EL PAPEL FACILITADOR O INHIBIDOR DE *PINUS PINEA* L. SOBRE ESPECIES AUTÓCTONAS DE MATORRAL MEDITERRÁNEO

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El papel facilitador o inhibidor de *Pinus pinea* L. sobre especies autóctonas de matorral mediterráneo

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A mis padres

Descubrir el mundo por uno mismo
Abarca toda la vida y
Nunca se consigue del todo
Incluso cuando todo conocimiento parece cercano
Encontramos una nueva etapa,
La que nos anima a continuar

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GENERAL SUMMARY

Negative and positive interactions operate simultaneously in plant communities and the balance between these two forces drives community structure and function. Mediterranean sand dune ecosystems are nutrient-poor systems in which vegetation is composed mainly by *Pinus pinea* L., *Juniperus phoenicea* L., *Osyris lanceolata* Hochst. & Steud. and several shrubs belonging to the Cistaceae. *P. pinea*, due to its abundance and size, is known to interact with the rest of the species. With these assumptions we investigated the possible impacts of *P. pinea* on several aspects: (1) seed germination and seedling performance of three Mediterranean shrubs (Cistaceae family), (2) study of the decomposition processes of various life forms at the study site, (3) intensity of competition between seedlings of *P. pinea*, *C. salvifolius* and *P. lentiscus* under different combinations and nutrient treatments in order to evaluate the different behavior under nutrient-rich or poor conditions and (4) evaluate the special relationship between *O. lanceolata*, a hemiparasitic dioecious shrub and *P. pinea*. A thin needle layer could improve seed germination and seedling performance of shrub species due to the maintenance of a safe site and supplying some nutrients. These same needles may retard decomposition processes, but the presence of *O. lanceolata* litter in the soil enhances litter decomposition by releasing nutrients and enhancing litter decay rates of other species. A well-developed root system allows *P. pinea* to cope with the rest of the species and morphological traits are more important in predicting changes in competition than ecophysiological traits. This doctoral thesis highlights the important role played by *O. lanceolata*, not only in the nutrient cycling, but also spreading at the study site due to the presence of a good host, *P. pinea*.

CAPÍTULO 1

Introducción general

INTRODUCCIÓN GENERAL.

El conocimiento de las interacciones que se establecen entre la planta y el suelo sobre el que se desarrolla es esencial para comprender el funcionamiento de los ecosistemas. Ya en los albores de las grandes civilizaciones de la antigüedad se describían las propiedades de los suelos como factores condicionantes del tipo de vegetación y de la productividad agrícola (Hillel, 1991). Durante siglos el conocimiento popular distinguió entre especies forestales que degradaban las condiciones del suelo, como las coníferas, y especies que las mejoraban, como la mayoría de las especies frondosas de hoja caduca, lo que llevó a favorecer las formaciones de bosque mixto (Rennie, 1955; Urbieta et al., 2008). Sin embargo, el papel de la vegetación como elemento formador de los suelos no fue explícitamente reconocido hasta finales del siglo XIX por Dokuchaev (1879; en Attiwill y Adams, 1993). Posteriormente, Jenny (1941) estableció los cinco factores formadores del suelo: material original, vegetación, relieve, clima y tiempo.

Los factores formadores del suelo son la pieza clave para entender la enorme heterogeneidad de tipos suelo tanto a nivel de paisaje como de perfil individual de suelo (Bardgett, 2004). La biota, junto con la vegetación constituye uno de los principales factores formadores de suelo; ambos pueden actuar como factores determinantes tanto en la formación del suelo como en el desarrollo del perfil edáfico.

Desde que el ser humano comenzó a interesarse por el entorno que lo rodeaba y el resto de organismos que lo habitaban, los estudios y observaciones sobre suelo y vegetación eran llevados a cabo por separado; en el primer caso por edafólogos y en el segundo caso por botánicos. Sin embargo, desde finales del siglo XX se viene realizando una labor multidisciplinar en el estudio de las interacciones planta-suelo (Finzi et al., 1998; van Breemen y Finzi, 1998; Copley, 2000; Wardle et al., 2004). La compleja red de interacciones que se establecen en la asociación planta-suelo hace que se desarrolle un entramado de relaciones que redundan en la estabilidad del sistema, así como desempeñan un papel crucial en el control de las propiedades y procesos del ecosistema (Hobbie, 1992; Binkley y Giardina, 1998).

Además de las relaciones planta-suelo, es importante tener en cuenta las relaciones planta-planta, añadiendo más complejidad si cabe a los ecosistemas. Las relaciones que se establecen entre las diversas especies vegetales pertenecientes a una comunidad dada pueden ser de tipo positivo o facilitador (Pugnaire, 2010) o de tipo negativo, hablando en este último caso de relaciones de competencia entre especies vegetales (Caldwell et al., 1991; Reynolds y D´Antonio, 1996).

Connell (1983) y Schoener (1983) concluyeron que las relaciones de competencia entre especies vegetales eran fuertemente asimétricas y mostraban una variación espacio-temporal. Desde entonces se han venido realizando diversos estudios sobre competencia entre plantas (Underwood, 1986; Goldberg y Barton, 1992), pero fue Fowler (1986) quien describió por primera vez unos resultados de competencia entre especies vegetales en ambientes áridos y semiáridos, según los cuales se definía la competencia por el agua como el principal factor limitante para la supervivencia y el crecimiento vegetal en este tipo de ecosistemas; esta misma autora mencionó la falta de información disponible para describir semejanzas y diferencias entre las interacciones planta-planta entre distintas zonas biogeográficas, como ocurría en el caso de las regiones mediterráneas.

Las regiones mediterráneas ocupan las latitudes medias entre los 30º y 40º norte y sur en 5 regiones del mundo: la Cuenca Mediterránea, California, Chile central, la región de El Cabo en Sudáfrica y las zonas suroeste y sur de Australia. La sequía estival es el principal factor que define el clima mediterráneo (Köppen, 1923). La vegetación mediterránea está compuesta fundamentalmente por árboles y arbustos esclerófilos (Orshan, 1983) con un sotobosque herbáceo o de matorral. Desde los años 60 del siglo pasado se ha puesto énfasis en los efectos de los factores abióticos (clima, fuego, deficiencias de nutrientes en el suelo y estrés hídrico) sobre este tipo de comunidades vegetales (Mooney y Conrad, 1977; di Castri, 1991).

En las regiones mediterráneas la presencia de grandes formaciones perennes de matorral hace pensar en la existencia de fuertes relaciones de competencia. Los individuos crecen muy próximos entre sí y se llegan a establecer solapamientos del dosel arbóreo-arbustivo (Lamont et al., 1984). Además de la sequía estival, la variabilidad del régimen de precipitaciones y la escasez de ciertos nutrientes en el suelo son las principales características que definen los ecosistemas mediterráneos (Kruger et al., 1983). Según Grubb (1985) y Tilman (1987), el hecho de que los recursos en el suelo sean limitantes conduciría a una intensa competencia entre las plantas, sugiriendo que en las comunidades mediterráneas la competencia por el agua y otros recursos del suelo puede ser muy fuerte (Mooney y Dunn, 1969).

La región mediterránea que se aborda en la presente tesis doctoral es la Cuenca Mediterránea y, en concreto, un ecosistema dunar perteneciente al Parque Nacional de Doñana

GERMINACIÓN DE SEMILLAS Y ESTABLECIMIENTO DE PLÁNTULAS

La germinación es un proceso clave para los ecosistemas desde el punto de vista de establecimiento y reclutamiento de nuevos individuos al sistema. Varios mecanismos regulan la germinación de semillas en su hábitat natural, algunos de los cuales son internos, mientras que otros son factores medioambientales externos, pudiendo cualquiera de ellos determinar si una semilla germinará o no. Sin embargo, la demostración en laboratorio de la existencia de un mecanismo regulador no es prueba de su actuación bajo condiciones naturales (Mayer y Poljakoff-Mayber, 1975).

En concreto, las especies leñosas pueden alterar las condiciones abióticas del entorno que las rodean, lo que puede repercutir en la composición de organismos del suelo. Cuando estos cambios en las condiciones tanto abióticas como bióticas del suelo repercuten en el crecimiento de la propia planta que está generando esos cambios o incluso en el *fitness* de sus propágulos, estamos hablando de un bucle de retroalimentación que puede llegar a limitar la composición de la comunidad y hasta la distribución de las especies (Catovsky y Bazzaz, 2000).

Las condiciones ecológicas presentes en un determinado hábitat afectarán la germinación (Capon y van Asdall, 1967; Vickery, 1967). Con respecto a esta afirmación, probablemente las condiciones microclimáticas existentes en las proximidades de la semillas serán los factores determinantes de la germinación, más que las condiciones climáticas per sé. Las semillas caen y pueden hacerlo directamente al suelo, sobre hojarasca o, en algunos casos, sobre zonas más o menos cubiertas por agua.

Las condiciones que prevalecen bajo estas circunstancias dependerán de la naturaleza del suelo, su composición química y su estructura físico-química y de la profundidad a la que queda la semilla en relación al suelo o bajo el agua. La profundidad influirá en la aireación así como en la penetración de la luz. La composición química del suelo, o del agua, puede afectar la germinación de diversas formas; por ejemplo, sobre el suelo puede existir una capa de mayor o menos grosor de hojarasca o materia orgánica parcialmente descompuesta que puede contener sustancias que inhiban la germinación; también puede presentar un alto contenido en sales que retardarán osmóticamente o evitarán la germinación.

Sin embargo, también cabe citar el caso de ambientes bastante restrictivos como pueden ser regiones áridas o semiáridas, donde la semilla puede caer en un sitio próximo a la copa de un árbol o arbusto frondoso o bajo un manto de hojarasca que permita reunir una serie de condiciones de humedad y temperatura óptimas para la germinación, además de evitar la alta radiación del entorno. Son los denominados *safe sites* que permiten el reclutamiento de nuevos individuos a la comunidad vegetal existente (Duncan et al., 2009; Graae et al., 2011).

Un ejemplo de lo citado en el párrafo anterior sería el caso de ecosistemas semiáridos pobres en nutrientes, como por ejemplo los sistemas dunares mediterráneos, donde una capa de acículas de pino piñonero de poco grosor no sólo no tendría consecuencias negativas sobre la germinación y posterior establecimiento de plántulas pertenecientes a diversas especies vegetales de matorral mediterráneo, como describieron Maestre et al. (2004) para la interacción *Pinus halepensis* - *Pistacia lentiscus*, sino que permitiría el establecimiento de un microhábitat con unas condiciones de humedad y temperatura beneficiosas para que las semillas germinen y las plántulas resultantes tengan una oportunidad de subsistir en las duras condiciones que imponen este tipo de sistemas naturales (Valera-Burgos et al., 2012). Maestre et al. (2003) describieron en otra especie del género *Pinus*, en concreto *P. halepensis*, que no había resultados significativos como para afirmar que esta especie favoreciera el establecimiento de especies arbustivas en ambientes semiáridos mediterráneos, aunque sí favorecía el de hierbas perennes.

DESCOMPOSICIÓN DEL MATERIAL VEGETAL

Los organismos descomponedores son esenciales para el correcto funcionamiento de los ecosistemas terrestres debido principalmente a que son ellos los encargados de la descomposición de la materia orgánica presente en el suelo, convirtiendo dicha materia orgánica en dióxido de carbono y otras sustancias nutritivas solubles que proporcionan recursos para el resto de la biota y disponible para los productores primarios. Todo lo anterior conlleva un importante papel en el ciclo de los nutrientes.

En total, entre el 80-90 % de la producción primaria entra al sistema del suelo como hojarasca y raíces y los primeros descomponedores de este material son bacterias y hongos. La fragmentación inicial y la ingestión de materia orgánica fresca por organismos detritívoros incrementan el área superficial para una posterior colonización microbiana de esta materia orgánica.

La mineralización de los nutrientes – el proceso mediante el cual los microorganismos del suelo rompen la materia orgánica tanto soluble como insoluble y la convierten en formas inorgánicas disponibles para los productores primarios – es un proceso de crucial importancia para el funcionamiento de los ecosistemas dado que, en muchos ecosistemas, determina directamente la disponibilidad de nutrientes para las plantas. Por ejemplo, en ecosistemas fértiles, como bosques deciduos, el aporte de nitrógeno a las plantas está fuertemente relacionado con las tasas de mineralización de nitrógeno en suelo (Nadelhoffer et al., 1985).

La mayoría del nitrógeno en el suelo (aprox. 96-98 %) se encuentra en la materia orgánica muerta presente en complejos poliméricos insolubles del tipo proteínas, ácidos nucleicos y quitina.

La fauna del suelo también afecta a los procesos que se desarrollan en el ciclo de los nutrientes vía alteración física del material en descomposición y del ambiente edáfico. Existen dos tipos de organismos encargados de este proceso: (1) los transformadores de la hojarasca que consumen detritus vegetal y desprenden este material digerido como pellets fecales, afectando con ellos las tasas de descomposición y la liberación de nutrientes y (2) los ingenieros del ecosistema que construyen estructuras físicas en el suelo que proporcionan hábitats para los microbios y otros organismos, y también alteran el movimiento de materiales a través de suelos y ecosistemas (Lavelle et al., 1995).

Los consumidores de la hojarasca son fundamentalmente animales tales como microartrópodos y macrofauna que consume el detritus vegetal y que liberan este material al suelo en forma de pellets fecales, que tienen un elevado ratio superficie:volumen comparado con la hojarasca inicial, lo que acelera su tasa de descomposición (Webb, 1977). Estos pellets fecales también proporcionan un ambiente favorable para el crecimiento microbiano, especialmente para las bacterias, ayudando de nuevo a incrementar las tasas de descomposición y la liberación de nutrientes (Hassall et al., 1987; Zimmer y Topp, 2002).

En ecosistemas forestales, la hojarasca es el más importante aporte de materia orgánica y nutrientes a la capa de humus. La cantidad y calidad de la hojarasca influencia la naturaleza de la comunidad microbiana, incluyendo su tamaño, composición, función y propiedades fisiológicas. La composición de la comunidad microbiana puede, a su vez, influenciar el proceso de descomposición, y los cambios químicos que subyacen durante el fenómeno de descomposición de la hojarasca. Con el conocimiento relativo a la composición química inicial de la hojarasca y los cambios químicos durante la descomposición, es posible predecir cómo variarán las tasas de pérdida de masa en los sucesivos estados de descomposición (Berg y McClaugherty, 2008).

Toda la hojarasca, sin importar de qué especie proceda, contiene esencialmente nutrientes tales como N, P, S, K, Ca, Mg, Mn y Fe, pero las concentraciones de dichos elementos dependen de la especie en concreto. Por ejemplo, la hojarasca perteneciente al género *Alnus* (fijadora de nitrógeno) presente altas concentraciones de nitrógeno (sobre un 3%); sin embargo, la hojarasca de coníferas es más pobre en nitrógeno (con frecuencia por debajo del 0.4%). La especie de la que procede la hojarasca es, por lo tanto, un factor importante a tener en cuenta para determinar los niveles de nutrientes en la hojarasca, pero el clima y la composición mineral del suelo, así como el material original del suelo y el humus adquieren una elevada importancia (Berg y McClaugherty, 2008). Siguiendo la idea anterior, en dos estudios llevados a cabo a finales del siglo XX, se determinó que en un ecosistema compuesto principalmente por *Pinus sylvestris*, la concentración de nitrógeno en dicho ecosistema parecía estar relacionada tanto con el clima (Berg et al., 1995) como con la concentración de nitrógeno en el humus (Berg et al., 1999).

Existe una serie de parámetros que relacionan la calidad de la hojarasca con la tasa de descomposición de la biomasa y han sido ampliamente estudiados, como por ejemplo la relación carbono:nutrientes, la concentración de lignina o la dureza de la hoja (Gallardo y Merino, 1992; Cornwell et al., 2008; Meier y Bowman, 2008). También puede llegar cierto punto en el cual no se sigue descomponiendo la hojarasca y ésta pasa a formar parte de la materia orgánica del suelo en forma de complejos estables, este proceso recibe el nombre de límite de descomposición y ha sido ampliamente descrito por Berg (2000).

Los ecosistemas dunares mediterráneos son sistemas pobres en nutrientes y los suelos sobre los que se desarrollan están caracterizados por su baja fertilidad, por lo que la producción primaria en este tipo de ecosistemas se encuentra limitada por la baja disponibilidad de nutrientes (Gallardo, 2001). Wright y Westoby (2003) describieron que las especies pertenecientes a hábitats pobres en nutrientes presentan una mayor capacidad de reabsorción de nutrientes antes de la abscisión foliar, lo que conduciría a una producción de hojarasca pobre en nutrientes en comparación con hábitats más prósperos desde el punto de vista de la disponibilidad de nutrientes en el suelo. La presencia de cutina y otras sustancias similares en las hojas de especies vegetales mediterráneas confiere impermeabilidad y resistencia a la degradación microbiana (Kolattukudy, 1980), un hecho que también frena la salida de nutrientes desde la hojarasca al sistema.

Se ha descrito en varios ecosistemas pobres en nutrientes la importancia de ciertas especies hemiparásitas en el reciclaje de nutrientes en dichos ecosistemas, ya que estas plantas toman agua y nutrientes de sus hospedadores y, salvo excepciones, producen una hojarasca más rica en nutrientes que el resto de especies vegetales que constituyen la comunidad (Quested et al., 2003; Quested et al., 2005; Quested, 2008). En este mismo sentido, esta misma autora describió el papel de una especie hemiparásita de ecosistemas pobres en nutrientes subárticos (*Bartsia alpina*) como una especie con un impacto positivo en la distribución de nutrientes en el sistema y aumentando la biodiversidad a escala local.

INTERACCIONES PLANTA-PLANTA

La mayoría de las relaciones que se establece entre las plantas son indirectas, salvando el caso de plantas parásitas por ejemplo, con un efecto directo sobre el rendimiento de la planta hospedadora, o el de las sustancias alelopáticas. Estas interacciones son, sin embargo, intensas y muy importantes para la dinámica de las comunidades vegetales. Algunos claros ejemplos son la competencia por recursos tales como la disponibilidad de agua en el suelo como por la luz (Pugnaire, 2010).

Si tenemos en cuenta por ejemplo un ambiente fuertemente estresante con un suelo seco y pobre en nutrientes, donde solamente plantas muy tolerantes a la sequía pueden crecer, como arbusto y árboles con un sistema radicular bien desarrollado para profundizar en el perfil del suelo, cuando un arbusto empieza a crecer, inmediatamente modifica su zona de influencia; en primer lugar la sombra que proyecta tampona en cierta manera las fluctuaciones diarias de temperatura y su hojarasca proporciona materia orgánica al suelo y la consecuente liberación de nutrientes. Por la noche, algunas plantas pueden humedecer la superficie del suelo mediante la extracción de agua desde capas muy profundas, en un proceso llamado *hydraulic lift* (Richards y Caldwell, 1987).

Desde que comenzaron a ser estudiadas las relaciones entre las plantas, las llamadas relaciones negativas (como la competencia por ejemplo) pasaron a tener un lugar preferente en dichos estudios y parecía ser que este tipo de interacciones gobernaban sobre la estructura y funcionamiento de las comunidades vegetales. Otro tipo de interacciones negativas que se establecen entre las plantas son las producidas por sustancias alelopáticas, sustancias segregadas por algunas plantas que inhiben la germinación de semillas o el crecimiento vegetal (Nilsson, 1994; Hewit y Menges, 2008; Oveisi et al., 2008).

Sin embargo, cada vez más estudios destacan también la importancia de las interacciones positivas, tales como la facilitación y las plantas “nodrizas” en particular. En este sentido, Padilla y Pugnaire (2006) describieron la utilidad de algunos arbustos en la restauración de ecosistemas, ya que actuaban como especies nodrizas que facilitaban el establecimiento y supervivencia de plántulas de otras especies vegetales.

Pugnaire (2010) determinó que todas las plantas muestran efectos positivos y negativos sobre sus vecinos y que el balance depende de muchos factores, incluyendo el nivel de recursos, la presencia de herbívoros o la frecuencia de perturbaciones. Lo que se observa en la realidad es el balance neto de las diversas interacciones; en cualquier momento los efectos positivos pueden predominar sobre los negativos o viceversa, dependiendo de las condiciones ambientales (Armas y Pugnaire, 2005).

Otro ejemplo de interacción existente entre plantas son las relaciones de parasitismo (Qasem, 2006). Las plantas parásitas son un grupo taxonómicamente diverso de angiospermas que dependen parcial o completamente de una especie hospedadora para obtener agua, carbono y nutrientes, los cuales adquieren mediante la fijación a las raíces o a la parte aérea del hospedador sirviéndose de una estructura especial que recibe el nombre de haustorio, que penetra en el xilema del hospedador y/o formando estrechas conexiones con el floema (Press y Phoenix, 2005).

El estudio de las interacciones químicas hospedador-parásito es un relativamente nuevo campo de investigación que cada vez recibe más atención por razones científicas y económicas. La existencia de plantas parásitas ha sido descrita desde tiempos remotos. Dioscórides (s. I a.C.) describió plantas pertenecientes a la familia Orobanchaceae, siendo el género *Orobanche* descrito por Linneo en 1793. En total unas 4000 especies de plantas parásitas agrupadas en 20 familias han sido descritas. Las principales familias de plantas parásitas son Scrophulariaceae, Orobanchaceae, Cuscutaceae, Viscaceae, Loranthaceae y Santalaceae (perteneciendo a esta última familia una de las especie que nos ocupa en la presente tesis doctoral: *Osyris lanceolata* Hocht. & Steud.).

Las plantas parásitas pueden ser divididas en hemiparásitas y holoparásitas, dependiendo de la presencia o ausencia de clorofilas. Las holoparásitas dependen de sus hospedadores para conseguir nutrientes y completar su ciclo de vida, ya que no pueden fijar carbono a través de la fotosíntesis. Las hemiparásitas toman de sus hospedadores solamente agua y nutrientes, y su parasitismo puede ser facultativo (Galindo et al., 2004).

La relación que se establece entre una especie parásita y su hospedador es extremadamente específica: cada especie parásita reconoce a su(s) hospedador(es). La especificidad de hospedador depende de un abanico amplio de factores, tales como la habilidad del parásito para reconocer y atacar a la planta hospedadora, romper las defensas del hospedador y la existencia de suficientes recursos en el hospedador como para asegurar la supervivencia del parásito (Galindo et al., 2004).

El parasitismo reduce con frecuencia el *fitness* de la especies hospedadora, lo cual conlleva a cambios en las interacciones de tipo competitivo que se establecen entre las especies hospedadoras y las no hospedadoras y una cascada de efectos en la estructura y diversidad de la comunidad (Pennings y Callaway, 2002). Los impactos en la comunidad vegetal se incrementan debido a que las plantas parásitas ejercen un doble efecto: parasitan y compiten con las plantas existentes en sus inmediaciones (Press y Phoenix, 2005).

ESTRUCTURA Y OBJETIVOS DE LA TESIS.

El objetivo principal de esta tesis doctoral es estudiar las interacciones ecológicas entre el pino piñonero (*Pinus pinea* L.) y diversas especies de matorral mediterráneo, así como comprender el funcionamiento de un ecosistema dunar mediterráneo en base a las interacciones entre las especies vegetales más abundantes en él y cómo se desarrolla el reciclaje de la materia orgánica de origen vegetal. Con esta investigación se pretende contribuir a una mayor comprensión de las interacciones planta-suelo y planta-planta en un ecosistema dunar mediterráneo, aumentar la capacidad para una adecuada toma de decisiones acerca de un manejo sostenible y completar la base de conocimiento existente acerca de estas interacciones en otros ecosistemas.

Para la elaboración de esta Tesis Doctoral se realizaron trabajos descriptivos y experimentales en condiciones de campo llevados a cabo en el Médano del Asperillo, entorno perteneciente al Parque Nacional de Doñana (provincia de Huelva), así como diversos ensayos de laboratorio, algunos de los cuales llevados a cabo en las instalaciones de la Universidad de Sevilla (Facultad de Biología y Servicio General de Invernadero) y otros en las instalaciones de centros de investigación europeos durante las 3 estancias breves de investigación llevadas a cabo en Francia (2009), Portugal (2010) e Inglaterra (2011).

El **capítulo 2** presenta una primera aproximación al estudio de las interacciones planta-planta y planta-suelo desarrolladas a lo largo de la presente tesis. En concreto se centra en el estudio de la germinación de diversas especies de matorral mediterráneo presentes en la zona de estudio (*Cistus salvifolius* L., *C. libanotis* L. y *Halimium halimifolium* (L.) Willk.) atendiendo a la presencia de una barrera física y/o extractos preparados a partir de acículas de pino piñonero en distintas concentraciones y el seguimiento del establecimiento de las plántulas emergidas de las mismas semillas bajo las mismas condiciones. Las sustancias presentes en los extractos pueden servir tanto como sustancias inhibidoras y/o estimuladoras de la germinación, así como la capa de acículas puede representar tanto la existencia de un microclima propicio para la germinación de semillas y posterior establecimiento de las plántulas como un impedimento a la entrada de luz y desarrollo de las plántulas.

