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EL MANEJO DEL SUELO PARA CONSERVAR LAS REDES DE MICELIO DE LOS HONGOS MICORRÍZICOS ARBUSCULARES Y SU RELACIÓN CON EL DESARROLLO DEL MAÍZ.

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Resumen

El uso intensivo de prácticas agrícolas ha resultado en efectos perjudiciales en el ambiente en general, por lo que los sistemas de cultivos sostenibles representan una alternativa a la agricultura convencional. Sin embargo, se requiere de una comprensión más profunda de las interacciones biológicas dentro de los agroecosistemas. Se realizaron experimentos de campo para evaluar la influencia de las prácticas agrícolas de fertilización, labranza y la diversificación de cultivos en el funcionamiento de la simbiosis establecida entre el maíz y las comunidades nativas de hongos micorrízicos arbusculares (HMA) en etapas tempranas de desarrollo, la competencia/facilitación con el frijol y su efecto sobre el rendimiento del cultivo. Se plantearon dos hipótesis: 1) Las prácticas que permitan mantener la integridad funcional de la red de micelio de los HMA desde el comienzo del ciclo con la reducción en la intensidad de las prácticas agrícolas, aumentará la eficiencia simbiótica de los HMA, lo que resultará en un balance entre la asimilación de C, mayor adquisición temprana de nutrientes y biomasa, y finalmente, en mayor rendimiento y calidad nutricional del grano, y 2) el tiempo de siembra del frijol afectará la competencia/facilitación temprana mediada por la red miceliar de los HMA en el cultivo intercalado maíz-frijol, por lo que se espera que la asignación de nutrientes sea mayor para la planta más desarrollada que se conecta a la red miceliar.

Para probar estas hipótesis se establecieron dos experimentos en parcelas agrícolas en el estado de Michoacán. En el primer experimento se combinaron tratamientos de labranza del suelo, fertilización y fungicida para modular el desarrollo y el funcionamiento temprano de los HMA y así evaluar las relaciones tempranas entre los HMA nativos y los parámetros de intercambio de gases, nutrición, crecimiento y rendimiento del maíz. Para el segundo experimento se utilizó la labranza para retrasar el desarrollo y el funcionamiento temprano de los HMA y se estableció un cultivo intercalado maíz-frijol en franjas, en donde el frijol se sembró en tres momentos diferentes (siembra temprana, simultánea y tardía) en relación con la siembra del maíz y se evaluaron las respuestas del crecimiento, nutrición y rendimiento de grano durante el desarrollo de ambos cultivos.

Los resultados obtenidos en el primer experimento mostraron que las relaciones funcionales predichas, que esperaban que una mayor colonización micorrízica e integridad de la red de micelio en el suelo resultaría en el aumento de la captación de N y P, el intercambio de gases, la biomasa, el rendimiento y la calidad del grano, solo se apoyaron al inicio, en la etapa de desarrollo V3

(tercera hoja desarrollada con collar visible). Estas relaciones se invirtieron en etapas posteriores cuando aumentó la colonización de raíces por HMA y otros hongos, especialmente en los tratamientos sin fungicida. La colonización por HMA y otros hongos se relacionó negativamente con el nitrógeno (N), y en menor proporción también con el P, de las plántulas y tuvo efectos menores durante el crecimiento vegetativo, pero disminuyó el rendimiento y el contenido de N del grano en un 10%. La fertilización mineral (118 (N), 95 (P), 47 (K), 5 (Ca), 8 (Zn), 4 (Fe), 3 (B), 1 (Cu)) compensó parcialmente el periodo de interacción negativa, pero el uso del fungicida evidenció la presencia de biota perjudicial para las plantas, aunque no se pudo separar si la mejoría nutricional se debió a la inhibición de los HMA, los otros hongos registrados, o ambos. Este primer experimento reveló la interacción temprana entre la disponibilidad de nutrientes, los HMA, otros hongos de las raíces y la nutrición del maíz, la cual provocó una interrupción en la relación funcional esperada entre los HMA y el maíz. Esa disrupción redujo el rendimiento sin causar síntomas de enfermedades o depresión del crecimiento, por lo que se concluye que el manejo intensivo convencional histórico parece estar promoviendo a largo plazo el mantenimiento de biota perjudicial en las raíces, en detrimento de la nutrición y el rendimiento del maíz.

