n = 3).

Field Gas Exchange and Leaf Water Potential Measurements (GUA6, GUA8, After Harvest 2)

In addition to monitoring $g_{s,max}$, gas exchange was measured once in June and July 2017 together with leaf water potential measurements to have concurrent measurements of both variables for the GUA6 and GUA8 genotypes (four plants per irrigation treatment and genotype).

Leaf water potential (Ψ_{leaf}) was undertaken immediately after gas exchange measurements with a Scholander-type pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, United States) in one fully expanded leaf per plant.

Leaf Hydraulic Vulnerability Curves (GUA6, GUA8, After Harvest 2)

Leaf hydraulic conductance (K_{leaf}, mmol m^{-2} s^{-1} MPa^{-1}) was measured after H2 (June of 2017) in fully developed, current year and sun-exposed leaves of WW plants of the GUA8 and GUA6 genotypes to obtain leaf hydraulic vulnerability curves (Ψ_{leaf} -K_{leaf}). To measure K_{leaf}, we used the Evaporative Flux Method (EFM, Scoffoni et al., 2012), with the results obtained by this method being similar to K_{leaf} measurements in olive achieved by the Dynamic Rehydration Method (DRKM, Blackman and Brodribb, 2011) as demonstrated by Hernández-Santana et al. (2016). Briefly, the method consists of measuring the flow rate of water through the leaf (mmol m^{-2} s^{-1}) and the corresponding Ψ_{leaf}. To achieve this, we sealed the pots containing the plants at the field in dark plastic bags containing wet paper towels inside to create a low-demand atmosphere. The plants were left to equilibrate at the laboratory for at least 30 min and then, to measure the leaf water flow, leaves were cut from the bagged plants under purified water and rapidly connected to a flowmeter consisting of silicon tubing containing purified, degassed water. The tubing was connected to a pressure transducer (PX26-005GV, Omega Engineering Ltd., Manchester, United Kingdom), which, in turn, was connected to a Campbell data logger CR1000 (Campbell Scientific Ltd., Shepshed, United Kingdom) which recorded water flow readings every 1 s. Reference tubing of different resistances was used to minimize measurement errors (Sack et al., 2011; Melcher et al., 2012). Once connected, the leaves were allowed to transpire inside a Li-Cor 6400-22 Opaque Conifer Chamber for at least 30 min with the photosynthetically active radiation (PAR) level set to 1,200 µmol m^2 s^{-1} using the Li-Cor 6400-18A RGB Light Source (both instruments were from Li-Cor, Lincoln, NE, United States) until the water flow was stable (coefficient of variation < 5% for the last 5 min). We chose EFM because using the Li-6400 gas analyzer also allowed us to measure the water vapor flux simultaneously with the liquid water flow. When both gas and liquid flows reached a steady state, leaves were removed from

the tubing and stored for equilibration in dark and halted transpiration conditions for at least 30 min. Then, Ψ_{leaf} was measured with a Scholander-type pressure chamber (PMS Instrument Company, Albany, OR, United States). The plants were left to gradually dehydrate in the field so that a wide range of hydraulic conductance and Ψleaf values were obtained.

Pressure–Volume Curves: Turgor Loss Point and Osmotic Pressure at Full Turgor (GUA6, GUA8, After Harvest 2)

We used one leaf from four plants for each irrigation treatment (WW, WS) and genotype (GUA6 and GUA8) to calculate pressure–volume curves (n = 4). Leaves were sampled in the morning of August 29, 2017 and were rehydrated for 24 h, then left to desiccate. Leaf weight and Ψleaf were measured many times during that desiccation period until the leaves reached minimum Ψ_{leaf} values of ca. -5 MPa. The turgor loss point (TLP, MPa) and osmotic pressure at full turgor (Π0, MPa) were calculated according to Sack and Pasquet-Kok (2017).

Photosynthetic Response Curves (GUA6, GUA8, After Harvest 2)

Four AN–Ci response curves (the response of net CO_2 assimilation to varying intercellular CO_2 concentration) were measured between 09:00 and 13:00 GMT on different days in July 2017 for the GUA6 and GUA8 genotypes and for each irrigation treatment (WW and WS) (four repetitions, 16 curves per genotype). Measurements were made using two LI-6400 portable photosynthesis systems (LI-COR, Lincoln, NE, United States) at 28°C (close to ambient temperature), saturating photosynthetic photon flux density (1,600 µmol m^{-2} s^{-1}) and an ambient CO_2 concentration (Ca) of between 50 and 1,150 µmol mol^{-1}. After steady-state photosynthesis had been achieved (usually after 20–40 min exposure to saturating PPFD), the response of A_N to varying Ci was measured by lowering Ca stepwise from 400 to 50 µmol mol^{-1}, returning to 400 µmol mol^{-1} and then increasing Ca stepwise from 400 to 1,150 µmol mol^{-1}. Each A–Ci curve comprised 16 measurements. The maximum carboxylation rate (V_{cmax}, µmol m^{-2} s^{-1}), maximum rate of electron transport (J_{max}, µmol m^{-2} s^{-1}) and mesophyll conductance to CO_2 (g_m, mol m^{-2} s^{-1}) were estimated by the curve-fitting method proposed by Ethier and Livingston (2004). Prior to curve analysis, CO_2 leaks in the chamber were corrected by following the procedure

described in Flexas et al. (2007). Rubisco kinetic parameters were taken from Bernacchi et al. (2002). Values of V_{cmax}, Jmax and gm obtained from the A–Ci curve analysis were recalculated at 25°C using the temperature dependence parameters specific for olive reported in Díaz-Espejo et al. (2006, 2007).

Foliar N (GUA6, GUA8, After Harvest 2)

Leaf samples were taken for N analysis from the H2 samples of all the genotypes and irrigation treatments. Enough current-year leaves were sampled to have at least 0.4 g of dry weight to analyze leaf N. Samples were washed in distilled water, dried at 70°C until constant weight, ground and passed through a 500 mm stainless-steel sieve. N concentration was determined by Kjeldahl method.

Data Processing and Statistical Analysis (AMK6, ACZ9, GUA6, GUA8)

Statistical analyses were performed to assess the effect of the irrigation treatment and genotype on leaf, root and the whole-plant RGR values, in addition to LMF, RMF, SLA, SRL, and NAR for H2. V_{cmax}, g_m, Π0, TLP, foliar N, leaf area – root area ratio (LA:RA) and leaf sapwood area ratio (LA:SA) were also estimated for the GUA6 and GUA8 genotypes after H2. One-way ANOVA was used in cases where more than two levels were compared, while the Student's t-test was used for comparisons between two levels. No transformations were needed to achieve normality. Differences were considered significant for values of $p < 0.05$. SigmaPlot software (version 12.0, Systat Software, Inc., San Jose, CA, United States) was used to conduct these analyses and provide best-fit curves to the dataset to determine the relationships between the different variables analyzed. In addition, two-way ANOVA was used to analyze the interaction between irrigation treatment and genotype RGR at leaf, root and plant level. We used a mixed model in which we included genotype, irrigation treatment and the interaction between both variables. Finally, we also used analysis of covariance (ANCOVA) that included the interaction term of each component related to RGR with irrigation treatment to test its effect on the relationships established for RGR. For these analyses we considered that variables were linearly related. These analyses were conducted with R software (R Core Team, version 3.4.3, 2018) using the "lm()" function.

Path analysis (structural equation modeling with no latent variables) was used to compare four alternative conceptual models to reveal the causal relationships that link hydraulic variables with A_N through their effect on g_s. We stated a priori the relationships among variables with a strong mechanistic or well-established and accepted empirical basis only (Shipley, 2000). The main underlying hypotheses were: (i) A_N is determined mainly by g_s, g_m, and V_{cmax} (Niinemets et al., 2009; Flexas et al., 2014; Perez-Martin et al., 2014); (ii) g_s is influenced by leaf hydraulic conductance (Brodribb and Holbrook, 2004; Sack and Holbrook, 2006; Scoffoni et al., 2016), LA:SA and LA:RA (Magnani et al., 2002; Addington et al., 2006; Martínez-Vilalta et al., 2009); and (iii) the major determinant of V_{cmax} is foliar nitrogen (Walcroft et al., 1997; Díaz-Espejo et al., 2006, 2007; Niinemets, 2012). We compared four models that differed according to whether K_{leaf}, LA:SA and LA:RA influence g_s directly (see **Figure 4A**), LA:SA and LA:RA are covariates (see **Figure 4B**), LA:RA effects on g_s are mediated by LA:SA (see **Figure 4C**) and LA:RA and LA:SA influence g_s through their impact on K_{leaf} (see **Figure 4D**). For the path analysis we have a total of 16 data points obtained from 16 plants, for each variable: 4 replicates × 2 genotypes × 2 treatments. All variables were measured or estimated on each of the 16 plants. To perform this analysis it is not important to consider or compare treatments or genotypes, but to provide estimates of the magnitude and significance of hypothesized causal connections between sets of variables. Although our small sample size (16 points for each variable) limits the complexity of the models and the strength of our conclusions, the results on how the hydraulic variables are related to each other and to g_s complement the simple regression analyses and comparisons conducted. All regression, covariance and variance relationships were determined and are shown in path diagrams. Gas exchange data used for the analysis were those measured in the A–Ci curves: average g_s and A_N obtained at 400 ppm CO_2 and vapor pressure deficit between 1.5 and 2 kPa. Leaf hydraulic conductance was estimated using the vulnerability curves and Ψ_{leaf} measured for those same plants around the time the data for the curves were obtained. The remaining variables were measured or calculated with the data from each plant. All variables were Ln-transformed before analysis to obtain linear relationships because structural equation modeling assumes linearity between variables and (approximate) multivariate normality (Shipley, 2000). Each path model

was fitted and compared with the observed results using maximum likelihood. We conducted a Confirmatory Factor Analysis to test whether the Fit Indices of the model were acceptable in terms of similarity between observed and predicted matrix [P-value (chi-square) > 0.05], discrepancy adjusted for sample size [Comparative Fit Index (CFI) > 0.9], and residuals of the model [Root Mean Square Error of Approximation (RMSEA < 0.06)]. Path analyses were conducted and diagrams prepared using the R packages "lavaan" (Rosseel, 2012) and "semPlot" (Epskamp, 2013).

Results

Variability in Plant Relative Growth Rates Among Genotypes (AMK6, ACZ9, GUA6, GUA8) and Irrigation Treatments

Due to the deficit irrigation, gs,max in the WS plants of the four genotypes selected was lower (around 37%) than that in WW plants, but only for the hottest and driest months (mid-June to September of 2016) (**Figures 1B–D**). In the remaining months, due to the lower evaporative demand of the experimental site (**Figure 1A**), the reduced irrigation applications were not sufficient to produce a marked reduction of $g_{s,max}$.

Although the irrigation protocol based on the reduction of $g_{s,max}$ provoked only moderate water stress conditions in the hottest months, it was enough to decrease RGR values of WS plants significantly and to different extents amongst genotypes compared to WW plants for the period from April 2016 (H1) to April 2017 (H2) in all genotypes (**Figure 2**). At the three levels considered, leaf, root and plant, there was a statistically significant interaction between the irrigation treatment and genotype ($p < 0.001$), i.e., the effect of irrigation depends on the genotype. Whereas GUA6 showed the highest RGR in WW plants, both in leaves (6.54 × 10-3± 0.42 × 10-3 g g-1 day-1; **Figure 2A**) and roots (7.90 × 10-3± 0.23 × 10-3 g g-1 day-1; **Figure 2B**), GUA8 presented the highest RGR in WS plants. Moreover the RGRroot of GUA8 was statistically similar ($p > 0.05$) between treatments, in contrast to the rest of the genotypes where RGR_{root} was significantly lower in WS than in WW plants ($p < 0.05$). Based on these findings, GUA8 was the genotype in which RGR was least affected by water stress.

