P +Fe

Efecto de la fuente de fósforo y de la inoculación con diferentes microorganismos sobre la absorción de hierro y fósforo por las plantas

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EFECTO DE LA FUENTE DE FÓSFORO Y DE LA INOCULACIÓN CON DIFERENTES MICROORGANISMOS SOBRE LA ABSORCIÓN DE HIERRO Y FÓSFORO POR LAS PLANTAS

Tesis Doctoral

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La presente Memoria de Tesis Doctoral titulada: "Efecto de la fuente de fósforo y de la inoculación con diferentes microorganismos sobre la absorción de hierro y fósforo por las plantas", realizada por la Diplomada en Ingeniería Técnica Agrícola por la Universidad de Sevilla y Licenciada en Enología por la Universidad de Cádiz, Dña. Ana Mª García López, ha sido realizada bajo mi dirección, y cumple las condiciones exigidas para optar al Grado de Doctor por la Universidad de Sevilla.

Para que así conste, firma la presente en Sevilla, a doce de mayo de dos mil diecisiete.

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LISTADO DE ABREVIATURAS UTILIZADAS

ADP: adenosina difosfato

AIC: akaike information criterion

AMF: hongos micorrízicos arbusculares

ANOVA: analisis de la varianza

ATP: adenosina trifosfato

BCA: agentes de control biológico

BS: *Bacillus subtillis*

C1: carbono posición 1

C18: columna C18

C3: carbono posición 3

C6: carbono posición 6

CA-Fe: Fe extraido con citrato ascorbato

CCE: calcium carbonate equivalent

CFU: colony forming units

Da: dalton

DM: dry matter

DTPA: ácido dietilentriaminpentaacetico

EC: electrical conductivity

Feca/Fecbd: óxidos de hierro pobremente cristalinos

Feca+Fecbd: óxidos de hierro

HPLC: high performance liquid chromatography

IAA: ácido indolacético

Ins6P: inositol 6 fosfato

IP: inorganic phosphorus

MRP: molibdate reactive phosphorus

OP: organic phosphorus

P-Al: fosfatos de aluminio

P-Ca: fosfatos de calcio

P-Fe: fosfatos de hierro

PGN: p-nitrophenyl-b-D-Glucopiranoside

PGPR: rizobacterías promotoras del crecimiento de plantas

Pi: fosforo inorgánico

PiH+Co: cotransportador de fosfato

PMM: microorganismos movilizadores de fósforo

PNP: p-nitrophenyl phosphate

Po: fosforo orgánico

RH: Relative humidity

RP: roca fosfatada

SOM: soil organic matter

spp: especies

RESUMEN

El fósforo (P) es un recurso no renovable y por ello es preciso una mayor eficiencia en el uso del P presente en el suelo y del aplicado como fertilizante. Para esta mayor eficiencia es necesario el desarrollo de diferentes alternativas. Es conocida la capacidad movilizadora de muchos microorganismos estos pueden ser útiles para promover la disolución de fosfatos de calcio insolubles o para aumentar la hidrólisis enzimática de P orgánico. La movilización de P e hierro (Fe) pueden llevarse a cabo de forma conjunta o de forma muy similar, es por ello que es interesante valorar como los microorganismos movilizadores de P pueden afectar también a la disponibilidad de Fe para las plantas, este último elemento (Fe) es un nutriente que puede resultar limitante en el desarrollo de cultivos para muchos suelos principalmente calcáreos. En este contexto, parece razonable plantear una investigación centrada en un empleo efectivo de P, usando para ello formas de P poco disponibles para las plantas haciendo uso de la manipulación de la rizosfera con microorganismos movilizadores de P y estableciendo cuáles son los mecanismos que explican sus efectos sobre la nutrición fosfatada de las plantas e indirectamente sobre la nutrición férrica.

En el primer capítulo de esta Tesis Doctoral, se hace una introducción general a la problemática del P y el Fe en la agricultura, con información sobre la importancia que los microorganismos rizosféricos pueden tener sobre la movilización de estos nutrientes en la rizosfera. En este capítulo, se definen los objetivos de esta Tesis Doctoral, centrándonos en el uso sostenible de los microrganismos que debe implicar, además de una mejora en la nutrición de las plantas, otros beneficios adicionales en su aplicación.

En el segundo capítulo se describe el efecto que tiene la inoculación con *Trichoderma asperellum* T34, sobre la disponibilidad de P en plantas de pepino (*Cucumis sativus* L. cv. Tropico). Los tratamientos incluían tres fuentes de P diferentes en solubilidad (KH_2PO_4, fitato y roca fosfatada). Para simular las condiciones que pueden darse en el suelo, el medio de cultivo consistió en una mezcla de arena silícea en parte recubierta de ferrihidrita como fuente de Fe sobre la que se adsorbió el P, y para para simular suelos calcáreos se uso arena calcárea. *Trichoderma asperellum* T34 aumentó el contenido total de P en las raíces de pepino independientemente de la forma P suministrada y mejoró la acumulación de P en la planta cuando el nutriente se aplicó como fitato (inositol-6-P) o roca fosfatada (RP, básicamente constituida por mineral tipo apatito). La actividad fitasa se vio incrementada con la inoculación de *Trichoderma asperellum* T34 explicando una mayor absorción cuando el P se administró como fitato. En cambio, cuando se suministró como roca fosfatada (RP) la solubilización se favoreció por la acidificación y el aumento de aniones orgánicos en el medio. Se concluyó que T34 puede mejorar la nutrición fosfatada en plantas cuando el medio contiene fitatos o fosfatos de Ca insolubles como formas de P dominantes.

En el tercer capítulo, se estudió la existencia de un umbral de Fe medido como citrato-ascorbato extraido (CA-Fe) para que la inoculación con el agente de biocontrol *Trichoderma asperellum* T34 sea efectiva. Se realizó un experimento en cámara de cultivo con condiciones controladas en cultivo de pepino (*Cucumis sativus* L. cv. Serena), en el que se investigaron dos factores; concentración de Fe creciente en un medio artificial de cultivo (0, 8, 16, 32.5, y 75 mg kg^{-1} de CA-Fe) y la inoculación o no

con *Trichoderma asperellum* T34. El Fe se aplicó como ferrihidrita adsorbida a parte de la arena silícea. El desarrollo de la biomasa aérea de las plantas disminuyó con la inoculación de T34, relacionándose con una disminución de la acumulación de Fe en las plantas. Sin embargo, con la dosis más alta de Fe (75 mg kg^{-1}), las diferencias en biomasa, altura de las plantas y el contenido y concentración de Fe en parte aérea entre plantas inoculadas y no inoculadas no fueron significativas. El umbral de Fe para que la inoculación sea beneficiosa en el medio fue de 37 mg kg^{-1} sin inoculación de T34, en cambio con T34 este umbral era de 65 mg kg^{-1}, lo que implica que, por debajo de este límite, se requiere suministro adicional de Fe para la inoculación con T34 debido a una posible competencia con el microorganismo.

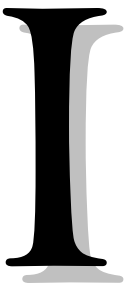
En el cuarto capítulo de la tesis se describe el efecto que la inoculación con diferentes microorganismos (*Bacillus subtillis, Bacillus amyloliquefaciens, Aspergillus niger* y *Trichoderma asperellum* T34) tienen sobre la absorción de P por las plantas y como esta absorción está afectada por el pH y el poder tampón de medios calcáreos de cultivo cuando se utilizan diferentes fuentes de P. Para ello se realizaron tres experimentos en medios de cultivo artificiales con plantas de pepino (*Cucumis sativus* L. cv. Tropico) para evaluar cómo los microorganismos pueden contribuir a la nutrición fosfatada de las plantas: (i) P suministrado a una dosis de 40 mg P kg^{-1} como RP en medio de cultivo con pH neutro; (ii) medio calcáreo tamponado a pH 8.5 y P suministrado como RP a 40 mg kg^{-1}, y (iii) medio calcáreo tamponado a pH 8.5 y P suministrado en dos formas (PR y KH_2PO_4) a una dosis de 200 mg kg^{-1}. Cada microorganismo actuó de manera diferente dependiendo del medio de cultivo, revelando

que la actividad movilizadora de P de los microorganismos y el incremento que provocan en la acumulación en planta está afectada por el pH y por la concentración de P en el medio. En general, los mejores resultados se obtuvieron con *Bacillus subtillis* que aumentó la absorción de P en medio silíceo y en medio calcáreo fertilizado con 200 mg P $kg^{-1.}$

En el quinto capítulo se valoró el efecto que tenía la presencia de óxidos de Fe sobre la adsorción de P por las plantas en medios de cultivo calcáreos cuando éstas eran inoculadas con *Bacillus subtillis* QST713. Para ello se realizó un experimento durante un mes con tres factores: (i) fuente de P, soluble (KH_2PO_4) aplicada a 100 mg kg^{-1}, o insoluble (RP, aplicada a dos concentraciones: 100 ó 200 mg kg^{-1}), presencia de óxidos de Fe (ferrihidrita) en el medio (0 o 300 mg kg^{-1} de Fe extraíble con citrato-ascorbato), e inoculación con *Bacillus subtilis* QST713. El microorganismo mejoró el desarrollo de las plantas e incrementó el contenido total de P y P absorbido por las plantas, adscrito posiblemente a la solubilización del P y al aumento en el crecimiento de las raíces. Este efecto fue independiente de la presencia de óxidos de Fe. También se vio mejorada la absorción de Fe por las plantas. Por lo tanto, *Bacillus subtilis* QST713 mejora la nutrición de P en plantas cultivadas en medios que poseen una alta capacidad de adsorción de P independientemente de la solubilidad de la fuente de P y de la presencia de superficies adsorbentes de P que reducen su disponibilidad para las plantas en el medio de cultivo.

En el sexto capítulo se estudió como las propiedades del suelo, especialmente aquellas propiedades relacionadas con la geoquímica de P del suelo, pueden interferir sobre el

efecto que la inoculación con *Trichoderma asperellum* T34 o *Bacillus subtilis* QST713 puede tener sobre la biodisponibilidad de P en las plantas. Se realizó un experimento utilizando como cultivo el trigo en cámara de cultivo con condiciones controladas. Los tratamientos incluían la inoculación con *Trichoderma asperellum* T34, *Bacillus subtilis* QST713 y un control sin inoculación, sobre una colección de doce suelos con diferentes características físico-químicas y en un rango de P Olsen de 4.8 a 8.7 mg kg^{-1}. De manera general, *B. subtilis* QST713 fue más efectivo que *Trichoderma asperellum* T34, incrementando la absorción de P por las plantas. En cambio, el desarrollo de biomasa de las plantas fue similar para ambos microorganismos. El efecto de los microorganismos en la acumulación de P en la planta se redujo con el aumento de P disponible en la planta y con una mayor proporción de óxidos de Fe poco cristalinos a óxidos cristalinos, y aumentó con la concentración de P orgánico hidrolizable por fitasas en varios extractos del suelo. La fosfatasa se correlacionó con la arcilla y el contenido en óxidos de Fe presentes en el suelo. Además, tanto las actividades enzimáticas medidas como la capacidad de acidificación estuvieron muy influidas por las propiedades de los suelos. Los resultados ponen de manifiesto que las propiedades de los suelos relacionadas con los mecanismos de movilización del P de los microorganismos son cruciales para explicar sus beneficios potenciales sobre los cultivos.



El Real Decreto de 17 de septiembre de 1845, de D. Pedro José Pidal, ministro de Gobernación sienta las bases en España de los estudios de doctorado tal como hoy los conocemos.

CAPÍTULO I INTRODUCCIÓN Y OBJETIVOS

1.1 Relevancia del fósforo y el hierro en la agricultura

El fósforo (P) y el hierro (Fe) se encuentran entre los dieciséis nutrientes esenciales para el crecimiento de las plantas. Son elementos de suma importancia en la agricultura, y su exceso o defecto provoca importantes desórdenes metabólicos. Si existe una deficiencia de estos elementos en los suelos cultivados o si aun existiendo una cantidad suficiente, la planta es incapaz de asimilarlos, el desarrollo del cultivo será inviable. Este hecho lleva en muchos casos a la necesidad de utilizar fertilizantes para solventar las deficiencias nutricionales de estos dos elementos para las plantas.

Según diversos autores (Runge-Metzger, 1995; von Uexküll y Mutert, 1995) en más del 30% de las tierras agrícolas del mundo, el rendimiento agrícola está limitado por la falta de P en el suelo. Según lo descrito por Kirkby y Johnston, (2008) la agricultura intensiva requiere entre 20-35 kg de P por hectárea (ha) y aunque esta cantidad sea aplicada como fertilizante, una mínima parte (raramente más del 25%) es aprovechado por los cultivos (Syers et al., 2007). Gran parte del P aplicado se fija en el suelo, reduciéndose drásticamente su disponibilidad para el cultivo. Estas reacciones de fijación son básicamente mecanismos de adsorción y precipitación y hacen que sólo una mínima parte del P total presente en el suelo sea aprovechado por las plantas (Delgado y Torrent, 1997; Delgado y Scalenghe, 2008).

En el caso del Fe la deficiencia es también un factor limitante en el crecimiento de las plantas. El Fe está presente en grandes cantidades en los suelos, pero su disponibilidad al igual que ocurre con el P es generalmente muy baja. Las plantas

pueden absorber el Fe en sus estados de oxidación Fe^{2+} (hierro ferroso) y Fe^{3+} (hierro férrico). La mayor proporción de Fe en la corteza terrestre está en forma férrica. En cambio, la forma ferrosa es fisiológicamente más importante para las plantas ya que esta forma es relativamente soluble, pero se oxida fácilmente a Fe^{3+}, que tiende a precipitar formando óxidos de muy baja solubilidad implicando una baja disponibilidad para las plantas en los suelos.

1.1.1 Fósforo.

El P es considerado el segundo macronutriente más importante después del nitrógeno (N) para el crecimiento y desarrollo de las plantas. Este elemento se ve involucrado en procesos metabólicos importantes como la producción de energía, fotosíntesis, glicólisis, respiración, activación y desactivación de enzimas, reacciones de óxido-reducción, metabolismo de los carbohidratos, y fijación de N. Además, es un elemento estructural de los ácidos nucleicos y de los fosfolípidos de las membranas. Por todo ello es considerado un factor limitante para la producción agrícola (Fageria y Baligar, 2005), especialmente en los cultivos de cereales (Zebarth et al., 2009, Luce et al., 2011). Se puede decir que el P es un elemento abundante existiendo un reservorio de $4x10^{15}$ toneladas métricas en la corteza terrestre. Este elemento se encuentra principalmente en la materia orgánica dispersa, en los sedimentos y rocas sedimentarias, y como mineral apatito de las rocas magmáticas (Keyzer, 2010). Pese a su relativa abundancia en la corteza terrestre (onceavo elemento más abundante), la disponibilidad de P es limitada para los cultivos debido a sus formas químicas dominantes y a sus reacciones en el suelo. Por otro lado la complejidad química y la

variabilidad espacial de este elemento en los suelos dificulta la identificación de las diferentes formas de P complicando la determinación de su disponibilidad para el cultivo (Hsu, 1966; Sawhney, 1973; Webber, 1978; Pierzynski et al., 1990). Diversos autores (Bieleski, 1973; Marschner, 1995; Yang y Finnegan, 2010) han descrito este fenómeno como "la paradoja del fósforo". Esta paradoja reside en que, a pesar de su relativa abundancia, la concentración de P en la solución del suelo es habitualmente inferior a 10^{-4} M y raramente excede de 10^{-2} M. Según resumió Kuo, (1996), el contenido de P total del suelo puede ser relativamente alto, típicamente de 200 a 5000 mg kg^{-1}, pero la capacidad del suelo de hacer disponible este P es muy variable. Esto explica que a pesar de que la reserva de P total en el suelo en algunos casos exceda ampliamente el requerido por el cultivo, estos puedan presentar deficiencia de P. La concentración total de P en los cultivos varía típicamente entre 0.05 y 0.50% de peso seco, siendo las semillas el principal reservorio de este macronutriente, almacenando en forma de ácido fítico. El P tienen la capacidad de formar complejos con la mayor parte de los cationes contenidos en la semilla, Ca^{2+}, Mg^{2+} y K^{+}, y también Fe^{2+} y Zn^{2+} (Maathuis, 2009) hecho de relativa importancia en el desarrollo del cultivo.

1.1.2 Hierro

El Fe es el cuarto elemento más abundante en la corteza continental después del Oxígeno (O), Silicio(Si) y Aluminio (Al), constituyendo alrededor del 15% de la masa de la corteza terrestre. Es, con diferencia, el micronutriente más abundante en los suelos, como constituyente de diversos minerales, entre los que se encuentran los filosilicatos, los óxidos e hidróxidos de hierro, y la siderita. En los suelos muy ricos en materia

orgánica este elemento aparece en forma complejada. El contenido de Fe en suelo suele variar entre el 1 y 5%, y en algunos casos, pueden hallarse valores cercanos al 10%.

Al igual que ocurre con el P, pese a su gran abundancia su baja solubilidad determina una baja biodisponibilidad del Fe. Las plantas requieren en algunos casos más de 1.5 kg de Fe por ha, en el caso de suelos bien aireados en condiciones óptimas la concentración de Fe^{3+} y Fe^{2+} libre es menor de 10^{-15} M. Esta concentración está muy por debajo del valor crítico para asegurar una adecuada nutrición de las plantas (Marschner, 1995). Según Lindsay y Schwab, (1982), para que las plantas tengan un crecimiento normal este valor se establece entre 10^{-9} y 10^{-4} M de Fe^{3+} en la disolución del suelo. Un hecho constatado es que la disponibilidad de formas de Fe asimilables es muy dependiente del pH del suelo. Así, a pH ácido las formas solubles de Fe son más abundantes. En cambio, en suelos alcalinos y calcáreos con pH superiores a 7 dominan las formas insolubles, y por tanto resulta poco disponible para las plantas. Esto contribuye a explicar su deficiencia en estos suelos. incluso en el caso que la concentración de hierro total en el suelosea alta.

La deficiencia de Fe en el suelo provocará desordenes fisiológicos importantes en las plantas. Este elemento desempeña un importante papel en varios sistemas enzimáticos, donde la hemina funciona como grupo prostético. Entre ellos figuran las catalasas, peroxidasas y varios citocromos. Está implicado también en los mecanismos respiratorios de las células. Otras enzimas como la ferredoxina son importantes en las reacciones de óxido reducción en la planta. Una función muy relevante es la biosíntesis

de la clorofila. Por ello, el efecto más evidente de la deficiencia de Fe en las plantas es la clorosis, que se manifiesta con un amarilleamiento internervial en las hojas jóvenes por falta de los pigmentos fotosintéticos que captan la luz, clorofila y carotenos.

1.2 El fósforo en el suelo

El P en el suelo puede estar en forma orgánica o inorgánica. Ambas pueden estar en la disolución del suelo o asociadas a partículas minerales o a compuestos orgánicos del suelo. Se ha descrito que el porcentaje de P total que corresponde a formas orgánicas varía habitualmente entre un 20 y un 80%. El P orgánico está asociado a la materia orgánica básicamente como ésteres del ortofosfato (Saavedra y Delgado, 2005; Recena et al., 2015).

Por otro lado, las formas disociadas del ácido ortofosfórico son las formas dominantes de P inorgánico. Se considera que estas son las formas de P disponibles para las plantas cuando están presentes en la disolución del suelo o equilibrio con la misma.

En la naturaleza, el P inorgánico procede de minerales primarios presentes en el suelo o de la descomposición y transformación del fósforo orgánico realizada por diferentes bacterias y hongos. Como se ha descrito en el apartado 1.1.1, tan solo una pequeña fracción de este P inorgánico es soluble y asimilable por las plantas. La concentración del ion ortofosfato en el suelo típicamente oscila entre 0.1 y 10 μM (Mengel y Kirkby, 2001). Estas concentraciones están muy por debajo de las concentraciones consideradas como óptimas para el crecimiento de las plantas (> 10 μM) (Föhse et al., 1988). Los iones presentes en la disolución del suelo dependen del

pH del mismo. Según si el suelo es ácido o alcalino se produce la disociación del ácido ortofosfórico (H_3PO_4) en $H_2PO_4^-$, HPO_4^{-2} o PO_4^{-3}, esta disociación además de al pH también está ligada a la presencia de iones de Ca, Fe, Al y Mg.

1.2.1 Materiales primarios de fósforo

Sin considerar el P acumulado en los seres vivos, el P es un elemento distribuido muy heterogéneamente en la corteza terrestre: la mayor parte se encuentra en depósitos de rocas fosfatadas (Delgado y Scalenghe, 2008). Estas rocas constituyen la fuente de P utilizada en la agricultura y en otras actividades del ser humano. De acuerdo con el American Geological Institute Glosary of Geology, (Gary y McAfee, 1974) una roca fosfatada es una roca sedimentaria compuesta principalmente de mineral fosfatado. El apatito es el décimo mineral más abundante de la tierra, el cual se forma bajo todas condiciones geológicas, ígneas, metamórficas, y sedimentarias variando su composición en función de su geoquímica originaria principalmente en sus formas clorapatito, fluorapatito e hidroxiapatito $Ca_5 (PO_4)_3$ Cl, F, OH.

Cerca del 80% de la producción mundial de roca fosfórica proviene de los depósitos de origen sedimentario marino, un 17% es de origen ígneo y de sus productos derivados de la meteorización y el resto proviene de los depósitos sedimentarios residuales y de tipo guano. Estas rocas son una fuente de fósforo y calcio importante para ser aplicados como fertilizante en los suelos, pero la disponibilidad para ser absorbidas por las plantas es baja, debido principalmente a su baja solubilidad principalmente en condiciones alcalinas, no liberando el suficiente fósforo para el crecimiento y desarrollo óptimo de las plantas. Por ello, es necesario su transformación

industrial en formas más solubles que son la base de los fertilizantes fosfatados comerciales habitualmente usados.

En los suelos, el apatito también puede ser una forma importante de P, es más significativo en suelos poco meteorizados. La acción de plantas y microorganismos acaba solubilizando lentamente el apatito, apareciendo más P orgánico en el suelo y, a medida que la meteorización se desarrolla aparecen más óxidos y formas solubles de Fe y Al, una parte importante del P se irá asociando a estos elementos (Figura 1). En los suelos, el P orgánico y el inorgánico se puede encontrar como formas adsorbidas a minerales del suelo, sobre todo a Fe y Al, ocluidas dentro de minerales formados en el suelo (típicamente óxidos), y precipitados como fosfatos metálicos cuya estabilidad y abundancia dependerá del pH del suelo (de Fe y Al en suelos ácidos, y de Ca en suelos básicos) (Ruíz et al., 1997; Delgado y Torrent, 2000; Saavedra y Delgado, 2005; Delgado y Scalenghe, 2008).

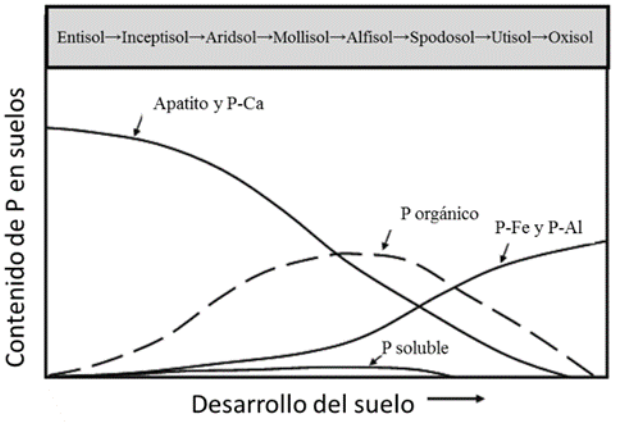


Figura.I.1 Distribución de las formas mayoritarias de P en el suelo relacionadas con el desarrollo del suelo. Adaptada de Foth y Ellis (1997).

1.2.2 Fósforo orgánico

El nivel de P orgánico en los suelos puede ser muy variable. En suelos de ámbito mediterráneo, con bajos contenidos en materia orgánica, se han descrito niveles que oscilan entre el 20 y el 70% del P total del suelo (Saavedra y Delgado, 2005; Recena et al., 2015). En suelos minerales se ha llegado a describir que el P orgánico puede representar hasta el 80% del P total del suelo (Dalal, 1977; Anderson, 1980). Un gran porcentaje de este P orgánico está asociado a moléculas de alto peso molecular, como lípidos, proteinas y glúcidos. Las principales formas de fosfatos orgánicos son ésteres del ortofosfato, básicamente monoésteres o diésteres como los ácidos nucleicos, siendo el monoester inositol-6-fosfato la forma química quizás más abundante en los suelos (Anderson, 1980, Turner et al., 2002, 2003). Tanto el inositol como los ácidos nucleicos parecen tener origen principalmente microbiano, estas moléculas proceden de la degradación de plantas, animales y microorganismos, acumulándose, sobre todo, en los horizontes más superficiales del suelo. El P orgánico podrá ser utilizado o reciclado por microorganismos del suelo para sus necesidades metabólicas o bien sufrir procesos de acumulación en dichos horizontes. Para que el P orgánico pueda ser utilizado por la planta, debe ser mineralizado, lo que implica la rotura de enlaces éster. Este es un proceso de hidrólisis enzimática realizado por enzimas fosfatadas, que pueden ser de origen microbiano o vegetal. La facilidad con que el P orgánico es hidrolizable varía según la fuente, describiéndose desde las formas más fácilmente hidrolizables y por tanto utilizables por la planta hasta las más resistentes a la mineralización y poco disponibles. El myo-inositol-6-fosfato pese a ser, uno de los estereoisómeros

dominantes en el suelo del inositol-6-fosfato, es poco disponible debido a su estable asociación con la fase sólida del suelo (Anderson, 1980; Jørgensen et al., 2015).

La mineralización de la fracción de P orgánico es crucial en suelos pobres en este nutriente, ya que con este proceso de transformación se libera fósforo inorgánico a la disolución del suelo. Esto contribuye al suministro de fósforo disponible para las plantas (Recena et al., 2015).

1.3 Reacciones de fósforo en el suelo

Las plantas absorben el fósforo de la disolución del suelo esencialmente como iones fosfato diácido o monoácido. A medida que la concentración en la disolución baja por la absorción de las plantas, hay una tendencia de desplazamiento del equilibrio existente con la fase sólida, promoviendo la liberación de P adsorbido o precipitado en formas relativamente solubles (Holford, 1997).

La cantidad de P presente en la disolución del suelo está determinada por una serie de reacciones. Estas reacciones son de: disolución-precipitación del P asociado a partículas minerales y de adsorción-desorción del P sobre superficies adsorbentes del suelo e hidrólisis de la materia orgánica (Matar et al., 1992; Comerford,1998; Hinsinger, 2001; Delgado y Scalenghe, 2008) (Figura 2). Además de estas reacciones, el poder tampón de los suelos juega un papel importante para mantener las concentraciones de P en la solución del suelo. El poder tampón de P es la capacidad que tiene el suelo para mantener constante la concentración de dicho elemento a medida que las plantas absorben P (Holford et al., 1980; Recena et al., 2015). Sin embargo, se ha constatado con frecuencia que la disponibilidad de P está inversamente relacionada a la capacidad

tampón, debido a que los suelos con mayor poder tampón son también los suelos donde mayor fracción de P se fija en formas no asimilables (Recena et al., 2017).

1.3.1 Reacciones de disolución-precipitación

Como se ha descrito en apartados anteriores, el pH del suelo determina el tipo de fosfato metal precipitado. En los suelos con pH básico, y particularmente los calcáreos, se pueden formar: fosfato monocálcico [$Ca(H_2PO_4)2H_2O$], fosfato dicálcico ($CaHPO_4 \cdot 2H_2O$), octocálcico [$Ca_8H_2(PO_4)6 \cdot 5H_2O$], tricálcico, e hidroxiapatito [Ca5(PO4)3(OH)] (Lindsay, 1979; Delgado et al., 2002). Se asume que otros apatitos presentes son minerales primarios no formados en los procesos de formación del suelo o a partir de la aplicación de fertilizante (Ruíz et al., 1997; Delgado y Torrent, 2000).

La precipitación de fosfatos de calcio es un proceso importante en suelos calcáreos afectando directamente la disponibilidad del fósforo aplicado como fertilizante al cultivo (Afif et al., 1993; Delgado et al., 2002). En suelos calcáreos del suroeste de España, Solis yTorrent (1989), Ruiz et al. (1997), López-Piñeiro y García-Navarro (2001), encontraron que el P precipitado con Ca, medido como extracción de HCl era la forma de P inorgánico más abundante. La precipitación de fosfatos de Ca empieza con fosfatos relativamente solubles y termodinámicamente inestables (monocálcicos), que se disuelven y vuelven a precipitar como fosfatos cada vez menos solubles y termodinámicamente más estables. Esta secuencia acaba con la formación de hidroxiapatito, la forma termodinámicamente estable en condiciones de suelos calcáreos. El hidroxiapatito es insoluble por lo que su contribución a la nutrición de las plantas es reducida (Delgado et al., 2002; Delgado y Scalenghe, 2008). Las condiciones

de manejo del suelo pueden ser relevantes afectando este proceso. La adición de materia orgánica puede disminuir le velocidad a la que se forman fosfatos de Ca termodinámicamente estables (Delgado et al., 2002; Saavedra et al., 2007).

En cambio, a pH ácido los precipitados formados son fosfatos de Fe y de Al (Lindsay et al., 1989), del tipo estrengita [$FePO_4 \cdot H_2O$], vivianita [$Fe_3(PO_4)2 \cdot H_2O$], variscita [$AlPO_4 \cdot H_2O$] y varios del grupo de la plumbogumbita [$(PbAl_3(P4)\ (P_3OH)(OH)_6$]. La reducida solubilidad de estos compuestos también condiciona su disponibilidad para las plantas.

1.3.2 Reacciones de adsorción-desorción

Diferentes constituyentes del suelo pueden actuar como adsorbentes del P. Esta adsorción se realiza esencialmente mediante reacciones de intercambio de ligandos, formándose complejos de esfera interior en superficies hidroxiladas de carga variable (Sposito, 1989). Existen diversos compuestos orgánicos e inorgánicos en el suelo que pueden contribuir a la adsorción del P. En suelos ácidos, la adsorción de P está generalmente atribuida a los "óxidos" e "hidróxidos" de Fe y Al (Figura 2). También se ha constatado la relevancia que pueden tener complejos organometálicos en la adsorción de P en suelos ácidos (Delgado y Torrent, 1997). En suelos formados bajo clima mediterráneo, los óxidos de Fe son las principales superficies adsorbentes (Peña y Torrent, 1984; 1990). En estas condiciones se considera que los óxidos de Fe cristalinos son los más relevantes como superficies adsorbentes. Sin embargo, la contribución de óxidos de Fe pobremente cristalinos parece que puede afectar significativamente el equilibrio del P inorgánico adsorbido con la disolución del suelo

(Domínguez et al., 2001; Recena et al., 2015; 2016). Los bordes hidroxilados de minerales de la arcilla, y la calcita también pueden actuar como superficies adsorbentes, generalmente con menor relevancia que los óxidos.

La adsorción de P también dependerá de la cantidad de materia orgánica del suelo, ya que la materia orgánica compite por los lugares de adsorción con el P (Saavedra et al., 2007). La materia orgánica presente en los suelos puede actuar también como adsorbente del P de la solución. La capacidad de esta materia orgánica para adsorber P depende de la complejación de cationes de Fe, Al y Ca. En ausencia de dichos cationes, la materia orgánica compite por los lugares de adsorción de P (Afif et al., 1995).

La adsorción de P por las superficies adsorbentes también está afectada por otras propiedades del suelo, como la salinidad o la composición de cationes de la disolución del suelo (Delgado et al., 2010). Además, el equilibro del P en el suelo puede verse afectado por variaciones del pH. Los cambios en el pH pueden favorecer la disolución de los precipitados de fosfato cuando las formas precipitadas dejen de ser termodinámicamente estables (Jurinak et al., 1986). También afectan la adsorción de iones de P a otros constituyentes del suelo (Rajan et al., 1996) y/o la retención en compuestos orgánicos que complejen el Ca, Fe o Al.

1.3.2.1 Óxidos de hierro

El hierro (Fe) es uno de los elementos más abundantes en los suelos agrícolas. Este elemento se encuentra, entre otras formas, como óxidos o hidróxidos de hierro

(Schwertmann y Taylor, 1989). Los óxidos pueden ser heredados del material parental del suelo o pueden ser minerales de neoformación provenientes de la meteorización de minerales primarios en el propio suelo.

Existe una relación compleja entre los óxidos de Fe y otros compuestos orgánicos e inorgánicos del suelo. Parte de la materia orgánica en el suelo puede estar adsorbida a los óxidos de Fe. Estos óxidos suelen presentarse en el suelo, infiltrados en los propios agregados de arcilla o como recubrimiento de minerales primarios y secundarios. La interacción de los óxidos de hierro con las arcillas depende del pH. A pH bajos, los óxidos se precipitan sobre la superficie de los minerales arcillosos y, una vez formados estos revestimientos, son estables a pH elevados (Peacock y Rimmer, 2000). Por otro lado, los hidróxidos presentes en el suelo son el resultado directo de la oxidación del hierro ferroso, que con el tiempo es meteorizado y convertido en hidróxido férrico, $Fe(OH)_3$.

En general los óxidos e hidróxidos de hierro más comunes en los suelos son la goethita (FeOOH), la hematites (Fe_2O_3), la ferrihidrita (u óxido férrico hidratado) ($F_5O_8H•4H_2O$), el oxihidróxido férrico amorfo ($Fe_2O_3·nH2O$), la lepidocrocita (FeOOH), la maghemita (Fe_2O_3) y la magnetita (F_3O_4). Los tres últimos óxidos están habitualmente en menor proporción que los otros (Schwertmann, 1991). Todas estas formas difieren únicamente en la distribución espacial de la unidad estructural básica: el octaedro $Fe(O,OH)_6$ (Lindsay, 1985).

En muchos suelos ácidos, los óxidos, hidróxidos y oxihidróxidos de hierro constituyen un grupo de coloides que son capaces de adsorber cantidades importantes

de fosfato y metales traza y además tienen una importante influencia en las concentraciones de los mismos en la solución del suelo. También afectan la carga eléctrica y las propiedades de la superficie del suelo e incrementan la capacidad amortiguadora del mismo.

La adsorción de metales traza y de fosfatos se ve afectada por la presencia de las diferentes formas de hierro, por el pH y la materia orgánica (Zhang et al., 1997). En particular, la presencia de óxidos de hierro implica un aumento de la capacidad de adsorción de los fosfatos del suelo debido a un aumento del área superficial (Prasetyo y Gilkes, 1994). En ellos los grupos hidroxilos o moléculas de agua, son remplazadas por un anión fosfato, formándose un complejo binuclear bidentado (Reddy et al., 1999; Torrent, 1997; Colombo et al., 1994; Goldberg y Sposito, 1985; Parfitt y Russell, 1977; Parfitt et al., 1975).

La capacidad de adsorción de los fosfatos a los óxidos de hierro tiene generalmente, valores promedios 2,5 μmoles P m^{-2} y rangos de 1.5 a 3.5 μmoles P m^{-2} (Reddy et al., 1999; Torrent, 1997; Enyard, 1994; Torrent et al., 1990; Borggaard, 1983). Cuanto mayor sea la superficie específica de los óxidos, mayor será la capacidad de adsorber P por unidad de masa. Esta superficie específica aumenta cuanto menor es el grado de cristalinidad de los óxidos. Los complejos de fosfato formados son muy estables y dan lugar a tipos de cambio lento y una aparente irreversibilidad (histéresis) de la adsorción del fósforo, produciendo un almacenamiento de fosforo en los suelos, sedimentos y humedales a largo plazo (Reddy et al., 1999; Goldberg y Sposito, 1985; Parfitt et al., 1975). Los principales causantes de esta histéresis en la adsorción de P

son: (i) la lenta cinética de liberación del P, debida en parte a los microporos del suelo (Van der Zee y van Riemskijk, 1991) (ii) las formas de P que se adsorben (mayor histéresis en algunas formas de P orgánico) o que pueden precipitar, y (iii) el grado de saturación de las superficies adsorbentes en los suelos (Torrent y Delgado, 2001).

En el caso de los suelos típicos de ámbito Mediterráneo, pese a que los óxidos pobremente cristalinos representan un pequeño porcentaje de la totalidad de los óxidos de Fe, contribuyen más que los cristalinos al suministro de Fe a la planta (De Santiago y Delgado, 2006). Este hecho se ha confirmado en cultivos de suelos calcáreos (sorgo y soja; Loeppert y Hallmarck, 1985), (garbanzo y girasol; Del Campillo y Torrent, 1992), en olivo (Benitez et al., 2002) y en altramuz (De Santiago y Delgado, 2006, 2007). Esta mayor capacidad de ser movilizado el Fe presente en óxidos amorfos se atribuye a la mayor superficie específica de estos compuestos.