En el segundo experimento, la labranza redujo el desarrollo temprano de los HMA en las raíces y el suelo en maíz y frijol, pero aumentó el desarrollo temprano del maíz por su sensibilidad a la compactación del suelo del grupo Vertisol, la cual no se observó en el frijol. El tiempo de siembra fue más importante para el frijol que para el maíz, pero en ambos cultivos el mejor tiempo fue cuando se sembró primero el frijol. En el caso del frijol, la labranza reducida que preservó la red de micelio de los HMA mejoró la nutrición, aumentó la fijación de N en el frijol, la transferencia de N fijado hacia el maíz, el desarrollo y el rendimiento, sobre todo al sembrarlo antes o de forma simultánea al maíz. El maíz se desarrolló mejor con labranza y los tiempos de siembra no fueron relevantes. El frijol, pero no el maíz, tuvo una ventaja competitiva en el cultivo intercalado de maíz y frijol si se conservaban las redes.

Palabras clave: micorriza, labranza, nitrógeno, fósforo, intercambio de gases

Abstract

The intensive use of agricultural practices has resulted in detrimental effects on the environment in general, so that sustainable cropping systems represent an alternative to conventional agriculture. However, a deeper understanding of the biological interactions within agroecosystems is required. Field experiments were carried out to evaluate the influence of agricultural practices of fertilization, tillage and crop diversification on the functioning of the symbiosis established between maize and native communities of arbuscular mycorrhizal (AM) fungi at early stages of development, competition/facilitation with bean plants and their effect on crop yield. Two hypotheses were defined: 1) Practices that maintain the functional integrity of the mycorrhizal network from the beginning of the cycle with the reduction in the intensity of the practices will increase the symbiotic efficiency, which would result in a balance between the assimilation of C, a higher early acquisition of nutrients, biomass and finally in higher yield and nutritional quality of the grain. 2) bean sowing time will affect the early competition/facilitation mediated by the mycorrhizal mycelial network in maize-bean intercropping, so it is expected that the allocation of nutrients will be higher for the more developed plant connecting to an AM mycelium network.

Two experiments were established in agricultural plots in the state of Michoacán to test these hypotheses. In the first experiment, soil tillage, fertilization, and fungicide treatments were combined to modulate the development and early functioning of AM fungi and evaluate early relationships between native AM fungi and gas exchange, nutrition, growth, and yield parameters of maize. For the second experiment, tillage was used to create a delay in the development and early functioning of the AM fungi and a maize-bean intercropping was established where the beans were sown at three different times (early, simultaneous and late sowing) in relation to the sowing of maize and growth, nutrition and grain yield responses were evaluated during the development of both crops.

Results obtained in the first experiment showed that the predicted functional relationships expecting that increased root colonization and soil mycorrhizal network integrity would increase N and P uptake, gas exchange, biomass, yield and grain quality, were supported only at the beginning, at stage V3 (third leaf fully emerged and visible collar). These relationships turned out the opposite at later stages when the colonization of roots by AM fungi and other fungi increased, especially in the treatments without fungicide. Colonization by AM fungi and other fungi was negatively related to nitrogen, and to a lower extent also phosphorus, concentration in the seedlings.