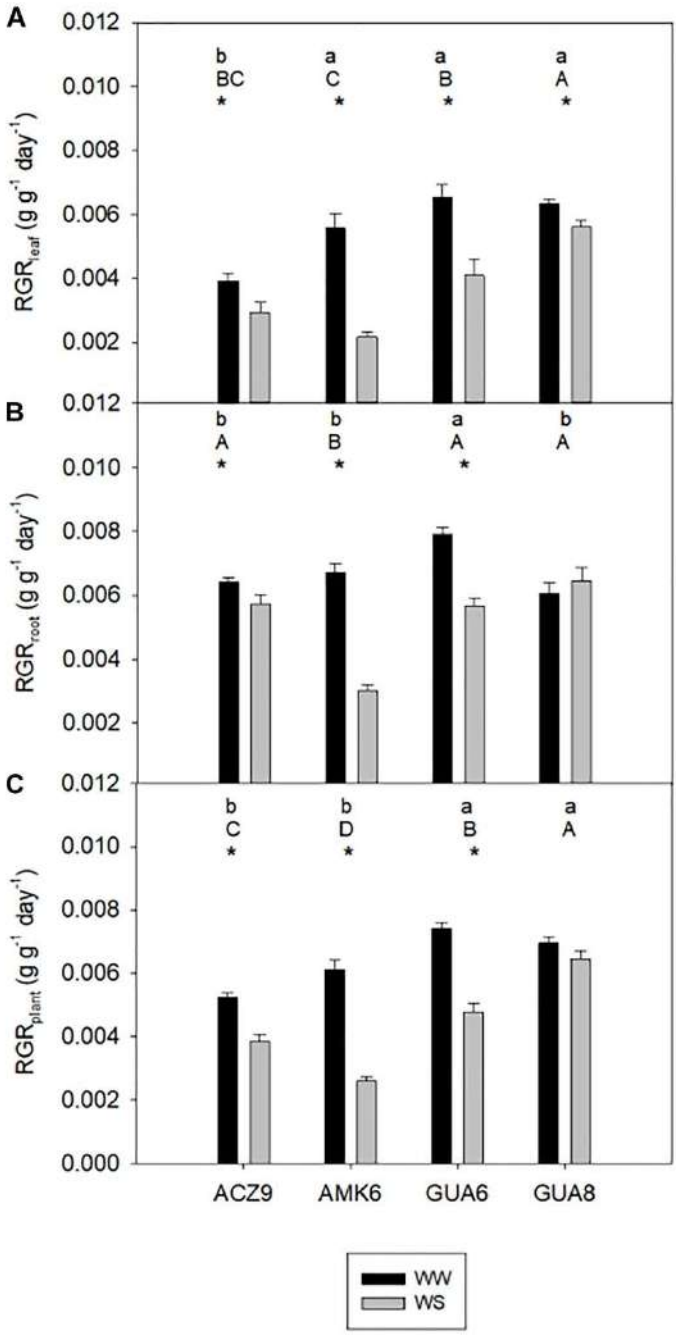


FIGURE 2 | Comparison of relative growth rate (RGR) from harvest 1 to harvest 2 of leaf (A), root (B), and the whole-plant (C) biomass for the different genotypes of well-watered plants (WW) and water-stressed plants (WS). Bars are the average of four plants ±1 SE. Lowercase letters indicate statistical differences among the genotypes for WW plants and capital letters for WS plants. The asterisks indicate significant differences ($p < 0.05$) between WW and WS plants for each genotype.

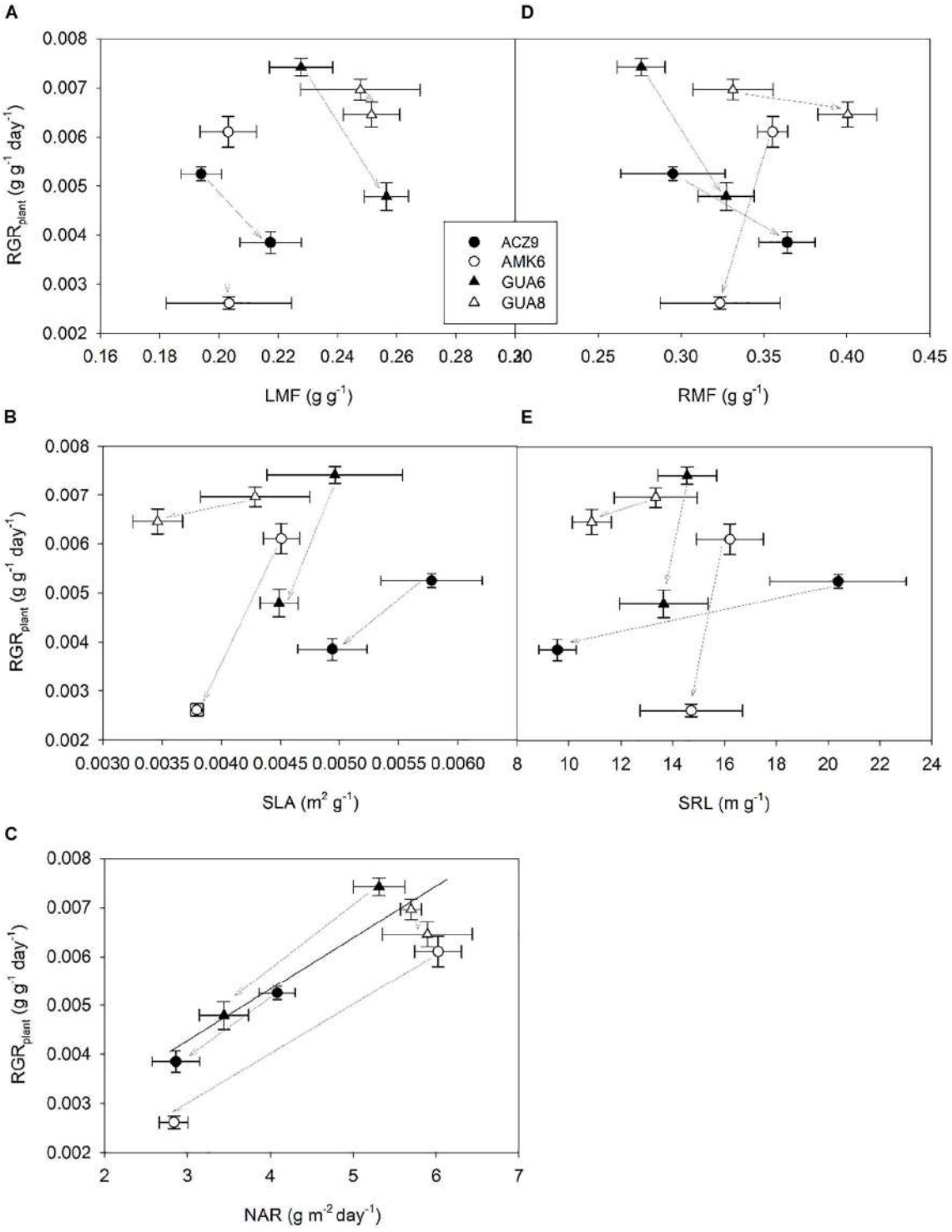


FIGURE 3 | Relationship between plant relative growth rate (RGRplant) (H1 to H2) and leaf mass fraction; LMF (A), specific leaf area; SLA (B), net assimilation rate; NAR (C), root mass fraction; RMF (D) and specific root length; SRL (E) for the different genotypes for well-watered (WW) and water-stressed (WS) plants. Each point is the average of four plants ±1 SE. Gray arrows indicate the change provoked by water stress (from WW to WS plants).

A regression analysis was conducted to relate RGR_{plant} with each of its components at the leaf level (LMF, SLA, and NAR) and corresponding parameters at the root level

(RMF and SRL) (**Figure 3**) by pooling together all genotypes and irrigation treatments. Variations in RGRplant were mainly explained by changes in NAR (**Figure 3C**) based on the strong correlation between parameters (R2 = 0.79; $p < 0.01$). The highest RGR_{plant} values were found for those genotypes with the highest NAR. ANCOVA revealed non-significant differences in the regression lines between WW and WS plants. The other traits studied related to carbon allocation (LMF and RMF) and anatomy (SLA and SRL), both for leaves and roots, were not significantly correlated with RGR_{plant}. While SLA and SRL showed similar patterns in each genotype, with both parameters reduced under WS conditions (**Figures 3B,E**), the different magnitude of the change for each genotype prevented a common trend from being identified.

The Role of Hydraulic Traits in GUA8 and GUA6 to Explain Relative Growth Rate Patterns

To further explain the above results showing NAR as the main parameter related to changes in RGR_{plant}, we focused on gas exchange dynamics at the leaf level of the GUA6 and GUA8 genotypes, including both irrigation treatments, which provided the most contrasting results in terms of growth for the different irrigation treatments. While GUA6 had the highest RGR under the WW conditions (although not significantly so), the same was true for GUA8 under the WS conditions. Pooling together the data for GUA6 and GUA8 to conduct a Path Analysis, we found two path models (**Figures 4A,C**) that were better than the other two (**Figures 4B,D**) in terms of fit statistics (see Materials and Methods section for further details). These two best-fitting models differed from each other in terms of how LA:RA impacted on g_s. In the model shown in **Figure 4A** the effect is direct, whereas in **Figure 4C** the impact is indirect and mediated by LA:SA. Here, the total variance explained for A_N was 0.88 and 0.85 for the models in a and c, respectively. The regression between LA:RA and gs or LA:SA was not significant in any case, meaning that LA:SA and K_{leaf} were the main variables controlling g_s. The path coefficient was lower in model a for K_{leaf} (0.42) than for the LA:SA path coefficient (0.49), whereas in model c this trend changed slightly (0.47 and 0.45 for the K_{leaf} and LA:SA path coefficients, respectively). Stomatal conductance was the variable determining A_N to the greatest extent across the models.

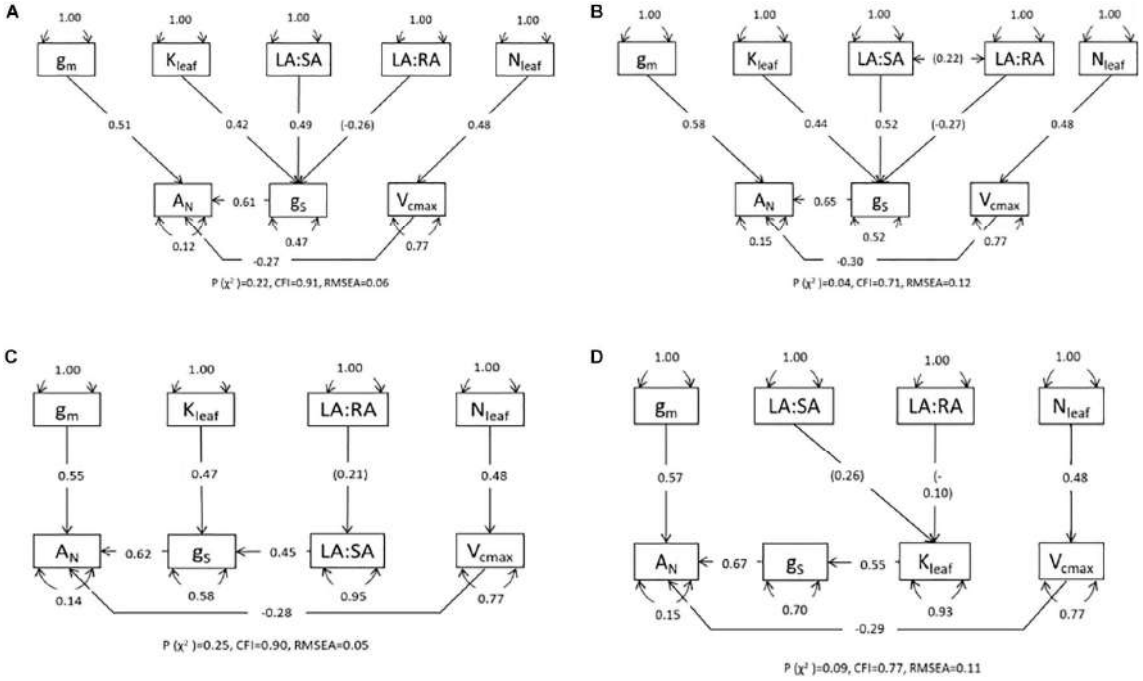


FIGURE 4 | Path diagrams for GUA6 and GUA8 from the plants measured after harvest 2 for the four models describing causal relationships with photosynthesis (A_N) mediated by stomatal conductance (g_s): (A) direct impact of leaf hydraulic conductance (K_{leaf}), leaf area to sapwood area ratio (LA:SA) and leaf area to root area ratio (LA:RA); (B) same as in (A) but both ratios represented as covariance; (C) direct effect of Kleaf but indirect effect of LA:RA mediated by LA:SA; and (D) indirect effect of LA:RA and LA:SA through their effect on Kleaf. Single-ended arrows and associated number indicate direct relationships and standardized parameter estimates of regression; double-ended arrows represent covariance; the curved double-ended arrows are the variances of each variable. In brackets are the non-significant parameters. V_{cmax}, maximum velocity of carboxylation; g_m, mesophyll conductance; N_{leaf}, foliar nitrogen. Overall fit statistics for each path model [p (χ2), CFI (Comparative Fit Index) and RMSEA (Root Mean Square Error of Approximation)] are shown at the bottom of each diagram.

