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IMPORTANCIA DE LAS LEVADURAS RIZOSFÉRICAS EN LA NUTRICIÓN DE MAÍZ

T E S I S

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RESUMEN

Las levaduras son habitantes comunes del suelo, pero la información sobre su ecología es limitada. En este estudio, la abundancia y la composición de la comunidad de las levaduras dominantes de la rizósfera fueron examinadas en diferentes agroecosistemas de maíz convencionales en dos sitios geográficos que difieren en las características del suelo. La identificación taxonómica molecular de las levaduras se realizó con la secuenciación del dominio D1D2.

Además, se evaluó la respuesta de crecimiento de plantas de maíz a la inoculación con las levaduras de la rizósfera *Cryptococcus flavus* y *Candida railenensis* en tratamientos sin y con micorrizas nativas, así como sin y con fertilización mineral de KH_2PO_4. Finalmente, se evaluaron las interacciones entre el hongo micorrícico arbuscular (HMA) *Rhizophagus irregularis* y las levaduras rizosféricas *Cr. flavus* y *C. railenensis* en cuanto al crecimiento de plantas de maíz y la absorción de P empleando marcadores de isótopos de P (^{32}P y ^{33}P).

Los resultados mostraron que las levaduras son comunes en todos los campos de maíz estudiados durante el ciclo completo de crecimiento. La mayor abundancia se obtuvo durante la etapa de floración del maíz. La abundancia de levaduras rizosféricas respondió negativamente al pH del suelo y la cantidad de Mg. Se encontraron ocho especies de levaduras de seis géneros. Cuatro de las ocho especies de levaduras solubilizaron $Ca_3(PO_4)_2$. Hubo una promoción de crecimiento de las plantas de maíz en suelo desinfectado inoculado con *C. flavus y Solicoccozyma aeria* con respecto al peso seco aéreo y con *C. railenensis* en peso seco de la raíz, pero solo en combinación con la fertilización mineral de P. Por otro lado, se observó la promoción y supresión del crecimiento de la planta de maíz después de la inoculación con una comunidad nativa de HMA con y sin fertilización con P inorgánico, respectivamente. La inoculación con *Cr. flavus* redujo la biomasa de la parte aérea de las plantas de maíz independientemente del estado micorrícico, pero solo sin la fertilización con P. La colonización de raíces con HMA se incrementó con *C. railenensis* sin fertilización de P, pero se redujo con *Cr. flavus* en combinación con la fertilización con P inorgánico.

El método de dilución de isótopos de P fue usado para medir la posible solubilización de fosfatos en el suelo por las levaduras de la rizósfera con la subsecuente absorción de P por la asociación con HMA. Los resultados mostraron un crecimiento de la planta después de la inoculación con *R. irregularis* y ambas especies de levaduras alteraron la longitud específica de la raíz del maíz (cm g^{-1} raíz seco). Las levaduras también aumentaron el contenido de P de las plantas, pero solo en las plantas micorrizadas. De manera más específica, las levaduras aumentaron la absorción de ^{32}P por las hifas de *R. irregularis*, pero no la absorción de ^{33}P por las raíces. Por otro lado, las levaduras no tuvieron efecto sobre el crecimiento extraradical de micelio de *R. irregularis*. La inoculación con levaduras no dio como resultado una dilución de isótopos de P medida como actividad específica (kBq ^{32}P o ^{33}P/mg ^{31}P), lo cual indica que el incremento en la absorción de P en plantas de maíz asociado con *R. irregularis* en combinación con las levaduras seguramente no tuvo que ver con la solubilización de P, mientras que la alteración en el crecimiento de la raíz y la longitud de raíz específica son mecanismos probables de interacciones.

En conclusión, las levaduras de la rizósfera con características promotoras de crecimiento vegetal son comunes en los agroecosistemas de maíz y muestran un gran potencial para mejorar la nutrición de P del cultivo mediante el aumento de la longitud de raíz y la toma de P por las hifas micorrícicas.

Palabras clave: Levaduras; hongos micorrícicos arbusculares; rizósfera, agroecología; suelo; maíz.

ABSTRACT

Yeasts are common soil inhabitants, but information on their ecology is limited. In this study, the abundance and community composition of dominating rhizosphere yeasts were examined in different conventional maize agroecosystems in two different geographic sites differing in soil characteristics. Molecular taxonomic identification of yeasts was performed by sequencing the D1D2 domain.

Moreover, the maize plant growth response to inoculation with the native maize rhizosphere yeasts *Cryptococcus flavus* and *Candida railenensis* was examined in non-mycorrhizal and mycorrhizal maize with and without mineral P fertilization as KH_2PO_4. Finally, a possible cooperation between the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* and the rhizosphere yeasts *Cr. flavus* and *C. railenensis* was evaluated in terms of maize plant growth and P uptake employing P isotope tracer monitoring.

The main results showed that yeasts were common in all maize agroecosystems throughout the complete growing cycle. The highest abundance was obtained during the flowering plant growth stage. The abundance of rhizosphere yeasts responded negatively to soil pH and Mg content. Eight yeast species from six genera were obtained. Four out of the eight yeast species solubilized $Ca_3(PO_4)_2$. When grown in disinfected soil, maize plant growth was promoted after inoculation with *Cr. flavus* and *Solicoccozyma aeria* in terms of shoot dry weight, and with *C. railenensis* in terms of root dry weight, but only in combination with mineral P fertilisation. Besides, maize plant growth promotion and suppression was observed after inoculation with a native community of AMF with and without mineral P fertilization, respectively. Inoculation with *Cr. flavus* reduced the shoot biomass of maize plants irrespective of the mycorrhizal status, but only without P fertilization. AM fungal root colonization was increased by *C. railenensis* without P fertilization, but was reduced by *Cr. flavus* in combination with mineral P fertilization.

The P isotope dilution method was used to measure possible phosphate solubilization in soil by the rhizosphere yeasts with subsequent P uptake by the mycorrhizal association. Main results showed strong plant growth promotion after inoculation with *R. irregularis* and both yeast species altered the specific root

length of maize (cm g^{-1} dry root). The yeasts also improved maize shoot P content, but only in mycorrhizal plants. More specifically yeasts improved AMF hyphal ^{32}P uptake, but not that of root ^{33}P uptake. On the other hand, yeasts had no effect on extraradical growth of *R. irregularis.* Inoculation with yeasts did not result in P isotope dilution measured as specific P isotope activity (kBq ^{32}P and ^{33}P/mg ^{31}P), indicating that the reported improved P uptake from AM associations in combination with yeasts was most likely not related to P solubilization, whereas alteration in root growth and specific root length seems more likely mechanisms of interactions.

In conclusion, rhizosphere yeasts with plant growth promoting traits are common in maize agroecosystems showing strong potential to improve crop P nutrition by increasing root length and AMF hyphal P uptake.

Keywords: Yeasts; arbuscular mycorrhizal fungi; rhizosphere; agroecology; soil; maize.

Capítulo 1:

Introducción y Marco teórico

1.1 INTRODUCCIÓN

El maíz es la principal fuente de alimento en México y como en todos los cultivos su producción agrícola requiere de la aplicación del uso de fertilizantes para potencializar su rendimiento, que comúnmente son aplicados en forma mineral. Sin embargo, las cantidades excesivas de fertilizantes inorgánicos que se aplican a los agroecosistemas causan impactos ambientales adversos en los ecosistemas terrestres y acuáticos (Tilman, 1999).

La producción de maíz es a menudo limitada por la baja disponibilidad de fósforo. Los fertilizantes fosfatados, derivados predominantemente de las rocas fosfóricas, se utilizan en sistema agrícolas convencionales para superar la deficiencia de P en el suelo y así hacer una contribución significativa a la producción de alimentos. Sin embargo, las reservas de roca fosfórica son un recurso finito y podrían agotarse en los próximos 50-100 años (Cordell *et al*., 2009). Además, la demanda de P se ha incrementado de tal forma que los precios han aumentado en el mercado, por lo que cada vez son menos accesibles para los productores.

Una herramienta alternativa al uso de fertilizantes inorgánicos, es la utilización de los microorganismos de la rizósfera capaces de agregar nutrientes a través de los procesos naturales de solubilización de fósforo inorgánico y orgánico.

Los microorganismos son un componente integral del ciclo de fósforo en el suelo y son importantes para la transferencia de este elemento a las plantas. Los microorganismos solubilizadores de fósforo, a través de diversos mecanismos de solubilización y mineralización, son capaces de convertir el P orgánico e inorgánico del suelo en forma de fosfatos para facilitar la absorción por las raíces de las plantas (Sharma *et al*., 2013; Richardson *et al*., 2011; Shen *et al*., 2011).

Las levaduras son hongos unicelulares distribuidos abundantemente en la rizósfera. La posibilidad de utilizar levaduras como agentes promotores del crecimiento de las plantas ha sido poco explotado en comparación con las bacterias y los hongos micorrícicos. Sin embargo, algunos estudios muestran que las levaduras del suelo tienen la capacidad de estimular el crecimiento, nutrición y

rendimiento de algunas plantas (Botha, 2011). Otros estudios han encontrado que la interacción de las levaduras con otros microorganismos puede resultar benéfico. Tal es el caso de la interacción con los hongos micorrícicos arbusculares (HMA), en donde se ha observado una interacción sinérgica, mejorando la asimilación del nitrógeno y fósforo de las plantas, así como aumentando la colonización micorrícica de la raíz (Boby *et al*., 2008; Gollner *et al*., 2006; Sampredro *et al*., 2004; Fracchia *et al*., 2003)

La mayoría de las investigaciones sobre levaduras del suelo se han enfocado en su identificación y enumeración, sin mucho énfasis en su función en el ecosistema. Sin embargo, hay estudios cuyos resultados muestran que una gran diversidad de interacciones complejas pueden ocurrir entre las levaduras y los factores bióticos y abióticos (Botha, 2011). La comprensión de la función de las levaduras del suelo en los procesos del ecosistema puede ser una clave para futuras prácticas agrícolas sostenibles.

En el presente proyecto se evaluó la importancia de las levaduras rizosféricas en agroecosistemas convencionales de maíz en México con el objetivo de: 1) Conocer su abundancia durante todo un ciclo de cultivo 2) Examinar cómo responden las levaduras rizósfericas a las características fisicoquímicas del suelo; 3) Identificar cuáles especies de levaduras dominan en la rizósfera de maíz; 4) Examinar la capacidad de las levaduras rizosféricas para solubilizar fósforo y mejora el crecimiento vegetal del de este cultivo; 5) Evaluar las interacciones entre el maíz, las levaduras de la rizósfera y los HMA en términos del rendimiento del crecimiento de las plantas afectadas por la fertilización con P; y 6) Evaluar la posible cooperación entre levaduras de la rizósfera, y HMA en cuanto a la adquisición de P para la nutrición de plantas, empleando el método de dilución de isótopos de P (^{32}P y ^{33}P).

1.2 MARCO TEÓRICO

1.2.1 Maíz

El maíz (*Zea mays* L.*)* es una gramínea anual originaria de Mesoamérica y representa uno de los cereales de mayor importancia en el mundo. Actualmente es el cereal más plantado mundialmente en volumen de producción, superando al trigo y al arroz (Ramos, 2013), debido a la diversificación en su uso. Puede utilizarse para consumo humano y pecuario; en la industria es utilizado para producir aceites, almidón, fructuosa, glucosa, dextrosa y botanas entre otros. También es empleado en la elaboración de algunas bebidas alcohólicas y otros productos utilizados como materia prima, en las industrias minera, textil, electrónica, farmacéutica y alimentaria (Ranum *et al*., 2014).

En México es el cultivo más importante y la principal fuente de alimento con una producción anual del de 18.2 millones de toneladas en una superficie de 8.5 millones de hectáreas (De la Rosa *et al*., 2006).

México posee la mayor diversidad genética de maíz, la cual se manifiesta en variación de caracteres morfológicos vegetativos, así como de espiga, mazorca y grano, y en la composición química del grano. De las 436 razas reportadas en el continente americano 50 se encuentran en México (De la Rosa *et al*., 2006)

La mayor diversidad de razas y variedades del maíz que se concentran en México, han formado parte del germoplasma con el que se han desarrollado variedades de alto rendimiento y adaptabilidad, así como la producción de híbridos para zonas de riego (Sánchez y Goodman, 1992).

El maíz puede ser encontrado en todos los estados, climas y altitudes de México. Se siembran diversas variedades y se consume de distintas formas distribuyéndose principalmente en 5 rubros: 57% de la producción nacional se destina al consumo humano, 26% a la alimentación del ganado, 11% elaboración de harinas, 4% mermas y 2% siembras (Ron *et al*., 2006; FIRA,1998). En años recientes su cultivo se ha extendido globalmente gracias a su capacidad de adaptación a diversos climas y su versatilidad.

Además, el maíz en nuestro país tiene importantes valores culturales, simbólicos y

espirituales, lo cual no ocurre en otros países, por lo que en México, el maíz no es un producto agrícola más y su cultivo se realiza con la esperanza de obtener mucho más que solamente ingresos monetarios, sin dejar de lado que existen grandes productores que destinan toda su producción al mercado (Polanco y Flores, 2008).

Al ser el maíz un producto básico para los mexicanos, su producción es un desafío para los agricultores para cubrir las necesidades de consumo nacional. Algunas limitaciones comunes que los agricultores enfrentan es la falta de agua; plagas y enfermedades ocasionadas por bacterias, hongos, virus y malezas; así como la disponibilidad de los nutrientes requeridos por el cultivo (Vázquez-Carrillo *et a*l., 2011).

1.2.2 Agroecosistemas de maíz

La producción de maíz se realiza principalmente en dos diferentes tipos de manejo: convencional y orgánico, aunque también hay con sistemas intermedios (Hellin *et al*., 2013).

El manejo convencional del maíz incluye el uso de fertilizantes inorgánicos y plaguicidas, así como semillas con alto potencial de rendimiento (híbridas). Por su parte, el manejo orgánico busca dar énfasis a la fertilidad del suelo y la actividad biológica restringiendo o eliminando el uso de agroquímicos, prefiriendo el uso de semillas criollas (Hellin *et al*., 2013).

La siembra de maíz en México se lleva a principalmente en dos niveles, a gran escala industrial y/o en pequeña escala, principalmente para autoconsumo, sin requerir de grandes inversiones o extensiones de tierra, como son las huertas familiares y la milpa (Polanco y Flores, 2008). La milpa es un agroecosistema mesoamericano practicado desde hace miles de años. Es un policultivo que incluye, además del maíz, asociaciones con frijol, calabaza, chile, jitomate, tomate verde y otras hortalizas (Benítez y Fornoni, 2013).

En la producción de maíz, el nitrógeno (N) y el fósforo (P) son frecuentemente deficientes para alcanzar altos rendimientos. En el manejo agrícola orgánico es común la aplicación de estos fertilizantes en forma orgánica, como el estiércol,

composta, etc. Sin embargo, se aplican grandes cantidades de fertilizantes inorgánicos en el manejo convencional. Particularmente, la fertilización excesiva con fósforo es común y muchos agricultores aplican innecesariamente altas cantidades de fertilizantes fosforados, ocasionado diversos problemas ambientales (Lehman y Taheri, 2017), como son la eutrofización de ríos, lagos y mares, pérdida de nutrientes del suelo y contaminación de los suelos (Stoate *et al*., 2009).

Un aspecto importante a considerar es que los recursos mundiales de P mineral no son renovables en una escala de tiempo humano (Scholz y Wellmer, 2013). Estas preocupaciones ambientales requieren el desarrollo de estrategias para mejorar la eficiencia del uso de P y racionalizar su uso en los agroecosistemas (Richardson *et al.,* 2011; Lehman and Taheri, 2017).

1.2.3 Fósforo

El fósforo (P) es uno de los nutrientes más importantes para el crecimiento de las plantas y también uno de los más limitados en el suelo. Desempeña un papel primordial en casi todos los procesos metabólicos de la planta. Algunos de los atributos asociados con la nutrición de fósforo son el desarrollo de raíces y tallos, la formación de flores y semillas, la madurez y producción de los cultivos, la fijación de N en leguminosas, la calidad de los cultivos y la resistencia a las enfermedades de las plantas (Khan *et al*., 2009).

El P se encuentra en el suelo como fósforo inorgánico u orgánico formando parte de la materia orgánica. El fósforo inórganico (Pi) se encuentra mas abundantemente en forma de apatitas, que son formas estables en cuanto a su solubilidad. Además, en el suelo, el Pi generalmente se asocia con otros compuestos como Ca, Fe y Al, cada uno de los cuales tiene características de solubilidad únicas que determinan la disponibilidad de fosfatos para la planta (Shen *et al*., 2011)

El fósforo orgánico, por otro lado, se encuentra en formas estabilizadas como inositol fosfatos y fosfonatos, formas activas como monoésteres, diésteres de ortofosfato y polifosfatos orgánicos (Condron *et al*., 2005; Turner *et al*., 2002).

De acuerdo a Shen *et al*. (2011) el fósforo inorgánico representa entre el 35% y 70% del total de fósforo presente en el suelo, y el orgánico entre el 30% y el 65%. En estudios de cronosecuencias de suelos, Richardson *et al*. (2004) observaron que a medida que evoluciona el suelo el contenido de fósforo orgánico aumenta a expensas del inorgánico. Se considera que alrededor del 80% del fósforo total estaría en forma orgánica y el contenido en la biomasa microbiana puede ser desde el 1% hasta más del 10%, siendo ésta una reserva importante por su alta tasa de renovación y evita que el P se fije en formas minerales poco solubles (Richardson, 2001; Seeling y Zasoski, 1993).

Debido a las fuertes y múltiples interacciones que el ión fosfato establece con los constituyentes del suelo, este se encuentra en bajas concentraciones y es poco móvil. La movilidad y la biodisponibilidad de los fosfatos en los suelos está principalmente limitada por la adsorción (es decir, la adhesión física o iones de fosfato en las superficies de otras moléculas), la precipitación en minerales de tipo apatita y la inmovilización en la materia orgánica como fitato (Vessey y Hinsinger, 2001). Por ello, la concentración de fósforo disponible en la solución del suelo generalmente está por debajo de 10 µM, que es muy inferior a la que se encuentra en los tejidos vegetales (Shen *et al*., 2011).