This N reduction had minor effects during vegetative growth but decreased the yield and grain N by 10%. Fertilization partially compensated the period of negative interaction but the improved nutrition with the application of fungicide revealed the presence of harmful biota, including AM fungi, although it was not possible to separate if the improved nutrition was due to the inhibition of AM fungi, other registered root fungi, or both. The early interaction between nutrient availability, AM fungi, other root fungi, and maize nutrition that caused a disruption in the hypothetical functional relationship between AM fungi and maize reduced yield without causing symptoms of diseases or growth depression. It is concluded that the historical conventional intensive management seems to be promoting in the long term the maintenance of the root biota detrimental to maize nutrition and yield.

In the second experiment, tillage reduced early development of AM fungi in the roots and in the soil in maize and bean, but increased the development and yield of maize because of its sensitivity to the soil compaction of the Vertisol group, an effect that was not observed in bean. Sowing time was more important for bean than for maize, but for both crops the best time was when bean was sown first. In bean, reduced tillage preserving mycorrhizal networks improved early nutrition, biomass, N fixation in bean, transfer of fixed N to maize, and yield, especially when sown before or simultaneously with maize. Maize development and yield, in turn, were better with tillage and sowing time was not relevant. Bean, but not maize, showed a competitive advantage by preserving the AM fungal network in the maize-bean intercrop.

Keywords: mycorrhiza, tillage, nitrogen, phosphorus, gas exchange

Capítulo I. Introducción general

1. Introducción general

Los hongos micorrízicos, pertenecientes al phylum Glomeromycota, establecen asociaciones simbióticas con el 70-90% de las familias de plantas terrestres, por lo que la asociación con micorrizas es la condición normal para la mayoría de las especies vegetales, incluyendo muchas especies de importancia agrícola (Hart y Reader, 2002; Mohammadi, 2011; Valentine et al., 2013, Hoysted et al. 2018). La asociación micorrízica se caracteriza por un intercambio bilateral de nutrientes, donde el hongo recibe carbono (C) fijado fotosintéticamente de la planta hospedera y le puede proporcionar a la planta nutrientes minerales, en particular fósforo (P), y agua, que son absorbidos de la solución del suelo y translocados rápidamente por el micelio extraradical (Mikkelsen et al., 2008; Gianinazzi et al., 2010). En este sentido, estos hongos llamados micorrízicos arbusculares (HMA), tienen una gran importancia tanto en los contextos agrícolas como en los ecológicos.

Los sistemas agrícolas, sin embargo, son particularmente alterados por las diferentes prácticas que se usan para facilitar el trabajo y promover el desarrollo del cultivo, pero que también pueden afectar a las comunidades de HMA y, en consecuencia, afectar los beneficios que les confieren a las plantas. Existen prácticas de uso común dentro de los sistemas agrícolas, como es el caso de la labranza, el monocultivo y la fertilización, que pueden tener efectos negativos en los HMA al inhibir su desarrollo y funcionamiento e incluso que pueden llegar a promover la proliferación de especies de HMA oportunistas que usan mucho carbono de las plantas sin transferirles nutrientes o agua (Johnson et al., 2015). Sin embargo, la crisis ambiental y la urgencia de generar sistemas agrícolas más sustentables promueven la transición hacia modelos de producción donde se busca aprovechar al máximo los mecanismos naturales de mantenimiento de la fertilidad en el suelo, dentro de los que se encuentran las asociaciones micorrízicas (Rillig et al., 2019). Por lo tanto, resulta primordial entender cómo se pueden maximizar los beneficios de las asociaciones micorrízicas en los sistemas productivos considerando el contexto actual mexicano donde todavía predomina el esquema convencional y de lo cual, aún falta mucho conocimiento para diseñar prácticas alternativas exitosas. Hasta ahora, los trabajos publicados en México se centran en inoculaciones de productos comerciales con un aislamiento de HMA, o una mezcla de ellos (Alarcón et al., 2012; Pellegrino et al., 2022), en los que se asume que las comunidades nativas no

son eficientes o que se puede mejorar la interacción con cepas de gran capacidad de promoción del crecimiento. Esto coincide con el resto del mundo, donde las prácticas de agricultura convencional ignoran cómo funcionan las interacciones biológicas y buscan reemplazar sus efectos de promoción con altas cantidades de fertilizantes. Existe un gran desconocimiento de la capacidad de promoción del crecimiento de las comunidades nativas de HMA y de las prácticas que la preservan o que pueden disminuirla