At the leaf level, we further assessed differences in gas exchange and related variables for the different genotypes and irrigation treatments. We observed that g_s was slightly higher in GUA6 than in GUA8 for all levels of leaf water potential (**Figure 5A**), and that A_N was similar between genotypes for all levels of g_s (**Figure 5C**). As a result, the water-use efficiency calculated for GUA8 was also higher than for GUA6, in the sense that, to assimilate 1 μmol of CO_2, GUA6 plants transpired more water than GUA8. Accordingly, g_m was higher for GUA8 than GUA6 (**Table 3**), with this difference more evident in WS plants ($p < 0.05$; GUA6: 0.15 ± 0.03 mol m^{-2} s^{-1}; GUA8 0.24 ± 0.02 mol m^{-2} s^{-1}). In addition, V_{cmax} and foliar N (**Table 3**) followed the same trends for g_m, with statistically significant differences ($p < 0.05$) seen in the foliar N of WS plants. The hydraulic vulnerability curves for both genotypes were similar over the range of Ψleaf values and followed a sigmoidal shape (**Figure 5B**). No significant differences between

irrigation treatments or genotypes were seen for the other hydraulic traits (Π0 and TLP) analyzed (**Table 3**).

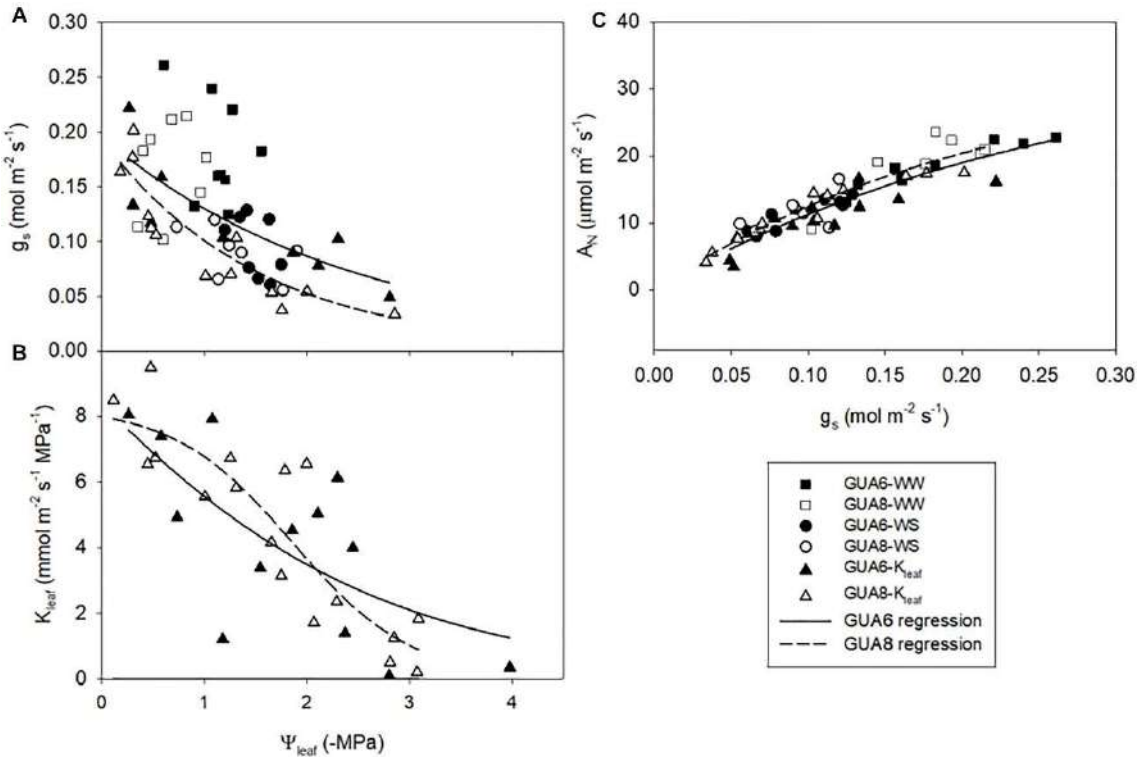


FIGURE 5 | Response curves of stomatal conductance; g_s (A) and leaf hydraulic conductance; K_{leaf} (B) to leaf water potential Ψ_{leaf}; and relationships between g_s and net photosynthesis rate; A_N (C) for GUA6 and GUA8 genotypes combing information for both well-watered (WW) and water-stress (WS) plants. Each point is one measurement per plant. Data in panels (A,C) were obtained from field-conducted A–Ci measurements with corresponding Ψ_{leaf} measurements (June and July 2017); and from the dry-down experiment to obtain the leaf hydraulic vulnerability curves shown in panel (B).

TABLE 3 | Average and standard errors of different variables measured in the genotypes GUA6 and GUA8, for well-watered (WW) and water-stressed plants (WS).

	GUA6		GUA8	
	WW	**WS**	**WW**	**WS**
Foliar N	3.68 ± 0.46	**3.71 ± 0.46**	3.92 ± 0.99	**5.83 ± 0.78**
g_m	0.22 ± 0.05	**0.15 ± 0.03**	0.27 ± 0.03	**0.24 ± 0.02**
V_{cmax}	213.75 ± 41.38	165.39 ± 16.80	199.54 ± 4.92	221.97 ± 15.86
LA:RA	0.74 ± 0.12	**0.53 ± 0.08**	0.63 ± 0.24	**0.28 ± 0.07**
LA:SA	8.72 ± 1.47	5.36 ± 0.77	6.56 ± 0.51[a]	3.35 ± 0.97[b]
Π_o	−1.43 ± 0.37	−1.36 ± 0.39	−1.41 ± 0.18	−1.33 ± 0.10
TLP	−2.09 ± 0.29	−2.37 ± 0.45	−2.09 ± 0.12	−2.28 ± 0.19

Numbers followed by different letters indicate significant differences between WW and WS plants for one genotype and bold numbers represent significant differences between the two genotypes for one irrigation treatment. Foliar N, leaf nitrogen (gN m^{-2}); g_m, mesophyll conductance (mol m^{-2} s^{-1}); V_{cmax}, maximum velocity of carboxylation (μmol m^{-2} s^{-1}); LA:RA, leaf area divided by root area (m m^{-2}); LA:SA, ratio between leaf area and sapwood area (cm^2 mm^{-2}); Π_O, osmotic pressure at full turgor (−MPa); TLP, turgor loss point (−MPa).

At whole-plant level, values of LA:SA and LA:RA ratios were always lower for GUA8 than GUA6 and for WS compared to WW. These differences were significant ($p < 0.05$) for the LA:RA ratio between the genotypes in WS plants, and between the irrigation treatments in the case of LA:SA for GUA8 (**Table 3**).

Discussion

Our results suggest that whole-plant hydraulic allometry adjustments together with shorter-term leaf physiological responses allowed the GUA8 genotype to buffer the impact of the drought stress experienced, leading to a RGR that was less-affected by water stress compared to the other olive genotypes tested. At the whole-plant level, the observed fine tuning of the supply-demand hydraulic system made this genotype more capable of extracting and transporting water. Also, as the total leaf area was lower, water transport capacity on a leaf specific basis was higher. At the leaf level, the greater photosynthetic capacity in GUA8 WS than in GUA6 WS plants (higher g_m and V_{cmax} in WS

plants, **Table 3**) also resulted in a slightly higher water-use efficiency for GUA8 under conditions of water stress (**Figure 5C**). Although it is difficult to estimate the below-ground biomass in adult trees grown under field conditions, more work is needed in adult trees to verify the patterns found in this study in juvenile olive seedlings growing in pots.

Differential Response of Relative Growth Rate to Water Stress in Olive Genotypes

Our results showed that although $g_{s,max}$ was only significantly reduced in summer, this decrease was sufficient to decrease RGR in different olive genotypes over a whole year (**Figure 1**). Such a long experimental period for this kind of study, coupled with long-term responses to soil and atmospheric drought as described here, are not usual. Although this experiment length adds value to the study, it could influence the results due to ontogenetic drift. As described by Rees et al. (2010), RGR is not totally size independent, because most plants become increasingly inefficient as they get larger because of self-shading, tissue aging, allocation to structural components, etc. However, such an effect is not likely to have happened in our study since there is no correspondence between the size of the plants (**Table 2**) and RGR (**Figure 2**) for either treatment. Despite belonging to the same species and sharing most of their water-stress response traits, differences were observed among the studied genotypes, with the GUA8 genotype having a significantly less-affected RGR as a result of decreased stomatal conductance in response to water stress (**Figure 2**). From the components of the RGR analysis, only physiological changes (NAR) were strongly and positively correlated to RGRplant among the genotypes (**Figure 3**). Similar patterns have been found in other woody species (Galmés et al., 2005; Shipley, 2006), particularly under the high radiation of field experiments in comparison to laboratory or greenhouse experiments (Shipley, 2002). Other plant growth components did not show a common pattern of change among the genotypes analyzed, although in general denser roots and leaf tissues were found for GUA8 than for the other genotypes, which is consistent with GUA8 being less affected by water stress. The influence of the different components on the decrease in RGR imposed by drought conditions has been shown to be strongly dependent on the

species in question, reflecting differences in response and adaptation to environmental constraints (Galmés et al., 2005).

Coordinated Response of Hydraulic Properties and Leaf Gas Exchange to Water Stress

We further assessed relationships between, and differences in, physiological parameters that might influence gas exchange and thereby explain why the RGR of GUA8 was less affected by water stress than GUA6. At the leaf level, and for both genotypes, the net photosynthesis rate was shown to be mainly limited by stomatal conductance (**Figures 4, 5**) as demonstrated for many other species, given that stomatal closure is one of the earliest responses to drought and the dominant limitation to photosynthesis under mild to moderate drought conditions (Flexas and Medrano, 2002). The relationship between stomatal conductance and leaf hydraulic conductance was strong (**Figure 4**), thus adding to a growing body of evidence reporting the coordination between water supply and demand at the leaf level (Sack and Holbrook, 2006; Scoffoni et al., 2016). Leaf hydraulic conductance determines the efficiency of the coordination between water supply and demand, and hence, it may determine the degree that the stomata can remain open to allow photosynthesis. In that sense, leaf hydraulic conductance has been increasingly recognized to play a central role in determining plant performance and productivity (Brodribb, 2009; Flexas et al., 2013).

At the plant level, we observed changes in the hydraulic allometry (as proposed by Maseda and Fernández, 2006) of WS plants compared to WW plants, with morphological adjustment being more evident in GUA8. These changes involved a decrease of leaf area to sapwood and root areas, which may reflect a tuning of the hydraulic structure of these individuals to increase water extraction and transport capacity under conditions of water deficit, thereby improving the supply of water to the leaves and the leaf-specific hydraulic conductivity of the plant (Martínez-Vilalta et al., 2009; Martin-StPaul et al., 2017). This, in turn, helps to maintain stomatal conductance (Addington et al., 2006) and photosynthesis (Zhou et al., 2016). WS GUA8 showed a significant increase in root area to leaf area ratio compared to that seen in WS GUA6. This change could contribute to improved plant hydraulic efficiency by helping to

maintain the plant water potential within a safe range, thereby reducing the risk of disruptive xylem embolism (Magnani et al., 2002) and a decline in below-ground hydraulic conductance (Johnson et al., 2018). In addition, olive plants have been shown to be very resistant to cavitation, including leaf xylem and coarse root xylem pathways (Rodríguez-Domínguez et al., 2018), so loss of xylem water transport capacity under our experimental framework were unlikely. However, pathways outside the xylem may have reduced K_{leaf} and, in turn, g_s (Scoffoni et al., 2017) under moderate water stress conditions.

Although homeostasis in response to a sudden perturbation can be achieved only through stomatal regulation, structural changes appear to play a central role in the plant's adjustment to prevailing environmental conditions over periods of months to years (Magnani et al., 2002). Indeed, the LA:SA ratio was also highly correlated to stomatal conductance, although this was not the case for the LA:RA ratio. Despite the lack of association between LA:RA and g_s, optimal allocation of resources between transpiring foliage and absorbing roots has been suggested to be coordinated with short-term regulation of g_s in response to drought (Magnani et al., 2002; Rodriguez-Dominguez and Brodribb, unpublished). A differential LA:RA response to water stress by the GUA6 and GUA8 genotypes, used in the path analysis, might underlie the lack of the relation between LA:RA and g_s, as mentioned above. New advances in root hydraulics that are just beginning to emerge (Cuneo et al., 2016; Poyatos et al., 2018; Rodriguez-Dominguez et al., 2018) will bring new possibilities to explore the impact of changes in LA:RA on stomatal conductance.