El **capítulo 3** se centra en el estudio de la descomposición de la hojarasca perteneciente a las especies más representativas del área de estudio (*P. pinea*, *Juniperus phoenicea* L., *C. salvifolius*, *H. halimifolium*, *Rosmarinus officinalis* L. y *Osyris lanceolata* Hochst. & Steud.), con el objetivo de profundizar en el funcionamiento del ecosistema estudiado desde el punto de vista de reciclaje de nutrientes y de cómo varían las tasas de descomposición y de liberación de nutrientes en función de la identidad de estas especies y de la zona donde se desarrolle este proceso de crucial importancia en ecosistemas pobres en nutrientes como son los sistemas dunares mediterráneos. En una primera aproximación se llevaron a cabo dos tipos de estudio de descomposición de hojarasca: directamente en el campo con bolsas de descomposición y una aproximación en laboratorio mediante el uso de microcomos.

Posteriormente se diseñó otro experimento en campo (**capítulo 4**) que permitía comparar dos zonas en el área de estudio: una cuya vegetación predominante estaba formada por *P. pinea*, fruto de plantaciones forestales durante la primera mitad del siglo XX, y otra con vegetación principalmente compuesta por *J. phoenicea* y *R. officinalis* (vegetación original del área de estudio).

El **capítulo 5** aborda las relaciones planta-planta a un nivel de interacción plántula-plántula mediante un experimento en invernadero. Las especies seleccionadas son *P. pinea*, *C. salvifolius* y *Pistacia lentiscus* L. Se trata de analizar las interacciones (competencia) que se producen entre estas especies (teniendo a *P. pinea* como la especie principal competidora) en dos tipos de tratamiento: un control regando todas las macetas con agua solamente y un tratamiento fertilizador añadiendo solución nutritiva Hoagland modificada. Las hipótesis de partida imponía el principio de que en ambientes ricos en nutrientes el nivel de competencia entre dos especies o individuos de la misma especies es mayor que en ambientes pobres en nutrientes. El objetivo fundamental de este capítulo se centra en analizar si el efecto de la diferente disponibilidad de nutrientes tendrá un efecto en la competencia tanto intra- como interespecífica.

En el **capítulo 6** se aborda la interacción entre plantas en un estudio de campo centrado en el estudio de un caso particular, el de la especie hemiparásita *O. lanceolata*, presente en el área de estudio y cuya población parece expandirse gracias al efecto *perching* facilitado por *P. pinea*. La hipótesis de partida es que una especie hemiparásita tendría un efecto negativo sobre la especie hospedadora. En nuestro estudio llevamos a cabo la experimentación para comprobar la hipótesis de partida en las dos zonas diferenciadas del área de estudio: la compuesta por *P. pinea* principalmente y la de vegetación más conservada compuesta por *J. phoenicea*. Ambas especies de coníferas son tenidas en cuenta como potenciales hospedadoras de *O. lanceolata*. Además de las medidas ecofisiológicas también se procede al muestreo de parcelas en las dos zonas escogidas para determinar la distribución de las distintas especies, tanto a nivel de individuo adulto como de establecimiento de plántulas, para determinar si existe “preferencia” por parte de la especie hemiparásita en cuanto a especie hospedadora.

La tesis concluye con un análisis conjunto de los resultados que permite obtener una visión global de las interacciones planta-suelo y planta-planta en ecosistemas dunares mediterráneos y una evaluación de las implicaciones que estas interacciones tienen en el funcionamiento del ecosistema.

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CAPÍTULO 2

Effects of *Pinus pinea* litter on seed germination and seedling performance of three Mediterranean shrub species

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Summary. *Pinus pinea* L. is a coniferous tree inhabiting the Mediterranean Basin, which appears widely distributed in the Iberian Peninsula, frequently associated to Mediterranean shrub species, such as *Cistus salvifolius* L., *C. libanotis* L. and *Halimium halimifolium* (L.) Willk. (Cistaceae). This study investigated the effects (both allelopathic and/or physical) of *P. pinea* needles on the seed germination and early growth of these shrub species. Laboratory experiments were carried out in order to assess an expected negative effect by using different treatments to seeds (physical layer and two aqueous extracts concentrations). Seed germination of all species was inhibited when treated with aqueous extracts and the degree of inhibition was higher when using the extract with the highest concentration, while no effect was observed with the physical layer treatment. Seedling performance was also sensitive to the different treatments, showing the most stressed values with the high concentration extract. The results from this study highlight the importance of allelopathy of *P. pinea* needles as a mechanism for controlling seed germination and seedling performance of some Mediterranean shrub species.

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2.1. INTRODUCTION

The Stone pine (*Pinus pinea* L.) is one of the most widely spread conifers in the Iberian Peninsula and it grows in almost all the countries of the Mediterranean Basin (Ovando et al., 2010). In Spain, the Stone pine covers a surface area of over 400,000 ha, which represents around 50 % of its world distribution range. Though it is a native species to the Iberian Peninsula, its distribution area has spread in the last two centuries favoured by afforestation policies, due to its ability to colonise rocky and sandy substrates or degraded soils, and due to its ecological plasticity, attractive shape and economic value (Iovieno et al., 2010).

The public environmental benefits of Stone pines were traditionally related to soil protection against surface erosion and the consolidation of coastal dunes, and more recently to the provision of shelter and food for local wildlife and recreation (Montero et al., 2004). However, little is known about the effects of this species on neighbouring woody species, particularly on their seed germination and seedling establishment of other species in the community.

Germination and seedling establishment are the most critical stages for plant population dynamics (Facelli, 1994) and an especially critical phase in the life cycle of plants inhabiting dry environments (Huang et al., 2004; Yang et al., 2010). Seed germination is a key process to the understanding of plant community functioning in ecosystems (Geissler & Gzik, 2010). The ecological conditions prevailing in a given habitat will affect germination. Seeds fallen in litter-covered microsites are favoured in arid ecosystems, because these sites may retain a high number of seeds and maintain soil moisture (Facelli & Pickett, 1991). Seeds in their natural habitat interact with other plants and wild animals. The interaction with other plants may be due to inhibitors, stimulators or modification of the micro-habitat (Lovett et al., 1989).

Coastal dune ecosystems of the south-west of Spain are nutrient-poor systems with shallow fertile soils (Gallardo, 2001). Litter decomposition rates are low (García-Plé et al., 1995), so Mediterranean pine forests are prone to accumulate relatively thick needle layers below theirs canopies (Izhaki et al., 2000). Many studies have been carried out on the effects of other *Pinus spp.* over some ecosystem properties, as incident light and water availability reduction caused by the pine canopy (Maestre et al., 2003).

Navarro-Cano et al. (2009) also showed a negative effect of *Pinus halepensis* Mill. on the seedling performance of *Stipa tenacissima* L.

The possible negative effect of *P. pinea* on the germination and seedling performance of Mediterranean shrub species, and in particular, species from the Cistaceae family, are not well documented. These species are well-adapted to live in the Mediterranean Basin, where heat-stimulated germination has been linked to the rupture of the seed coat in species with water-impermeable seeds (Herranz et al., 1998; Paula & Pausas, 2008). The three tested species in this study belong to the Cistaceae, of which two belong to the genus *Cistus*, whose species are known to be early colonisers, adapted to a spectrum of unpredictable disturbances characteristic of Mediterranean ecosystems (Troumbis & Trabaud, 1986).

This research was carried out in order to determine whether *P. pinea* has a negative effect on early settlement stages of three common Mediterranean shrub species; specifically, we wanted to distinguish between the effects of aqueous extracts and the presence of a physical layer on the germination and seedling growth of three species from the Cistaceae family. Thus the aim of this study was to determine the physical and chemical effects of Stone pine´s littered needles on germination and to study the seedling performance of the species from Cistaceae.

2.2. MATERIALS AND METHODS

2.2.1. *Sampling site*

The material was collected from the coastal dunes of El Asperillo (34^{o} 0´N 6^{o} 36´W), neighbouring the Doñana National Park (south-west Spain). The climate is Mediterranean type with oceanic influence and mild temperatures. Mean annual temperature is 16.8 ^{o}C. Average annual rainfall is 550 mm, concentrated between October and March. Vegetation consists on native dune scrub of *Halimium halimifolium* (L.) Willk., *H. calycinum* (L.) K.Koch., *Cistus libanotis* L., *C. salvifolius* L., *Rosmarinus officinalis* L., *Lavandula stoechas* Lam., *Cytisus grandiflorus* (Brot.) DC., *Stauracanthus genistoides* (Brot.) Samp., *Corema album* (L.) D. Don, *Juniperus oxycedrus* L. and *J. phoenicea* L., together with *Pinus pinea* (Muñoz Reinoso & Díaz Barradas, 1992).

2.2.2. *Target species*

Three of the most abundant shrub species such as *Cistus salvifolius*, *Cistus libanotis* and *Halimium halimifolium* representing Mediterranean dune systems, were selected for this study.

2.2.3. *Material collection*

Seeds were collected from different individuals of each of the 3 selected species in October 2008, placed in paper envelopes and were stored under laboratory conditions until used in December 2008. Stone pine´s needles were collected from the trees after shaking them, in order to avoid non-senescent plant material.

One of the most widely used bioassay techniques for studying allelopathy involves the preparation of aqueous extracts of plant leaf or root material and testing them on seed germination and seedling growth of different species under laboratory conditions (Inderjit & Dakshini, 1990).

Two hundred grams of Stone pine´s needles were soaked in 1 L of distilled water for 24 h at 25 ^{o}C and then filtered. The extract was diluted with distilled water to obtain concentrations of 3.4 and 10 g L^{-1} and these were stored in a refrigerator at 4 ^{o}C. These extract concentrations were chosen in order to simulate plant material left in the field after a low and high productive year, respectively (Emeterio et al., 2004). These concentrations are similar to those used by Fernandez et al. (2006) in a study involving *P. halepensis* about its potential allelopathic effect in the secondary succession.

Of each study species, 100 incised seeds were soaked in 1 % solution of Tetrazolium salt (2, 3, 5-triphenyl tetrazolium chloride) for 48 h under dark conditions and the viability of seeds was determined according to Moore (1973). A seed was considered viable when the embryo was stained light pink or red. This coloration shows that the embryo is alive, though does not necessarily mean that it is sufficiently active for germination (Egley & Chandler, 1978).

Trials were carried out using 90 mm plastic Petri dishes with Whatman® n^{o} 4 filter papers (Molina et al., 1991; Fernandez et al., 2006). One hundred seeds of each target species for each treatment were placed in Petri dishes (4 replicates in total), to which 2 ml of extract solution was added. The dishes were then regularly moistened with an equal volume of the same extract solution (1 ml) until the end of the experiment. For controls, distilled water was used. Toothpicks were used in order to simulate the effect of a physical layer similar to the possible effect of Stone pine´s needles in the field. Two variables were analysed for the three target species: germination of seeds (rate and response curve), and length of seedlings (radical and hypocotyl) during 8 weeks, with no germination after this time. Seeds were surface-decontaminated in sodium hypochlorite solution (4 % v/v) for 2 min and subsequently were immersed in 70 % (v/v) ethanol for 2 min. Seeds were then washed with distilled water and finally placed on filter paper in Petri dishes.

2.2.4. *Seed germination under different conditions*

Four hundred seeds of each of the 3 target species were distributed in groups of 25 in 16 Petri dishes with filter paper (4 replicates for each treatment for each species). Treatments were 0 (as control), 0 + toothpicks (as physical layer), and extract solutions (3.4 and 10 g L^{-1}, as allelopathic effect). Petri dishes were placed in a growth chamber (ASL Aparatos Científicos M-92004, Madrid) where conditions were 12 h light (60 µmol m^{-2} s^{-1}) under cyclic temperature conditions, ranging from 18 to 25 ^{o}C, the dark phase coinciding with the lowest temperature, and the light period coinciding with the 20-25 ^{o}C temperature interval. Forty-eight Petri dishes were monitored (16 per species).Water for the control and “0 + toothpicks” treatment and extract solutions for the other treatments were added to Petri dishes weekly. Seed germination was recorded daily for 8 weeks.

2.2.5. *Seedling performance*

All obtained seedlings were grown in pots filled with sand from the study site for two months. The experimental design was similar to that of seed germination studies. Pots were placed in a glasshouse and moistened weekly in the same way as explained for seed germination experiments. Radicle length and elongation of the aerial portion of seedlings of target species were recorded weekly from the Petri dishes and then we compared measurements derived from seedlings of the same age. Dry weight of seedlings at the end of the experiment was also recorded.

In order to determine the status of the photochemical apparatus of leaves of seedlings, the maximum quantum efficiency of photosystem (PS) II photochemistry (F_v/F_m) (Rohácek & Barták, 1999) seedling´s leaves was measured using a portable modulated fluorometer (mini-PAM, Walz, Germany) after 14 and 30 days from the first germination (n = 10, one measurement per plant). The darkness adaptation period lasted for 20 minutes for each measurement, using leaf-clips exclusively designed for this purpose. An actinic photosynthetic photon flux of 3000 µmol m^{-2} s^{-1} was used to determine the induction of fluorescence, which was recorded at wavelengths of ~650 nm. The value of (F_v/F_m) was calculated by using Equation 1, which is:

$$F_v/F_m = (F_m - F_0)/F_m, \quad (1)$$

In which F_m and F_0 are the maximum and minimum fluorescence in dark-adapted tissue, respectively. The values of F_m and F_0 were generated by the supporting software for the fluorometer.

2.2.6. *Data analysis*

All the statistical analyses were carried out using SPSS version 18.0 (SPSS, Chicago, IL, USA). Data on germination (%) were arcsine-transformed to ensure normality and homogeneity of variances and the means were compared using Tukey´s post hoc test. All the P-values were considered to be significant at $P \leq 0.05$. A two-way ANOVA was also carried out with species and treatments as factors.

Data on seedling performance, and shoot and root length were normally distributed and were analysed for each target species using one-way ANOVA. Comparisons of main effects were performed using the least significance difference test at $P < 0.05$.

Four parameters were determined: the germination onset, which is the time (in days) between the beginning of the experiment and the commencement of the germination, the final germination percentage at the end of the experiment (G), calculated over viable seeds, T_{50} (days required to reach 50 % of total germination) and, finally, percentage inhibition or stimulation (Jefferson & Pennacchio, 2003).

2.3. RESULTS

2.3.1. *Seed germination under different conditions*

Seed viability results were 86 %, 72 % and 71 % for *C. salvifolius*, *C. libanotis* and *H. halimifolium*, respectively.

Regarding germination, the different ANOVA tests applied showed significant differences between species and treatments. Interaction between these two factors (species and treatments) and between treatments in all the species (Fig. 2.1) was also significant (see Table 2.1).

		Germination level		**FG**		**T_{50}**	
	D.f.	**F-ratio**	**P-value**	**F-ratio**	**P-value**	**F-ratio**	**P-value**
Species	2	169.230	0.000	48.308	0.000	156.621	0.000
Treatments	3	29.000	0.000	10.154	0.000	110.000	0.000
Species * treatments	6	2.510	0.039	10.154	0.000	30.552	0.000

Table 2.1. Two-way ANOVA of effects of different treatments on germination level, first germination (FG) and on T_{50}.

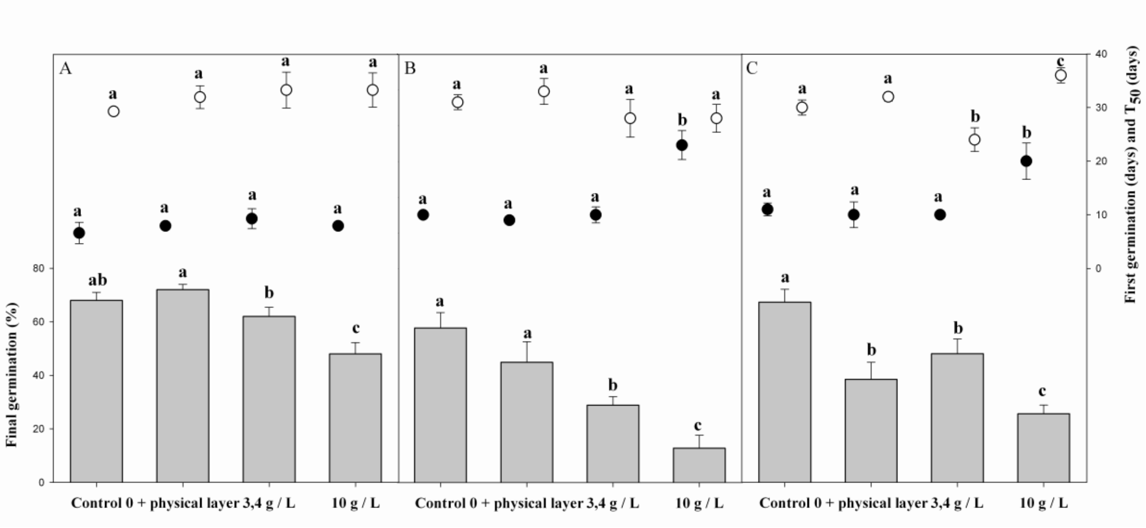

Fig. 2.1. Final germination (%), days to first germination (black symbols) and T_{50} (white symbols) in days for (A) *C. salvifolius*, (B) *C. libanotis* and (C) *H. halimifolium*, according to different treatments: Control (just moistened up with distilled water), “0 + physical layer” (watered and a simulated physical layer) and the two extract solutions (at low and high concentration). Values are means ± S.E. (n = 4). Bars and symbols (black and white) of each species with different letters are significantly different (P < 0.05) according to Tukey´s post hoc test.

The Stone pine needle aqueous extracts showed inhibitory effects on the seed germination of the three Cistaceae species (Fig. 2.2). The degree of inhibition increased at a high concentration (10 g L^{-1}). The treatment with 0 g L^{-1} + physical layer decreased the germination rate of *H. halimifolium* and *C. libanotis*, while no significant differences between this treatment and the control were observed for *C. salvifolius* (Fig. 2.2). In all cases, the high concentration of the needle extract significantly decreased the germination rate. In the case of *C. salvifolius*, the lower concentration of needle extract did not affect the final germination when compared with the control, but the lower concentration affected the other two species´ germination (Fig. 2.2).

Days needed for first germination did not differ in the case of *C. salvifolius*, while in the other two species significant differences were found for the high concentration of needle extract, with an average delay of 13 and 10 days for *C. libanotis* and *H. halimifolium*, respectively.

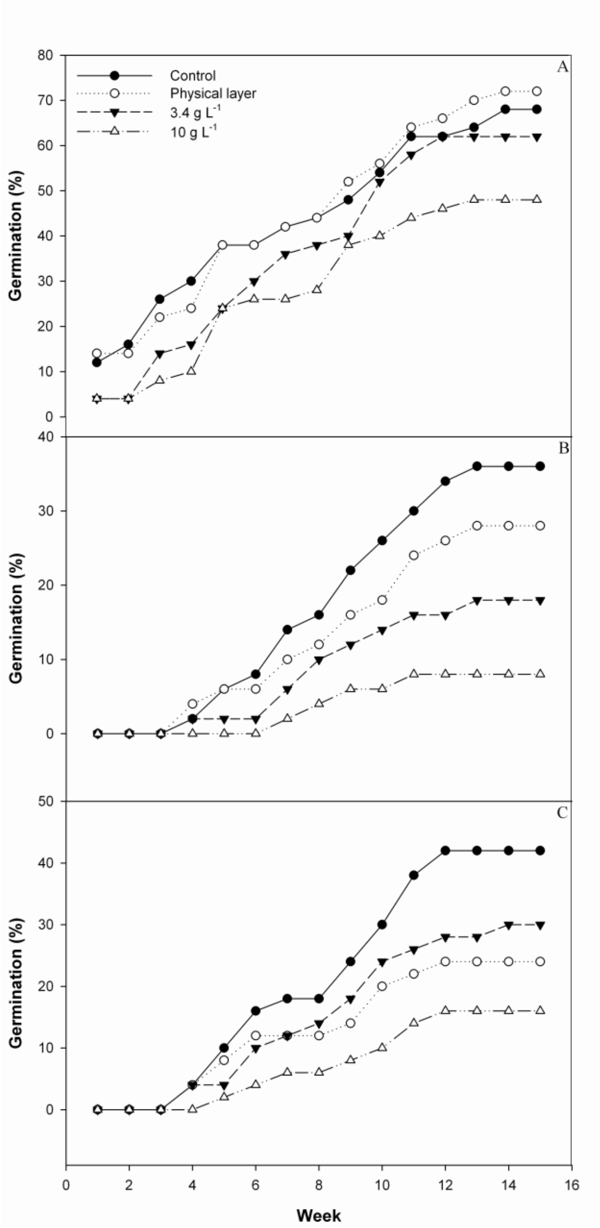

Fig. 2.2. Cumulative germination of the three Cistaceae species under different treatments: Control, simulated physical layer two extract solutions at low/high concentration. (A) *C. salvifolius*, (B) *C. libanotis* and (C) *H. halimifolium* for 8 weeks.

For both species belonging to the genus *Cistus*, no significant differences were found for T_{50} among the treatments, while a significant delay was observed for *H. halimifolium* when treated with the high concentration extract solution (Fig. 2.1).

The percentage of inhibition of both aqueous extracts of needles differed among the species, with C. *libanotis* being the most sensitive species to both extracts (Table 2.2).

	C. salvifolius	***C. libanotis***	***H. halimifolium***
3.4 g L^{-1}	- 9	- 50	- 29
10 g L^{-1}	- 29	- 78	- 62

Table 2.2. Percentage inhibition/stimulation (%) of the two different aqueous extract concentrations over the three Mediterranean shrub species.

2.3.2. *Seedling performance*

The elongation of hypocotyls in *C. salvifolius* decreased compared to control (Fig. 2.3). However, the effects of different treatments on radicle elongation differed among the species, with no negative effects observed in the case of *H. halimifolium* (Fig. 2.3).

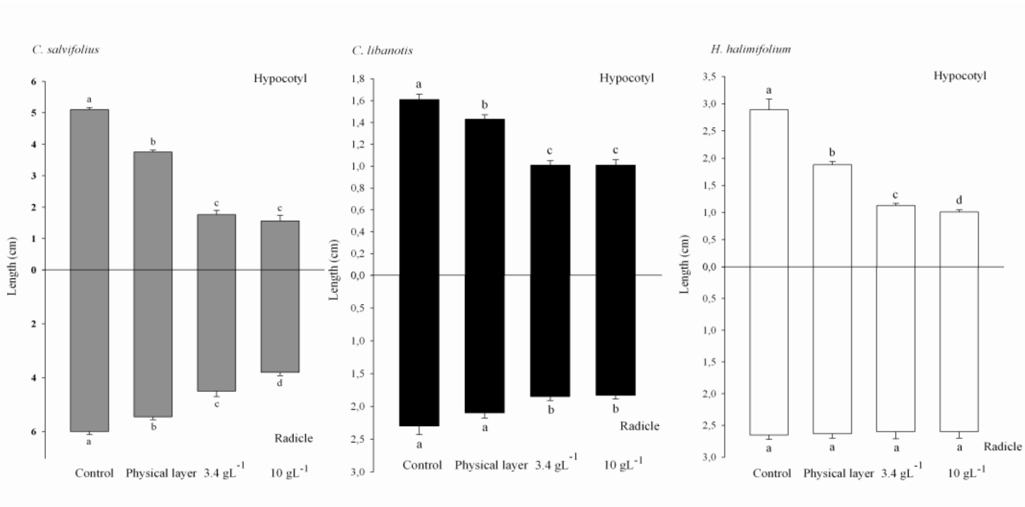

Fig. 2.3. Radicle and hypocotyls growth of *C. salvifolius*, *C. libanotis* and *H. halimifolium* for 8 weeks, growing under different conditions (control, physical layer and two extract solutions at low and high concentrations). Bars of each species with different letters are significantly different ($P < 0.05$) according to Tukey´s post hoc test.

The physical layer treatment showed no differences in radicle elongation for *C. libanotis* and *H. halimifolium*, while there were significant differences between this treatment and the control in *C. salvifolius*.

The responses of the seedlings´ dry weight to the needle extract concentrations were similar to those observed in radicle and hypocotyls length in all tested species. The extracts also had inhibitory effects on the maximum quantum yield by PSII (F_v/F_m) in the three species (Table 2.3); hence inhibition was significantly different at high concentrations (10 g L^{-1}).

Treatment ($g L^{-1}$)	**Dry weight (mg $seedling^{-1}$)**	**Chlorophyll fluorescence (F_v/F_m)**
Cistus salvifolius		
0	2.09 ± 0.11a	0.834 ± 0.004a
0 + Physical layer	2.03 ± 0.11a	0.821 ± 0.007b
3.4	1.67 ± 0.20b	0.816 ± 0.006b
10	0.98 ± 0.13c	0.776 ± 0.009c
P-value	<0.0001	<0.0001
Cistus libanotis		
0	1.97 ± 0.13a	0.818 ± 0.010a
0 + Physical layer	1.95 ± 0.08a	0.804 ± 0.004a
3.4	1.78 ± 0.06b	0.779 ± 0.006b
10	1.02 ± 0.06c	0.746 ± 0.008c
P-value	<0.0001	<0.0001
Halimium halimifolium		
0	1.85 ± 0.07a	0.827 ± 0.005a
0 + Physical layer	1.81 ± 0.07a	0.814 ± 0.003b
3.4	1.55 ± 0.11b	0.818 ± 0.006ab
10	0.73 ± 0.10c	0.747 ± 0.007c
P-value	<0.001	<0.0001

Table 2.3. Dry weight and chlorophyll fluorescence of *C. salvifolius*, *C. libanotis* and *H. halimifolium* grown in *P. pinea* needle extract-treated soils. The values in a column with different letters are significantly different ($P < 0.05$) according to Tukey´s post hoc test. Data are the mean (± SD) of the four replicates. The values in a column marked with different letters indicate a significant difference at $P < 0.05$.