1.3.2.2 Otras superficies adsorbentes de fósforo

Además de los óxidos de Fe y Al, otros minerales presentes en el suelo pueden actuar como adsorbentes del P. Entre ellos se encuentra la calcita la cual posee una reducida superficie específica (<1 $m^2\ g^{-1}$); (Sø et al., 2011) (Figura 2). La superficie especifica es el factor principal que hay que tener en cuenta en la adsorción de P (Madsen, 2001), junto a la actividad de $Ca^{2+,}$ $CO3^{2-}$ (Zachara et al., 1993) y H^+ (Somasundaran y Agar, 1967). Las moléculas de H_2O, HCO^{3-} o H^+ son sustituidas en la reacción de adsorción (Matar et al., 1992). Esta sustitución dependerá de la superficie ocupada y del número de grupos reactivos (Matar et al., 1992). En estas reacciones se produce un cambio de ligando con los grupos (OH)H coordinados de los iones situados

en el borde del cristal (White, 1981). En todo caso la adsorción de P por la calcita es inferior a la de los óxidos de Fe (Borrero et al., 1988; Solís y Torrent, 1989).

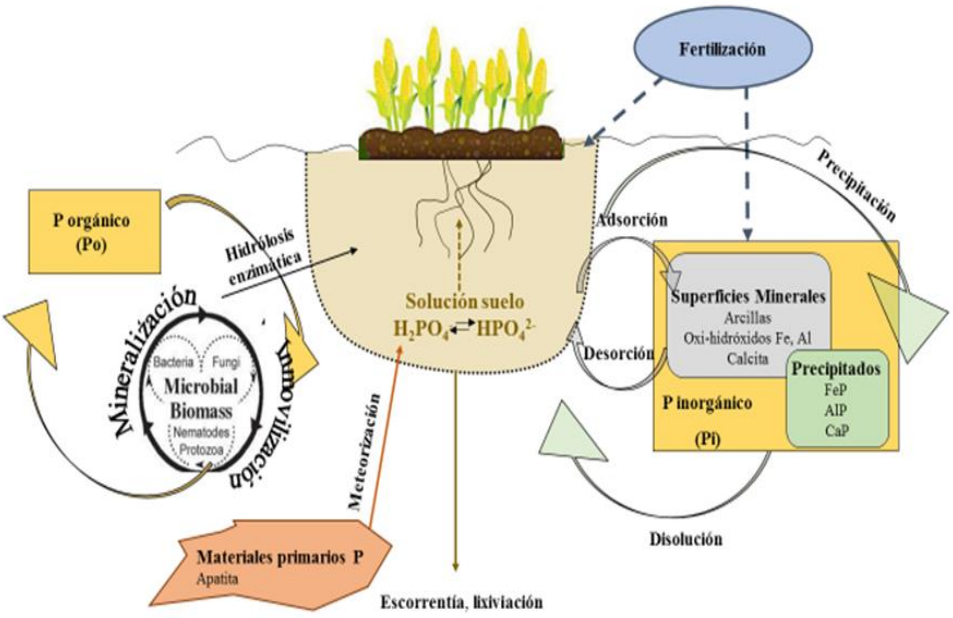


Figura I.2 Ciclo del P en el suelo.

1.4 Interacción del fósforo con otros nutrientes

La nutrición de las plantas no se puede contemplar de manera aislada, ya que hay interacciones entre los distintos nutrientes minerales. Estas interacciones pueden resultar en sinergias o antagonismos. El antagonismo implica que la presencia elevada de un nutriente afecta negativamente la absorción o utilización de otro. Este efecto entre nutrientes se puede dar a nivel del suelo, rizosfera o en la propia planta (Zhang et al,

2006). El antagonismo nutricional implica un desorden que no es producido por la falta del elemento en cuestión sino por un mal equilibrio con el resto de nutrientes en el suelo, en el interior de la planta o en ambos. Por tanto, el exceso de unos nutrientes pueden tener efectos sobre la adsorción de otros nutrientes, afectando de manera directa a de las plantas. De esta manera la fertilización excesiva con un determinado nutriente puede provocar la deficiencia de otros.

En el caso del P, un exceso de mismo interacciona negativamente con la mayoría de los micronutrientes (Fe, Mn, Zn y Cu), bien por la formación de precipitados en el suelo o por procesos metabólicos en las plantas que impiden la translocación de los nutrientes desde la raíz al resto de partes de la planta (Brown y Tiffin, 1962; Verma y Minhas, 1987; Zhu et al., 2002; James et al., 1995; Zhang et al., 2015).También se ha encontrado una disminución en la disponibilidad de azufre y calcio cuando se aplican grandes cantidades de fosfatos, en el caso del calcio por la formación de fosfatos insolubles.

1.4.1 Efectos del fósforo sobre la disponibilidad de hierro. Antagonismo fósforo - hierro

El P y el Fe son elementos que se encuentra en el suelo en una continua interacción. Las cantidades de óxidos de Fe presentes en el suelo afectaran de manera directa a la disponibilidad de P por su efecto en la dinámica de este último en el suelo. Por otra parte, la acumulación de P en el suelo afecta también la disponibilidad de Fe para las plantas. La adsorción de los iones fosfato a los óxidos de Fe ha sido ampliamente estudiada (Cornell y Schwertmann, 2003; Torrent, 1987; Schwertmann y

Taylor, 1989, Bigham et al., 2002) debido a la alta afinidad que el fosfato tiene por los grupos hidroxilos presentes en las superficies de estos minerales. Sin embargo, los iones fosfatos adsorbidos inhiben de manera general la disolución de Fe y por tanto la disponibilidad para las plantas (Borggaard,1991; Celi et al.,2003; Cornell y Schwertmann, 2003). Sánchez-Rodríguez et al,. (2013) observaron que el fosfato adsorbido en óxidos incrementaba la incidencia de la clorosis férrica cuando había poca concentración de óxidos de Fe en el medio de crecimiento. Estos autores concluyeron que la solubilidad de los óxidos de Fe, fuente de Fe para las plantas, disminuía al incrementarse la cobertura de su superficie por fosfato adsorbido. La adsorción de fosfato en los óxidos puede evitar la adsorción de diferentes agentes complejantes como ácidos orgánicos y sideróforos, que son cruciales para la liberación de Fe de las superficies de los óxidos de Fe como, citrato (Geelhoed et al., 1999) o ácido mugineico (Hiradate y Inoue, 1998).

Marschner y Schropp, (1977) demostraron que la aplicación de altas dosis de fertilizante fosfatado inducía la clorosis en viña y Sánchez-Rodríguez et al., (2013) constataron que la fertilización fosfatada disminuía el contenido en clorofila de olivos. Summer y Farina, (1986) y Fageria et al., (1990) también describieron como altas aplicaciones de P provocaban deficiencias de Fe a las plantas. Además de la disminución de la disolución de los óxidos por el fosfato adsorbido, otros mecanismos que explican de reducción del Fe por aplicación de P pueden ser la inhibición de la absorción de Fe por las raíces, inhibición del transporte de Fe desde las raíces a los

brotes, y la inmovilización interna del Fe en la planta (Ayued, 1970; Fageria, 2001; Elliott y Lauchli: 1985; Moraghan y Mascagni, 1991).

1.4.2 Antagonismo fósforo - zinc

En el caso del zinc (Zn) una alta disponibilidad de P para las plantas induce deficiencias de este micronutriente (Adriano y Murphy, 1970). Fageria, (2001) sugiere que en el caso de suelos calcáreos, donde el Zn es normalmente deficiente o poco asimilable por las plantas, la adición de P induce a la deficiencia de Zn. Estos autores consideran que la interacción ocurre a nivel metabólico en la planta.

El Zn es absorbido por las plantas como catión (Zn^{2+}) y el P como aniones de fosfato (sobre todo, $H_2PO_4^-$ ó HPO_4^{-2}). Estas formas químicas se atraen, lo que facilita la formación de enlaces químicos que pueden formarse en del suelo o en la raíz de la planta. Como consecuencia se produce una deficiencia de Zn inducida por P. Marschner, (2002) observó una concentración menor de Zn en brotes y un crecimiento de la planta retardado cuando había altas cantidades de P. Sánchez-Rodríguez et al., (2017) pusieron de manifiesto que la acumulación de Zn en planta se veía afectada no sólo por el nivel de Zn del suelo, sino también por la disponibilidad de P en el suelo, y que el Zn extraíble por DTPA sólo afectaba el rendimiento en suelos donde la disponibilidad de P no era limitante para el rendimiento del cultivo. Para Ova et al., (2015), hay una razón asociada a microorganismos del suelo en el antagonismo entre P y Zn ya que el efecto negativo del P en la absorción de Zn en trigo se debía, al menos en parte, a una inhibición de la colonización de las raíces por micorrizas.

1.5 El fósforo como recurso no renovable y la necesidad de una mayor eficiencia en su uso

El P es un elemento fundamental para el desarrollo de la vida y en particular ligado al desarrollo de la agricultura. Sin embargo, su fuente natural, mayoritariamente rocas fosfatadas, es finita. Hace 160 años se pusieron a la venta los primeros fertilizantes fosfatados y desde entonces su uso ha ido aumentando de manera progresiva. Por ello la reserva de P en el planeta ha ido disminuyendo considerablemente. Además de su esperado agotamiento, otro de los problemas es que las reservas de P están muy concentradas geográficamente, por lo que unos pocos países tienen el control de la oferta de esta materia prima. Casi el 90% de las reservas estimadas se encuentran en cinco países: Marruecos (el primer exportador mundial), China, Argelia, Siria y Sudáfrica, según el Servicio Geológico de los Estados Unidos.

Se estima que el crecimiento de la población mundial alcanzará los 9 billones de personas en 2050 (Bruinsma, 2009) y como consecuencia se espera un crecimiento proporcional de las necesidades de alimentos y, con ello, de los fertilizantes aplicados al suelo. Se prevé que en 2035 la demanda de estos fertilizantes superará la oferta (Cordell et al., 2009). Según Bruinsma, (2009) y Craswell et al., (2010), esta demanda en el año 2050 seráun 66 % superior al consumo en 2005/2007. Según la Iniciativa Mundial de Investigación del Fósforo, “todos los sistemas agrícolas modernos dependen de entradas continuas de fertilizantes fosfatados derivados de roca fosfórica”, un elemento no reemplazable, cuyas reservas, al ritmo actual de explotación, podrían agotarse en este siglo (Steen, 1998; Smil, 2000; Gunther, 2005; Cordell et al., 2009). Al

aumentar la demanda de fósforo los precios subirán y los costes de producción de fertilizante fosfatado también. Además, el progresivo agotamiento de reservas implicará el uso de fuentes de menor calidad. Por tanto, nos encontramos con un panorama en el que la búsqueda de alternativas al manejo tradicional de la fertilización fosfatada es necesaria. Resultan esenciales estrategias de fertilización más eficientes y un mayor reciclaje del P en los residuos agrícolas, industriales y urbanos (Cordell et al., 2009; Withers et al., 2014). En estas condiciones, la utilización de microorganismos con capacidad para solubilizar las reservas fosfóricas del suelo puede contribuir a un uso más eficiente del fertilizante fosfatado y del P acumulado en el suelo (Antoun, 2012; Zaidi et al., 2014). La participación de los microorganismos en la nutrición fosfatada de las plantas es un hecho reconocido desde hace ya muchas décadas. Sin embargo, la reciente preocupación por el P ha incrementado el potencial interés práctico de su uso. Además de ser en sí mismos, como biomasa, una reserva de P del suelo, los microorganismos pueden promover la mineralización del P orgánico y la solubilización de formas de P inorgánico poco solubles en el suelo. Este potencial uso debe ser una alternativa a investigar.

1.6 Mecanismos de adquisición del fósforo e hierro por plantas y microorganismos

Habida cuenta que en algunos casos el P y el Fe en la disolución del suelo puede ser escasa para cubrir las necesidades de cultivos y microorganismos, éstos han desarrollado mecanismos para la movilización y adquisición de P y Fe a partir de la fase sólida del suelo (Marschner et al., 2011). La movilización del P del suelo implica la

liberación de este elemento desde la fase sólida a la disolución del suelo. Si el P en disolución está en forma orgánica, debe ser además mineralizado para poder ser asimilado por las plantas. En el caso del Fe, este macronutriente se encuentra asociado a la materia orgánica o es secretado en la actividad biológica es por ello que es necesaria una reacción de complejación, que permita su movilización y utilización. Por otra parte, los microorganismos parecen ser más competitivos que las plantas en los procesos de adquisición de nutrientes como el P y el Fe, ya que pueden aprovechar nutrientes movilizados, no solo por ellos mismos sino, por las plantas. En el caso del Fe, los microorganismos son capaces de utilizar nutrientess complejado con compuestos exudados por la raíz y, además, descomponer estos exudados radiculares e inmovilizar los nutrientes como biomasa microbiana antes de que lleguen a la superficie de la raíz.

Los mecanismos para movilizar Fe de la fase sólida del suelo implican la acidificación, reducción y secreción de sustancias complejantes de Fe (Lemanceau et al., 2009; Robin et al., 2008; Sánchez-Alcalá et al., 2011). Esta secreción está frecuentemente asociada a la presencia de complejantes efectivos muy específicos del Fe llamados sideróforos. Se ha comprobado que las plantas pueden utilizar Fe complejado por sideróforos de origen microbiano, y viceversa los microorganismos pueden usar Fe proveniente de exudados de plantas. Bajo deficiencias de Fe, los microorganismos son capaces bien de incrementar la secreción de ácidos orgánicos o de producir sideróforos que complejan el escaso Fe^{3+} presente. Los sideróforos, producidos por los microorganismos son diversos, en el caso de los hongos producen ferricromos y en el caso de bacterias los sideroforos producidos son enterobactina,

piovardina y ferriozaminas. (Neilands, 1984). Los sideróforos bacterianos son usualmente una fuente pobre de Fe para las plantas (Marschner et al., 2011).

Los mecanismos responsables de la movilización microbiana del P mineral son la producción de ácidos orgánicos, la producción de protones (normalmente asociada a la asimilación de NH_4^+ y a los procesos respiratorios), y la producción de ácidos inorgánicos y CO_2. (Marschner, 2008, Marschner y Rengel, 2010, Marschner et al., 2011). Es conocido que similares estrategias son usadas por las plantas para mejorar y movilizar el P existente. Además, las plantas han desarrollado diferentes mecanismos de adaptación morfológica que le proporcionan una mayor adquisición del P y a su vez una mejora en la eficiencia de su utilización interna (Vance et al., 2003; Lambers et al., 2006). La secreción de ácidos orgánicos con capacidad complejante de metales y la acidificación contribuyen también a la movilización del Fe. Por otra parte, mecanismos específicos de movilización del Fe, como la reducción, podrían promover una movilización de P al disolver este metal de la superficie de óxidos, que son las principales superficies adsorbentes de P.

1.6.1 Enzimas

Como se ha descrito en apartados anteriores, el P orgánico que se encuentra en los suelos, necesita ser mineralizado para poder ser asimilado por las plantas y microorganismos rizosféricos. Esta mineralización es llevada a cabo por diversas enzimas de tipo fosfatasa y fitasa. Se estima que la mayor fuente de enzimas fosfatasas es de origen microbiano (García et al., 1992; Xu y Johnson, 1995) y que dentro de los

horizontes del suelo las mayores cantidades de esta enzima se encuentran en la rizosfera (Tarafdar y Junk 1987).

La mineralización de P implica la hidrólisis de enlaces, que libera ortofosfato. Esta rotura puede llevarse a cabo a través de la actividad de tres grupos de enzimas: (i) fosfatasas no específicas (fosfomonoesterasas, fosfodiesterasas y fosfotriesterasas), las cuales actúan mediante la catalización de la hidrólisis de los enlaces fosfoéster o fosfoanhidros (Vincent et al., 1992); (ii) fitasas, las cuales liberan el P del ácido fítico (inositol-6-fosfato) y (iii) fosfonatasas y liasas C-P, enzimas que llevan a cabo la rotura del enlace C-P. En el caso de las fitasas, catalíticamente, la mayoría de estas enzimas pertenecen a la familia de las fosfatasas ácidas de histidina. Este grupo de enzimas catalizan la hidrólisis del ácido fítico formando mono, di, tri, tetra y pentafosfatos de myo-inositol y fósforo inorgánico (Lei y Porres; 2003). Esta hidrólisis puede ser clasificada en dos grupos según el lugar de iniciación de la hidrólisis sobre el anillo de inositol. Las fitasas microbianas, especialmente las de origen fúngico, frecuentemente escinden el grupo fosfato en el carbono C1 ó C3 del anillo de inositol, y son llamadas 3-fitasas. Las fitasas vegetales actúan preferentemente sobre el carbono C6 y se denominan 6-fitasas.

Generalmente, las fitasas producidas por los hongos son extracelulares, mientras que en las bacterias comúnmente son endoenzimas. La mayoría de las fitasas tienen un pH óptimo de funcionamiento en torno a 4.5-6.0, pero en el caso de las fitasas de *Bacillus* spp. sus pH óptimos son neutros o alcalinos (Lei y Porres, 2003). Las

principales actividades enzimáticas corresponden aparentemente a las fosfatasas ácidas y a las fitasas (Quiquampoix y Mousain, 2005; Rodríguez et al., 2006).

La actividad fosfatasa en la rizosfera se considera una respuesta general de las plantas para movilizar P orgánico en repuesta a la deficiencia de P (Richardson et al., 2005). En un gran número de trabajos en ensayos in vitro ha sido confirmada la hidrólisis de sustratos orgánicos y liberación de P por la acción de fosfatasas extracelulares (George et al., 2008; Hayes et al., 1999; Tadano et al., 1993; Tarafdar y Claassen, 1988).Otros estudios también han demostrado como esta hidrólisis de fósforo orgánico y posterior utilización del ion ortofosfato por el sistema radicular también se produce en sistemas de plantas y rizobox.

1.6.2 Exudación ácidos orgánicos

En medios deficientes de P las plantas desarrollan diversos mecanismos de regulación, los genes encargados de movilizar el P del suelo y transportarlo al interior de las plantas son activados (Liu et al., 1998; Sas et al., 2001; Vance et al., 2003). Así, mecanismos como la exudación de carboxilatos por parte de las raíces empiezan a actuar (Figura 3) Se produce un aumento de la concentración de estas moléculas, los ácidos orgánicos son liberados a la rizosfera produciendo un significativo incremento, la concentración de los acidos organicos puede llegar a ser hasta diez veces mayor en el resto del volumen de suelo (Jones et al., 2003). Se asume que estos ácidos movilizan el P inorgánico de los fosfatos precipitados con Fe, Al y Ca, y promueven la desorción de P adsorbido en superficies adsorbentes por intercambio de ligandos (Gerke et al., 2000).

Estos ácidos orgánicos provienen del metabolismo de compuestos de alto peso molecular como carbohidratos, péptidos y lípidos (Baziramakenga et al., 1995). Dependiendo de las propiedades de disociación y del número de grupos carboxilo, los ácidos orgánicos tendrán carga negativa, por lo que pueden formar complejos estables con cationes metálicos Ca^{2+}, Mg^{2+}, Fe^{3+} yAl^{3+} lo que promueve la solubilización de compuestos precipitados de estos metales (Stevenson, 1967; Sagoe et al., 1998). Además, la acción de los ácidos orgánicos en la solubilización de fosfatos también puede atribuirse a que disminuyen el pH. Estas reacciones pueden prevenir la fijación de fosfatos añadidos al suelo como fertilizantes. Así, se ha demostrado que los ácidos orgánicos reducen la precipitación de fosfato por el hierro y el aluminio (Stevenson 1967; Moghimi y Tate, 1978; Illmer et al., 1995).

Dentro de la familia de los ácidos orgánicos de bajo peso molecular, el cítrico es el ácido orgánico que se puede encontrar con más asiduidad en la rizosfera, teniendo una alta efectividad en suelos calcáreos como complejante de calcio (Dinkelaker et al., 1989) e incluso en suelos ácidos como complejante de Fe y Al (Gardner et al. 1982). La efectividad de los diferentes ácidos movilizando nutrientes va a depender de factores como su forma y cantidad. El citrato y oxalato tienen mayor eficiencia respecto a otros ácidos orgánicos como malato, malonato, succinato, fumarato, acetato y lactato (Bar-Yosef, 1991).

Por otro lado, al igual que las plantas, los microorganismos solubilizadores de fosfatos normalmente producen también ácidos orgánicos. Se considera que la formación de estas moléculas, son el principal mecanismo de solubilización por parte de

los microorganismos (Rodriguez y Fraga 1999; Halder et al., 1990). Para la mayoría de las bacterias, se ha demostrado que la mayor parte de su capacidad para solubilizar el P inorgánico está estrechamente relacionada con la producción de ácidos orgánicos. Este hecho se debe a la acidificación asociada y la capacidad de estos compuestos para complejar iones Ca^{2+}, Fe^{3+} y Al^{3+} (Chuang et al., 2007).

De manera general, la solubilización por parte de los hongos se produce por la liberación de ácido cítrico, glucónico y oxálico. Chuang et al., (2007), demostró que la fuente de P insoluble utilizada en el medio de cultivo, determina el tipo de ácido orgánico producido por el hongo responsable de la solubilización. Es por ello que cuando el P era suministrado como Ca-P, el principal ácido orgánico producido era el ácido glucónico, mientras que cuando el P era suministrado como Al-P o Fe-P, la mayor producción de ácidos orgánicos correspondió al ácido oxálico.

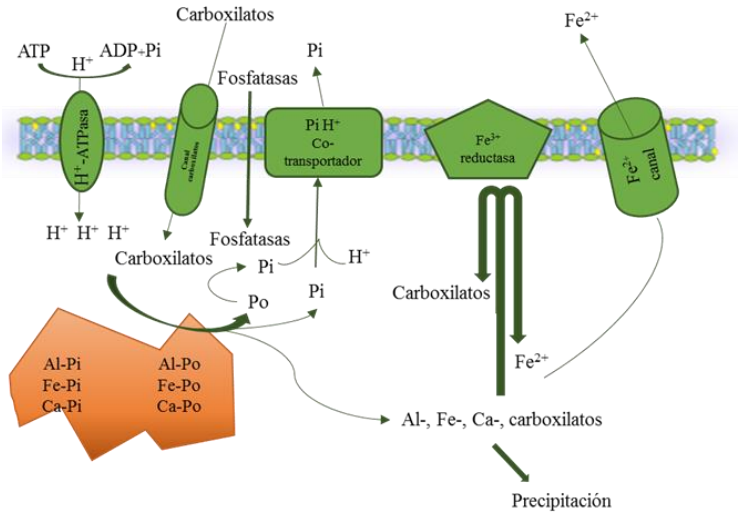


Figura I.3 Efecto de los carboxilatos y otros exudados radiculares sobre la movilización del P inorgánico (Pi) y el P órganico (Po). Adaptada de Lambers et al., 2006.

1.6.3 Mecanismos de adquisición del fósforo específicos de plantas

1.6.3.1 Raíces

La difusión es el principal mecanismo por el que el P llega a las raíces de las plantas (Jungk, 1994; Kirkby y Johnston, 2008). Este proceso, generalmente, es un proceso muy rápido que surge por gradiente de concentración entre la disolución del suelo y el citoplasma de las células radiculares (Schachtman et al., 1998; Amtmann et al., 2006). Ante una deficiencia de P en los tejidos, las plantas, responden con una mayor movilización de los productos provenientes de la fotosíntesis en a las raíces, generando un aumento en la proporción de la biomasa radicular respecto a la biomasa de la parte aérea (Hermans et al., 2006; Kirkby y Römheld, 2006). Al aumentar la biomasa radicular se produce una mayor cantidad de suelo rizosférico explorado, acompañado de cambios morfológicos como son la reducción en el crecimiento de la raíz primaria y la aparición de raíces secundarias acompañadas de una gran cantidad de pelos radiculares (Föhse y Jungk, 1983; Bates y Lynch, 1996; Gilroy y Jones, 2000; Lynch y Brown, 2001). Estos cambios morfológicos conllevan costes metabólicos (Hetrick, 1991) y están regulados hormonalmente (López-Bucio et al., 2002; Vance et al., 2003). Otra adaptación por parte de la planta, es la formación de estructuras radiculares especializadas denominados “cluster”. Este cambio morfológico se desarrolla principalmente en varias especies que crecen en suelos con poca disponibilidad de P.

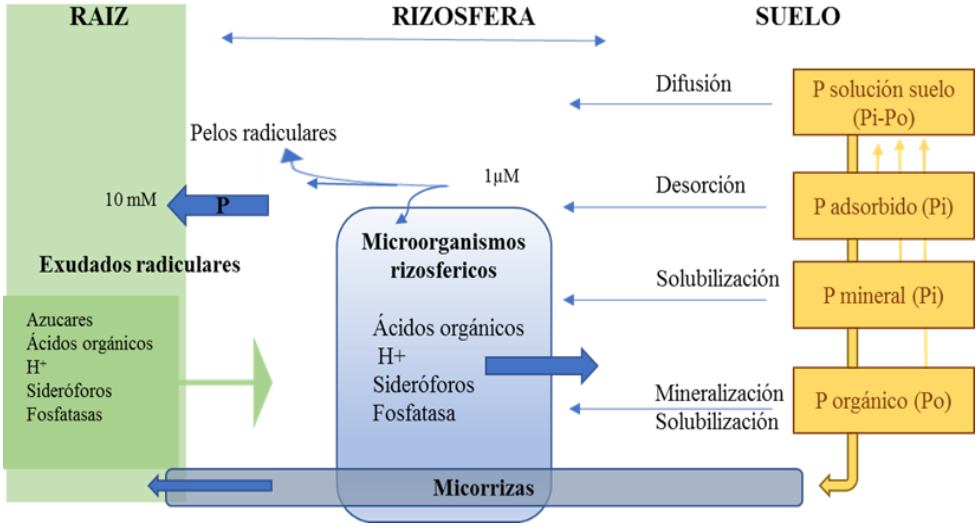


Figura I.4 Procesos físico-químicos que influyen en la disponibilidad del P en la rizosfera. Adaptada de Richardson, 2001.

1.6.3.2 Simbiosis con micorrizas

La simbiosis entre plantas y micorrizas se encuentran en casi todos los ecosistemas y pueden mejorar el crecimiento de las plantas a través de una serie de mecanismos que incluyen, un aumento de la captación de nutrientes, en particular, P, y otros micronutrientes esenciales tales como Zn, Cu K, Ca y Mg, en función del pH del suelo, (Clark y Zeto, 2000). Esta simbiosis también puede promover la protección contra otros tipos de estrés abióticos y bióticos y mejorar la estructura del suelo (Buscot, 2005; Smith y Read, 2008). Es un hecho bien conocido como los hongos micorríticos contribuyen significativamente a la nutrición fosfatada de las plantas, sobre todo en condiciones de deficiencia de P (Barea et al., 2008).

1.6.4 Mecanismos de adquisición de hierro específicos de plantas

Las plantas pueden clasificarse según los mecanismos desarrollados para la adquisición de Fe, en plantas de estrategia I o estrategia II (Chaney et al., 1972; Marschner et al., 1986; Römheld, 1987; Bienfait, 1988; Brown y Jolley, 1988; Hopkins et al., 1992; Marschner y Römheld, 1995). Las plantas de estrategia I, tienen desarrollados mecanismos específicos para la adquisición de Fe. Estas plantas potencian la actividad de la enzima Fe(III)-reductasa que se encuentra en la membrana plasmática radicular, la cual reduce el Fe (III) por el cual estas plantas obtienen hierro descomponiendo los quelatos de Fe (III) y reduciendo el Fe (III) a Fe (II) para que entre en la célula (Moog y Brüggemann, 1994; Robinson et al., 1999; Schmidt, 1999; Rombolà et al., 2002). Otro mecanismo por el cual estas plantas obtienen hierro es mediante la extrusión de H^+ por las raíces mediante la enzima H^+-ATPasa (Brown, 1978; Toulon et al., 1992). La extrusión de H^+ va unida a cambios morfológicos en la raíz, como son el incremento de raíces laterales y el engrosamiento de las zonas subapicales, que permiten una mayor absorción del Fe al aumentar la superficie de contacto (Kramer et al., 1980; Welkie y Miller, 1993; Schmidt, 2001). En último lugar, se produce la biosíntesis y excreción por parte de las raíces de ácidos orgánicos como oxalato, malato y citrato (Ström et al., 2005) azúcares reductores, aminoácidos, compuestos fenólicos y flavinas.

Las plantas de estrategia II o gramíneas tienen mecanismos que se basan en la biosíntesis y secreción en la zona radicular de agentes quelantes altamente específicos y efectivos para solubilizar el Fe (III) de los compuestos inorgánicos del suelo (Marschner y Römheld, 1995). Son denominados fitosideróforos y su actuación se basa

en la formación de un complejo Fe (III)–fitosideróforo, el cual es transportado mediante un trasportador específico situado en la membrana plasmática de las células apicales de la raíz (Curie et al., 2001), hacia el citoplasma de las células radiculares. (Takagi, 1976; Marschner, 1986, Ma et al., 1993; Von Wirén et al., 2000). Una vez que el complejo se encuentra en el citosol, el Fe (III) es liberado y el fitosideróforo se degrada o es excretado al exterior nuevamente. Bajo deficiencias severas de Fe, la producción de fitosideróforos representa el 50-90% del incremento de exudados en la punta de la raíz (Fan et al 1997, Marschner et al., 2011).

1.7 Uso de microorganismos en la agricultura

La rizosfera se extiende a solo unos pocos milímetros de las superficies radiculares (Bringhurst et al., 2001) pudiendo ser definida como cualquier volumen de suelo influenciado por las raíces de las plantas y/o asociado a las raíces y pelos radiculares, y a el material producido por la planta (Andrade et al., 1997; Mahaffee y Kloepper, 1997; Bringhurst et al., 2001). La rizosfera representa el volumen de suelo con mayor actividad microbiana debido a la secreción de exudados radiculares. Sin embargo, la estructura de las comunidades microbianas rizosféricas son diferentes a las del resto del suelo (Foster 1986, Marielly y Aragno, 1999, Gomes et al.,2001, Berg et al., 2002). En el suelo rizosférico la cantidad de aminoácidos y azucares provenientes de los exudados radiculares es mayor que en otras zonas del suelo, sirviendo como nutriente y fuentes de energía para los organismos que se encuentran en este. Como consecuencia de esto, a medida que nos acercamos a la superficie radicular el número de microorganismos aumenta, pudiendo alcanzarse $1,2x10^{8}$ células cm^{-3} o 10^{9}-10^{12}

células microbianas g^{-1} suelo a una distancia de 10 µm de la superficie de la raíz (Gray y Smith, 2005). La abundancia de exudados radiculares se explica en parte como mecanismo para movilizar nutrientes del suelo, y son también efectivos estimulando el crecimiento microbiano (Marschner et al., 2011).

De igual manera que los organismos rizosféricos se benefician de los exudados radiculares, las plantas obtienen beneficio de los microorganismos presentes en el suelo, dándose una estrecha relación suelo-planta-microorganismos (Figura 4). Las asociación microbianas con las raíces son complejas y pueden mejorar la habilidad de las plantas para adquirir nutrientes del suelo a través de varios mecanismos: i) un aumento de la biomasa radicular, bien por extensión directa (por ejemplo, asociaciones con micorrizas) o mediante la mejora de crecimiento de las raíces, y/o desarrollo de pelos radiculares (por ejemplo, rizobacterias que producen auxinas), (ii) una contribución directa a la disponibilidad de nutrientes, como fijación de N (por ejemplo, los rizobiosdiazotróficos) o por estimulación y/o contribución a los procesos metabólicos que movilizan nutriente de fuentes poco disponibles (por ejemplo, aniones orgánicos), (iii) un efecto indirecto sobre la disponibilidad de nutrientes por desplazamiento de los equilibrios de adsorción que producen un incremento en la transferencia de nutrientes a la solución del suelo, y (iv) a través de la recuperación de nutrientes de la biomasa microbiana de la rizosfera. (Gyaneshwar et al., 2002; Jakobses et al., 2005; Kucey et al., 1989; Richardson, 2007; Tinker, 1980).

En cambio, los microrganismos también pueden disminuir la disponibilidad de nutrientes, como el P, para las plantas por diferentes procesos: (i) inmovilización de P a

la biomasa microbiana, (ii) descomposición de compuestos movilizadores de P generados por las raíces, (iii) inhibición del crecimiento radicular o colonización micorrítica, y (iv) contrarrestando la disminución de pH inducida por la raíz por el consumo de protones (Marschner et al., 2011).

Se sabe que la manipulación de las comunidades microbianas asociadas a plantas puede contribuir a protegerlas de enfermedades o promover el crecimiento de las plantas mediante diferentes mecanismos (Chanway, 1998; Cook, 1990; Gilbert y Parke, 1993). Los bio-recursos se pueden definir como cualquier material orgánico aplicado al suelo para mejorar la calidad del suelo, el suministro de nutrientes y el crecimiento de las plantas (Owen et al., 2015).

Desde hace varias décadas las micorrizas han sido utilizadas como biofetilizantes y promotores del crecimiento y particularmente desde los años 50 también se han usado para mejorar la absorción de P (Koide y Mosse, 2004; Malusa et al., 2012). El estudio y utilización de bacterias promotoras del crecimiento (PGPRs) en agricultura es un campo ampliamente estudiado. Pueden ser divididas como PGPRs intracelulares, las cuales residen en el interior de las células de las plantas, y PGPRs extracelulares. Éstas últimas viven fuera de las células de las plantas y no producen nódulos, pero mejoran el crecimiento de las plantas por la producción de compuestos que favorecen el desarrollo de las plantas (como auxinas), mejorando la resistencia a enfermedades o mejorando la movilización de nutrientes del suelo (Gray y Smith, 2005). Las PGRPs extracelulares pueden dividirse en tres tipos en función del grado de asociación al sistema radicular de las plantas: bacterias que viven cerca pero no en contacto con las raíces, las que

colonizan el sistema radicular y las que viven en el espacio entre las células del cortex radicular (Vessey, 2003; Gray y Smith, 2005).

Otros organismos a tener en cuenta en su papel en la agricultura son los agentes de biocontrol (BCA). Sus mecanismos de actuación son complejos, y lo que se ha definido como biocontrol es la sinergia entre diferentes mecanismos de actuación para lograr el control de la enfermedad. Estos mecanismos se basan en la competencia entre el patógeno y el agente de biocontrol por los nutrientes del suelo y/o por el espacio ocupado. El agente de biocontrol tiene, la capacidad de producir metabolitos que impiden la germinación de esporas de los patógenos (los conocidos como fungicida), produce la muerte celular del patógeno (los conocidos como antibióticos) o poseen la capacidad de modificar el pH de la rizosfera, creando un ambiente hostil que impiden el desarrollo de los patógenos.

Son diversas las definiciones para estos biofertilizantes. La categoría de biofertilizantes es comúnmente referida a productos que contienen microorganismos del suelo e incrementan la disponibilidad y/o absorción de minerales y nutrientes para las plantas (Malusa et al., 2012). De acuerdo con la definición de Vessey, (2003), biofertilizantes son productos que contienen microorganismo los cuales aplicados al suelo o a semillas colonizan la rizósfera o el interior de las plantas y promueven el crecimiento al aumentar la disponibilidad de nutrientes primarios a las plantas. Owen et al., (2015), sin embargo, han clasificado estos productos comerciales como bio-inoculantes. Son formulaciones personalizadas que utilizan las funciones de los

microorganismos en una amplia gama de cultivos y tipos de suelo (Roesti et al., 2006; Ahmad et al., 2013).

Muchos biofertilizantes comerciales se basan principalmente en las rizobacterias promotoras del crecimiento de plantas (PGPR) que ejercen efectos beneficiosos sobre el desarrollo de las plantas a menudo relacionados con el incremento de la disponibilidad de nutrientes para la planta (Vessey, 2003). Sin embargo, no todas las cepas de PGPR ejercen un efecto positivo en el crecimiento de las plantas por el aumento de la disponibilidad de nutrientes a la planta (Zehnder et al, 2001). Otras PGPR parecen promover el crecimiento a través de la supresión de enfermedades, o por medio de la producción de fitohormonas y péptidos que actúan como bioestimulantes (Glick et al, 1998; Jiménez-Delgadillo, 2004; Mena-Violante y Olakle-Portugal, 2007).

1.7.1 Microorganismos para mejorar la adsorción de fósforo e hierro por las plantas

Dentro de la gran familia de microorganismos rizosféricos algunos de ellos contribuyen a mejorar la disponibilidad de P para las plantas gracias, en parte, a mecanismos que pueden estar implicados en un efecto de biocontrol o PGPRs, como la producción de hormonas o sideróforos que están íntimamente relacionados con la solubilización de P. Aquellos microorganismos con atributos específicos para movilizar el P inmovilizado en el suelo son denominados microorganismos movilizadores de fósforo (PMM) (Owen et al., 2015). Son varios los estudios realizados sobre la solubilización de fosfatos por parte dediferentes especies microbianas, tanto in vitro

como en condiciones de suelo (Kucey et al., 1989; Rodríguez y Fraga, 1999; Goldstein, 2000; Whitelaw, 2000; Vassilev et al., 2001) con el objetivo de conocer mecanismos desarrollados o las fuentes de nutrientes que estos microrganismos son capaces de solubilizar. Entre las bacterias con esta capacidad se destacan los géneros: *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium, Enterobacter, Pantotea, Klebsiella, Rhodobacter, Arthrobacter, Serratia* y *Erwinia*; en los hongos son de interés los géneros: *Aspergillus, Penicillium, Trichoderma, Fusarium* y levaduras (Khan et al., 2007; Khan et al., 2010, Zaidi et al., 2009).