1.2.3.1 Adquisición de fósforo por la planta

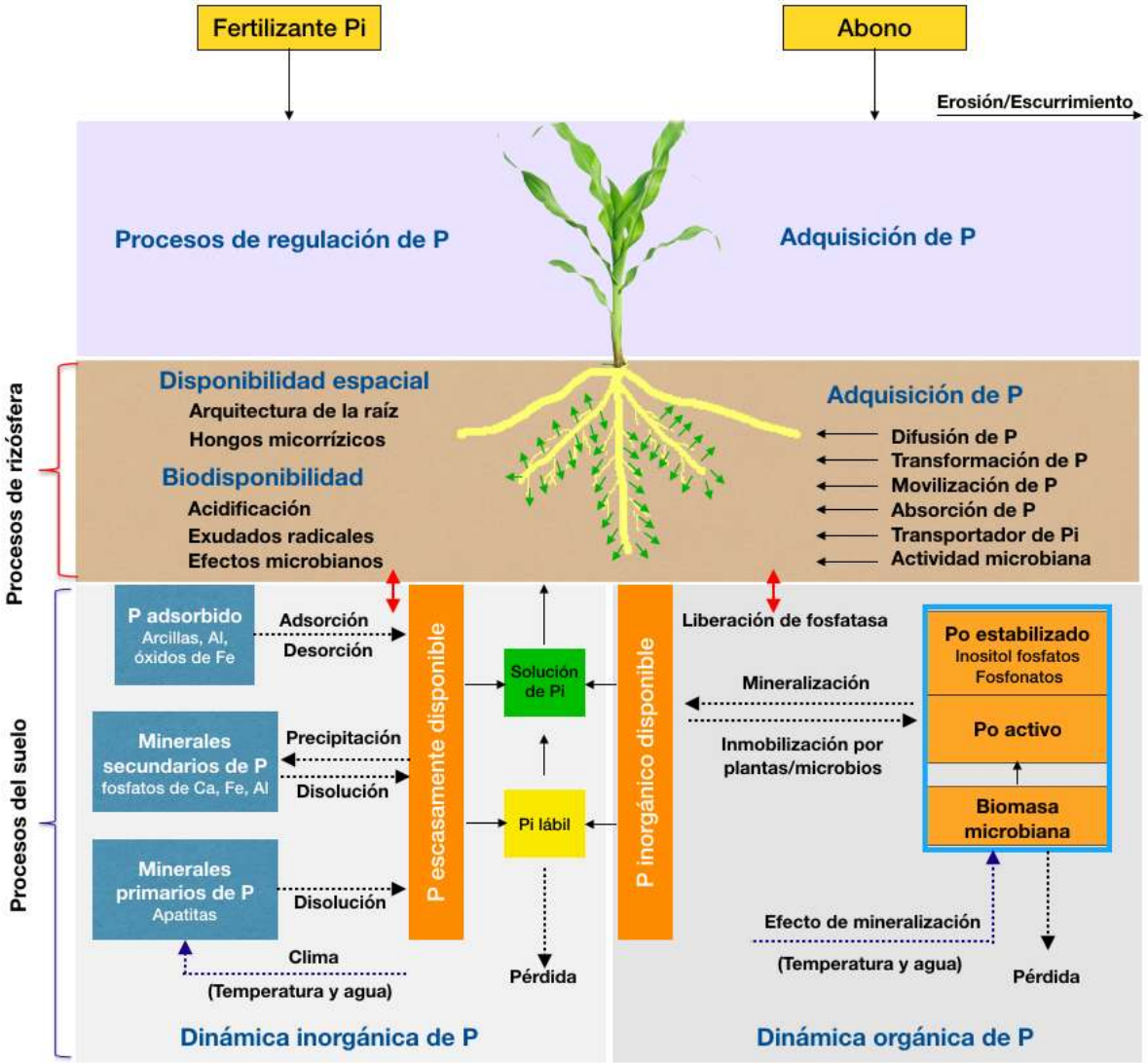
El fósforo es absorbido por las raíces de las plantas en forma de aniones ortofosfatos (principalmente $H_2PO_4^-$ y HPO_4^{2-}) presentes en la solución del suelo. En la mayoría de los suelos, la concentración de ortofosfatos en solución es baja y, por lo tanto, debe reponerse a partir de otras fuentes de P del suelo para satisfacer los requisitos de la planta (Richardson *et al*., 2009).

Las plantas han desarrollado diversos mecanismos fisiológicos y bioquímicos para enfrentar la escasez de fósforo disponible en el suelo, ya sea para forzar su solubilización o mejorar su absorción (Vence *et al*., 2003) (Figura 1).

Además, éstas pueden responder a la falta de P cambiando la arquitectura de su raíz, lo cual juega un papel importante en la maximización de la adquisición de P porque los sistemas de raíces con mayor área de superficie son capaces de explorar un volumen dado de suelo de manera más efectiva (Lynch y de Leij,

2012; Richardson *et al*., 2011). También pueden secretar ácidos orgánicos que solubilizan algunas formas de fósforo, de manera directa o indirecta al producir cambios en el pH del suelo o secretar fosfatasas, que ayudan a la mineralización de este elemento desde la materia orgánica (Shen *et al*., 2011). Ciertas especies o genotipos de plantas han desarrollado transportadores con alta afinidad por el fosfato, para capturarlo eficientemente a las bajas concentraciones en las que está en la solución del suelo (Shen *et al*., 2011). Estos mecanismos están bajo su propio control genético (Richardson *et al*., 2011).

Figura 1. Dinámica de P en el suelo/rizósfera-planta. Modificado de Shen *et al*., 2011



Las plantas pueden asociarse con microorganismos de la rizósfera, los cuales son parte integral del ciclo de fósforo en el suelo y con su actividad colaboran eficientemente en el incremento de la adquisición de P.

Se ha demostrado que una amplia gama de bacterias, hongos filamentosos y levaduras solubilizan el P inorgánico y orgánico incrementando la capacidad de la planta para adquirir el fósforo del suelo mediante diferentes mecanismos (Sharma *et al*., 2013). Algunos microorganismos de la rizósfera pueden incrementar el crecimiento de la raíz y el desarrollo de los pelos radiculares mediante efectos hormonales, ya sea por la producción de fitohormonas o alterando el balance hormonal de la planta, con lo que aumentan la capacidad de exploración y su superficie de absorción (Hayat *et al*., 2010; Richardson *et al*., 2009).

Ciertos microorganismos rizosféricos han sido reportados por su capacidad de secretar protones, ácidos orgánicos de bajo peso molecular y sideróforos, que solubilizan las formas precipitadas de fósforo inorgánico mediante acidificación, reacciones de intercambio de ligandos, o de quelación de iones metálicos, como el hierro, que están comúnmente asociados en el suelo con complejos de fósforo. También es conocida la producción de fosfatasas y fitasas por parte de estos microorganismos, que mineralizan el fósforo contenido en la materia orgánica haciéndolo disponible para la planta (Richardson *et al*., 2011; Ryan *et al*., 2001; Tarafdar y Claassen, 1988; Whitelaw *et al*., 1999).

La asociación entre los microorganismos solubilizadores de fósforo y las raíces es de naturaleza sinérgica, ya que los microorganismos proporcionan fosfato soluble y las plantas suministran compuestos de carbono originados en la raíz (principalmente azúcares), que pueden metabolizarse para el crecimiento microbiano (Richardson *et al.,* 2009). Por su parte, los HMA han sido ampliamente estudiados por su capacidad para transportar el P del suelo a su planta hospedera (Jeffries y Barea, 1994).

Los microorganismos solubilizadores de fósforo (MSF), junto con otra microflora rizosférica benéfica mejoran la producción de cultivos. Se han observado interacciones sinérgicas en el crecimiento de las plantas mediante la inoculación de MSF con microorganismos fijadores de nitrógeno tales como *Azospirillum*

(Belimov *et al*., 1995) y *Azotobacte*r (Kundu y Gaur, 1984) o con los HMA (Kim *et al*., 1998).

1.2.4. Rizósfera

La rizósfera es la zona crítica de interacciones entre las plantas, el suelo y los microorganismos. Las raíces pueden modificar enormemente el ambiente de la rizósfera a través de sus diversas actividades fisiológicas, particularmente la exudación de compuestos orgánicos como el mucílago, ácidos orgánicos, fosfatasas y algunas sustancias específicas de señalización, que son factores clave de varios procesos de la rizósfera (Philippot *et al*., 2013). Los procesos químicos y biológicos en la rizósfera no solo determinan la movilización y adquisición de nutrientes del suelo y de la dinámica microbiana, sino que también controlan la eficiencia en el uso de nutrientes de los cultivos y así influencian profundamente la productividad de los cultivos (Lynch y de Leij, 2012; Hinsinger *et al*., 2009).

En la rizósfera, las raíces son capaces de liberar cantidades significativas de exudados. El proceso en sí depende en forma general de la especie de la planta (Rengel, 2002), presencia de microorganismos (Lynch y de Leij, 2012), estatus nutricional (Wittenmayer y Merbach, 2005) así como de la disponibilidad de oxígeno, edad, medio de desarrollo y otras condiciones de crecimiento (Gibbs *et al.,* 1998).

La composición y cantidad de compuestos exudados por la raíz, dependen marcadamente de la fenología de la planta (Oliveros-Bastida *et al*., 2009). Por ejemplo, en el maíz, la cantidad total de compuestos liberados por gramo de peso seco de raíz, disminuyen con su desarrollo y se ha estimado que estos cambios son una consecuencia de alteraciones en el balance de carbohidratos, aminoácidos y aminas, mientras que el balance de otros compuestos constitutivos, no afectan esta cantidad de compuestos liberados, como lo es el caso de los ácidos orgánicos (Gransee y Wittmayer, 2000). Algunos estudios se han llevado a cabo para entender esta diferencia en la exudación entre plantas jóvenes y adultas, donde se ha podido demostrar que ciertas características fisiológicas,

como la capacidad de distribución del carbono asimilado por la raíz hacia el tallo, son factores fundamentales (Oliveros-Bastida *et al*., 2009). Sobre esto, se ha observado que la asimilación de carbono desde un medio de crecimiento en plantas jóvenes es traslocado a la raíz, mientras que en plantas adultas, el mismo es traslocado de manera preferencial en el tallo, con lo cual, la distribución de carbono entre los diferentes órganos influye también en los compuestos orgánicos exudados (Palta y Gregory, 1997).

Por otro lado, los compuestos en los exudados juegan un papel importante en la determinación de la estructura de la comunidad microbiana en la rizósfera (Grayston *et al*., 1995). La composición de las especies de microorganismos de la rizósfera puede ser alterado por la producción de exudados de las raíces, que alteran el estado de nutrientes posteriormente a través de la descomposición y mineralización de sustancias orgánicas, y por medio de la formación de materia orgánica del suelo (Hodge y Millard, 1998). Los microorganismos, al ser un componente vital de la rizósfera y la biomasa total, así como la composición de las poblaciones microbianas afectan marcadamente las interacciones entre plantas y el ambiente del suelo (Bowen y Rovira, 1999).

Los microorganismos rizosféricos participan en la transformación biogeoquímica de elementos como el carbono, nitrógeno y fósforo en el suelo, con lo cual se incrementan la disponibilidad de nutrientes, y contribuye de esta forma a la fertilidad del suelo, al crecimiento de las plantas al liberar elementos esenciales para la nutrición vegetal y al mantenimiento de los ciclos de nutrientes (van der Heijden *et al*., 2008). La estructura y diversidad de las comunidades microbianas en la rizósfera puede variar entre las especies de plantas. Incluso las diferentes zonas de una raíz en una misma planta pueden contener diferentes comunidades microbianas, reflejando diferencias cuantitativas y cualitativas en la exudación de la raíz (Morgan *et al*., 2005). Por lo tanto, las poblaciones microbianas de la rizósfera de una misma especie de planta que crecen en el mismo lugar pueden mostrar grandes variaciones, tanto espacial como temporalmente.

Las comunidades microbianas pueden interactuar sinérgicamente degradando moléculas complejas lo cual es realizado por poblaciones mixtas de

microorganismos de tal suerte que una población le ofrece a otra un sustrato más simple (Johansson *et al*., 2004). También existen interacciones antagónicas entre los microorganismos, como son la competencia por factores limitantes (C, N, P y Fe), competencia por los sitios de la colonización y los nutrientes suministrados por semillas y raíces, la producción de sustancias inhibidoras o tóxicas produciendo efectos microbiocidas, el parasitismo que puede implicar la producción de enzimas extracelulares y la predación (Whipps, 2001).

1.2.5 Levaduras en la rizósfera

Las levaduras son hongos polifiléticos clasificados dentro de los Ascomicetos y Basidiomicetos, cuyo crecimiento asexual se produce predominantemente por brotación o fisión, y no forman sus estados sexuales dentro o sobre un cuerpo fructífero (Botha, 2011). Estos microorganismos se encuentran ampliamente distribuidos en el ambiente natural (Xin *et al.,* 2009; Mestre *et al.,* 2011; Bura *et al.,* 2012; Xu *et al*., 2012). Las levaduras se encuentran presentes en la rizósfera en grandes poblaciones, en comparación con la cantidad de poblaciones que se encuentran en partes más lejanas del suelo (Badr El-Din *et al.*, 1986; Cloete *et al.*, 2009). Algunos estudios indican que las levaduras son muy abundantes en la rizósfera en comparación con otros grupos de hongos (Xu *et al*., 2012).

Las levaduras pueden afectar la estructura del suelo, ya que son capaces de producir compuestos poliméricos extracelulares que hacen que las partículas del suelo se unan entre sí (Botha, 2006) y además pueden intervenir en el reciclaje de nutrientes (Botha, 2011). Así, una amplia diversidad de nutrientes simples son fácilmente asimilables por la mayoría de las levaduras que actúan como saprófitos. Las levaduras crecen en las inmediaciones de las plantas, utilizando nutrientes procedentes de la materia en descomposición, frutas?, y la rizósfera (Kurtzman y Fell, 1998). La posibilidad de utilizar las levaduras como agentes promotores del crecimiento vegetal ha sido poco explorada en comparación con las bacterias y los hongos micorrícicos. Sin embargo, algunos estudios muestran que las levaduras del suelo tienen la capacidad de estimular el crecimiento, nutrición y rendimiento de algunas plantas como maíz, betabel, buchú? y habas

(Nassar *et al*., 2005; El-Tarabily, 2004; y Cloete *et al*., 2009). Otros estudios han encontrado que la interacción de las levaduras con otros microorganismos puede resultar benéfica para las plantas. Tal es el caso de la interacción con los HMA, en donde se ha observado una interacción sinérgica, aumentando la asimilación del nitrógeno y fósforo, así como aumentando la colonización micorrícica de la raíz (Boby *et al.*, 2008).

Entre las funciones mas relevantes de las levaduras en el suelo se encuentran la mineralización de la materia orgánica, el mejoramiento del crecimiento de las raíces ya sea directamente o por la estimulación de la colonización de las raíces con HMA y la protección contra hongos patógenos de la raíz (Botha, 2011).

La solubilización de fosfatos insolubles por las levaduras también ha sido reportada en estudios *in vitro* (Nakayan et al., 2013; Xiao et al., 2013). Este tipo de estudios son de gran utilidad para conocer mejor el papel que juegan las levaduras en el suelo.

1.2.6 Hongos micorrícicos arbusculares

Los HMA establecen las simbiosis con mayor trascendencia para la producción de agrosistemas con las raíces de la mayoría de las plantas terrestres (Gianinazzi *et al*., 2010). Los HMA no tienen una clara especificidad entre los simbiontes, lo cual se refleja en su distribución global, asociadas a muy distintas especies de plantas y en ecosistemas muy diversos (Smith y Read, 2008). El micelio externo de un mismo hongo puede interconectarse con los sistemas radicales de diferentes plantas que cohabitan un mismo suelo (Mikkelsen *et al*., 2008) dando como resultado la formación de una extensa red de conexiones de gran importancia para el funcionamiento de los ecosistemas (van der Heijden y Horton, 2009).

La importancia de los HMA en los procesos biogeoquímicos se ha centrado principalmente en el P, ya que es bien conocido que estos hongos transportan P inmóvil del suelo a la planta hospedera, incrementando la superficie de absorción y el área de exploración de las raíces (Jeffries y Barea, 1994). Sin embargo, aún no está claro si los HMA pueden solubilizar formas orgánicas (Joner, 2000) y minerales de P (Bagyaraj et al., 2015).

La asociación micorrícica desempeña un papel crucial en secuestrar el fósforo de la solución del suelo y posteriormente almacenarlo como gránulos de polifosfato en las estructuras fúngicas del suelo y las raíces, proporcionando la base para el intercambio de P y C entre los HMA (Larsen *et al*., 2015).

Se ha demostrado que los HMA aumentan la actividad de la fosfatasa alcalina en la rizósfera (Bagyaraj *et al*., 2015), pero se sabe poco sobre la influencia de los HMA sobre la solubilización de P mineral por otros organismos del suelo.

Dado que los HMA promueven la nutrición de P en la planta hospedera transportando el P a través de sus hifas, es importante saber cómo otras biotas del suelo afectan esta función clave de los HMA (Larsen *et al*., 2015). La tecnología de marcadores de isótopos P combinada con el uso de sistemas de crecimiento compartimentados con espacios libres de raíces (Jakobsen *et al*.,1994) ha mejorado el conocimiento sobre las interacciones entre los HMA y otras biotas del suelo. Empleando el método de isótopos desarrollado por Jakobsen (1994), se ha examinado cómo el transporte de P a través de las hifas de los HMA está afectado por microorganismos agentes de control biológico (*Trichoderma harzianum*, Green et al., 1999; *Burkholderia cepacia*, Ravnskov *et al*., 2002*; Pseudomonas flourescens*, Ravnskov *et al*., 1999), promotores del crecimiento vegetal (*Aspergillus niger*, Medina *et al*., 2006), patógenos de la raíz (*Fusarium culmorum*, Larsen *et al*., 1998) y animales del suelo (Collembola, *Folsomia candida*, Larsen y Jakobsen, 1996). Las conclusiones generales de estos experimentos sugieren que el transporte de P por la hifas de los HMA relativamente no se ve afectado por otros microorganismos. Esto puede deberse probablemente a la ventaja ecológica de que los HMA reciben energía directamente de su planta hospedera lo cual hace que estos hongos sean competidores superiores en comparación con la rizósfera saprotrófica en la que habitan los microorganismos (Larsen *et al*., 2015).

1.2.7 Interacciones entre levaduras y HMA

Existen pocos estudios respecto a las interacciones entre levaduras y HMA. La principal interacción reportada es la capacidad de las levaduras o sus exudados para estimular la longitud de la hifas de los HMA, con lo cual aumentan las

posibilidades de contacto entre las hifas de los HMA y las raíces de las plantas, y en consecuencia hay un aumento en el establecimiento de las micorrizas (Boby *et a*l., 2007; Sampedro *et al*., 2004; Fracchia *et al*., 2003; Larsen y Jakobsen, 1996). Por otro lado, en su estudio Gollner *et al*., (2006) encontraron que la doble inoculación de maíz con levaduras y HMA resultó en un aumento de la biomasa en la parte aérea, dependiendo de la combinación de las especies de levadura y el aislado de HMA.

1. 3 HIPÓTESIS Y OBJETIVOS

1.3.1 Hipótesis

Las levaduras de la rizósfera son abundantes en los agroecosistemas de maíz y contribuyen a la adquisición de fósforo promoviendo la nutrición de este cultivo.

1.3.2 Objetivos

Objetivo general

Evaluar la importancia de levaduras rizosféricas como facilitadoras de fósforo para la nutrición del cultivo de maíz.