2.4. DISCUSSION

P. pinea displays allelopathic activity and influences the emergence and seedling performance of *C. salvifolius*, *C. libanotis* and *H. halimifolium*, which are some of the most representative shrub species from sandy ecosystems of Western Mediterranean region. The effects of the laboratory simulation varied depending on the treatment, the target species and the growth attribute measured.

The degree of inhibition was related to the concentration of the extracts used. Besides the inhibition of seed germination observed among the different treatments, there were some cases of inhibition in the radicle and hypocotyl elongation of seedlings. These results are in agreement with those reported by other studies for a number of plant species of different families, whose allelochemicals negatively affect neighbouring species: Asteraceae (Escudero et al., 2000), Gramineae (Emeterio et al., 2004), Fabaceae (Rashid et al., 2010), even synthetic compounds (Chiapusio et al., 1997).

Different studies have shown that the presence of allelochemicals in the soil may be determined by many factors, such as the density of leaves littered, the rate of decomposition of plant material, the neighbouring plant species and rainfall (Saxena et al., 1996). Soil type and its pH are also important (Saxena et al., 1996) in determining whether or not allelopathic substances are present in the soil and if they are in threshold concentrations to affect other plants (Jefferson & Pennacchio, 2003).

The needles of *P. pinea* may play an important role not only by releasing allelopathic substances to the soil, but also acting as a physical layer. This study shows that seed germination of *C. salvifolius* and *C. libanotis* was not affected by the presence of the toothpick physical layer which simulated the effect of litter in the field; *H. halimifolium*, in contrast, exhibited an inhibition pattern when compared with the control, similar to the one of low concentration needle extract. *Cistus* species have, in general, hard-coated seeds impervious to water, whereby germination is improved by disturbances which expose seeds to different agents that contribute to alter the seed coat (Corral et al., 1990; Roy & Sonie, 1992). Most seeds with impermeable seed coats germinate equally well in light and darkness over a range of temperatures after the seed coat becomes permeable; this could explain why there were no differences on seed germination between the control and the physical layer treatment for *C. salvifolius* and *C. libanotis* (Fig. 2.1).

Fernandez et al. (2006) described the allelopathic effect of *Pinus halepensis*, limiting the establishment of other species, either by reducing interspecific competition or affecting their growth. In our case, *P. pinea* showed allelopathic effects on both seed germination and seedling performance. Although some studies have reported stimulatory effects on shoot growth (Tefera, 2002; Fernandez et al., 2006), the inhibitory effect of the great variety of aqueous extracts from different plant species has been widely described (Facelli & Pickett, 1991; Emeterio et al., 2004; Fernandez et al., 2006; Hewitt & Menges, 2008). Radicle and hypocotyl elongation were clearly inhibited at a higher concentration of needle extracts, comparable to other studies, such as *Carduus nutans* on pasture plants in New Zealand (Wardle et al., 1991), *Artemisia* *princeps var. orientalis* (Kil & Kyeong, 1992), *Medicago arborea* (Bousquet-Melou et al., 2005), *Pinus halepensis* (Alrababah et al., 2009). The reduction in growth of radicle and hypocotyl of the target species when treated with aqueous extracts of both concentrations suggests *P. pinea*´s allelopathic potential. Rashid et al. (2010) also found this behaviour when comparing seedling growth of *Bidens pilosa* and *Lolium perenne* grown in soil treated with aqueous extracts of a plant with allelopathic potential in contrast with seedlings grown on untreated soil.

Radicle length was reduced in both *Cistus* species by needle extracts of *P. pinea*, but not in *H. halimifolium*. The inhibitory effect on radicle length by allelochemicals have been reported in other studies (Emeterio, 2004; Shui et al., 2010), it may influence seedling establishment in the field due to the differential access to water sources, more pronounced when water resources are limited which occurs frequently in the Mediterranean region. Hence, a poor development of the radicle system could affect the ability of *C. salvifolius* and *C. libanotis* to put up with water deficit, especially during summer. In this study, reduction values of 34 % in radicle elongation were observed for *C. salvifolius* when comparing the control and with 10 g L^{-1} aqueous extract. The absence of effects on *H. halimifolium* radicle also supports the plastic character of this species (Zunzunegui et al., 2000).

Dark-adapted values of (F_v/F_m) reflect the potential quantum efficiency of PSII, Maxwell and Johnson (2000) found them to be a sensitive indicator of plant photosynthetic performance, their measured optimal values being ~0.83 for most plant species (Johnson et al., 1993). Values of <0.80 indicate that plants have been exposed to stress (Rachmilevitch et al., 2006). Considering this, *C. salvifolius* and *H. halimifolium* seedlings grown in the soil watered with 10 g L^{-1} needle extracts and those of *C. libanotis* grown in the soil watered with ≥3.4 g L^{-1} needle extracts were under stress conditions. These results lead to the conclusion that *P. pinea* needles possess a chemical phytotoxic effect to these three shrub species. Marwood et al. (2003) and Rashid et al. (2010) found similar values in the (F_v/F_m) of *Myriophyllum spicatum* for *Bidens pilosa* and *Lolium perenne*, respectively. The observed lower values of photochemical efficiency in seedlings treated with aqueous extracts are related to lower dry weight and with a lower development of shoot and root system.

2.5. CONCLUSIONS

The current study highlights the importance of *P. pinea* in modifying the environmental conditions, which plays an important role in inhibiting seed germination and seedling recruitment of a number of Mediterranean shrub species by allelochemicals. Although the two species belonging to the genus *Cistus* showed no differences in seed germination with the physical layer treatment, radicle and hypocotyl elongation were more sensitive to the different treatments. The results derived from this study will allow us to develop long-term field experiments in order to compare the glasshouse experiments with natural situations as, for example, delimiting plots in soils under pine canopies and far from them and study germination and seedling performance of the target species.

A thin needle layer in the soil could improve seed germination and seedling establishment of several Mediterranean shrub species because this litter layer may retain a moisture level, which could be beneficial for seed germination in a semiarid ecosystem, similar to the study site. A thick litter layer could avoid seed reach the soil and may represent a great source of allelochemicals, which can inhibit seed germination and seedling establishment.

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CAPÍTULO 3

Three different approaches for estimating litter decomposition rate of four woody Mediterranean species: the role of a hemiparasitic plant species

CAPÍTULO 3

Three different approaches for estimating litter decomposition rate of four woody Mediterranean species: the role of a hemiparasitic plant species

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Summary. Mediterranean sand dunes are nutrient-poor ecosystems dominated by evergreen and semideciduous scrubs. In *El Asperillo* (Huelva, SW Spain), *Pinus pinea L.* has been widely used in plantations and competes directly with Mediterranean shrub species. It produces litters with high amounts of recalcitrant compounds which decompose slowly and form a thick layer of needles in the soil. This study was carried out in order to compare decomposition rates of *P. pinea* litter with three other representative plant species of the study site and to assess the possible factors controlling the decomposition process. We also investigated the possible important role of the hemiparasitic shrub *Osyris lanceolata Hochst. & Steud.* in the nutrient cycling. We developed three different approaches: litter-bag experiment in the field, microcosm experience in the laboratory and NIRS (near-infrared spectroscopy). We found that *P. pinea* was the species with the lowest decomposition rate among the studied species and we found out the importance of *O. lanceolata* in the nutrient cycling at the ecosystem level because senescent leaves kept high amounts of nitrogen.

Este capítulo reproduce el siguiente artículo: **Valera-Burgos J, Kazakou E, Díaz-Barradas MC, Zunzunegui M (2012)** Three different approaches for estimating litter decomposition rate of four woody Mediterranean shrub species: the role of a hemiparasitic plant species (Submitted to *Plant and Soil*).

3.1. INTRODUCTION

Litter decomposition is a key process in the nutrient cycling of most terrestrial ecosystems (Swift et al., 1979; Couteaux et al., 1995; Chapin et al., 2002) and a major pathway by which terrestrial vegetation modifies the soil environment (Swift et al., 1979). It is controlled by three main factors: climate, litter quality and the nature and abundance of the decomposer organisms (Aerts, 1997; Swift et al., 1979).

At a global scale, evapotranspiration rate was determined as the best index explaining the species decomposition rates among well-defined climatic sites, although moisture and temperature in the soil were found much more suitable to predict decomposition rates at a local scale (Berg et al., 1993; Rovira & Vallejo, 1997). So the same type of litter may have different decomposition rates according to different climatic conditions (Gallardo, 2001).

However, at the local scale, litter quality prevails as the regulator factor under favourable conditions (Lavelle et al., 1993; Couteaux et al., 1995; Swift et al., 1999). More precisely, Vitousek & Howarth (1991) pointed out that plants with low amounts of nutrients would produce nutrient-poor litter with a high C-to-nutrient ratio, so this litter decomposes more slowly and high amounts of nutrients remain in the soil for a long period. In addition, some authors described a high concentration of poliphenols in Mediterranean plant tissues, which affects negatively not only the decomposition process, but also the nutrient use efficiency as a possible source of resorption for plants (Aerts & De Calluwe, 1997; Gallardo, 2001).

Recent studies have shown the importance of several plant traits affecting decomposition processes: specific leaf area (SLA), related to net assimilation rate (Reich et al., 1992, 1997) and is related to plant relative growth rate (RGR: Reich et al., 1997; Poorter & Garnier, 1999); leaf dry matter content (LDMC), related to nutrient retention within the plant (Poorter & Garnier, 1999; Ryser & Urbas, 2000); leaf nitrogen content (LNC) and leaf carbon content (LCC) in green leaves which are strongly correlated to the nitrogen-rich compounds involved in the carbon-fixation process (Evans, 1989) and, consequently, the carbon-to-nitrogen ratio (C/N) and potential decomposability of litter.

Mediterranean ecosystems are nutrient-poor systems and present low-fertile soils, so primary production is limited by low availability of nutrients. In these ecosystems a positive feedback loop may be acting, by which a nutrient-poor soil produces vegetation with nutrient-poor litter, which implies a low decomposition rate and, finally, a low availability of nutrients in the soil (Gallardo, 2001). In particular, Gallardo & Merino (1993) described Mediterranean ecosystems of the southwest of Spain as nutrient-poor systems where litter decompose very slowly.

Quested et al. (2003, 2005) described the importance of a hemiparasitic plant (*Bartsia alpina* L.) in the nutrient cycling in a nutrient-poor ecosystem, showing that this species produces nutrient-rich litter which is linked to plant growth enhancement. The same authors pointed out the possible same effects of other hemiparasitic plants in other nutrient-poor communities, as it has been described by Press & Graves (1995) in the Mediterranean zones of the south-western coastal region of Australia and parts of South Australia.

There are several approaches to estimate litter decomposition: (1) directly in the field by using the nylon mesh bag technique (Bocock & Gilbert, 1957); (2) directly in the laboratory with litterbags incubated in microcosms (Taylor & Parkinson, 1988) and (3) indirectly with the NIRS system (Joffre et al., 1992).

Litter bags are a technique used to study decomposition processes where plant material is incubated and monitored over time. Although this method creates a microenvironment, it is easily used to study the dynamics of decomposition in samples of known initial weight which can be recovered in field conditions (McTiernan et al., 2003; Berg & Laskowski, 2006).

Near infrared reflectance spectroscopy (NIRS) is an analytical technique that allows determining chemical constituents in different plant tissues and in the soil with a wide range of concentrations (Gillon et al., 2004; Côuteaux et al., 2005; Schimann et al., 2007). The NIRS technique is rapid and non-destructive with exact and reproducible measurements of chemical components in organic materials (McTiernan et al., 2003).

In the present study we investigated the decomposition of four types of leaf litter in a Mediterranean ecosystem of southwest Spain. The main aim of this work was to evaluate the importance of chemical properties of the Mediterranean leaf litter and functional traits in predicting litter decay rates and to determine. To develop this main goal we developed some other purposes:

1. Determine decomposition rate of the target species related to their decomposability under controlled conditions in the laboratory (incubation in microcosms) and comparing then these results with those from the field experiment to establish if climatic conditions impose its effects on decomposition processes.

2. Compare decomposability of the target species with their initial chemical characteristics in order to find out if these characteristics affect decomposition processes /decomposition rate.

3. Evaluate whether decomposition rates were related to several plant traits, measured before tissue senescence.

This study was based on the initial hypothesis that *Osyris lanceolata Hochst. & Steud.*, as a hemiparasitic plant species, could have an important role in the nutrient cycling, as has been described in other studies on parasitic plants (Quested et al., 2003; Quested et al., 2005).

The target species would react in different ways through the decomposition process based on environmental characteristics and theirs chemical properties.

3.2. MATERIALS AND METHODS

3.2.1. *Study site*

The field experiment was carried out in “El Asperillo” (34^{o}0´N 6^{o}36´W), in the Doñana National Park (south-west Spain). The climate is Mediterranean type with oceanic influence and mild temperatures. Mean annual temperature is 16.8 ^{o}C. Average annual rainfall is 550 mm, concentrated between October and March (Fig. 3.1). Vegetation consists of native dune scrub of *Halimium halimifolium* (L.) Willk., *H. calycinum* (L.) K.Koch., *Cistus libanotis* L., *C. salvifolius* L., *Rosmarinus officinalis* L., *Lavandula stoechas* L., *Cytisus grandiflorus* (Brot.) DC., *Stauracanthus genistoides* (Brot.) Samp., *Corema album* (L.) D.Don, together with *Juniperus oxycedrus*, *J.*

phoenicea, *Pinus pinea* (Díaz-Barradas & Muñoz-Reinoso, 1992) and *Osyris lanceolata*.

3.2.2. *Climatic conditions at the study site*

Temperature and precipitation data during the field experiment period (from August 2009 until May 2010) were collected and due to differences related to past years we also included mean data from the last 50 years. Precipitation fallen during the incubation period in the field was 2 times higher than the mean (data not shown).

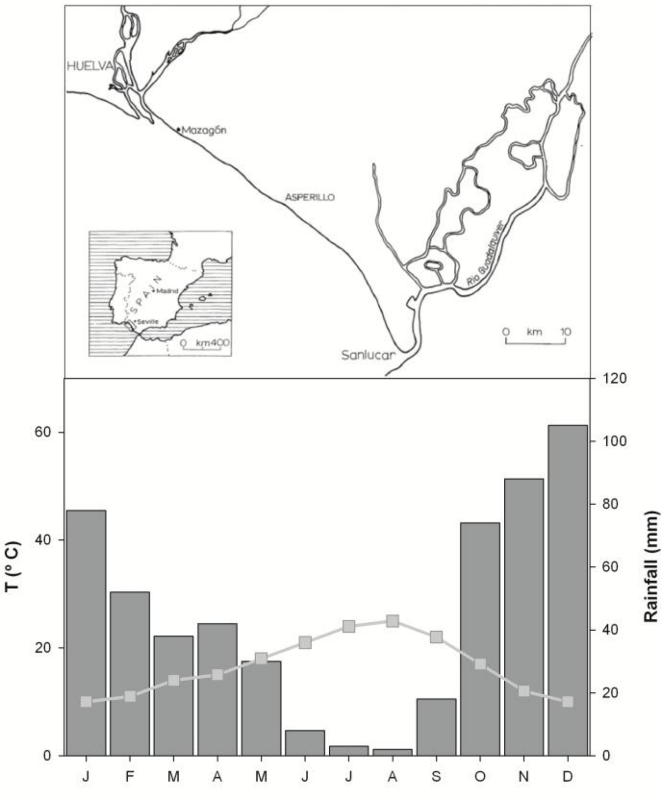

Fig. 3.1. Ombrotermic diagram of El Asperillo (Huelva, SW Spain) and map of the study site.

3.2.3. *Target species*

We selected some of the most representative species at the study site: the Stone pine [*Pinus pinea* L. (*Pinaceae*)], a native Mediterranean tree species widely distributed in the Iberian Peninsula who creates a thick layer of needles in the surface beneath its canopy, *Osyris lanceolata* Hocht. & Steud. (Santalaceae), a hemiparasitic dioecious shrub species (Herrera, 1988) which may play an important role in the nutrient cycling, *Cistus salvifolius* L. (Cistaceae) and *Halimium halimifolium* (L.) Willk. (Cistaceae) one of the most abundant plant families in the Mediterranean Basin, according to Muñoz-Reinoso & Díaz-Barradas (1992).

3.2.4. *Collection of material*

Traits of living leaves were measured on 10 replicate samples per species during summer 2009 on the youngest, fully-expanded, well-lit leaves of each species (cf. Garnier et al., 2001) for methods. Additional leaf material of comparable characteristics was collected for spectral analysis.

The litters were collected in summer for all the species. For *P. pinea* it was easy to select senescent needles directly from the trees, while for the rest of the species it was difficult to collect the needed material, so that dead leaves that dropped after gently shaking the plants were collected. Dead leaves were carefully cleaned, air-dried and stored in the laboratory.

3.2.5. *Traits measurements*

Leaf traits were measured on each species, using standardized procedures (Cornelissen et al., 2003). Specific leaf area (SLAgreen) and leaf dry matter content (LDMCgreen) were calculated as the ratio between leaf area and leaf dry mass, and between leaf dry mass and saturated fresh mass, respectively. Leaf nitrogen content (LNC) and leaf carbon content (LCC) in green leaves were determined with an elemental analyser (model EA 1108; Carlo Erba Instruments, Milan, Italy).

3.2.6. *Decomposition dynamics and decay rate (k)*

We established three approaches with plant material collected: we prepared several litter-bags with the litters collected and placed them (1) directly in the field at the study site (Fogel & Cromack, 1977; Rapp & Leonardi, 1988) and (2) in microcosms in the laboratory under humidity and temperature controlled conditions (Taylor & Parkinson, 1988); and (3) we also studied the spectral characteristics of both green and senescent leaves by using the Near-Infrared Spectrometry (NIRS).

(1) *Decomposition in the field*

Three grams (±0.1 g) of litter of the 4 target species were weighed into litterbags made of nylon of 1 mm mesh (Northern Mesh, Oldham, UK). We prepared 10 litterbags per species and they were placed at the study site from August 2009 until May 2010 (272 days) in order to compare these results with those obtained from the microcosm experience.

(2) *Decomposability in microcosms*

In order to study the effect of litter quality alone on litter potential decomposition or litter decomposability we measured litter decomposition under standardized conditions by keeping all other environmental factors constant, in microcosms, under controlled temperature and humidity conditions. Microcosms, as simplified analogues of natural ecosystems, allow the study of litter decomposition by controlling temperature and humidity, with similar soil conditions and decomposer populations, while maintaining a sufficiently natural situation so that results of laboratory tests may be extrapolated to the field situation with confidence (Taylor & Parkinson, 1988). The microcosm type used for this experiment was described by Taylor & Parkinson (1988). Each microcosm chamber, 15 cm high, was made of a 15 cm diameter polyvinylchloride pipe, fitted with a lid and a sealed bottom. The lid could be opened to allow gas exchange and the plug at the bottom could be removed to drain excess water. A grid, 2 cm above the bottom, divided the chamber into two unequal parts: a usable space of 1.5 L capacity and a drainage compartment of 33 mL. One kilogram of soil, of known water-holding capacity, was placed on the grid. The soil was a 3:1 mixture of mineral soil and surface organic horizon taken from the common garden where the experiment was conducted (Kazakou et al., 2006).

The soil was moistened up to 80 % of field capacity. The microcosms were kept in the dark at 22 ^{o}C throughout the experiment and watered once a week to maintain constant soil moisture during incubation. Two litter samples per species were removed from the microcosm at the end of 1, 2, 4, 6 and 8 weeks. Soil particles were carefully removed from the litter bags and the litter samples were weighed after drying for 48 h at 55 ^{o}C. Prior to the experiment, two litter samples of 2 g from each species were weighed, dried in an oven for 48 h at 55 ^{o}C and weighed again in order to correct the initial mass for the water content of litter.

The percentage of oven-dried litter mass remaining is denoted LMR hereafter. To compare the decomposability of the different species, the single negative exponential model proposed by Olson (1963) was fitted to the LMR of each litter during the course of the experiment: LMR = $100e^{-k_{pot}t}$, where k_{pot} is the potential decomposition rate constant (litter decomposability) over time t in days; and %MR is expressed as a percentage of the original mass.

We also determined carbon and nitrogen concentrations from the beginning of the experiment and, during the whole decomposability process after litter sampling, until the end of the incubation period in microcosms.

(3) *Spectrometry measurements*

Near-infrared reflectance measurements were made with a near infrared reflectance spectrophotometer (NIRSystems 6500) in green leaves. Each sample was packed into a sample cell having a quartz-glass cover. For each sample, one reflectance measurement of monochromatic light was made from 400 to 2500 mm to produce a spectrum with 1050 data points at 2-nm intervals over this range. Reflectance (R) was converted to absorbance [A = log (1/R)]. Data analysis was conducted using the ISI software system (Shenk & Westerhaus, 1991). The samples of green leaves and initial litters of each of the four studied species were dried, ground and scanned as explained above. The aim was to explore if the spectra of the initial litters and of the green leaves contain information on the decomposability of the litter, and especially if the spectral data of the green foliage are related to the decay parameters of the litter.

Calibrations were performed using the partial least squares regression (PLS) method (Shenk & Westerhaus, 1991). This method uses all the spectral information, unlike stepwise regression type methods that only use a small number of wavelengths. PLS regression is recognized as a very powerful tool for developing models from spectroscopic data (Shenk & Westerhaus, 1991). PLS calibrations for k were then carried out using the first and second derivative data from the whole spectrum (400-2500 nm) and from the near-infrared region (1100-2500 nm) of the green leaves spectra. After comparison of the results of various treatments, the calibration that gave the best-fitted k values was selected. From the spectral data of the green leaves and the initial litters, we predicted the k values using equations obtained from previous studies on the same species or on species of the same families as in our study (Perez Harguindeguy et al., 2000).

3.2.7. *Estimating the litter decomposition rate*

The ash content of all the litter samples was predicted by NIRS (Joffre et al., 1992), which enabled the ash-free litter mass remaining (LMR hereafter) to be determined for each. The LMR values for each species were fitted to a mathematical model assuming that the litters were composed of one compartment with different rates of decomposition:

$$LMR = 100e^{-kt} \text{ (Olson, 1963)}$$

Where k is the rate constants over time t for the whole litter and LMR is expressed as a percentage of the original mass and the time in days. This decay rate was calculated for the three types of experimentation (field, microcosm and NIRS).

3.2.8. *Data analyses*

All variables used in this study were normally distributed. We used one-way ANOVA to compare mass loss and decay rates between species under field and microcosms conditions. Differences among groups were determined by using Tukey-test with a $p<0.05$.

We also used a two-way ANOVA with species and treatments (field, microcosms and NIRS) as independent variables and decomposition rate as dependent variable.

To study the potential relationships between the selected variables we chose Pearson correlations, and we developed a PCA in order to show differences between attributes and species by using LDMC, SLA, LNC, LCC and C/N.

We used the SPSS Statistics 17.0 software for Windows (Chicago, IL, USA).

3.3. RESULTS

(a) Differences in decomposition rate and traits between the studied species

One-way ANOVA showed that there were three groups related to decomposability under optimal conditions in microcosms; thus, *O. lanceolata*, as the species with greater decomposability, followed by *H. halimifolium* and the group composed by *P. pinea-C. salvifolius* with similar values of decomposability (Table 3.1). A similar pattern was observed for the species in the field, with the exception that in this case the three groups were composed by *P. pinea*, *C. salvifolius-H. halimifolium* and the third one by *O. lanceolata*.

Species	Mean (microcosms)	Mean (field)
P. pinea	70.9 ± 3.17^{a}	78.3 ± 5.11^{a}
C. salvifolius	69.4 ± 4.14^{a}	71.2 ± 3.82^{b}
H. halimifolium	63.8 ± 5.27^{b}	68.7 ± 8.04^{b}
O. lanceolata	41.2 ± 3.37^{c}	57.2 ± 4.98^{c}

Table 3.1. Litter mass remaining (%) under optimal conditions in microcosms and natural conditions in the field. a-b-c show Tukey test results in order to distinguish between groups related to decomposability in microcosms ($p<0.005$).

Litter mass remaining in the field showed the same behaviour as under optimal conditions in the microcosms, with *P. pinea* as the species with the greater percentage of mass remaining after the 9-month incubation period in the field, and *O. lanceolata*, as the species with higher decomposability among the 4 studied species (Table 3.1).