En ensayos in vitro se han aislado bacterias solubilizadoras de P del género *Pseudomonas, Bacillus* y *Acetobatcter* que producen ácido indolacético, contribuyendo al crecimiento de las plantas. También se ha demostrado que la inoculación con hongos y bacterias solubilizadoras de P pueden incrementar el rendimiento o el crecimiento de las plantas, tanto en condicionesde invernadero como en condiciones de campo (Chuang et al., 2007; Vassilev et al., 2007; Valverde et al., 2007; Wasule et al., 2007; Widada et al., 2007).

Los microorganismos son capaces de hidrolizar un amplio rango de sustratos que contienen P orgánico en cultivos in vitro y cuando son añadidos al suelo (Adams y Pate, 1992; Macklon et al., 1997; Richardson et al., 2005). De hecho, se han demostrado los beneficios adicionales de la inoculación microbiana para la utilización de P orgánico por plantas bajo condiciones controladas de cultivo (Richardson et al., 2001).

La exudación de compuestos quelantes producidos por microorganismos (sideróforos) aumenta la solubilidad del Fe en la rizosfera y por tanto favorece la absorción de Fe por parte de las plantas (Crowley et al., 1988, Crowley, 1992; Walter et al., 1994, Chen et al., 1998; Shenker, 1999; Masalha et al., 2000; Siebner-Freibach et al., 2003). En cambio, esta liberación de Fe no va acompañada de un aumento de la disponibilidad de P para las plantas, como sería lo esperable. No hay evidencias actuales que puedan demostrar que los sideróforos aumenten la disponibilidad de P en el suelo. Sin embargo, varios autores han descrito el aumento de sideróforos en organismos solubilizadores de P (Vassilev et al., 2006; Caballero-Mellado et al., 2007; Hamdali et al., 2008).

Tanto hongos como bacterias producen sideróforos, sustancias de bajo peso molecular (generalmente menor de 1500 Da) que se unen fuertemente al Fe (III) y pueden suministrar dicho elemento a las células microbianas. Pueden ser tanto intra como extracelulares. Un factor muy influyente en la biosíntesis de estos sideróforos es el pH externo. Su producción disminuye al bajar el pH y se incrementa si éste asciende (Eisendle et al., 2004). De ahí la importancia de éstos compuestos en la biodisponibilidad del Fe en medios calcáreos (Kraemer, 2004). Hay muchos trabajos que explican la complejación de Fe por los sideróforos. Shenker et al. (1999), estudiaron la rizoferrina, sustancia secretada por *Rhizopusarrizus,* que es un hongo muy común en la rizosfera. Otros investigadores han usado directamente los sideróforos microbianos en aplicaciones foliares para remediación de clorosis debida a la falta de Fe en la planta

encontrando, en algunos casos, un efecto similar al de quelatos comerciales (Bucheli-Witschel y Egli, 2001; Fernández et al., 2005).

Si la concentración de sideróforos y fitosideróforos es similar, el Fe es complejado preferiblemente por sideroforos microbianos. Incluso estos pueden complejarlos de los complejos Fe-fitosideróforos. Además, el Fe proveniente de los complejos Fe-fitosideróforos o de los complejos Fe-citrato es una buena fuente de Fe para las bacterias, las cuales disminuyen la producción de sus propios sideróforos, aprovechado el Fe ya complejado por compuestos segregados por las plantas (Marscher y Crowley, 1998).

Hay numerosos estudios sobre inoculación con PGPRs que movilizan nutrientes. Sin embargo, estos microrganismos muestran alta movilización de nutrientes in vitro, mejorando también la toma de nutrientes en suelos esterilizados pero su efecto en campo es muy variable (Gyaneshwar et al., 2002). Fundamentalmente esto es debido a la pobre persistencia del inoculo en el suelo por la competición con las comunidades microbianas nativas. Esto da lugar a una rápida disminución de sus poblaciones en la rizosfera (Maschner y Crowley, 1996).

1.7.1.1 *Bacillus* spp.

Bacillus subtilis es una bacteria Gram positiva que se encuentra con asiduidad en los suelos. Se ha descrito su protección frente enfermedades causadas por bacterias y hongos patógenos (Choudhary y Johri, 2009; Pérez-García et al., 2011; Zamioudis y Pieterse, 2012; Lakshmanan et al., 2013). Su aplicación se pude realizar en raíces, bien

por inoculación o por aplicación directa de las esporas sobre la superficie de las raíces o por aplicación directa al suelo, dando protección directa a las plantas frente a enfermedades y también promoviendo el crecimiento de las plantas.

Se ha demostrado que *Bacillus amyloliquefaciens* y *Bacillus subtilis* sintetizan diferentes compuestos los cuales producen un aumento en el desarrollo de la planta, como giberelinas y ácido indolacético (Turner y Backman, 1991), fitasas extracelulares (Idriss et al., 2002), quitinasas (Manjula y Podile, 2001; Wang et al. 2002) y peptinas antifúngicas (Leifert et al., 1995, Pinchuk et al., 2002, Raaijmakers, et al., 2002; Walker et al., 1998). Además, los suelos tratados con *Bacillus* incrementan los niveles de citoquininas en plantas.

Bacillus juega un importante papel en proporcionar P a la nutrición de las plantas (Kurdish, 2001, Menkina, 1950, Roi et al., 2001, Roi et al., 2004). Ciertas cepas de *Bacillus subtilis* son eficaces en mineralizar compuestos de P orgánico y de disolver fosfato cálcico (Roi et al., 2004).

1.7.1.2 *Trichoderma* spp.

En el caso de los hongos*, Trichoderma* es una de los géneros de hongos más estudiado como organismos de biocontrol mediante la estimulación de los mecanismos de defensa de la planta y que también posee actividad como promotor de crecimiento. (Harman y Bjorkman, 1998).

Los mecanismos de biocontrol han sido ampliamente estudiados y son conocidos. En cambio, el mecanismo responsable de la mejora del crecimientode las plantas al ser

tratadas con cepas de *Trichoderma* no ha sido tan estudiado. Altomare et al. (1999) ha descrito la capacidad de potenciar el crecimiento de las plantas y el biocontrol de *Trichoderma harzianum* T22 para solubilizar los minerales insolubles in vitro, incluyendo roca fosfatada. Esta movilización se atribuye a la solubilización, quelación y a procesos de reducción, que también juegan un papel importante en el biocontrol de enfermedades.

OBJETIVOS

Bajo la premisa de que el P es un recurso no renovable y se hace precisa una mayor eficiencia en la utilización del P del suelo y del P aplicado como fertilizante. Es preciso, por tanto, valorar como formas de P en el suelo consideradas poco disponibles, pueden ser utilizadas por las plantas. Microorganismos y plantas han desarrollado mecanismos similares de movilización de P del suelo. En determinadas condiciones los mecanismos de los microorganismos pueden ser más eficaces que los de las plantas e incluso, pueden modificar los mecanismos movilizadores de las plantas incrementando su eficacia.

En este contexto, el objetivo principal de esta tesis doctoral fue evaluar el efecto que la inoculación con microorganismos rizosféricos, que proporcionan beneficios adicionales como biocontrol de enfermedades y PGPRs, lograban tener sobre la nutrición fosfatada de las plantas y sobre la nutrición férrica cuando eran suministradas fuentes de fósforo poco asimilables por las plantas. Se establecieron los siguientes objetivos específicos:

- Estudiar la utilización por las plantas de diferentes fuentes de fósforo, algunas de ellas consideradas poco disponibles como fosfatos de calcio insolubles y fitato.

- Establecer los mecanismos por los cuales las plantas y microorganismos pueden movilizar fósforo de fosfatos de Ca insoluble o formas de fósforo orgánicas.

- Establecer los umbrales de Fe por debajo de los cuales *Trichoderma asperellum* T34 es efectivo.

- Valorar el efecto de los óxidos de Fe presentes en el medio sobre la absorción de fósforo por las plantas a partir de fosfatos de calcio y sobre la posible contribución de los microorganismos a la absorción por las plantas de P.

- Determinar los parámetros edáficos que pueden tener un repercusión positiva o negativa sobre el efecto que la inoculación de microorganismos solubilizadores de fosfatos puedan tener sobre la absorción de P en las plantas.

II

Los ascomicetos han sido aislados de lugares extremos, desde rocas de las planicies de la Antártida hasta las profundidades del mar.

RESUMEN

Se estudió la contribución de *Trichoderma asperellum* T34 sobre la absorción de P por las plantas a partir de formas escasamente fito-disponibles tales como fitatos y fosfatos de Ca insolubles. Se realizaron dos experimentos con pepino (*Cucumis sativus* L. cv. Tropico) sobre arena silicea con dos factores: (i) fuente P; KH_2PO_4, fitato (Ins6P) y roca fosfatada (PR), y (ii) inoculación con T34. También se usaron cultivos in vitro en medio líquido de T34. T34 aumentó el contenido total de P en raíces de pepino independientemente de la forma P utilizada y mejoró la captación total de P por las plantas cuando el P se suministró como Ins6P o PR. El aumento de la actividad fitasa observada con la inoculación de T34 contribuye a explicar su influencia favorable sobre la absorción de P cuando fue suministrada como Ins6P. La solubilización de los fosfatos de Ca procedentes de PR fue favorecida por un ligero efecto acidificante y por el aumento de la concentración de aniones orgánicos promovida por el hongo en el medio de cultivo de las plantas. Se puede concluir que T34 puede mejorar la nutrición de P en plantas cultivadas en medios que contienen fitatos o fosfatos de Ca insolubles como formas dominantes de P.

CAPÍTULO II CAPTACIÓN DE FÓSFORO POR LAS PLANTAS PROCEDENTE DE FUENTES POCO DISPONIBLES AFECTADAS POR *TRICHODERMA ASPERELLUM* T34

Título: **Plant uptake of phosphorus from sparingly available P-sources as affected by *Trichoerma asperellum* T34**

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Plant uptake of phosphorus from sparingly available P- sources as affected by *Trichoderma asperellum* T34

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The contribution of *Trichoderma asperellum* T34 to the plant uptake of phosphorus (P) from sparingly phytoavailable forms such as insoluble calcium (Ca) phosphates and phytates was studied. Two experiments with cucumber (*Cucumis sativus* L.) on siliceous sand were performed involving two factors, namely: (i) P source, viz. KH_2PO_4, phytate (Ins6P), and phosphate rock (PR), and (ii) inoculation with T34. Liquid pure cultures of T34 were also used. T34 increased the total content in P of cucumber roots irrespective of the particular P form and enhanced total P uptake by plants with P supplied as Ins6P or PR. The increased phytase activity observed with T34 contributes to explain its favourable influence on the uptake of P supplied as Ins6P. Solubilization of Ca phosphates from PR was favoured by the slightly acidifying effect and the increased organic anion concentration promoted by the fungus in the plant growth media. It can be concluded that T34 can improve P nutrition in plants grown on media containing phytates or insoluble Ca phosphates as dominant P forms.

Key words: phosphorus, *Trichoderma*, rhizosphere, Ca phosphates, phytase.

Introduction

The consequences of the depletion of P resources have aroused increasing interest in recent years, especially after the rapid increase of phosphate rock prices in 2007 and 2008 (Cordell et al. 2009, Gilbert 2009). Efficient use of P can help to make agriculture sustainable in a scenario of increasing prices of fertilizers owing to the rapid depletion of available reserves (Cordell et al. 2009, Ryan et al. 2012). Also, unlocking residual P forms bound to soil is an essential stage towards a sustainable future for agriculture (Stutter et al. 2012).

Soil microorganisms can facilitate the mobilization of soil P through various mechanisms (Wang et al. 2012). Many rhizobacteria and fungi possess mechanisms to mobilize Fe, and hence Fe-bound P, including chelator release, acidification (Marschner et al. 2004, Lemanceau et al. 2009) and reduction processes (Sánchez-Alcalá et al. 2011). Acidification by plant growth-promoting bacteria facilitates uptake of P from poorly soluble —and hence largely unavailable— Ca phosphates by plants (Browne et al. 2009, Singh and Reddy 2011). Also, rhizosphere microflora is known to promote the hydrolysis of organic P forms by releasing hydrolytic enzymes, which facilitates their use as P sources by plants (Hariprasad and Niranjana 2009, Patel et al. 2010). The mobilization of P from poorly soluble Ca phosphates and stable organic forms such as phytates can be important in many environments, where both sparingly available P forms frequently account for most P present in soil (Celi et al. 2001, Saavedra et al. 2007). Increasing the acquisition of P by plants from these residual soil P forms by microbial inoculation can contribute to a more sustainable management of non-renewable P resources and to improve the productivity of P-poor soils, which expand on one third of the agricultural land in the World (MacDonald et al. 2011).

Biological control agents can be effective in mobilising P in the rhizosphere, which warrants their use for integrated management of plant diseases and P supply to plants (Vassilev et al. 2006), thus contributing to a more sustainable agricultural production. The soil fungi *Trichoderma* spp. are efficient, widely used biological control agent (Howell 2003, Schuster and Schmoll 2010) that enhances nutrient uptake by plants (Yadav et al. 2009, de Santiago et al. 2013). In spite of the effects described by Altomare et al. (1999) or Rudresh et al. (2005), little information about contribution of *Trichoderma* to P uptake by plants is available. Although the positive contribution of P-mobilizing microorganisms to plant nutrition is well-known, evidences of mobilization from specific P forms and mechanisms involved in this process are most often limited to pure cultures of microorganisms and rarely observed in plant growth studies (Singh and Reddy 2011, Wang et al. 2012). For accurate assessment of their use to improve plant nutrition, it is required: (i) more direct evidences of the effect of microorganisms on plant P uptake from specific P forms present in soil, (ii) a deeper knowledge about mechanisms involved in P mobilization in plant growth media, and (iii) how these mechanisms interact with P-mobilizing mechanisms of plants.

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This work was undertaken in order to study the contribution of *Trichoderma asperellum* T34, an effective biocontrol agent (Segarra et al. 2007), to plant uptake of P from sparingly phytoavailable forms such as insoluble Ca phosphates and phytates by plants, and also to elucidate the mechanisms behind this potential effect in plant growth media with a view to facilitating the integrated use of the fungus in biocontrol and fertilization strategies.

Materials and methods

Experimental setup

Two sets of experiments were performed, namely: (i) cultivation of T34 in liquid media, in order to assess its P-mobilizing mechanisms and (ii) inoculation experiments with plants grown in artificial growth media to assess how T34 can contribute to P nutrition of plants from different P sources.

Cultivation of T34 in liquid media

Trichoderma asperellum T34 was cultivated in Czapek liquid media containing P from various sources in order to examine the potential enzyme production and acidification abilities of the fungus. The media contained sucrose (30 g L^{-1}), $NaNO_3$ (2 g L^{-1}), $MgSO_4$ (0.1 g L^{-1}), KCl (0.5 g L^{-1}) and $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}), and had a pH of 7.8. A completely randomized experiment with 3 replications was performed by using an identical T34 inoculation rate (100 conidia mL^{-1}) and a single factor (P source) with 4 different treatments, namely: (i) phosphate applied as KH_2PO_4 at full strength of 0.23 g L^{-1} P, which is the usual concentration for this type of medium, (ii) KH_2PO_4 at 23 mg L^{-1} P (1/10 strength KH_2PO_4), (iii) ground (< 0.1 mm) phosphate rock (PR, essentially apatite) at 0.23 g L^{-1} P, and (iv) phytate (*myo*-inositol-6-*hexakis*phosphate Na salt, Ins6P) at 0.23 g L^{-1} P. The low KH_2PO_4 treatment (23 mg L^{-1} P) was included to study responses of T34 to suboptimal phosphate concentrations in solution that were greater than that expectable with an insoluble P source (RP). Cultivation runs were done in 250 mL glass flasks.

After 7 days of cultivation, the media were passed through a cellulose nitrate filter of 0.22 µm pore size and used to determine fungal biomass, enzyme activities in solution, pH, molybdate reactive P (MRP) according to Murphy and Riley (1962), and the concentration of major organic anions produced (particularly those capable of mobilizing Fe and P such as oxalic and citric).

Inoculation experiments with plants

Experiments with cucumber plants (*Cucumis sativus* L. cv Tropico) were performed by using an artificial growth medium consisting of siliceous sand previously sieved through 0.5 and 1 mm mesh in order to ensure adequate aeration and hydraulic conductivity. The sand was washed several times with 0.2 M Na_2CO_3 to improve dispersion and remove impurities. The source of Fe in the growth media was ferrihydrite, which was supplied by coating part of the siliceous sand with this oxide according to Rahmatullah and Torrent (2000). The concentration of Fe bound to ferrihydrite in the growth media, estimated as citrate-ascorbate extractable Fe, was 200 mg kg^{-1} Fe medium. The pH of the media was 8 and the electrical conductivity (EC) in all (1:5 medium:water extract) lower than 100 µS m^{-1}.

Two different experiments were conducted under exactly the same plant growth conditions by using a completely randomized design with five replications (one pot per replication and one plant per pot) and two factors, namely: P source, with three different treatments (KH_2PO_4, Ins6P and PR [<0.1] mm), and T34 (tests with and without inoculation [control]).

Phosphorus from KH_2PO_4 and Ins6P was supplied in the form of compounds adsorbed on Fe oxides covering part of the siliceous sand in order to imitate the major fractions of these P forms in soils which are usually bound to Fe oxides as dominant sorbent surfaces, whereas PR was carefully mixed with the growth media before transplanting. This growth medium was intended to mimic the dynamics of two major soil P forms not readily available, namely: sparingly soluble Ca phosphates and phytates (Saavedra and Delgado 2005). The P rate used in all the cases was 6.4 mg of P per kg of growth medium, equivalent to 2.56 mg pot^{-1} P (viz., 2–3 times the amount of P expected to be absorbed by each plant). Phosphate and Ins6P were adsorbed on siliceous sand coated with Fe oxides by immersing 1 kg of sand in 1.5 L of the same nutrient solution used in the experiment, which contained 5.25 and 6 mg L^{-1} P as KH_2PO_4 and Ins6P sodium salt, respectively. Phosphorus concentration in surrounding

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solution was determined daily and nutrient solution with P replaced by a new one with the same P forms and concentration until the desired adsorbed P amount was obtained. After adsorption, P concentration in equilibrium with siliceous sand coated with Fe oxide was 1.5 mg L^{-1} P and 3 µg L^{-1} P for KH_2PO_4 and Ins6P, respectively. Then, to check that amounts of sorbed P were those estimated, and to know the dominant forms in which P was retained in the medium, a sequential extraction with 0.1M NaOH + 1 M NaCl and 1 M HCl was performed. The first extraction step, which was intended to desorb P from Fe oxides, recovered 53% of inorganic phosphate and 45% of Ins6P, the second step released the remaining sorbed P —mostly precipitated P, which indicates that a substantial fraction of the inorganic phosphate or InsP6 supplied precipitated as Ca salts (Ca provided by nutrient solution) as it can be expected in soils with similar pH (e.g. Lindsay (1979) for inorganic phosphate, or Celi et al. (2001) for InsP6). Phosphorus in nutrient solution used to adsorb P on Fe oxides and in the extracts of sequential extraction were determined colorimetrically (Murphy and Riley 1962), following persulphate digestion (Díaz-Espejo et al. 1999) in the case of those samples treated with Ins6P.

Inoculation with T34 was carried out by immersing roots in a water suspension containing 10^3 conidia mL^{-1} before transplanting (de Santiago et al. 2009), followed by addition of 10^4 conidia g^{-1} medium after transplanting. The microorganisms were supplied in 20 mL of a water suspension containing $2 \cdot 10^5$ conidia mL^{-1} and prepared according to Segarra et al. (2007), a micropipette was used to apply the conidia at four points around each plant.

Seeds were sown in 35 mL polyvinyl multipots that were filled with perlite and irrigated with de-ionized water. After 28 days, plants were transplanted in the growth medium and allowed to grow for a further 26 days. Each pot (350 mL, 5.5 cm diameter, 15 cm high polystyrene cylinder) was filled with 400 g of siliceous sand (80 g with adsorbed Fe oxides) and used to grow a single plant. The experiment was conducted in a growth chamber with a photoperiod of 14 h, a 25/23 °C day/night temperature, 65% RH and 22 w m^{-2} light intensity. Pots were irrigated daily with of a Hoagland type nutrient solution without P and Fe. The total volume of solution applied per pot was 300 mL, and composition of the nutrient solution was (all concentrations in mmol L^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), H_3BO_3 (0.024), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002) and H_2MoO_4 (0.0005). The pH of the nutrient solution was 6.

After the experiments, plant dry matter (DM) in shoots and roots was measured, and P and Fe concentrations in plant shoots and roots were determined. Rhizospheric growth medium, sampled as the medium retained by roots after hand-shaken them on a table during 30 s, was used to determine alkaline phosphomonoesterase, acid and alkaline phytase, T34 density and the concentration of low-molecular weight organic anions.

Enzyme activity determination

Phosphomonoesterase activity was determined as the amount of p-nitrophenol released from 5 mM p-nitrophenyl phosphate according to Tabatabai and Bremner (1969). Acid and alkaline phosphomonoesterase were determined at pH 5.5 and 9, respectively, in the Czapek media used for liquid cultivation of T34, and at pH 9 in the plant growth media. Phytase activity in the plant growth media and in the filtered Czapek media was determined by incubating the media in MES buffer at a volume ratio of 1:1 at 37 °C for 60 min. The final concentrations in the assay were 15 mM MES and 2 mM Ins6P —the latter was added as a substrate for the enzyme. The pH was 5.5 and 7.5 for acid and alkaline phytase, respectively. All solutions used were previously passed through a cellulose nitrate filter of 0.22 µm pore size for sterilization. After incubation, the reaction was stopped by adding an identical volume of 10% trichloroacetic acid, and the suspensions centrifuged at 10 000 g for 10 min. The supernatants were used to determine molybdate reactive P according to Murphy and Riley (1962), phytase activity was expressed in enzyme units (1 µmol of molybdate reactive P released per min) per kg of growth medium. P sorption on Fe oxides present in the plant growing media during hydrolysis of Ins6P was assessed by incubation in a KH_2PO_4 solution containing 4.2 mg P L^{-1} in order to measure the fraction of phosphate recovered after the phytase activity assay (George et al. 2005). P concentrations after incubation of the growth media with Ins6P were corrected for the ratio of P recovery in the media.

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Organic anion determination

Organic anions were extracted from the plant growth media (Radersma and Grierson 2004) by using water at a 1:1 ratio with the sand and shaking in a end-over-end shaker at 4 Hz for 1.5 h. Then, the suspensions were centrifuged at 10 000 g for 10 min and the supernatants passed through filters of 0.45 µm pore size. With the Czapek media, the filtrates obtained from the 0.22 µm filters were directly used for analysis. Organic anions were separated by high performance liquid chromatography on an HPLC Varian ProStar 410 instrument equipped with a C18 column from Varian (250 mm × 34.6 mm, 8 µm particle size) at 45 °C, using isocratic elution with 98% 25 mM KH_2PO_4 at pH 2 and 2% methanol as carrier at a flow rate of 0.8 mL min^{-1} (injected volume = 20 µL). Organic anions were detected at 215 nm, using a Varian 486 335 photo-diode array detector. Individual stock standard solutions (acetic, oxalic, citric, malic, fumaric and succinic) were prepared from Sigma chemicals and combined to obtain a dilute working-strength standard.

Plant analysis

Plant shoots and roots were separated and their dry mass (DM) was determined after drying to constant weight in a forced-air oven at 65 °C. Dried plant material was ground to pass through a 1 mm sieve prior to mineralization. An aliquot of 0.25 g was mineralized in porcelain crucibles in a furnace at 550 °C for 8 h. Then, 10 mL of 1 M HCl was used to dissolve ash and heated at 100 °C for 15 min, the digest being used to determine P and Fe. Phosphorus was determined according to Murphy and Riley (1962) and Fe by atomic absorption spectrometry. Certified plant material was also analysed in parallel to check for complete recovery of nutrients with this procedure. Seeds were also analysed with the same method in order to determine nutrient concentrations. Total uptake by plants was calculated as the total amount present in aerial parts and roots minus the total amount in seeds (Delgado and Torrent 1997).

Determination of T34 density in growing media

The density of T34 in the rhizospheric media after the experiments was determined by dilution plating, using the *Trichoderma* semi-selective medium of Chung and Hoitink (1990) according to Borrero et al. (2012). To this end, 5 g of growth medium was suspended in 90 mL of a 1 g L^{-1} pyrophosphate solution in water. The suspension was shaken and a 10-fold dilution series from 10^{-1} to 10^{-4} was prepared with water agar (1 g L^{-1}). Then, the suspensions were pipetted onto three plates per dilution. Colony forming units (CFU) were counted 7 days after plating and expressed as CFU mL^{-1} growth medium. No CFU was detected in the treatments without T34 inoculation.

Statistical analysis

An analysis of variance was performed by using the General Linear Model procedure in Statgraphics Plus 5.1 (StatPoint, 2000) to identify the effects of the studied factors on the different variables measured in the experiments. Previously, data were checked for normal distribution and homoscedasticity by using the Kolmogorov–Smirnov test and Levenne test, respectively, as implemented with the previous software. Any data not passing either test required additional transformation (*transformed data = data*^{b}, being *b* the slope of the relation between the logarithms of standard deviation and the logarithm of the mean for each treatment), which was effective to achieve ANOVA requirements. Each replication of the experiment with plants was considered a separate block in order to exclude the variation associated with repetition of the experiment (de Santiago et al. 2009). Only one factor (P source) was used in the analysis of variance of T34 CFU in the growing media because no CFU was detected in non-inoculated media. Means were compared via Tukey's test; by exception, when interactions between factors were significant, the main effects could not be assessed in a combined analysis, so it was impossible to compare factor means between treatments (de Santiago et al. 2013). A mean comparison for the combined effect of factors was performed when interaction was significant. Regressions and comparison between linear regressions were done by using the same model and software.

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Results

Cultivation of T34 in liquid media

Development of *Trichoderma asperellum* T34 in the liquid media, measured as the amount of biomass produced during incubation, differed depending on the P source; thus, it was higher with InsP6 and the more dilute KH_2PO_4 than with the other P sources (Table 1). Significant phosphomonoesterase and phytase activities were observed in all the liquid media, the 1/10 KH_2PO_4, PR and Ins6P treatments exhibiting the highest phosphomonoesterase activity per gram of fungal biomass (Table 1). By contrast, phytase activity peaked with the full strenght KH_2PO_4 concentration but was not significantly different from that observed with InsP6 (Table 1). No significant differences in low-molecular weight organic anions, mostly oxalate (>99% of anions detected; beside oxalate, only trace amounts of citric acid were observed; results not shown) —between P sources were observed (Table 1). In all cases, pH was decreased relative to its initial value in the liquid culture media, and significant differences between PR and Ins6P were observed (Table 1). MRP concentrations decreased in the following sequence: KH_2PO_4 (0.23 g L^{-1}) > Ins6P > 1/10 KH_2PO_4 (23 mg L^{-1}) > PR (Table 1).

Table 1. Effect of various P sources on *Trichoderma asperellum* T34 biomass, molybdate reactive P (MRP) concentration and pH, organic anions and enzyme production in liquid cultures of the fungus (Czapek media)

					Phosphomonoesterase		Phytase	
Source of variation	Biomass	MRP	pH	Sum of organic anions	Acid	Alkaline	Acid	Alkaline
P source[a]	g L^{-1}	mg L^{-1}		mmol g^{-1} biomass	mg pNP h^{-1} g^{-1} biomass		EU g^{-1} biomass	
Phosphate rock	0.62 ± 0.02 c	3.1 ± 1.4 d	7.07 ± 0.16 a	37 ± 2	3.7 ±0.1 a	4.3 ± 0.8 a	0.7 ± 0.1 c	5.8 ± 1.1 c
Ins6P	1.54 ± 0.1 a	159 ± 4 b	6.43 ± 0.1 b	36 ± 7	2.7 ± 0.5 a	3.3 ± 0.6 a	46.0 ± 7.8 a	54.2 ± 7.5 a
1/10 KH_2PO_4	1.82 ± 0.34 a	17 ± 0.5 c	6.82 ± 0.22 ab	46 ± 8	2.7 ± 0.4 a	2.5 ± 0.6 a	3.3 ± 0.4 b	10.6 ± 1.2 b
KH_2PO_4	0.98 ± 0.1c b	227 ± 1 a	6.72 ± 0.06 ab	36 ± 7	1.3 ± 0.4 b	0.2 ± 0.1 b	66.7 ± 13.7 a	72.9 ± 15.3 a

Means ± standard error, n = 3; a = all sources were supplied at a concentration of 0.23 g P L^{-1}, by exception, 1/10 Phosphate was applied at 23 mg P L^{-1}; Ins6P = myo-inositol-6-hexakisphosphate; pNP = p-nitrophenol released; EU = enzyme units (in µmol P min^{-1}) released; MRP = molybdate reactive P as determined according to Murphy and Riley (1962); Means followed by different letters within a column are significantly different as per Tukey's test at $p < 0.05$ for each factor

Experiments with plants

Plant DM was significantly lower with PR than with the other two sources ($p < 0.001$; Tables 2 and 3). The P concentration of shoots was lower with InsP6 ($p < 0.01$); however, the total P content of roots with this P source was not significantly different from that obtained with KH_2PO_4 and PR (Table 3). Phosphorus source also affected the Fe concentration of roots and total Fe content in shoots and roots (Tables 2 and 3), Ins6P decreasing the Fe concentration and total content of roots relative to KH_2PO_4 (Table 3).

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Table 2. Significance (*p* values) in the analysis of variance of the different variables studied in cucumber plants (*Cucumis sativus* L.) and the plant growth media

Source of variation	Plants									Growth media						
	Dry matter			P concentration		Total P		Fe concentration		Total Fe		Phosphatase	Phytase		Oxalate	pH	CFU
	Shoots	Roots	Total	Shoots	Roots	Shoots	Roots	uptake	Shoots	Roots	Shoots	Roots		Alkaline	Acid			
P source	0.0000	0.0013	0.0000	0.0021	0.5158	0.0000	0.0220	0.0001	0.5280	0.0258	0.0014	0.0070	0.8427	0.0000	0.0000	0.0008	0.0050	0.2549
T34	0.0076	0.0544	0.0087	0.0056	0.6974	0.1584	0.0210	0.0577	0.6021	0.4827	0.0063	0.0255	0.8922	0.3611	0.0147	0.0952	0.0392	
P source × T34	0.2991	0.0684	0.1907	0.0653	0.6161	0.0373	0.3871	0.0497	0.7383	0.7952	0.3113	0.0703	0.0340	0.0111	0.0518	0.0247	0.9478	

CFU = colony forming units; *p* values < 0.05 indicate a significant effect of the source; CFU was 0 for non-inoculated plants; therefore, only one source (P source) was considered in the analysis of variance

Table 3. Effects of the main factors on plant development, and concentration and total content of P and Fe in plants

P source	Dry matter (g plant^{-1})			P (g kg^{-1})		Total P		Fe		Total Fe (µg plant^{-1})	
	Shoots	Roots	Total	Shoots	Roots	Roots (mg plant^{-1})	Shoots (mg kg^{-1})	Roots (g kg^{-1})	Shoots	Roots
Phosphate rock	0.70 ± 0.05 b	0.12 ± 0.01 b	0.83 ± 0.06 b	0.99 ± 0.04 a	0.93 ± 0.09	0.11 ± 0.02 b	66 ± 23	1.05 ± 0.10 a	48 ± 19 b	135 ± 110 b
Ins6P†	0.84 ± 0.07 a	0.16 ± 0.01 a	0.99 ± 0.08 a	0.87 ± 0.02 b	0.86 ±0.07	0.13 ± 0.01 ab	62 ± 25	0.76 ± 0.09 b	60 ± 33 ab	134 ± 104 b
KH2PO4†	0.95 ± 0.06 a	0.18 ± 0.02 a	1.13 ± 0.07 a	1.00 ± 0.04 a	0.88 ± 0.06	0.14 ± 0.01 a	70 ± 31	1.01 ± 0.09 a	67 ± 23 a	184 ± 108 a
Inoculation										
+ T34	0.88 ± 0.05 a	0.16 ± 0.01	1.04 ± 0.06 a	0.91 ± 0.02 b	0.94 ± 0.07	0.14 ± 0.01 a	67 ± 24	0.98 ± 0.09	64 ± 27 a	166 ± 110 a
– T34	0.78 ± 0.05 b	0.14 ± 0.01	0.93 ± 0.07 b	1.00 ± 0.04 a	0.84 ± 0.04	0.11 ± 0.01 b	66 ± 22	0.90 ± 0.07	53 ± 25 b	137 ± 106 b

Means ± standard error; n = 20 for P source; n = 30 for inoculation; Ins6P = myo-inositol-6-hexakisphosphate; † = Adsorbed on Fe oxides; The effects were only considered when the factor concerned exhibited no significant interaction; Means followed by different letters within a column are significantly different as per Tukey's test at $p < 0.05$ for each factor

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Acid phytase in the growth media after cultivation was greater with InsP6 than with the other P sources ($p < 0.001$; Table 4). pH was also significantly affected by P source (Table 2), with Ins6P leading to a lower value than PR ($p < 0.01$; Table 4).

Table 4. Effects of the main factors on properties of the plant growing media

P source	Acid phytase (EU) kg^{-1}		pH	CFU
Phosphate rock	0.5	± 0.1 b	7.84 ± 0.04 a	9318 ± 2335
Ins6P†	3.8	± 0.2 a	7.64 ± 0.06 b	9740 ± 2482
KH2PO4†	1.2	± 0.3 b	7.73 ± 0.06 ab	5155 ± 1112
Inoculation				
+ T34	2.1	± 0.4 a	7.69 ± 0.04 b	
– T34	1.6	± 0.4 b	7.78 ± 0.04 a	

CFU = colony forming units; EU =enzyme units (amount of enzyme hydrolysing 1 µmol of P in 1 min); Ins6P = myo-inositol-6-hexakisphosphate; Means ± standard error: n = 20 for P source; n = 30 for inoculation; † = Adsorbed on Fe oxides; The effects were only considered when the factor concerned exhibited no significant interaction; Means followed by different letters in a column were significantly different as per Tukey's test at; $p < 0.05$ for each factor

Inoculation with T34 increased DM ($p < 0.01$), total content of Fe in shoots ($p < 0.01$), and total contents of P and Fe in roots ($p < 0.05$) (Tables 2 and 3). Total P uptake was linearly related to total Fe uptake. However, linear regression had significantly lower slopes ($p< 0.05$) with T34 than without it (Fig. 1), thus revealing that P uptake was less markedly dependent on Fe uptake in the presence of T34 than in its absence.

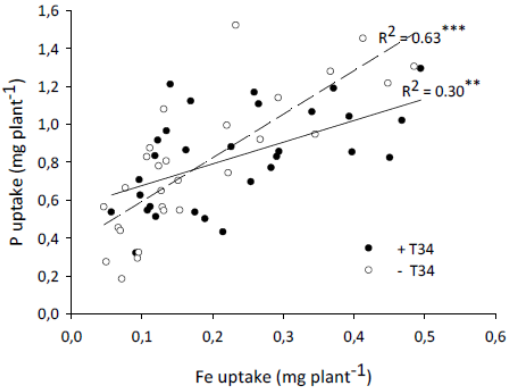

Fig. 1. Relationships between total P uptake and total Fe uptake by cucumber plants. The dotted line represents the linear regression for plants without T34 inoculation (Y = 0.36 + 2.3 X) and the solid line that for inoculated plants (Y = 0.56 + 1.15 X). The intercept and slope of both regression lines were significantly different at $p < 0.05$ **.

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T34 increased acid phytase activity and decreased pH after cultivation in the growth media ($p < 0.05$, in both cases; Tables 2 and 4). The effect of T34 on alkaline phytase, total content of P in aerial parts and total P uptake by plants was dependent on P source as suggested by the significant interaction between both factors ($p < 0.05$; Table 2). With Ins6P, T34 led to a P uptake and total P content of aerial parts not significantly different from those obtained with KH_2PO_4 (Table 5).