Muchos microorganismos benéficos del suelo son sensibles a las perturbaciones mecánicas asociadas a la labranza convencional, incluyendo los HMA (Brito et al., 2012; Mathew et al., 2012). Por ejemplo, la perturbación del suelo es un factor que, a través de la remoción de las capas superficiales en el perfil del suelo, puede alterar considerablemente el micelio extraradical de los HMA (Jasper et al., 1989; Evans y Miller, 1990). Las hifas extraradicales de los HMA pueden extenderse varios centímetros desde la superficie de las raíces de las plantas hasta la matriz del suelo, creando una red de hifas extraradicales (ERH) que puede colonizar otras plántulas, explorar el suelo y trasladar nutrientes a la planta (Miller et al., 1995; Merrild et al., 2013; Gottshall et al., 2017). Sin embargo, esta red hifal es muy susceptible a los movimientos de suelo y la remoción de capas que van asociadas al arado. En diferentes estudios, en algunas ocasiones se redujo la colonización por HMA debido a la alteración de la estructura del suelo, pero en todas las ocasiones se redujo la absorción de P (Evans y Miller, 1990; Fairchild y Miller, 1988, 1990; McGonigle et al., 1990a). Lo anterior se ha tomado como evidencia de que la alteración del micelio extraradical puede reducir la absorción de P por una planta en desarrollo (Miller et al., 1995).

Esto puede tener importantes repercusiones ya que, al destruirse la red de hifas con cada ciclo de cultivo, los HMA utilizarían el C vegetal para restablecer la red de micelio micorrízico y habría una capacidad de captación de P baja hasta que la red de exploración se restituyera. Las plantas invertirían entonces más C en el mantenimiento de los HMA con baja compensación en forma de nutrientes o agua durante el restablecimiento de la red miceliar, por lo que el crecimiento vegetal podría reducirse temporalmente. Por lo tanto, el costo en términos de C para mantener a los HMA que aún van a construir la red de micelio en una etapa inicial de establecimiento podría afectar el crecimiento de la planta en comparación a la conexión rápida a una red de micelio sin alteración en suelos sin labranza (Van der Heijden y Horton, 2009; Olsson et al., 2010; Gavito et al., 2019).

Esto es un factor clave debido a la importancia de la nutrición temprana en las plantas, ya que varios estudios han mostrado que el suministro temprano de P a la planta es crítico para el rendimiento óptimo de algunos cultivos (Grant et al., 2001). Particularmente, el rendimiento del maíz es altamente sensible a la absorción temprana de P (Barry y Miller, 1989; Miller, 2000a), y a su vez la adquisición temprana de P se ha relacionado al desarrollo del micelio intraradical y la integridad del micelio extraradical de los HMA, lo que implica una conexión rápida a una red de micelio micorrízico (Evans y Miller, 1988, Miller et al., 1995; Gavito y Miller, 1998). Existen, no obstante, algunos reportes (McGonigle y Miller, 1993; McGonigle y Miller, 1996; Galvez et al., 2001; Gavito y Miller, 1998) donde observaron beneficios en la nutrición de P en plántulas pero que no se tradujo en un mayor rendimiento en etapa de cosecha. Sin embargo, estos reportes coinciden en que es probable que algunos otros factores, como la temperatura, humedad, el tipo y la textura del suelo, contenido de nutrientes y materia orgánica del suelo, esquemas de manejo, el tiempo de exposición al manejo sin labranza, (Rhoton, 2000; Kabir 2005), ajenos al desempeño de los HMA, impidan ver sus beneficios ya en la cosecha. Por lo tanto, su evaluación es importante a escala local en las condiciones del campo mexicano y, además, evaluando etapas intermedias del desarrollo de las plantas hasta ahora desconocidas por enforcarse en los rendimientos finales. Dado que los componentes del rendimiento (número y peso de granos) quedan definidos en determinadas fases del desarrollo, un manejo adecuado para el logro de elevados rendimientos dependerá del conocimiento riguroso de cada una de ellas y de los factores ambientales que las afectan (Ortas, 2008).