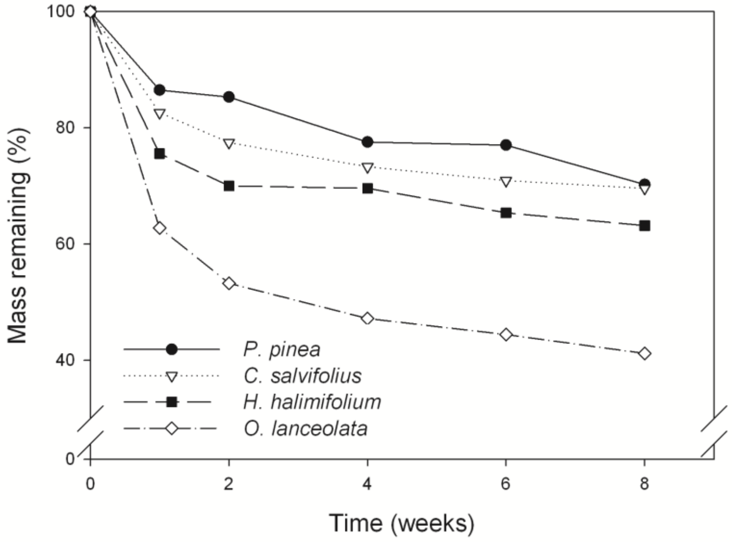

Fig. 3.2. Litter mass remaining percentage (%) of the studied species incubated (a) at the study site and (b) microcosms. Symbols show significant differences ($p<0.05$) in mass remaining percentage between the initial and the final period of incubation (A-B for *P. pinea*; a-b for *C. salvifolius*; 1-2 for *H. halimifolium* and I-II for *O. lanceolata*).

The highest percentage of mass remaining when finished the experiment was shown by *P. pinea*, while the opposite behaviour was shown by the hemiparasitic shrub *O. lanceolata* (Fig. 3.2).

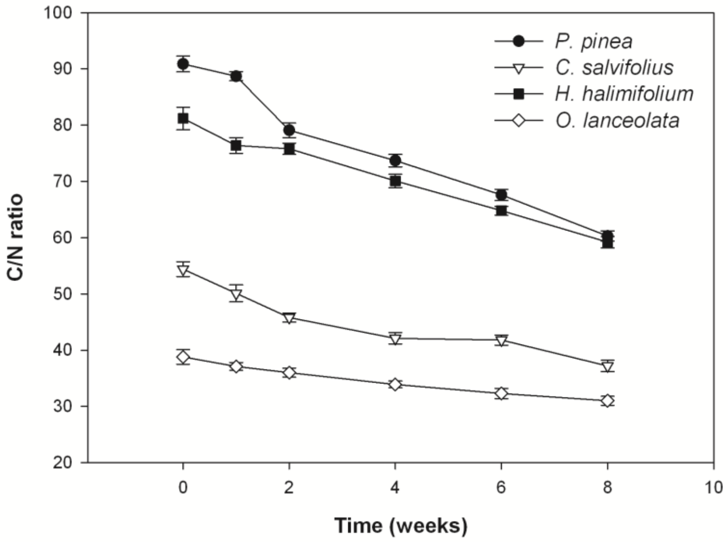

Fig. 3.3. Carbon-to-nitrogen ratio among the 8-week incubation period in microcosms for the 4 target species. Symbols shows significant differences among the 8 weeks after a repeated one-way ANOVA (n = 3, $p<0.05$; A-B-C-D-E for *P. pinea*; I-II-III for *C. salvifolius*; 1-2-3 for *H. halimifolium* and a-b-c for *O. lanceolata*).

H. halimifolium showed intermediate values between the group *P. pinea*-*C. salvifolius* and *O. lanceolata*. Likewise this shrub species showed similar mass remaining values between weeks 2 and 4, with a soft mass loss towards the last week of incubation under microcosm conditions. For *P. pinea* the highest weight loss occurred in the last week of incubation.

Litter decomposition rate (k) of all the species was decreasing gradually among the 8 weeks of incubation in microcosms, with the highest values observed in *O. lanceolata*.

(b) Differences of decomposition rates between field, microcosm incubation and NIRS predictions

Litter decomposability predicted values were significantly related to the observed values after litter incubation under microcosm conditions and in the field (Fig. 3.4).

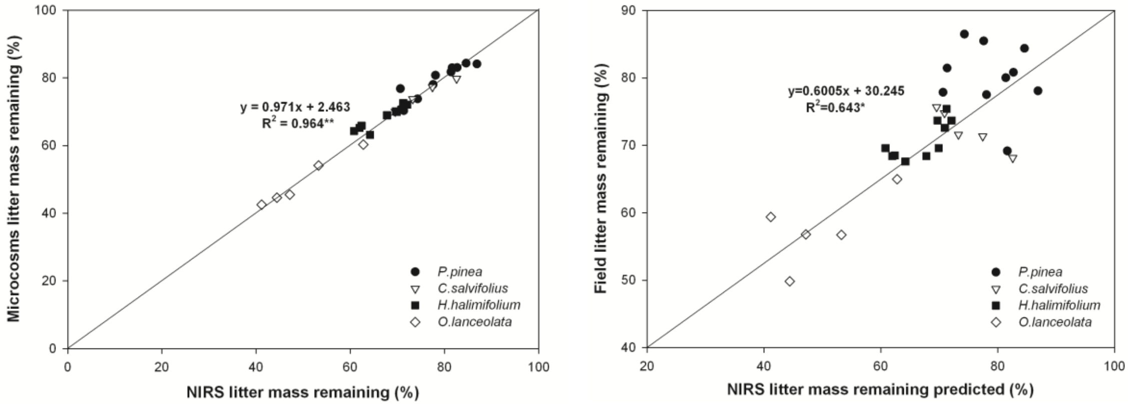

Fig. 3.4. Relationship between litter mass remaining percentages in microcosms vs. predicted by the NIRS (on the left) and incubated in the field vs. the NIRS (on the right).

Two-way ANOVA showed that there were interaction between species and the three approaches of estimating litter decomposability and there were differences between the different species and these species would not behave in the same way under changes in climatic conditions (Table 3.2).

Factors	gl	RMS	F	p
Species	3	0.798	178.004	0.000
Treatment	1	0.376	83.950	0.000
Species x Treatment	3	0.093	20.800	0.000
Error	52	0.004		

Table 3.2. Two-way ANOVA to test differences between species and treatments (microcosms vs. field conditions) related to decay rate.

Comparing decomposition rate in microcosms and in the field we observed that, with the exception of *C. salvifolius*, k was significantly greater in microcosms than in the field, especially *O. lanceolata* (Fig. 3.5).

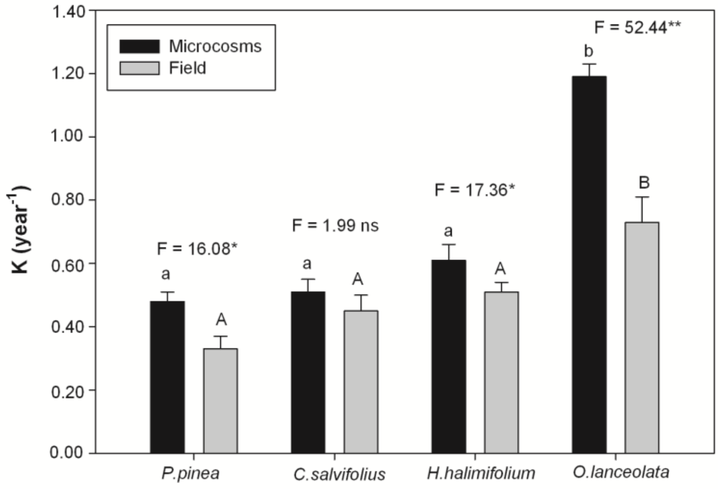

Fig. 3.5. Decay rate (year-1) between litter incubated under microcosms conditions vs. litter incubated in the field for the 4 species (ns: no significant; *: p<0.05; **: p<0.005). Letters show the Tukey-test results to prove if there were differences related to decay rate in each treatment (a-b: microcosms; A-B: field).

(c) Links between leaf, litter traits and species decomposition rate

All the leaf traits were significantly different among the 4 species, with the LDMC, LNC and C/N ratio as the variables the most different among the studied species (Table 3.3).

A significant decrease of carbon and nitrogen concentrations was observed over the 8-week incubation period in microcosms, so that we also observed a significant decrease in the C/N ratio over the 8 weeks (Fig. 3.3).

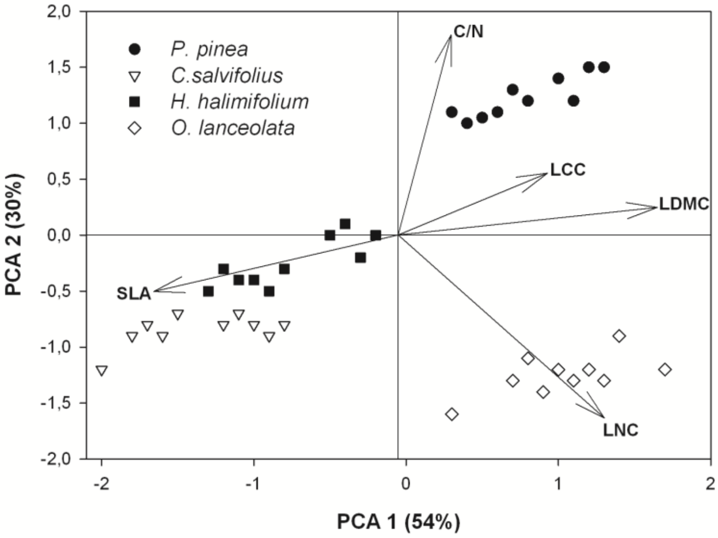

Fig. 3.6. Principal Component Analyses (PCA) for species and variables (C/N; LCC; LDMC; LNC; SLA).

Variable	gl	RMS	F	p
LMR	3	842.425	66.452	0.000
K	3	0.274	57.845	0.000
SLA	3	39.297	49.304	0.000
LSM	3	205881.381	38.904	0.000
LDMC	3	31473.694	740.072	0.000
LNC	3	26.100	1151.421	0.000
LCC	3	2981.484	6.948	0.001
C/N	3	2157.364	315.714	0.000

Table 3.3. One-way ANOVA for different characteristics and functional traits for the 4 species (LMR: litter mass remaining in the field; k: decomposition rate; SLA: specific leaf area; LDMC: leaf dry matter content; LNC: leaf nitrogen content; LCC: leaf carbon content; C/N: carbon-to-nitrogen ratio).

We found a strong relation between decay rate and leaf nitrogen content and the carbon to nitrogen ratio for the 4 species. From Fig. 3.7 we observed the positive relation between the initial litter nitrogen content and the decay rate in the field, with *O.*

lanceolata as the species with higher decomposition rate and high litter initial nitrogen content.

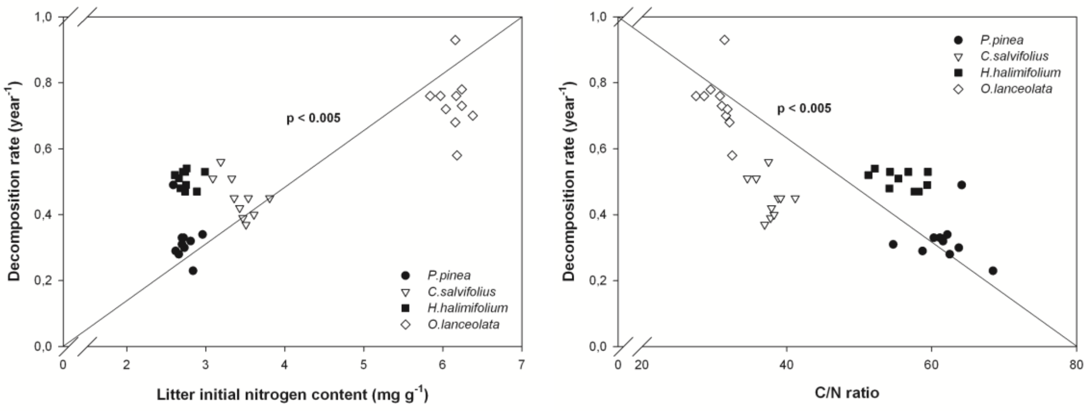

Fig. 3.7. Relationship between decomposition rate (year^{-1}) and litter initial nitrogen content (on the left) and C/N ratio (on the right).

We also observed a negative relationship between decomposability rate and carbon-to-nitrogen ratio (Fig. 3.7).

The first axis of the PCA accounted for nearly 54 % of variation in the data, while the second axis accounted for 30 % (Fig. 3.6). Axis I was determined by two traits: SLA and LDMC, both opposed to each other (Table 3.4) making the difference between *P. pinea*-*O.lanceolata* versus the two *Cistaceae* species. Although LDMC was negatively related with decay rate (the opposite SLA), *O. lanceolata* showed higher LDMC values, but its decomposition rate was the highest among the species. The second axis was determined by LNC and C/N, separating *P. pinea* from the other 3 species, with *O. lanceolata* as the species with a higher LNC. Decomposition rate (k) was positively related to initial nitrogen content and negatively related to initial C/N (Table 3.4).

	SLA	**LDMC**	**LNC**	**K-9 months (field)**
Initial litter composition				
Initial C	0.52**	-0.52**	0.16 ns	0.01ns
Initial N	0.34*	-0.45**	0.78**	0.87**
Initial C/N	-0.16 ns	0.09 ns	-0.82**	-0.81**
Decomposition rate				
K – 9 months (field)	0.32*	-0.87**	0.81**	---

Table 3.4. Pearson coefficients between functional parameters and chemical composition of initial litters and decomposition ate (n = 40, except for decomposition rate, n = 24; ns = non significant; *: $p<0.05$; **: $p<0.005$).

3.4. DISCUSSION

3.4.1. *Differences of decomposition rates between studied species*

P. pinea showed the lowest k values. This tree species possesses high amounts of structural substances as hemicelluloses and lignin, which delay decomposability process (Fioretto et al., 1998). Berg & McClaugerthy (2008) described a range of 223-288 mg g^{-1} of lignin in *P. pinea* needles, while Gallardo & Merino (1993) pointed out 13, 8 mg g^{-1} for *H. halimifolium* and 14, 1 mg g^{-1} for *C. libanotis*. On the other side, several studies show the negative relationship between short and long-term decomposability rate versus lignin content and other polymeric substances with high amounts of carbon and, in general, a high C/N ratio (Meentemeyer, 1978; Palm & Rowland, 1995; Aerts, 1997; Mesquita et al., 1998).

LMR (%) showed by *C. salvifolius* were similar to *P. pinea* and lower than *H. halimifolium*, maybe due to high amounts of cutin on the leaf surface. Kolattukudy (1980) pointed out that cutin, an important component in leaves of Mediterranean plant species, confer impermeability and resistance to microbial degradation and Gallardo & Merino (1993) described greater values in *C. libanotis* (14,3 mg g^{-1}), a species related to *C. salvifolius*, to those observed in *H. halimifolium* (9,2 mg g^{-1}) in relation to cutin content in leaves. This fact would explain decomposability differences among the species.

Results obtained in *O. lanceolata*, being the species that showed the greatest values of k, are in agreement with those found by several authors in other hemiparasitic species (Quested et al., 2003; Quested et al., 2008), who described the important role played by that species in the nutrient cycle of the ecosystem where they appear. Senescent leaves of that hemiparasitic species have high amounts of nutrients which are released to the soil and can be reutilized for the rest of plant species presented in the ecosystem (Quested et al., 2003; Quested et al., 2005; Quested, 2008).

Decomposition process has been described as a two-phase process: a first phase, where the most soluble fraction and a second phase, where the most recalcitrant substances start to decompose little by little (Swift et al., 1979; Melillo et al., 1982; Gallardo & Merino, 1993; Aerts & De Caluwe, 1997). Berg & Staaf (1980) demonstrated the loss of more soluble substances in needles of *P. sylvestris* in the first 100-150 days since litter fall (first phase), while the most recalcitrant substances began to decompose two years later (second phase). This pattern is shown by *P. pinea* during the first 3 months of the incubation period, but we can´t develop any idea of the second phase because our study lasted 9 months. Otherwise, the rest of the species did not show the same pattern with these two phases, but a gradual decomposition process.

3.4.2. *Differences of decomposition rates between field, microcosms incubation and NIRS predictions*

Although Mediterranean ecosystems present high temperatures during almost all the year, a fact that would benefit the decomposition process, the rainfall does not behave in the same way, low during summer in this kind of systems; this pattern in addition with the fact that these ecosystems are high climatic variability systems (Martínez-Alonso et al., 2007) make difficult the establishment of favourable conditions to decomposition process.

K values obtained after litter incubation in the field were not similar with those observed in other studies of Mediterranean species. Schlesinger & Hassey (1981) pointed out k values of -0.18 year^{-1} and -0.19 year^{-1} for two Californian Chaparral species, while Gallardo & Merino (1993), described k values of -0.16 for *H. halimifolium* and -0.14 for *C. libanotis*. The fact that k values observed in the field in the current study are greater than those values observed in other studies (*P. pinea* = -0.33, *C. salvifolius* = -0.45, *H. halimifolium* = -0.51 and *O. lanceolata* = -0.73) may be due to the fact that the incubation period in the field (August 2009-May 2010) matched with a rainfall period 2,2 times greater than mean annual rainfall. As Kruger et al. (1983) described, short-term unpredictable periods of favourable conditions for decomposition can occur in Mediterranean ecosystems.

According to Kruger et al. (1983), optimal temperature and humidity for bacterial growth do not overlap in Mediterranean ecosystems, which could be related to low decomposability rates and mineralization of organic matter present in the soil of Mediterranean ecosystems and just highest in unpredictable and short-term periods. All of this suggests low nutrient availability, which lead the heterogeneity of strategies carried out by different Mediterranean species to get great values of nutrient use efficiency (Gallardo et al., 2009). Nutrient resorption capacity of a leaf is strongly related to nutrient content in green leaves (Aerts, 1996) and defer between sites with different nutrient availability, with a higher resorption capacity for species from nutrient-poor habitats (Wright & Westoby, 2003).

Quested (2003) found nitrogen concentrations in *Bartsia alpina* litter (a hemiparasitic plant species) three times higher to non-parasitic species in the same ecosystem, which favours higher decomposability rates and mineralization and, therefore, higher nutrient availability in such ecosystems. Nitrogen and carbon analyses show higher nitrogen amounts (3 times) in *O. lanceolata* leaves *vs.* the other 3 species, which supports the idea.

Similar values were found for *C. salvifolius* and *H. halimifolium* when comparing them in the model proposed by the NIRS, which can be interpreted as similar values for species belonging to the same family (Cistaceae) and the fact that this species may share some chemical characteristics that lead to a similar decomposability behaviour.

We observed a positive relationship when comparing decomposability predicted by the NIRS with that observed in the field, but greater when comparing predicted decomposability by the NIRS with results obtained from microcosm conditions, due to in field conditions disfavoured climatic conditions impose its presence.

In relation to initial characteristics of the litter NIRS predict a decomposability rate and a percentage of mass remaining similar to that observed after incubation period of litter in microcosms. So the current study shows that NIRS is a good method to predict decay rate by using spectral characteristics of the litter in these Mediterranean species (Joffre et al., 1992; Gillon et al., 1993).

Fortunel et al., (2009) found a similar result in a study which included 20 species, with a good correlation between decay rate of the litter incubated in microcosms and decay rate predicted by the NIRS ($R^2 = 0.85^{***}$).

3.4.3. *Links between leaf, litter traits and species decomposition rates*

C/N ratio along the 8-week period in microcosms, showed a decrease for all the species along the process. This decrease has been described in other ecosystems and for other species (Gallardo & Merino, 1993; Vivanco & Austin, 2008). *P. pinea* showed the highest decrease in the C/N ratio. The fact that *O. lanceolata* possessed a higher nitrogen content in leaves versus the other species would explain the more gradual decrease of this ratio, with significant differences observed just between the initial period and final of incubation, because carbon and nitrogen would be released gradually.

Initial nitrogen content and the C/N were the best predictors of the decay rate, as has been observed in numerous studies (Aber et al., 1990; Aerts et al., 1997).

Initial chemical composition of the litters was strongly related to some of functional traits of green leaves: SLA and LDMC. Plants with high SLA and low LDMC gives nitrogen-rich litter, which decomposes faster; the opposite occurs with species with low SLA and LNC and a high LDMC, which creates nitrogen-poor litters, which decomposes slowly (Cortez et al., 2007). By this reason, both decay rate and released nitrogen are related to green leaves traits through decomposition process.

LDMC represents leaf structural characteristics, which are likely to survive through senescence and influence litter structure and chemistry. LDMC depends on tissue density (Garnier & Laurent, 1994), which is in turn determined by the proportions of mesophyll vs. vessels and fibres (Garnier & Laurent, 1994). So, LDMC can be considered as a best predictor of decomposability.

There are so many studies that pointed out the important correlation between decomposability and functional traits of the plant community, as LDMC, LCC and LNC (Kazakou et al., 2006; Fortunel et al., 2009). Values found in the present study and in other previous studies support the hypothesis of "afterlife" effects of functional traits of green leaves in litter quality and its decomposability (Hobbie & Chapin, 1996; Cornelissen, 1996; Wardle et al., 1998; Quested et al., 2003).

3.5. CONCLUSIONS

The extensive use of *P. pinea* with plantation purposes delays nutrient cycling of ecosystems, due to two fundamental factors: 1) high amounts of needles fall down from the tree and remain in the soil and 2) decay rates of this litters is lower than observed in other species from the same ecosystem.

Our results provide evidence that some leaf traits can be used as good predictors of litter decomposability, with C/N ratio and LNC as the best predictors. *O. lanceolata*, as a hemiparasitic species, has an important role in the nutrient cycling of the studied ecosystem by enhancing litter decomposability of the rest of the species and releasing high amounts of nitrogen kept in the litters. NIRS is a powerful tool to predict decomposability according to initial chemical properties of the leaves in these Mediterranean woody species.

3.6. ACKNOWLEDGEMENTS

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CAPÍTULO 4

Does hemiparasitic plant litter enhance decomposability of other species in a nutrient-poor environment?

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Does hemiparasitic plant litter enhance decomposability of other species in a nutrient-poor environment?

Summary. We investigated the hypothesis that hemiparasites accelerate nutrient cycling in nutrient-poor communities. Hemiparasites take nutrients up from their hosts, thus producing high quality litter that release nutrients to the soil and enhancing litter decomposition processes. If hemiparasites would not do that, these nutrients would remain in host tissues or would decompose slowly as poor quality litter. This hypothesis was tested using species from a Mediterranean community where the root hemiparasite *Osyris lanceolata* is abundant. We set up a field litterbag experiment using litterbags containing litter of the different species alone and *Osyris* litter together other Mediterranean shrub litters. The N content of green leaves, and the N and C content of leaf litter were measured in the hemiparasitic plant species, five commonly co-occurring species and one species belonging to other community. Fresh leaves of the hemiparasite had greater N concentrations than leaves of the rest of the species. This difference was even more marked in litter, with hemiparasite litter containing about 10 mg g^{-1} N, between two to three times as much N as in the litter of commonly co-occurring species. *Osyris* litter decomposed faster and lost between 5 and 25 times more N than that of the rest of the species over the course of the experiment. Mixtures of co-occurring species and hemiparasite litter showed significantly more mass loss than expected, while nutrient release was the same than expected. It is concluded that hemiparasites have the potential to enhance decomposition and nutrient cycling in nutrient-poor environments.

Este capítulo reproduce el siguiente artículo: **Valera-Burgos J, Zunzunegui M, Díaz-Barradas MC (2012)** Does hemiparasitic plant litter enhance decomposability of other species in a nutrient-poor environment? (Submitted to *Annals of Botany*).

4.1. INTRODUCTION

Most of the 90% of net aboveground primary production returns to the soil as litter and constitutes the major resource for soil decomposers (Swift et al., 1979), rather than being consumed by herbivores (Odum, 1960; McNaughton et al., 1989). Plant litter has important effects in structuring plant communities (Grime, 1979; Facelli & Pickett, 1991), as well as being a major determinant of C and nutrient storage and fluxes through ecosystems (Chapin, 1993; Wardle et al., 1997). Plant litter therefore has the potential to be an important factor mediating plant-plant interactions, and may in some cases play a more profound role in structuring communities than it does in nutrient cycles (Facelli & Pickett, 1991).

It has been described that several plants belonging to nutrient-poor environments have low nutrient concentrations, mainly nitrogen, which imposes low nutrient losses from the plant because of high resorption processes (Berendse & Jonasson, 1992; Eckstein et al., 1999; Aerts & Chapin, 2000). These nutrient-poor communities often possess low leaf nutrient concentrations and plants growing under these conditions tend to conserve more resources and maintain their leaves for a long time, which affect negatively the nutrient cycling (Heal et al., 1981; Hobbie, 1992; Cornelissen, 1996; Cornelissen et al., 1999).

However there are many plants, hemiparasites, in this type of ecosystems that obtain their nutrients, carbon and water from a different plant (host), although they are able to photosynthesise (Press, 1989). These plants have generally high concentrations of nutrients in their leaves, which imply high quality litter than litter from surrounding species (Killingbeck, 1993; Hooper & Vitousek, 1997; Quested et al., 2003). Hemiparasites are frequently abundant in nutrient-poor habitats and can account for a significant proportion of the total nutrients entering the decomposition subsystem (Quested et al., 2005). Hemiparasites thus have the potential to influence ecosystem properties in a very different way from other species (Press, 1998; Phoenix & Press, 2005).

Most of decomposition studies try to determine decomposition of litter for one species by studying litter decomposition of this species alone, but litter of a species seldom decomposes in monoculture in natural systems, so it is more and more necessary the study of decomposition of litter implying different types of litters, at least the most representative functional groups. This is the main reason why is so interesting the study of decomposition of mixing litters where at least one of the species is hemiparasite or N-fixer. Nutrient-rich litter of a hemiparasite, for example, might impact on ecosystem function via interaction with other species´ litters. Mixing litters is a good way to show whether different species interact during decomposition, in terms of alteration of decomposition rates, nutrient loss and timing of nutrient release in comparison with the same litters decomposing (Gustafson, 1943; Chapman et al., 1988; Fyles & Fyles, 1993; Wardle et al., 1997; Salamanca et al., 1998; Hoorens et al., 2002, 2003). Such interactions can be substantial and may either enhance or retard overall decomposition and nutrient release.