Table 5. Effect of the significant interactions between main factors on properties of the plant growth media and concentration and total content of P in plant

P source	Inoculation	Total P uptake‡	Total P shoots	Phospho-monoesterase	Alkaline phytase	Organic anions
		mg plant^{-1}		mg pNP kg^{-1} h^{-1}	EU kg^{-1}	mmol kg^{-1}
Phosphate rock	+ T34	0.77 ± 0.08 bc	0.73 ± 0.06 ab A§	5.21 ± 1.33	2.0 ± 0.1 a	47 ± 15 a A
	– T34	0.59 ± 0.07 c	0.62 ± 0.07 b B	4.02 ±0.08	2.5 ± 0.3 a	32 ± 10 ab B
Ins6P†	+ T34	0.79 ± 0.07 abc A	0.77 ± 0.07 ab A§	3.72 ± 0.57	1.5 ± 0.07 b A	6 ± 2 c
	– T34	0.54 ± 0.10 c B	0.56 ± 0.09 b B	4.86 ± 1.1	1.2 ± 0.07 c B	16 ± 5 ab
KH2PO4†	+ T34	0.92 ± 0.09 ab	0.87 ± 0.08 a	4.11 ± 0.83	1.3 ± 0.1 bc	7 ± 1 bc B
	– T34	1.01 ± 0.07 a	0.98 ± 0.06 a	5.07 ± 0.93	1.2 ± 0.09 c	12 ± 2 ab A

Mean ± standard error, n = 10 ; Ins6P = myo-inositol-6-hexakisphosphate; EU = enzymatic units (amount of enzyme hydrolysing 1 µmol P in 1 min); pNP = p-nitrophenol ; † = Adsorbed on Fe oxides; Means followed by a different lower case letter in a column were significantly different as per Tukey's test at $p < 0.05$ for the six combinations of treatments; means followed by a different upper case letter in a column were significantly different as per Tukey's test at $p < 0.05$ for the same P source; §P = 0.0761 and $p = 0.0590$ for Ins6P and PR, respectively.; ‡ = Estimated as total P in aerial parts and roots minus P present in seeds. Total P in shoots was occasionally greater than total P uptake because P in seeds was not counted for P determination in aerial parts

Inoculation increased total P in aerial parts and total P uptake in the plants supplied with Ins6P (Table 5). In inoculated plant growth media, total P uptake and P in shoots with PR was not significantly different from those with KH_2PO_4 (Table 5). Phosphomonoesterase and organic anions in the growing media after cultivation were also affected by T34, but an opposite trend with PR vs other two P sources was observed explaining the significant interaction in both variables (Table 2). Phosphomonoesterase activity was linearly related to root development, measured as DM ($R^2 = 0.32$; $p < 0.001$, n = 60), and to CFU of T34 in the media ($R^2 = 0.34$; $p < 0.05$, n = 30; non-inoculated pots excluded). The contents in organic anions —oxalate accounted for the only one detected— were decreased by T34 with KH_2PO_4 and Ins6P, which were the two P sources leading to the lowest concentrations of organic anions in the growth media, meanwhile the opposite effect was observed with PR (Table 5).

Discussion

Effect of T34 on plant development and nutrient uptake

Trichoderma asperellum T34 increased the total content of P in roots of cucumber (Tables 2 and 3) irrespective of the particular P form used to supply this nutrient; also, it enhanced total P uptake by plants when the phosphorus was supplied as Ins6P or PR (Tables 2 and 5). This reveals a favourable effect of the fungus on the use of not readily phytoavailable P forms (e.g., Ins6P, poorly soluble Ca phosphates) by plants. The improvement of P uptake by T34 is in agreement with previous works with other *Trichoderma* spp. which revealed their abilities for solubilising insoluble phosphates (Altomare et al. 1999, Rudresh et al. 2005). In addition to P, T34 increased the total content of Fe in shoots and roots of cucumber plants (Tables 2 and 3), which suggests improved Fe uptake in media with restricted availability of this nutrient by effect of their ferrihydrite concentration and pH (de Santiago et al. 2009).

The favourable effect of the fungus on development of cucumber plants measured as DM yield might be related to the improved P and Fe uptake observed with T34 in media with restricted availability of both nutrients (Table 3). Changes in phytohormone profile in plants (e.g. indole-3-acetic acid or 1-aminocyclopropane-1-carboxylic acid; Verma et al. 2007, Martínez-Medina et al. 2011) can also contribute to the effect of T34 increasing plant development. However, this effect seems improbable because there was not a significant increase in plant DM by T34 when the most effective P source (KH_2PO_4) was only taken into account ($p = 0.4668$). T34 did not promoted positive effects on P ($p = 0.2403$) or Fe uptake ($p = 0.3943$ and $p = 4012$ for total Fe in shoots and roots, respectively) by plants with KH_2PO_4. Thus, increased development by T34 seemed to be related to positive effects on P and Fe nutrition more clearly observed in plants supplied with PR and Ins6P. Although an increased uptake of other nutrients different from P and Fe by T34 is possible, which can also contribute to the effect of the

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fungi increasing plant development (Yedidia et al. 2001, de Santiago et al. 2013), it seems unlikely because a sufficient supply of these nutrients is guaranteed through nutrient solution.

Iron mobilization strategies of plants and microorganisms involve the exudation of organic anions with Fe-complexation abilities (e.g., citrate) and acidification (Jones 1998, Marschner et al. 2011). This additionally contributes to increasing P availability to plants through desorption from oxides or dissolution of precipitated phosphates. Therefore, P uptake by plants may be related to Fe uptake by plants. However, the relationship between P uptake and Fe uptake was found to differ depending on whether the growth medium was inoculated with T34 (Fig. 1). This result suggests the involvement of different Fe and P release mechanisms when T34 is present in the growth medium, e.g. more specific mechanisms for Fe release by the fungus (e.g., siderophore production; (de Santiago et al. 2009), which can also provide more available Fe to plants with less P release from oxides.

Enzymatic hydrolytic activities in growing media

The improved P uptake by plants in T34-containing media where P was supplied in organic form (Ins6P) might be related to production of extracellular hydrolytic enzymes involved in the P cycle. This hydrolytic capacity was reflected in the phytase activity and MRP concentration observed in liquid pure cultures of T34 with Ins6P as the P source (Table 1) and in the increased acid phytase activity in plant growing media containing the fungus (Table 4). Also, alkaline phytase activity was increased by T34, but only when P was supplied to plant growth medium as Ins6P.

The effect of T34 on phosphomonoesterase activity in the plant growing media showed different trends depending on P source (significant interaction) and peaked with PR (Table 5). This P source was expected to lead to the lowest MRP concentrations in solution judging by the results with the liquid culture (Table 1). There is contribution of plants to such activity in the rhizosphere because 32% of all phosphomonoesterase activity was explained by root development, measured as DM; on the other hand, 34% of this enzymatic activity was explained by T34 development, measured as CFU in the media. An increased phosphomonoesterase activity, which is related to the hydrolysis of monoester phosphates other than Ins6P, suggests the presence of an additional mechanism for T34 to facilitate P nutrition by plants in soil. This activity is known to be involved in the turnover of organic P in the rhizosphere (Turner and Haygarth 2005).

Phosphomonoesterase activity due to T34 was seemingly more strongly affected by the inorganic P concentration in solution than by P source. Thus, it was lower in the liquid T34 culture with the source leading to the highest MRP concentration in the medium (Table 1); by contrast, phytase activity was apparently more markedly affected by the dominant P form present in the medium (Table 1). Phytase activities were greatest with Ins6P and full strength KH_2PO_4 in the T34 liquid cultures. It is known that phytase activity can increase at increased substrate concentration, but also at increased KH_2PO_4 in pure cultures (Chun et al. 2007). These results are consistent with those in the plant growing media, where acid phytase activities peaked with Ins6P (Table 4). Adsorption of fungal (acid) phytases on Fe oxides markedly reduces their activity (George et al. 2005). This activity is known to be increased by Ins6P by effect of adsorption of this P form on Fe oxides decreasing adsorption of phytase and increasing its catalytic activity over time as a result (Giaveno et al. 2010). This contributes to explain the increased acid phytase activity observed with Ins6P as P source in the plant growth media containing Fe oxides. Although inorganic P is also adsorbed on Fe oxides, sorbent surfaces have a higher affinity for Ins6P than for inorganic P (Berg and Joern 2006) as revealed in the present study by the much lower P concentration in equilibrium with InsP6 (3 μg L^{-1}) than with phosphate (1.5 mg L^{-1}) adsorbed on ferrihydrite. This accounts for the increased inhibition of acid phytase adsorption and hence the greater activity of this enzyme in solution observed with Ins6P relative to KH_2PO_4 (Table 4). An increased affinity of oxides for Ins6P relative to inorganic phosphate accounts for the decreased Fe accumulation in roots with the former P source compared to KH_2PO_4 since Ins6P inhibits adsorption of complexing organic compounds released by roots or T34 on Fe oxides, a requirement for efficient Fe release from Fe-rich particles (Borer et al. 2009).

Acidification and organic anions in growth media

The increased use of P from PR might be related to the acidification of the rhizosphere and/or the production of low-molecular weight organic anions facilitating dissolution of Ca phosphates (Reyes et al. 1999, Hariprasad and Niranjana 2009). These effects were observed in the pure culture of T34 (Table 1); also, acidification of the rhizosphere and increased concentration of organic anions with PR by T34 was observed in the plant growing media (Tables 4 and 5).

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Oxalate was the dominant organic anion in the plant growth media. This is unsurprising since oxalate is typically the dominant anion exuded by roots of cucumber (El-Baz et al. 2004) and other plants (Jones 1998, Ström et al., 2005) under conditions of Fe or P deficiency. Furthermore, microbial degradation in the rhizosphere can have a lesser effect on oxalate than on other organic anions (Ström et al. 2001). A much higher organic anion concentration was found with PR than with Ins6P or KH_2PO_4 (Table 5). In spite of the organic anion production by T34 observed in the pure liquid culture (Table 1), the effect of the fungus was not always positive in plant growth media. In these media, T34 increased the concentration of organic anions with PR, thus contributing to dissolve PR, but decreased it with Ins6P and KH_2PO_4 as revealed by the significant interaction between both factors (Tables 2 and 5). This can be explained at least in part by the production of organic anions by plants. Plants and T34 seemingly increased organic anions concentrations to a greater extent with PR than with other P sources because this strategy is essential to dissolve sparingly soluble phosphates (Ström et al. 2005). With other P sources, T34 improves P uptake by plants through other mechanisms (e.g., hydrolytic activity with Ins6P); this can lead to a decreased exudation of organic anions by roots in order to mobilize P, which is highly costly in energetic terms (Ström et al. 2002, 2005).

Conclusions

Trichoderma asperellum T34 increased the total content in P of cucumber roots and enhanced total P uptake by plants when the phosphorus was supplied as Ins6P or PR. This was mainly explained by the production of extracellular hydrolytic enzymes involved in the P cycle, rhizosphere acidification, and increased organic anion concentration with T34. Although these results are promising for the potential usefulness of T34 for integration in biocontrol and P fertilization strategies, further research in soils and field conditions are still required.

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III

Hierro, su nombre deriva del anglosajón "iren", cuyo símbolo, Fe, se acortó de la palabra latina "ferrum" que significa firmeza.

RESUMEN

La inoculación con agentes de biocontrol pueden afectar a la absorción de hierro (Fe) por parte de las plantas. El objetivo de esta investigación fue estudiar la necesidad de definir un umbral de Fe en los medios de cultivo para la inoculación con el agente de biocontrol *Trichoderma asperellum* T34. Se realizó un experimento completamente al azar con pepino (*Cucumis sativus* L. cv. Serena) con dos factores: concentración de Fe en el medio de crecimiento en forma de ferrihidrita (0, 8, 16, 32,5 y 75 mg kg^{-1} de extracto de citrato-ascorbato Fe (CA-Fe), y la inoculación de plantas con T34. La materia seca (DM) de las partes aéreas del pepino se vio disminuido por T34. Esto fue relacionado con una disminución de la acumulación de Fe en las plantas, siendo más significativo en las partes aéreas que en las raíces. Sin embargo, con la concentración más alta de Fe (75 mg kg^{-1}), las diferencias en rendimiento de materia seca, alturas de las plantas, y el contenido y concentración de Fe en los brotes entre las plantas inoculadas y no inoculadas no fueron significativas. El umbral de CA-Fe en el medio para el rendimiento de materia seca de pepino fue de 37 mg kg^{-1} sin T34. Con T34, este umbral fue de 65 mg kg^{-1}, lo que implica que, por debajo de este límite, se requiere un suministro adicional de Fe para la inoculación con T34.

CAPÍTULO III UMBRALES DE DISPONIBILIDAD DE HIERRO PARA LA INOCULACIÓN CON *TRICHODERMA ASPERELLUM* T34 EN PEPINO

Título: **Iron availability thresholds for the inoculation of cucumber with *Trichoderma asperellum* T34**

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Iron availability thresholds for the inoculation of cucumber with *Trichoderma asperellum* T34

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Abstract

Inoculation with biocontrol agents can affect iron (Fe) uptake by plants. The objective of this research was to study the necessity of defining a Fe threshold in growth media for the inoculation with the biocontrol agent *Trichoderma asperellum* T34. A completely randomized experiment with cucumber (*Cucumis sativus* L.) was performed involving two factors: Fe rate in the growth medium in the form of ferrihydrite (0, 8, 16, 32.5, and 75 mg kg^{-1} of citrate-ascorbate-extractable Fe (CA-Fe), and plant inoculation with T34. Dry matter (DM) of aerial parts of cucumber was decreased by T34. This was related to a decreased accumulation of Fe in plants, more in aerial parts than in roots. However, at the highest Fe rate (75 mg kg^{-1}), differences in DM yield, plant height, and the content and concentration of Fe in shoots between inoculated and noninoculated plants were not significant. The threshold of CA-Fe in the medium for DM yield of cucumber was 37 mg kg^{-1} without T34. With T34, this threshold was 65 mg kg^{-1}, which implies that, below this limit, additional Fe supply is required for inoculation with T34.

Key words: biocontrol / *Cucumis sativus* / fertilization / ferrihydrite / micronutrients

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1 Introduction

Iron (Fe) is an essential element for microorganisms and plants. Although it is relatively abundant in soils, Fe availability is constrained by the low solubility of Fe^{III} species and the slow dissolution of Fe minerals, particularly under nonacidic conditions (*Kraemer* et al., 2006; *Lemanceau* et al., 2009). An accurate definition of Fe sufficiency thresholds, below which the deficiency of this nutrient can be expected, is necessary for a sustainable management of Fe nutrition depending on plant sensitivity to Fe deficiency (*de Santiago* and *Delgado*, 2006). Thresholds have been defined on the basis of readily reducible Fe (*e.g.,* citrate-ascorbate-extractable Fe), mainly related to poorly crystalline oxides, which are usually dominant sources of this nutrient for plants in calcareous soils (*de Santiago* et al., 2008a, b).

Plants and microorganisms have developed similar mechanisms to mobilize Fe from the solid phase and take up Fe, based on the release of Fe chelators (siderophores) (*Sharma* and *Johri*, 2003; *Khan* et al., 2006; *Jin* et al., 2006), acidification (*Lemanceau* et al., 2009), and reduction processes (*Valencia-Cantero* et al., 2007; *Rakshit* et al., 2009; *Marschner* et al., 2004; *Sánchez-Alcalá* et al., 2011). Alteration of the Fe availability by these mechanisms is more evident in the rhizosphere, where microbial density and activity is greater due to root exudation (*Marschner* et al., 2004; *Sánchez-Alcalá* et al., 2011; *Violante* et al., 2005; *Robin* et al., 2008).

The manipulation of the rhizosphere by inoculating with microorganisms may lead to the control of soil-borne diseases (biocontrol) and to an improved plant nutrition (*Vassilev* et al., 2006; *Zuo* and *Zhang*, 2011; *de Santiago* et al., 2011). Interaction between the Fe-acquisition mechanisms of the different organisms in the rhizosphere may lead to intense competition for this nutrient (*Winkelmann*, 2007), which is known to be one of the mechanisms explaining the biological control of many soil-borne plant diseases (*Mercado-Blanco* and *Bakker*, 2007; *Chaiharn* et al., 2009), and also can result in an improved (*Lemanceau* et al., 2009; *Violante* et al., 2005; *Renshaw* et al., 2002) or decreased Fe uptake (*Marschner* et al., 2011) by plants.

The soil fungus *Trichoderma* spp. is an efficient and widely used biological control agent (*Howell*, 2003; *Schuster* and *Schmoll*, 2010; *Fontanelle* et al., 2011) which increases Fe uptake by plants (*Haas* et al., 2008; *Hoyos-Carvajal* et al., 2009; *de Santiago* et al., 2009; *Segarra* et al., 2010). However, potential benefits from the inoculation with the biocontrol agent *Trichoderma asperellum* T34 on Fe nutrition of plants seem to be related to soil conditions, in particular Fe availability (*de Santiago* et al., 2011). Thus, it seems necessary to clarify under which conditions the potential use of biocontrol agents can promote negative or positive effects on plant nutrition, in particular, how Fe uptake by plants can be affected depending on the availability of this nutrient in soil. The particular case of the contributory effect of T34 on Fe uptake by cucumber (*Cucumis sativus* L.) plants in a calcareous growth medium differing in the concentration of poorly crystalline Fe oxides concentration was studied in this work

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with the main objectives of: (1) clarifying the potential effect of T34 on the sufficiency threshold of Fe in the growth media for plants, and (2) if it is necessary to define a threshold of Fe for the inoculation with T34. Both objectives are focused on the sustainable management not only of the biocontrol agent T34, but also of the Fe nutrition of cucumber plants.

2 Material and methods

2.1 Experimental design

A completely randomized experiment with five replications was performed involving two factors: Fe rate in the growth medium in the form of ferrihydrite (0, 8, 16, 32.5, and 75 mg kg^{-1} of citrate-ascorbate-extractable Fe), and plant inoculation with T34 (inoculated and noninoculated with T34). Each replication corresponded to a pot with one plant. The experiment was performed twice at different times under the same growth conditions in a growth chamber.

The growth medium was prepared by carefully mixing siliceous sand (> 99% quartz) with calcareous sand (> 99.5% $CaCO_3$). Both sands were sieved, and particles above 0.15 mm diameter were selected in order to obtain good aeration and hydraulic conductivity in the medium. 380 g of a mixture of calcareous (75%) and siliceous (25%) sand was placed in each pot. Siliceous sand was previously washed with a solution containing 0.27 M Na citrate + 0.11 M $NaHCO_3$ + 2% Na dithionite to reduce the concentration of Fe oxides (*de Santiago* and *Delgado*, 2006). The aim of this treatment was to achieve a citrate-ascorbate-extractable Fe concentration of less than 3 mg kg^{-1}. The Fe concentration of the calcareous sand was negligible. Iron enrichment of the medium was achieved by introducing a fraction of the siliceous sand coated with ferrihydrite, after washing with diluted Na_2CO_3 (*de Santiago* et al., 2009).

For inoculation with T34, a commercial inoculum provided by Biocontrol Technologies S.L. (Barcelona, Spain) was used; this product contained 11.3% of dry conidia and 88.7% of inert material. Inoculation was performed following the method of *Segarra* et al. (2007) for cucumber by immersing plant roots in a suspension in water containing 10^3 conidia mL^{-1}. After transplanting, the medium was inoculated with 10^4 conidia mL^{-1} growth medium; this inoculation was carried out by applying 20 mL of a conidia suspension in water (1.375×10^5 conidia mL^{-1}) with a micropipette on the growth-medium surface in five points around the plants (*de Santiago* et al., 2009).

2.2 Plant material and growth conditions

Cucumber (*Cucumis sativus* L. cv. Serena) was used for the experiments. Although it can be considered an Fe-efficient plant, symptoms of Fe-deficiency chlorosis can be observed when it is grown in strongly calcareous growth medium (*Bacaicoa* and *García-Mina*, 2009). It is an important horticultural crop in which the use of T34 is recommended to control soil-borne diseases (*Segarra* et al., 2007). Seeds were germinated in peat, and 15 d after germination individual plants were transplanted to pots (polystyrene cylinder: 350 mL, 5.5 cm diameter, 15 cm height). The experiments were conducted in a growth chamber over a period of 27 d under the same controlled environmental conditions: photoperiod of 14 h, a 25°C/23°C day/night temperature, 65% RH, and 22 W m^{-2} light intensity. Plants were irrigated daily with a Fe-free Hoagland-type nutrient solution. The composition of the solution was (all concentrations in mM): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KH_2PO_4 (1), KCl (0.05), H_3BO_3 (0.009), Mn Cl_2 (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002), and H_2MoO_4 (0.0005). 300 mL of the nutrient solution were applied per pot during the growth period. The pH of the nutrient solution ranged between 5.5 and 6.

2.3 Plant analysis

Chlorophyll was measured in triplicate in the last completely expanded leaf at the end of each experiment using a Minolta SPAD-502 (Minolta Camera Co, Ltd., Osaka, Japan). Accurate correlation between SPAD units and leaf chlorophyll concentration was previously checked (Chlorophyll (mg [kg fresh weight]$^{-1}$) = 0.3 ln (SPAD) – 0.48; R = 0,85; $P < 0.1\%$, n = 18).

The shoots and roots of each plant were separated and their dry weight was determined after drying in a forced-air oven at 65°C until constant weight (approximately 48 h). Dried plant material was ground to pass a 1-mm sieve prior to dry-combustion mineralization. Aliquots of 0.25 g were then mineralized in porcelain crucibles in a furnace at 500°C for 8 h. After that, 10 mL of 1 M HCl were used to dissolve ashes, heated at 100°C for 15 min, and Fe, Cu, Mn, and Zn were determined using atomic-absorption spectrometry (Unicam Solaar M Ficher Scientific, Madrid, Spain) in the digest. Certified plant material was also analyzed to assess complete recovery of nutrients by this procedure.

2.4 Growing-media analysis

β-glucosidase activity, as a general measure of microbial activity involved in the carbon cycle in the growth medium (*de Santiago* et al., 2008b), was determined at the end of both experiments in the rhizospheric growth medium as the amount of PNP (*p*-nitrophenol) formed from PGN (*p*-nitrophenyl-β-D-glucoside) (*Eizavi* and *Tabatabai*, 1988).

At the end of the experiments, the densities of T34 in the rhizospheric medium were determined by dilution plating using the *Trichoderma* semiselective medium of *Chung* and *Hoitink* (1990), as described by *Borrero* et al. (2012). The growth medium (five replicates) was suspended in 90 mL of a pyrophosphate solution in water (1 g L^{-1}). The suspension was shaken and a tenfold dilution series (from 10^{-1} to 10^{-4}) was prepared with water agar (1 g L^{-1}). Suspensions were pipetted onto three plates per dilution. Colony-forming units (CFU) were counted 7 d after plating and expressed as CFU per mL of growth medium sampled. No CFU were detected in treatments without T34 inoculation.

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2.5 Statistical analysis

An analysis of variance was performed to identify the effects of the two factors on SPAD readings, dry-matter (DM) production (shoots and roots), nutrient concentrations in the aerial part and roots, and β-glucosidase activity in the growth medium. Only Fe rate was considered in the analysis of variance of T34 CFU in the growth medium because no CFU were detected in noninoculated growth medium. In the analysis of variance, each replication of the experiment was considered a separate block in order to exclude the variation associated with the repetition of the experiment. The General Linear Model procedure in Statgraphics Plus 5.1 was used (*Statpoint*, 2000). Linear and quadratic responses (L and Q) to Fe rate were considered in the model. In a preliminary analysis, all terms (factors and interactions) were included. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from the final models (*Borrero* et al., 2012). Means were compared with Tukey's test, except when the interaction between factors was significant; in this case, the main effects could not be evaluated in a combined analysis and the mean comparison between treatments of each factor could not be performed. Regressions to fit total DM yield and total Fe accumulation in plants to Fe rate were performed with the same software. Data were fitted to an exponential rise to maximum equation with three parameters, $y = a + b \times (1 - \exp[-c \times x])$, and the maximum adjustable DM was considered $a + b$; sufficiency Fe-threshold values were considered those to achieve 90% of the maximum DM (*Black*, 1993).

3 Results

Overall, β-glucosidase activity increased with increasing Fe rates, but Fe in the growth medium did not affect T34 CFU at the end of the experiments (Tab. 1).

The development of cucumber plants, measured as shoot-DM yield, shoot height, and root-DM yield increased at higher Fe rates in the form of ferrihydrite in the growth medium. In addition, the chlorophyll concentration of leaves measured using SPAD was positively affected by Fe supply (Tab. 2). In general terms, the total content of studied nutrients in the aerial part of plants was higher at increased Fe rates (Tab. 3). However, Fe concentration in the aerial parts was not increased by Fe addition and the concentration of Cu and Mn decreased at increased Fe rates (Tab. 4). The decreasing response of Zn concentration in shoots to increasing Fe rates was different in T34-inoculated and noninoculated media, as revealed by the significant interaction observed between both factors (Tab. 4). In roots, increased Fe rates accounted for increased contents of Fe, Mn, and Zn, increased Fe concentrations, and decreased concentrations of Mn (Tabs. 5 and 6); for total Cu content and concentration the response to increasing Fe rates was significantly affected by T34, as revealed by the significant interaction between both factors.

Table 1: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the β-glucosidase activity and T34-colony-forming units (CFU) in the growth medium at the end of the experiments.

	β-glucosidase activity[a]		CFU[c]	
	– T34	T34	+ T34	
Fe rate / mg kg^{-1}	/ µg PNP g^{-1} h^{-1}			
0	10.88 ± 3.29[b]	13.72 ± 1.99	15795 ± 3322	
8	8.21 ± 1.79	9.88 ± 2.93	10130 ± 3526	
16	15.45 ± 3.59	13.60 ± 4.73	11126 ± 3547	
32.5	24.66 ± 8.55	19.62 ± 6.95	11605 ± 2669	
75	24.06 ± 4.33	16.57 ± 3.38	15846 ± 2574	
T34 inoculation	16.63 ± 2.27	14.82 ± 1.97	12900 ± 1386	
ANOVA results[d]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	11.05	0.13	0.7	41.04
Fe rate (Q)	NI		2.21	14.96
T34	1.93	16.82		
Fe rate (L) × T34	NI			
Fe rate (Q) × T34	NI			

[a] Measured as the amount of PNP (*p*–nitrophenol) formed from PGN (*p*–nitrophenyl-β-D-glucoside)
[b] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[c] CFU, colony-forming units per g of growing medium; only Fe rate was considered in the analysis of variance of T34 CFU in the growth medium because no CFU were detected in noninoculated growth medium.
[d] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For the two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

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Table 2: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the DM yield and chlorophyll meter readings (SPAD) of cucumber grown on a calcareous growth medium.

	Shoots				Roots		SPAD	
	DM		height		DM			
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ g plant^{-1}		/ cm		/ g plant^{-1}		/ arbitrary units	
0	0.94 ± 0.04[a]	1.09 ± 0.10	14.0 ± 1.43	16.4 ± 2.60	0.34 ± 0.03	0.37 ± 0.04	3.37 ± 0.92	4.28 ± 0.73
8	1.32 ± 0.10	1.05 ± 0.08	21.2 ± 2.15	15.2 ± 2.25	0.47 ± 0.04	0.46 ± 0.07	10.99 ± 1.04	11.89 ± 1.47
16	1.56 ± 0.13	1.18 ± 0.10	23.9 ± 2.98	18.3 ± 2.66	0.64 ± 0.10	0.47 ± 0.04	17.33 ± 1.11	14.92 ± 2.27
32.5	1.79 ± 0.10	1.48 ± 0.11	26.7 ± 2.57	23.3 ± 2.79	0.74 ± 0.10	0.65 ± 0.07	23.67 ± 1.33	20.60 ± 1.47
75	2.13 ± 0.15	1.99 ± 0.11	27.1 ± 2.54	24.3 ± 2.14	0.71 ± 0.08	0.76 ± 0.11	29.11 ± 0.83	28.23 ± 0.75
T34 inoculation	1.54 ± 0.08a	1.37 ± 0.07b	22.5 ± 1.23a	19.7 ± 1.20b	0.58 ± 0.04	0.55 ± 0.04	16.74 ± 1.42	16.34 ± 1.34
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	121.28	< 0.01	24.15	< 0.01	43.04	< 0.01	400	< 0.01
Fe rate (Q)	NI		8.25	0.51	12.43	0.07	57.35	< 0.01
T34	7.36	0.8	4.65	03.37	0.9	34.59	1.29	25.91
Fe rate (L) × T34	NI		NI		NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant were removed from final models.

Table 3: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the total content of micronutrients in the shoots of cucumber grown on calcareous growth medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ µg plant^{-1}		/ µg plant^{-1}		/ µg plant^{-1}		/ µg plant^{-1}	
0	27.32 ± 3.45[a]	25.77 ± 4.24	4.05 ± 0.32	3.45 ± 0.25	27.42 ± 1.75	27.26 ± 3.26	11.05 ± 0.49	11.40 ± 1.18
8	50.34 ± 10.39	27.44 ± 5.20	5.89 ± 0.46	4.14 ± 0.49	31.92 ± 1.95	27.87 ± 3.22	14.25 ± 0.93	10.90 ± 0.94
16	47.64 ± 8.05	32.59 ± 6.51	6.05 ± 0.52	4.48 ± 0.55	34.23 ± 1.50	34.48 ± 3.05	14.74 ± 0.83	12.61 ± 1.00
32.5	49.44 ± 4.84	31.94 ± 4.70	6.42 ± 0.40	5.34 ± 0.52	36.32 ± 2.97	33.94 ± 3.29	15.17 ± 0.90	14.29 ± 1.20
75	61.88 ± 6.87	60.96 ± 6.55	7.05 ± 0.57	6.80 ± 0.51	40.69 ± 4.79	36.69 ± 2.31	16.67 ± 0.95	16.86 ± 0.94
T34 inoculation	46.96 ± 3.50a	36.20 ± 3.06b	5.88 ± 0.25a	4.89 ± 0.27b	34.07 ± 1.40	32.19 ± 1.41	14.35 ± 0.45	13.31 ± 0.56
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	57.58	< 0.01	44.21	< 0.01	23.29	< 0.01	39.37	< 0.01
Fe rate (Q)	NI		4.05	4.71	NI		NI	
T34	18.89	< 0.01	12.69	0.06	1.8	18.3	3.59	6.13
Fe rate (L) × T34	NI		NI		NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors, $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

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Table 4: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the concentration of micronutrients in the shoots of cucumber grown on calcareous growth medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}	
0	28.72 ± 3.13[a]	24.17 ± 3.36	4.30 ± 0.29	3.32 ± 0.34	29.63 ± 2.41	25.32 ± 2.05	11.83 ± 0.50	10.41 ± 0.41
8	40.13 ± 9.60	26.14 ± 4.09	4.53 ± 0.27	4.10 ± 0.49	24.99 ± 1.74	26.29 ± 1.39	10.94 ± 0.45	10.49 ± 0.44
16	31.48 ± 4.98	26.30 ± 4.12	3.91 ± 0.18	3.74 ± 0.29	22.99 ± 1.88	29.52 ± 1.90	9.75 ± 0.52	10.76 ± 0.62
32.5	28.42 ± 3.40	21.04 ± 1.87	3.61 ± 0.16	3.59 ± 0.20	20.27 ± 1.25	23.31 ± 1.67	8.50 ± 0.27	9.68 ± 0.42
75	31.32 ± 5.27	31.86 ± 4.12	3.38 ± 0.24	3.47 ± 0.24	18.56 ± 0.99	18.91 ± 1.47	7.95 ± 0.34	8.64 ± 0.51
T34 inoculation	32.12 ± 2.61a	25.92 ± 1.62b	3.95 ± 0.12	3.64 ± 0.14	23.36 ± 0.94	24.52 ± 0.90	9.82 ± 0.28	9.96 ± 0.24
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	1.70	19.51	7.09	0.92	48.8	< 0.01	63.72	< 0.01
Fe rate (Q)	NI		NI		NI		4.82	03.09
T34	8.75	0.4	3.02	8.55	2.01	15.96	0.6	44.13
Fe rate (L) × T34	NI		NI		NI		8.14	0.54
Fe rate (Q) × T34	NI		NI		NI		4.57	3.54

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

Table 5: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the total content of micronutrients in the roots of cucumber grown on calcareous growing medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ µg plant^{-1}		/ µg plant^{-1}		/ µg plant^{-1}		/ µg plant^{-1}	
0	53.51 ± 4.48[a]	61.35 ± 7.87	1.99 ± 0.18	2.03 ± 0.27	5.05 ± 0.48	4.74 ± 0.51	2.44 ± 0.19	2.92 ± 0.30
8	88.62 ± 10.94	85.97 ± 12.16	2.46 ± 0.24	2.69 ± 0.39	5.60 ± 0.47	5.74 ± 0.75	3.96 ± 0.42	4.24 ± 0.54
16	117.04 ± 12.62	82.75 ± 6.72	3.03 ± 0.39	2.64 ± 0.20	7.70 ± 1.21	5.56 ± 0.44	5.17 ± 0.82	4.50 ± 0.37
32.5	187.14 ± 26.40	129.52 ± 15.94	3.69 ± 0.43	3.17 ± 0.29	8.21 ± 1.00	7.13 ± 0.65	5.37 ± 0.54	5.21 ± 0.42
75	200.02 ± 15.81	198.11 ± 57.69	3.57 ± 0.48	4.75 ± 0.38	7.33 ± 0.79	7.90 ± 0.98	5.92 ± 0.45	6.43 ± 0.45
T34 inoculation	128.32 ± 10.57	120.10 ± 7.07	2.93 ± 0.18	3.09 ± 0.19	6.73 ± 0.39	6.27 ± 0.35	4.54 ± 0.28	4.71 ± 0.25
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	113	< 0.01	55.11	< 0.01	24.77	< 0.01	61.88	< 0.01
Fe rate (Q)	20.29	< 0.01	3.58	0.06.2	12	0.08	13.05	0.05
T34	2.98	8.70	0.41	52.26	2.24	13.84	0.12	72.6
Fe rate (L) * T34	NI		4.77	3.17	NI		NI	
Fe rate (Q) * T34	NI		4.23	4.26	NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analysed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

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Table 6: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the concentration of micronutrients in the roots of cucumber grown on calcareous growth medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}	
0	159.86 ± 9.56[a]	168.75 ± 13.68	6.07 ± 0.57	5.85 ± 0.77	15.20 ± 1.28	13.33 ± 1.07	7.39 ± 0.52	8.13 ± 0.50
8	184.43 ± 13.21	183.95 ± 8.57	5.21 ± 0.29	5.89 ± 0.61	12.14 ± 1.09	12.82 ± 0.90	8.78 ± 1.25	9.47 ± 0.91
16	192.33 ± 10.86	177.42 ± 9.79	4.97 ± 0.34	5.66 ± 0.30	12.04 ± 0.86	12.02 ± 0.92	8.40 ± 0.72	9.68 ± 0.63
32.5	261.71 ± 18.35	197.65 ± 8.46	5.20 ± 0.36	4.97 ± 0.37	11.53 ± 0.92	11.28 ± 0.87	7.65 ± 0.45	8.36 ± 0.72
75	291.35 ± 14.84	295.63 ± 12.26	4.53 ± 0.34	7.32 ± 1.03	10.61 ± 0.81	10.78 ± 0.86	8.81 ± 0.72	10.57 ± 1.75
T34 inoculation	217.56 ± 9.38	204.53 ± 8.23	5.21 ± 0.19b	5.95 ± 0.31a	12.33 ± 0.49	12.00 ± 0.42	8.22 ± 0.35	9.25 ± 0.46
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	158.18	< 0.01	0.25	61.56	15.79	0.01	2.99	08.72
Fe rate (Q)	NI		4.92	02.91	4.55	03.56	NI	68.13
T34	3.16	7.88	5.58	02.04	0.22	63.78	3.96	5.36
Fe rate (L) × T34	NI		8.35	0.49	NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

The effect of T34 on β-glucosidase activity was not significant (Tab. 1). Inoculation with T34 decreased the development of the aerial part of cucumber plants (DM yield and height), but did not affect root DM and SPAD meter readings (Tab. 2). In addition, T34 decreased the total content of Fe and Cu in the aerial parts, whereas the effect on Mn and Zn contents was not significant (Tab. 3). Inoculation with T34 decreased Fe concentration in the aerial parts, while its effect on Zn concentrations varied depending on the Fe rate, as revealed by the significant interaction between both factors (Tab. 4). Inoculation with T34 did not affect the total content and concentration of nutrients in roots, with the exception of Cu; its effect on total Cu content and concentration in roots was different depending on the Fe rate, as evidenced by the significant interaction between both factors (Tabs. 5 and 6).

The maximum DM yield estimated from the regression fitting total DM production and Fe rate in the media without T34 inoculation (2.86 g plant^{-1}) was almost equal to that obtained with the highest Fe rate (mean 2.84 g plant^{-1}, Tab. 2). Sufficiency CA-Fe threshold for noninoculated plants was 37 mg kg^{-1}; for inoculated plants, it was necessary 65 mg kg^{-1} of CA-Fe in the growth media to achieve the same total dry matter (Fig. 1). Except for 0 and 75 mg CA-Fe kg^{-1}, the total DM yield and the total Fe uptake by plants was always lower with T34 inoculation than without inoculation (Fig. 1).

4 Discussion

The increased development and chlorophyll concentration of cucumber plants at increased Fe rates (Tab. 2; Fig. 1) demonstrates that this nutrient was a limiting factor for plant development and that ferrihydrite was an efficient Fe source for plants grown on a calcareous medium, in agreement with previous findings (*de Santiago* and *Delgado*, 2007). According to the DM-response curve to Fe rate in the growth medium (Fig. 1), the threshold below which additional Fe supply is necessary was 37 mg CA-Fe kg^{-1}. This reveals a greater Fe-acquisition efficiency than of Fe-chlorosis-sensitive plants grown in calcareous soils which can suffer this deficiency below 140 mg CA-Fe kg^{-1} (de *Santiago* and *Delgado*, 2006; *de Santiago* et al., 2008a).

As expected, increased Fe rates also resulted in increased total Fe content in the aerial parts and roots of plants (Tabs. 3 and 5), thus revealing increased Fe availability in the medium. However, the effect of Fe addition on Fe concentration in plant shoots was not significant (Tab. 4), in agreement with previous findings demonstrating that Fe concentration in aerial parts or leaves is not always related to Fe-deficiency symptoms (*de Santiago* and *Delgado*, 2006). In contrast to what was observed in aerial parts, Fe concentration in roots was increased at increased ferrihydrite concentration in the growth medium (Tab. 6). This can be explained in terms of an increased Fe mobilization from the growth medium and reveals that besides mobilization and accumulation in roots, Fe transport to and within aerial parts is a limiting factor.