Carbon Balance at the Leaf Level

Despite gs and A_N being very similar in the two genotypes, gs was slightly higher in GUA6 than in GUA8, although this was not reflected in A_N. This resulted in a better instantaneous water use efficiency for GUA8 than GUA6, which could be advantageous under conditions where water is scarce. Indeed, GUA8 exhibited leaf gas exchange traits which enhanced the net photosynthesis rate for a given gs. This was observed in terms of changes in V_{cmax} and g_m. A larger gm is an interesting solution for plants under water stress (Barbour et al., 2010; Flexas et al., 2016), since it reduces that drawdown in CO_2

from the intercellular spaces to the chloroplastic sites of carboxylation, without an increase in transpiration. This is even more important if V_{cmax} has increased, as in the case of GUA8, since a higher V_{cmax} demands more CO_2. Therefore, an orchestrated enhancement of both V_{cmax} and gm is necessary to yield the desired goal of increasing A_N under water stress conditions. This physiological strategy has been reported as being typical of Mediterranean species with sclerophyllous leaves (Flexas et al., 2013; Peguero-Pina et al., 2015a, 2017). Moreover, the mechanism has not only been shown in angiosperms but also in gymnosperms (Peguero-Pina et al., 2015b), and is now accepted as a typical characteristic of species living in arid and semi-arid environments.

The high concentration of leaf N, as measured in this study for GUA8, confirms that this increase in nitrogen is not a mechanism for storing this macronutrient. The prime goal of the increase in N is directed to an enhancement of V_{cmax} and subsequently the A_N. The increase of N is putatively driven by the decrease in SLA, since a larger mass is concentrated by leaf surface area. Foliar N is mainly allocated to the photosynthetic apparatus of the leaf (Rubisco, electron transport, and chloroplasts) (Evans, 1989). This obviously has a direct impact on V_{cmax} and J_{max}, but it is also likely to affect g_m. Although we have no data on the anatomy of the leaves, an increase of SLA and N have been reported to enhance the surface area of chloroplasts exposed to the intercellular spaces, thus improving the liquid component of mesophyll conductance, which is usually the most limiting factor for gm (Tosens et al., 2012; Tomas et al., 2013; Flexas and Díaz-Espejo, 2015).

Conclusion

We showed here that genotypes belonging to the olive species can exhibit different RGRs in response to water stress. Although differences among genotypes within species are usually smaller than differences among species, two main adjustments to improve the net photosynthesis rate were identified in one of the genotypes (GUA8) used in this study, allowing it to maintain or even increase growth rate under mild water stress conditions. First, at the whole-plant level, a hydraulic allometry adjustment took place as a result of the decrease in the ratios of the areas of leaf-root and leaf-sapwood, the latter being also strongly related to stomatal conductance. Secondly, at the leaf level we

identified an increase in CO_2 fixation for a given stomatal conductance that was brought about by an adjustment of traits optimizing CO_2 fixation (higher mesophyll conductance and leaf N favoring maximum carboxylation rate). We also found that the leaf hydraulic conductance plays an important role in controlling stomatal conductance. Multi-scale studies such as the present one can be of great help to provide information on alternative opportunities to generate more drought-tolerant varieties.

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00291/full#supplementary-material

References

Anderegg, W. R. L., Klein, T., Bartlett, M., Sack, L., Pellegrini, A. F. A., Choat, B., et al. (2016). Meta-analysis reveals that hydraulic traits explain cross-species patterns of drought-induced tree mortality across the globe. Proc. Natl. Acad. Sci. U.S.A. 113, 5024–5029. doi: 10.1073/pnas.1525678113

Barbour, M. M., Warren, C. R., Farquhar, G. D., Forrester, G. U. Y., and Brown, H. (2010). Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination. Plant Cell Environ. 33, 1176–1185. doi: 10.1111/j.1365-3040.2010.02138.x

Bernacchi, C. J., Portis, A. R., Nakano, H., von Caemmerer, S., and Long, S. P. (2002). Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiol. 130, 1992–1998. doi: 10.1104/pp.008250

Blackman, C. J., and Brodribb, T. J. (2011). Two measures of leaf capacitance: insights into the water transport pathway and hydraulic conductance in leaves. Funct. Plant Biol. 38:118. doi: 10.1071/FP10183

Blum, A. (2017). Osmotic adjustment is a prime drought stress adaptive engine in support of plant production. Plant Cell Environ. 40, 4–10. doi: 10.1111/pce.12800

Brodribb, T. J., Holbrook, N. M., Zwieniecki, M. A., and Palma, B. (2005). Leaf hydraulic capacity in ferns, conifers and angiosperms: impacts on photosynthetic maxima. New Phytol. 165, 839–846. doi: 10.1111/j.1469-8137.2004.01259.x

Brodribb, T. J., Feild, T. S., and Jordan, G. J. (2007). Leaf maximum photosynthetic rate and venation are linked by hydraulics. Plant Physiol. 144, 1890–1898. doi: 10.1104/pp.107.101352

Brodribb, T. J. (2009). Xylem hydraulic physiology: the functional backbone of terrestrial plant productivity. Plant Sci. 177, 245–251. doi: 10.1016/j.plantsci.2009.06.001

Brodribb, T. J., and Holbrook, N. M. (2003). Stomatal closure during leaf dehydration, correlation with other leaf physiological traits 1. Plant Physiol. 132, 2166–2173. doi: 10.1104/pp.103.023879

Brodribb, T. J., and Holbrook, N. M. (2004). Diurnal depression of leaf hydraulic conductance in a tropical tree species. Plant Cell and Environment 27, 820–827. doi: 10.1093/treephys/tps028

Brodribb, T. J., and Holbrook, N. M. (2006). Declining hydraulic efficiency as transpiring leaves desiccate: two types of response. Plant Cell Environ. 29, 2205–2215. doi: 10.1111/j.1365-3040.2006.01594.x

Brodribb, T. J., and Jordan, G. J. (2008). Internal coordination between hydraulics and stomatal control in leaves. Plant Cell Environ. 31, 1557–1564. doi: 10.1111/j.1365-3040.2008.01865.x

Buckley, T. N. (2005). The control of stomata by water balance. New Phytol. 168, 275–292. doi: 10.1111/j.1469-8137.2005.01543.x

Burnett, A. C., Rogers, A., Rees, M., and Osborne, C. P. (2016). Carbon source–sink limitations differ between two species with contrasting growth strategies. Plant Cell Environ. 39, 2460–2472. doi: 10.1111/pce.12801

Chen, J.-W., Zhang, Q., Li, X.-S., and Cao, K.-F. (2010). Gas exchange and hydraulics in seedlings of Hevea brasiliensis during water stress and recovery. Tree Physiol. 30, 876–885. doi: 10.1093/treephys/tpq043.

Cuneo, I. F., Knipfer, T., Brodersen, C. R., and McElrone, A. J. (2016). Mechanical failure of fine root cortical cells initiates plant hydraulic decline during drought. Plant Physiol. 172, 1669–1678. doi: 10.1104/pp.16.00923

Díaz-Espejo, A., Fernández, J. E., Torres-Ruíz, J. M., Rodríguez-Domínguez, C. M., Pérez-Martín, A., et al. (2018). "The olive tree under water stress: fitting the pieces of response mechanisms in the crop performance puzzle," in Water Scarcity and Sustainable Agriculture in Semiarid Environment, eds I. F. García Tejero and V. H. Durán Zuazo. Amsterdam: Elsevier. doi: 10.1016/B978-0-12-813164-0.00018-1

Díaz-Espejo, A., Nicolás, E., and Fernández, J. E. (2007). Seasonal evolution of diffusional limitations and photosynthetic capacity in olive under drought. Plant Cell Environ. 30, 922–933. doi: 10.1111/j.1365-3040.2007.001686.x

Díaz-Espejo, A., Walcroft, A. S., Fernández, J. E., Hafidi, B., Palomo, M. J., Girón, I. F., et al. (2006). Modeling photosynthesis in olive leaves under drought conditions. Tree Physiol. 26, 1445–1456. doi: 10.1093/treephys/26.11.1445

Epskamp, S. (2013). semPlot: Path Diagrams and Visual Analysis of Various SEM Packages' Output. R Package Version 0.3.3. Available at: https://github.com/SachaEpskamp/semPlot

Ethier, G. J., and Livingston, N. J. (2004). On the need to incorporate sensitivity to CO2 transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. Plant Cell Environ. 27, 137–153. doi: 10.1111/j.1365-3040.2004.01140.x

Evans, J. (1989). Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78, 9–19 doi: 10.1007/BF00377192

Fernández, J. E. (2014). Plant-based sensing to monitor water stress: applicability to commercial orchards. Agric. Water Manage. 142, 99–109. doi: 10.1016/j.agwat.2014.04.017

Flexas, J., Díaz-Espejo, A., Berry, J. A., Cifre, J., Galmés, J., Kaldenhoff, R., et al. (2007). Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. J Exp Bot. 58, 1533–1543. doi: 10.1093/jxb/erm027

Flexas, J., Díaz-Espejo, A., Conesa, M. A., Coopman, R. E., Douthe, C., Gago, J., et al. (2016). Mesophyll conductance to CO2 and Rubisco as targets for improving intrinsic water use efficiency in C3 plants. Plant Cell Environ. 39, 965–982. doi: 10.1111/pce.12622

Flexas, J., and Medrano, H. (2002). Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Ann. Bot. 89, 183–189. doi: 10.1093/aob/mcf027

Flexas, J., Carriquí, M., Coopman, R. E., Gago, J., Galmés, J., Martorell, S., et al. (2014). Stomatal and mesophyll conductances to CO2 in different plant groups: underrated factors for predicting leaf photosynthesis responses to climate change? Plant Sci. 226, 41–48. doi: 10.1016/j.plantsci.2014.06.011

Flexas, J., and Diaz-Espejo, A. (2015). Interspecific differences in temperature response of mesophyll conductance: food for thought on its origin and regulation. Plant Cell Environ. 38, 625–628. doi: 10.1111/pce.12476

Flexas, J., Niinemets,Ü., Gallé, A., Barbou, M. M., Centritto, M., Díaz-Espejo, A., et al. (2013). Diffusional conductances to CO2 as a target for increasing photosynthesis and photosynthetic water-use efficiency. Photosynth. Res. 117, 45–59. doi: 10.1007/s11120-013-9844-z

Galmés, J., Cifre, J., Medrano, H., and Flexas, J. (2005). Modulation of relative growth rate and its components by water stress in Mediterranean species with different growth forms. Oecologia 145, 21–31. doi: 10.1007/s00442-005-0106-4

Gortan, E., Nardini, A., Gascó, A., and Salleo, S. (2009). The hydraulic conductance of Fraxinus ornus leaves is constrained by soil water availability and coordinated with gas exchange rates. Tree Physiol. 29, 529–539. doi: 10.1093/treephys/tpn053

Hernández-Santana, V., Fernández, J. E., Rodríguez-Domínguez, C. M., Romero, R., and Díaz-Espejo, A. (2016). The dynamics of radial sap flux density reflects changes in stomatal conductance in response to soil and air water deficit. Agric. For. Meteorol. 218–219, 92–101. doi: 10.1016/j.agrformet.2015.11.013.