The hypothesis that nutrient-rich parasite litter in nutrient-poor environments, which is a high quality litter, has the ability to increase decomposition rates of the neighbour species and increase nutrient release, mainly nitrogen, from their litters has been supported by microcosm studies (Quested et al., 2002)., but it is unknown whether this effect is important under natural conditions.

According to the mass ratio hypothesis (Grime, 1998), species effects are proportional to their contribution to the community, such that emergent effects are not important. However, it is well established that litter of different species interacts during decomposition, often giving decomposition rates and nutrient losses that are substantially different from those expected based on single species values (Gustafson, 1943; McTiernan et al., 1997; Wardle et al., 1997; Finzi & Canham, 1998; Wardle et al., 2003; Gartner & Cardon, 2004).

The principal aims of the present study are to assess the possible positive effects of hemiparasite plant litter on the nutrient cycling and nutrient release in a nutrient-poor environment related and if there are differences in decomposition rates attending to different vegetation areas at the study site. Hemiparasite litter alone and in combination with that of other species is investigated. We focus on nitrogen because it is the nutrient that most commonly limits plant growth in nutrient-poor environments. Specifically we consider the following questions:

1. Does hemiparasite litter interact in term of mass loss and N release with that of other species?

2. Does hemiparasite litter decompose more rapidly than that of co-occurring species?

4.2. MATERIALS AND METHODS

4.2.1. *Study site*

The present study was carried out in El Asperillo (34º0´N 6º36´W) at Doñana National Park (south-western Spain). The climate is of Mediterranean sub-humid type with an oceanic influence and mild temperatures. Mean annual temperature is 16.8ºC. Average annual rainfall is 550 mm, although it is subject to high variability, ranging from 170 to 1028 mm during the last 25 years (Fedriani & Delibes, 2009). Precipitation is concentrated from October to March (García Novo, 1997). Vegetation consists of native dune shrub including *Halimium halimifolium*, *Halimium calycinum, Cistus salvifolius*, *Rosmarinus officinalis*, *Lavadula stoechas*, *Cytisus grandiflorus*, *Stauracanthus genistoides*, *Corema album*, *Juniperus oxycedrus* and *J. phoenicea*, together with *Pinus pinea*. This ecosystem was originally composed by *J. phoenicea* but during the twentieth century *P. pinea* was introduced with plantation purposes.

4.2.2. *Target species*

Litter species were chosen on the basis of being widespread and common in the study area and having litter with contrasting physical and chemical properties: the tree *P. pinea*, *J. phoenicea*, the scrubs *C. salvifolius* and *H. halimifolium* (both belonging to the Cistaceae), *Osyris lanceolata* (a hemiparasitic shrub species), and *R. officinalis*. We also used *Pistacia lentiscus* litter as “standard litter” in the experiment, because this species does not belong to the study site.

4.2.3. *Collection of material*

Green leaf material and undecomposed leaf litter was collected in July 2010, at the end of the growth season for Mediterranean species. Only leaves were collected for all the species, as the majority of litter shed from these species consists of leaves, and woody litter has very different decomposition dynamics and spatial arrangement. Care was taken to collect freshly senesced, undecomposed litter. Litter was air-dried in the laboratory of a minimum of 5 days and stored in the laboratory.

4.2.4. *Field litterbag experiment*

We chose two different zones at the study site, one of them with *J. phoenicea* and *R. officinalis* as dominant vegetation (fragments of the ancient vegetation at the study site in a difficult-access zone) and another one with *P. pinea* as the best represented plant species at the selected zone. We set up a litter-bag experiment with the collected material. For this purpose we prepared 10 individual nylon litter-bags of 1 mm mesh for each species (10 x 15 cm) and 10 mixed nylon litter-bags of 1 mm mesh for each of the different target species plus *Osyris* litter. Litter-bags were deposited in each zone (5 litterbags of each type per zone) and were picked up one year later. We also prepared litter-bags containing *P. lentiscus* litter (10 litterbags with *P. lentiscus* litter alone plus 10 litterbags with *P. lentiscus* + *Osyris* litter), as we selected this species as standard species in our study, due to its absence at the study site and we also left 5 litter-bags at the site from where we collected *P. lentiscus* plant material.

4.2.5. *Decay rate*

The annual decomposition constant *k* (Olson, 1963) for the exponential relationship was calculated using the equation:

$$\ln (x_1/x_0) = kt,$$

Where x_0 is the original amount of litter, x_1 is the amount of litter remaining after time *t*, and *t* is the time (in years). The *k* values represent a 1-year period.

4.2.6. *Initial litter quality*

A subsample of litter from each species was dried for 48 h at 65ºC for the determination of initial litter quality. This subsample was analyzed for carbon (C) and nitrogen (N) using an elemental analyzer (Carlo Erba, Milano, Italy).

4.2.7. *Response variables*

Observed (*O*) and expected (*E*) mass remaining of litter mixtures was expressed as a fraction of initial litter mass. Expected mass remaining was defined as the mean mass remaining of the component litter species decomposing in isolation weighted by their relative initial mass in the mixture (Gartner & Cardon, 2004; Lecerf et al., 2007). The difference between *O* and *E* indicates deviation from additivity.

4.2.8. *Data analysis*

Data were tested for normality and equality of variance and, when necessary, where $\log_e$-transformed before analysis. Mass remaining, k, and percent C and N needed to be $\log_e$-transformed to meet assumptions of normality. One-way analysis of variance (ANOVA) was carried out in order to compare mass remaining, k, and percent element remaining in litter after 12 months, followed by Tukey tests. All analyses were performed using SPSS 18.0 for Windows.

4.3. RESULTS

4.3.1. *Litter chemistry*

As expected, the N concentration in fresh leaves of the hemiparasitic species *Osyris lanceolata* was about two to three times higher (about 11.52 % N) in comparison with commonly co-occurring species and the standard species. This pattern was even more marked in leaf litter, with the hemiparasite litter containing between 9.45% and 10.37% from juniper and pinewood respectively, and co-occurring species containing between 2.36% and 4.78% N. *P.lentiscus* litter also showed low N concentrations. This difference is also reflected in the C:N ratio, with the values for co-occurring species being even double than those of the hemiparasitic species (Fig. 4.1). There were no differences in N content when comparing species´ litter alone *vs.* mixed litterbags nor in the pinewood neither in the juniper wood (see Fig. 4.2).

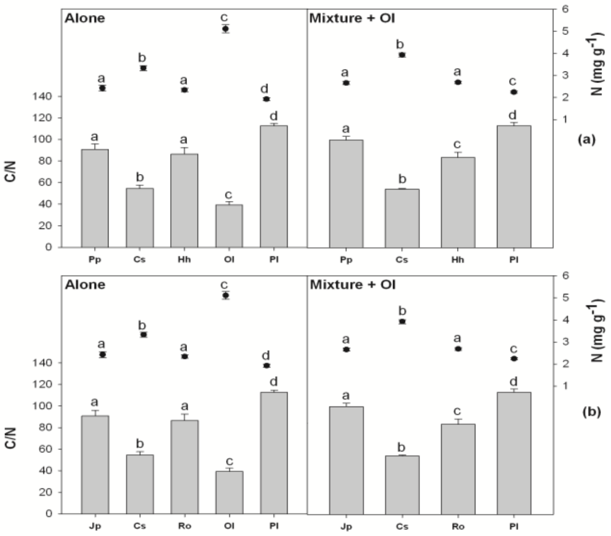

Fig. 4.1. Leaf Nitrogen Content and C : N ratio for the target species in (a) pinewood and (b) juniper. Letters indicate significant differences (Tukey test, P<0.05). Species codes as in Fig. 4.1. Pp: *Pinus pinea*; Jp: *Juniperus phoenicea*; Cs: *Cistus salvifolius*; Hh: *Halimium halimifolium*; Ol: *Osyris lanceolata*; Pl: *Pistacia lentiscus*; Ro: *Rosmarinus officinalis*.

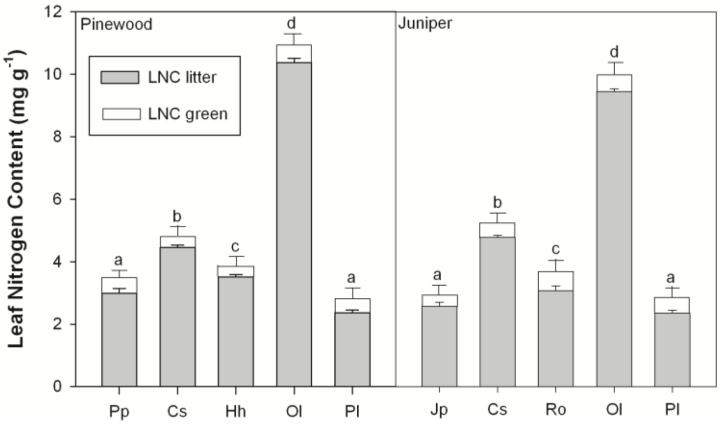

Fig. 4.2. Leaf Nitrogen content in the target species. Pp: *Pinus pinea*; Cs: *Cistus salvifolius*; Hh: *Halimium halimifolium*; Ol: *Osyris lanceolata*; Jp: *Juniperus phoenicea*; Ro: *Rosmarinus officinalis*; Pl: Pistacia lentiscus. LNC was analysed with one-way ANOVA (F = 57.8, P<0.0005). Letters indicate significant differences (Tukey test, P<0.05). Pp: *Pinus pinea*; Jp: *Juniperus phoenicea*; Cs: *Cistus salvifolius*; Hh: *Halimium halimifolium*; Ol: *Osyris lanceolata*; Pl: *Pistacia lentiscus*; Ro: *Rosmarinus officinalis*.

4.3.2. *Field litterbag experiment*

Osyris lanceolata had lost substantially and significantly more mass at all combinations than litter of the co-occurring species (see Fig. 4.3) and lost aprox. 43 % of its initial nitrogen content over the course of the experiment (F = 175.45, P<0.001). In contrast, *Pistacia lentiscus* lost only 7.6% of its initial N content. When the initially very different N concentrations are taken into account (Table 4.1), *Osyris* litter lost 25 times more nitrogen than *P. lentiscus* over the course of the experiment.

	Mature leaf N(%)	**Litter N (%)**	**Litter C:N ratio**
Pinewood			
Pinus pinea	2.99 (0.45)	2.77 (0.15)	90.74 (7.23)
Cistus salvifolius	4.45 (0.10)	3.84 (0.37)	54.54 (5.25)
Halimium halimifolium	3.51 (0.30)	2.67 (0.30)	84.08 (6.58)
Osyris lanceolata	10.37 (0.50)	5.94 (0.16)	39.29 (1.28)
Juniper			
Juniperus phoenicea	2.58 (0.45)	2.13 (0.03)	92.31 (8.05)
Cistus salvifolius	4.54 (0.20)	3.87 (0.25)	56.48 (1.56)
Rosmarinus officinalis	3.07 (0.45)	2.58 (0.13)	108.46 (6.57)
Osyris lanceolata	9.89 (0.27)	6.23 (0.18)	42.05 (2.25)
Standard species			
Pistacia lentiscus	2.36 (0.10)	2.18 (0.06)	112.46 (0.95)

Table 4.1. N concentration of mature leaves and N concentration/C:N ratio of leaf litter of the different species taken into account in the current study. SEs are given *in parentheses* after the mean (n=5).

There was evidence that mass loss in litter mixtures differed significantly from what would be expected on the basis of single species decomposition when *Osyris* litter was presented. Decomposition constants significantly differed among litter types within sites (Fig. 4.4), demonstrating direct litter quality effects based on different plant species composition, with higher decomposition constants when *Osyris* litter was presented. Over the course of the experiment (360 days) litter of the hemiparasite lost significantly more mass than any other species (F = 385.5, P<0.0005) (Fig. 4.3).

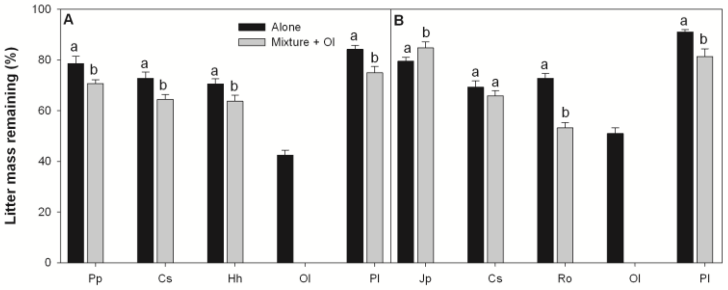

Fig. 4.3. Litter mass remaining (%) in (A) Pinewood and (B) Juniper. Letters indicate significant differences (Tukey test, P<0.05). Species codes as in Fig. 4.1. Pp: *Pinus pinea*; Jp: *Juniperus phoenicea*; Cs: *Cistus salvifolius*; Hh: *Halimium halimifolium*; Ol: *Osyris lanceolata*; Pl: *Pistacia lentiscus*; Ro: *Rosmarinus officinalis*.

Litter of *P. lentiscus* decomposed significantly slower when decomposing at the study site (K = 0.19) compared when decomposed at the site of origin (K = 0.25), but decomposition rate was increased when decomposing with *O. lanceolata* litter (K = 0.29).

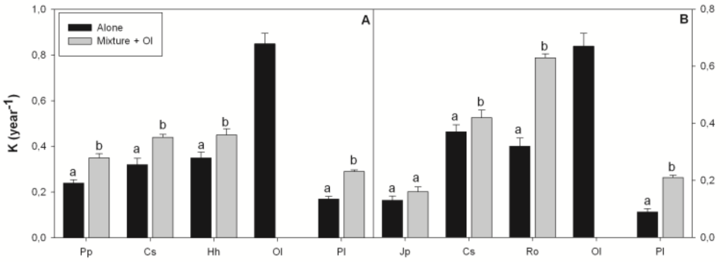

Fig. 4.4. Litter decay rate in (A) Pinewood and (B) Juniper. Letters indicate significant differences (Tukey test, P<0.05). Species codes as in Fig. 4.1. Pp: *Pinus pinea*; Jp: *Juniperus phoenicea*; Cs: *Cistus salvifolius*; Hh: *Halimium halimifolium*; Ol: *Osyris lanceolata*; Pl: *Pistacia lentiscus*; Ro: *Rosmarinus officinalis*.

The bivariate relationship between observed and expected litter mass remaining revealed frequent detectable deviations of individual litter mixtures from the 1 : 1 line, thus providing evidence of nonadditive breakdown (Fig. 4.5). Only 17.4 % of litter mixtures decomposed additively (i.e., observed and expected litter mass remaining differed by <0.01). The other litter mixtures decomposed either faster (73.9 %) than expected as a result of synergistic responses (points below the 1 : 1 line on fig. 4.5) or slower (8.7 %) than expected as a result of antagonistic responses (points above the 1 : 1 line on Fig. 4.5) (Fig. 4.5).

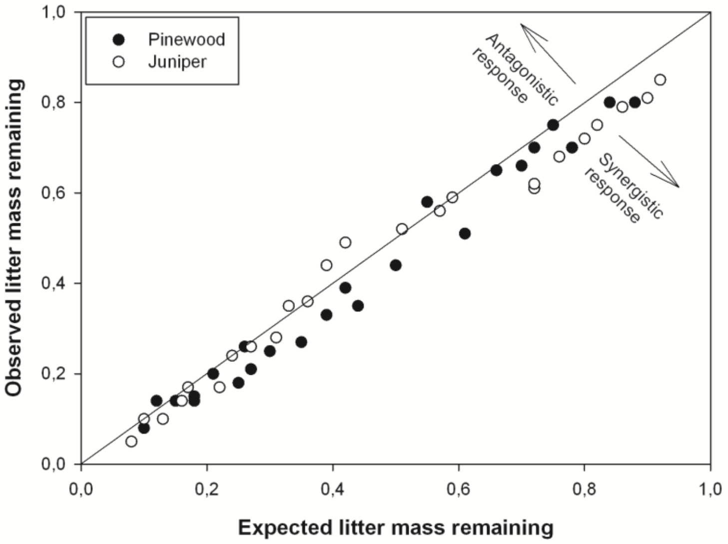

Fig. 4.5. Observed vs. expected litter mass remaining of the 50 individual mixtures of litter species incubated at the study site. The 1 : 1 axis (solid line) represents additivity. Negative deviations from additivity (below the 1 : 1 line) indicate synergistic response (acceleration of litter breakdown), and positive deviations (above the 1 : 1 line) indicate antagonistic responses (deceleration of litter breakdown) of litter mixtures.

4.4. DISCUSSION

4.4.1. *Quantity and timing of nutrient release from litter*

In answer to our first question, *Osyris* litter interacted with litter of co-occurring species in terms of increasing decomposition constants and nutrient release; the majority of the nitrogen was released from hemiparasite litter, and surrounding plants are likely to take it up, supporting the idea that hemiparasitic plants have a substantial direct impact on the nutrient cycling of ecosystem where they appear, by increasing nutrient availability to other plants (Quested et al., 2003, 2005). Our second question was also answered in the affirmative; litter of the hemiparasite decomposed faster and lost up to 25 times more nitrogen than litter of the studied species over the course of the experiment under field conditions. This second assumption was reported by several authors who found high N concentrations in the leaves of parasitic angiosperms (Lamont, 1983; Michelsen et al., 1998) and in hemiparasite litter (Quested et al., 2002), which would explain the positive effects of hemiparasite litter in the nutrient cycling of nutrient-poor environments.

There was evidence that mass loss in litter mixtures differed significantly from what would be expected on the basis of single species decomposition. Observed and calculated expected N contents, however, differed significantly just for the hemiparasitic species. In this case, we found that *Osyris* litter released much more N when decomposing in litter mixtures than when decomposing alone, which has been described for *Bartsia alpina* by Quested et al. (2002). They described the behavior of this hemiparasitic species belonging to a nutrient-poor ecosystem, which released more N when decomposing in litter mixtures. These authors also pointed out that perennial parasites N concentration in leaf material was lower after senescence when compared with annual parasites, but we found that the perennial hemiparasite *O. lanceolata* contained high amounts of N in leaf material after senescence.

Since *Osyris lanceolata* litter contains up to 3 times more N, and has a C:N ratio which is between 25% and 45% of that of the co-occurring species, it is not surprising that *Osyris* litter decomposed much more quickly than the co-occurring species litters, given that N concentration is positively, and C:N ratio negatively, correlated with decomposition rates in the early stages of decomposition (Taylor et al., 1989; Cotrufo et al., 1995; Aerts & de Caluwe, 1997). Total N losses over the course of the experiment from *Osyris* litter decomposing alone were between 3 and 25 times higher than for any of the studied species. The link between nutrient content and nutrient losses has been demonstrated in several studies (Berg, 1986). The production of such nutrient-rich, quickly decomposing litter may well have the potential to affect nutrient cycling in a nutrient-poor environment.

4.4.2. *Affinity relationships between litter and microsite*

Although the idea of specificity between plant and soil biota affecting litter decomposition is appealing, it has been difficult to detect in natural ecosystems (Vivanco & Austin, 2008). High quality litter may decompose faster in a fertile site due to interactions between soil resource availability and litter resource availability, independent of its origin (e.g. priming effect), but true affinity effects occur only when a lower quality litter decomposes faster in its own low fertility environment, demonstrated here in the case of all species when decomposing in the mixture with *O. lanceolata* litter, even for the *exogenous* litter belonging to *P. lentiscus*.

Some studies have provided indirect evidence that decomposer communities may be specialized to a characteristic litter type of a given ecosystem. For example, in a regional-scale study by Gholz et al. (2000), litter from broadleaved trees decomposed faster than pine litter in a broadleaved forest. We observed that for *P. lentiscus*, which represented the standard litter in the current study, there were differences in decomposition of its litter when compared both sites (original vs. study site), so we can affirm the assumption exposed above. However, *P. lentiscus* litter in the mixture with *O. lanceolata* litter decomposed even faster than *P. lentiscus* litter at the original place of plant material collection.

4.4.3. *Implications for ecosystems*

Several studies have shown the importance of hemiparasite litter enhancing availability of nutrients to soil organisms and other plants in some nutrient-poor ecosystems, such as sub-arctic heath communities (Quested et al., 2003, 2005). Although hemiparasitic species produce N-rich, fast decomposing litter, their effects on ecosystem function are very different. Hemiparasites take nutrients from their host plants, and transform a proportion of them into nutrient-rich leaf litter, thus releasing much more nitrogen and other nutrients to the soil, being further available for other organisms. It is likely that long-lived, perennial hemiparasites increase the resource patchiness across sites where they occur. Increased local patchiness could enhance local plant and decomposer community biodiversity (Sulkava & Huhta, 1998; Anderson et al., 2004).

4.5. CONCLUSIONS

The current study highlights the importance of root hemiparasitic species as a key factor of nutrient cycling in nutrient-poor environments by enhancing litter decomposition processes (Press, 1998). It is the first evidence of the important role of the hemiparasitic species *O. lanceolata* for the nutrient cycling in a Mediterranean sand dune ecosystem. However, this first assumption needs further experimentation about litter mixtures and the effects on plant growth and whether the enhancement of litter decomposition processes depends on parasite abundances and litter inputs on plant communities.

4.6. ACKNOWLEDGEMENTS

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CAPÍTULO 5

Seedling competition between *Pinus pinea* and two Mediterranean shrub species (*Cistus salvifolius* and *Pistacia lentiscus*) under different nutrient conditions

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Seedling competition between *Pinus pinea* and two Mediterranean shrub species (*Cistus salvifolius* and *Pistacia lentiscus*) under different nutrient conditions

Summary. The competitive ability among the tree species *Pinus pinea* L. *v.* the Mediterranean shrub species *Cistus salvifolius* L. and *Pistacia lentiscus* L. under different nutrient and competition treatments were evaluated. In particular, we explored the hypothesis that the studied species would show different responses attending to a nutrient-poor or rich- environment and whether the plant is growing alone *v.* intra- or interspecific competition. For this purpose, we selected seedlings of the target species and we set up a 1-year factorial experiment with different levels of nutrients and competition combinations. We measured ecophysiological (photosynthesis and chlorophyll fluorescence) and morphological (biomass) variables. *P. pinea* showed significant differences in relation to photosynthetic rate, with the most significant difference during the summer period, while no significant differences were found for the other species. In general, plants showed similar values of F_v/F_m, with the exception of *P. pinea* in summer in the interspecific competition with *P. lentiscus*. As we measured biomass, we observed an intense belowground competition (as a result from the competition index used), mainly between *P. pinea* and *P. lentiscus* and in the case of intraspecific competition for *C. salvifolius*. Our results highlight the idea that *P. pinea* is a well-adapted species to nutrient-poor soils.

Este capítulo reproduce el siguiente artículo: **Valera-Burgos J, Zunzunegui M, Díaz-Barradas MC, Oppo F (2012)** Seedling competition between *Pinus pinea* and two Mediterranean shrub species (*Cistus salvifolius* and *Pistacia lentiscus*) under different nutrient conditions (Submitted to *Physiologia Plantarum*).

5.1. INTRODUCTION

The Stone pine (*Pinus pinea L.*) is a coniferous tree species, which has been widely used for afforestation purposes in the Iberian Peninsula during the XIX and XX centuries (Iovieno et al. 2010) in order to fix sand dunes mainly. Many Mediterranean ecosystems have been modified due to these plantations and these modifications affect not only the diversity and distribution of plant species but also plant interactions in nutrient-poor soils, such as sand dune ecosystems of the south-west of Spain.

All natural soils exhibit heterogeneity in nutrient supply (Jackson & Caldwell 1989; Caldwell et al. 1991; Caldwell & Percy 1994; Richard et al. 2000), so that plant-available nutrients in soil are usually non-uniformly distributed (Robertson et al. 1993; Pickett et al. 2000; Hodge 2004). It is worth of giving importance to ecosystems with limiting soil resources, because they are more suitable to develop studies on belowground competition as most plant interactions is believed to occur belowground (Casper & Jackson 1997; Weiner et al. 1997).

Casper & Jackson (1997) described the influence of belowground interactions and competitive ability on the distribution and abundance of plants in nutrient-poor habitats, where nutrients and water are typically limiting plant growth (Boorman 1982; Berendse 1990; Weigelt et al. 2000). Nutrient-poor soils, like Mediterranean sand dunes, impose limitations on plant growth and development, as indicated by studies of plant responses to nutrient additions (Ingestad 1982; Kruger 1987; Witkowski et al. 1990), litter decomposition processes (Mitchell et al. 1986), and N allocation patterns (Stock et al. 1987). In order to put up with such limitations, plants exhibit traits that allow them to survive under these adverse conditions, for example high allocation to roots, low allocation to stems, nutrient conservation via low tissue N content and/or long-lived leaves (Smith et al. 1999).

Previous studies have demonstrated that different species exhibited a wide range of morphological as well as physiological root plasticity (Einsmann et al. 1999; Hodge 2004; Forde & Walch-Liu 2009). It has been described that many plants modify some traits, such as root-to-shoot ratios in response to environmental limitations as shading or low nutrient availability for example (Reynolds & Dantonio 1996; Aphalo et al. 1999).