The improved development of cucumber plants contributes to explain the increased total contents of other nutrients in aerial parts and roots (Tabs. 3 and 5). However, the concentrations of Cu, Mn, and Zn in shoots and roots decreased at increased Fe rates (Tabs. 4 and 6). This can be ascribed at

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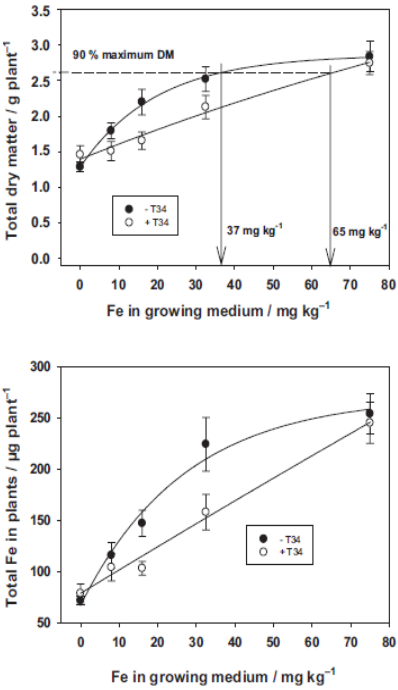

Figure 1: Total dry-matter (DM) yield and total iron accumulation in plants as a function of citrate-ascorbate-extractable Fe concentration in the growth medium. Error bars indicate twice the standard error (n = 10). Dry matter and Fe accumulation in plants was fitted to Fe concentration in the media using an exponential rise to maximum equation with three parameters, $y = a + b\ (1 - \exp[-c\ x])$, and the maximum adjustable maximum DM was considered $a + b$; R^2 was always > 0.99 and $P < 5\%$.

least in part to antagonism with Fe (*Heitholt* et al., 2003; *Assimakopoulou*, 2006) or, in the case of Zn, to adsorption on Fe oxides (*Montilla* et al., 2003).

Although the initial microbial activity in the growth medium was expected to be minimal due to the absence of hydrocarbon sources and to the heating of siliceous sand to adsorb ferrihydrite, some β-glucosidase activity was observed at the end of the experiment even in noninoculated pots (Tab. 1) indicating the development of microorganisms in the rhizosphere of plants. Microbial activity, measured as β-glucosidase activity, increased at increased Fe rates (Tab. 1). This can be related to an increased microbial activity with improved plant development because β-glucosidase activity was correlated with DM of shoots ($r = 0.38$, $P < 0.1\%$). This is in agreement with previous findings by *Valé* et al. (2005) with other herbaceous plants. Beside this, the positive effect of Fe on microbial activity can be also explained in terms of the reduced Fe availability which may be a limiting factor for microbial-biomass development. The number of CFU of T34 was not affected by Fe rate (Tab. 1), which contrasts with the effect on general microbial activity measured as β-glucosidase activity. Inoculation with T34 did not affect this enzymatic activity (Tab. 1) in agreement with *de Santiago* et al. (2013) indicating that β-glucosidase activity was affected by other rhizospheric microorganisms. The number of CFU of T34 was not correlated with DM of plants, thus revealing that T34 development was not affected by plant development contrasting with general microbial activity estimated as β-glucosidase activity. This contributes to explain the lack of effect of Fe rate on T34 CFUs. Also, this nonsignificant effect of Fe rate on T34 CFUs may indicate that T34 is more effective in the acquisition of Fe than other rhizospheric microorganisms.

Overall, inoculation with T34 reduced the development of the aerial parts of plants (DM yield and shoot height, Tab. 2). This can be related to a decreased Fe uptake by plants since inoculation with T34 reduced Fe concentration and the total content of Fe in shoots (Tabs. 3 and 4). However, differences in DM yield, height, and Fe content and concentration in shoots between inoculated and noninoculated plants were not significant when control without Fe or the highest Fe rate were only taken into account (Tab. 2, Fig. 1). At 0 Fe rate the extreme Fe deficiency accounted for a very serious limitation of plant development and masks other potential depressing factors. At other Fe rates, a negative effect of T34 on plant development can be ascribed to stress promoted by the fungus or other competition different from Fe. However, the absence of negative effect at the highest Fe rate in the medium may reveal a competition for Fe between T34 and plants in Fe-poor medium and the greater efficiency of T34 in the acquisition of this nutrient. The decreased total DM yield in T34 inoculated plants at Fe rates between 8 and 32.5 mg kg^{-1} was related to a decreased total Fe uptake by plants (Fig. 1) which contributes to support the hypothesis of a competition by Fe between plants and T34. The Fe-acquisition strategy of this fungus, mainly based on the release of siderophores (*Haas* et al., 2008), must be more efficient than the strategy of cucumber plants mainly based on the excretion of low-molecular-weight organic acids (*Kamilova* et al., 2006). Overall, it is accepted that microbial siderophores, such as those released by T34, show a much higher affinity towards Fe than chelators derived from nongraminaceous plants (*Marschner* et al., 2011).

The results contrast with previous studies reporting positive effects of T34 on Fe uptake by plants susceptible to Fe deficiency and grown in media with citrate-ascorbate-extractable Fe concentrations in the form of ferrihydrite above 150 mg kg^{-1} (*de Santiago* et al., 2009). These positive results were ascribed to the production of siderophores by T34. Fungal siderophores can be used as a source of Fe by cucumber plants at least in part through an increased reduction rate in roots (*Wang* et al., 1993; *Hördt* et al., 2000). However, the production of fungal siderophores can be increased under conditions of restricted Fe availability to take up Fe more efficiently (*Winkelmann*, 2007). A higher production of fungal siderophores in Fe-poor media, such as those with less than 75 mg kg^{-1} of CA-Fe for cucumber, in addition to their higher affinity towards Fe, could result in a decreased Fe uptake by plants with less effective acquisition mechanisms. This may also explain why T34 decreased Fe uptake by cucumber in a

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growth medium with less than 75 mg Fe kg^{-1}, but not by lupins in a medium with 150 mg Fe kg^{-1} in the form of ferrihydrite (*de Santiago* et al., 2009).

The effect of T34 on plant development cannot be ascribed to other nutrients. Nutrient concentrations were high enough to cover plant and microbial biomass needs. Furthermore, T34 tended to decrease the antagonistic effect of Fe supply on Zn and Cu accumulation in plants: the decreasing response of Zn concentration in shoots to increasing Fe rates was more marked in noninoculated plants (Tab. 3), and T34 enhanced Cu concentration in roots (Tab. 5). Thus, it seems that T34 contributed to improve Cu and Zn nutrition in cucumber, which may be ascribed to the positive effect of siderophores produced by T34 on the uptake of other nutrients by plants (*Altomare* et al., 1999).

As stated above, sufficiency Fe-threshold for noninoculated cucumber below which Fe fertilizer should be applied was 37 mg kg^{-1} of CA-Fe. To achieve the same total DM production by inoculated cucumber plants, a minimum CA-Fe in the growth medium of 65 mg kg^{-1} is required (Fig. 1). Below this value, Fe fertilization must be considered in order to overcome a potential decrease of plant growth due to competition with T34. However, T34 increases the uptake of Fe by wheat (Strategy II) in a growth medium with an Fe concentration as low as 15 mg kg^{-1} of citrate-ascorbate-extractable Fe (*de Santiago* et al., 2011). Siderophores exuded by wheat have a much higher affinity towards Fe than the chelates derived from plants such as cucumber (*Kraemer* et al., 2006). Thus, the potential risk of competition between T34 and plants for Fe in growth media and hence the Fe threshold in the media for the inoculation with this microorganism seems to be related not only to the Fe concentration in the media, but also to the Fe-acquisition strategy of plants which determines, among other factors, plant susceptibility to Fe-deficiency chlorosis.

5 Conclusions

The results reveal the need to consider potential interactions between plants and microorganisms used in rhizosphere manipulation. Nutrient thresholds in growth media or soil above which inoculation does not promote a decreased uptake of the nutrient must be known, and fertilizer applied if competition between plants and microorganisms can be expected. Thresholds are only valid for a particular situation and they have to be newly adjusted for every plant–microorganism–substrate combination. It can be inferred that rhizosphere manipulation, including biocontrol strategies, must be integrated with nutrient-management strategies.

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IV

El fósforo, no se encuentra en estado elemental, siempre combinado fundamentamente en forma de fosfato, siendo descubierto por Henning Brand al calentar una muestra de orina evaporada.

RESUMEN

Los microorganismos rizosféricos pueden aumentar la disponibilidad de P en las plantas. El objetivo de este trabajo fue esclarecer los efectos de dos rizobacterias promotores del crecimiento de las plantas y agentes de biocontrol (*Bacillus subtilis* QST713 y *B. amyloliquefaciens* FZB24), un agente de biocontrol (el hongo *Trichoderma asperellum* T34), y *Aspergillus niger* CBS513.88 sobre la absorción de P por las plantas a partir de fosfatos de Ca insolubles. Se realizó un experimento con cultivo microbiano en medios líquidos. Se realizaron también tres experimentos con plantas de pepino (*Cucumis sativus* L. cv. Tropico) en tres diferentes medios: medio silíceo fertilizado con 40 mg P kg^{-1} de roca fosfórica (PR), medio calcáreo suministrado con el mismo fertilizante, y medio calcáreo fertilizado con KH_2PO_4 o PR a 200 mg P kg^{-1}. A pesar de la solubilización observada de PR en el cultivo líquido, no todos los microorganismos mejoraron la absorción de P en las plantas con esta fuente de P. El efecto de cada microorganismo también difirió dependiendo del medio de cultivo, revelando que la actividad solubilizadora de P se ve afectada por el pH y la concentración de P en el medio. De manera general, los mejores resultados se obtuvieron con *Bacillus subtilis* QST713 que aumentó la absorción de P por las plantas en el medio de cultivo silíceo y en el medio calcáreo fertilizado con 200 mg P kg^{-1}. Este efecto positivo sobre la nutrición de P de las plantas fue el resultado no sólo de una mayor solubilización de P, sino también de una mayor capacidad por parte de las plantas para absorber P. Los otros microorganismos estudiados proporcionaron resultados menos prometedores a pesar de las estrategias de movilización de P que mostraron en los cultivos líquidos

(acidificación y exudación de aniones orgánicos). Por lo tanto, estos no pueden ser los únicos mecanismos que contribuyen a la toma de P por parte de las plantas. Nuestros resultados evidencian la capacidad de *B. subtilis* QST713 para mejorar el uso de PR como una fuente de P para cultivos en suelos calcáreos o para mejorar la utilización por las plantas del P residual presente en forma de fosfatos de Ca.

CAPÍTULO IV EFECTO DE VARIOS MICROORGANISMOS SOBRE LA ABSORCIÓN DE FÓSFORO PROCEDENTE DE FOSFATOS DE CALCIO INSOLUBLES EN PLANTAS DE PEPINO

Título: **Effect of various microorganisms on phosphorus uptake from insoluble Ca-phosphates by cucumber plants**

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Effect of various microorganisms on phosphorus uptake from insoluble Ca-phosphates by cucumber plants

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Abstract

Rhizospheric microorganisms can increase P availability to plants. The objective of this work was to elucidate the effects of two plant growth promoting rhizobacteria and biocontrol agents (*Bacillus subtilis* QST713 and *B. Amyloliquefaciens* FZB24), a biocontrol agent (the fungus *Trichoderma asperellum* T34), and *Aspergillus niger* CBS513.88 on P uptake from insoluble Ca-phosphates by plants. An experiment involving microbial cultivation in liquid media and three involving cultivation of cucumber plants in a siliceous growing medium fertilized with 40 mg P kg^{-1} as phosphate rock (PR), a calcareous medium supplied with the same fertilizer, and one fertilized with KH_2PO_4 or PR at 200 mg P kg^{-1} were conducted. In spite of the observed PR solubilization in liquid culture, not all the microorganisms improved P uptake by plants from this P source. The effect of each microorganism also differed depending on the plant-growing medium, revealing that its P-solubilizing activity was affected by pH and P concentration in the medium. Overall, best results were obtained with *Bacillus subtilis* QST713 which increased P uptake from the siliceous growing medium and the calcareous medium fertilized with 200 mg P kg^{-1}. Improved P nutrition of plants was the result not only of increased P solubilization, but also of enhanced ability of plants to absorb P. The other microorganisms studied provided less promising results despite the P mobilizing strategies they exhibited in the liquid culture (acidification and organic anion exudation). Therefore, these cannot be the only mechanisms contributing to P uptake by plants. Our results support the ability of *B. subtilis* QST713 to enhance the use of PR as a P source for calcareous soils or to improve uptake of residual P in the form of sparingly soluble Ca-phosphates.

Key words: P availability / Ca-phosphates / P fertilization / microorganisms

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1 Introduction

In a scenario of depleting phosphate rock reserves, which constitute a finite source of P fertilizer (*Cordell* et al., 2009; *van Vuuren* et al., 2010), it has become increasingly important to improve the efficiency of P fertilization in P-poor soils (*Ryan* et al., 2012) and the availability to plants of P reserves in P-rich soils (*Stutter* et al., 2012). These reserves consist largely of sparingly soluble Ca-phosphates in calcareous soils (*Delgado* and *Torrent*, 2000; *Saavedra* and *Delgado*, 2005), which span roughly one third of all agricultural land in the world. Also, an increasing price of P fertilizer can be partially offset by decreasing fertilizer manufacturing costs such as by using unprocessed phosphate rock (PR) (*Vassilev* et al., 2009; *Vassileva* et al., 2010). Direct use of PR can also alleviate environmental concerns arising from P fertilizer production (*Enamorado* et al., 2009). However, PR is typically composed of highly insoluble Ca-phosphates (usually of the apatite type) that are especially inefficient as a P source for calcareous soils.

Rhizospheric microorganisms can promote solubilization of insoluble Ca-phosphates and hydrolysis of organic P forms, thus, increasing P availability to plants (*Oliveira* et al., 2009). Mobilization of P in the rhizosphere is a common mechanism of plant growth promoting rhizobacteria (PGPR) for enhancing plant development (*Vessey*, 2003; *Richardson* et al., 2009). Ca-phosphate solubilization by microorganisms involves acidification and organic acid production (*Pérez* et al., 2007; *Hariprasad* and *Niranjana*, 2009). Organic acids have been frequently found in P solubilization studies with rhizobacteria (*Patel* et al., 2010) and fungi (*Vazquez* et al., 2000; *Reyes* et al., 2001; *Vassilev* et al., 2001).

PGPR have other potential benefits for plants derived from stimulation of plant growth by production of plant hormones (auxins), improving uptake of other nutrients, or reducing biotic or abiotic plant stress without pathogenic effects (*Welbaum* et al., 2004; *Lugtenberg* and *Kamilova*, 2009; *Hayat* et al., 2010; *Bhattacharyya* and *Jha*, 2012; *Lakshmanan* et al., 2013). Thus, manipulating the plant rhizosphere with microorganisms, additionally having positive agricultural effects such as reducing biotic stress (*e.g.*, plant disease) and improving plant nutrition, might be an effective approach to promoting cost-effective integrated management practices (*Vassilev* et al., 2006; *de Santiago* et al., 2013). The micro-

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organisms to be used to this end should favour P mobilization from dominant P forms present or supplied to soil, such as insoluble Ca-phosphates, and efficiently colonize the plant rhizosphere.

Strains of *Bacillus amyloliquefaciens* have been demonstrated to promote the growth of inoculated roots (*Fan* et al., 2011) and to be an effective biocontrol agent (*Yuan* et al., 2012) with P-mobilizing capabilities (*Ramírez* and *Kloepper*, 2010). Certain isolates of the PGPR *Bacillus subtilis* have been found to enhance P uptake by plants (*Orhan* et al., 2006; *Mena-Violante* and *Olalde-Portugal*, 2007) and to be effective biocontrol agents of plant diseases (*Lim* and *Kim*, 2010). The biocontrol abilities of commercial strains QST713 of *B. subtilis* (*Lahlali* et al., 2011) and FZB42 of *B. amyloliquefaciens* (*Chowdhury* et al., 2015) are well-known, this being the main argument for their widely promoted merchandizing. However, although expected, there is no clear information available on the P-mobilizing abilities of either of these *Bacillus* strains which may be of interest for the promotion of cost-effective integrated management practices.

These positive effects on P uptake have been also observed with many fungal isolates (*Yadav* and *Tarafdar*, 2003; *Singh* and *Reddy*, 2011; *Nakayan* et al., 2013). *Trichoderma asperellum* T34 is a recently commercialized strain which has been demonstrated to be an effective biocontrol agent able to increase the uptake of micronutrients by plants (*de Santiago* et al., 2009; *de Santiago* et al., 2013). However, although other isolates of *Trichoderma* spp., such as *T. harzianum*, is known to mobilize P (*Altomare* et al., 1999), there is no reference to the potential use of T34 for improving P nutrition of plants. Another common fungus, *Aspergillus niger*, has been recognized as one of the most effective microorganisms for PR solubilization (*Goenadi* and *Sugiarto*, 2000; *Abd-Alla* et al., 2001) ascribed, at least in part, to its high capability for the production of organic acids (*Grewal* and *Kalra*, 1995; *Schneider* et al., 2010). Although it has been studied as a P-mobilizing organism in organic substrates (*Vassilev* et al., 2007) and different soils (*Li* et al., 2015), little is known about its use as a P-mobilizing microorganism or other potential benefits it may have in soil. Strain CBS513.88, in particular, has been widely used in industry for organic acid production (*Hertz-Fowler* and *Pain*, 2007).

The effectiveness of P-solubilizing microorganisms may depend on the preparation and formulation of soil microbial inoculants (*Bashan*, 1998; *Vassilev* et al., 2001, 2007), and on the application method (*Malusá* et al., 2012). Furthermore, different responses to their use can be obtained depending on the medium where they are assayed. Mobilization from specific P forms and mechanisms involved in this process have been usually studied using pure microorganism cultures and not in plant-growing media [solubilization of PR by *T. harzianum* (*Altomare* et al., 1999) or *B. subtilis* (*Roi* et al., 2004; *Swain* et al., 2012)]. Processes that appear to be effective in the laboratory may not be operable in soil. Overall, there is little information on the reactions of P in different soils and, frequently, experiments are designed inappropriately and do not allow a specific response to P mobilization to be confirmed (*Jakobsen* et al., 2005; *Richardson* and *Simpson*, 2011). In addition, the efficiency of microorganisms in improving plant nutrition may be constrained by environmental conditions, particularly by soil properties (*de Santiago* et al., 2013). Thus, it can be hypothesized that full insight into the P-solubilizing potential of rhizosphere microorganisms should be obtained with a set of experiments performed with different steps: (1) pure cultivation to assess potential mechanisms involved in the mobilization of specific P forms, and (2) experiments in soil or growing media with different properties to assess how these properties or interaction with plant roots affect their efficiency. Among soil properties, pH is a crucial factor affecting P dynamics and microorganism activity (*Sarwar* et al., 1992; *Bashan*, 1998) and, therefore, studies involving media with different pH and buffering capacity (*e.g.*, calcareous and non-calcareous) should be performed to clarify how P solubilization is affected. The effects on Ca-phosphate solubilization in calcareous media are very relevant for assessing practical benefits because the solubility of these compounds is limited in these media (*Delgado* and *Scalenghe*, 2008). Furthermore, the capability of mobilizing a specific P form can only be assessed in artificial growing media, in which a given P compound can be introduced and investigated alone. This is not possible in soil where many different P forms (organic and inorganic) are present.

The purpose of this work was to investigate the effect of two commercial PGPR and biocontrol agents (*B. subtilis* QST713 and *B. amyloliquefaciens* FZB42), a commercial fungus used as a biocontrol agent (the fungus *T. asperellum* T34) and the fungus *A. niger* (CBS513.88) on P uptake from insoluble Ca-phosphates by plants. It was expected to confirm their potential benefits on P nutrition of plants and to obtain deeper knowledge of the P-mobilizing mechanisms and the influence of the artificial growing medium, whether calcareous or otherwise, as well as the effect of the P-fertilizer rate used on their mobilizing ability. To this end, different types of experiments, involving different media, P sources and rates, were performed. P-solubilizing capability along with additional known benefits will demonstrate the relevance of these microorganisms in the context of integrated management practices in agriculture.

2 Material and methods

2.1 General experimental setup

According to the defined hypothesis, in the present study, two types of experiment were performed:

(1) one experiment in liquid pure culture of microorganisms with different P sources (PR and KH_2PO_4) was used to assess their capability of mobilizing P from PR, and potential mechanisms involved (*Experiment 0*).
(2) three experiments in artificial growing media with plants were used to assess how the microorganisms can contribute to P nutrition of plants:
(a) *Experiment 1*: in non-buffered siliceous media at a near-neutral pH and P supplied as PR at a rate of 40 mg P kg^{-1}.
(b) *Experiment 2*: in buffered calcareous media at pH 8.5 and P supplied in the same form as that in Experiment 1.

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(c) *Experiment 3*: in calcareous media at pH 8.5 and P supplied in two forms (PR and KH_2PO_4) at a higher rate than used in the two previous experiments (200 mg P kg^{-1})

Experiments 1 and 2 were intended to assess microbial effects on P uptake by plants as affected by pH and buffering capacity of soil. Of particular relevance is the verification of this activity in calcareous media. Experiment 3 will provide information on the effect of microorganisms on the P uptake by plants from soluble (*e.g.*, typical P fertilizer) and non-soluble (PR) P sources applied to calcareous media. Comparison of Experiments 2 and 3 will also provide information on the consequences of different P rates on the mobilizing activity of the microorganisms studied.

2.2 Cultivation of microorganisms in liquid media (Experiment 0)

A completely randomized experiment with 3 replications was performed in liquid Czapek medium involving two factors, namely:

(1) Inoculation with different microorganisms, with four treatments: *B. subtilis* strain QST 713 (Serenade Max, Bayer CrospScience), *B. amyloliquefaciens* strain FZB24 (Rhizovital, Agrichem S.A), *A. niger* (CBS513.88), and *T. asperellum* T34 (Biocontrol Technologies), and
(2) P source in the medium, with two treatments: KH_2PO_4 at 0.23 g P L^{-1} (the usual concentration for this type of medium), and ground (< 0.1 mm) phosphate rock (PR, essentially apatite) at the same rate.

Czapek liquid medium was adjusted to pH 7.8 and contained the following substances, with all concentrations in g L^{-1}: sucrose (30), $NaNO_3$ (2), $MgSO_4$ (0.1), KCl (0.5) and $FeSO_4 \cdot 7\ H_2O$ (0.01). Cultures were grown in 250 mL glass flasks inoculated with 100 conidia (fungi) or spore (bacillus) per mL of media.

After 7 d, each medium was passed through a 0.22 μm cellulose-nitrate filter to determine alkaline phosphatase activity in solution, pH, and molybdate reactive P (MRP) according to *Murphy* and *Riley* (1962), and the concentrations of major organic acids formed.

2.3 Inoculation experiments with plants in artificial growing media (Experiments 1 to 3)

Three experiments were performed at different times using cucumber (*Cucumis sativus* L) grown in artificial growing media. All experiments were conducted in accordance with a completely randomized design with 5 replications, each replication consisting of one pot containing a single plant.

Experiments 1 and 2 involved a single factor, namely inoculation with the microorganisms T34, *B. subtilis* QST713, *B. amyloliquefaciens* FZB24 or *A. niger* CBS513.88, in addition to a control treatment without inoculation. The growing medium was siliceous sand at pH 7.5 in Experiment 1. In Experiment 2, the growing medium was calcareous (pH 8.5) and prepared by mixing siliceous sand (70%) with calcareous sand (30%). Phosphorus was applied as PR (the same compound used in the liquid cultures) at a rate of 40 mg P kg^{-1} growing medium.

Experiment 3 was performed in the same growing medium as the Experiment 2 (*viz*., calcareous sand buffered at a basic pH) but involved two factors, namely:

(1) P source, which was either KH_2PO_4 (soluble P) or PR (< 0.1 mm, insoluble P) at an identical rate (200 mg P kg^{-1}), and
(2) microorganism (T34, *B. subtilis* QST713, *B. amyloliquefaciens* FZB24, or no inoculation). *Aspergillus niger* was excluded on the grounds of the results obtained in Experiments 1 and 2.

The lower P rate used (40 mg P kg^{-1}) in Experiments 1 and 2 was intended to mimic the usual P rate for many crops or the amount of P related to insoluble Ca-phosphates (hydroxyapatite type) in P-poor soils. On the other hand, the higher rate used in Experiment 3 was intended to mimic P fertilization in intensive crops (*Delgado* et al., 2002) or an amount of P frequently found in well-fertilized calcareous soils (*Saavedra* and *Delgado*, 2005). In all the experiments, P fertilizer was applied by mixing with growing medium before transplanting.

Artificial growing media consisted of variable combinations of siliceous and calcareous sand in order to mimic calcareous and non-calcareous soils. Siliceous and calcareous sand were passed through 0.2 and 0.5 mm sieves in order to ensure good aeration and hydraulic conductivity in the growing medium. The siliceous sand was washed with dilute $NaCO_3$ to disperse impurities and rinsed with de-ionized water, whereas the calcareous sand was only washed with de-ionized water.

2.4 Plant cultivation

Seeds were germinated in peat and irrigated with de-ionized water. After 14–15 d (two true leaves stage), the cucumber plants were transplanted into 350 mL pots (15 cm high × 5.5 cm diameter polystyrene cylinders) containing 400 g of growing medium. Previously, roots were washed with water to eliminate any peat residue.

After inoculation, pots were placed in a growing chamber with a photoperiod of 14 h, a 25/23°C day/night temperature regime, 65% of relative humidity, and 22 w m^{-2} light intensity for another 28 d. Pots were irrigated on a daily basis with a P-free Hoagland-type nutrient solution at pH 6 containing the following ingredients, with all concentrations in mmol L^{-1}: $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), Fe-EDDHA (0.02), H_3BO_3 (0.024), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002), and H_2MoO_4 (0.0005).

2.5 Inoculation in experiments with plants

Inoculation was done identically in all experiments but procedures slightly differed between inoculants. In all the cases,

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and to avoid different effects due to the inoculation system, commercial products were used for each microorganism and inoculation was done using suspensions of viable cells in de-ionized water. Inoculation with T34 was done by immersing plant roots in a suspension of water containing 10^3 conidia per mL before transplanting, followed by 10^4 conidia per g growing medium after transplanting (*de Santiago* et al., 2009). A volume of 20 mL of conidial suspension in water (2×10^5 conidia per ml), prepared according to *Segarra* et al. (2007), was applied to the surface of each growing medium at four different points around the plants. Inoculation with *B. subtilis* strain QST 713 (Serenade Max, Bayer CrospScience) and *B. amyloliquefaciens* strain FZB24 (Rhizovital, Agrichem S.A) was performed by adding 2×10^4 and 3.75×10^6 colony forming units (CFU) per gram of growing medium, respectively, according to the manufacturerl's recommendations. A volume of 20 mL of aqueous suspension containing 4×10^5 and 7.5×10^7 CFU per mL, respectively, was applied to the surface of each soil at different points around the plants after transplanting. *Aspergillus niger* (CBS513.88) was inoculated by applying 20 mL of a conidial suspension in water (2×10^7 conidia per mL) to the surface of the growing medium in order to obtain a final concentration of 10^6 conidia per g growing medium. Application of inoculum suspensions on soil surface mimics the usual broadcast application usually recommended for herbaceous crops for many microorganisms (*Malusá* et al., 2012) and also manufacturer's recommendations.

The control treatment involved the application of the same volume (20 mL) of water than that of suspension used to inoculate plants; it was applied at different points around the plants. The supply of nutrients with inoculation treatments was considered negligible when compared with the supply of a Hoagland type solution, as the nutrient content of commercial products was very low (it was not included in the specifications) and the dilution of the product was very high in the suspension used. In addition, displacement of solution in the growing media after the first irrigation with the Hoagland-type solution ensured achieving very similar nutrient concentrations (except P) in solution.

2.6 Analysis of plants

Plant shoots and roots were separated and dried to constant weight (approx. 48 h) in a forced-air oven at 65°C to determine dry mass (DM). Dry plant material was ground and an aliquot of 0.25 g mineralized in a furnace at 550°C for 8 h, the resulting ash being dissolved in 1 M HCl with heating at 100°C for 15 min. Phosphorus in the digest was determined by using the colorimetric procedure of *Murphy* and *Riley* (1962). Certified plant material was also analysed in parallel to confirm quantitative recovery of nutrients with this procedure. Total P uptake by plants was calculated as the amount of P present in shoots and roots minus that in seeds.

2.7 Analysis of growing media

Alkaline phosphatase activity was determined in the liquid culture medium and rhizospheric growing medium at the end of each experiment by measuring the amount of *p*-nitrophenol released from 5 mM *p*-nitrophenyl phosphate, using the method of *Tabatabai* and *Bremner* (1969).

Low molecular weight organic acids (LMWOA) were extracted from the rhizospheric growing medium according to *Radersma* and *Grierson* (2004). To this end, 5 g of fresh medium were shaken with 5 mL of de-ionized water at 250 rpm for 1.5 h, followed by centrifugation at 10,000 *g* for 10 min and passage of the supernatant through a 0.45 μm filter. LMWOA in these extracts and in the filtered liquid culture medium were determined by using a HPLC Varian ProStar 410 and a Varian 486 335 photo-diode array detector in combination with a 50 mm × 4.6 mm C18 column of 8 μm particle size and 100 Å pore size. The mobile phase was a solution containing 25mM KH_2PO_4 adjusted to pH 2 with H_3PO_4 and 2% v/v methanol, and was circulated at 0.8 ml/min. The injected volume was 20 mL, and LMWOA were detected at 215 nm.

The pH of the rhizospheric growing medium was determined after extraction with water in a 1:2.5 ratio. Bicarbonate-extractable P (P_{bic}) P in the rhizospheric growing medium after cropping was determined colorimetrically (*Murphy* and *Riley*, 1962) after extraction with 0.5 M $NaHCO_3$ at pH 8.5 for 30 min. Total available P after cropping was taken to be combination of the amount absorbed by cucumber plants and that extractable with bicarbonate present in the medium after cropping.

Microbial density in the media was determined by dilution plating, using Na-pyrophosphate ($Na_4P_2O_7 \cdot 10\ H_2O$) extraction of the rhizospheric growing medium and various semi-selective culture media. *Trichoderma* spp. were isolated by using the semi-selective medium of *Chung* and *Hoitink* (1990) as described by *Borrero* et al. (2012); *Bacillus* spp. were isolated on nutrient–agar medium after heating the suspension at 80°C for 10 min according to *Tuitert* et al. (1998). *A. niger* was isolated on potato dextrose–agar medium amended with 50 mg L^{-1} streptomycin and 50 mg L^{-1} chlortetracycline hydrochloride. Colony-forming units (CFUs) were counted 7 d after plating and expressed as CFU/g growing medium.

2.8 Statistical analysis

The effects of potentially influential factors on the different variables measured in the experiments were assessed by analysis of variance (ANOVA), using the General Linear Model (GLM) in the software Statgraphics Plus 5.1 (*StatPoint*, 2000). Previously, data were checked for normal distribution and homoscedasticity by using the Kolmogorov–Smimov test and Levenne test, respectively, as implemented with the previous software according to *Acutis* et al. (2012). Any data not passing either test required additional transformation.

The results obtained in the liquid cultures (Experiment 0) were subjected to one-way ANOVA for each P source since this was the only effective way of obtaining a normal distribution of data. Those for plants (Experiments 1 and 2) required one-way ANOVA to assess the effect of the microorganisms on the target variables. Finally, the results of the other experiment with plants (Experiment 3) were subjected to two-way ANOVA in order to elucidate the effects of the two factors in-

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volved (*viz.*, P source and microorganism). Only one factor (P source) was considered in the ANOVA of CFU in the growing medium in this experiment with plants because no CFU were detected in the non-inoculated medium. Significance was assessed at $P < 0.1$

When the interaction between factors in Experiment 3 was significant, main effects could not be assessed in a combined analysis; thus, only their combined effect were assessed (*Borrero* et al., 2012; *de Santiago* et al., 2013). Regressions were performed by using the GLM model in Statgraphics.

3 Results

3.1 Liquid cultures (Experiment 0)

All microorganisms studied were found to acidify the liquid culture media and produce large amounts of low molecular weight organic acids (LMWOA) to an extent depending on the particular P source used (Fig. 1). Thus, *B. Subtilis* QST713, *B. Amyloliquefaciens* FZB24, and *A. niger* CBS513.88 lowered pH by 2–3 units with P supplied as PR but only by less than 1 unit—and with no significant differences—with soluble phosphate (Fig. 1). T34 was the only microorganism exhibiting similar acidification capabilities with both P sources (Fig. 1).

All microorganisms were similarly effective in producing LMWOA with PR as P source, but the fungi proved to be more effective than both *Bacillus* species with soluble P (Fig. 1). Molybdate reactive P concentrations (MRP) with PR in the medium well above the expected equilibrium values for apatite-like minerals (PR) confirms the ability of these microorganisms to mobilize P from sparingly soluble Ca-phosphates in this type of culture (Fig. 1). Phosphatase activity was strongly influenced by P source, being much greater overall with PR. The two *Bacillus* species were the most effective microorganisms in producing these enzymes (Fig. 1).

3.2 Siliceous growing medium fertilized with a low P rate (Experiment 1)

Inoculation with microorganisms significantly affected the dry matter (DM) in root (Table 1). *Bacillus subtilis* QST713 doubled root DM relative to the control treatment (no inoculation) and similarly to T34. By contrast, *B. amyloliquefaciens* FZB24 and *A. niger* CBS513.88 led to root DM values not significantly different from those of non-inoculated plants (Table 1). *Bacillus subtilis* QST713 was the only microorgan-

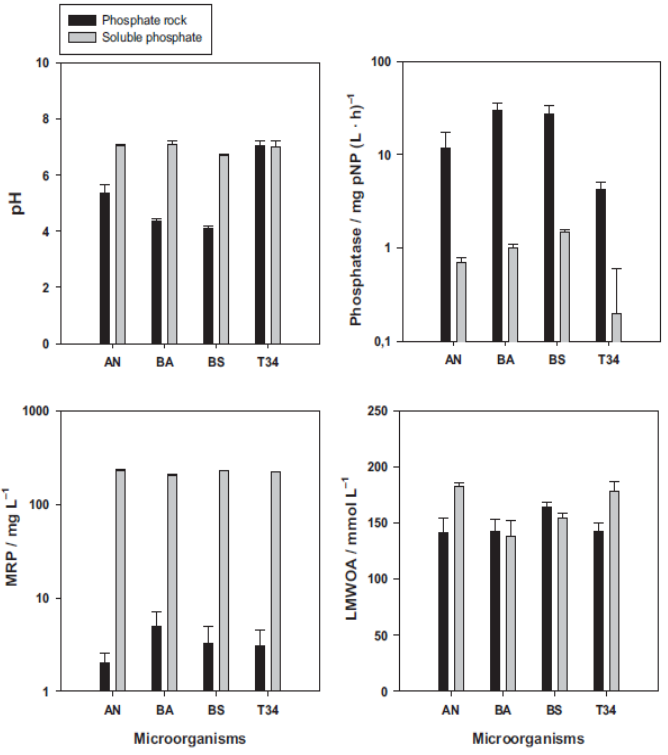

Figure 1: Effect of microorganisms with different P sources on the pH, alkaline phosphatase, low molecular weight organic acids (LMWOA), and molybdate reactive P (MRP) in liquid culture of the microorganisms (Experiment 0). AN: *Aspergillus niger*; BA: *Bacillus amyloliquefasciens*; BS: *Bacillus subtilis*; T34: *Trichoderma asperellum* T34; pNP: *p*-nitrophenol released. One-way ANOVA performed for each P source independently; with PR the effect of "microorganism" was significant for pH ($P < 0.001$) and phosphatase ($P < 0.05$); with soluble phosphate the effect of "microorganism" was significant for phosphatase ($P < 0.05$), MRP ($P < 0.01$), and LMWOA ($P < 0.05$). Error bars indicate one standard error.

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ism studied in the siliceous growing medium significantly increasing total P in shoots and roots, total P uptake, and available P relative to the control. For all these variables, except total P in roots, T34 led to non-significantly different results from those obtained with *B. subtilis* QST713 and with control without inoculation (Table 1). DM in shoots, root-to-shoot ratio (data not shown), P concentration in shoots and roots (data not shown), bicarbonate extractable P in the growing medium (P_{bic}), and phosphatase activity exhibited no significant differences between treatments (Table 1). Although the concentration of LMWOA in the medium was significantly affected by the presence of the microorganisms, the highest concentrations were not those for the treatments resulting in maximal plant development or P uptake (Table 1). All microorganisms significantly decreased pH in the medium relative to the control without inoculation (Table 1).

Total P uptake by plants and total P in shoots increased linearly with increasing DM in roots (R^2 = 0.83, and R^2 = 0.67, respectively; P < 0.001). Total P uptake decreased exponentially with increasing bicarbonate extractable P in the rhizospheric growing media after crop (R^2 = 0.37; P < 0.01) and logarithmically with increasing LMWOA concentration (R^2 = 0.34; P < 0.01). pH and LMWOA explained 50% of the variance in total P uptake by plants (Y = 18.7 – 2.1 pH – 1.4 log LMWOA; P < 0.001). Finally, DM in roots decreased logarithmically with increasing concentration of LMWOA (R^2 = 0.41; P < 0.001).