Objetivos particulares

1. Caracterizar las poblaciones de levaduras en la rizósfera del cultivo de maíz en diferentes agroecosistemas en tres etapas fenológicas del ciclo del cultivo.

2. Evaluar las funciones promotoras de las levaduras rizosféricas en el crecimiento vegetal del cultivo de maíz.

3. Evaluar el efecto de las levaduras en diferentes escenarios de fertilización mineral y orgánica.

4. Evaluar las interacciones entre las levaduras y los hongos micorrícicos arbusculares del cultivo de maíz.

CAPÍTULO 2

Plant growth promotion traits of rhizosphere yeasts and their response to soil characteristics and crop cycle in maize agroecosystems

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Plant growth promotion traits of rhizosphere yeasts and their response to soil characteristics and crop cycle in maize agroecosystems

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ABSTRACT

Yeasts are common soil inhabitants, but information about their ecology is limited. Here we examined the abundance of rhizosphere yeasts in six conventional maize agroecosystems in two different geographic areas in Mexico differing in soil characteristics and agricultural practices. In order to examine the plant growth promotion potential of maize rhizosphere yeasts a collection of yeast species was obtained, which were identified taxonomically in terms of sequencing of the D1D2 domain. Main results showed that yeasts were present in all maize fields during the complete growing cycle, though highest during flowering. The abundance of rhizosphere yeasts responded negatively to soil pH and amount of Mg. The maize rhizosphere yeast collection obtained included eight species from six genera with the Ascomycota species *Meyerozyma guillermondii* and *Candida railenensis* as the most frequent. Four out of the eight yeast species solubilised $Ca_3(PO_4)_2$, whereas none of the yeasts solubilised $FePO_4$. Maize plant growth was promoted after inoculation with *Cryptococcus flavus* and *Solicoccozyma aeria* in terms of shoot dry weight and with *C. railenensis* in terms of root dry weight, but only in combination with mineral P fertilisation. In conclusion, rhizosphere yeasts with plant growth promotion traits are common in maize agroecosystems, where soil physico-chemical characteristics and plant growth stage seem to determine their abundance.

1. Introduction

The rhizosphere is a biological hot spot in the soil where interactions between roots and soil biota take place, which regulate plant and soil nutrient dynamics (Lynch and de Leij, 2012). Rhizosphere microorganisms play an important role in soil fertility since they are involved in the cycling of nutrients like phosphorus and nitrogen, which are required for plant growth (Philippot et al., 2013). Plant growth promoting traits of soil microorganisms include nitrogen fixation by diazotrophic bacteria and phosphate solubilisation by bacteria and fungi (Larsen et al., 2014).

Yeasts are a polyphyletic fungal group belonging to the Ascomycota and Basidiomycota (Kurtzman et al., 2011). Many yeasts are common soil inhabitants (Botha, 2006) in both natural (Yurkov et al., 2012) and agricultural ecosystems (Sláviková and Vadkertiová, 2003). Some of the most frequently isolated soil yeasts belong to the genera *Candida, Cryptococcus, Rhodotorula, Lipomyces, Sporobolomyces, Trichosporon, Pichia, Saccharomyces, Debaryomyces, Aureobasidium* and *Williopsis* (Yurkov et al., 2012; Sláviková and Vadkertiová, 2003).

Though yeasts are abundant in soil (Xu et al., 2012) limited information is available about their ecology and the role of soil yeasts in agroecosystems is largely unknown (Botha, 2006). However, known functional traits of soil yeasts include organic matter decomposition, phosphate solubilisation, root growth promotion, soil aggregation and biocontrol of root pathogens, and yeasts also serve as prey for other soil biota (Botha, 2011). More specifically, plant growth promotion by soil yeasts has been reported in rice (Amprayn et al., 2012), sugar beet (El-Tarabily, 2004) and maize (Sarabia et al., 2017a; Nakayan et al., 2013; Gollner et al., 2006; Nassar et al., 2005), which has been linked to P solubilisation (Nakayan et al., 2013), increased root growth induced by indole acetic acid (Nassar et al., 2005) and improved P uptake (Sarabia et al., 2017b).

Integration of soil microorganisms, including yeasts, in agroecosystems is pivotal for the development of strategies to improve crop nutrient use efficiency for key plant nutrients like phosphorus, which is often limiting plant growth due to its low mobility in soil (Richardson

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et al., 2011). However, in order to integrate plant beneficial soil microorganisms in agroecosystems, profound knowledge about their ecology is required. This includes information about their abundance, functional traits, interactions with other soil biota and how they respond to the soil environment and agricultural practice (Larsen et al., 2014).

Maize is worldwide an important cereal crop for human consumption and animal fodder (Bennetzen and Hake, 2009) and in Mexico maize is the main basic food crop. In the present study, we examined the response of maize rhizosphere yeasts to soil physico-chemical characteristics and plant growth stage in conventional maize agroecosystems in Mexico as well as their *in-vitro* P solubilisation traits and plant growth promotion potential under different mineral P fertilization scenarios.

2. Materials and methods

2.1. Study areas

Six different rain-fed maize fields in Mexico were selected according to agricultural practice and geographic site. Field 1 (20° 38.32′N 101° 28.11′W 1733 m above sea level (masl)), field 2 (20° 39.36′N 101° 24.22′W 1719 masl) and field 3 (20° 45.11′N 101° 23.51′W 1747 masl) were located in Irapuato, Guanajuato and field 4 (19° 26.80′N 98° 46.30′W 2989 masl), field 5 (19° 26.81′N 98° 46. 24′W 2994 masl) and field 6 (18° 28.79′ N 98° 47. 88′W 2512 masl) in Estado de Mexico (State of Mexico). All six maize fields had been cultivated with monoculture maize for more than ten years before sampling of rhizosphere soil in the autumn 2013. Characteristics of the six maize agroecosystems are presented in Table 1.

2.2. Soil physico-chemical characteristics

Before rhizosphere soil sampling, representative soil samples between rows were collected in a zig-zag design for physico-chemical parameters, soil type, pH, P, N, K, Ca, Mg, Na, Fe, Zn, Mn, Cu, organic matter content, clay, carbonates and base saturation according to the soil fertility analyses service provided by INIFAP (Instituto de Investigaciones Forestales, Agrícolas y Pecuarias, Celaya, Mexico) (Supplementary material Table 1).

2.3. Rhizosphere soil sampling

In each of the six fields, an X sampling design was employed with the four extreme points and the center as the sampling points resulting in five samples. In each field the outermost 3 m of the four sides were excluded to avoid neighbour effects. Sampling of maize plants was performed at three plant growth stages, including vegetative (v6, six leaves stage, July 2013), flowering (both female and male reproductive organs visible, September 2013) and senescence growth stages (entire aboveground plant system completely dry, November 2013). From each of the five sampling points from all three vegetative growth phases in all six fields complete 30 × 30 × 30 cm blocks of soil were excavated with the plant stem as the centre and transferred to laboratory facilities for subsequent sample processing and analyses. Excessively wet soil blocks were left to dry for some days outside to facilitate soil mixing and sampling. Stones, large debris and macro fauna were removed from the soil, which thereafter was homogeneously mixed. Approximately 100 g soil from each of the five soil blocks was mixed in a composite sample, which was divided in two subsamples each with approximately 250 g soil. Soil samples were kept in the refrigerator (4 °C) until further processing, but with a maximum of two days storage after sampling.

Table 1
General features of the maize fields examined.

Feature	Study sites	
Field	1, 2, 3	4, 5, 6
State	Guanajuato	State of Mexico
City	Irapuato	Texcoco
Altitude (m above sea level)	1700–1750	2500–3000
Climate	Dry temperate	Subhumid temperate
Farming system	Rainfed maize monoculture	Rainfed maize monoculture
Fertilisation type and level (kg N ha^{-1})	Synthetic and organic (30–200)	Synthetic and organic (150)
Seed variety	Hybrid	Land race
Cultivation	Tractor	Tractor, animal
Pest, disease and weed control	Chemical, mechanic	Chemical, manual
Maize yield (ton ha^{-1})	10	2

2.4. Quantification and isolation of yeasts

From each of the six maize fields two rhizosphere soil (80 g) samples obtained from the composite soil sample were individually suspended in 720 ml sterile millipore water. The soil suspensions were subjected to serial dilutions (10^{-1}–10^{-10}). From each dilution, 0.1 ml sample was plated in duplicate on sterile Saboraud media with chloramphenicol (65 g sabouraud agar and 0.1 g chloramphenicol in 1 l Milipore water) and incubated at 28 °C for 3–5 days, after which yeasts colonies were counted and the abundance of yeasts in soil expressed as colony forming units (CFU) g^{-1} soil dry weight. Ten colonies were randomly isolated from plates representing the three plant growth stages from both geographic sites, transferred to potato dextrose agar (PDA) (39 g of PDA in one liter of Milipore water) and stored at 4 °C for further species identification.

2.5. Taxonomic identification of yeasts

Pure yeast isolates were grouped into morphotypes according to color and texture based on fresh cultures on PDA. Genomic DNA was extracted from solid cultures on Saboraud medium after 4 days of incubation at 28 °C. The cells were collected and placed in 1.5 mL microcentrifuge tubes containing 450 μL of NTES lysis buffer (250 mM NaCl, 200 mM Tris–HCl pH 8.5, 25 mM EDTA, 0.5% SDS and 0.01% β-mercaptoethanol). The cells were broken by vortexing the suspension for 2 min at maximum speed. A volume of phenol-chloroform (25:25, pH 8.0) was added, vortexed for 1 min and centrifuged to extract the organic phase. After RNAse A (Invitrogen™) treatment, a volume of phenol-chloroform was added and the DNA was precipitated with 0.5 volumes of isopropanol at − 20 °C. Pellets were washed with ethanol (70% v/v) and resuspended in 20 μL of sterile deionized water.

The primer pair NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell, 1993) were used to amplify the D1/D2 domain of the 26 S ribosomal DNA gene. The amplifications were carried out in 30 μL reaction volume containing 25 ng of template DNA, 1 μM of each primer and Platinum® PCR SuperMix (Invitrogen, USA). PCR reactions were done using a Veriti® Thermal Cycler (Thermo Fisher Scientific) instrument with the following amplification schedule: one initial denaturing cycle at 94 °C for 6 min, followed by 35 cycles of denaturing at 94 °C for 1 min, alignment at 62 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Both isolated DNA integrity and amplification products were analyzed in 1% agarose gels stained with SYBR® safe (Thermo Fisher Scientific). The amplification products (600 pb) were purified and sequenced by Macrogen Inc. (Rockvill, MD, USA). The obtained sequences were submitted to GenBank with accession numbers KY952838 to KY952876.

The sequences of the D1D2 domain of the yeasts were individually analyzed within the database of the NCBI GenBank by means of the algorithm BLASTn. Sequences displaying a similarity of 98% or higher with study sequences were selected for creating a local database from

which multiple alignments were constructed using the software ClustalX V2.0.11 (http://www.clustal.org/clustal2/) employing predetermined parameters. The resulting alignment was manually adjusted and an analysis of the pattern of nucleotide substitution was carried out in the software MEGA V6.06 (Tamura et al., 2013). The chosen algorithm was Kimura-2 with gamma distributed plus invariant sites. A phylogenetic tree was built in MEGA by the Maximum Likelihood method. The statistical significance was assessed by bootstrap with 1000 iterations. Bayesian analyses were performed with the computer program MrBayes v3.2.5 (Ronquist et al., 2012) implementing the GTR +I+G model of nucleotide substitutions. Two independent analyses of two parallel runs and four chains were carried out for 2,000,000 generations. Analyses were initiated from a random tree and trees were sampled every 500th generation. The first 25% of the resulting trees were eliminated as burn in. Both runs were pooled and a consensus tree and posterior probabilities (PP) were calculated. The trees obtained were edited using FigTree V1.4.3 (Rambaut, 2014).

2.6. P solubilisation assay

All pure yeast isolates were examined for phosphate solubilisation of tricalcium diphosphate and iron phosphate using the National Botanical Research Institute's phosphate growth medium (NBRIP) according to Nautiyal (1999). In each agar plate four yeast colonies obtained from four day old colonies were transferred and incubated at 28 °C for 7 days, and after this period halos, which refer to a circular transparent area around the yeast colony center differing in colour from the other part of the agar plate, were used to score for phosphate solubilisation.

2.7. Plant growth assay

A greenhouse pot experiment was carried out with the maize hybrid DK-2061. A fully factorial two-way design was used: 1) Maize rhizosphere yeasts (without, *Candida railenensis* MSRY-115, *Crypococcus flavus* MSRY-75, *Solicoccozyma aeria* MSRY-131 and *Clavispora lusitaniae* MSRY-59) and 2) Mineral P (without, KH_2PO_4 and $Ca_3(PO_4)_{2)}$. The yeasts selected for this assay differed in P solubilisation activity with *Cr. flavus* and *S. aeria* as examples of yeasts without P solubilisation activity and *C. railenensis* and *Cl. lusitaniae* as examples of of yeasts with P solubilisation traits. The two P sources differed in terms of solubility, with KH_2PO_4 readily soluble in the soil solution, whereas $Ca_3(PO_4)_2$ needs to be solubilised before becoming biologically available. Each of the fifteen treatments had five replicates giving a total of 75 experimental units.

Soil was obtained from the experimental field station of the National Agricultural University of Mexico, Campus Morelia, Michoacán, Mexico. Soil texture was clayish consisting of 53.2% clay, 27.3% silt and 19.5% sand and with the following chemical characteristics: 2.7% organic matter, 23.2 mg kg^{-1} inorganic nitrogen, 5.8 mg kg^{-1} available phosphorus (Olsen P) and pH (H_2O) 7.3. Soil was mixed with quartz sand (1:1, w:w) and sterilized in an autoclave. Plant nutrients were mixed into the soil (mg kg^{-1} dry soil): K_2SO_4, 75.0; $CaCl_2 \times 2H_2O$, 75.0; $CuSO_4 \times 5H_2O$, 2.1; $ZnSO_4 \times 7H_2O$, 5.4; $MnSO_4 \times H_2O$, 10.5; $MgSO_4 \times 7H_2O$, 45; $Na_2MoO_4 \times 2H_2O$, 0.18; NH_4NO_3, 86.2. The mineral phosphorus KH_2PO_4 and $Ca_3(PO_4)_2$ (200 mg P per pot) was mixed into the soil in the respective treatments.

Yeasts were inoculated by mixing 1×10^6 cells per gram of soil into the soil prior to sowing. Plants were watered daily by weight to 70% of the water holding capacity. Nitrogen (30 mg) was applied in terms of NH_4NO_3 every second week during the plant growth period. Plants were harvested 8 weeks after sowing. The shoot was separated from the root and both were dried for 48 h at 70 °C immediately after harvesting for determination of dry weight.

2.8. Statistical analyses

To summarize variation in soil physico-chemical properties across plots and to assess their similarity, we performed a principal component analysis (PCA). Soil variables were previously scaled to unit variance, and yeast abundance was also projected on the PCA coordinates to qualitatively assess its association with soil variables. Only the first two principal components were extracted as they explained 79.2% of the total variance. Analysis was performed using 'rda' function in the 'vegan' library for R (Oksanen et al., 2017). We further tested the possible links between physico-chemical soil properties and yeast abundance using Pearson correlations. Pearson partial correlations were then used to test for the association of each of these variables with yeast abundance after controlling for the other significant variables ($p = 0.05$).

To assess the effects of sites and growth stages on yeast abundances while accounting for the non-independency of the data taken from the same field, we fitted linear mixed-effect models using the function "lme" in the "nlme" library for R (Pinheiro et al., 2016). Site, stage and their interaction were included as fixed effects, while field was included as a random effect on the intercept of the model. Yeast abundance was log-transformed to meet homogeneity of variance assumption. Given the balanced nature of the dataset, significance of each term in the model was tested with Wald tests using the "anova.lme". Tukey´s all-pair comparisons within each factor with bonferroni-corrected *p*-values were performed using "glht" function in the "multcomp" library for R (Hothorn et al., 2008).

Variables from the plant growth assay (shoot and root dry weight and root-shoot ratio) were examined in terms of two-way ANOVA with "yeasts" and "phosphorus" as the two factors. Prior ANOVA, variance homogeneity was tested with Bartlett test. Comparison of treatment means was done with post-hoc LSD. The ANOVA was performed with the software JMP version 13.1.

3. Results

3.1. Soil characteristics and abundance of yeasts

Fields from the same geographic site were more similar, in terms of soil physic-chemical characteristics, than fields from different geographic sites (Fig. 1). All soils were loamy except in field 3, which was a clay soil. Soil pH was in the range 5.9–7.1, soil P content 1.2–9.4 ppm, N content 12.0–23.9 ppm and soil organic matter 1.5–2.8, which are common values for agricultural soils in Mexico (Supplementary material Table 1).

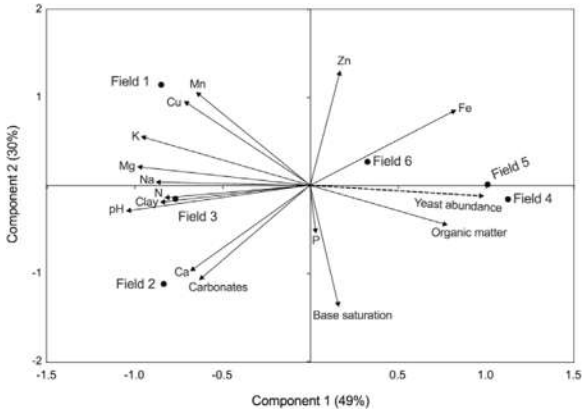

Fig. 1. Combined scatter and component weight plot of the principal component analysis of soil physico-chemical characteristics and abundance of rhizosphere yeasts from maize agroecosystems in Guanajuato (Field 1, 2 and 3) and State of Mexico (Field 4, 5 and 6).

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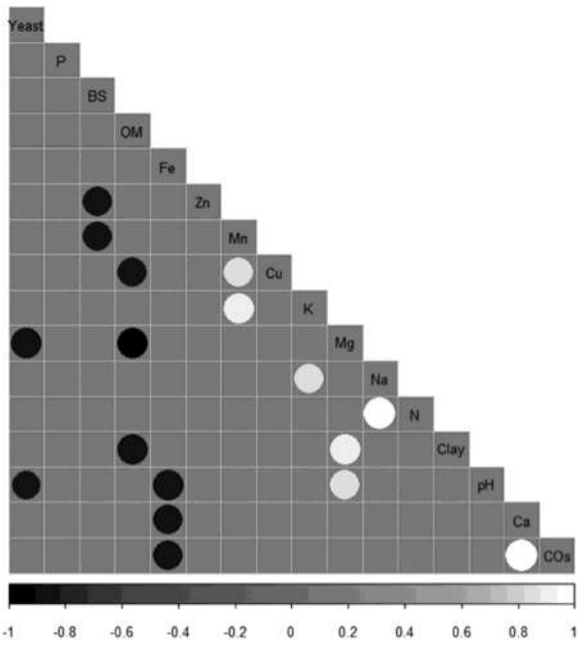

Fig. 2. Pearson correlation analysis of physicochemical soil variables including clay, macro- (N, P and K) and micronutrients (Fe, Zn, Cu, Mn, Mg, Na, Ca), pH, base saturation (BS), carbonates (COs), organic matter (OM) and abundance of rhizosphere yeasts. Only significant correlations are shown ($p < 0.05$). Black and white circles represent negative and positive correlations, respectively.