Rajaniemi (2002) pointed out that root system size is positively associated with belowground competitive ability.

Mediterranean vegetation is dominated by evergreen sclerophyllous trees and shrubs (Specht 1969; Orshan 1983) with an herbaceous or shrubby understory. The presence of dense, evergreen shrubland stands suggests that competition may be strong in Mediterranean-type ecosystems (Vilà & Sardans, 1999). Moreover, differences in rainfall among consecutive years, summer drought and differential availability of some nutrients in the soil are three main characteristics of Mediterranean ecosystems (Di Castri et al. 1988; Kruger et al. 1983). There are several authors that maintain the idea of high intense competition between plants in soils with limited resources (Grubb 1985; Tilman 1987), while others suggest that competition would be less intense under intense abiotic stress conditions (Grime 1973; Huston & Smith 1979).

Shrublands dominate much of the Mediterranean region, have extended in recent decades and are projected to further expand in many areas, including the southern and central Iberian Peninsula as a result of land-use change, fire, and drought (Vázquez & Moreno 2001; Mouillot et al. 2002; Acacio et al. 2009). Several species of *Cistus* are among the dominant species in the siliceous, relatively mesic soils of the western Mediterranean. *Pistacia lentiscus* is occurring in a wide variety of habitats, from open communities in garrigues to close communities in more mesic and shaded sites (Díaz-Barradas & Correia 1999). Pine woodlands have been planted in many areas of coastal on Mediterranean mountains and might establish a strong competition for underground resources with shrublands (Granados Corona et al. 1983; Fernández et al. 2006).

The present study was carried out in order to examine seedling competition in an experimental design of nutrient treatments. We evaluated the competitive ability of *P. pinea* compared to the Mediterranean shrub species *C. salvifolius* and *P. lentiscus* by exploring several physiological and morphological traits. In particular, we tested the hypothesis that the differences in phenotypic plasticity of physiological, morphological and allocation traits explain the differences in competitiveness between the Stone pine and scrubland vegetation and that this competition is less intense in nutrient-poor environments.

5.2. MATERIALS AND METHODS

5.2.1. *Plant material and experimental design*

Seedling plants of two native Mediterranean plant species [*Cistus salvifolius* L. (Cistaceae) and *Pistacia lentiscus* L. (Anacardiaceae)] were grown in direct competition with *Pinus pinea* L. (Pinaceae) seedlings. Seedlings ages were ten, eight and seven months for *P. pinea*, *P. lentiscus* and *C. salvifolius*, respectively and they were provided by a nursery garden belonging to the Andalusian government located in Higuera de la Sierra (province of Huelva, SW Spain).

The experiment was carried out in a semi-open greenhouse belonging to the University of Seville, located on the top of the Faculty of Biology, with a transparent plastic roof to keep off rainwater and without walls to ensure light intensity, temperature and humidity inside the greenhouse was similar to the external environment. All plants were watered twice a week during summer and once a week the rest of the year before sunrise in order to avoid drought effects. We used 20 x 19 cm pots in the current study. The soil was a 3:1 mixture of sand and organic substrate in order to reproduce the same conditions of the original habitats of the target species.

5.2.2. *Competition experiment*

This experiment took place under high-light conditions as described above. Plants of the three species were planted in an additive design where a single plant individual served as control. There were intra- and interspecific competition treatments (i.e. *P. pinea vs.* one shrub species) always consisting of two plant individuals, with 10 replicates. There were two levels of nutrients: one control with plants only watered and one treatment with plants fertilized weekly with 100 mL of a 2-fold modified Hoagland solution [$NH_4(H_2PO_4)$, KNO_3, $Ca(NO_3)*4H_2O$, $MgSO_4*7H_2O$, H_3BO_3, $MnCl_2*4H_2O$, $ZnSO_47H_2O$, $CuSO_4*5H_2O$, $H_2MoO_4*H_2O$]. We have an N = 160 (10 replicates per treatment x 8 levels of competition x 2 levels of nutrient). The experiment lasted 1 year.

5.2.3. *Morphological and allocation measurements and nutrient analysis*

Throughout the competition experiment plant height was recorded at regular intervals. At the end of the experiment all plants were harvested and divided into aerial part and roots. In the case of *P. pinea* needles were measured instead of leaves. Leaf fresh weight was determined immediately. Leaf area was measured with Midebmp (Ordiales-Plaza, 2000). Leaf area and dry weights were used to calculate the following parameters: specific leaf area (SLA; leaf area per unit dry weight), leaf area ratio (LAR; the amount of leaf area per unit total plant mass), leaf weight ratio (LWR; leaf mass per total plant mass), stem weight ratio (SWR; stem mass per total plant mass), root weight ratio (RWR; root mass per total plant mass) and root/shoot ratio (root dry mass per shoot dry mass).

5.2.4. *Gas exchange measurements*

Leaf gas-exchange measurements were performed by means of an open system compact gas exchange analyzer (LCi-portable photosynthesis, ADC, UK), which calculates net photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gs). Water use efficiency (WUE) was calculated as the ratio of CO_2 uptake per H_2O transpired or A/E. Measurements were conducted on leaves from terminal shoots and all results were expressed on an area basis, calculated using Midebmp (Ordiales-Plaza 2000), from scanned images of the leaves from each measured shoot. Three measurements were conducted per plant and the mean value per plant was calculated for statistical analysis.

Photosynthesis measurements were always carried out on leaves of the same ontogenic status, since ontogenic status can influence the capacity for photosynthetic acclimation (Frak et al. 2001).

5.2.5. *Chlorophyll fluorescence a measurements*

Photochemical efficiency of chlorophyll a (photosystem II) was measured using a portable modulated fluorometer (MINI-PAM, Walz Effeltrich, Germany) following the modulated pulse-amplitude technique (Bilger et al. 1995). Calculations followed Schreiber et al. (1995). Maximum photochemical efficiency of chlorophyll a (F_v/F_m) was measured on leaves previously adapted to darkness for 20 min (three twigs per x individuals). The average value of the three F_v/F_m measurements was calculated for each individual (n = 8). Photochemical efficiency of chlorophyll a was measured at solar midday on sunny days, the time of maximum environmental stress. Effective photochemical efficiency of PSII ($\Delta F/F_m'$) was determined at 400 µmol m^{-2} s^{-1} and 1500 µmol m^{-2} s^{-1}.

5.2.6. *Harvest and calculation*

At the end of the experiment, which lasted from March 2009 until March 2010, all plants from all treatments were harvested and taken to the laboratory. Roots were then washed free of sediments and divided into shoots-leaves and roots and their fresh weights were determined. Finally, samples were oven dried at 80 ^{o} C for 48 h, and then their dry weights (g per plant) recorded.

5.2.7. *Data analyses*

Normal distribution of data was tested with Kolmogorov-Smirnov test. One-way multivariate analyses of variance (MANOVA) were carried out to determine the effects of treatment (pattern of nutrient supply and neighborhood) on yield. Separate analyses were conducted for each species as target, with treatment as the independent variable, and above- and belowground biomass as dependent variables. This was followed by the corresponding univariate analyses of variance (ANOVA). A univariate ANOVA was used to analyze the effect of treatment on total biomass. Significant differences between means were determined using Bonferroni multiple-means comparison tests at $P<0.05$. All statistical analyses were carried out by using the SPSS Statistics 18.0 software for Windows (Chicago, IL, USA).

To investigate the effect of treatment on the intensity of competition in both treatments, mean competitive intensity (CI) was calculated for the target plants. As an index of absolute competitive intensity, we used the mean reduction in yield in the presence of a competitor compared with mean yield in the absence of a competitor. An equation of Kadmon (1995) was adapted to calculate CI (in g) experienced by plants grown with a competitor (intraspecific competitor):

$$CI = [2Yi,sp1 - Yc,sp1]/2 \qquad \text{(equation 1)}$$

CI experienced by both species when grown with an interspecific competitor:

$$CI = [(Yi,sp1 + Yi,sp2) - Yc,sp1]/2, \qquad \text{(equation 2)}$$

Where Y = yield, i denotes plants grown alone, c denotes plants grown in competition (and therefore always two plants), sp1 is the target plant species and sp2 is the competitor species. Biomass data from each replicate target plant of a given species, in each species combination, were matched by nutrient distribution with the mean biomass for plants of the same species grown alone. For the interspecific competition treatment, CI was calculated for each species, as it was possible to separate their belowground parts.

If plants grown together are unaffected by competition, CI is zero. Positive CI values indicate that plants are competing, and negative values indicate that they benefit from each other´s presence. To determine whether CI differed significantly from zero, one-sample t-tests were carried out. The effects of treatment (pattern of nutrient supply and neighbor identity) on CI were investigated using one-way ANOVA with treatment as independent variable and CI as dependent variable. Significant differences between mean values were determined using Bonferroni multiple-means comparison tests at $P<0.05$.

5.3. RESULTS

5.3.1. *Plant growth*

In most cases root weight and root-to-shoot ratios were higher in control pots for all species, except in the case of *P. pinea* and *P. lentiscus* under interspecific competition, in which case both species showed that their root system was significantly increased in the nutrient-rich soil pot (Fig. 5.1). For the stem and leaf weight ratios, we observed the higher values in nutrient-rich soil pots, except for the interspecific combination *P. pinea – P. lentiscus* (Fig. 5.1).

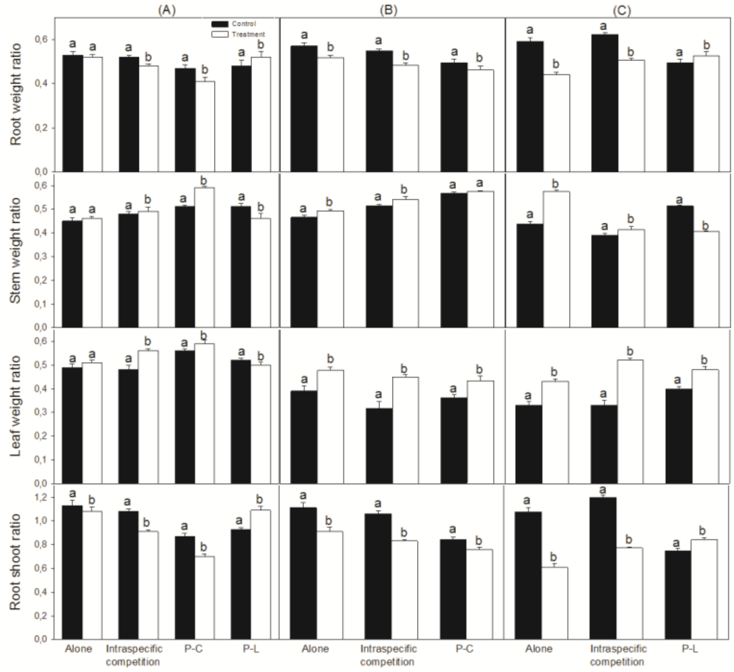

Fig. 5.1. The effects of no nutrient addition (control) and treatment on root, stem and leaf weight ratio and root-to-shoot ratio in *P. pinea* (A), *C. salvifolius* (B) and *P. lentiscus* (C) as target plants. Graphs show mean values for either single plants (Alone = treatment without competition) or summed weights for two plants (Intra- or interspecific competition; P-C: interspecific competition between *P. pinea* and *C. salvifolius*; P-L: interspecific competition between *P. pinea* and *P. lentiscus*). Letters indicate significant differences between treatments at the P<0.05 level, following Bonferroni multiple-means comparison tests.

Specific leaf area (SLA) and leaf area ratio (LAR) exhibited significant differences between treatments and species (Table 5.1). Increasing nitrogen availability had no effect on SLA and LAR of *P. lentiscus*, whereas both variables increased in *P. pinea*. *C. salvifolius* exhibited a decrease in SLA with increasing nitrogen.

Species	**Pot**	**SLA**	**(m^2kg^{-1})**	**LAR**	**(cm^2g^{-1})**
		Control	Treatment	Control	Treatment
Pinus pinea	Alone	7.82±1.05aA	9.25±0.92bA	30.05±4.85aA	48.75±2.96bA
	Intraspecific competition	8.12±1.45aA	9.52±1.07bA	30.78±5.12aA	49.12±2.87bA
	Interspecific competition (*Cistus*)	8.05±1.74aA	9.93±0.98bA	31.32±3.96aA	48.15±3.02bA
	Interspecific competition (*Pistacia*)	8.25±1.42aA	9.58±0.78bA	31.98±2.78aA	47.95±2.19bA
Cistus salvifolius	Alone	18.45±1.78aA	12.75±1.45bA	89.75±5.45aA	74.85±4.72bA
	Intraspecific competition	16.95±2.25aA	13.05±1.45bA	90.95±3.75aA	75.12±5.25bA
	Interspecific competition	17.45±1.75aA	12.89±1.34bA	91.09±4.75aA	74.85±3.96bA
Pistacia lentiscus	Alone	12.87±1.47aA	13.09±1.52aA	41.25±3.41aA	39.58±2.87aA
	Instraspecific competition	12.55±2.55aA	12.85±1.94aA	40.89±4.45aA	40.42±3.12aA
	Interspecific competition	12.96±1.96aA	12.52±1.48aA	41.37±3.96aA	40.98±2.57aA

Table 5.1. Specific Leaf Area (SLA) and Leaf Area Ratio (LAR) mean values (± standard deviation) of the three target species and treatments (competition and nutrient); lower and upper case superscript letters indicate significant differences between nitrogen treatments and species, respectively, (ANOVA), $P<0.05$; n = 10).

Plant biomass was significantly affected by competition in both treatments (control and nutrient) (Table 5.2, Fig. 5.2). For *P. pinea* as target, CI differed from zero in control (intraspecific neighbour; t = 6.816, d.f. = 6, P = 0.001: interspecific neighbour; t = 3.815, d.f. = 7, P = 0.025, Fig. 3a) and in the nutrient treatment (intraspecific neighbour; t = 7.125, d.f. = 6, P = 0.002: interspecific neighbour; t = 5.895, d.f. = 6, P = 0.001). For *C. salvifolius* as target, CI only differed significantly from zero in control with an interspecific neighbour (t = 4.514, d.f. = 6, P = 0.003, Fig. 3b) and in nutrient treatment with both intra- and interspecific neighbour (intraspecific; t = 2.782, d.f. = 7, P = 0.024: interspecific; t = 3.218, d.f. = 7, P = 0.016). For *P. lentiscus* as target, CI differed from zero in a similar way to that of *C. salvifolius*: in control with an interspecific neighbour (t = 3.879, d.f. = 6, P = 0.006, Fig. 3c) and in nutrient treatment with both intra- and interspecific neighbour (intraspecific; t = 2.915, d.f. = 7, P = 0.012: interspecific; t = 3.874, d.f. = 7, P = 0.007). To summarize, *P. pinea* competed with neighbours in both conditions (control and nutrient treatment), whereas *C. salvifolius* and *P. lentiscus* competed with neighbours in nutrient treatment and with an interspecific neighbour in control.

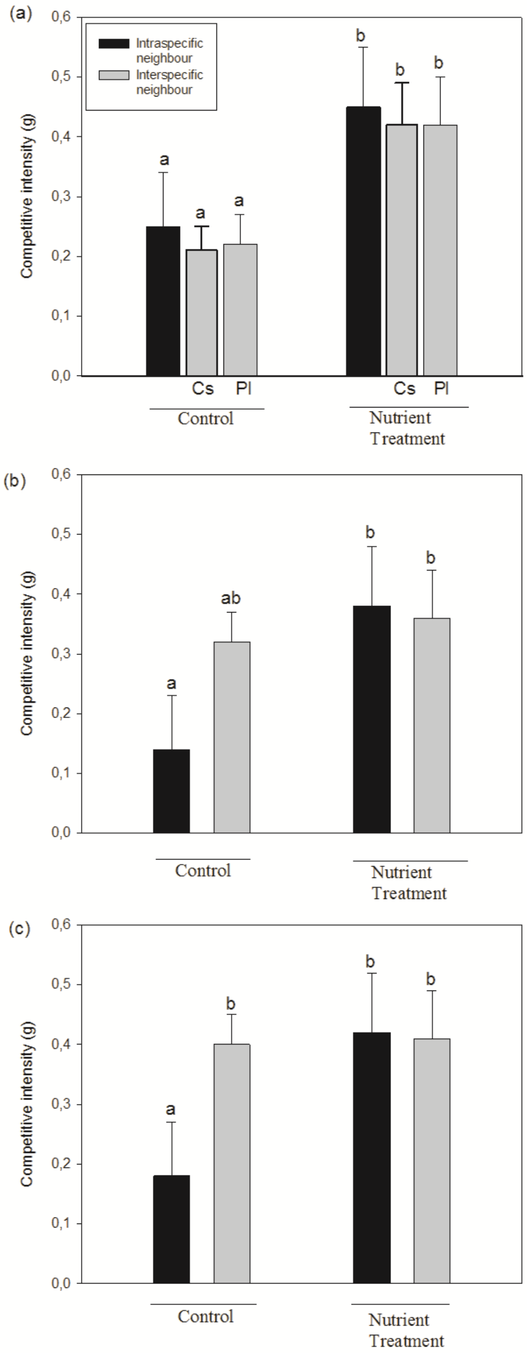

Fig. 5.2. Mean (+ 1 SE) competitive intensity in treatments with *P. pinea* (a), *C. salvifolius* (b) and *P. lentiscus* (c) as target plants. Treatment represents nutrient addition (Treatment in the graph) and Control represents no nutrient addition (Control in the graph). Letters indicate significant differences in mean CI values between treatments at the P<0.05 level, following Bonferroni multiple-means comparison tests. See Table 5.2 for associated analyses.

(a)	***P. pinea* as the target plant**	**d.f.**	**MS**	***F*-value**	***P***
Dependent Variable	Source	4	0.725	3.785	0.029
CI	Treatment Error	27	0.174		
(b)	***C. salvifolius* as the target plant**	**d.f.**	**MS**	***F*-value**	***P***
Dependent Variable	Source	3	0.669	3.458	0.032
CI	Treatment Error	27	0.185		
(c)	***P. lentiscus* as the target plant**	**d.f.**	**MS**	***F*-value**	***P***
Dependent Variable	Source	3	0.684	3.323	0.025
CI	Treatment Error	26	0.219		

Table 5.2. The effect of treatment on competitive intensity (CI) with *P. pinea* (a), *C. salvifolius* (b) and *P. lentiscus* (c) as target plant. Results of univariate analyses of variance. D.f. = degrees of freedom, MS = mean square. See Fig. 2 for means and standard errors.

5.3.2. *Chlorophyll fluorescence*

There were significant differences in F_v/F_m and Φ PSII in summer for *P. pinea* in the interspecific competition, with the highest values in the control. We also observed the same behavior with the effective quantum yield of PSII (Fig. 5.3).

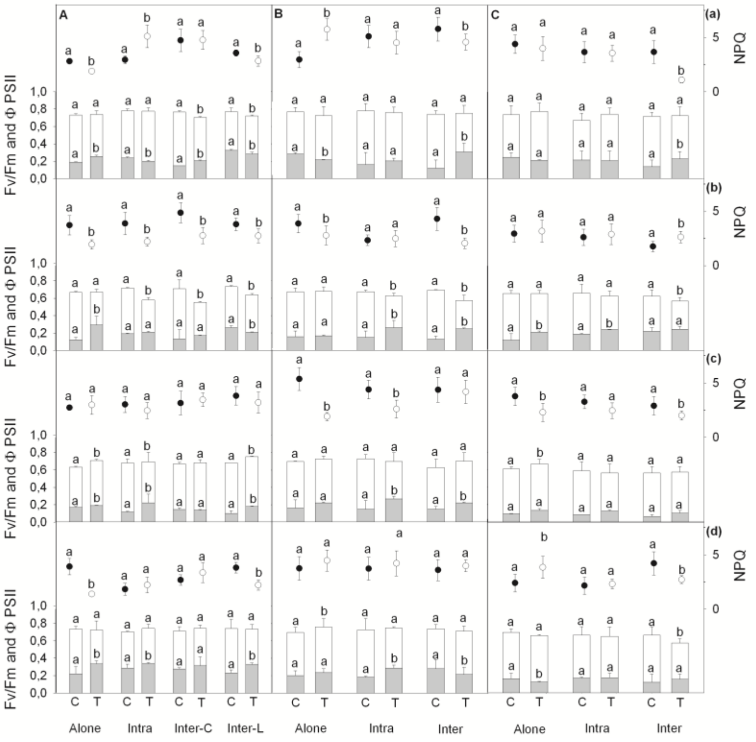

Fig. 5.3. Fv/Fm, Φ of PSII and quenching of chlorophyll fluorescence (NPQ) for (A) *P. pinea*, (B) *C. salvifolius* and (C) *P. lentiscus* in (a) summer, (b) autumn, (c) winter and (d) spring. Letters indicate significant differences between control (C) and treatment (T) (ANOVA P<0.05), mean ± s.e.

Plants showed similar values of F_v/F_m in April and August and there were no significant differences between both samplings in relation to control *v.* treatment (Fig. 5.3).

5.3.3. *Gas exchange*

Photosynthetic rate was significantly increased by nitrogen treatment in *P. pinea* in summer, while for the rest of the species there were no significant different among seasons and treatment (Fig. 5.4).

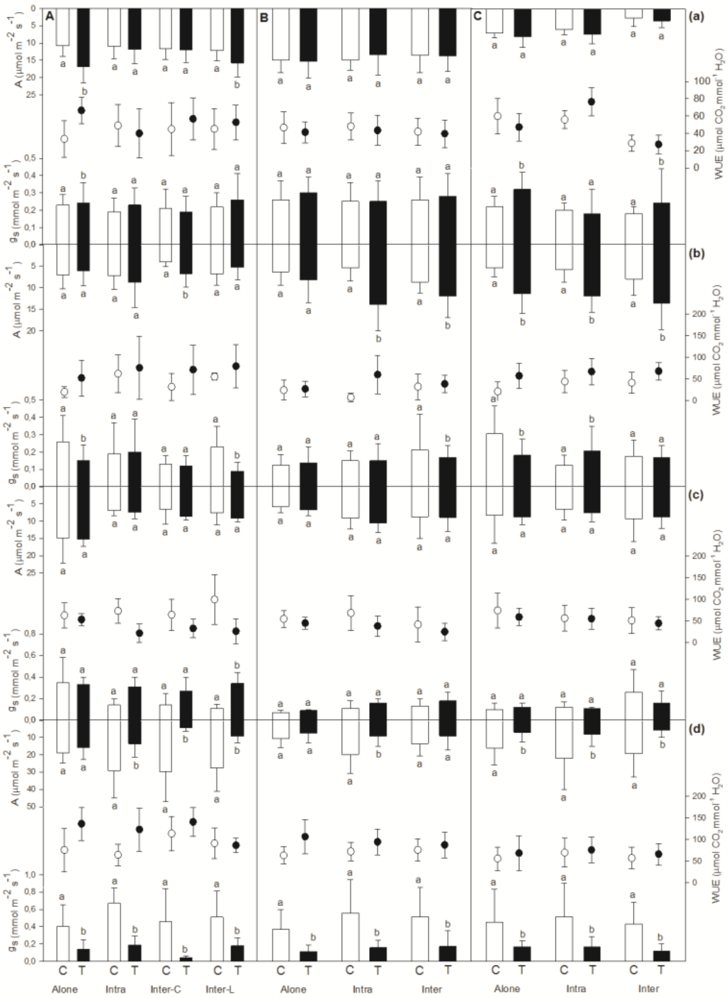

Fig. 5.4. Photosynthetic rate, A; leaf conductance to water vapour, g_s and water use efficiency (WUE) for (A) *P. pinea*, (B) *C. salvifolius* and (C) *P. lentiscus* in (a) summer, (b) autumn, (c) winter and (d) spring. Letters indicate significant differences between control (C) and treatment (T) (ANOVA P<0.05), mean ± s.e.

In general, photosynthetic rate values were higher for *P. pinea* and *C. salvifolius* than for *P. lentiscus*, and this difference was more evident in spring and summer (Fig. 5.4). *P. lentiscus* also showed the lowest values of water-use efficiency (WUE) when compared with the other target species.

5.4. DISCUSSION

The prediction that competition intensity would be greater under nutrient-rich conditions (Robinson et al. 1999) was upheld for *P. pinea*, *C. salvifolius* and *P. lentiscus* (Fig. 2). Day et al. (2003) described an intense competition between *Briza media* and *Festuca ovina* under nutrient-rich conditions; Robinson et al. (1999) and Hodge et al. (1999) proposed that root proliferation in nutrient patches may have evolved as a competition mechanism, because nutrients can be more effectively depleted from discrete patches. In fact, this response has been shown for a range of crop species and also, using artificial substrates, for Douglas-fir (Friend et al. 1990), lodgepole pine (Coutts & Philipson 1977), and Stika spruce (Coutts & Philipson 1976), as we observed for *P. pinea* (another coniferous species).