3.3 Calcareous growing medium fertilized with a low P rate (Experiment 2)

The inoculation with microorganisms had no significant effects on plant growth (measured as DM production), total P uptake by plants, LMWOA (Table 2), P concentration (data not shown), and total amount of P in shoots and roots. *Bacillus subtilis* QST713 significantly increased bicarbonate extractable P in the medium after cropping, and total available P, relative to the other treatments (Table 2). All microorganisms decreased the root-to-shoot ratio of plants (P < 0.05; data not shown) and lowered the pH of the medium relative to the control without inoculation (Table 2). Although phosphatase activity in the medium differed significantly between microorganisms, none led to significant differences in this enzyme from the control treatment (Table 2). Total P uptake by plants increased linearly with increasing DM in roots (R^2 = 0.76; P < 0.001).

3.4 Calcareous growing medium fertilized with a high P rate (Experiment 3)

Bacillus subtilis QST713 increased DM in shoots and roots, and total DM in cucumber relative to the control without inoculation and T34, but had no significantly different effects from those of *B. amyloliquefaciens* FZB24 (Table 3). *Bacillus subtilis* QST713 was the only microorganism which significantly increased P concentration in shoots in comparison to control (P < 0.05; data not shown). Total P in shoots was increased by *B. subtilis* QST713 as compared with the control treatment. P uptake and total P in roots were also increased by *B.*

Table 1: Effect of the different microorganisms on cucumber development (DM yield), total content of P in cucumber plants, bicarbonate-extractable P in the rhizosphere (P_{bic}), available P, pH, low molecular weight organic acids (LMWOA), and alkaline phosphatase activity in the siliceous growing media in which plants were grown with a P supply of 40 mg kg^{-1} (Experiment 1).[ab]

Microorganism	DM Shoots	DM Roots	Total DM	Total P Shoot	Total P Root	P uptake[c]	P_{bic}	Available P	pH	LMWOA	Alkaline Phosphatase
	/ g plant^{-1}			/ mg plant^{-1}			/ mg kg^{-1}			/ mmol kg^{-1}	/ mg pNP (kg · h)$^{-1}$
A. niger	0.58	0.14	0.72	0.53 (–0.32)	0.16	0.59 (–0.32)	1.55	3.02	7.35	73 (1.84)	17.6
B. amyloliquefasciens	0.8	0.13	0.98	0.73 (–0.16)	0.11	0.82 (–0.21)	1.18	3.24	7.34	58 (1.72)	20
B. subtilis	1	0.28	1.28	1.19 (0.04)	0.32	1.39 (0.11)	1.09	4.57	7.36	38 (1.52)	17.5
T. asperellum T34	1	0.21	1.22	0.91 (–0.06)	0.18	0.98 (–0.02)	1.37	3.82	7.32	45 (1.62)	12.2
No inoculation	0.62	0.12	0.74	0.63 (–0.21)	0.16	0.67 (–0.17)	1.58	3.27	7.45	66 (1.82)	12
LSD[d]	0.404	0.100	0.050	0.408 (0.205)	0.092	0.437 (0.257)	0.534	1.07	0.103	29.3 (0.247)	8.58
P value	0.1262	0.0347	0.1013	0.0336	0.003	0.0299	0.2724	0.0530	0.0262	0.085	0.2698

[a]Transformed data between brackets (logarithmic transformation in all the cases).
[b]DM, dry matter; LMWOA, low molecular weight organic acids; P_{bic}, bicarbonate-extractable molybdate-reactive P in the growing medium.
[c]Estimated as total P in aerial parts and roots minus P present in seeds. Total P in shoots was occasionally greater than total P uptake because P in seeds was not counted for P determination in aerial parts.
[d]LSD, least significant difference.

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Table 2: Effect of the different microorganisms on cucumber development (DM yield), total content of P in cucumber plants, bicarbonate-extractable P in the rhizosphere (P_{bic}), available P, pH, low molecular weight organic acids (LMWOA), and alkaline phosphatase activity in the calcareous growing media in which plants were grown with a P supply of 40 mg kg^{-1} (Experiment 2).[ab]

Microorganism	DM Shoots	DM Roots	Total DM	Total P Shoot	Total P Root	P uptake[c]	P_{bic}	Available P	pH	LMWOA	Alkaline Phosphatase
	/ g $plant^{-1}$			/ mg $plant^{-1}$			/ mg kg^{-1}			/ mmol kg^{-1}	/ mg pNP (kg · h)$^{-1}$
A. niger	0.47	0.08	0.55	0.46 (–0.38)	0.12	0.47	2.17 (0.32)	3.35	7.81	58	18.0 (1.17)
B. amyloliquefasciens	0.64	0.11	0.75	0.68 (–0.24)	0.21	0.79	2.78 (0.43)	4.74	7.79	38	43.4 (1.55)
B. subtilis	0.78	0.12	0.90	0.68 (–0.20)	0.12	0.69	6.32 (0.78)	8.05	7.80	67	54.8 (1.68)
T. asperellum T34	0.88	0.16	1.04	0.96 (–0.12)	0.10	0.95	2.97 (0.46)	5.34	7.78	48	25.4 (1.39)
No inoculation	0.84	0.14	0.98	1.21 (0.02)	0.06	1.17	2.09 (0.31)	5.01	8.01	37	32.9 (1.51)
LSD[d]	0.619	0.136	0.806	0.607 (0.360)	0.136	0.768	0.596 (0.186)	2.16	0.092	44.6	28.7 (0.331)
P value	0.3255	0.6309	0.6055	0.2504	0.2752	0.3052	0.0008	0.0066	0.0045	0.5248	0.0635

[a]Transformed data between brackets (logarithmic transformation in all the cases).
[b]DM, dry matter; pNP: *p*-nitrophenol released LMWOA; low molecular weight organic acids; MRP, bicarbonate-extractable molybdate-reactive P.
[c]Estimated as total P in aerial parts and roots minus P present in seeds. Total P in shoots was occasionally greater than total P uptake because P in seeds was not counted for P determination in aerial parts.
[d]LSD, least significant difference.

subtilis QST713 in relation to the control treatment, but they were not significantly different from those obtained with *B. amyloliquefaciens* FZB24 (Table 3). LMWOA in the medium was increased by T34 relative to the other treatments. On the other hand, alkaline phosphatase exhibited no significant differences between inoculation treatments. All variables showed higher values with soluble phosphate than with PR (Table 3)—by exception, alkaline phosphatase and CFU (data not shown) were unaffected by P source.

The effect of inoculation with *B. subtilis* QST713, *B. amyloliquefaciens* FZB24, and *T. asperellum* T34 on pH was found to depend on the particular P source as revealed by the significant interaction between both factors (Fig. 2). With PR, only T34 lowered the pH. With soluble phosphate, both *Bacillus* species lowered it to a greater extent than did T34.

Total P in shoots and total P uptake increased linearly with increasing DM in roots ($R^2 = 0.86$ and $R^2 = 0.88$, respectively; $P < 0.001$ in both cases). When only PR was considered as P source in this experiment, P in shoots and total P uptake also increased linearly with increasing DM in roots ($R^2 = 0.89$ and $R^2 = 0.88$, respectively; $P < 0.001$ in both cases); also, P uptake increased with increasing P_{bic} after crop ($Y = 1.26\ X^{0.44}$; $R^2 = 0.49$; $P < 0.001$) and with decreasing LMWOA ($Y = 0.48\ X^{-0.65}$; $R^2 = 0.27$; $P < 0.05$). Low molecular weight organic acids in the growing media fertilized with PR decreased with increasing bicarbonate extractable P after cropping [$Y = 1/(0.25 + 0.33\ X)$; $R^2 = 0.52$; $P < 0.001$)].

4 Discussion

All the studied microorganisms were able to mobilize P from sparingly soluble Ca-phosphates in liquid pure media. This ef-

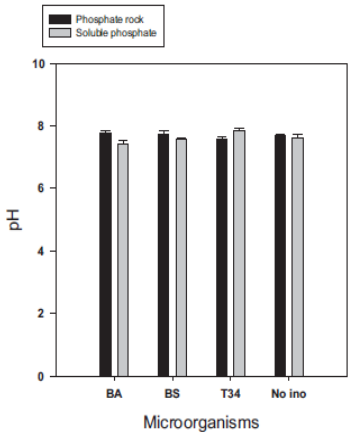

Figure 2: Interaction of P source and microorganisms on the pH in the calcareous growing medium in which cucumber plants were fertilized with 200 mg P kg^{-1} (Experiment 3). BA: *Bacillus amyloliquefasciens*; BS: *Bacillus subtilis*; T34: *Trichoderma asperellum* T34; No ino: Control without microorganism inoculation. The effect of the interaction between factors on pH was significant ($P < 0.01$). Error bars indicate one standard error.

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Table 3: Effect of the different microorganisms on cucumber development (DM yield), total content of P in cucumber plants, bicarbonate-extractable P in the rhizosphere (P_{bic}), available P, low molecular weight organic acids (LMWOA), and alkaline phosphatase activity in the calcareous growing media were plants where grown with a P supply of 200 mg kg^{-1} (Experiment 3).[abc]

Factors	DM Shoots	DM Roots	Total DM	Total P Shoot	Total P Root	P uptake[d]	P_{bic}	Available P	LMWOA	Alkaline Phosphatase
	/ g plant^{-1}			/ mg plant^{-1}			/ mg kg^{-1}		/ mmol kg^{-1}	/ mg pNP (kg · h)$^{-1}$
Microorganism										
B. amyloliquefaciens	1.44	0.26	1.70	1.67	0.33	1.90	5.06 (0.42)	9.74 (0.89)	4.37 (0.55)	23.7
B. subtilis	1.61	0.28	1.89	2.19	0.36	2.44	9.39 (0.63)	15.50 (1.03)	3.18 (0.43)	23.2
T. asperellum T34	1.12	0.21	1.33	1.31	0.27	1.47	4.32 (0.07)	7.20 (0.60)	8.35 (0.81)	23.3
No inoculation	1.25	0.22	1.48	1.51	0.25	1.66	5.63 (0.32)	9.78 (0.83)	3.96 (0.48)	26.8
LSD[e]	0.301	0.057	0.366	0.364	0.085	0.421	4.449 (0.216)	4.461 (0.201)	3.133 (0.197)	5.13
P value	0.0185	0.0647	0.0185	0.0009	0.0168	0.0012	0.0047	0.0106	0.0054	0.4540
P source										
Phosphate rock	1.09	0.17	1.26	1.03	0.22	1.14	0.78 (–0.20)	3.62 (0.52)	2.47 (0.33)	25.8
Soluble phosphate	1.64	0.31	1.96	2.40	0.42	2.71	11.94 (1.01)	17.78 (1.17)	7.28 (0.80)	22.9
LSD[e]	0.222	0.040	0.252	0.264	0.063	0.307	3.196 (0.154)	3.827 (0.154)	2.279 (0.144)	3.729
P value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1210

[a]Only the effect of the main factors in the ANOVA has been considered since the interaction between both factors were not significant for these variables.
[b]Transformed data between brackets (logarithmic transformation in all the cases.
[c]DM, dry matter; pNP; p-nitrophenol released LMWOA; low molecular weight organic acids; Pbic, bicarbonate-extractable molybdate-reactive P in the growing medium
[d]Estimated as total P in aerial parts and roots minus P present in seeds. Total P in shoots was occasionally greater than total P uptake because P in seeds was not counted for P determination in aerial parts.
[e]LSD, least significant difference.

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fect was related to acidification and LMWOA production. However, as hypothesized, the effect of each microorganism differed depending on the properties of plant growing media and P rate used. Overall, the best plant cultivation results were obtained with *B. subtilis* strain QST713. In all growing media, *B. subtilis* QST713 was efficient in mobilizing P because it increased available P relative to the control without inoculation. On the other hand, T34 promoted effects similar to that obtained with QST713 in terms of P uptake by plants and plant development in the siliceous medium, whereas *B. amyloliquefaciens* FZB24 was as efficient as QST713 in calcareous medium with the high P rate.

Effects of QST713 are consistent with the well-known P-mobilizing abilities of other isolates of *B. subtilis* (*Roi* et al., 2004; *Orhan* et al., 2006; *Mena-Violante* and *Olalde-Portugal*, 2007; *Swain* et al., 2012). This mobilization can be explained, at least in part, by the high acidification capacity of this bacillus. Acidification was observed in pure liquid cultures (Experiment 0) and in the plant growing medium with PR as P source. At high P rates in the calcareous medium, acidification by *B. subtilis* strain QST713 was less evident as the likely result of a greater P status in the rhizosphere, which can decrease acidification according to data from studies in pure liquid culture.

B. subtilis strain QST713 increased P uptake by plants in the siliceous medium and in the calcareous medium fertilized with a high P rate (Experiments 1 and 3). However, this microorganism did not increase P uptake in the calcareous medium treated with a low P rate (Experiment 2) despite the increased P availability in the medium. Therefore, enhanced P uptake by this microorganism is not only the result of increased P solubilization, but also, seemingly, of the improved ability of plants to absorb P in siliceous medium and in the calcareous supplied with a high P rate. Enhanced P uptake may be achieved by boosting root development by *B. subtilis* (*Roi* et al., 2004; *Lim* and *Kim*, 2010). This is consistent with the increased root DM with this microorganism in media, in which it also increased P uptake by plants. Increased root development is a common strategy to improve P uptake by plants in P-deficient media (*Sun* et al., 2014; *Wang* et al., 2014), which is consistent with the positive relationships observed between root DM and P uptake by plants. The absence of a positive effect of *B. subtilis* strain QST713 on root DM in the calcareous medium supplied with a low P rate supports the hypothesis of enhanced root development acting as a driving force for an effect of microbial inoculants on P uptake. This may have resulted from factors limiting root growth and activity in a calcareous P-poor medium. Nutrient concentration and pH in media are major factors affecting the effect of rhizosphere microorganisms on plant development (*Sarwar* et al., 1992; *Bashan*, 1998). This contributes to explain different effects of microorganisms on root development depending on P supply and type of growing medium, which in the calcareous P-poor medium was reflected in decreased root to shoot DM ratio.

In siliceous medium, P absorption by plants led to depletion of mobilized P in the growing media as revealed by increased P uptake with decreasing bicarbonate-extractable P in the medium (P_{bic}) after crop. On the contrary, in calcareous medium fertilized with a high P rate, the positive relationship between P uptake and P_{bic} when P was applied as PR suggests that P uptake from PR can be partially related to P mobilization in the growing medium.

Microbial production of LMWOA was not significantly affected by the P concentration of the pure liquid cultures as it was similar in the presence of both soluble phosphate and PR. With a low P rate, LMWOA concentration in rhizospheric media was similar in siliceous and calcareous growing medium. However, in calcareous growing media, LMWOA concentrations were roughly one order of magnitude lower at high P fertilizer rates (Experiment 3) than at low rates (Experiment 2). Thus, P rate was more relevant in affecting LMWOA than was the type of growing medium. The difference observed between low and high P rates in the calcareous medium can be attributed to the response of cucumber plants depending on P rates supplied to the growing medium. LMWOA exudation is a response of plants to P deficiency in the growing medium (*Jones*, 1998; *Walker* et al., 2003) and LMWOA release by cucumber roots is known to be triggered as a nutrient mobilizing strategy (*Kamilova* et al., 2006). Decreased LMWOA concentration with increasing total P uptake in the siliceous and in the calcareous medium fertilized with PR at the high rate supports the hypothesis of decreased LMWOA exudation by plants with increasing P availability in the medium. Thus, LMWOA exudation may be a response to low P availability but not an effective mechanism for increasing P availability to plants under these conditions in agreement with *Ström* et al. (2005). The present study does not provide evidence of a LMWOA-mediated effect of P-mobilizing microorganisms on P uptake by plants, which contradicts classical theories explaining the effects of some PGPR such as *Bacillus* on P plant nutrition (*Rodríguez* and *Fraga*, 1999; *Chen* et al., 2006) or secretion of oxalic, succinic, and citric acids by *A. niger* during phosphate solubilization (*Khan* et al., 2010; *Li* et al., 2015).

The other microorganisms provided less promising results than did *B. subtilis* strain QST713 despite the P mobilizing strategies they used in the liquid culture. Substantial amounts of CFU were found in all experiments with plants irrespective of P source (data not shown). Therefore, the non-significant differences of *B. amyloliquefaciens* strain FZB24, *A. niger* CBS513.88 and T34 with the control treatment (no inoculation) cannot be ascribed to deficient rhizosphere colonization and contradicts evidences observed for other strains [*e.g.*, *Li* et al., 2015 (*A. niger*); *Xu* et al., 2015 (*B. amyloliquefaciens*)]. T34 showed an intermediate P mobilizing ability in the siliceous medium (available P) and no effect in calcareous media. This can be likely ascribed to a lower acidification capacity when compared with *B. subtilis* strain QST713 as observed in liquid culture with PR as P source. Bicarbonate extractable P with *B. amyloliquefaciens* strain FZB24 was not significantly different from that with *B. subtilis* strain QST 713 in Experiment 3, which demonstrated that P-mobilization capability is likely to be related to its acidification ability observed in pure liquid culture. This mobilization and enhancement of root development may explain why P uptake with *B. amyloliquefaciens* strain FZB24 was not significantly lower than that with *B. subtilis* strain QST713 in this experiment

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(Table 3). In the other media, root DM was not significantly different between either of the strains of *Bacillus* spp. However, in the siliceous medium, P uptake with *B. amyloliquefaciens* strain FZB24 was lower than with *B. subtilis* strain QST713. Possible explanations for this difference could be that, at low P rates, enhanced root development did not necessarily correspond with increased P uptake because the P transporters genes expression can be depressed by this microorganism (*Talboys* et al., 2014).

5 Conclusions

Phosphate rock solubilization by the microorganisms studied in pure liquid culture did not necessarily guarantee a corresponding effect in plant-growing media. The pH, related to carbonate content, and P concentration in the medium were crucial factors affecting benefits of the microorganisms studied on P uptake by plants. The major effects were obtained with the PGPR *B. subtilis* strain QST713, which facilitated P solubilization from sparingly soluble Ca-phosphates and improved P nutrition of cucumber plants in siliceous media and also in calcareous media fertilized with a high P rate. This study holds promise for using PR as a P source for calcareous soils or for improving the efficiency of residual P in the form of sparingly soluble Ca-phosphates. In addition, P mobilizing strategies, such as acidification, and LMWOA or enzyme production are not the only reasons for success with rhizospheric microorganisms facilitating P nutrition of plants, which must involve additional, more complex interactions between microorganisms and plants, which are likely to be associated with alteration of root growth.

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Bacillus subtilis es utilizada tradicionalmente para la elaboración de un alimento japonés, llamado Natto, consistente en un derivado de la soja, resultante de la fermentación de su semilla por parte de la bacteria.

RESUMEN

En este trabajo, se estudiaron los efectos de *Bacillus subtilis* cepa QST713 sobre la absorción de P por parte de la planta de pepino (*Cucumis sativus* L. cv. Tropico) a partir de varias formas de P de diferente solubilidad y cómo el potencial efecto del microorganismo se podía afectar por la presencia de óxidos de Fe. El experimento realizado involucró tres factores: (i) fuente de P (KH_2PO_4 a 100 mg kg^{-1}, y roca fosfatada [PR] a 100 o 200 mg kg^{-1}); (ii) inoculación de plantas con QST713 (inoculado y no inoculado); y (iii) óxido de Fe (ferrihidrita) en el medio de cultivo (0 o 300 mg kg^{-1} de concentración de citrato-ascorbato extraíble Fe). La ferrihidrita disminuyó el rendimiento de materia seca en las plantas en más del 50%. La inoculación con QST713 aumentó el crecimiento de la planta y la acumulación total de P por plantas y la absorción de P, estimado como P total en partes aéreas y raíces menos el P presente en las semillas. En general, QST713 aumentó la captación de P en un 40%, siendo el efecto independiente de la presencia de ferrihidrita y de la fuente de P. La mayor absorción de P observada puede atribuirse a la solubilización de P y al aumento del crecimiento de las raíces. Por lo tanto, QST713 mejora la nutrición de P en plantas cultivadas en medios con una alta capacidad de adsorción de P independientemente de la solubilidad del compuesto utilizado como fuente de dicho nutriente.

CAPÍTULO V EFECTO DE *BACILLUS SUBTILIS* SOBRE LA ABSORCIÓN DE FÓSFORO EN PEPINO AFECTADO POR ÓXIDOS DE HIERRO Y POR LA SOLUBILIDAD DE LA FUENTE DE FÓSFORO

Título: **Effect of *Bacillus subtilis* on phosphorus uptake by cucumber as affected by iron oxides and the solubility of the phosphorus source**

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Effect of *Bacillus subtilis* on phosphorus uptake by cucumber as affected by iron oxides and the solubility of the phosphorus source

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In this work, we examined the effects of *Bacillus subtilis* strain QST713 by assessing plant P uptake from variably P compounds. The experiment performed involved three factors: (i) P source (KH_2PO4 at 100 mg kg^{-1}, and phosphate rock [PR] at 100 or 200 mg kg^{-1}); (ii) plant inoculation with QST713 (inoculated and non-inoculated); and (iii) Fe oxide (ferrihydrite) in the growth medium (0 or 300 mg kg^{-1} concentration of citrate–ascorbate-extractable Fe). Ferrihydrite decreased dry matter yield in plants by more than 50 %. Inoculation with QST713 increased plant growth, and total accumulation of P by plants and P uptake, estimated as the total P in aerial parts and roots minus P present in seeds. Overall, QST713 increased P uptake by 40 %, the effect being independent of the presence of ferrihydrite and P source. The increased P uptake observed can be ascribed to solubilization of P and to increased root growth. Therefore, QST713 improves P nutrition in plants grown on media with a high P adsorption capacity irrespective of the solubility of the P compound.

Key words: phosphorus, *Bacillus subtilis*, rhizosphere, Ca phosphates, Fe oxides.

Introduction

Although agricultural soils usually contain substantial amounts of total P, P deficiency is a widespread problem in agriculture (Holford 1997, Wang et al. 2011) because most of total P is present as low plant-available forms owing to its reactions in soil (Delgado and Scalenghe 2008, Ryan et al. 2012). Among such reactions, precipitation of metal phosphates and P adsorption onto Al- and Fe-oxides, are known to play a crucial role in the dynamics and availability of this nutrient in soils (Schwertmann and Taylor 1989, Guzman et al. 1994, Saavedra and Delgado 2005). A high P fixing capacity in soils such as those with high contents of Fe oxides decreases the efficiency of applied fertilizer in increasing P availability in soil (Ryan et al. 2012).

Today's agriculture relies on regular inputs of phosphate fertilizer derived from phosphate rock, a non-renewable, strategic resource (Whiters et al. 2014). The predicted future scarcity of which is raising fertilizer prices and emerging as a critical issue that may affect global food security in the near future (Gunther 2005, Cordell et al. 2009, Cordell and Neset 2014, Amundson et al. 2015). This requires an efficiently management of available P resources (Simpson et al. 2011, Withers et al. 2014), through a better understanding of the biogeochemical cycle of P, the design of more rational fertilization schemes, as well as the use of more efficient biological resources such as crops with an increased capacity to use it (Kochian 2012, Heppell et al. 2015). The manipulation of the rhizosphere by inoculation with microorganisms capable of improving P nutrition of crops can also contribute to the efficient management of P resources (Patel et al. 2010, Wang et al. 2011, García-López et al. 2015).

Rhizosphere microbial communities can improve uptake of some nutrients such as P and hence it may contribute to overcome nutritional deficiencies (Aseri et al. 2008, Zuo and Zhang 2011, de Santiago et al. 2013). Some P-solubilizing microorganisms have been recommended for use as inoculants by virtue of their ability to increase P availability to plants by mobilizing P from scarcely available forms such as insoluble Ca phosphates or organic P forms naturally present in or applied to soil (Richardson 1994, 2001, Illmer et al. 1995, Whitelaw et al. 1999, Oliveira et al. 2009). Rhizospheric microorganisms can have additional benefits such as plant growth promotion (PGPR) or facilitating biocontrol of soil-borne diseases (Vassilev et al. 2006, de Santiago et al. 2011). The benefits of microbial inoculants on crops can provide a basis for their use in cost-effective integrated agricultural practices. Some commercial strains of *Bacillus subtilis* have proved effective PGPR and biocontrol agents (Yuan et al. 2012); others, have been found to facilitate solubilization of P compounds (Orhan et al. 2006, Mena-Violante and Olalde-Portugal 2007).

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Phosphorus availability to plants is related to the solubility of metal phosphates present in soil —Ca phosphates in alkaline soils, mainly— and to P desorption from sorbent surfaces such as Fe oxides. The action of P-mobilizing microorganisms may be affected by the sorption capacity of the growing medium and the solubility of P forms in it. The influence of the solubility of P compounds may be better understood by elucidating the role of scarcely soluble Ca phosphates in calcareous media in supplying P to inoculated plants. These P compounds are major residual sources of P unavailable to plants in calcareous soils. Although the literature on P-mobilizing organisms is growing steadily, there is still no unambiguous information about the main P forms being mobilized and how their mobilization is related to soil properties affecting P dynamics in soil, and particularly to Fe oxides. Microbial mechanisms enhancing Fe uptake by plants through dissolution of Fe minerals, such as release of complexing agents, acidification, or Fe(III) reduction (Lemanceau et al. 2009), may contribute to the uptake of P bound to Fe compounds by plants (Ghosh et al. 2015). Thus, an increased Fe uptake by inoculated plants with P-mobilizing microorganisms may reveal that improved P nutrition is ascribed to the release of P bound to Fe compounds. The main objective of this research was to understand the effect of inoculation with PGPR *Bacillus subtilis* QST713 on P uptake by plants and whether the effect is influenced by the presence of Fe oxides (ferrihydrite) in the growing medium or the solubility of the P source applied. QST713 was selected because it is a commercial strain of *B. subtilis* effective in promoting plant growth and in the biocontrol of some relevant soil-borne diseases (Lahlali et al. 2011). Thus, evidence of its effectiveness as a P-mobilizing inoculant under adverse conditions (e.g., in media with scarcely soluble dominant P forms and a high P adsorption capacity) is bound to support its use in cost-effective integrated management practices contributing to agricultural sustainability in the near future.

Material and methods

Experimental design

An experiment with cucumber plants (*Cucumis sativus* L. cv Tropico) grown in an artificial medium was performed. This type of medium was used to assess P mobilization from specific P forms because the wide range of compounds present in agricultural soils precludes identifying the origin of P absorbed by plants. The growing medium was a mixture of siliceous (70%) and calcareous (30%) sand. Before use, both sands were sieved between 0.5 and 1 mm to facilitate aeration and increase hydraulic conductivity. Siliceous sand was washed several times with diluted Na_2CO_3 to disperse and remove clay minerals and Fe oxides, whereas calcareous sand was washed with deionized water. The final pH of the growing medium, measured in water at a 1:2.5 growing medium/solution ratio, was 8.5 —a typical value for the calcareous soil the growing medium was intended to mimic.

The experiment, which was performed with 5 replications following a completely randomized design, involved three factors, namely: (i) P source, which was either a soluble form (KH_2PO_4) applied at 100 mg kg^{-1} medium or an insoluble form (phosphate rock, PR; sieved at 0.1 mm, and consisting mainly of apatite-type compounds) applied at two different rates (100 or 200 mg kg^{-1}); (ii) plant inoculation with *Bacillus subtilis* (inoculated and non-inoculated); and (iii) Fe oxide (ferrihydrite) in the growth medium (0 or 300 mg kg^{-1} citrate–ascorbate-extractable Fe). Fe oxide enriched media were achieved by using part of the siliceous sand (180 g) coated with ferrihydrite according to Rahmatullah and Torrent (2000). Each experimental replication consisted of a cylindrical pot (polystyrene cylinder: 350 ml, 5.5 cm diameter, 15 cm height) containing 0.4 kg of growing medium and with one plant of cucumber. Previously, seeds were germinated in peat and one plant was transplanted to each pot 15 d after germination. Plants were grown in a chamber under constant controlled environmental conditions, namely: a 14 h photoperiod, a 25/23 °C day/night temperature, 65% RH and a 22 W m^{-2} light intensity. Plants were irrigated with a P-free Hoagland-type solution containing the following nutrients (all concentrations in mmol l^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), Fe-EDDHA (0.02), H_3BO_3 (0.024), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002) and H_2MoO_4 (0.0005). The pH of the nutrient solution was 6.

The plants were inoculated with *B. subtilis* strain QST 713 (Serenade ASO, Bayer Cropscience) by adding 2×10^7 colony forming units (CFU) per kilogram of growing medium. To this end, a volume of 20 ml of an aqueous suspension containing 4 10^8 CFU l^{-1} was applied to the soil surface divided over four points around each plant after transplanting. Non-inoculated plants received an identical volume of de-ionized water that was applied in the same manner. The amount of nutrients supplied by the inoculant was negligible. Also, using Hoagland solution, which contains high nutrient concentrations, offset any potential differences in nutrient supply by effect of its rapidly replacing the solution in the sandy growing media after irrigation.

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Plant analysis

After 28 days of growth, plants were harvested, and the shoots and roots of each plant separated and dried at 65 °C to constant weight in a forced-air oven. Then, dry matter (DM) in shoots and roots was measured, and P and Fe concentration in both organs were determined. To this end, dried plant material was ground to pass through a 1-mm sieve and an aliquot of 0.25 g was placed in a porcelain crucible for mineralization by dry combustion in a furnace at 550 °C for 8 h. After combustion, the ash was dissolved in 10 ml of HCl (1 mol l^{-1}) and the resulting solution heated at 100°C for 15 min to recover all nutrients. Phosphorus in the digest was determined by using the colorimetric procedure of Murphy and Riley (1962), and Fe by atomic absorption spectrometry on a Unicam Solaar M instrument from Thermo Fisher Scientific (Madrid, Spain). Certified material (1 sample in each combustion run) was also analysed in parallel to assess total recovery of nutrients present in the plant material. Total P uptake by plants was estimated as that present in plant tissues minus that in seeds.

Growing medium analysis

Alkaline phosphatase activity at the end of the experiment was determined in the rhizospheric growth medium according to Tabatabai and Bremner (1969), i.e., as the amount of PNP (*p*-nitrophenol) formed from pNPP (*p*-nitrophenyl phosphate).

Organic anions (OA) were extracted from rhizospheric soil by shaking an amount of 5 g of sample with 5 ml of NaOH (0.1 mol l^{-1}) at 4 Hz during 1.5 h (Baziramakenga et al. 1995, Radersma and Grierson 2004). After shaking, suspensions were centrifuged at 10,000 *g* for 10 min and the supernatants were filtered through Whatman 42 filter paper. The filtrate from each NaOH extract was acidified to pH 2–3 with H_2SO_4 (1 mol l^{-1}) and the supernatant passed through a filter of 0.45 µm pore size. Organic anions were separated by high performance liquid chromatograph (HPLC) on an HPLC Varian ProStar 410 instrument furnished with a C18 column (Varian, 250 mm × 34.6 mm, 8 µm particle size), using isocratic elution with 98 % of H_2SO_4 (5 mmol l^{-1}) at pH 2 plus 2 % methanol at 0.8 ml min^{-1} as carrier solution and a 20 µl injected volume. Organic anions were detected at 215 nm, using a Varian 486 photo-diode array detector. Individual standard solutions of acetic, oxalic, citric, malic, fumaric and succinic acid, all from Sigma (Barcelona, Spain), were also used.

At the end of the experiments, the density of *Bacillus* spp in each rhizospheric medium was determined by dilution plating. *Bacillus* spp were isolated on nutrient–agar medium after heating the suspension at 80 °C for 10 min according to Tuitert et al. (1998).

Statistical analysis

The effect of microorganisms on the different measured variables was assessed by analysis of variance (ANOVA), using the General Linear Model (GLM) in the software Statgraphics Plus 5.1 (StatPoint 2000). Differences between means were assessed with Tukey's test at $p < 0.05$. Significant interactions between factors precluded evaluation of main effects in a combined analysis and hence comparing means between treatments for each main factor. When interactions were significant, mean comparison for the combined effect of factors was performed as recommended by Borrero et al. (2012) and de Santiago et al. (2013). Normality and homoscedasticity were always previously ascertained by using the Smirnoff–Kolmogorov and Levene test, respectively (Acutis et al. 2012), and data transformed when either or both tests were not passed. Regressions were also done with Statgraphics Plus 5.1.

Results

As expected, application of the most soluble P form (KH_2PO4) increased P concentration in shoots and roots, total P in shoots and roots and P uptake (Tables 1 and 2). Enriching the growing medium with ferrihydrite resulted in markedly decreased root growth, total P content in roots and shoots, and P uptake. In contrast ferrihydrite resulted in significantly increased P concentration in roots and shoots, and accumulation of organic anions (OA) in the medium. Significant interactions between ferrihydrite enrichment and P source were observed for total P content in shoots, P uptake, and total Fe and total dry matter in shoots (Table 1). The effect on total P and Fe in shoots was only significant with KH_2PO4, whereas that on DM yield was significant with both this P source and PR at 100 mg kg^{-1} (Table 3). Overall, the most negative consequences of enriching the growing media with Fe were observed with P applied in its most soluble form. None of the factors studied affected hydrolytic enzyme activity or CFU in the rhizosphere at the end of the experiment (Table 1). Inoculation with *B. subtilis* significantly increased DM yield in roots (Tables 1 and 2). The microorganism significantly increased total P in shoots and roots, and also total P uptake (Tables 1 and 2); in fact, P uptake was increased by 40 % on average relative to the control without inoculation (Table 2). By contrast, the effects of *B. subtilis* on P concentration in plants were not significant (Table 1).

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Table 1. Significance (*p* values) in the analysis of variance of the different variables studied in cucumber plants (*Cucumis sativus* L.) and growing media

Source of variation	Plant												Growing media		
	Dry matter			P concentration		Total P		Fe concentration		Total Fe		Phosphatase	OA	pH	CFU
	Shoots	Roots	Total	Shoots	Roots	Shoots	Roots	uptake[‡]	Shoots	Roots	Shoots	Roots				
Factors															
A: P source	0.073	0.562	0.099	0.000	0.001	0.000	0.023	0.000	0.333	0.085	0.117	0.226	0.203	0.128	0.001	0.842
B: Oxide Fe	0.000	0.000	0.000	0.031	0.005	0.000	0.000	0.000	0.320	0.000	0.000	0.017	0.793	0.000	0.647	0.515
C: Inoculation	0.058	0.043	0.029	0.870	0.673	0.017	0.038	0.009	0.230	0.000	0.051	0.847	0.162	0.000	0.000	
Interactions																
AB	0.016	0.053	0.010	0.292	0.355	0.000	0.103	0.000	0.944	0.815	0.028	0.073	0.775	0.426	0.084	0.341
AC	0.576	0.916	0.601	0.711	0.085	0.170	0.354	0.162	0.019	0.154	0.273	0.620	0.642	0.932	0.039	
BC	0.289	0.482	0.376	0.943	0.874	0.172	0.541	0.299	0.660	0.437	0.444	0.394	0.613	0.071	0.950	
ABC	0.419	0.283	0.449	0.233	0.482	0.804	0.675	0.890	0.243	0.979	0.383	0.396	0.132	0.217	0.008	

CFU = colony forming units; OA = organic anions; *p* values < 0.05 indicate a significant effect of the source; ‡ = estimated as the total P in aerial parts and roots minus P present in seeds. CFU was 0 for non-inoculated plants.

Table 2. Effect of the main factors on plant development, concentration and total content of P and Fe and organic acid in growing media.

Source of variation	Dry matter			P concentration		Total P		Fe concentration		Total Fe		OA
	Shoots	Roots	Total	Shoots	Roots	Shoots	Roots	Uptake[‡]	Roots	Shoots	Roots	
	g plant^{-1}			g kg^{-1}		mg plant^{-1}		g kg^{-1}	μg plant^{-1}		mmol kg^{-1}
P source												
PR 100	0.30±0.03	0.06±0.01	0.35±0.04	1.3±0.2b	1.05±0.09b	0.34±0.03b	0.06±0.01b	0.28±0.03b	0.69±0.13	33±4	0.03±0.01	98±12
PR 200	0.35±0.03	0.06±0.01	0.41±0.03	1.1±0.1b	1.30±0.16b	0.35±0.02b	0.07±0.01ab	0.30±0.02b	0.61±0.08	36±3	0.03±0.00	96±15
KH_2PO_4	0.54±0.09	0.16±0.04	0.68±0.12	2.3±0.2a	1.70±0.17a	1.13±0.18a	0.25±0.05a	1.22±0.22a	0.64±0.09	61±11	0.09±0.01	113±17
Inoculation												
+ *B. subtilis*	0.44±0.05	0.10±0.02a	0.55±0.07a	1.5±0.2	1.32±0.11	0.67±0.12a	0.14±0.03a	0.69±0.15a	0.49±0.05b	49±7	0.05±0.01	75±8b
– *B. subtilis*	0.35±0.04	0.07±0.01b	0.41±0.06b	1.6±0.2	1.34±0.14	0.52±0.09b	0.09±0.02b	0.49±0.11b	0.81±0.10a	37±5	0.04±0.01	133±14a
Fe rate (mg kg^{-1})												
0	0.55±0.05a	0.13±0.02a	0.69±0.07a	1.4±0.2b	1.15±0.07b	0.84±0.13a	0.17±0.03a	0.89±0.16a	0.41±0.02b	59±7a	0.05±0.01a	80±5b
300	0.23±0.02b	0.03±0.00b	0.26±0.02b	1.7±0.2a	1.57±0.17a	0.34±0.03b	0.05±0.01b	0.28±0.04b	0.94±0.10a	15±3b	0.03±0.00b	124±15a

Means ± standard error; n = 20 for P source and 30 for inoculation; PR = phosphate rock; OA = organic anions; ‡ = estimated as the total P in aerial parts and roots minus P present in seeds. Only the effects of factors were considered when the factor was not involved in a significant interaction. Means followed by a different lowercase letter in a column were significantly different according to Tukey's test ($p < 0.05$) for each factor.