The principal component analysis of the soil characteristics showed a clear separation of the two sites along component 1, explaining 49% of the total variation (Fig. 1). The main soil variables explaining this separation was organic matter, with higher values in fields from the State of Mexico, whereas Mg, Na, N, clay and pH were higher in fields located in Guanajuato. Abundance of yeasts was positively associated with component 1, with higher values for fields in the State of Mexico. Component two accounted for 30% of the total variation and is associated mainly to base saturation, which was higher in field 2, 3 and 4 whereas Zn associated with field 1,5 and 6 (Fig. 1). Overall component one and two accounted for 79% of the total variation.

The correlation analysis confirmed the negative association of yeast abundance with pH and Mg^{2+} content, which correlated positively with each other (Fig. 2). Also several significant correlations between the different soil physico-chemical variables measured were found. The correlation between yeast and soil Mg or pH was not significant after the correlation with the other was taken into account (partial correlation between yeasts and Mg $= -0.63$, $p = 0.25$, $n = 6$; partial correlation between yeasts and pH $= -0.24$, $p = 0.70$, $n = 6$). These results show high collinearity of pH and soil Mg as possible predictors of yeast abundance.

Yeast abundance during the full crop cycle in all six maize fields was in the range 6.3×10^3–1.1×10^6 cfu g^{-1} soil dry weight. Significant individual factor effects of "Site" ($p = 0.0117$) and "Plant growth stage" ($p = 0.0400$) was found, whereas the interaction between factors was non-significant ($p = 0.2087$). On average, yeast abundance was 7 fold higher in maize fields in the State of Mexico compared to Guanajuato. Yeast abundance in the rhizosphere of flowering plants was the highest; significantly higher than in the rhizosphere of plants in the vegetative growth stage, but not significantly different from the abundance in the rhizosphere of senescent maize plants (Fig. 3).

3.2. Yeast identification

A total of 36 pure yeast isolates were obtained from different fields and plant growth stages. Eight yeast morphotypes were recovered

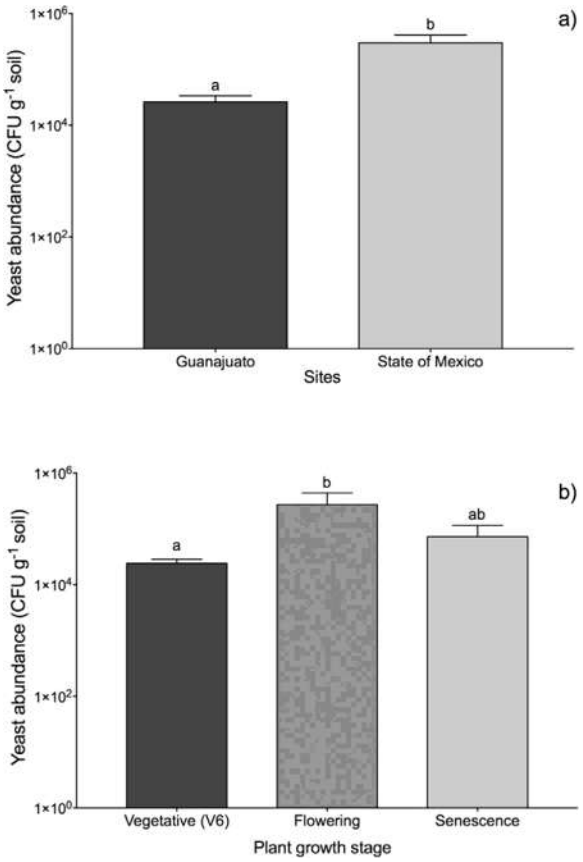

Fig. 3. Abundance of rhizosphere yeasts in six maize fields in terms of treatment factors means for a) site (Guanajuato and State of Mexico, $n = 9$) and b) plant growth stage (vegetative, florescence and senescence, $n = 6$). Different letters indicate significant effects between treatments.

differing mainly in colony color, texture and shape, which were molecularly identified to species level, with an identification match in the range 99–100% (Table 2). Isolates of *C. railenensis* and *M. guillermondii* were the most abundant species in the yeast collection (Table 3).

3.3. P solubilisation assay

Four of the yeast species solubilised $Ca_3(PO_4)_2$ at different levels, whereas none of the eight yeast species solubilised iron phosphates (Table 3). A clear halo zone was formed around the colonies after 7 days of incubation on solidified NBRIP medium supplemented with calcium phosphate, indicating the phosphate-solubilising capacity of the yeast isolates.

3.4. Greenhouse maize plant growth assay

Significant "Yeast × P fertilization" interactions were obtained for both shoot ($p = 0.0220$) and root ($p = 0.0105$) dry weight. In terms of shoot dry weight *S. aeria* in combination with KH_2PO_4 increased the shoot dry weight by 27% compared to the similar treatment without yeast inoculation, whereas *Cr. flavus* in combination with $Ca_3(PO_4)_2$ increased this variable by 24% also when compared to the corresponding treatment without yeast inoculation (Fig. 4a). Concerning the root dry weight, the treatments with *C. railenensis* and $Ca_3(PO_4)_2$ increased the root dry weight by 28% (Fig. 4b).

4. Discussion

Yeasts are known to be abundant and diverse soil inhabitants in

Table 2
Characteristics of yeast species isolated from maize rhizosphere soil in relation to their synonyms, phylum, Gene bank match % and colony morphology.

Yeast species	Synonym	Phylum	Match %	Colony morphology
Candida railenensis	*Apiotrichum osvaldii*	Ascomycota	99–100	White to cream, smooth
Clavispora lusitaniae	*Saccharomyces carmosousae*	Ascomycota	99	Cream, smooth
Cryptococcus flavus	*Torula flava* *Chromotorula flava* *Rhodotorula flava* *Rhodotorula tokyoensis var. Flava* *Cryptococcus flavus* *Saitozyma flava*	Basidiomycota	99	Yellow-brown, smooth
Filobasidium globisporum	None	Basidiomycota	99	White-gray, mucoid
Meyerozyma caribbica	*Torula fermentati* *Candida fermentati* *Pichia caribbica*	Ascomycota	99–100	Light-gray, smooth
Meyerozyma guilliermondii	*Pichia guilliermondii* *Yamadazyma guilliermondii* *Endomyces lacteus* *Endomyces lacticolor* *Endomycopsis guilliermondii*	Ascomycota	99–100	Tannish-white, smooth
Solicoccozyma aeria	*Torulopsis aeria var, Aeria* *Torula aerius* *Torula aeria* *Torulopsis aeria* *Paratorulopsis aeria* *Cryptococcus aerius* *Cryptococcus albidus*	Basidiomycota	99–100	Pale brownish-yellow, smooth
Symmetrospora coprosmae	*Sporobolomyces coprosmae*	Basidiomycota	99	Orange-red, smooth

Table 3
Phosphate solubilization traits of maize rhizosphere yeasts.

Yeast	Number of isolates tested	$Ca_3(PO_4)_2$ solubilisation	$FePO_4$ solubilisation
Candida railenensis	14	+	–
Clavispora lusitaniae	1	+	–
Cryptococcus flavus	1	–	–
Filobasidium globisporum	1	–	–
Meyerozyma caribbica	3	–	–
Meyerozyma guilliermondii	10	+	–
Solicoccozyma aeria	4	–	–
Symmetrospora coprosmae	2	+	–

+ and – respectively indicates yeasts with and without P solubilisation traits

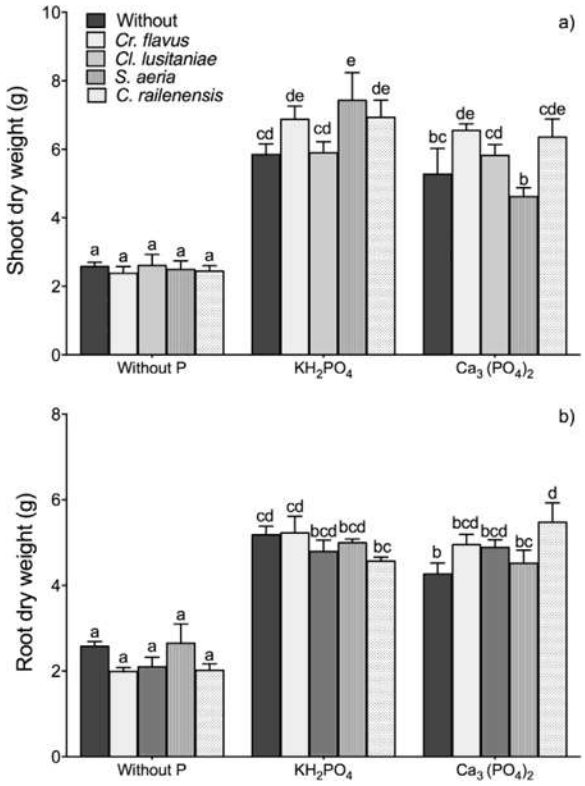

Fig. 4. Effects of individual inoculation with the rhizosphere yeasts *Cryptococcus flavus*, *Clavispora lusitaniae*, *Solicoccozyma aeria* and *Candida railenensis* on shoot (a) and root (b) dry weight of maize plants grown for eight weeks in different mineral P fertilisation settings (without, KH_2PO_4 and $Ca_3(PO_4)_2$ ($n = 5$). Different letters indicate significant effects between treatments.

natural ecosystems (Yurkov et al., 2016, Connell et al., 2008, Wuczkowski and Prillinger 2004,), whereas information on yeasts in agricultural soil is limited (Sláviková and Vadkertiová 2003). Here we found yeasts to be abundant in the maize rhizosphere throughout the growing cycle in all six maize fields examined differing mainly in geographic site and soil characteristics. These results are in accordance with the few other studies on soil yeasts in agricultural fields with different crops (Xu et al., 2012, Sláviková and Vadkertiová 2003, Gomes et al., 2003).

Our results showing that yeast abundance was highest during flowering followed by senescence, is similar to the results from Gomes et al. (2003) who showed that yeasts were among the most abundant rhizosphere fungi in the senescence maize growth phase. In the present study, the peak of yeast abundance in the flowering stage coincided with the full root development and high soil humidity since the flowering growth stage fell within the rainy season. In the fully developed root system, organic matter in the form of dead roots and root exudates should be more abundant and thus provide more nutrients for the yeast populations than in any of the other plant growth phases. However, the underlying mechanism explaining the peak in yeast abundance during the flowering growth stage needs to be further addressed.

In total 8 yeast morphotypes were obtained from maize rhizosphere soil of which all were identified to species level, representing 8 species from 7 genera. The number of yeast species recovered in the present study coincides with Sláviková and Vadkertiová (2003) who recovered 7 yeast species from maize fields in Slovakia. However, the soil yeast diversity was not fully explored in the present study, which included

only cultivable yeasts and also a limited number of samples. Hence, the yeast collection obtained in the present study merely represent the most common and abundant species in the maize rhizosphere. In a field study exploring fungal communities with a non-cultivable metagenomic approach, 23 yeast species from ten genera were recovered from pea rhizosphere soil (Xu et al., 2012).

Yeast populations clearly differed in abundance in the two geographic areas studied with highest population density in Guanajuato with more intense maize production. However, it is important to note, that the experimental design employed does not allow separating effects from geographic area and agricultural practice. Also the soil physicochemical characteristics differed between the geographic sites, where particularly pH and Mg showed strong correlation with yeast abundance, though also with strong collinearity between these two soil variables. Similarly, Vreulink et al. (2007) reported that soil yeast abundance correlated negatively with soil pH. Effects of pH on the abundance of yeasts in the soil may be related to direct pH effects, but may also be associated with indirect effects caused by reduction in the abundance of bacteria when decreasing pH. In general bacteria respond negatively to the reduction in soil pH, which may result in increased abundance of fungi when escaping competition for nutrients from bacteria (Rousk et al., 2010).

Phosphate solubilisation is among the functional traits reported for soil yeasts (Nakayan et al., 2013; Xiao et al., 2012; Hesham and Mohamed, 2011; Al-Falih, 2005), which is supported by the present study, where four out of the eight yeast species solubilised $Ca_3(PO_4)_3$. However, none of the yeasts solubilised $FePO_4$. Nakayan et al., (2013) suggested that the potential of soil yeasts to solubilise phosphate may vary at the genus and species levels, as also revealed in the present study.

Yeasts have been reported to improve the shoot and root growth in maize plants, which has been linked to their P solubilization traits (Nakayan et al., 2013; Hesham and Mohamed, 2011). In our study, the observed increase in plant growth performance by *Cr. flavus* and *S. aerea* is most likely not linked to P solubilisation since these two yeast species did not present P solubilising traits. In addition, *C. railanensis* and *Cl. lusitaneae* presented P solubilising traits, but did not promote plant growth. However, for *C. railanensis* and *Cl. lusitaneae* we cannot rule out the possibility that they also solubilised P in soil, since it could have been consumed by themselves or other soil microorganisms. More detailed studies including measurements of different soil P pools including microbial P, organic P and mineral P are needed to better understand the possible role of P solubilisation in soil by yeasts.

The observed plant growth promoting effects of rhizosphere yeasts depended on fertilisation with mineral P. Plants grown in soil with P limitation were not affected by inoculation with yeasts, while the three cases of yeast plant growth promotion were obtained in plants not limited by P due to abundant mineral P fertilisation. The plant growth promotion with *Cr. flavus* was only obtained in combination with the insoluble P source $Ca_3(PO_4)_2$, but since *Cr. flavus* do not solubilise $Ca_3(PO_4)_2$ further support that other mechanisms than P solubilisation were responsible for the plant growth promotion by *Cr. flavus*. On the contrary, plant growth suppression in maize when grown in P limited soil after inoculation with the same isolate of *Cr. flavus* as in the present study has been reported (Sarabia et al., 2017a). These contrasting results may be linked to the maize genotype, which was the only main experimental difference between these two studies.

Besides the plant growth promotion traits of rhizosphere yeasts shown in the present study, yeasts have been shown to provide other plant beneficial traits, like plant health and soil fertility promotion, which should be integrated in agroecosystems (Botha, 2011).

In conclusion, here we show that rhizosphere yeasts with plant growth promotion potential are common in maize agroecosystems throughout the complete crop cycle, with highest population densities in low input systems with acid soil. In addition, the maize plant growth promotion by inoculation with yeasts was obtained only in combination with mineral P fertilization. Future conservation and management of such plant growth promoting yeasts in agroecosystems require further information on how they perform in different agricultural settings including tillage, crop rotation, fertilisation and pest management.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.rhisph.2018.04.002.

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CAPÍTULO 3.

Mineral phosphorus fertilization modulates interactions between maize, rhizosphere yeasts and arbuscular mycorrhizal fungi

Marcela Sarabia, Pablo Cornejo, Rosario Azcón, Yazmín Carreón-Abud, John Larsen

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Mineral phosphorus fertilization modulates interactions between maize, rhizosphere yeasts and arbuscular mycorrhizal fungi

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ABSTRACT

Yeasts are abundant and diverse in rhizosphere soil, but information about their importance in agroecosystems is limited. Here we examined maize plant growth response to inoculation with the native maize rhizosphere yeasts *Cryptococcus flavus* and *Candida railenensis* in non-mycorrhizal and mycorrhizal maize without and with mineral P fertilization as KH_2PO_4. Maize plant growth promotion and suppression was observed after inoculation with a native community of arbuscular mycorrhizal (AM) fungi with and without mineral P fertilization, respectively. Inoculation with *Cr. flavus* reduced shoot biomass of maize plants irrespective of the mycorrhizal status, but only without P fertilization. AM fungal root colonization was increased by *C. railenensis* without P fertilization, but was reduced by *Cr. flavus* in combination with mineral P fertilization. In conclusion, our results show that mineral P fertilization strongly modulates interactions between maize, rhizosphere yeasts and AM fungi, which are important to take into account when integrating such root associated microorganisms as biofertilizers in agroecosystems.

1. Introduction

Maize is worldwide an important cereal crop for human consumption and animal fodder (Bennetzen and Hake, 2009) and like other crops requires application of phosphorus (P) when grown in soil below the critical P value for optimal growth (Tang et al., 2009). In conventional agriculture P is applied as mineral fertilizers; however, excessive application of phosphate fertilizers causes environmental problems such as eutrophication of water bodies (Lehman and Taheri, 2017). Although P is abundant in soils both in inorganic and organic soil P pools it is a strong plant growth limiting factor due to the formation of mineral complexes, phytate and other organic recalcitrant compounds, resulting in a general low P availability in soil (Sawers et al., 2017; Shen et al., 2011).

Plant P uptake from low P soil is facilitated by production and exudation of organic anions, alteration of the architecture of the root system increasing the root-soil surface area and root associations with P mobilizing microorganisms (Richardson et al., 2009b; Shen et al., 2011; Seguel et al., 2013; Cornejo et al., 2017). However, efficient soil P capture by roots depends on physicochemical parameters, environmental factors and biological interactions that take place in the soil (Richardson et al., 2009a).

Roots associate with a broad range of beneficial microorganisms including bacteria and fungi with plant growth promoting traits related to solubilisation, mobilization, uptake and transport of P by plants (Richardson et al., 2011).

Yeasts are single celled polyphyletic saprotrophic fungi abundant and diverse in agricultural soil (Slávková and Vadkertiová, 2003; Xu et al., 2012), but yet little is known about their importance in agroecosystems (Botha, 2011). Plant growth promotion has been reported after inoculation with soil yeasts, which may be related to their P solubilizing traits and production of plant growth regulating hormones (Nakayan et al., 2013; Xiao et al., 2013).

Most plants including maize form symbiotic root associations with AM fungi, which are obligate biotrophic fungi from Glomeromycota (Smith and Read, 2008), providing key ecosystem services in agroecosystems related with soil fertility and plant health and nutrition (Gianinazzi et al., 2010). Especially the importance of AM fungi in host P nutrition is well recognized (Smith and Smith, 2011). In maize AM associations have resulted in both plant growth promotion and plant growth suppression depending on maize genotype (Kaeppler et al., 2000; Sawers et al., 2017), environmental factors such as soil P levels

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(Grant et al., 2005) and farming practices such as tillage and crop rotation (Gavito and Miller, 1998).

Microbial interactions in the rhizosphere in relation to plant growth and nutrition have been examined extensively (Philippot, 2013; Hol et al., 2014; Larsen et al., 2009, 2015). However, limited information is available on the interactions between AM fungi and rhizosphere yeasts when associated with maize (Gollner et al., 2006) and information on how mineral P fertilization affects plant growth response to dual fungal inoculation with yeasts and AM fungi is missing. Since yeasts and AM fungi provide different functional traits in relation to plant P nutrition it is possible that their combined effects will result in improved plant growth in low P soil.