In the current study, all plants were grown under the same conditions until the beginning of the experiment. Then, plants under nutrient treatment responded to enriched soil in the same way as ruderal plant species respond similarly to locally enriched environment (Chapin 1980; Friend et al. 1990). However, there are several studies which support the hypothesis that nutrient-rich soil zones may not have an impact on root growth (Caldwell 1994). Although there are several studies that describe the root growth of some plant species under nutrient-rich soil conditions (Eissenstat 1991; Griffin et al. 1995), we observed that, in most cases, root growth was enhanced under nutrient-poor conditions, supporting the idea that the higher the nutrient availability the lower the root growth. Under our experimental conditions, the three target species produced more shoot dry matter than root dry matter and also the root/shoot ratio was higher in the control, with the exception of *P. pinea* when competing with *P. lentiscus*, indicating that more nutrients were provided for shoot growth per unit root dry weight. Physiological as well as morphological changes in the root system in response to nutrient availability, for example, increased nutrient uptake rates of roots in nutrient-rich soil, may be important in the adaptation of plants to soils that are heterogeneous in nutrient availability (Caldwell et al. 1992).

However, there is a lack of studies that assess root size measures to link them to belowground competition, with the exception of those of Caldwell et al. (1985), Aerts et al. (1991), Cahill & Casper (2000) and Cahill (2003). Another recent study, Bartelheimer et al. (2008) suggested that the amount of root biomass is of high importance for the competitive effect on a subject plant. Our results support the idea exposed above (see Fig. 5.1, Table 5.2).

The examined species differ in their strategies and life forms, *C. salvifolius* being a small shrub, *P. pinea* forms open pine forests on dune habitats, while *P. lentiscus* usually grows under the canopy of *P. pinea*. While physiology seems to be of little relevance, plasticity in morphological traits was of comparatively higher importance for adaptation to different environmental conditions in the studied plants. This is in agreement with findings than in a nutrient-limited environment growth is more hampered than photosynthesis (Poorter & Villar 1997; Poorter & Nagel 2000). According to Bouwer (1962, 1963) growth of belowground parts is promoted if water or mineral nutrients are short (Poorter & Nagel 2000). An indicator of this allocation pattern is the root/shoot ratio (Gower et al. 1992; Reynolds & Dantonio 1996; Perkins & Owens 2003), and this ratio was higher in control *v.* treatment, with the exception of *P. pinea* when competing with *P. lentiscus*.

Usually intraspecific competition is expected to be greater than interspecific competition (Hodge et al. 2000; Olson & Blicker 2003) because plants of the same species require the same environmental conditions. We can observe this behavior when comparing the cases of *P. pinea* and *C. salvifolius*, but not when comparing *P. pinea* and *P. lentiscus* (see Fig. 1, Table 2), probably due to the fact that these species showed an equal competitive effect on each other in relation to nutrient uptake.

The results of the present study indicate different growth strategies of the examined species. It has been suggested that, even in open dune ecosystems, high elongation rates may provide a competitive advantage for seedlings in occupying space and shading out competing plants (Peperkorn et al. 2005), and we observed that in all cases shoot elongation rate was higher in the nutrient treatment compared to the control, with the exception of *P. lentiscus* when competing with *P. pinea* (Fig. 5.1). The observed differences in root and shoot weight ratios among the target species were enhanced with increasing nitrogen supply. *P. pinea* showed the lowest differences between control *v.* nutrient treatment, indicating that an increase in N-availability has almost no effect on shoot elongation (Fig. 5.1). These low differences highlight that *P. pinea* is a well-adapted species to nutrient-poor soils (Richardson 1998).

According to our results, several authors (Poorter 1990; Van der Werf 1996; Hunt & Cornelissen 1997) found that slow-growing species usually presents lower SLA than fast-growing species. We observed the highest values of SLA in *C. salvifolius*, with *P. pinea* showing the lowest ones.

As it has been described in other studies, physiological parameters seem to be less plastic and give less information than morphological and allocation traits in competitive experiments (Valladares et al. 2000; Peperkorn et al. 2005). As we observed in the current study, measurements of chlorophyll fluorescence and photosynthetic rates did not give clear differences among treatments during the whole year.

To summarize, we can conclude that the development of a good root system provides an advantage in order to compete with individuals from the same species or to cope with interspecific competitors.

5.5. ACKNOWLEDGEMENTS

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CAPÍTULO 6

Effects of *Osyris lanceolata* (Santalaceae) on the physiological status of *Pinus pinea* and *Juniperus phoenicea* in a sand dune ecosystem

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Effects of *Osyris lanceolata* (Santalaceae) on the physiological status of *Pinus pinea* and *Juniperus phoenicea* in a sand dune ecosystem

Summary. *Osyris lanceolata* Hochst. & Steud. (Santalaceae) is an evergreen hemiparasitic plant species present in the Mediterranean Basin with a wide host range, which parasitizes the root system of many plants species thanks to a structure called haustoria. We investigated the influence of *O. lanceolata* on the ecophysiological behaviour of two coniferous species widely distributed in sandy nutrient-poor ecosystems in the SW of Spain, *Pinus pinea* L. and *Juniperus phoenicea* L. A, E, G_s, F_v/F_m and Φ PSII were not different between infected and uninfected plants. Mean water potentials of *O. lanceolata* were three to four times higher than those of host species, a behavior that could be interpreted as a rapid way to take nutrients and water from its hosts. Our results highlight that *O. lanceolata* affect softly the photosynthetic characteristics of its host species and possesses a very efficient mechanism of extraction of water and nutrients through the haustoria.

Este capítulo reproduce el siguiente artículo: **Valera-Burgos J, Zunzunegui M, Díaz-Barradas MC (2012)** Effects of *Osyris lanceolata* (Santalaceae) on the physiological status of *Pinus pinea* and *Juniperus phoenicea* in a san dune ecosystem (Submitted to *Journal of Experimental Botany*).

6.1. INTRODUCTION

Parasitic plants are an extensive group of species which interact with a host in order to obtain water and nutrients from them and, thus influencing the growth of their hosts by removing resources and by affecting photosynthesis at the leaf or whole-plant level (Watling & Press, 2001). There are two main groups of parasitic plants: Holoparasites, which present lack of chlorophyll and compete directly with the host for fixed carbon, act as extra sinks for host photosynthates and can enhance host photosynthetic productivity through this increased demand (Jeschke et al., 1994, 1995, 1997; Jeschke & Hilpert, 1997; Hibberd et al., 1998) and hemiparasites, which take some resources from their host plants but are also autotrophic, which makes source-sink effects on photosynthesis difficult to quantify (Press et al., 1999; Watling & Press, 2001). Whereas extraction of carbon is likely to increase host photosynthesis, and stimulation of host photosynthesis has been observed in some host-hemiparasite associations (Clark & Bonga, 1970; Cechin & Press, 1993), non-sink related interactions with the host can suppress host photosynthesis at both the leaf and whole-plant level (Graves et al., 1989; Cechin & Press, 1993; Gurney et al., 1995; Hibberd et al., 1996; Frost et al., 1997).

Although there is a large volume of literature, the ecology of belowground parasitic (including those sometimes referred to as hemiparasitic) species is still almost unknown. This poor knowledge is due to the fact that studying belowground connections between the parasite and their host is difficult (Dostalek & Münzbergová, 2010). The relations of parasitic plants with potentially limiting resources of the physical environment (water, nutrients) are mediated, and to some degree also buffered, by the physiology of their hosts (Kuijt, 1969).

The most studied hemiparasitic plant genus, *Striga*, has long been the subject of many studies in order to find out the mechanisms by which hemiparasites limit host photosynthesis, but less is known about other hemiparasites. Commonly, plants infected by *Striga* have lower stomatal conductances than uninfected plants (Gurney et al., 1995; Taylor et al., 1996; Frost et al., 1997), restricting diffusion of CO_2 into leaves and leading to lower rates of photosynthesis. Direct impacts on host photosynthetic metabolism have also been implicated in the suppression of host photosynthesis. For example, both carboxylation efficiency and electron transport capacity can be affected by parasite infection (Cechin & Press, 1993; Watling & Press, 2000; Shen et al., 2007; Cameron et al., 2008). Rubisco content was lower in rice plants infected by *Striga hermonthica* (Del.) Benth. than in uninfected plants (Watling & Press, 2000), but other studies have found no significant differences in the activity of key photosynthetic enzymes between infected and uninfected plants (Smith et al., 1995; Cameron et al., 2008). Lower chlorophyll concentrations have also been reported in plants infected by the root hemiparasite *Rhinanthus minor L.* than in uninfected plants (Cameron et al., 2008).

Root parasites from Santalales are a little-explored group which includes important weeds and endangered species (Musselman & Haynes, 1996; Marvier & Smith, 1997; Libby & Bloom, 1998). In Santalales there are taxa with extremely narrow host ranges, such as *Rafflesia* R.Br. ex Gray (Ismail, 1988), and species with quite broad host ranges, such as *Osyris alba* L. (Qasem, 2006).

Osyris L. is a genus of woody root hemiparasites in Santalaceae occurring from Portugal to India including northern and East Africa (Heide-Jørgensen, 2008). Depending on opinion there are 2-4 species. The leaves are evergreen and the flowers unisexual. It has been described that many *Osyris spp* parasitize evergreen and summer drought-deciduous shrubs of many species (Herrera, 1984). Species in the genus seem to be capable of using a wide range of angiosperms as hosts (Herrera, 1984; Qasem, 2006). As a xylem-tapping root hemiparasitic plant species, develops haustoria in order to absorb and process various nitrogenous and other solutes from the xylem of a range of common natural hosts.

To date, photosynthesis studies of hemiparasitic associations have mostly focused on root parasites from the genera *Striga* and *Rhinanthus* and a few mistletoes species. In this study we examined the impacts of infection by the root hemiparasite *Osyris lanceolata* Hochst. & Steud. (Santalaceae), an autotrophic and xylem-tapping plant species on two Mediterranean plant species. We conducted a field experiment to investigate whether *O. lanceolata* has a negative impact on its host species and try to find out by which mechanisms this hemiparasitic plant species benefits from its hosts.

6.2. MATERIALS AND METHODS

6.2.1. *Study site*

We conducted the study on the coastal dunes of El Asperillo (34^{o} 0´N 6^{o} 36´W), in the Doñana National Park (south-west Spain). The climate is Mediterranean type with oceanic influence and mild temperatures. Mean annual temperature is 16.8 ^{o}C. Average annual rainfall is 550 mm, concentrated between October and March. Vegetation consists of native dune scrub of *Halimium halimifolium (L.) Willk.*, *H. calycinum (L.) K.Koch.*, *Cistus libanotis L.*, *C. salvifolius L.*, *Rosmarinus officinalis*, *Lavandula stoechas*, *Cytisus grandiflorus*, *Stauracanthus genistoides*, *Corema album*, *Juniperus oxycedrus* and *J. phoenicea*, together with *Pinus pinea* (Muñoz-Reinoso & Díaz-Barradas, 1992).

6.2.2. *Study species*

Pinus pinea L. and *Juniperus phoenicea* L. are two coniferous species well established in the Iberian Peninsula. They are abundant in Mediterranean sand dune ecosystems and, in particular, *P. pinea* is widely distributed at the study site thanks to afforestation purposes during the last century, while *J. phoenicea* is restricted to a small difficult-access area and represents the ancient vegetation before the afforestation. Both species appear to be parasitized by *Osyris lanceolata* Hochst. & Steud., a hemiparasitic dioecious shrub which has increased its population at the study site during the twentieth century, whose seedlings may survive for up to one year on their own resources, but eventually die if they fail to establish haustorial connections with a host. It is often possible in the field to identify confidently the host of an adult *O. lanceolata* plant (Herrera, 1988).

6.2.3. *Plant material and growth conditions*

We selected two zones at the study site related to the most representative coniferous species, one of them with *P. pinea* and the other one with *J. phoenicea*. At both sites we selected ten host individuals and ten *O. lanceolata* individuals as parasites by direct observation of the root system. We also selected ten *P. pinea* and ten *J. phonicea* without a parasite in order to have a control in the study. All measurements were carried out in summer 2011 in order to test the behavior of the host-parasite association under summer-drought conditions, due to that as the parasite take water from the host it is the ideal season to investigate this behavior.

6.2.4. *Gas exchange*

In situ measurements of photosynthesis were made on leaves of infected host branches and uninfected control branches using a portable gas-exchange analyzer (LCi-portable photosynthesis, ADC, UK), which measures net photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gs). Measurements were made between 930 and 1130 h, under saturating light conditions (PPFD between 1500 and 1800 µmol m^{-2} s^{-1}).

6.2.5. *Water potential*

The leaf water potential of terminal shoots of infected host branches, uninfected control and parasite branches was measured at pre-dawn and midday. As the terminal portion of the shoots was leafy on all treatments, these stems were selected rather than leafless stems. Cut shoots were immediately inserted into a Scholander-type pressure chamber (Manofrigido, Portugal) and pressure applied until sap was expressed from the cut end.

6.2.6. *Chlorophyll content*

Leaf chlorophyll content was measured using the portions of stem on which photosynthesis measurements had been made. Samples were weighed and then ground in a mortar and pestle with a small amount of acid-wash sand in 2 mL of 80% (v/v) ice-cold acetone saturated with Na_2CO_3. The homogenate was then transferred to a centrifuge tube. The pestle and mortar were rinsed three times with 2 mL of 80% acetone and each rinsing was added to the centrifuge tube. Extracts were made up to 8 mL using 80% acetone and then centrifuged at 2500g for 10 min to remove the sand and other debris. Absorbance of the supernatant was measured at 663 and 646nm. Chlorophyll concentration was calculated as follows: chlorophyll a (mg L^{-1}) = 12.21 $A_{663} – 2.81 A_{646}$, chlorophyll b (mgL^{-1}) = 20.13 $A_{646} – 5.03 A_{663}$ (Chapman, 1988).

6.2.7. *Chlorophyll fluorescence*

We measured the efficiency of PSII under ambient light conditions, commencing at pre-dawn, and at 2-hourly intervals from 1 h before sunrise until 1 h after sunset. Measurements were made with a Mini-PAM portable chlorophyll fluorometer fitted with a 2030-B leaf-clip holder (Heinz Walz GmbH, Effeltrich, Germany). All stems were exposed to similar light conditions for these measurements.

6.2.8. *Proline*

Proline concentration was estimated using the acid-ninhydrin method (Bates et al., 1973). Fully expanded leaves were excised and immersed in liquid nitrogen while being transferred from the greenhouse to the laboratory. Tissues were stored at -25 ºC in a freezer until they were analysed. Two 0.5 g leaf or tissue samples were taken from each plant and were ground with liquid nitrogen. Sulphosalicylic acid (10 mL, 3%) was added and the extract was then vacuum filtered through Whatman no. 2 filter paper. Then, 2 mL of the filtered extract were taken for the analysis, to which 2 mL of acid-ninhydrin and acetic acid were added. The mixture was oven incubated at 100 ºC for 1 h and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 mL toluene and absorbance was read at 517 nm, using toluene as a blank. Proline concentration was calculated using L-proline (Panreac) for the standard curve.

6.2.9. *Statistical analysis*

All tests were conducted by SPSS (version 18.0, SPSS Inc., Chicago, IL, USA). Means of physiological variables between uninfected and infected plants were compared using Student´s *t*-test. Correlation analysis was conducted to test the relationships between photosynthesis and stomatal conductance.

6.3. RESULTS

6.3.1. *Gas exchange*

Infection with *O. lanceolata* had no significant effect on the gas exchange characteristics of both *P. pinea* and *J. phoenicea* (Fig. 6.1). Plants from *P. pinea* and *J. phoenicea* had assimilation rates that were almost the same to those of infected plants (Fig. 6.1a). The non-significant lower rates of assimilation in the infected plants were accompanied by lower stomatal conductance (Fig. 6.1b) and lower rates of transpiration (Fig. 6.1c). Uninfected plants from *P. pinea* had non-significant lower C_i than infected plants, but *J. phoenicea* showed the opposite pattern (Fig. 6.1d). There were linear relationships between assimilation rate and stomatal conductance in both uninfected and infected plants of each species (Fig. 6.2). Although uninfected plants always had higher assimilation rates for a given stomatal conductance than infected plants, there were no significant differences. Instantaneous water use efficiency was similar for uninfected and infected plants (data not shown).

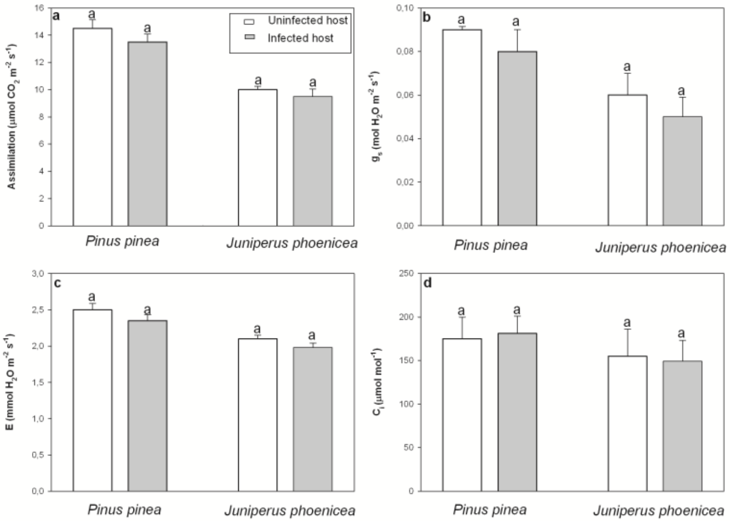

Fig. 6.1. (a) Assimilation rate (A), (b) stomatal conductance (g_s), (c) transpiration rate (E) and (d) intercellular CO_2 concentration (C_i) for *Pinus pinea* and *Juniperus phoenicea* plants either uninfected (open bars) or infected with *Osyris lanceolata* (solid bars). Values are means + s.e. (n=10). Within each graph letters indicate that means are significantly different at α=0.05.

We also observed high transpiration rates values for *O. lanceolata* compared to those recorded from host and control plants. Values of transpiration rate for the parasite ranged between 12 to 16 mmol m^{-2} s^{-1} when parasitized *P. pinea*, whose values ranged from 2 to 2.5 mmol m^{-2} s^{-1} (Fig. 6.1c), and between 10 to 14 mmol m^{-2} s^{-1} when parasitized *J. phoenicea*, whose values ranged from 1.8 to 2.2 mmol m^{-2} s^{-1} (Fig. 6.1c).

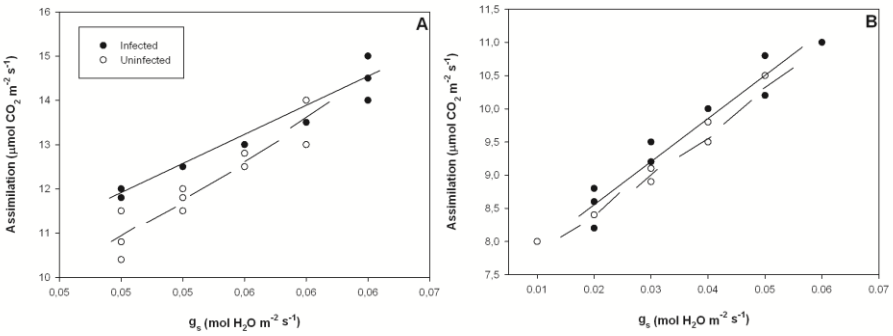

Fig. 6.2. Relationships between photosynthesis and stomatal conductance (gs) of *Pinus pinea* and *Juniperus phoenicea* plants either uninfected (open symbols; broken line) or infected with *Osyris lanceolata* (closed symbols; solid line). Correlation coefficients are 0.83 and 0.85 for *P. pinea* (uninfected and infected plants, respectively) and 0.75 and 0.78 for *J. phoenicea* (uninfected and infected plants, respectively) (P<0.05).

6.3.2. *Water potential*

Mean pre-dawn and midday water potentials of control plants were for *P. pinea*: -2.0 ± 0.1 MPa and -14.5 ± 1.5 MPa, respectively and for *J. phoenicea*: -1.5 ± 0.1 MPa and -11.5 ± 0.5 MPa, respectively. There were no significant differences in either pre-dawn or midday water potentials of uninfected and infected plants for both target species. Mean pre-dawn and midday water potentials of *O. lanceolata* plants always seemed to be three to four times higher than those water potential of their hosts, reaching even -45.5 ± 4.5 MPa at midday.

6.3.3. *Chlorophyll content*

The amounts of total chlorophyll, chlorophyll a and chlorophyll b were not significantly different between uninfected and infected plants for both target species (Fig. 6.3).

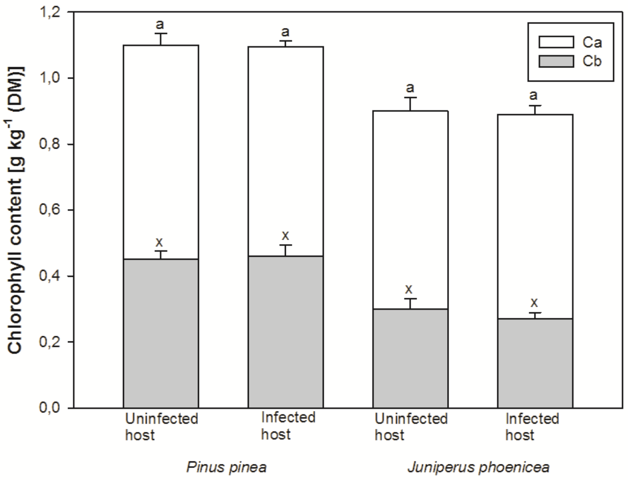

Fig. 6.3. Chlorophyll concentration (chlorophyll a and chlorophyll b) of *Pinus pinea* and *Juniperus phoenicea* plants either uninfected or infected with *Osyris lanceolata*. Values are means + s.e. (n=10). Within the graph different letters indicate that means are significantly different at α=0.05.

6.3.4. *Chlorophyll fluorescence*

F_v/F_m and ΦPSII of both *P. pinea* and *J. phoenicea* showed a similar pattern regardless of the infection treatments (Fig. 6.4). There were no significant differences in the photochemical efficiency of the PSII.

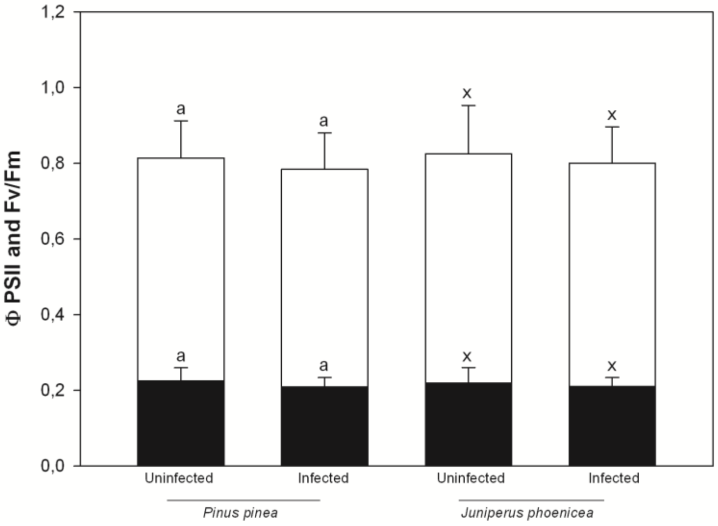

Fig. 6.4. Mean values + s.e. of the Φ PSII and F_v/F_m of dark-adapted leaves for *Pinus pinea* and *Juniperus phoenicea* plants either uninfected or infected with *Osyris lanceolata*. Within the graph letters indicate that means are significantly different at α=0.05.

6.3.5. *Proline*

Both *J. phoenicea* and *P. pinea* showed nearly 10 µmol g^{-1} dry weight of proline in plant tissues, in contrast to the high amounts observed in *O. lanceolata* haustoria and plant tissues, with 180 and 160 µmol g^{-1}, respectively (Fig. 6.5).

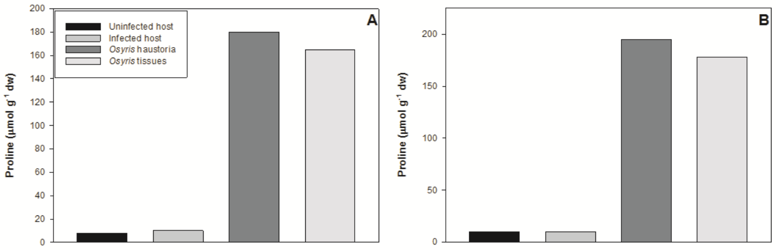

Fig. 6.5. Proline accumulation in host tissues of (A) *Pinus pinea* and (B) *Juniperus phoenicea* plants uninfected or infected with *Osyris lanceolata*, and also proline accumulation in parasite haustoria and tissues.

6.4. DISCUSSION

6.4.1. *Impact of O. lanceolata on host photosynthesis*

Hemiparasites have been frequently observed to have a negative impact on host photosynthesis (Watling & Press, 2001). However, in this study we observed that host photosynthesis was not significantly affected when compared with photosynthesis of control plants (Fig. 6.1a). Press & Seel (1996) found that in the *Rinanthus minor* (hemiparasitic plant species) - *Poa alpina ssp. vivipara* association, *P. alpina* plants accumulated less biomass and had lower shoot:root ratios than uninfected controls, but the parasite did not influence rates of photosynthesis in the host. It is worth of saying that it is difficult to identify the mechanism(s) by which the parasite affects host photosynthesis. In the current study, no differences were found in chlorophyll concentration between control and infected plants, suggesting that nitrogen extraction by the parasite had little impact on hosts.