Existe además una evidencia creciente de que el uso del C por parte de los HMA puede compensarse con un incremento en la tasa de intercambio de gases por las plantas durante la fotosíntesis, ya que ésta es a menudo mayor en plantas micorrizadas que en plantas no-micorrizadas (Valentine et al., 2001; Amaya-Carpio et al., 2009; Zhu et al., 2012). Si bien existe la compensación del uso del C por efecto de la colonización de los HMA, la perturbación del micelio de los HMA causada por las prácticas agrícolas como la labranza convencional en el suelo podría exacerbar la demanda de C por parte de los HMA al tener que reconstruir la red miceliar en cada ciclo de cultivo. Podría crearse una salida de C fotosintético que es excesiva para ciertos cultivos y es poco probable que en todos los casos esté totalmente compensada por la estimulación de la fotosíntesis (Gavito et al., 2019). En esos casos, la salida excesiva de C fotosintético para reconstruir la red de micelio, sin que los HMA pudieran retornar a cambio, nutrientes o agua a su hospedero, podría llevar a una

depresión temporal del crecimiento de la planta hasta que se restableciera nuevamente la red de exploración del suelo. Por ello es importante evaluar el uso que hacen los HMA del C fotosintético con relación a la adquisición de nutrientes en los diferentes cultivos, particularmente la evaluación del efecto de la alteración del micelio de los HMA, causada por la labranza, sobre las tasas fotosintéticas, la nutrición y el crecimiento del maíz; el cual es un cereal de gran importancia en el mundo y principalmente en México.

La conversión a los sistemas de manejo agrícola de cultivos sin labranza o con labranza reducida podría reducir los efectos negativos del manejo convencional para los HMA. Sin embargo, hasta la fecha no hay estudios de campo suficientes que hayan evaluado experimentalmente la relación entre el desarrollo de los HMA y la nutrición y el desempeño fotosintético del maíz. La literatura se concentra en los efectos de la labranza en la nutrición y el crecimiento del maíz sin ahondar en el balance del intercambio de carbono y nutrientes.

Los agricultores que utilizan la técnica de labranza de conservación a menudo también siembran cultivos intercalados o en rotación además del cultivo principal, para maximizar la utilización del suelo y de la mano de obra, disminuir el riesgo de pérdida por plagas y enfermedades, y proporcionar beneficios a las especies cultivadas (Enyi, 1973; Macheru-Muna et al., 2010). Comúnmente se cultivan cereales con leguminosas de grano, las cuales aportan elementos nutritivos a los suelos (Macheru-Muna et al., 2010). Cuando los cultivos son complementarios, como en la inclusión de leguminosas apropiadas en los sistemas de cultivo basados en cereales, se permite una utilización más eficiente de los recursos disponibles en el suelo y se proporcionan beneficios a las especies cultivadas, lo que puede resultar en un rendimiento mayor en los cultivos, respecto a cuando se cultivan por separado (Willey, 1979; Macheru-Muna et al., 2010).