In this study we examined interactions between maize, rhizosphere yeasts and AM fungi in terms of plant growth performance as affected by mineral P fertilization. We tested two hypotheses: 1) Possible maize plant benefit from inoculation with either rhizosphere yeasts or AM fungi will be highest without mineral P fertilization, and 2) Dual inoculation with rhizosphere yeasts and AM fungi will result in plant growth promotion to a higher extend than from single fungal inoculations.

2. Materials and methods

2.1. Soil

Soil was obtained from the experimental field station of the National Agricultural University of Mexico, Campus Morelia, Michoacán, Mexico. Soil texture was clayish consisting of 53.2% clay, 27.3% silt and 19.5% sand and with following chemical characteristics: 2.7% organic matter, 23.2 mg kg^{-1} inorganic nitrogen, 5.8 mg kg^{-1} available phosphorus (Olsen P) and pH (H_2O) 7.3. Soil was mixed with quartz sand (1:1, w:w) and disinfected in an autoclave. A full basic mineral fertilization except P was applied to the soil so that P was the only nutrient limiting plant growth (mg kg^{-1} dry soil): K_2SO_4, 75.0; $CaCl_2 \times 2H_2O$, 75.0; $CuSO_4 \times 5H_2O$, 2.1; $ZnSO_4 \times 7H_2O$, 5.4; $MnSO_4$ x H_2O, 10.5; $MgSO_4 \times 7H_2O$, 45; $Na_2MoO_4 \times 2H_2O$, 0.18; NH_4NO_3, 86.2. Phosphorus was mixed into the soil as KH_2PO_4 (200 mg P pot^{-1}) according to the experimental design.

2.2. AM fungi

AM fungi inoculum was obtained from a maize field (19° 35.91'N 101° 41.31'W, 2056 m above sea level), which was subsequently propagated in maize trap cultures under greenhouse conditions. The original native AM fungi population was composed by thirteen species: *Funneliformis mosseae, Acaulospora spinosa, Acaulospora scrobiculata, Acaulospora splendida, Gigaspora aff. albida, Scutellopspora dipurpurescens, Acaulaspora remmi, Rhizophagus irregularis, Diversispora spurca, Gigaspora decipiens, Scutellospora calospora* and *Pacispora aff. franciscana*. From the final maize trap culture only spores of the AM fungi *Gi. margarita*, *Gi. decipiens* and *R. irregularis* were recovered to be used as inoculum (Alvarado, 2015). Inoculation with these selected native AM fungi was performed prior to sowing mixing 50 g of the inoculum into the soil:sand mixture to the respective treatments with AM fungi. The AMF soil:sand inoculum contained root segments colonized with AMF, spores and mycelium as well as other root and soil associated microorganisms.

2.3. Yeasts

The yeasts examined, *Cryptococcus flavus* (MSRY-75, GenBank accession number: KY952865) and *Candida railenensis* (MSYR-124, GenBank accession number: KY952856), were originally isolated from maize rhizosphere soil. Yeasts were obtained from the yeast culture collection of the Agroecology group at Universidad Nacional Autonoma de Mexico, Campus Morelia. Both yeast species are common and abundant inhabitants of the maize rhizosphere in maize agroecosystems in Mexico and have been found to promote maize plant growth of two other maize genotypes. The two yeasts differ in the sense that only *C. railenensis* is able to solubilize tricalcium phosphate *in vitro* (Marcela Sarabia, unpublished data).

Yeast inoculum was made from four days old potato dextrose agar (PDA) cultures, which had been grown at 25 °C. Yeast cells were suspended in sterile Milli-Q water and the cell density was counted by microscopy using a haemocytometer. Yeasts were inoculated by homogeneously mixing 1×10^6 cells g^{-1} of the respective yeasts into the soil sand mix prior sowing.

2.4. Experimental design

A growth chamber pot experiment with the maize hybrid DK 2042 ® was carried out with three factors in a fully factorial randomized design: 1) Maize rizosphere yeasts (without, inoculation and with *Cr. flavus* (MSRY-75) or *C. railenensis* (MSRY-124); 2) AM fungi (two levels, without and with inoculation with a native population from maize rizosphere soil) and 3) Mineral phosphorus (without and with KH_2PO_4 (200 mg P pot^{-1})) giving a total of 12 treatments each with five replicates ($N = 60$).

2.5. Experimental conditions

Plants were grown in one liter pots with 800 g soil:sand mix in a growth chamber with 20 °C and 12 hour photoperiod. Pots were watered by weight to 70% of the water holding capacity every day. Nitrogen was applied as NH_4NO_3 to all plants every second week after sowing as aqueous solutions each time with 50 mg N giving a total of 200 mg N applied during the plant growth period.

2.6. Harvest and analyses

Plants were harvested 8 weeks after sowing. The shoot was separated from the root and dried for 48 h. at 70 °C to obtain the shoot dry weight. Roots were washed in tap water.

Two gram of root subsamples were used to measure AM fungi root colonization according to Giovanetti and Mosse (1980). The rest of the roots were dried to obtain the total root dry weight.

2.7. Statistics

All data were subjected to statistical analyses with generalized linear models (GLM) with the software R Core Team 2016. Tukey analyses were used for post hoc treatment comparisons for significant factor effects and interactions between factors.

3. Results

3.1. Shoot dry weight

Significant "Yeast x P" and "AM fungi x P" interactions were observed for shoot dry weight (Table 1). In general P fertilization markedly increased shoot dry weight (Table 1). Inoculation with *Cr. flavus* reduced shoot dry weight with 30% in plants without P fertilization compared to that of plants without yeast inoculation, whereas inoculation with *C. railenensis* increased shoot dry weight by 16% in plants with P fertilization compared to that of plants without yeast inoculation ($p > 0.05$) (Fig. 1). Inoculation with AM fungi without P fertilization increased shoot dry weight by 53% compared to plants without AM fungi inoculation; whereas in combination with P fertilization AM fungi inoculation resulted in a 53% reduction in shoot dry weight compared to that of plants without AM fungi inoculation (Fig. 2).

Table 1
Treatment means of plant growth characteristics of eight weeks old maize plants as affected by the factors yeast (without, *Cryptococcus flavus* and *Candida railenensis*), AM fungi (without and with) and P (without and with). A generalized linear model analyses (GLM) is presented for the variables shoot and root plant dry weight, and AMF root colonization of eight weeks old maize plants ($n=5$).

Yeast	AM fungi	P	Shoot dry weight (g)	Root dry weight (g)	AM fungi root colonization (%)
–	–	–	0.68 (0.07)	0.13 (0.02)	–
	–	+	7.24 (0.28)	2.04 (0.24)	–
	+	–	1.35 (0.20)	0.33 (0.07)	65.40 (3.19)
	+	+	3.00 (0.29)	0.80 (0.15)	25.40 (4.86)
Cr. flavus	–	–	0.63 (0.10)	0.12 (0.03)	–
	–	+	6.67 (0.37)	1.73 (0.15)	–
	+	–	0.83 (0.07)	0.24 (0.09)	46.75 (4.35)
	+	+	3.35 (0.18)	0.85(0.06)	20.60 (0.75)
C. railenensis	–	–	0.78 (0.07)	0.15 (0.03)	–
	–	+	8.05 (0.78)	2.14 (0.27)	–
	+	–	1.04 (0.15)	0.33 (0.06)	57.00 (1.08)
	+	+	3.88 (0.45)	1.12 (0.25)	38.20 (2.11)
	Analysis of variance				
	Yeasts (Y)		0.12	0.40 ***	
	AMF (A)		**	*** –	
	Phosphorus (P)		***	*** ***	
	Y x A		0.59	0.59 –	
	Y x P		**	0.47 ***	
	A x P		***	*** –	
	Y x A x P		0.15	0.80 –	

** $p < 0.01$.
*** $p < 0.001$.

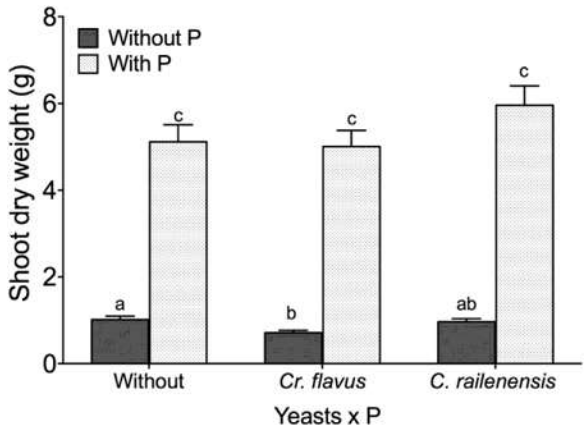

Fig. 1. Factor treatment means for shoot dry weight of eight weeks old maize plants for the interaction "Yeast (without, *Cr. flavus* and *C. railenensis*) x P fertilization (without and with)". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

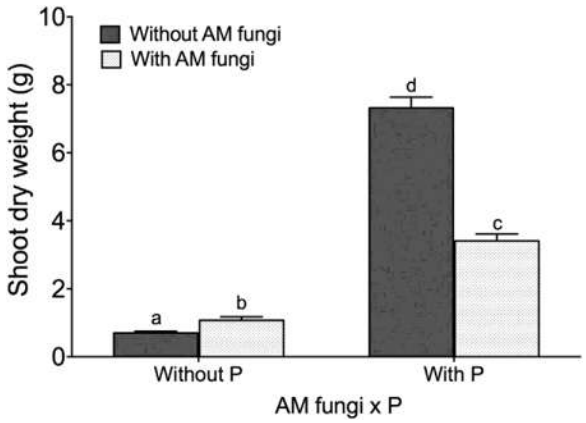

Fig. 2. Factor treatment means for shoot dry weight of eight weeks old maize plants for the interaction "AM fungi (without and with) x P fertilization (without and with)". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

3.2. Root dry weight

A significant "AM fungi x P" interaction was obtained for root dry weight (Table 1) and as for shoot dry weight in general P fertilization resulted in marked increased root dry weight (Table 1).

Inoculation with AM fungi without P fertilization increased root dry weight by 56% compared to plants without AM fungi inoculation; whereas in combination with P fertilization AM fungi inoculation resulted in a 53% reduction in root dry weight compared to that of plants without AM fungi inoculation (Fig. 3).

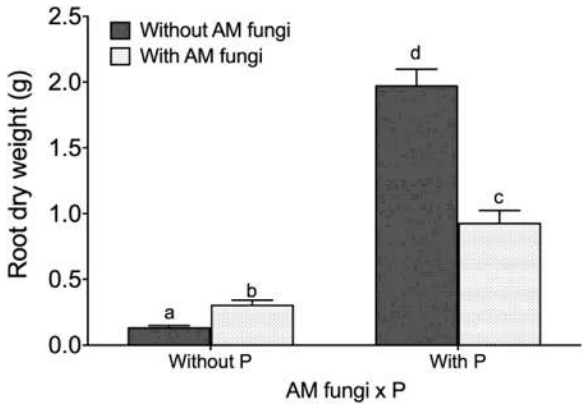

Fig. 3. Factor treatment means for root dry weight of eight weeks old maize plants for the interaction "AM fungi (without and with) x P fertilization (without and with)". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

3.3. AM fungus root colonization

At harvest eight weeks after AM fungi inoculation non-inoculated plants remained non-colonized and were therefore not included in the statistical analysis for AM fungal root colonization.

A significant "Yeast x P" interaction was found for AM fungi root colonization (Table 1). P fertilization reduced the AM fungi root

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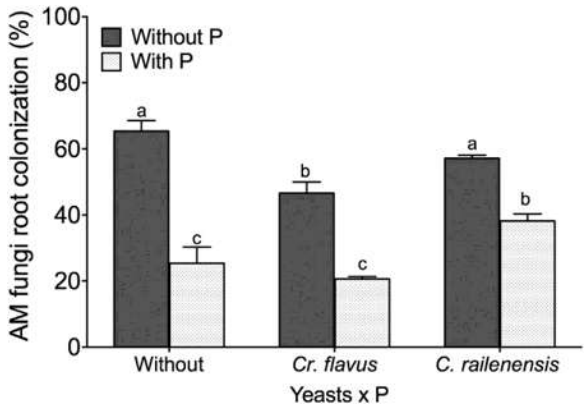

Fig. 4. Factor treatment means for AM fungi root colonization of eight weeks old maize plants for the interaction "Yeast (without, *Cr. flavus* and *C. railenensis*) x P fertilization (without and with)". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

colonization by 61%, compared to that of the plants without P fertilization (Fig. 4). Compared to plants without yeast inoculation, inoculation with *Cr. flavus* decreased AM fungi root colonization by 40% in plants without P fertilization, whereas inoculation with *C. railenensis* increased AM fungi root colonization by 34% in P fertilized plants (Fig. 4).

4. Discussion

Here we show that mineral P fertilization strongly modified interactions between maize, rhizosphere yeasts and AM fungi. Our first hypothesis that maize plant growth promotion by yeasts and AM fungi would be stronger without P fertilization was only true for AM fungi, whereas oppositely *C. railenensis* caused a reduction in plant growth. Consequently also the second hypothesis that dual inoculation with yeasts and AM fungi would result in improved plant growth compared to that of individual inoculation was rejected.

The observed plant growth reduction after inoculation with *Cr. flavus* is in contrast to other studies on plant response to soil yeasts, where plant growth promotion has been reported in rice (Amprayn et al., 2012), sugar beet (El-Tarabily, 2004) and maize (Nassar et al., 2005; Gollner et al., 2006; Nakayan et al., 2013). Since plant growth reduction was not observed in combination with P fertilization competition between *Cr. flavus* and maize roots for limited P sourced is a likely mode of interaction.

The observed plant growth promotion by AM fungi in maize in low P soil is well known though depending on maize genotype (Sawers et al., 2017; Kaeppler et al., 2000). Also the plant growth suppression by AM fungi inoculation in combination with mineral P fertilization has been thoroughly discussed by Johnson and Graham (2013), suggesting a parasitic association between AM fungi and their host plant when the carbon cost to maintain the symbiosis exceed the benefit from the improved P nutrition. In the present study only low and high P levels were examined, calling for further information on how maize plants respond to AM fungi in a full P fertilization gradient. Also P fertilization markedly reduced the AM fungi root colonization, which however, is a well-known response of AM associations to P fertilization (Thomson et al., 1992; Liu et al., 2000; Gosling et al., 2013).

These findings suggest that in maize agroecosystems it is important to develop crop nutrition strategies to improve P use efficiency assuring mutualistic maize mycorrhizal associations specially in terms of reducing the input of mineral P fertilizers. In order to integrate ecosystem services provided by native populations of AM fungi in agroecosystems (Gianinazzi et al., 2010), besides P fertilization, also require attention to other agricultural practices such as tillage and pest management that affects the performance of AM fungi (Larsen et al., 2014).

Mineral P fertilization also clearly altered the outcome of the interactions between yeasts and AM fungi in the present study, where *Cr. flavus* reduced the AM fungi colonization without P fertilization and *C. railenensis* increased AM fungi root colonization in combination with P fertilization. The reduction in AM fungi root colonization by *Cr. flavus* without P fertilization may be as a consequence of competition for P, whereas the underlying mechanism for the increase in AM fungal root colonization in P fertilized plants by *C. railenensis* needs to be further addressed. Other studies have also shown strong effects of soil yeasts on formation of AM fungal root and soil colonization (Fracchia et al., 2003; Gollner et al., 2006). Fracchia et al. (2003) reported increased AM fungal root colonization of clover and soybean in association with *Funneliformis mosseae* syn. *Glomus mosseae* and *Gigaspora rosea* after inoculation with *Rhodotorula mucilaginosa*. In general interactions between yeasts and AM fungi seem to be complex depending on fungal genotypes and plant and soil environmental conditions, which should de further addressed to optimize the joint use of both potentially plant-growth beneficial microorganisms.

In conclusion, our results show that mineral P fertilization strongly modulates interactions between maize, rhizosphere yeasts and AM fungi, which should be considered for strategies to improve P use efficiency in maize agroecosystems.

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CAPÍTULO 4

Rhizosphere yeasts improve P uptake of a maize arbuscular mycorrhizal association

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Rhizosphere yeasts improve P uptake of a maize arbuscular mycorrhizal association

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ABSTRACT

Plant roots associate with microorganisms to acquire phosphorus (P) from the soil including arbuscular mycorrhizal (AM) fungi and phosphate solubilizers such as yeasts. However, information on interactions between AM fungi and yeasts is limited. The objective of the present study was to examine possible cooperation between the AM fungus *Rhizophagus irregularis* and the rhizosphere yeasts *Cryptococcus flavus* and *Candida railenensis* in terms of maize plant growth and P uptake employing P isotope tracer monitoring. Compartmented growth units with root-hyphal (RHC) and hyphal (HC) mesh bags allowed studying interactions between *R. irregularis* and yeasts with and without roots. The P isotope dilution method was used to measure possible phosphate solubilization in soil by the rhizosphere yeasts with subsequent P uptake by the AM association. Plants were grown for 8 weeks in low P soil under controlled growth chamber conditions. Main results showed strong plant growth promotion after inoculation with *R. irregularis* and both yeast species altered the specific root length of maize. The yeasts also improved maize shoot P content, but only in mycorrhizal plants. More specifically yeasts improved AM hyphal ^{32}P uptake, but not that of root ^{33}P uptake. On the other hand, yeasts had no effect on extraradical growth of *R. irregularis*. Inoculation with yeasts did not result in P isotope dilution measured as specific P isotope activity, indicating that the reported improved P uptake from AM associations in combination with yeasts was most likely not related to P solubilization, whereas alteration in root growth and specific root length seems more likely mechanisms of interactions. We conclude that rhizosphere yeasts promote P nutrition of arbuscular mycorrhizal maize linked to improved specific root length and AM hyphal P uptake.

1. Introduction

Phosphorus is one of the main limiting nutrient for plant growth because of its adsorption chemistry and formation of mineral complexes with different elements such as Ca, Al and Fe making phosphate immobile in soil (Shen et al., 2011). Hence, in agroecosystems, crops require application of P as organic or mineral fertilizers depending on crop production system. However, P is often applied in excessive amounts to avoid potential limitations to plant growth. Excessive fertilization with P can have adverse eutrophication effects on water bodies, mainly as a result of soil run off after periods with heavy rain fall (Lehman and Taheri, 2017). Another important aspect to consider is that world mineral P resources are non-renewable on a human time scale (Scholz and Wellmer, 2013). These environmental concerns are calling for the development of strategies to improve P use efficiency in agroecosystems (Richardson et al., 2011; Lehman and Taheri, 2017).