Hunt, R., Causton, D. R., Shipley, B., and Askew, A. P. (2002). A modern tool for classical plant growth analysis. Ann. Bot. 90, 485–488. doi: 10.1093/aob/mcf214

Jackson, R. B., Mooney, H. A., and Schulze, E. D. (1997). A global budget for fine root biomass, surface area, and nutrient contents. Proc. Natl. Acad. Sci. U.S.A. 94, 7362–7366. doi: 10.1073/pnas.94.14.7362

Johnson, D. M., Domec, J. C., Carter Berry, Z., Schwantes, A. M., McCulloh, K. A., Woodruff, D. R., et al. (2018). Co-occurring woody species have diverse hydraulic strategies and mortality rates during an extreme drought. Plant Cell Environ. 41, 576–588. doi: 10.1111/pce.13121

Lo Gullo, M. A., Nardini, A., Trifilò, P., and Salleo, S. (2005). Diurnal and seasonal variations in leaf hydraulic conductance in evergreen and deciduous trees. Tree Physiol. 25, 505–512. doi: 10.1093/treephys/25.4.505

López-Sampson, A., Cernusak, L. A., and Page, T. (2017). Relationship between leaf functional traits and productivity in Aquilaria crassna (Thymelaeaceae) plantations: a tool to aid in the early selection of high-yielding trees. Tree Physiol. 37, 645–653. doi: 10.1093/treephys/tpx007

Magnani, F., Grace, J., and Borghetti, M. (2002). Adjustment of tree structure in response to the environment under hydraulic constraints. Funct. Ecol. 16, 385–393. doi: 10.1046/j.1365-2435.2002.00630.x

Martínez-Vilalta, J., Cochard, H., Mencuccini, M., Sterck, F. J., Herrero, A., Korhonen, J. F. K., et al. (2009). Hydraulic adjustment od scots pine across Europe. New Phytol. 184, 353–364. doi: 10.1111/j.1469-8137.2009.02954.x

Martin-StPaul, N. K., Limousin, J. M., Vogt-Schilb, H., Rodríguez-Calcerrada, J., Rambal, S., Longepierre, D., et al. (2013). The temporal response to drought in a Mediterranean evergreen tree: comparing a regional precipitation gradient and a throughfall exclusion experiment. Glob Chang Biol. 19, 2413–2426. doi: 10.1111/gcb.12215

Martin-StPaul, N., Delzon, S., and Cochard, H. (2017). Plant resistance to drought relies on early stomatal closure. Ecol. Lett. 20, 1437–1447. doi: 10.1111/ele.12851

Maseda, P. H., and Fernández, R. J. (2006). Stay wet or else: three ways in which plants can adjust hydraulically to their environment. J. Exp. Bot. 57, 3963–3977. doi: 10.1093/jxb/erl127

Melcher, P. J., Michele Holbrook, N., Burns, M. J., Zwieniecki, M. A., Cobb, A. R., Brodribb, T. J., et al. (2012). Measurements of stem xylem hydraulic conductivity in the laboratory and field. Methods Ecol. Evol. 3, 685–694. doi: 10.1111/j.2041-210X.2012.00204.x

Mencuccini, M. (2014). Temporal scales for the coordination of tree carbon and water economies during droughts. Tree Physiol. 34, 439–442. doi: 10.1093/treephys/tpu029

Nardini, A., and Salleo, S. (2000). Limitation of stomatal conductance by hydraulic traits: sensing or preventing xylem cavitation? Trees 15, 14–24. doi: 10.1007/s004680000071

Nardini, A., Tyree, M. T., and Salleo, S. (2001). Xylem cavitation in the leaf of Prunus laurocerasus and its impact on leaf hydraulics. Plant Physiol. 125, 1700–1709. doi: 10.1104/pp.125.4.1700

Nevo, E., Fu, Y. B., Pavlicek, T., Khalifa, S., Tavasi, M., Beiles, A., et al. (2012). Evolution of wild cereals during 28 years of global warming in Israel. Proc. Natl. Acad. Sci. U.S.A. 109, 3412–3415. doi: 10.1073/pnas.1121411109

Niinemets, Ü. (2012). Optimization of foliage photosynthetic capacity in tree canopies: towards identifying missing constraints. Oecologia 32, 505–509.

Niinemets, U., Diaz-Espejo, A., Flexas, J., Galmés, J., and Warren, C. R. (2009). Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. J. Exp. Bot. 60, 2249–2270. doi: 10.1093/jxb/erp036

Peguero-Pina, J. J., Sancho-Knapik, D., Flexas, J., Galmés, J., Niinemets,Ü., Gil-Pelegrín, E., et al. (2015b). Light acclimation of photosynthesis in two closely related firs (Abies pinsapo Boiss. and Abies alba Mill.): the role of leaf anatomy and mesophyll conductance to CO2. Tree Physiol. 36, 300–310. doi: 10.1093/treephys/tpv114

Peguero-Pina, J. J., Sisó, S., Flexas, J., Galmés, J., Niinemets,Ü., Sancho-Knapik, D., et al. (2017). Coordinated modifications in mesophyll conductance, photosynthetic potentials and leaf nitrogen contribute to explain the large variation in foliage net assimilation rates across Quercus ilex provenances. Tree Physiol. 37, 1084–1094. doi: 10.1093/treephys/tpx057

Peguero-Pina, J. J., Sisó, S., Sancho-Knapik, D., Díaz-Espejo, A., Flexas, J., Galmés, J., et al. (2015a). Leaf morphological and physiological adaptations of a deciduous oak (Quercus faginea Lam.) to the Mediterranean climate : a comparison with a closely related temperate species (Quercus robur L.). Tree Physiol. 36, 287–299. doi: 10.1093/treephys/tpv107

Pérez-Martín, A., Michelazzo, C., Torres-Ruiz, J. M., Flexas, J., Fernández, J. E., Sebastiani, L., et al. (2014). Regulation of photosynthesis, stomatal and mesophyll conductance under water stress acclimation

and recovery in olive trees: correlation with gene expression of carbonic anhydrase and aquaporins. J. Exp. Bot. 65, 3143–3156. doi: 10.1093/jxb/eru160

Poyatos, R., Aguadé, D., and Martínez-Vilalta, J. (2018). Below-ground hydraulic constraints during drought-induced decline in Scots pine. Ann. For. Sci. 75:100. doi: 10.1007/s13595-018-0778-7

Reddy, K. R., Brand, D., Wijewardana, C., and Gao, W. (2017). Temperature effects on cotton seedling emergence, growth, and development. Agron. J., 109, 1379–1387 doi: 10.2134/agronj2016.07.0439

Rees, M., Osborne, C. P., Woodward, F. I., Hulme, S. P., Turnbull, L. A., Taylor, S. H., et al. (2010). Partitioning the components of relative growth rate: how important is plant size variation? Am. Nat. 176, E152–E161. doi: 10.1086/657037

Reich, P. B. (2014). The world-wide 'fast–slow'plant economics spectrum: a traits manifesto. J. Ecol. 102, 275–301. doi: 10.1111/1365-2745.12211

Rodríguez-Domínguez, C. M., Carins Murphy, M. R., Lucani, C., and Brodribb, T. J. (2018). Mapping xylem failure in disparate organs of whole plants reveals extreme resistance in olive roots. New Phytol. 218, 1025–1035. doi: 10.1111/nph.15079

Rosseel, Y. (2012). Lavaan: an R package for structural equation modeling. J. Stat. Softw. 48, 1–36. doi: 10.3389/fpsyg.2014.01521

Ruane, J., Sonnino, A., Steduto, P., and Deane, C. (2008). Coping With Water Scarcity: What Role for Biotechnologies? Land and Water Discussion Paper. Rome: FAO.

Rugini, E. (1984) In vitro-propagation of some olive (Olea europaea sativa L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24, 123–134. doi: 10.1016/0304-4238(84)90143-2

Sack, L., and Pasquet-Kok, J. (2017). PrometheusWiki contributors, "Leaf pressure-volume curve parameters," PrometheusWiki.

Sack, L., Bartlett, M., Creese, C., Guyot, G., Scoffoni, C., and PrometheusWiki contributors (2011). "Constructing and operating a hydraulics flow meter." PrometheusWiki.

Sack, L., Ball, M. C., Brodersen, C., Davis, S. D., Des Marais, D. L., Donovan, L. A., et al. (2016). Plant hydraulics as a central hub integrating plant and ecosystem function: meeting report for 'Emerging Frontiers in Plant Hydraulics' (Washington, DC, May 2015). Plant Cell Environ. 39, 2085–2094. doi: 10.1111/pce.12732

Sack, L., Cowan, P. D., Jaikumar, N., and Holbrook, N. M. (2003). The 'hydrology' of leaves: co-ordination of structure and function in temperate woody species. Plant Cell Environ. 26, 1343–1356. doi: 10.1046/j.0016-8025.2003.01058.x

Sack, L., and Holbrook, N. M. (2006). Leaf hydraulics. Annu. Rev. Plant Biol. 57, 361–381. doi: 10.1146/annurev.arplant.56.032604.144141

Scoffoni, C., Albuquerque, C., Brodersen, C. R., Townes, S. V., John, G. P., Bartlett, M. K., et al. (2017). Outside-Xylem vulnerability, not xylem embolism, controls leaf hydraulic decline during dehydration. Plant Physiol. 173, 1197–1210. doi: 10.1104/pp.16.01643

Scoffoni, C., Chatelet, D. S., Pasquet-Kok, J., Rawls, M., Donoghue, M. J., Edwards, E. J., et al. (2016). Hydraulic basis for the evolution of photosynthetic productivity. Nat. Plants 2:16072. doi: 10.1038/nplants.2016.72

Scoffoni, C., McKown, A. D., Rawls, M., and Sack, L. (2012). Dynamics of leaf hydraulic conductance with water status: quantification and analysis of species differences under steady state. J. Exp. Bot. 63, 643–658. doi: 10.1093/jxb/err270

Shipley, B. (2000). Cause and Correlation in Biology: A User's Guide to Path Analysis, Structural Equations, and Causal Inference. Oxford: Oxford University Press.

Shipley, B. (2002). Trade-offs between net assimilation rate and specific leaf area in determining relative growth rate: relationship with daily irradiance. Funct. Ecol. 16, 682–689. doi: 10.1046/j.1365-2435.2002.00672.x

Shipley, B. (2006). Net assimilation rate, specific leaf area and leaf mass ratio: which is most closely correlated with relative growth rate? A meta-analysis. Funct. Ecol. 20, 565–574. doi: 10.1111/j.1365-2435.2006.01135.x

Sterck, F., and Zweifel, R. (2016). Trees maintain a similar conductance per leaf area through integrated responses in growth, allocation, architecture and anatomy. Tree Physiol. 36, 1307–1309. doi: 10.1093/treephys/tpw100

Tomas, M., Flexas, J., Copolovici, L., Galmés, J., Hallik, L., Medrano, H., et al. (2013). Importance of leaf anatomy in determining mesophyll diffusion conductance to CO2 across species: quantitative limitations and scaling up by models. J. Exp. Bot. 64, 2269–2281. doi: 10.1093/jxb/ert086

Tosens, T., Niinemets,Ü., Vislap, V., Eichelmann, H., and Castro-Díez, P. (2012). Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in Populus tremula: how structure constrains function. Plant Cell Environ. 35, 839–856. doi: 10.1111/j.1365-3040.2011.02457.x

Trentacoste, E. R., Contreras-Zanessi, O., Beyá-Marshall, V., and Puertas, C. M. (2018). Genotypic variation of physiological and morphological traits of seven olive cultivars under sustained and cyclic drought in Mendoza, Argentina. Agric. Water Manage. 196, 48–56. doi: 10.1016/j.agwat.2017.10.018

Turner, N. C. (2017). Turgor maintenance by osmotic adjustment, an adaptive mechanism for coping with plant water deficits. Plant Cell Environ. 40, 1–3. doi: 10.1111/pce.12839

United Nations (2015). Transforming Our World: The 2030 Agenda for Sustainable Development. New York, NY: United Nations

Walcroft, A. S., Whitehead, D., Silvester, W. B., and Kelliher, F. M. (1997). The response of photosynthetic model parameters to temperature and nitrogen concentration in Pinus radiata D. Don. Plant Cell Environ. 20, 1338–1348. doi: 10.1046/j.1365-3040.1997.d01-31.x

Xiong, D., Douthe, C., and Flexas, J. (2018). Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. Plant Cell Environ. 41, 436–450. doi: 10.1111/pce.13111

Zhou, S. X., Medlyn, B. E., and Prentice, I. C. (2016). Long-term water stress leads to acclimation of drought sensitivity of photosynthetic capacity in xeric but not riparian Eucalyptus species. Ann. Bot. 117, 133–144. doi: 10.1093/aob/mcv161

Ápendice 2

Evaluation of early vigor traits in wild olive germplasm

L. León, **P. Díaz-Rueda**, A. Belaja, R. De la Rosa, C. Carrascosa, JM. Colmenero-Flores

Scientia Horticulturae, Volume 264, 5 April 2020, 109157, https://doi.org/10.1016/j.scienta.2019.109157

Abstract

The control of the olive tree vigor is a critical factor to ensure adequate production and longevity of high-density olive cultivation. To deal with this problem, we intend to take advantage of the genetic variability present in the wild subspecies of Olea europaea to identify genotypes of reduced vigor with optimal adaptability to diverse soils and adverse environmental conditions. In this work, early vigor traits have been characterized in different wild olive subspecies that could potentially be used as rootstocks to control the vigor of grafted cultivars. Significant differences between genotypes were obtained for all vigor traits and high values of broad sense heritability were obtained for most of them, indicating strong genetic effect and therefore good possibilities for selection. Compared to 'Arbequina' and 'Picual' cultivars, some of the wild genotypes evaluated showed a clearly lower vigor at the end of the experimental period. These genotypes could be tested as dwarfing rootstocks for high-density olive plantations.

Keywords

Crop wild relatives; Heritability; *Olea europaea*; Rootstock; Variability

Introduction

Crop wild relatives could represent interesting sources of variability for agronomic traits difficult to find in cultivated materials, such as resistance to pest and diseases and abiotic stresses, yield and quality traits. Thus, in the last years, the use of crop wild relatives

germplasm has been extensively promoted in breeding programs of several fruit trees and nut crops (Aradhya et al., 2015).