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Bacillus subtilis decreased organic acid accumulation in rhizospheric soil by 43% relative to non-inoculated plants (Table 2). Inoculation, however, had no significant effect on phosphatase hydrolytic activity in the rhizosphere.

Table 3. Effect of the interaction between P source and Fe rate on various properties of the plants and growing media

		Dry matter		Total Phosphorus			Total Fe
P source	Fe rate (mg kg^{-1})	Total	Shoots	Shoots	Roots	P uptake[z]	Shoots
		g plant^{-1}		mg plant^{-1}			µg plant^{-1}
PR 100	0	0.47±0.05b	0.39±0.04b	0.39±0.04b	0.08±0.01bc	0.36±0.05b	41.04±5.63b
	300	0.24±0.03c	0.2±0.03c	0.28±0.03b	0.04±0.01c	0.2±0.03b	23.7±4.22b
PR 200	0	0.52±0.02ab	0.44±0.02ab	0.39±0.02b	0.08±0.01b	0.36±0.03b	41.6±2.42ab
	300	0.3 ±0.04bc	0.26±0.03bc	0.31±0.03b	0.05±0.01bc	0.25±0.04b	29.96±5.07b
KH_2PO_4	0	1.07±0.12a	0.83±0.09a	1.73±0.17a	0.38±0.05a	1.96±0.21a	94.4±13.79a
	300	0.24±0.05c	0.22±0.04c	0.45±0.09b	0.07±0.04bc	0.39±0.11b	23.76±5.21b

Mean ± standard error, n = 10; PR = phosphate rock; [z] = estimated as the total P in aerial parts and roots minus P present in seeds
Means followed by a different lowercase letter in a column were significantly different according to Tukey's test at a $p < 0.05$ (for combinations of treatments)

Table 4. Effect of the significant interaction between P source and inoculation with *Bacillus subtilis* on Fe concentration in shoots and pH in growing media

		Fe concentration	pH
P source	Inoculation	Shoots (mg kg^{-1})	
PR 100	+ *B .subtilis*	104ab	9.33bc
	– *B.subtilis*	106ab	9.66a
PR 200	+ *B.subtilis*	96ab	9.27c
	– *B.subtilis*	107ab	9.36bc
KH_2PO_4[†]	+ *B.subtilis*	137a	9.26c
	– *B.subtilis*	94b	9.47ab

Mean, n = 10; PR = phosphate rock; Means followed by a different lowercase letter in a column were significantly different according to Tukey's test at $p < 0.05$ (for combinations of treatments)

The effect of the microorganism on Fe concentration in shoots was found to depend on P source as revealed by the significant interaction between both factors (Table 1); this was especially so with P applied as KH_2PO_4 (Table 4). The effect of the microorganism on soil pH in the rhizosphere was also dependent of P source (Table 1), and significant with KH_2PO_4 and with PR at 100 mg kg^{-1} (Table 4).

Phosphorus uptake was related to total Fe in plants (data normalized by logarithmic transformation; $R^2 = 0.33$; $p < 0.001$). However, the relationship was highly significant for inoculated plants, but very weak for non-inoculated plants (Fig. 1). Phosphorus uptake and total Fe in plants were related to DM in roots ($R^2 = 0.52$ and $R^2 = 0.43$, respectively; both $p < 0.001$); there was, however, no significant differences in the regressions between inoculated and non-inoculated plants (results not shown).

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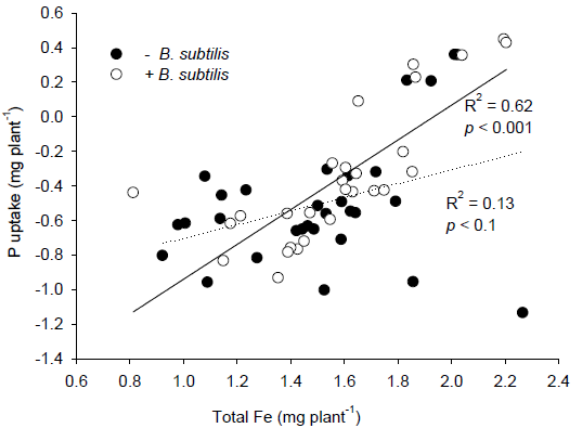

Fig. 1. Log-normalized relationship between P uptake by plants and total Fe in plants; solid line for plants inoculated with *Bacillus subtilis* QST713 and dotted line for non-inoculated plants

Discussion

Phosphorus availability to plants was found to be restricted to a similar extent by a poorly soluble P source and by the presence of sorbent surfaces (Fe oxides) in the media when P was applied as soluble phosphate (Table 3). Despite the decreased amount of Fe oxides present in the growing media relative to natural soils, unsaturation of sorbent sites on Fe oxides considerably reduced the efficiency of applied P. This finding confirms that solubilization of insoluble P forms or blocking of P applied to sorbent surfaces has crucial effects on P uptake by plants and hence on fertilizer efficiency. Based on the results, P from poorly soluble forms, which are gradually released through mobilizing mechanisms acting in the rhizosphere, is less prone to irreversible adsorption onto sorbent surfaces than are soluble P forms.

Inoculation with *B. subtilis* QST713 proved efficient towards mobilizing P; thus, total P in shoots and roots, and P uptake, were increased relative to the control without inoculation irrespective of P source and the presence of sorbent surfaces. This is consistent with the well-known P-mobilizing abilities of other strains of this microorganism (Roi et al. 2004, Orhan et al. 2006, Mena-Violante and Olalde-Portugal 2007). Based on our results, mobilization seemingly resulted at least partially from its acidifying effect; thus, the microorganism lowered the pH slightly in two of the three P treatments. However, the increased P uptake with *B. subtilis* QST713 in inoculated media enriched with ferrihydrite suggests that the potential benefits of the microorganism for P use by may be in part due to blocking of sorption sites on Fe oxides through release of organic compounds forming stable complexes such as siderophores with Fe; in fact, other strains of this microorganism are known to release such compounds as a Fe-mobilizing strategy (Sansinenea and Ortiz 2011, Zhao et al. 2011, Walia et al. 2014). These organic compounds compete for sorption sites with P, thus decreasing P adsorption and increasing P absorption by plants. The hypothesis of a siderophore-mediated increased P availability is supported by the evidence of increased Fe accumulation in shoots with *B. subtilis* QST713 when increased amounts of P were adsorbed on Fe oxides, i.e. with KH_2PO_4 (Table 4). Microbial siderophores are known to increase Fe uptake by plants (de Santiago et al. 2013). Also, P adsorption onto Fe oxides is known to decrease Fe uptake by plants by preventing adsorption of Fe-complexing organic compounds and hindering P dissolution as a result (Sánchez-Rodríguez et al. 2013). However, the increased Fe concentration in shoots in inoculated plants when P source was KH_2PO_4 and the increasing trend in total Fe in shoots (p = 0.0506) suggest that microbial siderophores are more efficient in competing for sorption sites on Fe oxides with P than Fe-complexing compounds released by plants. On the other hand, the decreased concentration of low-molecular weight organic acids in inoculated media suggests that the nature of these microbial

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Fe-mobilizing compounds is different from the OA excreted by roots. The compounds, usually referred to as "siderophores", are especially efficient and specific in complexing Fe relative to low-molecular organic acids excreted by plants (de Santiago et al. 2009, 2013).

Bacillus subtilis QST713 was effective in improving growth of cucumber plants measured as total DM . The increased growth may have been the consequence of enhanced plant nutrition. The assumption that P availability influences DM yield is supported by the fact that Fe oxides restricted the uptake by plants of applied P. P adsorption on Fe oxides implies that part of adsorbed P remains unavailable to plants (Delgado and Scalenghe 2008). Thus, P adsorption on Fe oxides results in decreased P concentration in solution and uptake by plants in Fe-enriched media. This explaines the lower DM yield in Fe-enriched media when compare with media without Fe oxides. However, increasing root growth is a common plant strategy for improving P uptake in P-deficient media (Sun et al. 2014, Wang et al. 2014); also, it is consistent with the positive relationship between root DM and P uptake by plants in observed this work. The increase in root DM resulting from the presence of *B. subtilis* was independent on any factors affecting P availability in the growing medium (viz., P source or ferrihydrite). Therefore, *B. subtilis* increased plant growth —more markedly in roots (43 %) than in shoots (29 %)— in media with a high or low P availability. This suggests that *B. subtilis* plays a direct role in increasing DM yield that is not necessarily mediated by improved P availability to plants. Thus, the enhanced root growth with *B. subtilis* QST713 increases P uptake by plants irrespective of the P availability level in the growing medium. This assumption is consistent with previous evidence of improved growth in plants inoculated with other *B. subtilis* strains resulting from their production of auxins (Roi et al. 2004, Lim and Kim 2009). The results are also consistent with those of Orhan et al. (2006) and Turan et al. (2007), who found other *Bacillus* strains to increase P uptake through improved root growth.

Organic anions exudation is one response of microorganisms and plants to P scarcity in the growing medium (Jones 1998, Walker et al. 2003). Also, exudation of organic anions by cucumber roots is known to be a mobilizing strategy triggered under nutrient deficiency conditions (Kamilova et al. 2006). This may account for the increased organic anion concentrations observed here when P availability to plants was decreased by application of ferrihydrite (Table 2). The fact that oxalate was the only organic anion detetected in most of the growing media (results not shown) is consistent with previous works showing that this compound is the main anion released by plant roots under P-deficient conditions (Jones 1998, Ström et al. 2005). Organic anions released by plants or bacteria can compete for sorption sites and facilitate P desorption as a result; also they can enhance dissolution of sparingly soluble phosphates (Ström et al. 2002, 2005). However, inoculation with *B. subtilis* decreased the concentration of organic acids in the rhizospheric medium. This can be ascribed to consumption of this C source by *B. subtilis* QST713, which is consistent with previous evidence that inoculation with microorganisms can decrease the concentration of OA in the rhizosphere (García-López et al. 2015). Therefore, the benefits of *B. subtilis* QST713 on P nutrition of plants cannot be ascribed to the production of low-molecular weight organic acids; rather, the acids exuded by plant roots can indirectly improve P uptake by plants by acting as C sources for the microorganism.

Low-molecular organic acids play a prominent role in an Fe acquisition strategy for dicotyledonous plants. Thus, the decreased organic anions concentrations observed with QST713 provide additional evidence for its contribution to Fe uptake by plants via other, more effective Fe-mobilizing mechanisms such as the above-described release of siderophores. A significant relationship between P uptake and total Fe in plants was observed with *B. subtilis* but not in non-inoculated plants (Fig. 1). This likely reveal that different mechanisms affecting Fe and P availability to plants may be involved when plants are inoculated with QST713. Thus, P uptake in inoculated plants was more significantly related to Fe uptake than it was in non-inoculated plants (Fig. 1). This result suggests that P mobilization in inoculated media is more markedly dependent on Fe-mobilizing mechanisms than it is in non-inoculated media.

Conclusions

Bacillus subtilis QST713 was effective in increasing P uptake by plants irrespective of the solubility of the P source applied or the adsorption capacity of the growing medium. This benefit on P uptake can be ascribed to an increased P mobilization from growing media and to an enhanced root growth. The increased Fe accumulation in plants grown in ferrihydrite-enriched media reveals a link between Fe and P acquisition strategies, and also that P uptake is improved by adsorption of siderophores onto Fe oxides.

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VI

Una taza de suelo puede contener tantos microorganismos como personas viven en la Tierra.

RESUMEN

La eficiencia en la inoculación con microorganismos movilizadores de P en el suelo puede verse afectada por las propiedades del suelo incluyendo la microbiota natural presente en el mismo. El objetivo de esta investigación fue estudiar la contribución de *Trichodermaa asperellum* T34 y *Bacillus subtilis* QST713 a la disponibilidad de P en suelos en plantas de trigo y cómo este potencial efecto puede verse afectado por las propiedades del suelo. Para ello se realizó un experimento completamente al azar que incluyó dos factores: (i) suelo (12, con un rango de valores de P Olsen que osciló entre 4.8 y 8.7 mg kg^{-1}), y (ii) inoculación de plantas con T34 o BS (inoculada y no inoculada). La inoculación con microorganismos afecto a la concentración de P en la parte aérea y a la fosfatasa alcalina en el suelo rizosférico. El efecto de T34 sobre la absorción de P y la materia seca (DM) de la parte aérea en relación al testigo sin inocular fue explicado en un 82% y 74%, respectivamente, por el P Olsen, la relación entre el Fe en óxidos poco cristalinos y el Fe en óxidos cristalinos (Feca/Fecbd), y el P hidrolizable por fitasa en el extracto de NaOH. En el caso de B. subtilis, para la absorción de P por las plantas en relación al testigo sin inocular, el 92% de la variación se explicó por el P hidrolizable por fitasas en extractos de NaOH, P Olsen, carbonatos, la relación Fe_{ca}/Fe_{cbd} y el P soluble en citrato. La mayoría de estas propiedades contribuyó a explicar el 87% de la variación en la relación de DM en parte aérea de plantas inoculadas *de B. subtilis* en respecto a DM en parte aérea del testigo sin inocular. En resumen, todos estos resultados serán útiles para definir una recomendación de inoculación más precisa en el

futuro, teniendo en cuenta que las propiedades del suelo son cruciales para explicar sus beneficios potenciales en los cultivos.

CAPÍTULO VI COMO AFECTAN LAS PROPIEDADES DEL SUELO A LA CAPACIDAD DE MOVILIZACIÓN DEL FÓSFORO DE *BACILLUS SUBTILIS* QST713 Y *TRICHODERMA ASPERELLUM* T34

Título: **Phosphorus mobilization by *Bacillus subtilis* QST713 and *Trichoderma asperellum* T34 as affected by soil properties**

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Introduction

Only a small portion of total P present in soil is readily available to plants and microorganisms (Hisinger, 2001; Delgado and Scalenghe, 2008; Richardson et al., 2011). This is the result of P reactions in soils, which in agricultural lands determines a low efficiency of applied P fertilizer (Saavedra and Delgado, 2005; Delgado and Scalenche, 2008; Ryan et al., 2012). Consequently, most of the historically applied P to soil remains in non-bioavailable forms (Delgado and Torrent, 1997; Recena et al., 2017), which constitutes a "legacy" (Withers et al., 2014) not contributing to agricultural productivity. This P legacy includes recalcitrant inorganic (e.g. metal phosphates) and organic P (non-readily hydrolysable). The enhancement of the use of these non-readily available forms by plants, and the achievement of greater efficiencies in the use of P fertilizer (Cordell et al., 2009; Ryan et al., 2012) are major issues in achieving a more sustainable use of this non-renewable strategic resource (Ryan et al., 2012; Withers et al., 2014). To this end, it is required the design of more rational fertilization schemes (Recena et al., 2016), as well as the use of more efficient biological resources such as crops with an increased capacity to use P (Shen et al., 2011; Kochian, 2012, Heppell et al., 2016). Within these biological resources, it should be also considered the inoculation with microorganisms able to mobilize native and soil P legacy and any insoluble source of P applied as fertilizer (Jones and Oburger, 2011; Owen et al., 2015; García-López et al., 2016).

The use of P-mobilizing microorganisms has gained attention in the last decades as a way of increasing agricultural productivity and sustainability in P-poor soils. Literature

release on this topic is abundant (Kim et al., 1998; Gunes et al., 2009; Singh and Reddy, 2011; Nakayan et al., 2013). However, agriculture needs cost effective integrated measures. This means that the exclusive use of microorganisms for improving P acquisition by plants is unrealistic without additional benefits from inoculation such plant disease biocontrol or any other way of promotion of plant growth. This is why the use of biological control agents effective in mobilising nutrients has gained interest. This will allow an integrated management of plant diseases and nutritional deficiencies, which are two major stresses constraining agricultural sustainability (Vassilev et al., 2006; de Santiago et al., 2011; 2013). In this regard, *Trichoderma* spp is a known biocontrol agent, which also has proved effective as plant-growth-promoting microorganism (Harman and Bjorkman, 1998; Vassilev et al., 2006). *Trichoderma asperellum* T34 has proved effective increasing Fe (de Santiago et al., 2009; 2011), and P uptake by plants (García-López et al., 2015). *Bacillus subtilis* is a plant-growth-promoting bacteria, and certain isolates of this PGPR has been found to enhance P uptake by plants (Orhan et al., 2006; Mena-Violante and Olakle-Portugal, 2007; García-López et al., 2016; García-López and Delgado, 2016) and to be an effective biocontrol agent of plant diseases (Lim and Kim, 2010; Lahlali et al., 2011).

The efficiency of microorganisms in improving plant nutrition may be constrained by environmental conditions, particularly soil properties (de Santiago et al., 2013). A wide range of several factors like soil, crop, environment, management or fertility are involved in the final efficacy of any fungal or bacterial strain utilised as inoculant (Heijnen et al., 1992; Rodriguez-Navarro et al., 1991; van Elsas and Heijnen, 1990; Heijnen and

Marinissen, 1995). In addition, the size and competitiveness of indigenous microbial populations may be determinant for the success in the establishment of microbial inoculants (Adholeya et al., 2005; Owen et al., 2015). Most of the studies reporting the benefits of a given P-mobilizing microorganism has been performed on a specific soil. Thus, new insight is required on the soil factors affecting the success of P-mobilizing microorganisms as biofertilizers for supporting their practical use. This success may be not only ascribed to the establishment of the inoculant in the rizhosphere, but also to microbial mechanisms to solubilize inorganic P or to hydrolyse organic P. These mechanisms have been shown to depend not only on the microorganism, but also they can be triggered by particular conditions in the plant growing medium (García-López et al., 2016).

This work is thus aimed at studying the potential contribution of microorganisms to P supply to plants as affected by soil properties, and particularly by those properties related to P dynamics in soil which may determine the efficiency of microbial mechanisms in mobilizing P. To this end, two different free-living, a fungus, *Trichoderma asperellum* T34 and a bacteria*, Bacillus subtillis* strain QST713, were selected because based on previous evidences (García-López et al., 2016) it is expectable that their P-mobilizing abilities are differently affected by soil properties. Both are commercial strains effective in promoting plant growth and biocontrol of some relevant soil-borne diseases, which have proved effective P-mobilizing microorganisms (García-López et al., 2015; 2016; García-López and Delgado, 2016). Particular emphasis will be put on soil

properties usually determined in routinary soil fertility studies focused on the practical application of results in assessing the potential efficiency of studied microorganisms.

Materials and methods

A set of 12 soil samples were selected for this study encompassing representative soils from different agricultural lands from Spain and including different orders according to Soil Taxonomy (Soil Survey Staff, 2010): Mollisols, Entisols, Inceptisols, Alfisols, and Vertisols. This selection involves representative soils developed under Mediterranean climate (Recena et al., 2016), which were used use for olive orchards, vineyards, and for field crops (mainly wheat-sunflower rotation). Samples were collected from the surface horizon, ground to 6 mm for plant cultivation, and for soil analysis, a portion of the soil was ground to 2 mm.

Soil analysis

General soil properties were assessed by the following methods: densimeter for particle size analysis (Gee and Bauder, 1986), dichromate oxidation for soil organic matter (SOM) (Walkley and Black, 1934), calcimeter method for total Ca carbonate equivalent (CCE), and the electrical conductivity (EC) and pH was measured at a soil:water ratio of 1:2.5. Nutrient availability index determined were DTPA extraction for micronutrients according to Lindsay and Norvell (1978), and bicarbonate extraction for P according to the Olsen method (Olsen et al., 1954). Iron ascribed to poorly crystalline Fe oxides was determined after reduction with citrate-ascorbate, and sequentially a reduction with citrate-bicarbonate-dithionite was done to assess the amount of Fe

ascribed to crystalline oxides (de Santiago and Delgado, 2006). In both extracts, Fe was determined by atomic absorption spectrometry.

In addition, main P fractions in soils were studied. Total inorganic P (Pi) was determined as the molibdate reactive P (MRP) after extraction with 0.5 M H2SO4, and total organic P as the increase in 0.5 M H_2SO_4 extractable MRP after soil calcination (Kuo, 1996). Main P fractions were studied according to the sequential fractionation scheme described by Recena et al. (2015). According to this scheme, main identified fractions are: (i) adsorbed P plus soluble metal phosphates (two first steps, NaOH and citrate-bicarbonate extractions), (ii) P ascribed to hydroxyapatite (citrate extractable, C-P), (iii) P occluded in poorly crystalline and crystalline Fe oxides (reductant soluble, citrate-ascorbate and citrate-bicarbonate-dithionite extractable, respectively), and (iv) residual Ca phosphates (acetate and HCl extractable). Phytase hydrolisable P was determined in all the P fractions as described by Recena et al. (2016). In all the fractionation extracts, and after enzyme hydrolysis, MRP was determined by the colorimetric method of Murphy and Riley (1962).

Experimental setup

A completely randomized experiment with three replications was performed twice at different times under the same growing conditions in a growing chamber. The experimental design involved two factors, namely: soil (12), and inoculation with microorganisms (control without inoculation*, Trichoderma asperellum* T34 and *B. subtillis* QST713). Each replication consisted of one pot containing one plant of wheat.

Seeds were pre-germinated in petri plate at 8º C and darkness during 15 d, and after that germinated in perlite. Seedlings were transplanted to pots 15 days after emergence, and grown for 33 additional days in 106 mL cylindrical pots (3 cm diameter; 15 cm height) containing 110 g of soil. Previously, roots were thoroughly washed with deionized water to eliminate any perlite residue.

Inoculation with T34 (Biocontrol Technologies, Barcelona, Spain) was made in two steps: by immersing plant roots in a suspension of water containing 10^6 conidia per L before transplanting, and by applying by 10^7 conidia per kg of growing medium after transplanting (de Santiago et al., 2009). The latter step involved the application of 20 mL of conidial suspension in water ($2 \cdot 10^8$ conidia L^{-1}) prepared according to Segarra et al. (2007) in the surface of each pot at four different points around the plants. Inoculation with B. subtillis strain QST713 (Serenade Max, Bayer CrospScience, Paterna, Spain) was performed by adding $2 \cdot 10^7$ colony forming units (CFU) per kg of growing medium after transplanting by applying 20 mL of aqueous suspension containing $4 \cdot 10^8$ CFU L^{-1} in surface of each soil at different points around the plants.

Plants grew in growing chamber under constant controlled environmental conditions during the experiment involving a photoperiod of 14 h, 23°C/20°C day/night temperature, 65% RH, and 22 W m^{-2} light intensity. Plant were fertigated with a P-free Hoagland-type nutrient solution with the following composition (all concentrations in mmol L^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), Fe- EDDHA (0.02), H_3BO_3 (0.024), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.006), and H_2MoO_4 (0.0005). pH of the nutrient solution was adjusted to 6 before irrigation.

Soil analysis after cultivation

β-glucosidase activity and alkaline phosphatase activity were determined in the rhizospheric soil at the end of both experiments. The former as the amount of pNP (p-nitrophenol) formed from PGN (p-nitrophenyl-b-D-Glucopiranoside) according to Eizavi and Tabatabai (1988), and the second activity as the amount of pNP released from 5 mM p-nitrophenyl phosphate using the method of Tabatabai and Bremner (1969).

Colony forming units (CFU) were determined by dilution plating after sodium pyrophosphate extraction of the rhizospheric soil. Trichoderma spp. was isolated by using the semi-selective medium of Chung and Hoitink (1990) as described by Borrero et al. (2012), and Bacillus spp. were isolated on nutrient–agar medium after heating the suspension at 80 ºC for 10 min according to Tuitert et al. (1998) Three plates per dilution ratio were used, and CFU were counted after 4 days. No CFU were detected in treatments without inoculation.

Extraction of organic anions from rhizospheric soil was done by shaking 5 g of rhizospheric soil in 5 mL 0.1 M NaOH for 1.5 h at 4 s^{-1} (Baziramakenga et al., 1995; Radersma and Grierson, 2004). After extraction, suspensions were centrifuged at 10,000 g for 10 min, the supernatant filtered through a 0.45 µm cellulose filter, and the filtrate acidified to pH 2–3 with 1 M H2SO4. High performance liquid chromatographic (HPLC) separation of organic acids was done with a HPLC Varian ProStar 410 equipped with a C18 column (Varian, 250 mm x 34.6 mm, and 8 µm particle size). Elution was isocratic with 98 % 5 mM H_2SO_4 at pH 2 + 2 % methanol as carrier solution at a flow rate of 0.8 mL min^{-1}, and 20 µL of injection volume. Organic anions were detected at 215 nm using a Varian 486 photo-diode array detector. Standard solutions of acids (acetic,

oxalic, citric, malic, fumaric, and succinic) were prepared as individual stock solutions, using Sigma acids (Sigma, Barcelona, Spain). The pH of the rhizospheric soil was determined after extraction with water at a 1:2.5 ratio.

Plant analysis

Plants were harvested including roots, which were separated from soil and thoroughly washed. After that, shoots and roots were separated, and both organs dried to constant weight (48 h) in a forced-air oven at 65ºC. After drying and determination of dry matter (DM) in each organ, dry plant material was ground and an aliquot of 0.25 g mineralized in a furnace at 550 ºC for 8 h. Resulting ashes were dissolved in 1 M HCl with heating at 100 ºC for 15 min. In the remaining solution, P was determined by the colorimetric procedure of Murphy and Riley (1962). Certified plant material was also analysed in parallel to confirm the complete recovery of nutrients with this procedure. Total P uptake by plants was calculated as the amount of P present in shoots and roots minus that in seeds.

Statistical analysis

Analysis of variance (ANOVA) was performed by using the General Linear Model procedure in Statgraphics Centurion XVI (StatPoint, 2013) to identify the effects of the studied factors on the different variables measured in the experiments. Previously, normal distribution and homoscedasticity were assessed by Kolmogorov–Smirnov and Levenne test, respectively. If necessary, data were potentially transformed to meet both criteria (transformed data = data^{-b}, being b the slope of the relation between the

logarithms of standard deviation and the logarithm of the mean for each treatment). Results from both replications of the experiment were jointly analysed, with each replication deemed as a separate block in order to exclude the variation associated with the repetition of the experiment (de Santiago et al., 2009). If data transformation did not allowed to meet the normality or homoscedasticity criteria, the effect of factors was assessed by the Kruskal-Wallis non-parametric test; in this cases, the effect of the interaction cannot be assessed. Only one factor (soil) was taken into account in the ANOVA of CFU in soil because no CFU was detected in non-inoculated soil. Means differences were assessed via Tukey's test ($P < 0.05$). When interactions between factors were significant, the main effects could not be assessed in a combined analysis, and consequently it was not possible to compare means of main factors (de Santiago et al. 2013). The significant interaction means that the effect of each inoculation treatment on a given variable differed depending on the soil. In that case, multiple regression analysis were performed to assess which soil properties explained the ratio of the observed value for inoculated plants to the value of non-inoculated plants (control). On this relative basis, the effect of microorganism can be assessed along different environments (soils) (Black, 1993). This analysis was done independently for T34 and for B. subtilis. The model dimension, i.e. number of independent variables to be included, was selected according to the Akaike information criterion (AIC; Akaike, 1974) and the accuracy of the model checked by the Cp statistic of Mallows (Gilmour et al., 1996). As additional requirement, independent variables included in the model were significant according to the t statistic at $P < 0.05$. When correlated independent variables

were included in the models to meet the best AIC, it was assumed that the sign of the coefficient does not have explicative value. In addition, other multiple regressions and correlation analysis was performed. Regression and correlation analyses were also done using Statgraphics Centurion XVI software.

Results

Soil properties

The studied soils showed a wide range of properties. In particular, they ranged widely in those properties related to the P cycle in soil, such as different P forms and fractions, the Ca carbonate equivalent (CCE), and Fe oxides (Table 1). Regarding the Fe oxide mineralogy, Fe bound to crystalline oxides was dominant, accounting on average for 86 % of Fe in oxides. Olsen P values ranged from 4.8 to 8.7 mg kg^{-1}; these values amounted to a minor fraction of inorganic P in soils, which varied from 0.18 to 0.52 g kg^{-1}.

Effects of soil and microorganisms

Soil significantly affected root DM and P concentration, meanwhile in the rhizospheric soil, it significantly affected alkaline phosphatase and β-glucosidase activities, and CFU of both microorganisms in inoculated pots (Table 2). Inoculation with microorganisms had a significant effect on P concentration in shoots and alkaline phosphatase in the rhizospheric soil (Table 2). These both variables were higher in control and with B. subtilis than with T34 inoculation (Tables 3 and 4).

The significant effect of soil on different variables was assessed by the correlations of variables with some soil properties (Table 5). Root DM was positively correlated with

Tabla VI.1 Main properties of studied soils.

	Clay	SOM	CCE	pH	EC	Fe_{ca}	Fe_{cbd}	IP	OP	C-P	NaOH-P_{phy}	P_{phy}	Olsen P
	——g kg^{-1}——				dS m–1	——g kg^{-1}——							—mg kg^{-1}—
Mean	326	10.3	270	8.00	0.13	0.96	5.67	0.34	0.3	0.092	4	40	6.8
SD	152	4.0	261	0-15	0.04	0.62	3.05	0.12	0.08	0.079	2.4	25	1.3
Range	157 –640	5.5 –20	0 – 723	7.7 – 8.8	0.10 –0.23	0.25 –2.02	2.12 –11.33	0.18 – 0.52	0.18 – 0.47	0.028 – 0.27	1.1 –10.3	15 – 99	4.8 – 8.7

SOM, soil organic matter, CCE, equivalent Ca carbonate equivalent, EC, electrical conductivity, IP, inorganic P estimated as the sum of MRP in fractionation scheme; OP, organic P estimated as organic P fraction sum; C-P, citrate soluble P in the fractionation scheme; ca subindex; citrate-ascorbate extractable; cbd subindex, citrate-bicarbonate-dithionite extractable; NaOH-P_{phy}, hydrolisable P with phytase in the NaOH extract of the P fractionation scheme; P_{phy}, sum of hydrolisable P with phytase in all the fractions of the P fractionation scheme

Tabla VI.2 Significance (p values) in the analysis of the variation (ANOVA and non-parametric tests) of the effect of soil, inoculation with microorganisms, and their interactions on different properties of plant and rizhospheric soil after wheat cultivation

Source of variation	Shoot DM	Root DM	P concentration shoots	P concentration roots	P uptake	Soil pH	Organic acids	Alkaline phosphatase	β-Glu	CFU	
										BS	T34
Soil (S)	0.0000	0.0000	0.3885	0.0146	0.0000	0.0000	0.0000	0.0000	0.0000	0.0120	0.0436
Microorganism (M)	0.0328	0.3282	0.0158	0.3109	0.0620	0.0968	0.1767	0.0017	0.3990		
S x M	0.0121	0.3574	0.5729	0.6671	0.0498	0.0264	0.0001	0.2059			

DM, dry matter; β -Glu, β -Glucosidase activity; CFU, colony forming units; BS, *Bacillus subtilis*; T34, *Trichoderma asperellum* T34

Transformations for ANOVA: log for DM roots, P uptake, organic acids, and T34 CFU; square root for BS; $X^{-0.5}$ for P concentration in shoots

Effect of factors on β-Glu was assessed by the Kruskal-Wallis non-parametric test; in this case, the effect of the interaction cannot be assessed

Effect on CFU was analyzed separately for each microorganism with soil as the unique factor

Tabla VI.3 Effect of different inoculation treatments on wheats plants

Inoculum	Shoot DM	Root DM	P concentration shoots	P concentration roots	P uptake
	————g plant^{-1}————		————g kg^{-1}————		mg plant^{-1}
Control without inoculation	0.64 ± 0.01	0.13 ± 0.01	1.13a ± 0.04	1.03 ± 0.04	0.75 ± 0.03
Bacillus subtilis QST713	0.67 ± 0.01	0.14 ± 0.01	1.13a ± 0.04	1.06 ± 0.03	0.78 ± 0.03
Trichoderma asperellum T34	0.68 ± 0.02	0.13 ± 0.01	1.01b ± 0.03	1.00 ± 0.03	0.70 ± 0.03

Mean ± standard error, n=72 for inoculum

DM, dry matter

P uptake estimated as the amount of total P in shoot and roots minus P presents in seeds.

Means followed by the same latter within the same column are not significantly different according to the Tukey test ($P < 0.05$); mean comparison is not performed when interaction between main factors is not significant

Tabla VI.4 Effect of different inoculation treatments on soil properties after wheats plants

Inoculum	pH	OA	Alkaline phosphatase	β-Glu	CFU
		mmol kg^{-1}	————mg pNP (kg h)$^{-1}$————		
Control without inoculation	8.66 ± 0.05	22.7 ± 2.2	149a ± 6.5	73 ± 5.8	nd
Bacillus subtilis QST713	8.62 ± 0.04	25.9 ± 2.2	145a ± 6.1	80 ± 5.2	$6.3\ 10^{7}$ ± $5.7\ 10^{6}$
Trichoderma asperellum T34	8.72 ± 0.05	21.4 ± 1.4	132b ± 10.5	84 ± 7	$5\ 10^{3}$ ± $2\ 10^{3}$

Mean ± standard error, n=72 for inoculum

OA, organics anions; β-Glu, β -Glucosidase activity; pNP, *p*-nitrophenol released; CFU, colony forming units; nd, not detectable

Means followed by the same latter within the same column are not significantly different according to the Tukey test ($P < 0.05$); mean comparison is not performed when interaction between main factors is not significant

Tabla VI.1 Correlation coefficients of variables significantly affected by the "soil" factor in ANOVA with soil properties

	SOM	CCE	pH	EC	Olsen P	Clay	C-P	OP	$Fe_{ca}+Fe_{cbd}$	Fe_{ca}/Fe_{cbd}
Root DM	0.37***	– 0.1ns	– 0.37***	– 0.348***	0.252***	– 0.486***	0.034ns	0.182**	– 0.1ns	– 0.04ns
β-Glu	0.412***	– 0.228***	– 0.085ns	0.077ns	0.179**	0.364***	0.416***	0.427***	0.344***	– 0.316***
Phos	0.353***	– 0.383***	– 0.191**	0.165*	– 0.139*	0.465***	0.467***	0.310***	0.389***	– 0.069ns
CFU BS†	0.139ns	– 0.127ns	– 0.080ns	0.2063ns	– 0.284*	0.273*	– 0.032ns	– 0.082ns	0.165ns	0.229ns
CFU T34†	– 0.0293ns	– 0.117ns	0.059ns	– 0.39ns	– 0.01ns	0.06ns	– 0.082ns	0ns	0.234ns	0.026ns

n = 216, except for CFU, which was 72 (inoculated treatments for each microorganism); †Correlation analysis only for pots inoculated with the corresponding microorganism.

SOM, soil organic matter; CCE, Ca carbonate equivalent; EC, electrical conductivity in 1:5 soil extracts; OP, organic P estimated as organic P fraction sum; C-P, citrate soluble P in the fractionation scheme; ca subindex; citrate-ascorbate extractable; cbd subindex, citrate-bicarbonate-dithionite extractable; DM, dry matter; β -Glu, β -Glucosidase activity; Phos; alkaline phosphatase activity; CFU, colony forming units; BS, *Bacillus subtilis*; T34, *Trichoderma asperellum* T34

***, **, *, ·, significant at *p* values of 0.001, 0.01, and 0.05, respectively

SOM and Olsen P, meanwhile its correlation with CCE, pH, EC, and clay content was negative. β-glucosidase positively correlated with SOM, Olsen P, clay, Fe in oxides (Fe_{ca} + Fe_{cbd}), and some P fractions (citrate soluble and organic). These properties were also correlated with alkaline phosphatase. In addition, this enzymatic activity was negatively correlated with Olsen P and pH, and positively with EC.

Only CFU of B. subtilis was correlated with soils properties: it was negatively correlated with Olsen P, and positively with clay content of soil (Table 5), and 88 % of its variance was explained by β-glucosidase, EC, SOM, and clay content (Figure 1).

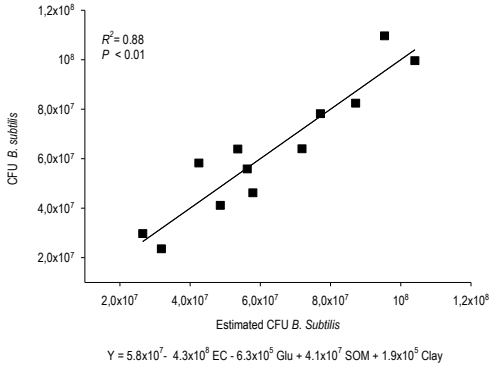

Figura VI.1 Estimation of colony forming units (CFU) of *Bacillus subtilis* QST713 as a function of electrical conductivity (EC), β-glucosidase activity (Glu), organic matter (SOM) and clay content in soil. All the variables used in the model were significant at P < 0.05.