Plants use different strategies to acquire P including exudation of organic anions, alteration of root architecture improving root-soil surface area and the formation of associations with biotrophic mycorrhizal fungi and free living plant growth promoting rhizosphere microorganisms such as P solubilizing bacteria and fungi (Richardson et al., 2009; Shen et al., 2011; Lehman and Taheri, 2017).

Improved host P nutrition by AM fungi is well documented both for natural ecosystems and agroecosystems with the external mycelium playing a key role in P uptake from the soil solution due to the strong expansion of the plant-soil interface (Smith and Smith, 2011). However, solubilization of phosphate by AM fungi either from mineral (Bagyaraj et al., 2015) or organic (Joner et al., 2000) forms is most likely indirect via AM fungi associated microorganisms. Indeed, such microbial cooperation was reported by Zhang et al. (2016) showing that the P

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solubilizing bacterium *Rahnella aquatilis* improve solubilization of organic P when associated with the AM fungus *Rhizophagus irregularis*.

P solubilization from rhizosphere microorgansims including plant growth promoting bacteria and fungi other than AM fungi have been suggested as a way to access plant insoluble P pools (Vassilev et al., 2014). Most studies on P solubilization of microorganisms are based on *in-vitro* assays with different forms of mineral P, which however most likely does not reflect what happens in the natural soil environment (Bashan et al., 2013).

Among P solubilizing fungi, soil yeasts represent a relative unexplored group of fungi (Botha, 2011) and this in spite of their high abundance and diversity in agroecosystems (Xu et al., 2012). Yeasts are single celled polyphyletic fungi from Ascomycota and Basidiomycota common in agricultural soil (Sláviková and Vadkertiová, 2003; Xu et al., 2012) with reported potential to promote plant growth through P solubilization (Nakayan et al., 2013; Xiao et al., 2013).

Microbial interactions in the rhizosphere is a key factor determining plant and soil nutrient dynamics (Richardson et al., 2009; Philippot et al., 2013; Hol et al., 2014; Larsen et al., 2015), and should be considered in strategies to improve nutrient efficiency in agrocosystems. Studies on interactions between rhizosphere microorganisms are manifold as revealed from a large body of reviews on this topic (Linderman, 1992; Whipps, 2001; Barea et al., 2002, 2005; Morgan et al., 2005; Barea et al., 2013; Larsen et al., 2015). However, information of interactions between AM fungi and soil yeasts is yet limited (Fracchia et al., 2003, 2004; Sampedro et al., 2004; Gollner et al., 2006; Boby et al., 2008) and though both groups of fungi are abundant in agroecosystems, studies on their possible interactions in relation to plant P nutrition is missing.

Here we examined possible co-operation between two rhizosphere yeasts, *Cryptococcus flavus* or *Candida railenensis*, and the *Rhizophagus irregularis*-maize arbuscular mycorrhizal association in terms of plant P nutrition employing the P isotope dilution method. Our hypothesis was that P solubilization in soil by yeasts will result in improved P transport by the external mycelium of *R. irregularis*.

2. Materials and methods

2.1. Experimental design

A full-factorial pot experiment with maize was carried out in a growth chamber with three factors: (1) AM fungus (two levels, without and with inoculation with *R. irregularis*, (2) Maize rhizosphere yeasts (three levels, without inoculation and inoculation with *Cr. flavus* or *C. railenenis*) and (3) Mineral phosphorus (two levels, without and with TCP ($Ca_3(PO_4)_2$). Each of the resulting 12 treatment had five replicates giving a total of 60 experimental units.

2.2. Biological materials

The *Zea mays* L maize inbred line Oh43, which is highly mycorrhizal responsive when grown in soil with low P (Sawers et al., 2017), was used in this experiment. Seeds were kindly provided by Dr. Ruairidh Sawers from Langebio, Cinvestav in Mexico.

The yeasts examined, *Cr. flavus* (GenBank accession number: KY952865) and *C. railenensis* (GenBank accession number: KY952856), were originally isolated from maize rhizosphere soil (Marcela Sarabia, unpublished data). Yeasts were obtained from the yeast culture collection of the Agroecology group at Universidad Nacional Autonoma de Mexico, Campus Morelia. Both yeast species are common and abundant inhabitants of the maize rhizosphere in maize agroecosystems in Mexico. The two yeasts differ in the sense that only *C. railenensis* is able to solubilize tricalcium phosphate *in vitro* (Marcela Sarabia, unpublished data).

Yeast inoculum was made from four days old potato dextrose agar (PDA) cultures, which had been grown at 25 °C. Yeast cells were suspended in sterile Milli-Q water and the cell density was counted by microscopy using a haemocytometer. Prior inoculation, viability of the yeast cells was determined by serial dilution plating on PDA. Viability of the yeast cells applied to the seed at sowing was 68% for *Cr. flavus* and 41% for *C. railenensis* and viability of yeast cells applied to the mesh bags three weeks after sowing was 80% for *Cr. flavus* and 87% for *C. railenenssis*.

Crude soil inoculum of the AM fungus *R. irregularis* (BEG87) including mycelium, spores and root segments was obtained from a pot trap culture with subterranean clover.

The growth substrate used was a 1:1 (w/w) mixture of a calcareous (pH 6.0 (H_2O)) low P (8 mg kg^{-1} Olsen P) Typic Hapludalf sandy loam (Soil Survey Staff, 2010) and quartz sand, which was partially sterilized by 15 kGy gamma irradiation. Before sowing, aqueous solutions of the following nutrients were supplied to the soil, allowed to dry and then evenly mixed into the soil (mg kg^{-1} dry soil): K_2SO_4, 370.31; $CaCl_2 \times 2H_2O$, 75.0; $CuSO_4 \times 5H_2O$, 2.1; $ZnSO_4 \times 7H_2O$, 5.4; $MnSO_4 \times H_2O$, 10.5; $MgSO_4 \times 7H_2O$, 405.43; $Na_2MoO_4 \times 2H_2O$, 0.18; NH_4NO_3, 285.71.

2.3. Experimental set-up

Pots (2L) were filled with 1.7 kg soil sand mix. AM fungus inoculum (10%, w/w) was mixed into the soil in the corresponding treatments with *R. irregularis* and the treatments without *R. irregularis* were left non-inoculated. Two empty tubes (200 ml) were inserted in the soil in each pot oppositely to the center, where three seeds were sown. Yeasts were inoculated directly on the seeds (10^6 cells $seed^{-1}$). Two weeks after seedlings emergence plants were thinned to one seedling per pot. Three weeks after sowing, when the mycorrhizal association was supposed to be established, the tubes were removed and replaced by mesh bags made from nylon mesh (Streno, Farum, Denmark) of the same volume and shape. One mesh bag with 25 µm mesh diameter, which allowed the passage of hyphae, but not roots (hyphal compartment HC) and the other with 400 µm mesh diameter in order to allow root access (root-hyphal compartment RHC). Each set of mesh bags were filled with 150g soil that had been labeled with carrier free solutions of 2.07 kBq $^{33}P\ g^{-1}$ soil for the RHC and 2.18 kBq $^{32}P\ g^{-1}$ soil for the HC. The isotopes had been mixed with the soil 3 weeks earlier in order to obtain a more uniform labeling of the plant available soil P pools. Just before filling the mesh bags, the labeled soil was fertilized with TCP (100 mg P per mesh bag) (Merck, Darmstadt, Germany) or not. Inoculation of the HC and RHC with *Cr. flavus* and *C. railenenis* (10^5 cells g^{-1} soil) was performed by adding 4 ml inoculum at 5 cm soil depth in the center of each mesh bags.

2.4. P isotope dilution

The P isotope dilution method (Barea et al., 2007) was used to test possible P solubilization of the TCP added or from the soil P pool, after inoculation with rhizosphere yeasts by calculating the shoot specific activity of $^{33}P/^{31}P$ and $^{32}P/^{31}P$ obtained from the isotope uptake from the RHC and HC mesh bags, respectively.

2.5. Plant growth conditions

The pots were placed completely randomized in a growth chamber at a 14/10 h light/dark cycle at 25/18 °C and 500 µmol $m^{-2}\ s^{-1}$ photosynthetically active radiation (PAR; 400–700 nm). Plants were watered daily by weight to 70% of the water holding capacity. Starting three weeks after sowing nitrogen was applied weekly as an aqueous solution of NH_4NO_3 corresponding to 50 mg N five times applying in total 250 mg N to each pot so that plants were not limited in nitrogen. Radioactivity in the plants was monitored every second day with a Mini 900 GM-monitor (Thermo Fisher Scientific, MA, USA) in order to determine the harvest date.

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2.6. Harvest and measurements

Plants were harvested eight weeks after sowing. The mesh bags with the labeled soil were removed from the pots and stored at −20 °C until further analyses. The shoot was separated from the root and dried for 24 h at 80 °C to obtain the shoot dry weight. Roots were washed in tap water. One gram root subsamples were used to measure AM fungus root colonization according to Giovannetti and Mosse (1980). Total root length was determined by line intersect method (Newman, 1966). The rest of the roots were dried to obtain the total root dry weight.

Ground samples of shoots and roots were digested in a 4:1 mix of nitric acid and perchloric acid and the ^{32}P and ^{33}P content in the digests was measured with a PerkinElmer Tri-Carb 2910TR Liquid Scintillation Counter (PerkinElmer, MA, USA).The same digests were analyzed for P concentration according to Murphy and Riley (1962).

When the soil from the HC and RHC mesh bags was no longer radioactive, one gram of freeze dried soil samples were used for fatty extraction of whole cell fatty acids according to the method of Sasser (1990). To enable quantification of the extracted fatty acid methyl esters, a known amount of an internal standard, nonadecanoate fatty acid methyl ester 19:0 was added to each sample. The AM fungus biomarker fatty acid 16:1ω5 and the general fungus biomarker18:2 ω6,9 were used to quantify *R. irregualris* and the yeasts, respectively, in both RHC and HC mesh bags, according to Larsen et al. (1998). Fatty acid identification was performed using the software Sherlock Version 6.0 (MIDI Inc., Delaware, USA) and fatty acid analysis was performed with the Agilent gas chromatograph7890B (Agilent, Santa Barbara, USA).

Also root dry weight, root length and AM fungus root colonization was measured in the RHC.

2.7. Statistical analyses

All data were subjected to multi factorial analyses of variance (ANOVA) performed with the software JMP (version 13.1) (SAS Institute Inc., Cary, USA). Prior to ANOVA, data were tested for variance homogeneity and if needed data were log-transformed. In order to obtain variance homogeneity for the variables shoot ^{33}P content, specific shoot ^{33}P uptake and shoot ^{33}P specific activity the basic three way design was split in two separate ANOVAs with and without *R. irregularis* and with TCP and yeast as the main factors. For the variables AM fungus soil biomass, shoot ^{32}P content, specific ^{32}P uptake and shoot ^{32}P specific activity two way ANOVAs with the factors yeast and TCP were performed.

3. Results

3.1. Shoot and root dry weight

Each of the factors yeast, *R. irregularis* and TCP significantly affected shoot dry weight of the maize plants, but interactions between factors were not detected (Table 2). Plants inoculated with *C. railenensis* had 26% greater shoot dry weight than plants without yeasts, whereas the effect of *Cr. flavus* was not significant (Fig. 1a). Inoculation with *R. irregularis* increased the shoot dry weight by 237% (Fig. 1b), while the addition of TCP to the mesh bag soil resulted in 18% greater shoot dry weight than recorded in plants without TCP (Fig. 1c).

For root dry weight significant two-way interactions were obtained for "*R. irregularis* × yeasts" and "yeast × TCP" (Table 2). In non-mycorrhizal plants, inoculation with *Cr. flavus* decreased the root dry weight with 31% compared to the treatment without yeast inoculation, whereas in mycorrhizal plants yeasts, had no effect on root dry weight (Fig. 2a). In treatments without TCP, *C. railenensis* increased the root dry weight with 38% compared to the treatment without yeast inoculation, whereas yeasts had no effects on root dry weight in treatments with TCP (Fig. 2b).

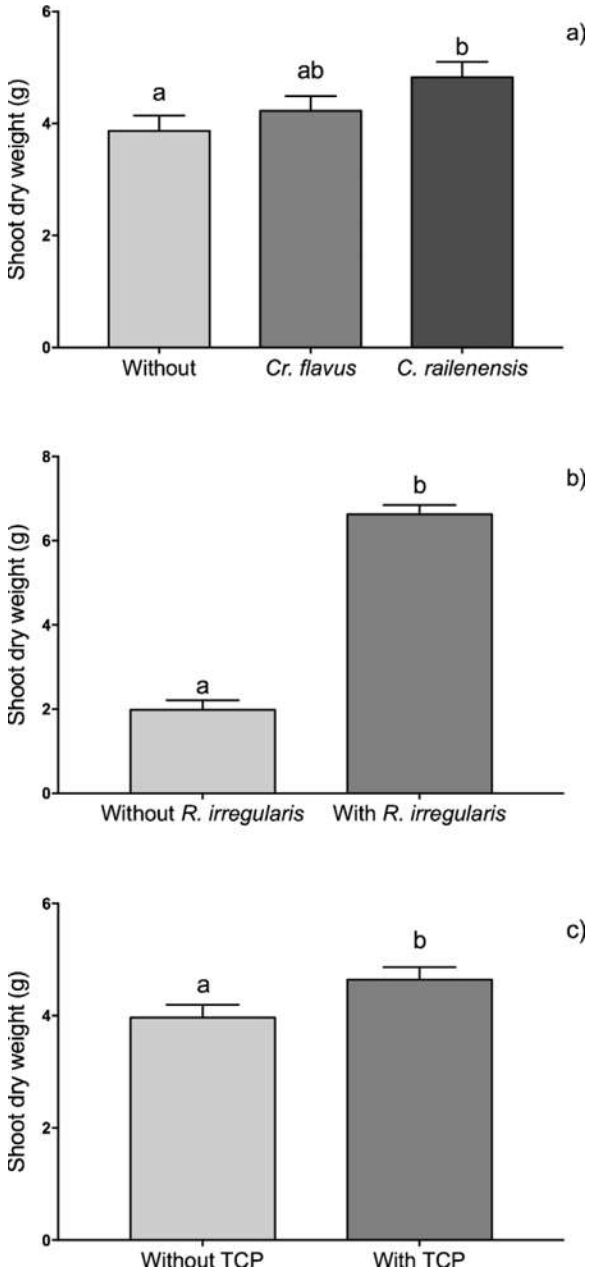

Fig. 1. Factor treatment means for shoot dry weight of eight weeks old maize plants of the individual factors (a) Yeast (without, *Cryptococcus flavus* and *Candida railenensis*), (b) *Rhizophagus irregularis* (without and with) and (c) TCP (without and with). Different letters indicate significant differences between treatments and error bars represent standard error of the mean.

3.2. AM fungal root colonization

Treatment means for AM fungal root colonization in plants inoculated with *R. irregularis* were in the range 89–98% and the only significant treatment effect was by *C. railenensis* that increased AM fungal root colonization by 6% over the level in the non-yeast treatment (Table 1).

3.3. Total and specific root length

For total root length, significant two-way interactions were obtained for "*R. irregularis* × yeasts" and "*R. irregularis* × TCP" (Table 2). Average total root length was 130% greater in *R. irregularis* colonized plants than in non-mycorrhizal plants (Table 1). Inoculation with *Cr.*

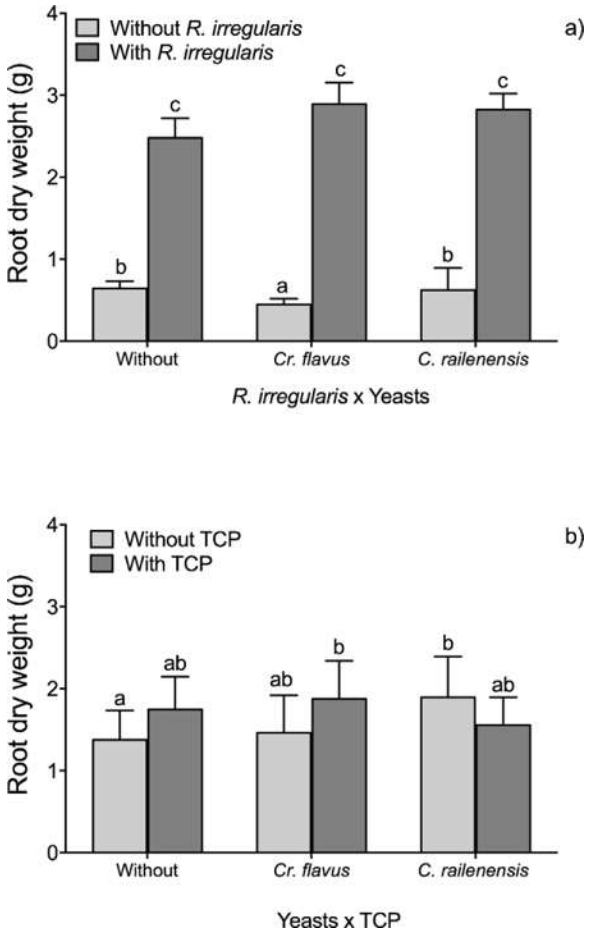

Fig. 2. Factor treatment means for root dry weight of eight weeks old maize plants of the treatments from the interactions (a) "*R. irregularis* (without and with) × Yeast (without, *Cryptococcus flavus* and *Candida railenensis*) and (b) "Yeast (without, *Cr. flavus* and *C. railenenis*) × TCP (without and with) ". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

flavus and *C. railenensis* increased total root length in mycorrhizal plants with 54% and 34% respectively, over the level in the non-yeast treatment, whereas yeasts had no effects in non-mycorrhizal plants (Fig. 3a). Application of TCP in the mesh bag soil increased the total root length with 64%, but only in non-mycorrhizal plants (Fig. 3b).

In order to obtain variance homogeneity for the variable specific root length it was necessary to perform ANOVAs with the data from the treatments without and with *R. irregularis* seperately. In both cases yeasts increased the specific root length, but to a higher extent in non-mycorrhizal than mycorrhizal plants (Fig. 4a, b). Application of TCP increased the specific root length of non-mycorrhizal plants by 25% (Table 1).

3.4. Total shoot P content

A significant "*R. irregularis* × yeast" interaction was observed for shoot P content (Table 2). Shoot P content was increased with 20% and 29%, by *Cr. flavus* and *C. railenensis,* respectively, but only in mycorrhizal plants (Fig. 5). On average, the shoot P content was 277% higher in mycorrhizal plants than in non-mycorrhizal plants (Table 1). Amendment of soils in mesh bags with TCP overall increased the shoot P content by 21% compared with the treatments without TCP (Table 1).