Olea europaea subsp. *europaea* var. *europaea* and var. *sylvestris* constitute the botanical varieties of cultivated and wild olives respectively, both of them widely spread throughout the whole Mediterranean basin. Besides the subsp. *europaea*, five other subspecies are included within the *Olea europaea* species, based on morphology and geographical distribution, including subsp. *cuspidata* and *laperrinei* spread in tropical and subtropical regions, subsp. *guanchica*, only recognized in the Canary Islands as well as the only two polyploid subspp *maroccana* (6n) and *cerasiformis* (4n) present in south-western Morocco; and Madeira archipelago, respectively (Green, 2002; Besnard et al., 2013).

The potential use of olive wild genetic resources also known as oleasters as a source of genetic variability for important agronomic traits has been suggested, particularly regarding resistance to specific adverse biotic and abiotic environmental conditions (Lavee and Zohary, 2011; Hernández-Santana et al., 2019). Agronomic evaluation of progenies involving wild parents showed higher vigor, shorter juvenile period and more abundant flowering than progenies from cultivated materials (Klepo et al., 2013). Improved oil composition for some important quality components has been also reported (Hannachi et al., 2009; León et al., 2018). However, wild olives also transmit undesirable fruit traits to its descendants (particularly lower fruit size and oil content), which indicates the need of additional back-cross generations and, therefore, slow down the selection process (Klepo et al., 2014). The need for a longer selection period compared with the cultivated germplasm has limited the potential use of wild relatives as parents in olive breeding programs up to now.

Rootstock selection allows a straightforward way of valorizing wild genetic resources (Warschefsky et al., 2016). In fact, grafting on wild materials was extensively used in the antiquity as a propagation technique in olive. Thus, the analysis of ancient olive trees in several countries reveals that grafting by using seedling, pre-selected clonal rootstocks or oleasters has been a practice widely used in the Middle East

(Barazani et al., 2014) and at a lower extent in central (Lazović et al., 2016) and western part (Ninot et al., 2018) of Mediterranean Basin.

However, it should be noted that olive trees are currently mainly grown on their own roots and rootstocks are exceptionally used in olive compared to other fruit tree species. The use of rootstocks has been recommended to cope with adverse biotic factors such as soilborne disease (Porras Soriano et al., 2003; Bubici and Cirulli, 2012) and abiotic environmental conditions such as preventing frost injury (Pannelli et al., 2002) or iron chlorosis caused by Fe deficiency (Alcántara et al., 2003). The use of rootstocks to control the vigor of the grafted cultivar has also been tested in several works (Baldoni and Fontanazza, 1990; Pannelli et al., 2002; Romero et al., 2014; Rugini et al., 2016), particularly due to the spreading of high density hedgerow olive orchards in recent years and the need for increasing the number of cultivars suitable for this new growing system (Rallo et al., 2013).

The use of wild genetic resources as rootstocks may represent a new source of variability for important agronomic traits. In olive, it has been only studied as a way to improve resistance to Verticillium wilt of susceptible cultivars (Arias-Calderón et al., 2015; Jiménez-Fernández et al., 2016). However, to the best of our knowledge, a comprehensive evaluation of vigor traits in olive wild materials and the potential use of wild genetic resources as rootstocks for vigor control has not been attempted so far in olive.

The aim of this work was the characterization of early vigor traits in different wild olive subspecies representing a wide range of genetic variability of the species *Olea europaea* in order to study the variability and heritability for these traits and the relationships between them, and to identify genotypes that could be potentially used as rootstocks to optimize intensive olive cultivation.

Materials and methods

Early vigor parameters were studied in 43 olive wild genotypes from different subspecies and areas of origin:

- *Olea europaea* subsp. *europaea* var. *sylvestris*: 17 genotypes from open pollination of two trees coming from different provenances in Andalusia (11 from Cádiz (ACZ) province (South-West of the region) and 6 from Jaén (AJA) province (East of the region)), and 7 from open pollination of one tree of the area of Marrakech (AMK) in Morocco.

- *Olea europaea* subsp. *guanchica* (GUA): 9 genotypes from open pollination of one tree of Canary Island

- *Olea europaea* subsp. *cuspidata* (CUS): 1 genotype from open pollination of one tree of South Africa

- Hybrids *O. europaea* subsp. *cuspidata* x *europaea* var. *sylvestris* (CEH): 9 genotypes from crosses.

Homogeneous plants of each genotype were initially obtained from *in-vitro* seedlings germinated in 100 % strength Rugini medium (Rugini, 1984) without hormones. Uninodal segments were further micropropagated in 100 % strength Rugini medium (Rugini, 1984) supplemented with 1 mg/l zeatin. *In-vitro* explants were incubated in a room chamber under 16 h light photoperiod (34 µmol $m^{-2}s^{-1}$ intensity with light-emitting diode (LED) 70 % red plus 30 % blue) at 25 ± 2 °C. Explants were grown up to 7−8 cm height and transferred for 21 days to rooting medium, consisting on 50 % strength Rugini medium supplemented with α-naphthalacetic acid (0.8 mg / l). Rooted seedlings were acclimatized *ex-vitro* for 3 weeks, transplanted to 1 L pots and grown under greenhouse conditions.

Afterwards plants were established in the field at the experimental farm of IFAPA Centre “Alameda del Obispo” in Cordoba (Spain) in May 2015 at 4 × 2 m spacing in a randomized design with 6 replicates per genotype. Plants of ‘Arbequina’ and ‘Picual’ cultivars obtained by standard vegetative propagation of semi-hardwood stem cutting were included for comparison. Standard cultural practices were followed, with no pruning to allow free development of plants and irrigation by in-line drip to avoid water stress of plants.

Vigor traits measurements were taken in Summer (July) and Winter (January). In Summer 2015 (S15) the following traits were measured: plant height (PH), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), maximum internode length (IM), total number of nodes (NN) and number of branches (NB), and length of the longest branch (BL). Plant height, diameter of the main axis at different heights and length of the longest branch measurements were repeated in Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18). Finally, canopy volume (CV) was also calculated in Winter 2018 from measurements of canopy height and width for final categorization of plant vigor.

Descriptive statistics and variability box and whisker plots were obtained for the evaluated agronomic traits. Analysis of variance was carried out to test differences between genotypes and broad sense heritability was estimated as $h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_E)$, where σ^2_G is the variance between genotypes and σ^2_E is the residual variance. Spearman's rank correlation between characters was calculated from average values per genotype.

Results

Overall, a wide variability was observed for all vigor traits measured at different time points. This variability was maintained throughout the evaluation period for some of them such as PH, or gradually increased for others such as DG (**Figure 1**). Average PH increased from 94 cm PH-S15 to 199 cm PH-W18, while DG increased from 6 mm DG-S15 to 39 mm TDG-W18.

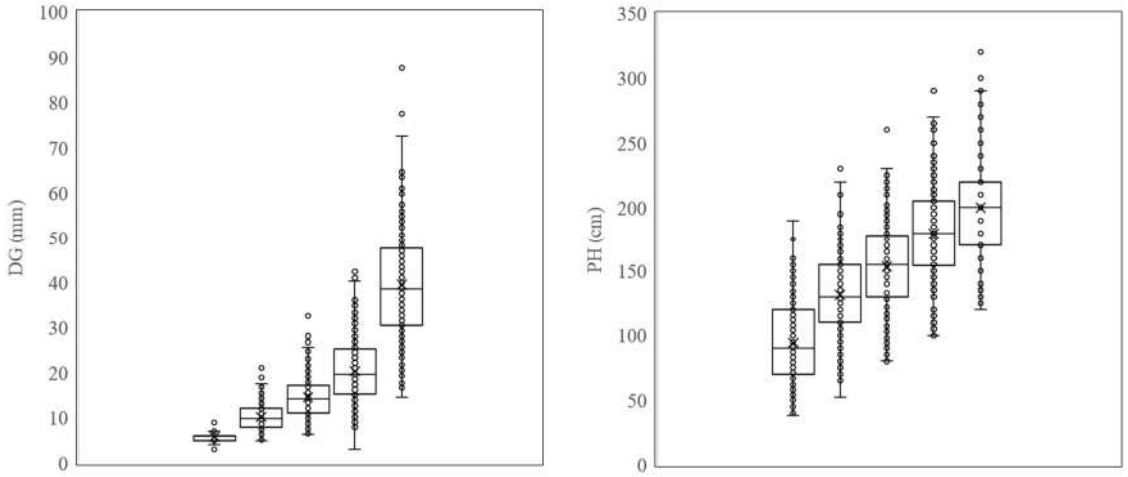


FIGURE 1 | Box and whisker plots for diameter of the main axis at ground level (DG) and plant height (PH) measured sequentially during the evaluation period (from left to right: Summer 2015, Winter 2016, Summer 2016, Winter 2017 and Winter 2018).

Significant differences between genotypes were obtained for all vigor traits, being the variance component between genotypes higher than the error variance for most of them (**Table 1**). As a consequence, high values of broad sense heritability were obtained for most of the evaluated vigor traits. It should be noted that the highest heritability values were obtained at the beginning of the evaluation period just after planting in the field, but heritability values decreased throughout the evaluation period for those traits measured sequentially due to a higher proportional increase of error variance as the plants grew older. For instance, heritability values for PH decreased from 0.93 in S15 to a half 0.47 in W18.

TABLE 1 | Variance components and heritability of vigor traits measured during the evaluation period.

	PH-S15	**PH-W16**	**PH-S16**	**PH-W17**	**PH-W18**
Genotype	1017,05	697,88	649,20	764,21	762,25
Error	72,39	255,47	404,22	571,94	846,73
h2	0,93	0,73	0,62	0,57	0,47
	BL-S15	**BL-W16**	**BL-S16**	**BL-W17**	
Genotype	58,73	214,85	278,26	356,11	
Error	38,35	204,11	333,89	469,62	
h2	0,60	0,51	0,45	0,43	
	DG-S15	**DG-W16**	**DG-S16**	**DG-W17**	**DG-W18**
Genotype	1,45	5,77	13,39	25,07	80,98
Error	0,47	3,50	8,70	23,84	73,65
h2	0,75	0,62	0,61	0,51	0,52

		DL-W16	DL-S16	DL-W17	DL-W18
Genotype		4,30	7,72	16,92	44,58
Error		2,26	5,26	15,20	44,18
h2		0,66	0,59	0,53	0,50
			DC-S16	**DC-W17**	**DC-W18**
Genotype			7,56	13,70	43,04
Error			4,59	11,92	46,48
h2			0,62	0,53	0,48
	IM-S15				
Genotype	76,90				
Error	36,34				
h2	0,68				
	NN-S15				
Genotype	113,04				
Error	20,46				
h2	0,85				
	NB-S15				
Genotype	99,88				
Error	40,85				
h2	0,71				

Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB), Summer 2015 (S15). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18).

Average data per genotype indicated also a high variability for vigor traits among genotypes and a general reduction on the differences between genotypes as the plants grew older (**Table 2**). As an example, coefficient of variation values for PH decreased from 34.2 % in S15 to 15.1 % in W18. Only for diameter of the main axis measured at ground level (DG) variability among genotypes remained similar during the evaluation period, with coefficients of variation from 21.3 to 26.5 %. In any case, a general wide variability and, therefore, possibilities for selection according to vigor characterization could be inferred for all the evaluated traits and periods.

TABLE 2 | Mean, minimum, maximum and coefficient of variation for vigor traits (average values per genotype) measured during the evaluation period on 43 wild genotypes.

	PH-S15	PH-W16	PH-S16	PH-W17	PH-W18
Mean	93,8	130,9	153,1	179,3	199,5
Min	41,8	79,2	97,5	115,8	150,8
Max	163,7	197,5	219,5	253,8	270,0
CV (%)	34,2	20,7	17,5	16,3	15,1
	BL-S15	**BL-W16**	**BL-S16**	**BL-W17**	
Mean	16,9	40,9	57,0	82,2	
Min	4,5	13,8	19,7	28,6	
Max	43,2	88,2	103,8	117,8	
CV (%)	47,6	38,8	32,6	25,5	
	DG-S15	**DG-W16**	**DG-S16**	**DG-W17**	**DG-W18**
Mean	5,8	10,2	14,5	20,3	39,4
Min	3,5	5,9	8,1	9,9	18,7
Max	9,2	16,6	25,9	37,4	67,6
CV (%)	21,3	24,8	26,5	26,5	24,5
		DL-W16	**DL-S16**	**DL-W17**	**DL-W18**
Mean		7,3	10,4	15,1	30,1
Min		3,4	4,6	7,1	15,5
Max		11,5	16,6	26,5	44,6
CV (%)		29,7	28,3	29,2	23,8
			DC-S16	**DC-W17**	**DC-W18**
Mean			6,4	9,5	22,6
Min			1,3	2,3	8,4
Max			12,8	20,3	41,5
CV (%)			47,3	42,4	31,3

	IM-S15
Mean	37,9
Min	27,0
Max	63,8
CV (%)	23,9
	NN-S15
Mean	39,0
Min	20,2
Max	68,3
CV (%)	27,7
	NB-S15
Mean	23,7
Min	3,8
Max	48,7
CV (%)	43,9

Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (DG, DL, DC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB), Summer 2015 (S15). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18).