Estas diferentes y múltiples plantas hospederas pueden conectarse a través de la red miceliar de los HMA en el suelo y la contribución de los socios que conforman esta relación puede variar en los beneficios que brindan (Weremijewicz et al., 2016; Hart et al., 2012). La asimetría de tamaño y edad entre las plantas hospederas puede ser un factor importante; por ejemplo, la transferencia de P puede ser mayor a plantas grandes que proporcionan la mayor cantidad de C, y suprimir el crecimiento de los individuos más pequeños por una deficiencia de P (Weiner, 1990). Dado lo anterior, evaluar el posible efecto de sembrar las plantas en diferentes tiempos sobre el transporte

mediado por la red de HMA a ambas especies de plantas en el cultivo intercalado puede permitir visualizar estrategias de manejo de esta técnica dentro de un esquema de reducción de labranza para maximizar el establecimiento y crecimiento de las plantas.

1.1 Hongos micorrízicos arbusculares (HMA)

Los HMA surgieron hace 350-460 millones de años (Simon et al., 1993) y aún conservan su morfología hasta la actualidad, probablemente como consecuencia de su estilo de vida simbiótico (Giovannetti y Gianinazzi-Pearson, 1994). En México se han registrado 160 especies de HMA, distribuidas en 34 géneros, 13 familias y cinco órdenes en Glomeromycota (Montaño et al., 2012; Chimal-Sánchez et al., 2015; Polo-Marcial et al., 2021). Estos hongos son simbiontes obligados de plantas y no pueden cultivarse en ausencia de una planta hospedero, ya que en ausencia de ésta su crecimiento y desarrollo es muy limitado; en cambio, la presencia de la raíz permite una colonización de entre el 60-90% del sistema radical bajo condiciones favorables (Bonfante y Perotto, 1995; Smith y Read 1997; Logi et al. 1998). La colonización de raíces por los HMA puede darse a partir de esporas, hifas y fragmentos de raíces colonizadas, que representan las principales fuentes de inóculo o propágulos (Smith y Read 1997). El proceso de colonización se inicia a partir de una hifa de penetración originada a partir de los propágulos que se encuentran en el suelo y activan su crecimiento bajo condiciones adecuadas de humedad, temperatura, o señales químicas favorables. Al entrar en contacto con la planta, las hifas del micelio primario forman una hifa especializada llamada apresorio o hifopodio, que le sirve de sostén en la fase primaria de penetración a las células corticales de la raíz (Bonfante y Perotto, 1995; Fernández, 2003).

La colonización fúngica ocurre de manera continua en dos sentidos, hacia el interior y exterior de la raíz. Dentro de la raíz, la hifa terminal se diferencia en arbúsculos dicotómicamente ramificados dentro de ciertas células corticales, estos son los sitios de intercambio de nutrientes durante la simbiosis (Smith y Smith 1996; Fernández, 2003). Una vez que las hifas de HMA han penetrado las raíces, comienza a desarrollarse el micelio extrarradical en el suelo, explorando el suelo hasta varios centímetros desde las raíces del hospedero, lo cual es importante para la adquisición y translocación de nutrientes del suelo a sus plantas hospedadoras, la colonización de nuevas plantas y la producción de esporas (Smith y Read 1997; Jakobsen et al. 1992; van der Heijden et al. 2015).

Las hifas y los arbúsculos se consideran el estado activo de la HMA, responsable del intercambio de nutrientes entre la planta y el hongo (Smith y Smith 1996). El micelio extraradical a menudo se encuentra asociado a esporas asexuales que se forman para la supervivencia en condiciones ambientales adversas (Smith y Read, 1997; Bentivenga et al. 1997, Sanders, 1999; Staddon y Fitter, 2001).