3.5. Shoot ^{33}P content, specific shoot ^{33}P uptake and specific activity (RHC)

For shoot ^{33}P content, specific shoot ^{33}P uptake and specific activity (^{33}P/^{31}P) variance homogeneity could not be obtained for the ANOVA including all three factors. However, variance homogeneity was obtained when two separate ANOVAs for treatments without and with *R. irregularis* were performed with the factors yeast and TCP, but no significant effects were obtained for these variables (Table 3). The root length in the RHC (Table 4) used to calculate the specific shoot ^{33}P uptake showed the same pattern as for the total root length (Table 1)

3.6. Shoot ^{32}P content, specific shoot ^{32}P uptake and specific activity (HC)

Shoot ^{32}P content of mycorrhizal plants was significantly affected by yeast and TCP (Table 5). Inoculation with *Cr. flavus* increased the shoot ^{32}P content by 46% compared to the level of the non-yeast treatment (Fig. 6). Application of TCP increased the shoot ^{32}P content with 29% compared to the treatments without TCP in terms of factor treatment means (Table 6). The average background value of ^{32}P shoot content for the non-mycorrhizal control treatment plants was 1.18 kBq (S.E. 0.21). Shoot ^{32}P uptake per unit AM fungus biomarker and shoot specific activity (^{32}P/^{31}P) were not significantly affected by treatments (Table 3). The background amount of the AM fungus biomarker fatty acid 16:1ω5 in soil from non-mycorrhizal treatments was on average 1.53 nmole g^{-1} soil (S.E 0.06). Neither yeast nor TCP had significant effects on the amount of 16:1ω5 (Table 6).

3.7. Yeast soil density in mesh bags

An attempt to estimate yeast population density in soil mesh bags

Table 1
Treatment means of the plant growth characteristics shoot and root dry weight, AMF root colonizacion (AMF root col.), total and specific root length and shoot P content of eight weeks old maize plants as affected by the factors yeast (without, *Cryptococcus flavus* and *Candida railenensis*), *Rhizophagus irregularis* (without (−) and with (+)) and tricalcium diphosphate (TCP) (without and with) (n = 5).

Yeast	*R. irregularis*	TCP	Shoot dry weight (g)	Root dry weight (g)	AMF root col. (%)	Total root length (m plant^{-1})	Specific root length (m root dwt^{-1})	Shoot P content (mg plant^{-1})
Without	–	–	1.63	0.49	0	30.10	62.61	2.04
	+	–	5.48	2.27	89	88.26	41.39	7.39
	–	+	2.43	0.80	0	66.47	79.85	2.91
	+	+	5.93	2.69	92	93.93	34.52	8.18
Cr. flavus	–	–	1.30	0.36	0	30.90	86.27	1.74
	+	–	5.96	2.57	91	137.78	53.95	7.52
	–	+	1.76	0.54	0	62.15	110.62	2.09
	+	+	7.88	3.21	95	142.73	44.96	11.17
C. railenensis	–	–	2.21	0.64	0	55.96	88.96	2.70
	+	–	7.23	3.15	96	135.57	42.94	10.07
	–	+	2.58	0.61	0	62.90	105.51	3.10
	+	+	7.29	2.50	96	110.62	44.20	10.09

Table 2
P values from three way analysis of variance (ANOVA) of plant growth parameters of the factors *Rhizophagus irregularis*, yeast and tricalcium diphosphate (TCP) and their interactions ($n = 5$). For the variable specific root length two separate ANOVAs were performed with the factors yeast and tricalcium phosphate for treatments without[†] and with[‡] *R. irregularis*, respectively ($n = 5$).

Factors	Shoot dry weight (g)	Root dry weight (g)	AMF root colonization (%)	Total root length (m plant^{-1})	Specific root length (m root dwt $^{-1}$)	Shoot P content (mg plant^{-1})
R. irregularis (Ri)	***	***	–	***	See footnotes	***
Yeasts (Y)	*	0.154	**	*		*
Tricalcium diphosphate (TCP)	*	*	0.348	0.161		*
Ri × Y	0.087	*	–	*		*
Ri × TCP	0.677	0.188	–	*		0.793
Y × TCP	0.449	*	0.157	0.174		0.561
Ri × Y × TCP	0.409	0.918	–	0.985		0.425

* $p = 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
[†] ANOVA for treatments without *R. irregularis*: Y, *; TCP,*; Y × TCP, $p = 0.914$.
[‡] ANOVA for treatments with *R. irregularis*: Y, **; TCP, 0.064; Y × TCP, $p = 0.230$.

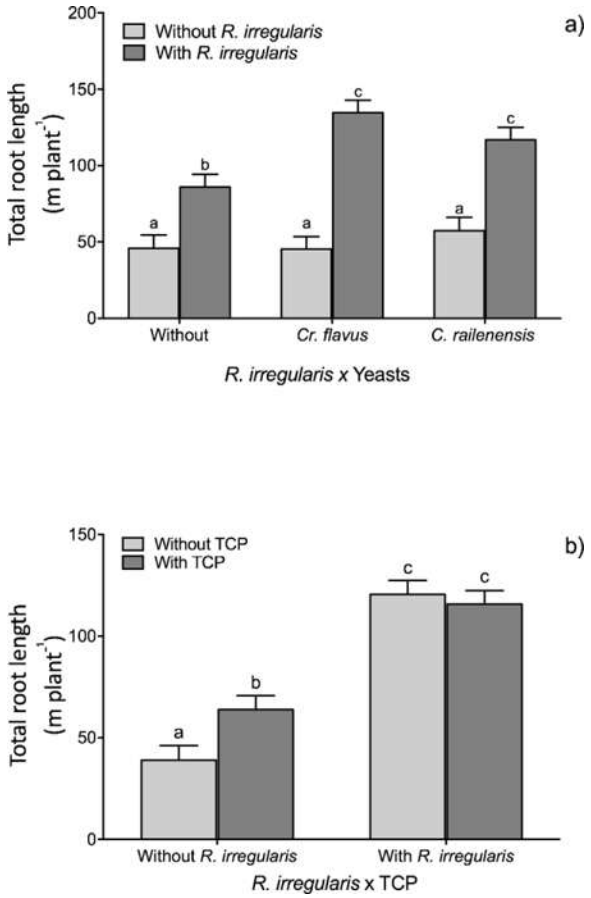

Fig. 3. Factor treatment means for total root length of eight weeks old maize plants of the treatments from the interactions (a) "*R. irregularis* (without and with) × Yeast (without, *Cryptococcus flavus* and *Candida railenensis*) "and (b) "*R. irregularis* × TCP". Different letters indicate significant treatment effects and error bars represent standard error of the mean.

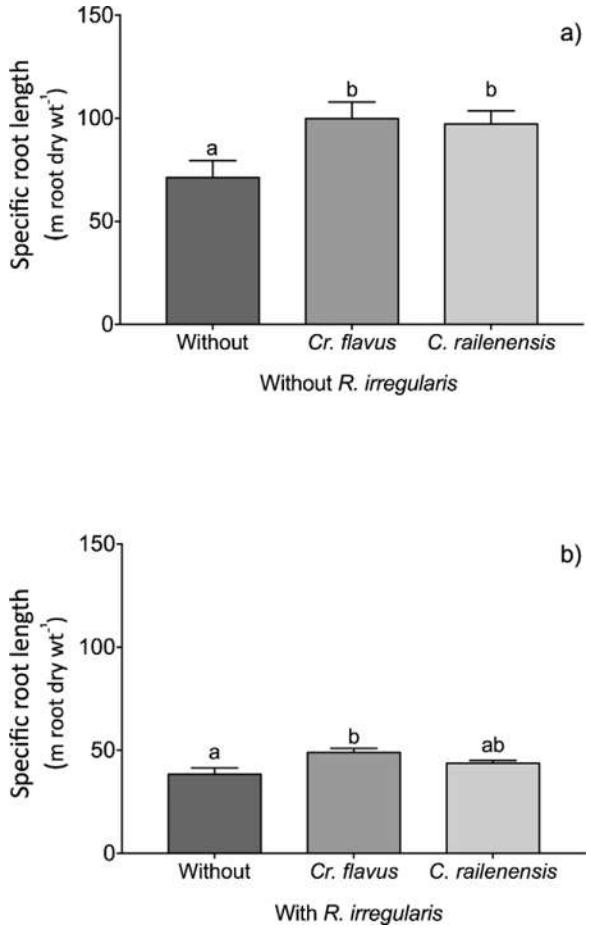

Fig. 4. Factor treatment means for specific root length of eight weeks old maize plants of the factor yeast (without, *Cryptococcus flavus* and *Candida railenensis*) (a) without *R. irregularis* and (b) with *R. irregularis*. Different letters indicate significant treatment effects and error bars represent standard error of the mean.

using the biomarker fatty acid 18:2ω6,9 at harvest five weeks after inoculation failed, since no significant effects of yeast were recorded for this variable (data not shown).

4. Discussion

The present study show that dual inoculation with either one of the rhizosphere yeasts *C. railenensis* or *Cr. flavus* and the AM fungus *R. irregularis* improved maize P nutrition. To our best knowledge this is the first report showing that rhizosphere yeasts can improve AM hyphal P uptake. The underlying mechanisms for this do not seem to be related with P solubilization by the yeasts, rejecting our main hypothesis. Instead, changes in maize specific root length appear to be the main mechanism for the observed effects of yeasts on maize P nutrition. However, the underlying mechanisms for the improved AM hyphal P uptake after yeast inoculation needs to be further addressed.

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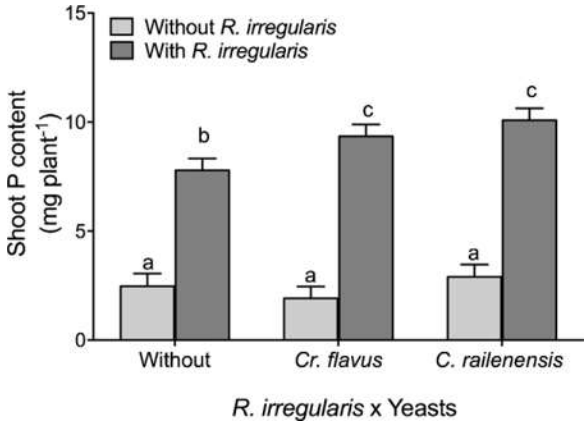

Fig. 5. Factor treatment means for shoot P content of eight weeks old maize plants of the treatments from the interaction "*R. irregularis* (without and with) × Yeast (without, *Cryptococcus flavus* and *Candida railenensis*). Different letters indicate significant treatment effects and error bars represent standard error of the mean.

4.1. Plant growth and P uptake

The improved maize growth and P uptake in response to individual and combined inoculation with the rhizosphere yeasts *C. railenensis* or *Cr. flavus* and the AM fungus *R. irregularis* is supported by reports that soil yeasts can promote growth of crops such as rice (Amprayn et al., 2012), sugar beet (El-Tarabily, 2004) and maize (Nassar et al., 2005; Gollner et al., 2006; Nakayan et al., 2013). Improved growth of maize due to inoculation with yeasts has been linked to P solubilization (Nakayan et al., 2013) and to increased root growth induced by indole acetic acid (Nassar et al., 2005). In the present study both yeasts increased shoot dry weight independent of *R. irregularis* inoculation and TCP application and both yeasts also improved total root length, but only for mycorrhizal plants. Both yeast species also improved plant P content, but had no significant effects on root ^{33}P uptake independent of TCP application, which suggests that neither of the yeasts *C. railenensis* or *Cr. flavus* solubilized TCP in soil. The observations that both yeasts had positive effects on maize P uptake and that only *C. railenensis* could solubilize P *in vitro* suggest that the improved P uptake in soil was not caused by solubilization of TCP. Accordingly, a previous study ascribed observed P responses in maize and lettuce after inoculation with three different yeasts to their common production of IAA and not to P solubilization, which could be demonstrated *in vitro* for only one of the three yeasts (Nakayan et al., 2013). In the present study the enhanced dry weight-specific root length of yeast-inoculated plants would furthermore have increased the root surface area for exploration of soil P.

The observed plant growth promotion by AM fungi in maize is well known, though depending on maize genotype (Kaeppler et al., 2000) and agricultural practice (Gavito and Miller, 1998) and the strong growth response in the Oh43 genotype used here is in agreement with Sawers et al. (2017). Also the *R. irregularis* induced reduction in specific root length, is a well-known maize response when associating with AM fungi (Kothari et al., 1990; Hetrick, 1991; Hao et al., 2008), reducing root exudation (Jones et al., 2004) and resulting in more energy allocated to the symbiotic association.

Table 4
P values from two separate analysis of variance (ANOVA) of the factors yeast and tricalcium diphosphate (TCP) were performed for treatments without (−) and with (+) *Rhizophagus irregularis*, respectively for all variables, except root length†, which was performed as a three way ANOVA with the factors yeast, *R. irregularis* and TCP (n = 5).

Factors	Root length (m)	Shoot ^{33}P content (kBq plant^{-1})	Specific shoot ^{33}P uptake (kBq m^{-1} root)	Shoot SA ^{33}P/^{31}P (kBq ^{33}P/mg ^{31}P)
− *R.irregularis*	See foot			
Yeast	note	0.266	0.192	0.101
TCP		0.094	0.123	0.291
Yeast × TCP		0.455	0.393	0.072
+ *R.irregularis*				
Yeast		0.083	0.276	0.384
TCP		0.151	0.816	0.783
Yeast × TCP		0.479	0.519	0.608

*p = 0.05.
**p < 0.01.
***p < 0.001.
† Three way ANOVA for the variable root length: Y, 0.161; *R. irregularis* (*Ri*) ***; TCP, 0.105; Y × *Ri*, p = *; Y × TCP, p = *; *Ri* × TCP, p = **; Y × *Ri* × TCP, p = 0.789.

4.2. Effects of yeasts on root and soil colonization by R. irregularis

Inoculation with *C. railenensis* slightly increased root colonization with *R. irregularis*, but on the other hand *R. irregularis* soil colonization was unaffected by yeast inoculation. Other studies have shown strong effects of soil yeasts, reporting both increase (Fracchia et al., 2003; Scervino et al., 2008) and decrease (Gollner et al., 2006) in AM fungus root and soil colonization. Overall, this suggests complex interactions between AM fungi and rhizosphere yeasts most likely depending on environmental plant and soil conditions as well as fungal species.

4.3. AM hyphal P transport

Here for the first time we show that rhizosphere yeasts can improve AM hyphal P transport. However, in similar studies using P tracer isotopes, Larsen and Jakobsen (1996) and Ravnskov et al. (1999) also reported increased hyphal P uptake of *Glomus intraradices* (BEG87) after application of commercial bakers dry yeast containing inoculum of the yeast *Saccharomyces cerevisiae*. The underlying mechanisms responsible

Table 3
Treatment means of root length in root hyphal compartment (RHC), shoot content of ^{33}P, specific uptake from RHC and specific ^{33}P activity as affected by the factors yeast (without, *Cryptococcus flavus* and *Candida railenensis*), *Rhizophagus irregularis* (without (−) and with (+)) and tricalcium diphosphate (TCP) (without (−) and with (+)) (n = 5).

Yeast	*R.irregularis*	TCP	Root length (m)	Shoot ^{33}P content (kBq plant^{-1})	Specific shoot ^{33}P uptake (kBq m^{-1} root)	Shoot SA ^{33}P/^{31}P (kBq ^{33}P/mg ^{31}P)
Without	−	−	2.57	2.06	0.88	1.00
	+	−	4.58	22.85	4.92	2.96
	−	+	2.21	2.04	0.94	0.61
	+	+	5.61	23.31	4.06	2.81
Cr. flavus	−	−	1.12	0.66	0.81	0.29
	+	−	4.40	25.12	5.64	3.40
	−	+	1.68	2.78	2.03	1.36
	+	+	6.94	35.46	5.15	3.20
C. railenensis	−	−	2.57	2.29	0.90	0.87
	+	−	6.31	30.18	4.86	2.97
	−	+	1.47	4.01	3.49	1.27
	+	+	6.30	34.15	5.48	3.43

Table 5
Treatment means of AM fungi soil biomass, shoot content of ^{32}P, specific uptake from the hyphal compartment (HC) and specific ^{32}P activity (SA) from eight weeks old maize plants as affected by the factors yeast (without, *Cryptococcus flavus* and *Candida railenensis*) and tricalcium diphosphate (TCP) (without (−) and with (+)) (n = 5).

Yeast	TCP	AMF soil biomass (nmole 16:1ω5 g soil^{-1})	Shoot ^{32}P content (kBq plant^{-1})	Specific shoot ^{32}P uptake (kBq nmole^{-1} 16:1ω5)	Shoot SA $^{32}P/^{31}P$ (kBq ^{32}P/mg ^{31}P)
Without	–	21.55	19.97	1.79	2.62
	+	18.72	24.60	1.31	3.04
Cr. flavus	–	29.51	25.80	1.03	3.54
	+	23.49	37.24	1.84	3.34
C. railenensis	–	28.89	27.71	1.43	2.80
	+	16.28	30.18	2.03	3.02

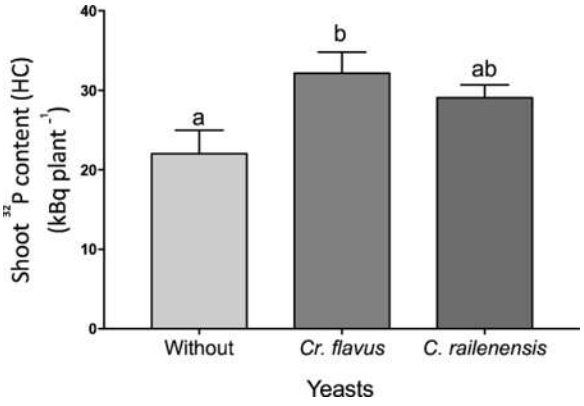

Fig. 6. Factor treatment means for shoot ^{32}P content of eight weeks old maize plants of the factor yeast (without, *Cyptococcus flavus* and *Candida railenensis*) for mycorrhizal plants only. Different letters indicate significant treatment effects and error bars represent standard error of the mean.

for the promotion of AM hyphal P uptake by *C. railenensis* and *Cr. flavus* in the present study seems not to be linked with improved AM fungal biomass and remains to be clarified.

The contribution of P uptake from mycelium of *R. irregularis* alone was in the same range as that of mycorrhizal roots. Pearson and Jakobsen (1993) reported similar results, suggesting a feed-back plant mechanism suppressing root uptake when sufficient P is available in the plant tissue. Supporting this explanation Smith et al. (2011) reported reduced expression patterns of the Pi transporters in the direct uptake pathway in AM mycorrhizal associations.

4.4. Yeast population density

Both *C. railenensis* and *Cr. flavus* have high content of the fungal biomarker fatty acid 18:2ω6,9 (data not shown), which was employed to measure the yeast population density. Similarly, Larsen et al. (1998) used 18:2ω6,9 to estimate population density of saprotrophic fungi. However, at harvest 8 weeks after seed inoculation and 5 weeks after inoculation in the RHC and HC mesh bags, no difference was found in the amounts of 18:2ω6,9 in treatments with and without yeasts. These findings indicate that populations of *C. railenensis* and *Cr. flavus* declined over the time course of the experiment. Another possibility could be that the fungus biomarker fatty acid method employed is too general and that the fungal background values were too large for discrimination of the yeast inoculants. However, the strong effects of yeast inoculation on maize plant performance and P uptake strongly indicates that the yeasts, at some point before harvest had been active in the soil environment. Nevertheless, in future studies yeast population density in soil should be examined with more specific methods like real time PCR.