Spearman's rank analysis showed significant correlations among the different vigor parameters (**Figure 2**). In general, correlation values between different vigor traits measured at the same timepoint were higher than comparisons made at different timepoints. Similarly, for any given vigor trait correlation values were higher between consecutive timepoints than between more distanced measurements. However, some traits such as NB-S15 and PH-W18 showed always slight correlations with the other evaluated vigor parameters (r<0.60). Canopy volume (CV), calculated in Winter 2018 showed significant correlation with all the evaluated vigor traits, the highest values (> 0.80) been observed with traits measured from Summer 2016, i.e. one year after planting in field. Among them, CV showed the highest correlation with diameter of the main axis measured at 50 cm from ground in Winter 2018 (DL-W18, r = 0.901).

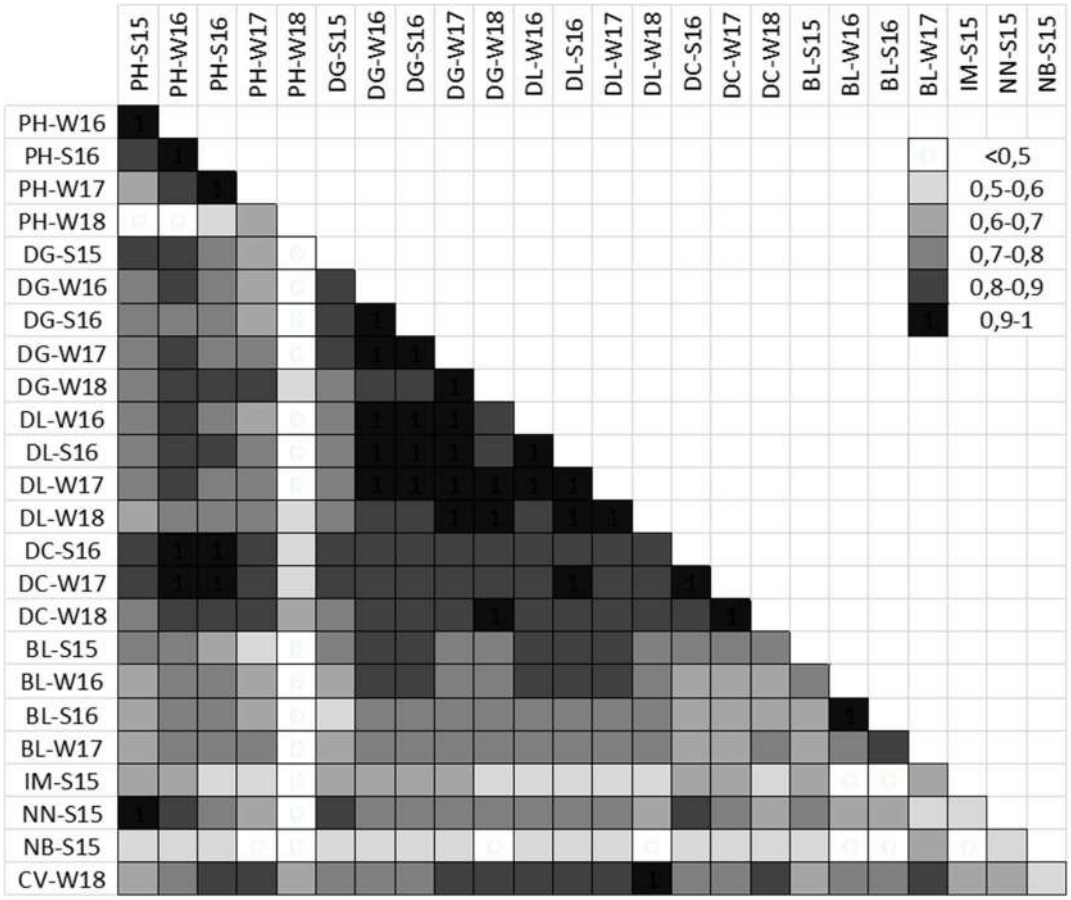


FIGURE 2 | Spearman's rank correlations between average vigor traits per genotype measured during the evaluation period. Plant height (PH), length of the longest branch (BL), diameter of the main axis at different heights (TDG, TDL, TDC measured at ground level, 50 cm and 100 cm from ground, respectively), Maximum internode length (IM), total number of nodes (NN), number of branches (NB). Summer 2015 (S15), Winter 2016 (W16), Summer 2016 (S16), Winter 2017 (W17) and Winter 2018 (W18).

Final classification of genotypes according to values on CV-W18 and TDL-W18 showed some clear differences regarding the origin of the plant materials (**Figure 3**). Thus, the lowest vigor was obtained in sylvestris genotypes from Jaen (Spain) and Marrakech (Morocco) provenances, while the highest vigor was obtained in *sylvestris* genotypes from Cadiz (Spain) and genotypes from crosses *cuspidata* x *sylvestris*. However, a wide variability was obtained in all cases, particularly in genotypes from *guanchica* origin. Compared to 'Arbequina' and 'Picual' cultivars, included as controls for comparison, some of the wild genotypes evaluated showed a clearly lower vigor at the end of the experimental period.

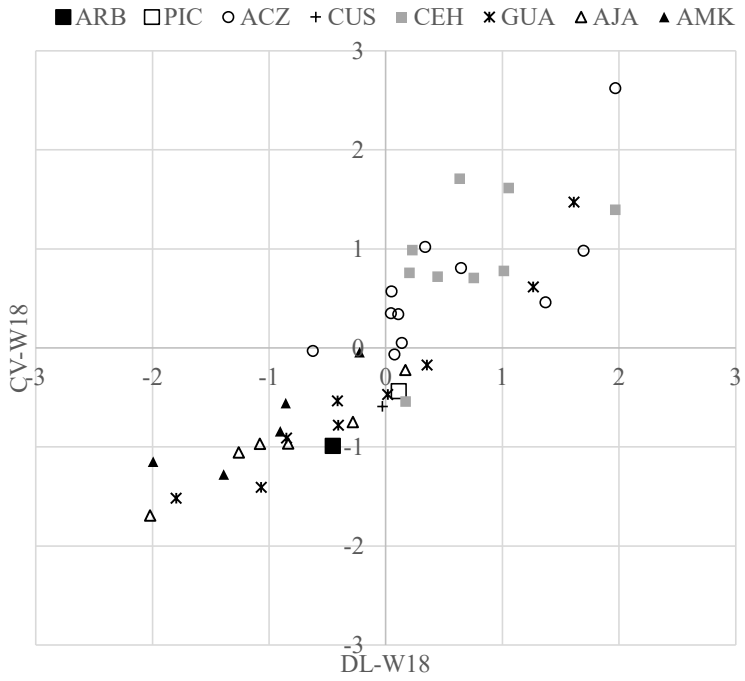


FIGURE 3 | Scatter plot of average data per genotype for diameter of the main axis at 50 cm from ground and canopy volume measured at the end of the experimental period in Winter 2018, TDL-W18 and CV-W18, respectively. Data are grouped according to the origin of the genotypes, including 'Arbequina' and 'Picual' as controls for comparison. Data were normalized by the mean and SD for each trait.

Discussion

Only a few early-bearing, highly productive, low vigor and weeping habit cultivars such as 'Arbequina' and 'Arbosana' are considered suitable for high density olive hedgerow orchards (De la Rosa et al., 2007), which limits the potential expansion of this modern growing system. The use of dwarfing rootstocks has been widely used to control tree

vigor in many fruit species (Gregory et al., 2013), which allows design of specific growing systems and planting densities according to rootstock vigor (Cummins and Aldwinckle, 1983), but this approach has been scarcely studied in olive.

A wide intraspecific variability for vigor traits has been reported from the evaluation in cultivar collections (Del Río et al., 2005; Tous et al., 2005). A quite similar variability has been found in this work in genotypes from different subspecies and areas of origin. Thus, for instance, a coefficient of variation of 24.8 % was obtained from the analysis of trunk diameter ten years after planting in 61 cultivars evaluated at the World Olive Germplasm of IFAPA in Córdoba compared to 24.5 % obtained in 43 olive wild genotypes in this work. A high heritability and strong genetic effect could be also inferred from the evaluation of vigor traits in these cultivar collections. However, significant environmental influence has also been observed for some cultivars, which underlines the need of extensive testing in different environments for adequate selection. In fact, a high correlation has been reported between olive tree vigor and different soil and landscape characteristics such as thickness of the soil root zone, clay and cation exchange capacity and distance to mountain summit (Gálvez et al., 2004).

The whole results of vigor evaluation should be considered in terms of variability, heritability and correlation among them. A high correlation between 17 different vigor parameters was also observed in the characterization of olive progenies from crosses between different cultivars, which allows the selection of a group of them as the best growth descriptors based on their influence by parent genotype (Hammami et al., 2011). Moreover, a high influence of plant age on growth habit traits was also determined and early characterization at the time of planting in the field was suggested as the most appropriate age for evaluating seedling growth parameters (Hammami et al., 2011). Early vigor measurements at the seedling stage before planting in the field was also proposed in olive progenies as a tool for selection for short juvenile period (De la Rosa et al., 2006). In this work however, measurements taken early after planting showed high heritability and variability among genotypes, but lower correlation with final characterization of the genotypes. The results obtained suggest that measurements taken from one year after planting could be considered more efficient for selection.

Among the wild genotypes evaluated in this work, some of them showed a clear lower vigor than 'Arbequina' at the end of the experimental period and could be considered potentially interesting dwarfing rootstocks. Future works should be carried out to test whether the differential vigor in the evaluated wild materials is extensible to the grafted cultivars of interest. Previous works testing single cultivars grafted on a range of potential interesting rootstocks indicate that the intrinsic vigor of some rootstock is transferred to the grafted cultivar. However, opposite results were also found in some cases, although it should be noted that explicit evaluation of vigor of the rootstock used was not carried out but based on previous literature (Troncoso et al., 1990; Romero et al., 2014). Similarly, different vigor control ability has been obtained for some rootstocks depending on the cultivar grafted, while a general dwarfing effect was observed for some others (Pannelli et al., 2002). These partly contradictory results suggest that further research is still necessary to guarantee adequate choice of rootstocks. Again, the environmental conditions should be also carefully considered in testing. For instance, water availability showed a marked effect on growth and production parameters that obscure the potential rootstock effect in experiments with reciprocal scion/rootstock combination of three olive cultivars (Lavee and Schachtel, 1999; Hernández-Santana et al., 2019). Moreover, the effect of grafting itself has been showed to control vigor by reducing plant size of self-grafted genotypes (Rugini et al., 2016), although no effect of self-grafting has also been reported for some cultivars (Pannelli et al., 2002).

Several mechanisms have been suggested as responsible of the dwarfing potential of olive rootstock, mainly related to the hydraulic architecture of the different scion/rootstock combinations (Nardini et al., 2006), as also reported for other fruit tree species (Cohen et al., 2007). The hydraulic characterization of the wild plant materials showing contrasting dwarf effect in our work could provide valuable information in future.

Conclusions

A wide variability for early vigor traits has been obtained from the evaluation of wild olive germplasms from different origins. In particular, low vigor and potentially

interesting dwarfing effect was obtained in some sylvestris genotypes from Jaen (Spain) and Marrakech (Morocco) provenances and some genotypes from guanchica origin. Future works should be carried out to test whether the differences in growth traits between the studied plant materials are translated into vigor control of grafted materials and, moreover, whether it represents a general effect or it is limited to particular scion/rootstock combinations. Moreover, the potential effect of the rootstock on other important agronomic traits of the grafted cultivar, such as earliness of bearing, productivity, fruit and oil traits, should be also evaluated to guarantee adequate choice of rootstock. The use of rootstocks as a tool to control vigor in olive could open new possibilities for future olive growing, mainly regarding high-density hedgerow olive orchards where only a very limited number of cultivars have been successfully adapted up to now.

References

Alcántara, E., Cordeiro, A. M., and Barranco, D. (2003). Selection of olive varieties for tolerance to iron chlorosis. *J. Plant Physiol.* 160, 1467–1472. doi: 10.1078/0176-1617-01013.

Aradhya, M. K., Preece, J., and Kluepfel, D. A. (2015). Genetic conservation, characterization and utilization of wild relatives of fruit and nut crops at the USDA germplasm repository in Davis, California. *Acta Hortic.*, 95–104. doi: 10.17660/ActaHortic.2015.1074.14.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., and Belaj, A. (2015). Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. *Crop Prot.* 75, 25–33. doi: 10.1016/j.cropro.2015.05.006.