Shen et al. (2010) found out in the *Cassytha pubescens* - *Cytisus scoparius* association that the lower rates of photosynthesis for infected plants involve a reduced demand for the products of the light reactions, making plant more vulnerable to photodamage, as they observed significant downregulation of PSII efficiency in infected plants relative to uninfected controls. In the present study we observed no differences in the photochemical efficiency of the PSII for both coniferous species (Fig. 6.4), supporting the idea that *O. lanceolata* did not affect the photosynthetic activity of its hosts.

6.4.2. *Survive in a summer-constrained habitat*

Tennakoon et al. (1997) described the high production of proline in the haustoria of *Santalum acuminatum* (Santalaceae), taken together with the evidence of massive subsequent export of the compound in parasite xylem sap, supporting the idea that this compound is of particular interest in relation to the suggested general role of proline in osmotic relations of plants (Samaras et al., 1995). There are several substances, such as proline and some polyols, which function in osmotic adjustment of tissues of a wide range of plant species (Popp & Smirnoff, 1995; Samaras et al., 1995). In the current study we found negligible amounts of proline in host tissues, in contrast to the high amounts observed in the haustorium and parasite tissues (Fig. 6.5), supporting the idea exposed above .

Proline is also known to accumulate in nitrogen-fixing root nodules of legumes where it has been suggested to act as an energy source for bacteroids (Zhu et al., 1992). The observed high amounts of proline in the haustoria of *Osyris* would not mean the implication of the parasite as an energy donor. In this case, the most accepted alternative would be that proline synthesized in, and exported from, the haustorium contributes significantly to the osmotic relations of the whole plant of *Osyris*, as it has been described for mannitol in other hemiparasites (Smith et al., 1969; Nour et al., 1984; Press et al., 1986). In fact, Press et al. (1986) reported concentrations of more than 150 mg/g dry weight of mannitol, accounting for more than 75% of total soluble sugars in *Striga*, *Orobanche* and *Lathraea*.

There were no significant differences in chlorophyll concentrations between uninfected and infected plants, presumably as a result, suggesting that the parasite is not sequestering significant amounts of N from the host plant, presumably as a result of a lack of vascular connectivity between the host and the parasite, as it has been described for the genus *Plantago* for example (Cameron et al., 2006; Cameron & Seel, 2007).

The high transpiration rates in *Osyris* will maintain a strong water potential gradient between parasite and host; these rates have therefore been interpreted as an important mechanism for acquiring water and solutes from the host sap (Fisher, 1983; Stewart & Press, 1990). In particular, they may be necessary, also with the proline content, to generate the water potential gradients required to allow transport of solutes across the haustorium.

There were no significant differences in water potentials between uninfected and infected plants, and *O. lanceolata* showed markedly more negative values of water potential, as it has been described in other hemiparasites which have more negative water potentials than their hosts (Press et al., 1988; Tesitel et al., 2010).

The transpiration rates of hemiparasitic angiosperms have been reported for many ecosystems, and are characteristically high in comparison to those recorded for associated non-parasitic plants (Press et al., 1990). Press et al. (1993) described transpiration rates which ranged from 10 to 25 mmol m^{-2} s^{-1} in *Bartsia trixago* (Scrophulariaceae) and *Parentucellia viscosa* (Scrophulariaceae) compared with rates of between 7 and 12 mmol m^{-2} s^{-1} for six host species. Large differences in transpiration rates have been also found between mistletoes and their hosts, with a quotient of parasite/host transpiration ranged from 1.5 to 7.9 (Ullmann et al., 1985). We found a quotient of parasite/host transpiration from 5.5 to 6.4.

6.4.3. *Carbon gain by the hemiparasite*

While carbon gain by the host occurs only through photosynthesis by host tissues, carbon gain by the hemiparasite occurs both through parasite photosynthesis and also by passive uptake of organic carbon contained within xylem fluids (Raven, 1983; Press et al., 1987). Thus, transpiration rate may play a key role in increasing parasite carbon gain. By exactly the same host-xylem mechanism, mineral uptake of nutrients should also be a function of xylem sap flux through the haustoria.

The high transpiration rates and greater leaf conductances of hemiparasites would result in higher photosynthetic rates if carbon dioxide diffusion were limiting photosynthesis. Yet photosynthetic rates of hemiparasites are not greater than those of their hosts(Hollinger, 1983; Goldstein et al., 1989; Marshall et al., 1994). Hemiparasites have a lower photosynthetic rate, despite having a higher intercellular carbon dioxide concentration, because their photosynthetic capacity is lower than that of their host (Ehleringer & Marshall, 1995).

6.5. CONCLUSIONS

To summarize, infection with the hemiparasite *O. lanceolata* did not affect photosynthesis and the water status of *P. pinea* and *J. phoenicea*. The hemiparasitic plant species exhibited high transpiration rates, strong negative water potentials and high amounts of proline, which implies the existence of a well-established mechanism in order to take up water and nutrients from its hosts.

6.6. ACKNOWLEDGEMENTS

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CAPÍTULO 7

Discusión general

DISCUSIÓN GENERAL.

El entramado de relaciones intra- e interespecíficas que se establece en los ecosistemas terrestres atiende a la diversidad de especies que lo componen, pudiendo diferenciarse dos subsistemas, la parte aérea (*aboveground*) y el suelo o parte subterránea (*belowground*). De la interrelación que se establezca entre ambos subsistemas depende el desarrollo de las interacciones entre los distintos componentes de ambos subsistemas. En la presente Tesis Doctoral se aborda el estudio de las relaciones entre las plantas (componente principal del subsistema aéreo) desde distintos puntos de vista: germinación y establecimiento de plántulas, interacción entre diversos tipos funcionales, relaciones de competencia y facilitación; pero también se hace una aproximación a la interrelación de las plantas con el suelo (subsistema subterráneo) desde el punto de vista del estudio de la descomposición de la hojarasca y reciclaje de nutrientes en el sistema objeto de estudio.

Todo el conjunto de interrelaciones hace que se establezca una comunidad vegetal formada por especies dunares, matorral mediterráneo (fundamentalmente especies pertenecientes a la familia Cistaceae), una especie arbustiva de carácter hemiparásito (*Osyris lanceolata* Hochst. & Steud.) y 2 especies arbóreas, una natural (*Juniperus phoenicea* L.) y otra que, aunque presente desde hace bastante tiempo en la cuenca mediterránea, ha sido introducida por políticas forestales (*Pinus pinea* L.), la cual modifica la estructura de la comunidad a distintos niveles.

El pino piñonero (*Pinus pinea* L.) aparece como especie descrita en el registro polínico en el ecosistema objeto de estudio desde hace varios miles de años (Caratini y Viguier, 1973). Sin embargo, ha sido durante el siglo XX cuando ha visto aumentada su población debido a la política de reforestación llevada a cabo en la zona con el principal objetivo de frenar el avance de las dunas. Fruto de esta reforestación intensiva, se ha incurrido en un exceso del nivel de competencia entre esta conífera y el resto de especies vegetales mediterráneas que aparecen en los ecosistemas dunares del suroeste de la Península Ibérica. Por lo tanto, esta Tesis trata de dar respuesta y explicar algunos aspectos que contribuyen al establecimiento de esta comunidad a distintos niveles (suelo y parte aérea) y en distintas etapas del desarrollo.

La germinación del banco de semillas y el establecimiento de plántulas son dos de los factores clave para las comunidades vegetales, más aún en un ambiente donde las condiciones restrictivas del entorno suelen imponer diferentes tipos de estrés sobre la comunidad vegetal como ocurre en los sistemas dunares bajo clima mediterráneo, siendo la zona de estudio (El Asperillo) un ejemplo característico. La expansión de *P. pinea* está incidiendo en este proceso de muy diversas formas: por un lado, y como había sido descrito en el capítulo 2 de la presente Tesis Doctoral, la presencia de una barrera física en el suelo formada por el depósito de acículas que caen de los pinos puede tener efectos tanto positivos como negativos; entre los efectos positivos, destacaríamos la existencia de una capa de acículas de un grosor moderado que permita una buena aireación en la superficie del suelo así como la llegada de radiación solar a la parte más inferior de dicha capa de acículas, produciendo un microambiente que permita retener una humedad y unas condiciones adecuadas para la germinación y establecimiento de plántulas en las inmediaciones de los individuos de *P. pinea*; de hecho en el capítulo 2 se presentan los resultados de la germinación y supervivencia de plántulas de 3 especies arbustivas mediterráneas (*Cistus salvifolius*, *C. libanotis* y *Halimium halimifolium*) en las que no se observan diferencias significativas en cuanto a tasa de germinación y supervivencia de plántulas comparadas con el control (Valera-Burgos et al., 2012), si bien es cierto que la tendencia es a menor porcentaje de germinación cuando existe la capa de acículas, por el simple hecho de existir dicha barrera física. Entre los efectos negativos tendríamos los derivados de la presencia de una gruesa capa de acículas que impediría la llegada de una radiación solar suficiente para la germinación de las semillas y el crecimiento de las posibles plántulas emergidas.

Cuando nos referimos a la capa de acículas depositada sobre el suelo también cabe mencionar el efecto químico de estas acículas sobre la germinación y posterior establecimiento de plántulas. Los resultados del capítulo 2 arrojan que los posibles efectos alelopáticos derivados de un año de baja productividad (3.4 g L^{-1}) de acuerdo con Emeterio et al. (2004) no afectan significativamente a la germinación y establecimiento de plántulas de las especies objeto de estudio, sino que más bien favorecen dichos procesos, posiblemente debido a un aumento significativo del nivel de nutrientes en un sustrato pobre en nutrientes propio de los sistemas dunares mediterráneos. Sin embargo, cuando el extracto de acículas empleado era de 10 g L^{-1} (equivalente a un año de elevada productividad) sí que se afectaban negativamente tanto la tasa de germinación como el establecimiento de plántulas de las 3 cistáceas empleadas en el estudio (Valera-Burgos et al., 2012).

Como se ha referido en párrafos anteriores, los sistemas dunares mediterráneos son ambientes pobres en nutrientes (Gallardo, 2001). Esta asunción es un aspecto bastante importante de la zona de estudio que nos ocupa en la presente Tesis Doctoral. Las especies vegetales mediterráneas tienden a reabsorber gran parte de los nutrientes presentes en las hojas antes de la abscisión foliar y, por lo tanto, la hojarasca que se genera es pobre en nutrientes; este hecho, unido a la presencia de sustancias impermeabilizantes como cutina y suberina (Gallardo y Merino, 1993), hecho bastante común en especies de ambientes mediterráneos, confieren mayor resistencia a la degradación microbiana, por lo que finalmente tenemos una baja entrada de nutrientes al ecosistema vía descomposición de la hojarasca. Por lo tanto, es un aspecto fundamental el comprender los procesos que controlan la descomposición o el retorno de nutrientes de la biomasa al suelo.

Como puede observarse en el capítulo 3 el pino piñonero mostró las tasas de descomposición más bajas tanto en condiciones óptimas de descomposición en microcosmos (con el 70.9 % de masa remanente al final de la incubación) como directamente en la zona de estudio (con el 78.3 % de masa remanente). Existen varios estudios en los que se demuestra que las coníferas suelen presentar bajas tasas de descomposición (Berg, 1986; Prescott, 1995; Berg et al., 2007; Zhang et al., 2008), lo que supone una escasa reintroducción de nutrientes al sistema debido a que el pino piñonero es una de las especies más representativas del ecosistema estudiado, fruto de las repoblaciones forestales llevadas a cabo en la zona durante el siglo XX.

Las cistáceas presentaron una dinámica de descomposición intermedia entre *P. pinea* y *Osyris lanceolata* Hochst. & Steud. (Santalaceae), una especie hemiparásita presente en el área de estudio y que juega un papel bastante importante en la dinámica de la comunidad vegetal a la que pertenece como se ha ido observando en los diversos capítulos de la presente Tesis Doctoral. Gallardo y Merino (1992, 1993) describieron que las especies mediterráneas, principalmente la pertenecientes a la familia Cistaceae, contienen una serie de sustancias que les confieren impermeabilidad y favorece su supervivencia en ambientes bastante estresantes en cuando a radiación y disponibilidad hídrica se refiere. Estas sustancias impiden en cierta medida el ataque bacteriano y fúngico de su hojarasca, por lo que la descomposición y correcto reciclaje de nutrientes se ve ralentizado.

En el capítulo 3 se llevó a cabo un estudio de la descomponibilidad de una serie de especies representativas de la zona de estudio incluyendo *P. pinea*, árbol predominante de la comunidad vegetal estudiada, *Cistus salvifolius* y *Halimium halimifolium* como representantes de una familia muy extendida en la cuenca mediterránea (Cistaceae) y *O. lanceolata*. En dicho capítulo se observó cómo *P. pinea* fue la especie que presentó los valores más bajos de descomposición y liberación de nutrientes, como ha sido descrito en otras coníferas (Berg, 1986), seguida de las cistáceas debido fundamentalmente a la presencia de cutina y suberina en sus hojas (Gallardo y Merino, 1993).

El papel de la especie hemiparásita *O. lanceolata* en las relaciones interespecíficas se puso de manifiesto en los capítulos 3 y 4. En el primero de ellos se observó cómo dicha especie presentaba los valores más altos de descomposición, quedando aproximadamente el 41% de masa remanente en microcosmos y el 57% en el campo. Quested et al. (2003, 2005) demostraron que la especie hemiparásita *Bartsia alpina* incrementaba el retorno de nutrientes al ecosistema del que formaba parte, un ecosistema caracterizado, precisamente, por la baja disponibilidad de nutrientes; de la misma forma, se observó que favorecía la descomposición de hojarasca de otras especies presentes en el mismo ecosistema utilizando bolsas de hojarasca mixta. Un hecho similar fue observado en *O. lanceolata* en el capítulo 4.

El último supuesto expuesto en el párrafo anterior fue demostrado en el capítulo 4, donde se pudo observar cómo la hojarasca de las especies vegetales más representativas del ecosistema estudiado se descomponía más rápidamente en presencia de la hojarasca de *O. lanceolata* que cuando lo hacían cada una de ellas por separado. Resultados similares del efecto de mezclar hojarasca de distintas especies han sido descritos en diversos estudios (Chapman et al., 1988; Gartner y Cardon, 2004; Quested et al., 2005; Lecerf et al., 2007), donde se ha comprobado una aceleración en la descomposición de especies cuya hojarasca se descomponía más lentamente cuando estaban aisladas que en mixturas con hojarasca de especies que presentaban una mayor tasa de descomposición.

Además de los aspectos tratados en los capítulos 2-4, la interacción entre las especies vegetales que componen la comunidad vegetal es un importante factor a tener en cuenta la hora de entender el funcionamiento del ecosistema. En este sentido, las relaciones de competencia que se establecen entre plántulas en los primeros años de vida en el caso de las especies de porte arbóreo y arbustivo presentes en el ecosistema estudiado (capítulo 5) nos da idea de esta importancia. La especie arbórea más representativa de la comunidad estudiada, *P. pinea*, constituye un fiel exponente de competidor potencial debido en primer lugar a su porte (sombreado), también al desarrollo de un sistema radicular bastante eficiente, así como la capa de acículas que se deposita en el suelo, que como se comentaba en párrafos anteriores puede tener efectos tanto neutros-positivos como negativos. Hay diversos estudios centrados en los efectos de coníferas sobre la vegetación presente en el ecosistema del que forman parte (Coutts y Philipson, 1976; Friend et al., 1990).

La interacción y posibles relaciones de competencia entre especies vegetales alcanza además un aspecto llamativo si se tienen en cuenta ambientes pobres en recursos frente a ricos en recursos, siendo la competencia mayor en el último de los casos, siguiendo las dos principales teorías en este sentido: la estrategia CSR (competidores-tolerantes al estrés-ruderales), que indica que la competencia se incrementa con la productividad (Grime, 1977) y la teoría R (hipótesis del ratio de recursos) que indica que la competición es consistente con el gradiente de productividad pero que se incrementa por agua y nutrientes (*belowground*) y por luz (*aboveground*) cuando los recursos bajo el suelo están más disponibles (Tilman, 1985, 1988). Los resultados obtenidos en el capítulo 5 permiten describir distintas asunciones del proceso competitivo: en primer lugar, que existe una diferenciación entre la parte áerea frente a la parte subterránea (Kruger, 1987; Aerts et al., 1991; Hodge, 2004; Peperkorn et al., 2005) en cuanto a la asignación de recursos entre las distintas combinaciones (*P. pinea*, *C. salvifolius* y *Pistacia lentiscus* aislados y en competencia intra- e interespecífica) y también dependiendo del nivel de recursos presente en la maceta.

Cabe citar en este contexto que es de esperar que la competencia intraespecífica sea superior a la competencia interespecífica (Hodge et al., 2000; Olson y Blicker, 2003), debido principalmente a que dos individuos de la misma especie presentarán los mismos atributos o muy similares y competirán por los mismos recursos, aunque sí es cierto que podemos encontrarnos con distintas especies que pertenezcan al mismo tipo funcional y compitieran como si se tratase de la misma especie.

El pino piñonero resultó ser la especie más competitiva de las 3 tenidas en cuenta para el estudio. Los mayores niveles de competición se produjeron en las raíces (*belowground competition*), siendo el mayor nivel de competencia el encontrado entre *P. pinea* y *P. lentiscus* (CI = 0.45). Diversos autores han referido la competencia bajo la superficie del suelo como el punto de vista más importante entre dos especies o individuos de la misma especie que comparten un mismo espacio vital (Aerts et al., 1991; Robinson et al., 1999; Cahill, 2003), sin obviar lógicamente la competencia de la parte aérea (*aboveground competition*) debida principalmente a la radiación lumínica.

Reubicando las ideas expuestas en los párrafos precedentes sí que se puede indicar el caso de *P. lentiscus*, cuya intensidad de competencia se vio incrementada en el caso de la existencia de un vecino conespecífico cuando crecían en un ambiente rico en nutrientes; sin embargo en el control (sin adición de nutrientes) la competencia interespecífica era claramente superior a la intraespecífica.

No todas las interacciones del pino piñonero estudiadas en este trabajo son de tipo negativo, ya sea por inhibición o impedimento de la germinación y desarrollo de plántulas o por competencia por los recursos. En este sentido conviene apuntar la relación de tipo facilitador/positivo que tiene *P. pinea* con respecto a la especie hemiparásita *O. lanceolata* (Santalaceae), en el llamado efecto *perching*. Dicho efecto facilitador consiste en que *O. lanceolata* es un arbusto dioico cuyas semillas son dispersadas por aves , principalmente *Sylvia atricapilla* y *Turdus merula* (Herrera, 1984), que encuentran en los pinos un buen sitio de refugio para poder alimentarse de los frutos de la especie hemiparásita y, posteriormente, defecar en las inmediaciones de los pinos. Dentro de estas deyecciones se encuentran las semillas de *O. lanceolata* que quedan en las inmediaciones de un buen hospedador potencial que facilitará en buena medida la supervivencia de nuevas plántulas y el crecimiento de esta santalácea.

Aunque el género *Osyris* ha sido descrito como un género de plantas hemiparásitas de un espectro más o menos amplio de hospedadores (Herrera, 1988; Qasem, 2006), en el ecosistema objeto de estudio se observa que *O. lanceolata* aparece asociado a *P. pinea* principalmente, debido fundamentalmente a la razón expuesta en el párrafo anterior; existiendo también una zona más conservada donde no se llevaron a cabo tareas de repoblación forestal con *P. pinea*, apareciendo la vegetación primigenia, compuesta fundamentalmente por sabinar (*Juniperus phoenicea* L.) y romero (*Rosmarinus officinalis* L.), encontrándose aquí individuos de *O. lanceolata* asociados a la sabina pero sin nuevo reclutamiento de plántulas, debido a que el pino piñonero acapara la práctica totalidad de las mismas.

A pesar de que muchos estudios describen los efectos negativos que tienen las plantas parásitas sobre sus hospedadores (Gurney et al., 1995; Shen et al., 2007; Cameron et al., 2008), en esta Tesis Doctoral en las medidas ecofisiológicas llevadas a cabo no se han obtenido resultados significativos que indiquen que *O. lanceolata* perjudica a su hospedador.

Un aspecto importante que se ha puesto de manifiesto en el capítulo 6 es la gran cantidad de prolina que se sintetiza en el haustorio y que se distribuye al resto del individuo que parasitiza, más si cabe porque los niveles de prolina en los hospedadores en este estudio son prácticamente nulos. Diversos autores han descrito que la prolina, osmolito compatible utilizado por las plantas en condiciones de estrés hídrico, se encuentra presente en un gran número de especies parásitas (Ehleringer y Marshall, 1995; Pate et al., 1994; Tennakoon et al., 1997) y, de hecho, tiende a sintetizarse en el haustorio de la propia planta parásita, como ocurría en el caso de *O. lanceolata*. Dicho osmolito compatible permite mantener un potencial hídrico tal que asegura el trasvase de agua y nutrientes desde el hospedador al parásito; unido lo anterior al hecho de que *O. lanceolata* mostró elevadas tasas de transpiración, se puede describir *O. lanceolata* como una auténtica máquina capaz de mantener un aporte continuo de las sustancias necesarias para su crecimiento y desarrollo desde el hospedador. Comportamientos similares en cuanto a la tasa de transpiración han sido descritos en otras especies parásitas, principalmente muérdagos y herbáceas (Press et al., 1987; Press et al., 1988), pero *O. lanceolata* no presentó bajos niveles de eficiencia en el uso del agua como en estos otros casos.

El complejo mosaico de interacciones tanto a nivel subterráneo como aéreo se suceden en el espacio y en el tiempo actuando a distintas escalas, desde la fisiológica, el individuo, y la población para permitir el mantenimiento de la comunidad vegetal y sus estrategias para hacer frente al verano mediterráneo en un suelo dunar, con una capacidad muy baja de retención de agua.

INVESTIGACIONES FUTURAS

Por último, los datos presentados en esta Tesis Doctoral permiten tener un conocimiento más detallado sobre el desarrollo de las interacciones del pino piñonero con el resto del matorral mediterráneo que aparece en el ecosistema objeto de estudio y da idea de las posibilidades de realizar estudios futuros siguiendo la misma línea de acción, como por ejemplo:

Realizar estudios pormenorizados sobre el efecto de las acículas de *P. pinea* sobre la germinación y supervivencia de plántulas de las especies más abundantes de la zona de estudio, así como evaluar la diferente respuesta de cada especie y, del mismo modo, separar en detalle el efecto físico del químico.

Llevar a cabo estudios de descomposición de la hojarasca de todas las especies presentes en la zona de estudio durante un período de al menos dos años, de tal modo que se puedan separar las distintas fases del proceso de descomposición para cada especie.

Analizar cuáles son los compuestos que cada especie va liberando durante el proceso de descomposición de la hojarasca y las fracciones que se van acumulando en el suelo

Realizar experimentos de competencia entre las especies estudiadas no sólo comparando un suelo pobre frente a uno rico en nutrientes, sino estableciendo también un parcheado de nutrientes en el suelo, así como tener en cuenta otros índices de competencia y plasticidad fenotípica.

Estudiar en detalle la anatomía y la histología del haustorio, órgano del que se sirve la planta parásita para tomar nutrientes y agua de su hospedador. Además realizar un estudio más exhaustivo en cuanto a rango de hospedadores y ver cuáles son los principales efectos en cada hospedador.

Analizar la implantación de *Osyris* y el establecimiento de la interacción con el hospedador.

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Conclusions

CONCLUSIONS.

1. A thick needle layer of *P. pinea* may have a direct or indirect impact on seed germination and seedling performance of Mediterranean shrub species, by inhibiting both processes. However, a thin needle layer could improve seed germination by establishing temperature and moisture conditions favorable for this process creating a mircosite where seeds may germinate in a better way than exposed to environmental conditions in a constrained habitat as a Mediterranean sand dune ecosystem.

2. The high amounts of needles felt down, remain in the soil for a long time with low decay rates which imposes very low nutrient inputs to Mediterranean ecosystems. Besides, a thick needle layer limits the drop of other species´ litter to the soil.

3. Mediterranean shrubs possess high amounts of recalcitrant compounds, such as cutin and suberin, in their leaves, so their litters decomposes very slowly and not so many nutrients return to the nutrient cycling.

4. *O. lanceolata*, the hemiparasitic dioecious shrub, plays an important role in the nutrient cycling of this nutrient-poor ecosystem, by enhancing litter decomposability of the rest of the species and by releasing high amounts of nitrogen kept in the litters.

5. Near Infrared Spectroscopy (NIRS) stood out as a powerful tool to predict decomposability according to initial chemical properties of the leaves for Mediterranean species.

6. Seedling competition between *Pinus pinea* and Mediterranean shrub did not show differences related to the physiological status, while plasticity in morphological traits was of comparatively higher importance for acclimation to different environmental conditions.

7. An increase in nutrient availability did not enhance shoot elongation in *P. pinea* and under both treatments (with or without nutrient supply) it stood out as the best competitive species when competing with Mediterranean shrub, highlighting the idea that *P. pinea* is a well-adapted species to nutrient-poor soils.

8. The development of a good root system provides an advantage in order to compete with individuals from the same species or to cope with interspecific competitors.

9. Host photosynthesis was no affected by the presence of the hemiparasitic shrub *O. lanceolata*.

10. *O. lanceolata* was able to synthesize proline in the haustoria. This characteristic, and the possession of higher transpiration rates than the host, allow the parasite to maintain lower water potentials compared to the host and, thus, maintaining the transport from the host to the parasite.

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