4.5. P isotope dilution and effects of TCP on maize, R. irregularis and yeasts

No difference in specific activity ($^{32}P/^{31}P$ or $^{33}P/^{31}$) was observed after yeast inoculation to either of the RHC or HC mesh bags irrespective of TCP application. These findings indicate that *C. railenenis* and *Cr. flavus* most likely did not solubilize phosphate under the present experimental conditions. Similar results have been reported by Meyer et al. (2017) also employing the P isotope dilution method to examine possible P solubilization from calcium P rich sewage sludge by the plant growth promoting rhizobacterium (PGPR) *Pseudomonas protegens* (CHAO) in a plant assay with *Lollium multiflorum.* The PGPR *P. protegens* (CHAO) is known to solubilize P in *in-vitro* assay, but failed to do this under soil conditions (Meyer et al., 2017) as was the case for *C. railenensis* in the present study.

5. Conclusions

We conclude that promotion of maize plant growth and P nutrition by inoculation with the rhizosphere yeasts *C. railenensis* and *Cr. flavus* is linked to improved root growth and specific root length. Both yeasts also improved hyphal P uptake by *R. irregularis*, but this needs to be further addressed to explore possible mode of interaction.

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Table 6
P values from two way analyses of variance of the factors yeast and tricalcium diphosphate (TCP) and their interactions for the variables AMF soil biomass, shoot ^{32}P content, specific shoot ^{32}P uptake and shoot specific activity (SA) (n = 5).

Factors	AMF soil biomass (nmole 16:1ω5 g soil^{-1})	Shoot ^{32}P content (kBq plant^{-1})	Specific shoot ^{32}P uptake (kBq nmole^{-1} 16:1ω5)	Shoot SA $^{32}P/^{31}P$ (kBq ^{32}P/mg ^{31}P)
Yeast	0.539	*	0.833	0.054
TCP	0.139	*	0.439	0.491
Yeast × TCP	0.687	0.369	0.383	0.486

* p = 0.05.

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Capítulo 5:

Discusión general, conclusiones y perspectivas

5.1 DISCUSIÓN

Las levaduras de la rizósfera son un grupo de microorganismos que ha sido poco explorado (Botha 2011) en comparación con las bacterias y otros hongos. A pesar de ser abundantes y diversas en los agroecosistemas (Xu *et al.,* 2012), se conoce relativamente poco acerca de sus funciones y sus interacciones con otros microorganismos, como los HMA. En el presente proyecto se evaluó la abundancia de las levaduras rizosféricas de maíz, así como su importancia como facilitadoras de fósforo para la nutrición de este cultivo.

Abundancia de levaduras de la rizósfera de maíz

En este estudio se encontró que las levaduras son comunes y abundantes en la rizósfera durante todo el ciclo de crecimiento en los campos de maíz examinados, los cuales difieren en cuanto el sitio geográfico y las características del suelo, como se muestra en el capítulo 2. Los resultados encontrados coinciden con otros estudios sobre levaduras del suelo en campos agrícolas con diferentes cultivos (Xu *et al*., 2012, Sláviková y Vadkertiová 2003, Gomes *et al*., 2003).

La abundancia de levaduras fue mayor durante la etapa de floración, con respecto a las otras dos etapas fenológicas evaluadas, esto debido probablemente a que las condiciones ambientales fueron favorables para la nutrición de las poblaciones de levaduras, ya que la etapa de floración se llevó a cabo en la época de lluvias donde había gran humedad en el suelo, las raíces completamente desarrolladas aun proporcionaban algunos exudados y además comenzaban su fase de descomposición con algunas raíces muertas formando parte de la materia orgánica presente en el suelo. Sin embargo, el mecanismo que explica el pico en la abundancia de levaduras durante la etapa fenológica de floración encontrado en este estudio debe ser estudiado con más detalle.

Respecto a las características fisicoquímicas estudiadas, las levaduras respondieron solo al pH y Mg en donde se encontraron correlaciones negativas de forma colineal. De manera similar Vreulink *et al*. (2007) reportaron una correlacion negativa entre la abundancia de levaduras y el pH del suelo. Es conocido que las bacterias son menos abundantes en suelos con pH bajos (Rousk *et al*., 2010) lo cual pudo haber reducido la competencia entre levaduras y bacterias y explica por

qué las levaduras fueron más abundantes en suelo con bajo pH. Por otro lado, es muy probable que el efecto de Mg esté ligado al pH por la colinealidad encontrada, pero eso debe ser examinado con más detalle.

El número de especies de levaduras encontradas en el presente estudio (8 especies de 7 géneros) coincide con lo encontrado por Sláviková y Vadkertiová (2003) en donde aislaron 7 especies de levaduras de los campos de maíz en Eslovaquia. Xu *et al*. (2012) en un estudio de campo de chícharo en Dinamarca, exploró comunidades de hongos con un enfoque metagenómico no cultivable, encontrando 23 especies de levaduras de diez géneros. .

Debido a que en este estudio únicamente incluyó levaduras cultivables y un número limitado de muestras, lo más seguro es que la diversidad no se exploró en su totalidad. Sin embargo, para los fines de este estudio, trabajamos con aislados de levaduras con el objetivo de caracterizar sus funciones respecto a la solubilización de P y la promoción de crecimiento vegetal.

Las poblaciones de levaduras tuvieron diferencias en los dos sitios geográficos estudiados con respecto a su abundancia y composición de especies. *Candida railenensis* fue la única especie de las levaduras aisladas que fue encontrada en ambos sitios. El sitio geográfico de Guanajuato, con una producción intensiva en el cultivo de maíz, tuvo una menor abundancia, sin embargo este sitio tuvo la mayor riqueza de especies. Cabe señalar que el diseño experimental empleado no permite separar los efectos del sitio geográfico y la práctica agrícola.

Solubilización de P

En el capítulo 2 se muestra que de las ocho especies de levaduras aisladas cuatro solubilizaron $Ca_3(PO_4)_2$, lo cual respalda que la solubilización de fósforo es una de las características funcionales reportadas para las levaduras del suelo (Nakayan *et al*., 2013; Xiao *et al*., 2012; Hesham y Mohamed, 2011; Al-Falih, 2005). Nakayan *et al*., (2013) sugirieron que el potencial de las levaduras del suelo para solubilizar el fosfato puede variar de acuerdo al género y la especie, lo cual puede ser observado en el presente estudio.

Promoción de crecimiento vegetal

Algunos estudios han reportado que las levaduras mejoran el crecimiento de brotes y raíces en las plantas de maíz, relacionándolo con su capacidad para solubilizar P (Nakayan *et al*., 2013; Hesham y Mohamed, 2011). En nuestro estudio (capítulo 2), esta característica funcional parece no estar relacionada con el aumento en el crecimiento de las plantas inoculadas con *Cr. flavus* y *S. aerea* ,ya que estas dos especies de levadura no presentaron atributos solubilizantes de P. Por otro lado, *C. railanensis* y *Cl. lusitaneae,* levaduras que sí solubilizaron P, no promovieron el crecimiento de la planta; sin embargo, existe la posibilidad de que *C. railanensis* y *Cl. lusitaneae* pudieron haber solubilizado P en el suelo y que este haya sido consumido por ellos mismos o por otros microorganismos del suelo. En todo caso parece que el criterio de selección de microorganismos con capacidad de solubilizar P en caja Petri solo muestra un potencial de éstos, pero puede ser que esto no se vea reflejado al estar el microorganismo en el suelo interactuando con la planta y otros elementos del suelo, pues existen otros mecanismos que pueden tener para promover el crecimiento en las plantas (Bashan *et al*., 2013).

Se necesitan estudios más detallados que incluyan mediciones de los depósitos de P en el suelo para comprender mejor el posible papel de la solubilización de P en el suelo por las levaduras.

Los efectos de promoción de crecimiento en plantas inoculadas con levaduras rizosféricas dependieron de la fertilización con P mineral (Capítulo 2). Las plantas que crecieron en suelo sin P no se vieron afectadas con la inoculación de levaduras, mientras que los casos donde hubo una promoción de crecimiento de plantas con levaduras se obtuvieron en plantas que no estaban limitadas por P debido a la fertilización mineral.

Además de la característica de promoción del crecimiento de las levaduras de la rizósfera mostradas en el capítulo 2, se ha demostrado que éstas proporcionan otros atributos benéficos en la salud de las plantas y la fertilidad del suelo (Botha, 2011), por lo que es importante considerar su conservación y manejo en los agroecosistemas. La obtención de información adicional sobre cómo las levaduras se desempeñan en diferentes entornos agrícolas en relación con la labranza, la rotación de cultivos, la fertilización y el control de plagas ayudará a la

conservación y el manejo futuro de las levaduras rizosféricas beneficiosas para las plantas.

Interacciones entre levaduras rizosféricas de maíz y HMA

En el capítulo 3, se mostró que la fertilización mineral con P modificó fuertemente las interacciones entre el maíz, las levaduras de la rizósfera y los HMA. La primer hipótesis planteada en este trabajo de que la promoción del crecimiento con levaduras y HMA sería más fuerte sin la fertilización con P fue solo cierta para el caso de los HMA, mientras que la levadura *C. railenensis* opuestamente causó una reducción en el crecimiento de la planta. En consecuencia, también se rechazó la segunda hipótesis que habíamos planteado en esta fase de que la inoculación dual con levaduras y HMA daría como resultado un crecimiento mejorado en comparación con la de la inoculación individual.

La reducción del crecimiento observada después de la inoculación con *Cr. flavus* contrasta con otros estudios sobre la respuesta de las plantas a las levaduras del suelo, donde una promoción de crecimiento fue observada. Dado que la reducción del crecimiento no se observó en combinación con la fertilización P, puede ser que un modo probable de interacción sea la competencia entre la levadura y las raíces del maíz.

Por otro lado, el crecimiento observado en las plantas de maíz inoculadas con HMA en suelos con bajo contenido de P es bien conocida, aunque depende del genotipo del maíz (Sawers *et al*., 2017; Kaeppler *et al*., 2000). De igual manera, la supresión del crecimiento de las plantas mediante la inoculación con HMA en combinación con la fertilización mineral de P ha sido ampliamente discutida por Johnson y Graham (2013), sugiriendo una asociación parasitaria entre HMA y su hospedera cuando el costo del carbono para mantener la simbiosis supera el beneficio de la nutrición de P.

En el presente estudio, solo se examinaron niveles bajos y altos de P, por lo que sería importante tener más información respecto a cómo las plantas de maíz responden a los HMA en un gradiente de fertilización de P completo.

La fertilización con P redujo notablemente la colonización de HMA, que es una respuesta bien conocida de las asociaciones de HMA a la fertilización con P (Thomson *et al*., 1992; Liu *et al*., 2000; Gosling *et al*., 2013) (capítulo 3).

Estos hallazgos sugieren que en los agroecosistemas de maíz es importante desarrollar estrategias de nutrición de los cultivos para mejorar la eficiencia del uso de P, asegurando asociaciones de micorrizas de maíz mutualistas, especialmente en términos de reducción del aporte de fertilizantes minerales (Larsen *et al*., 2014).

En el capítulo 3 se muestra que la fertilización con P mineral también alteró claramente el resultado de las interacciones entre las levaduras y los HMA, donde la levadura *Cr. flavus* redujo la colonización de HMA en los tratamientos sin fertilización con P, lo cual pudo deberse a la competencia por el P. Por otro lado, *C. railenensis* aumentó la colonización de la raíz de HMA en combinación con la fertilización con P. El mecanismo subyacente para este resultado necesita ser más estudiado.

En el capítulo 4 se mostró que la inoculación doble con cualquiera de las levaduras de la rizósfera *C. railenensis* o *Cr. flavus* y el HMA *R. irregularis* mejoraron la nutrición de P en maíz. Hasta donde sabemos, este es el primer reporte que muestra que las levaduras de la rizósfera pueden mejorar la captación de P por parte de las hifas de los HMA. Los mecanismos no parecen estar relacionados con la solubilización de P por las levaduras. No obstante, los cambios en la longitud de la raíz específica del maíz parecen ser el mecanismo principal para los efectos observados de las levaduras en la nutrición de P en el maíz. Sin embargo, los mecanismos involucrados en la mejora de la absorción de P por las hifas de los HMA después de la inoculación de la levadura deben abordarse más a fondo.

Con respecto al crecimiento vegetal y la toma de P mostrado en el capítulo 4, se observó que las dos levaduras utilizadas (*C. railenensis* y *Cr. flavus*) aumentaron el peso seco aéreo, independientemente de la inoculación con *R. irregularis* y la aplicación de TCP, así como también mejoraron la longitud total de la raíz, pero solo para las plantas micorrizadas. Ambas especies de levadura mejoraron el contenido de P de la planta, pero no tuvieron efectos significativos sobre la absorción de ^{33}P por las raíces independientemente de la aplicación de TCP, lo que sugiere que ninguna de las levaduras solubilizaron TCP en el suelo. Las observaciones de que ambas levaduras aumentaron la absorción de P en el maíz

y que solo *C. railenensis* podría solubilizar P *in vitro,* sugiere que la absorción mejorada de P en el suelo no fue causada por la solubilización de TCP.

El efecto de incremento en la longitud de raíz específica de las plantas inoculadas con levaduras aumenta además el área de superficie de la raíz para la exploración del suelo P. La promoción del crecimiento de la planta por los HMA asociados al maíz es bien conocida, aunque depende del genotipo (Kaeppler *et al*., 2000) y las prácticas agrícolas (Gavito y Miller, 1998) así como de la respuesta de crecimiento del genotipo Oh43 utilizado en este experimento después de la inoculación con HMA, lo que coincide con Sawers *et al*. (2017). Por otro lado, la reducción inducida por *R. irregularis* en la longitud de raíz específica, es una respuesta de maíz bien conocida cuando se asocia con HMA (Kothari *et al*., 1990; Hetrick, 1991; Hao *et al*., 2008), reduciendo la exudación de la raíz (Jones *et al*. al., 2004), lo que resulta en más energía asignada a la asociación simbiótica.

La inoculación con *C. railenensis* aumentó ligeramente la colonización de la raíz con *R. irregularis*. Otros estudios han demostrado fuertes efectos de las levaduras del suelo, mostrando un aumento en la colonización de la raíces (Fracchia *et al*., 2003; Scervino *et al*., 2008)

En este trabajo, por primera vez, se demostró que las levaduras de la rizósfera pueden mejorar el transporte de P por medio de las hifas de los HMA (Capítulo 4). Sin embargo, en estudios similares con isótopos marcadores de P, Larsen y Jakobsen (1996) y Ravnskov *et al*. (1999) también informaron un aumento en la captación de P por las hifas de *Glomus intraradices* (BEG87) después de la aplicación de levadura seca comercial que contenía inóculo de la levadura *Saccharomyces cerevisiae*. Los mecanismos responsables para la promoción de la absorción de P en las hifas por las levaduras *C. railenensis* y *Cr. flavus* parece no estar relacionado con el mejoramiento en la biomasa de hongos HMA.

La contribución de la absorción de P únicamente por el micelio de *R. irregularis* estuvo en el mismo rango que el encontrado en raíces micorrizadas, resultados similares a los de Pearson y Jakobsen (1993), lo cual sugiere que existe un mecanismo de retroalimentación que suprime la absorción de raíz cuando hay suficiente P disponible en el tejido de la planta.

Con respecto a la densidad de levaduras, los fuertes efectos de su inoculación sobre el rendimiento de la planta de maíz y la absorción de P indican fuertemente

que las levaduras, en algún momento antes de la cosecha, habían estado activas en el ambiente del suelo. Sin embargo, es importante que en futuros estudios, la densidad de población de levaduras en el suelo sean examinadas con métodos más específicos, como la PCR en tiempo real.

En cuanto al método de dilución, no se observó diferencia en la actividad específica (^{32}P / ^{31}P o ^{33}P / ^{31}P) después de la inoculación con levadura en cualquiera de las bolsas de malla RHC o HC independientemente de la aplicación de TCP. Estos hallazgos indican que *C. railenenis* y *Cr. flavus* muy probablemente no solubilizaron el fosfato en las presentes condiciones experimentales. Resultados similares han sido reportados por Meyer *et al*. (2017), quienes también emplearon el método de dilución de isótopos P para examinar la posible solubilización de P a partir de lodos de aguas residuales ricos en calcio por la rizobacteria promotora del crecimiento vegetal (PGPR) *Pseudomonas protegens* (CHAO) en un ensayo de planta con *Lollium multiflorum*. Se sabe que *P. protegens* (CHAO) solubilizó P en el ensayo *in vitro*, pero no lo hizo en condiciones de suelo (Meyer *et al*., 2017) como fue el caso de *C. railenensis* en el presente estudio (capítulo 4).

En general, las interacciones entre las levaduras y los HMA parecen ser complejas dependiendo de los genotipos de hongos y de las condiciones ambientales de la planta y el suelo, lo cual debe ser considerado para optimizar el uso conjunto de ambos microorganismos en beneficio del crecimiento de las plantas.

5.2 CONCLUSIONES Y PERSPECTIVAS

Los resultados de la presente tesis muestran que las levaduras son habitantes asiduos en la rizósfera del maíz durante todo el ciclo de crecimiento de este cultivo en agroecosistemas, y que la abundancia y estructura de su comunidad dominante en la rizósfera depende, entre otras cosas, de las características fisicoquímicas del suelo. Además, las levaduras rizosféricas cuentan con importantes atributos funcionales relacionados con la adquisición de P y promoción de crecimiento del maíz.

Por otra parte, la fertilización con P mineral modula fuertemente las interacciones entre el maíz, las levaduras rizosféricas y los HMA, lo cual debería considerarse para futuras estrategias orientadas a mejorar la eficiencia del uso de P en los agroecosistemas de maíz.

La inoculación de las levaduras rizosféricas *C. railenensis* y *Cr. flavus* favorecieron el crecimiento de raíz y la longitud de raíz específica, además de mejorar la captación de P de las hifas de *R. irregularis*, pero esto necesita ser más estudiado en futuras investigaciones para explorar el posible modo de interacción.

Un análisis con un enfoque metagenómico es recomendable para conocer más acerca de la funcionalidad y diversidad de levaduras en los agroecosistemas de maíz, con lo cual, en la medida que se obtengan y se estudien más especies, será posible descubrir más tipos de interacciones y así aclarar aún más el papel de las levaduras en la rizósfera.

Teniendo en cuenta el efecto potencial de las levaduras de la rizósfera de maíz sobre la promoción de crecimiento de este cultivo, es importante realizar mas estudios que integren la información obtenida en esta investigación en sistemas agrícolas con experimentos de campo, lo cuál ayudaría además a entender de que manera las prácticas agrícolas afectan a las levaduras, y por otro lado generar estrategias para promover y conservar a estos hongos.

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