Both enzymatic activities in the rhizosphere increased with clay content, and decreased with CCE and the ratio of Fe in oxides ascribed to poorly crystalline oxides (Fe_{ca}/Fe_{cbd}) according to the multiple regression analysis); in addition, β-glucosidase increased with increased organic P concentrations in soil (Table 6).

Tabla VI.2 Best multiple regressions predicting alkaline phosphatase and β-glucosidase activity as a function of soil properties

Dependent variable	Regression	Significance
Alkaline phosphatase	$Y = 164 + 0.13\ Clay - 0.86\ CCE - 250\ Fe_{ca}/Fe_{cbd}$	$R^2 = 0.35$; $P < 0.001$
β-glucosidase	$Y = 64 + 0.01\ Clay - 0.77\ CCE - 399\ Fe_{ca}/Fe_{cbd} + 0.23\ OP$	$R^2 = 0.49$; $P < 0.001$

n = 216; t statistic significant for all the independent variables used in the linear model at $P < 0.001$

CCE, Ca carbonate equivalent; OP, organic P estimated as organic P fraction sum; ca subindex; citrate-ascorbate extractable; cbd subinidex, citrate-bicarbonate-dithionite extractable

Effect of microorganisms as affected by soil

The effect of microorganisms on shoot DM, P uptake, and pH and organic acid concentration in rhizospheric soil differed depending on the soil, as revealed by the significant interaction between both factors (Table 2). This interaction was explained on

a relative basis, using the ratio of the effect of the microorganism to the effect of non-inoculated control.

In the case of B. subtilis for P uptake, 92 % of the variation in the ratio was explained by the phytase hydrolysable P in NaOH extracts, Olsen P, CCE, the ratio Fe_{ca}/Fe_{cbd}, and citrate soluble P (Figure 2).

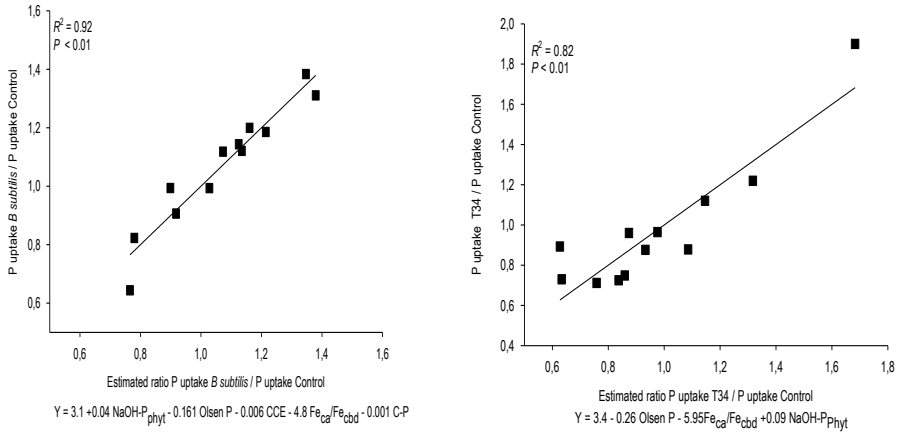

Figura VI.2 a) Estimation of the ratio of P uptake in *Bacillus subtilis* QST713 inoculated plants to that in non-inoculated control as a function of phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-Pphyt), Olsen P, Ca carbonate equivalent (CCE), the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Feca/Fecbd), and citate soluble P in the sequential fractionation scheme (C-P).
b) Estimation of the ratio of P uptake in *Thrichoderma asperellum* T34 inoculated plants to that in non-inoculated control as a function of phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-Pphyt), Olsen P, and the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Feca/Fecbd). All the variables used in the model were significant at P < 0.05.

Most of these soil properties (including OP instead citrate-soluble P in the model) contributed to explain 87 % of the variation in the ratio of shoot DM in *B. subtilis* inoculated plants to shoot DM in control (Figure 3). The sign of Olsen P and Feca/Fecbd in these both models did not have any predictive value since both variables were correlated (P < 0.05); on the other hand, properties ascribed to organic P contributed

positively to both ratios. Although differences in pH were minimal between B. subtilis and control, 86 % of the ratio between both treatments was explained by Olsen P, which contributed negatively, SOM, and organic P (Figure 4); these both later variables were correlated ($P < 0.05$).

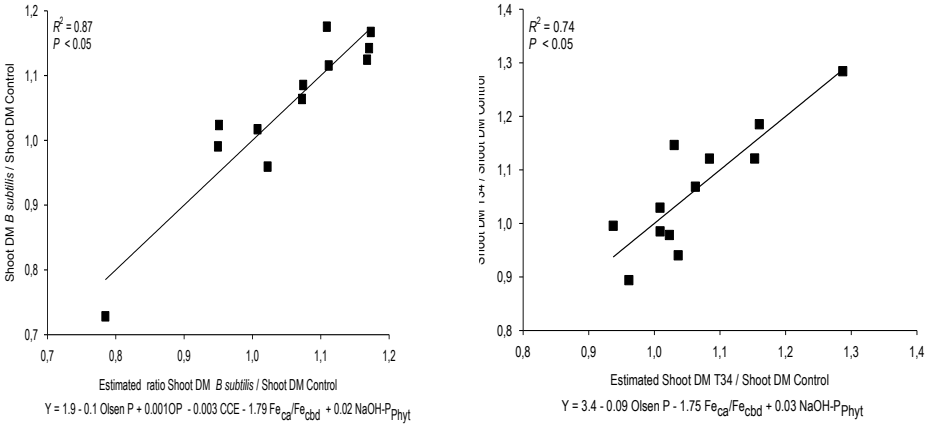

Figura VI.3 a) Estimation of the ratio of shoot dry matter (DM) in *Bacillus subtilis* QST713 inoculated plants to that in non-inoculated control as a function of Olsen P, total organic P (OP), Ca carbonate equivalent (CCE), the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Feca/Fecbd), and phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-Pphyt).
b) Estimation of the ratio of P uptake in *Thrichoderma asperellum* T34 inoculated plants to that in non-inoculated control as a function of Olsen P, the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Feca/Fecbd), and phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-Pphyt). All the variables used in the model were significant at P < 0.05.

On the relative basis defined above, Olsen P, the ratio of Fe in oxides ascribed to poorly crystalline oxides (Fe_{ca}/Fe_{cbd}), and phytase hydrolysable P in NaOH extracts explained an 82 % and 74 % of the variation in the effect of T34 on P uptake and shoot DM (Figure 2, and 3). The effect on pH was explained by Olsen P and the sum of phytase hydrolysable P in all the fractions in the sequential scheme used (Figure 4).

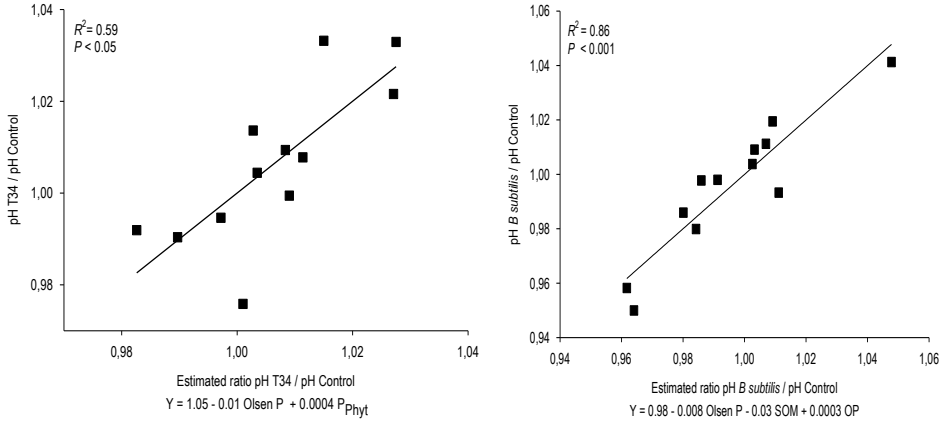

Figura VI.4 a) Estimation of the ratio of pH in rhizosphere in *Bacillus subtilis* QST713 inoculated pots to that in non-inoculated pots as a function of Olsen P, soil organic matter (SOM), and total organic P (OP).
b) Estimation of the ratio of pH in rhizosphere in *Thrichoderma asperellum* T34 inoculated pots to that in non-inoculated pots as a function of Olsen P, and the sum of phytase-hydrolysable P in all the extracts of the sequential fractionation scheme (Pphyt). All the variables used in the model were significant at P < 0.05.

Discussion

Effect of microorganisms on plant development and P uptake

Trichoderma asperellum T34 led to lower P concentration in shoots than *B. subtilis* and non-inoculated control. The effect was not ascribed to a dilution effect due to an increased DM accumulation in shoots, which was not significantly different from other treatments, or to an overall decreased P uptake. In addition, P concentration and DM in roots were not affected by inoculation with microorganisms. Thus, decreased P concentration in shoots with T34 this may reveal some change in the translocation of P from roots to shoots ascribed to this microorganism. This lower P concentration in T34 treated plants when compared with other treatments may be also the partial consequence of a lower phosphatase activity in the rizhosphere of T34-inoculated

plants. Although this microorganism produces P-hydrolytic enzymes, the hydrolytic activity may be affected by properties of plant growing media (García-López et al., 2015).

The effect of microorganisms on P uptake and plant development measured as shoot DM yield varied depending on the soil. In soils where inoculation did not increase these both variables, this was not ascribed to a fail in the colonization of rhizosphere by the microorganisms since CFU of both *B. subtilis* and T34 were detected in significant concentrations in all the inoculated pots. Soil properties affected CFU of B. subtilis, but not that of T34. The density of this latter microorganism was not correlated with biochemical or physicochemical properties of soil within the range studied. In the case of *B. subtilis*, CFU was negatively related to β-glucosidase activity at the end of the experiment. This evidenced that the density of this microorganism decreased with increased general microbial activity in soil. Interaction with indigenous microbial populations is crucial in explaining the effects of microbial inoculants (Vassileva et al., 2010). In this regard, competition of the studied strain of B. subtilis with other microorganism has proved to be a pivotal issue in explaining its benefits in inoculated growing media (Gossen et al., 2016). However, there was not any relationship between CFU and shoot DM yield or P uptake by plants for both microorganisms. Thus, the density of microorganisms was not significant explaining its potential benefits, which seemed to be more related to other soil properties, particularly, those related to P biogeochemistry (Figure 2 and 3).

Overall, *B. subtilis* was more effective in increasing P uptake than T34 as revealed by the value of the ratio of its effect to the effect of non-inoculated control. For the former inoculant, the ratio was greater than 1 in most of the cases, meanwhile for T34 it was lower than 1 in most of the cases (Figure 2). However, for shoot DM yield, both were effective in a majority of soils (Figure 3). This likely reveal that the growth promoting effect of B. subtilis may be related at least partially to an improved P nutrition, meanwhile in the case of T34 other factors should contribute to its growth promoting effect. It is worth noting that Olsen P of studied soils were in general below the threshold value for fertilizer response (Recena et al., 2016), and consequently DM yield must be limited by low P availability in soil. Thus, an improved P uptake by plants should be reflected in a greater DM yield. This is supported by the properties explaining the relative effect of both microorganisms when compared with non-inoculated control: for P uptake and shoot DM, explicative variables in the multiple regression models were almost the same and related to P biogeochemistry in soil (Figures 2 and 3).

Soils properties affecting the action of microorganisms

In previous studies, the action of *B. subtilis* was shown to be unrelated to the level of P availability in the artificial plant growing medium (García-López and Delgado, 2016). However, in the studied soils, the effect of both microorganisms on P uptake and shoot DM yield was affected by soil P availability (measured as Olsen P). It is reasonable to assume that microorganisms promote an increase P uptake with decreased P availability in soil. This agrees with the negative sign of Olsen P in the multiple regressions (Figure 2 and 3), despite the correlation of Olsen P with Fe_{ca}/Fe_{cbd}. In addition, the population

density of *B. subtilis* was negatively correlated with Olsen P, likely revealing that the microorganism was more competitive in soils with lower P availability level. This is in agreement with the known fact that increased P availability decrease populations of oligotrophic bacteria such as B. subtilis through its effect on root exudates (Koyama et al., 2014).

Positive effects of microorganisms on P uptake and shoot DM yield increased with increased concentrations in soil of phytase-hydrolysable P in NaOH extracts. This reveals that benefits of both microorganisms were ascribed at least partially to the hydrolysis of organic P which is consistent with their well-known hydrolytic enzyme production (García-López et al., 2015; 2016). However, for T34 other enzymes different from phosphatase assayed, likely phytases (García-López et al., 2015), should contribute to this effect. In addition, total organic P in soil contributes to explain positive effects of *B. subtilis* on shoot DM. According to Recena et al. (2016; 2017), organic P can be a source of P for plants. All these evidences reveal that positive contribution of these microorganisms to P uptake by plants can be ascribed at least partially to the hydrolysis of non-readily available organic P forms.

Mineralogy of Fe oxides were significant explaining the effects of both microorganisms on P uptake and shoot DM yield. In this regard, the ratio of Fe ascribed to poorly crystalline oxides to that ascribed to crystalline oxides (Feca/Fecbd) contributed to explain the effects of both microorganisms. This ratio has proved to be crucial explaining P availability to plants since P concentration in soil solution is expected to increase with increased ratios as the affinity of P for poorly crystalline oxides

is lower than for crystalline (Recena et al., 2017). Thus, P uptake by plants is expected to be enhanced at increased Feca/Fecbd. This makes less evident the contribution of studied microorganisms to P uptake at increased ratios of poorly crystalline oxides to crystalline oxides (Figure 2).

The relative effect of *B. subtilis* on P uptake and shoot DM was negatively affected by CCE. Thus, soil buffering capacity constrained the effect of this microorganism. This allow one to think that P mobilization by *B. subtilis* was related to acidification of rhizosphere in agreement with previous evidences (García-López and Delgado, 2016). This acidification should contribute to release precipitated metal phosphates. However, the contribution of poorly soluble Ca phosphates (citrate soluble, C-P) to P uptake seemed to be negative, perhaps revealing that when these forms were abundant, other forms such as organic P were preferentially mobilized. In addition, the phosphatase activity for all treatments was negatively correlated with CCE, which also contribute to explain the negative effect of this compound on the effect *of B. subtilis*. In the case of T34, its relative effect when compared with control was not affected by CCE. This perhaps reveals a decreased contribution of acidification mechanisms on P release by this microorganism in agreement with García-López et al. (2016).

Acidification capacity of both microorganisms was also affected by soil properties; it decreased with decreased Olsen P and with increased OP or with the sum of phytase hydrolysable P in all the fractions (Figure 4). This likely reveals that P availability level and concentration of hydrolysable organic P are factors explaining the triggering of P

mobilization based on acidification. In particular, acidification was decreased with increased concentration of potentially hydrolysable organic P.

Enzymatic activities were affected by soil properties. Both studied activities increased with SOM, likely ascribed to the positive effect of this soil property on microbial biomass (Moreno et al., 2016) and on root development. As expected, phosphatase was negatively correlated with Olsen P, revealing that this mechanism was less triggered with increased P availability in the soil. This activity increased with OP, likely evidencing that hydrolytic activity may be triggered by the presence of substrate, in agreement with previous evidences observed for T34 (García-López et al., 2015). When non-phytoavailable poorly soluble Ca phosphates are the dominant P forms in the plant growing media, it has been also observed an increased phosphatase activity (García-López et al., 2015) which may contribute to explain the positive correlation observed between this activity and citrate soluble P.

Phosphatase activity correlated positively with the soil P adsorption capacity, measured as clay or Fe in oxides. Several studied demonstrated that the presence of clay or iron oxides enhanced particular enzyme activities (Bayan and Eivazi, 1999; Allison, 2006; Shahriari et al., 2010). In addition, a decreased P concentration with increased adsorption capacity in soil may trigger the hydrolytic activity as observed previously by García-López et al. (2015) in pure cultures of T34. Since the P concentration in soil increase with increased Fe_{ca}/Fe_{cbd}, this explains the negative contribution of this ratio to the phosphatase activity in the rhizosphere (Table 6).

Conclusions

The density of both studied microorganisms (CFU) or biochemical properties ascribed to microbial activity did not explain the effect of both microorganisms on plant P uptake and DM yield relative to non-inoculated control. This relative effect was mainly explained by soil properties ascribed to P dynamics in soil. It decreased with increased plant available P and with increased ratio of poorly crystalline Fe oxides to crystalline oxides, and increased with the concentration of phytase-hydrolisable P in several soil extracts. Although potential effects on microbial community structure were not assessed in this study, it reveals that soil properties explaining the triggering of P-mobilizing mechanisms of microorganism are crucial in explaining their potential benefits on crops.

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VII

Martina Castells Ballespí, licenciada en Medicina, fue la primera mujer en alcanzar el doctorado el 4 de octubre de 1882.

CAPÍTULO VII DISCUSIÓN GENERAL

Los microorganismos juegan un papel importante en la nutrición de las plantas mejorando la disponibilidad y asimilación de nutrientes tan importantes como el P y Fe. La venta y comercialización de microorganismos que mejoran el crecimiento de las plantas (PGPRs), generalmente comercializados bajo el nombre de biofertilizantes, o que suministran protección o resistencia a enfermedades (agentes de biocontrol) viene desarrollándose desde hace décadas. En esta Tesis doctoral se ha comprobado el efecto de diferentes microorganismos rizosféricos en la acumulación de P y Fe en plantas cultivadas en medios artificiales y suelos de diferentes características. Se ha utilizado pepino como cultivo hortícola de relevancia donde la aplicación de un medio de producción con cierto coste como agentes de biocontrol puede estar justificado, así como trigo como cultivo extensivo muy extendido a nivel Mediterráneo. Por otra parte, nos hemos centrado sobre todo en microorganismos útiles como agentes de biocontrol con el objetivo de justificar beneficios adicionales en su uso que hagan del manejo integrado de estrese bióticos (enfermedades) o abióticos (deficiencias de nutrientes) prácticas más asumibles desde el punto de vista económico. Aunque la literatura sobre microorganismos movilizadores es extensa, rara vez se ha puesto énfasis en que haya diferentes tipos de beneficio, lo que contribuye a que su uso sea más conveniente desde un punto de vista económico.

De manera general la inoculación con *Trichoderma asperellum* T34 mejoró la asimilación de P por parte de las plantas. En el capítulo II, tanto el contenido total de P acumulado en raíz como el P tomado por las plantas se incrementaron respecto a las

plantas no inoculadas, principalmente cuando el P proviene de fuentes consideradas poco disponibles para las plantas. Esto implica que *Trichoderma asperellum* T34 presenta una capacidad general de movilizar P. Esto mismo fue descrito por Rudrhes et al., (2005), en cuyo trabajo se observó el incremento de crecimiento y rendimiento del cultivo del garbanzo debido a la inoculación con *Trichoderma* spp. en suelos deficientes en P, fertilizados con roca fosfatada. Altomare, et al. (1999) y Anusuya y Jayarajan, (1998) describieron como diferentes cepas de *Trichoderma*, poseían la capacidad de usar fosfato tricálcico (P insoluble) como fuente de P en cultivos in vitro.

En el caso de medios calcáreos, como se muestra en el capítulo IV, la asimilación de P por las plantas inoculadas con *Trichoderma asperellum* T34 no mostró diferencias significativas respecto a las no inoculadas cuando el P se aplicó como roca fosfatada (RP). La ausencia, en este caso, de efecto positivo en la nutrición fosfatada pone de manifiesto que las características de los medios de cultivo o de los suelos, en particular pH y capacidad tampón influyen en la capacidad del microorganismo para solubilizar P. Estos resultados eran esperables al ser *Trichoderma* spp. un hongo que tiene menor desarrollo en medios con pH básicos. Por otro lado, propiedades que tienen influencia en la geoquímica del P, en el suelo y que podrían afectar a los mecanismos de movilización, también condicionan el efecto sobre la absorción de P cuando inoculamos con *Trichoderma asperellum* T34, como se muestra en el capítulo VI.

La capacidad de movilizar P de fuentes poco disponibles por parte de microorganismos es atribuida a su actividad enzimática hidrolítica (capaz de hidrolizar P orgánico) y a la producción de ácidos orgánicos y acidificación del medio. Algunas

cepas de *Trichoderma* spp. desarrollan actividad fitasa y fosfatasa ácida (Kapri y Tewari, 2010; Nasi et al., 1999). Leitao et al. (2010) demostraron que *Trichoderma harzianum* poseía actividad hidrolítica de P, y que a partir de este microorganismo se podía purificar fosfatasa ácida. Tchameni et al. (2011) manifestó un aumento en la actividad fosfatasa de *Trichoderma asperellum* T34 cuando este era combinado con un AMF en cultivos de cacao. En la presente Tesis doctoral capítulo II se demuestra como *Trichoderma asperellum* T34 desarrolla una actividad fosfatasa y fitasa en cultivos in vitro. La producción de enzimas hidrolíticas extracelulares involucradas en el ciclo P se vieron incrementadas en los medios de cultivo de las plantas de pepino (*Cucumis sativus* L. cv. Tropico) que fueron inoculados con *Trichoderma asperellum* T34. Sin embargo, el resultado de la inoculación fue diferente atendiendo a la fuente de P aplicada. Cuando se aplicó el P como RP la actividad fosfatasa fue la mayor comparadas con otros tratamientos, mientras que cuando se aplicó fitato (Ins6P) como fuente de P la actividad fitasa se vio incrementada. Esto pone de manifiesto que, en situaciones donde el P es un nutriente limitante, el microorganismo aumenta la actividad fosfatasa ácida para movilizar P, según lo observado por Naseby y Lynch, (1998). En nuestro caso, la actividad fosfatasa aumenta en respuesta a la baja concentración de P en el medio como consecuencia de aplicar este nutriente en una forma muy poco soluble o como respuesta a un bajo nivel de P disponible para las plantas en el suelo, como quedó demostrado en el capítulo VI. Por otra parte, la actividad fitasa ácida aumenta en respuesta a la presencia del sustrato. Además, en el capítulo VI se comprobó que la actividad fosfatasa se correlacionó positivamente con la capacidad de

adsorción del P en el suelo, (arcilla u óxidos de Fe) como consecuencia de una menor concentración de P cuando aumenta la capacidad de adsorción, o por la retención de las enzimas en óxidos o minerales de la arcilla.

La mayor actividad enzimática fosfatasa y fitasa en los medios de cultivo de plantas en presencia de *Trichoderma asperellum* T34 nos haría pensar que es un mecanismo desarrollado por el hongo para favorecer la asimilación de P en el caso de restricciones de este nutriente o ante la presencia de P orgánico en el suelo. De esta capacidad, se deriva una mejora de la nutrición de las plantas. Por lo tanto, la inoculación con este hongo aportaría un beneficio adicional además del propio biocontrol de enfermedades.

En el capítulo II se describen otros mecanismos mediante los cuales se mejora la asimilación de P poco disponibles para las plantas. Se describe como se produce una acumulación de ácidos orgánicos (oxalato) cuando la fuente aplicada es RP. Es sabido que la acidificación de la rizosfera y/o la producción de aniones orgánicos de bajo peso molecular facilitan la disolución de fosfatos de Ca (Reyes et al., 1999, Hariprasad y Niranjana, 2009). La mayoría de las cepas estudiadas de *Trichoderma* acidifican su medio ambiente circundante mediante la secreción de ácidos orgánicos, como ácido glucónico, cítrico o fumárico. Estos ácidos orgánicos resultan del metabolismo de otras fuentes de carbono, principalmente de glucosa, y a su vez, son capaces de solubilizar fosfatos, micronutrientes y cationes minerales, incluyendo hierro, manganeso y magnesio. En este sentido, Zhao y Zhang (2015) describieron la presencia de tres ácidos orgánicos (oxálico, láctico y fórmico) en la solubilización de fosfatos en cultivo de pepino tras la inoculación con *Trichoderma asperellum* cepa Q1.

Se produce un aumento de la producción de ácidos orgánicos como respuesta de microorganismos y plantas a una deficiencia de P en el medio. En el caso de las plantas inoculadas con *Trichoderma asperellum* T34, las concentraciones de aniones orgánicos, en mayor medida cuando la fuente usada era RP. Esta estrategia, como se ha comentado anteriormente, es esencial para disolver fosfatos precipitados poco solubles (Ström et al., 2005).

Otro de los microorganismos rizosféricos de vida libre estudiados en la presente Tesis doctoral fue la bacteria *Bacillus subtilis* QST713. Este microorganismo mostró una capacidad importante para movilizar P de medios calcáreos y con una fuente poco disponible, roca fosfatada (capitulo IV). La capacidad de este microrganismo para la movilización de P fue estudiada en presencia y ausencia de óxidos de Fe (capítulos IV y V respectivamente) y en una amplia variedad de suelos (capítulo VI). Dichos óxidos interfieren de manera negativa en la disponibilidad de P debido a la adsorción del P en disolución a su superficie. En todos los capítulos, con presencia y ausencia de óxidos de Fe, o ante las diferentes propiedades de los suelos ensayados, se observó un aumento en el P total asimilado por las plantas y un aumento del contenido de P de las mismas cuando habían sido inoculadas con *Bacillus subtilis* QST713. De manera similar, Pindi et al., (2014), Raddadi et al., (2008) y Trivedi y Pandey, (2008) describieron la movilización de P por diferentes cepas de Bacillus, demostrando su capacidad solubilizadora, además de la producción de sideróforos y de ácido indolacético (IAA) como efectos adicionales.

Uno de los principales mecanismos descritos en la bibliografía de movilización de P de la rizosfera en presencia de bacterias es la producción de ácidos orgánicos de bajo peso molecular (láctico, oxálico, tartárico, fórmico, málico, cítrico, succínico, propiocico) (Chen et al., 2006; He y Sheng, 2006; Gulati et al., 2010; Zhu et al., 2011). Este hecho fue corroborado en el capítulo IV, en el que se describe como *Bacillus subtilis* QST713 segregó ácidos orgánicos, principalmente oxálico, en cultivos in vitro. En estos medios, *Bacillus subtilis* QST713 bajó el pH independientemente de la fuente de P utilizadas. Además, en el capítulo VI, el efecto de *B. subtilis* se vio afectado negativamente por el carbonato, limitando el poder tampón del suelo el efecto acidificante del microorganismo. Esto nos hace pensar que la acidificación de la rizosfera por parte de la bacteria tiene una consecuencia sobre la movilización de P de los suelos.

En medios calcáreos, cuando el P fue suministrado en forma de RP a una dosis de 40 mg kg^{-1}, cantidad inferior a las que suelen aplicarse como fertilizante en suelos para cultivo hortícola, el P asimilado por las plantas era similar en plantas inoculadas y no inoculadas por esta bacteria. Esto fue observado pese a la capacidad de segregar ácidos orgánicos por parte de la bacteria en cultivos in vitro. Este hecho está en contraposición a lo observado por Shahid et al (2015), donde se correlacionó la producción de ácidos orgánicos y la asimilación de P para el cultivo del girasol cuando las plantas eran inoculadas con bacterias.

La exudación de ácidos orgánicos por parte de las raíces de las plantas se ha descrito como un mecanismo que tienen para mejorar la asimilación de P, (Playsted et al. 2006; Abrahão et al. 2014). Según lo descrito por varios autores, esto se debe a que

los ácidos orgánicos provocan la disolución de P poco solubles (como fosfatos de Ca), o bien estas moléculas compiten con el P por los sitios de adsorción en las superficies adsorbentes como los óxidos de Fe. Este hecho fue corroborado en la presente Tesis, cuando se observó una mayor concentración de ácidos orgánicos en los medios de cultivo donde existía un bajo contenido de P o había presencia de óxidos de hierro (ferrihidrita). Curiosamente, la concentración de ácidos orgánicos era mayor en medios de cultivo de plantas no inoculados que en los inoculados. Esto pone de manifiesto que los ácidos orgánicos detectados procedían de las plantas en respuesta a un medio deficiente en P y que en medios inoculados o eran usados como fuente de carbono por los microorganismos, o bien la mejora en la absorción de P provocada por la inoculación disminuía la secreción de ácidos orgánicos por las raíces.

En los capítulos IV y V se describen correlaciones positivas entre la biomasa radicular generada y el contenido total de P de las plantas inoculadas, independientemente de los factores que pudiesen afectar a la disponibilidad de P (naturaleza de las fuentes de P usadas, presencia de óxidos de Fe). La inoculación con *Bacillus subtilis* QST713 promueve el crecimiento radicular lo que puede dar lugar a una mayor absorción de P por parte de las raíces. Esto puede contribuir a explicar las relaciones descritas entre desarrollo de raíz y absorción de P. Independientemente del nivel de disponibilidad de P en el medio de cultivo, esto puede deberse a un incremento en la producción de auxinas en plantas inoculadas (Roi et al., 2004, Lim y Kim, 2009). En el capítulo VI, se evidenció que el efecto sobre el crecimiento de las plantas por la inoculación con *B. subtilis* QST713 podría deberse a con una mejor nutrición de P,

mientras que en el caso de la inoculación con *Trichoderma asperellum* T34 la existencia de otros factores debían contribuir a su efecto positivo sobre el crecimiento las plantas.

También se observó un mayor desarrollo de las plantas cuando el microorganismo inoculado fue el hongo *Trichoderma asperellum* (capitulo II). Sin embargo, esteun mayor desarrollo de las plantas parece no estar ligado a la generación de fitohormonas ya que no hubo un aumento significativo de materia seca obtenida para las plantas inoculadas con *Trichoderma asperellum* T34 cuando la fuente de P era fosfato soluble (KH_2PO_4) que no limitaba la absorción del nutriente por el cultivo. El mayor aumento del desarrollo de las plantas podría estar relacionados con los efectos positivos sobre la nutrición de P y Fe, ya que se observaron más claramente en las plantas cuya fuente de P suministradas fueron PR e Ins6P.

Una posible estrategia para la adquisición de P en plantas inoculadas con microorganismos es la exudación de sideróforos microbianos. De Santiago et al. (2013) demostraron que los sideróforos microbianos aumentan la absorción de Fe en las plantas, pudiendo este hecho tener un efecto indirecto sobre el P. Los sideróforos pueden competir por los lugares de adsorción o contribuir a liberar Fe de los óxidos. Esto puede producir una disminución de la adsorción de P a los óxidos de Fe, lo que implicará una mayor disponibilidad de P para las plantas. Esta hipótesis de una mayor disponibilidad de P debido a la producción de sideróforos se apoya en la evidencia de que existe una mayor acumulación de Fe en la parte aérea de las plantas que fueron inoculadas con *Bacillus subtilis* QST713 cuando se usaron fuentes de P son muy solubles como KH_2PO_4 y que, por tanto, son muy rápidamente adsorbidas por los óxidos

de Fe (capítulo V). Ademas, la existencia de una relación significativa entre la absorción de P y el contenido total de Fe en las plantas inoculadas con *B. subtilis* QST713, revela que mecanismos que afectan la disponibilidad de Fe y P en las plantas pueden estar involucrados cuando las plantas son inoculadas con *Bacillus subtilis* QST713.

Parece existir una asociación ente los mecanismos de movilización de P y Fe desarrollados por las plantas en medios inoculados por microorganismos. Como se ha comentado con anterioridad en esta Tesis doctoral (capítulos I y V) se observó un comportamiento diferente para la asimilación de P y Fe según las plantas fueran inoculadas con microorganismos (*Trichoderma asperellum* T34 o *Bacillus subtilis* QST713) o la no inoculación de las plantas. Esto evidencia la existencia de mecanismos asociados a la liberación de Fe por parte de ambos microorganismos, pudiéndose deber a la acción de sideróforos microbianos, específicos que aumenta la liberación de Fe y que a su vez pueden contribuir a la movilización de P, probablemente debido a la liberación del P adsorbido a los óxidos de Fe cristalinos.

La diferente disponibilidad de Fe para las plantas inoculadas y no inoculadas por los microorganismos queda patente en varios capítulos de la presente Tesis (capítulos II, III y V). Las plantas cuyos medios de cultivo fueron inoculados con *Trichoderma asperellum* T34 o *Bacillus subtilis QST713* respectivamente, incrementaban el contenido total de Fe. Además, existen numerosas investigaciones en las que se describen como la inoculación con *Trichoderma asperellum* T34 aumenta la asimilación de Fe por las plantas cuando la fuente de Fe es ferrihidrita (de Santiago et al. 2009, 2011, 2013). Este hecho ha sido corroborado en la presente Tesis doctoral. Habida cuenta de que es

previsible que el microorganismo sea más eficiente que la planta en la absorción de Fe (por la especifidad de los sideróforos en complejar Fe), se puede plantear que en medios muy pobres en dicho nutriente haya competencia entre planta y *Trichoderma asperellum* T34 por este nutriente. Este efecto ha sido descrito por Marschner et al. (2011). Por ello puede ser interesante conocer si hay un umbral o cantidad mínima de Fe presente en el suelo para que el agente de biocontrol *Trichoderma asperellum* T34 actúe positivamente en la nutrición férrica de la planta. Esto se estudió en el capítulo III, y se observó que *Trichoderma asperellum* T34 disminuía la disponibilidad de Fe para las plantas de pepino por debajo de 75 mg Kg^{-1} de Fe extraido con citrato-ascorbato (Fe asociado a óxidos de baja cristalinidad) en el medio de cultivo. Se pudieron definir dos umbrales de Fe para evitar las deficiencias en pepino en este tipo de medio, 37 mg kg^{-1} en plantas no inoculadas con T34, y 75 mg kg^{-1} cuando las plantas eran inoculadas con *Trichoderma asperellum* T34. Por debajo de este valor la competencia entre plantas y T34, por este elemento puede influir de manera negativa en el cultivo. En cambio, de Santiago et al. (2009) constataron que en plantas de estrategia II como el trigo, *Trichoderma asperellum* T34 mejoraba la concentración de Fe en plantas con valores Fe extraíble por citrato-ascorbato de 15 mg kg^{-1} de Fe. Por tanto, es preciso tener presente la cantidad mínima de Fe en el suelo para que no exista competencia entre la planta y el microorganismo y por tanto para que la inoculación sea efectiva como agente de biofertilización. Esta concentración umbral dependerá de la eficiencia del cultivo en la adquisición de Fe.

VIII

Según su etimología "conclusión" proviene del latín "conclusĭo", "conclusiōnis" y este deriva del griego "ἐπίλογος", se forma a partir de "conclus" del verbo "concludere" que significa "cerrar" o "concluir".

CAPÍTULO VIII CONCLUSIONES

- Se verifico mediante cultivos in vitro, con bajas dosis de P o uso de fuentes de P poco disponibles (inositol-6-P y roca fosfatada) que *Trichoderma asperellum* T34 y *Bacillus subtilis* QST713 mostraron actividad enzimática hidrolítica de P (fosfatasa y fitasa) y exudación de ácidos orgánicos.
- El P asimilado por las plantas, cuando este procedía de fuentes poco disponibles (inositol-6-P y fosfatos de Ca insolubles), mejoró con la inoculación de *Trichoderma asperellum* T34 en las plantas de pepino (*Cucumis sativus* L. cv. Tropico) cultivadas en medios no calcáreos. Este efecto se asoció a la actividad enzimática hidrolítica (capacidad de hidrolizar P orgánico), la producción de ácidos orgánicos y la acidificación del medio.
- *Bacillus subtilis* QST713 mostró un efecto positivo en la asimilación de P por las plantas cuando este estaba presente en formas poco disponibles (roca fosfatada) o cuando se encontraba adsorbido a superficies adsorbentes (óxidos de Fe en forma de ferrihidrita). Se asume que este beneficio se asocia a la acidificación de la rizosfera y a la promoción del crecimiento radicular.
- Se verificó la segregación de ácidos orgánicos por parte de las plantas como mecanismo de respuesta a las deficiencias de P en el medio. Sin embargo, en los medios inoculados la concentración de ácidos orgánicos en la rizosfera podía disminuir, por su utilización como fuente de carbono por los microorganismos o por una menor exudación al mejorar la absorción de P por las plantas inoculadas.

- La inoculación con *Trichoderma asperellum* T34 y *Bacillus subtilis* QST713 incrementó el contenido total de Fe en las plantas cultivadas, por tanto, dichos microorganismos aportan beneficios adicionales sobre la nutrición férrica. Además, la existencia de mecanismos asociados a la liberación de Fe (sideroforos) por parte de los microorganismos estudiados favorecen la absorción de P por las plantas.
- Se definieron dos umbrales de Fe para evitar las deficiencias en pepino (*Cucumis sativus* L. cv. Serena) en este tipo de medio, 37 mg kg^{-1} en plantas no inoculadas con T34, y 75 mg kg^{-1} cuando las plantas eran inoculadas con *Trichoderma asperellum* T34.
- Las propiedades de los suelos relacionadas con la geoquímica del P, como nivel de P disponible, carbonatos, tipología de óxidos y P orgánico hidrolizable por enzimas, influyen de manera directa en la capacidad de los microrganismos para movilizar P y por tanto en el potencial beneficio sobre la nutrición fosfatada de las plantas.

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