Baldoni, L., and Fontanazza, G. (1990). Preliminary results on olive clonal rootstocks behaviour in the field. *Acta Hortic.*, 37–40. doi: 10.17660/ActaHortic.1990.286.2.

Barazani, O., Westberg, E., Hanin, N., Dag, A., Kerem, Z., Tugendhaft, Y., et al. (2014). A comparative analysis of genetic variation in rootstocks and scions of old olive trees – a window into the history of olive cultivation practices and past genetic variation. *BMC Plant Biol.* 14, 146. doi: 10.1186/1471-2229-14-146.

Besnard, G., Khadari, B., Navascués, M., Fernández-Mazuecos, M., El Bakkali, A., Arrigo, N., et al. (2013). The complex history of the olive tree: from Late Quaternary diversification of Mediterranean lineages to primary domestication in the northern Levant. *Proc. R. Soc. B Biol. Sci.* 280, 20122833. doi: 10.1098/rspb.2012.2833.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9.

Cohen, S., Naor, A., Bennink, J., Grava, A., and Tyree, M. (2007). Hydraulic resistance components of mature apple trees on rootstocks of different vigours. *J. Exp. Bot.* 58, 4213–4224. doi: 10.1093/jxb/erm281.

Cummins, J. N., and Aldwinckle, H. S. (1983). "Breeding Apple Rootstocks," in *Plant Breeding Reviews*, ed. J. Janick (Boston, MA: Springer US), 294–394. doi: 10.1007/978-1-4684-8896-8_10.

De la Rosa, R., Kiran, A. I., Barranco, D., and León, L. (2006). Seedling vigour as a preselection criterion for short juvenile period in olive breeding. *Aust. J. Agric. Res.* 57, 477. doi: 10.1071/AR05219.

De la Rosa, R., León, L., Guerrero, N., Rallo, L., and Barranco, D. (2007). Preliminary results of an olive cultivar trial at high density. *Aust. J. Agric. Res.* 58, 392. doi: 10.1071/AR06265.

Del Río, C., Caballero, J. M., and García-Fernández, M. D. (2005). Vigor. Banco de Germoplasma de Córdoba. En: Variedades de olivo en España (Libro II: Variabilidad y selec- ción). In: Rallo, L., Barranco, D., Caballero, J.M., Del Río, C., Martín, A., Tous, J., Trujillo, I. (Eds.), Junta de Andalucía. M.A.P.A. y Ediciones Mundi-Prensa, Madrid.

Gálvez, M., Parra, M. A., and Navarro, C. (2004). Relating tree vigour to the soil and landscape characteristics of an olive orchard in a marly area of southern Spain. *Sci. Hortic.* 101, 291–303. doi: 10.1016/j.scienta.2003.12.001.

Green, P. S. (2002). A Revision of *Olea* L. (Oleaceae). *Kew Bull.* 57, 91. doi: 10.2307/4110824.

Gregory, P. J., Atkinson, C. J., Bengough, A. G., Else, M. A., Fernández-Fernández, F., Harrison, R. J., et al. (2013). Contributions of roots and rootstocks to sustainable, intensified crop production. *J. Exp. Bot.* 64, 1209–1222. doi: 10.1093/jxb/ers385.

Hammami, S. B. M., León, L., Rapoport, H. F., and De la Rosa, R. (2011). Early growth habit and vigour parameters in olive seedlings. *Sci. Hortic.* 129, 761–768. doi: 10.1016/j.scienta.2011.05.038.

Hannachi, H., Sommerlatte, H., Breton, C., Msallem, M., El Gazzah, M., Ben El Hadj, S., et al. (2009). Oleaster (var. *sylvestris*) and subsp. *cuspidata* are suitable genetic resources for improvement of the olive (*Olea europaea* subsp. *europaea* var. *europaea*). *Genet. Resour. Crop Evol.* 56, 393–403. doi: 10.1007/s10722-008-9374-2.

Hernández-Santana, V., Díaz-Rueda, P., Díaz-Espejo, A., Raya-Sereno, M. D., Gutiérrez-Gordillo, S., Montero, A., et al. (2019). Hydraulic Traits Emerge as Relevant Determinants of Growth Patterns in Wild Olive Genotypes Under Water Stress. *Front. Plant Sci.* 10, 291. doi: 10.3389/fpls.2019.00291.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516.

Klepo, T., De la Rosa, R., Satovic, Z., León, L., and Belaj, A. (2013). Utility of wild germplasm in olive breeding. *Sci. Hortic.* 152, 92–101. doi: 10.1016/j.scienta.2012.12.010.

Klepo, T., Toumi, A., De La Rosa, R., LeÓn, L., and Belaj, A. (2014). Agronomic evaluation of seedlings from crosses between the main Spanish olive cultivar 'Picual' and two wild olive trees. *J. Hortic. Sci. Biotechnol.* 89, 508–512. doi: 10.1080/14620316.2014.11513113.

Lavee, S., and Schachtel, J. (1999). Interaction of cultivar rootstock and availability on olive tree performance and fruit production. *Acta Hortic.*, 399–414. doi: 10.17660/ActaHortic.1999.474.83.

Lavee, S., and Zohary, D. (2011). The potential of genetic diversity and the effect of geographically isolated resources in olive breeding. *Isr J Plant Sci* 59, 3–13.

Lazović, B., Adakalić, M., Pucci, C., Perović, T., Bandelj, D., Belaj, A., et al. (2016). Characterizing ancient and local olive germplasm from Montenegro. *Sci. Hortic.* 209, 117–123. doi: 10.1016/j.scienta.2016.06.022.

León, L., de la Rosa, R., Velasco, L., and Belaj, A. (2018). Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. *Front. Plant Sci.* 9, 232. doi: 10.3389/fpls.2018.00232.

Nardini, A., Gasco, A., Raimondo, F., Gortan, E., Lo Gullo, M. A., Caruso, T., et al. (2006). Is rootstock-induced dwarfing in olive an effect of reduced plant hydraulic efficiency? *Tree Physiol.* 26, 1137–1144. doi: 10.1093/treephys/26.9.1137.

Ninot, A., Howad, W., Aranzana, M. J., Senar, R., Romero, A., Mariotti, R., et al. (2018). Survey of over 4, 500 monumental olive trees preserved on-farm in the northeast Iberian Peninsula, their genotyping and characterization. *Sci. Hortic.* 231, 253–264. doi: 10.1016/j.scienta.2017.11.025.

Pannelli, G., Rosati, S., and Rugini, E. (2002). The effect of clonal rootstocks on frost tolerance and on some aspects of plant behaviour in Moraiolo and S. Felice olive cultivars. *Acta Hortic.*, 247–250. doi: 10.17660/ActaHortic.2002.586.46.

Porras Soriano, A., Soriano Martín, M. L., and Porras Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Prot.* 22, 369–374. doi: 10.1016/S0261-2194(02)00180-1.

Rallo, L., Barranco, D., Castro-García, S., Connor, D. J., Gómez del Campo, M., and Rallo, P. (2013). "High-Density Olive Plantations," in *Horticultural Reviews Volume 41*, ed. J. Janick (Hoboken, New Jersey: John Wiley & Sons, Inc.), 303–382. doi: 10.1002/9781118707418.ch07.

Romero, A., Hermoso, J. F., and Tous, J. (2014). Olive rootstocks to control' Arbequina IRTAI18' clone vigour results from a second one comparative trial. *Acta Hortic.*, 577–584. doi: 10.17660/ActaHortic.2014.1057.74.

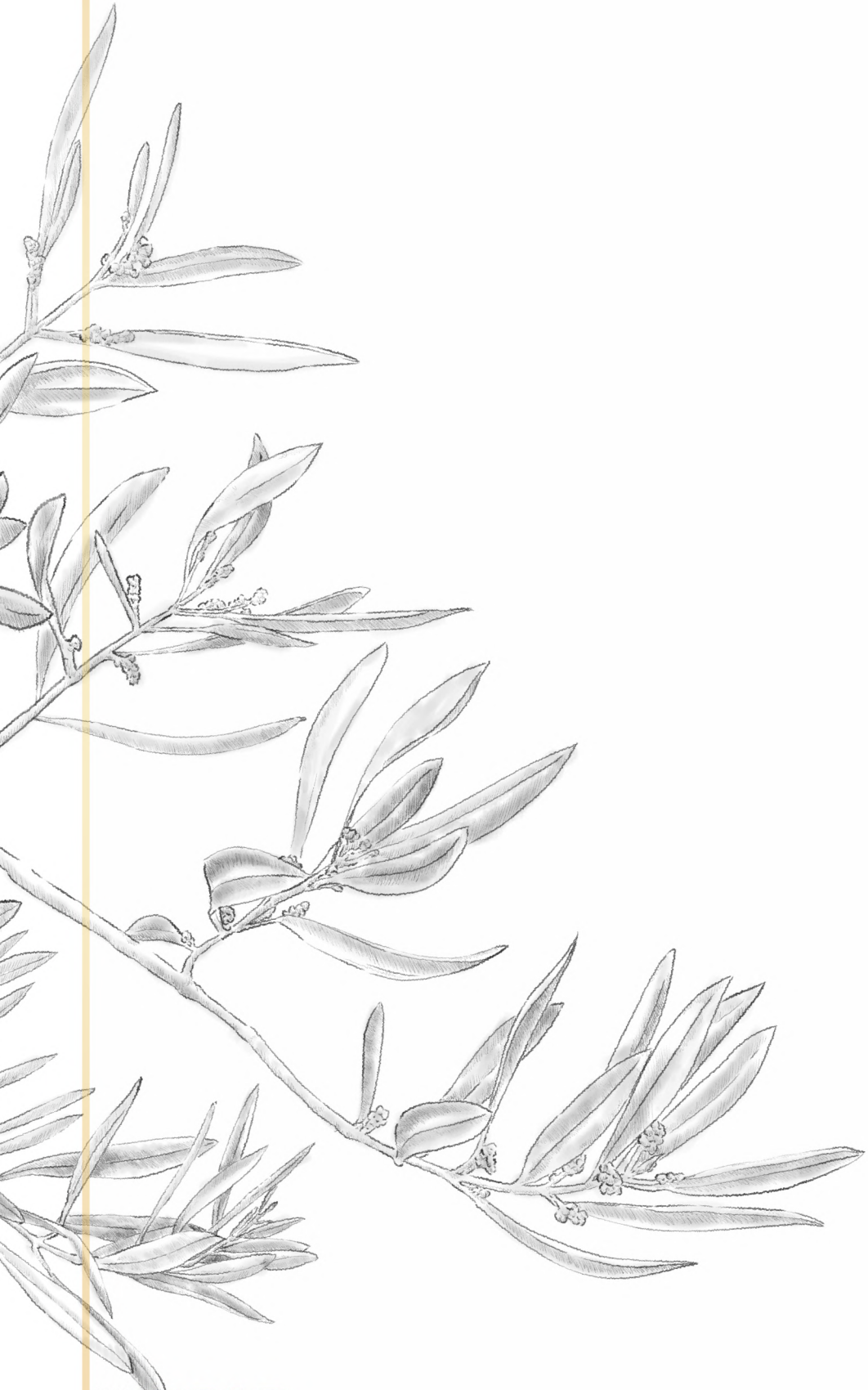
Rugini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Sci. Hortic.* 24, 123–134. doi: 10.1016/0304-4238(84)90143-2.

Rugini, E., Cristofori, V., and Silvestri, C. (2016). Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods. *Biotechnol. Adv.* 34, 687–696. doi: 10.1016/j.biotechadv.2016.03.004.

Tous, J., Romero, A., and Plana, J. (2005). Vigor. Banco de Germoplasma de Cataluña. en: variedades de olivo en españa (libro II: variabilidad y selección). In: Rallo, L., Barranco, D., Caballero, J.M., Del Río, C., Martín, A., Tous, J., Trujillo, I. (Eds.), Junta de Andalucía. M.A.P.A. y Ediciones Mundi-Prensa, Madrid.

Troncoso, A., Liñán, J., Prieto, J., and Cantos, M. (1990). Influence of diferent olive rootstocks on growth and production of 'Gordal sevillana.' *Acta Hortic.*, 133–136. doi: 10.17660/ActaHortic.1990.286.26.

Warschefsky, E. J., Klein, L. L., Frank, M. H., Chitwood, D. H., Londo, J. P., von Wettberg, E. J. B., et al. (2016). Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. *Trends Plant Sci.* 21, 418–437. doi: 10.1016/j.tplants.2015.11.008.



CSIC
Consejo Superior de Investigaciones Científicas

Universidad de Sevilla