1.1.1 Importancia de los HMA

Los HMA ubicuos en la naturaleza se consideran importantes no sólo para la nutrición y productividad de las plantas, sino también como componentes de los ciclos de nutrientes de los ecosistemas (Smith y Read, 2008). Este grupo de hongos tienen una importante función en las relaciones suelo-planta en el contexto de los ciclos de nutrientes, especialmente para el fósforo (P), cobre y zinc (relativamente inmóviles) y nitrógeno (N) (Smith y Read, 2008; Mohammadi, 2011). Además, entre sus efectos benéficos también se encuentran el mejoramiento de la agregación del suelo (Rillig y Mummey, 2006; Wilson et al., 2009), la retención de nutrientes (Cavagnaro et al., 2015), el incremento de la resistencia de las plantas al estrés hídrico (Augé et al., 2015), a la presencia de patógenos (van der Heijden et al., 1998) y a condiciones de toxicidad y acidez del suelo (Smith y Read, 2008; Mohammadi, 2011); además favorecen la diversidad florística y la productividad de los ecosistemas (Van der Heijden et al., 2008). Las asociaciones de las plantas con los HMA son de gran importancia y sin ellas muchas plantas no sobrevivirían, particularmente aquellas que se encuentran en suelos limitados en nutrientes.

1.1.2 HMA y nutrición vegetal

La asociación micorrízica se caracteriza por un intercambio bilateral de nutrientes, donde el hongo puede recibir de la planta hospedera compuestos de carbono (C) reducidos en forma de carbohidratos simples y/o ácidos grasos, y a cambio puede proporcionar a la planta nutrientes minerales como P, N, Zn y Cu, y agua, que son absorbidos de la solución del suelo y translocados rápidamente por el micelio extraradical (Mikkelsen et al., 2008; Gianinazzi et al., 2010; Řezáčová et al., 2017). Cuando las raíces se asocian con HMA, el micelio extraradical de estos hongos

permite una mayor exploración del suelo y, por tanto, mayor adquisición de nutrientes que de otra forma serían inaccesibles y que se transfieren rápidamente a las células corticales dentro de la raíz, resultando en una mejor nutrición y crecimiento de las plantas (Smith y Read, 2008; Smith y Smith, 2011).

Los hongos micorrízicos son responsables de una más eficiente absorción de nitrógeno (N) y fósforo (P) que suple las necesidades de las plantas en los ecosistemas naturales (Hobbie y Hobbie 2006; van der Heijden et al. 2006). Los HMA pueden incrementar sustancialmente la absorción del P presente en los suelos, al aumentar la superficie de absorción y el área de exploración de las raíces en el suelo y otros mecanismos, por lo que hasta el 70% del P total absorbido por las plantas micorrizadas podría obtenerse a través de los HMA (Li et al., 1991; Smith y Read, 2008). Aunque la transferencia de P desde el suelo a la planta hospedera se considera el principal beneficio de la simbiosis micorrízica arbuscular hacia la planta, la absorción de N por las plantas a través del micelio de HMA también se ha demostrado (Bücking y Kafle, 2015).

Los HMA son capaces de adquirir tanto NO_3^- como NH_4^+, y transferirlos a sus plantas hospederas, por lo que pueden incrementar sustancialmente la adquisición de N mineral del suelo por las plantas (Smith y Read, 2008; Hodge y Storer, 2015). Así mismo, al inmovilizar el N inorgánico en su micelio o al transferirlo a la planta, potencialmente limitan la desnitrificación y la lixiviación del N del suelo (Veresoglou et al., 2012). Además, se ha sugerido además que los HMA pueden favorecer la liberación del N de la materia orgánica (Hodge et al., 2001; Hodge y Storer, 2015), probablemente debido a la estimulación de la actividad microbiana a través de la deposición al suelo de sustancias orgánicas compuestas principalmente por C provenientes del micelio o de las raíces de la planta mejor nutrida (Marschner et al., 2001; Veresoglou et al., 2012; Hestrin et al., 2019). En este mismo sentido, se ha observado también mayor fijación simbiótica de N por las bacterias fijadoras en las plantas asociadas con estas bacterias en presencia de HMA, debido a la mejora en la nutrición (Linderman, 1992; Kaschuk et al., 2009). Así, la adquisición de N mediada por hongos micorrízicos arbusculares es particularmente importante cuando el N del suelo se encuentra limitado (Hodge y Storer, 2015; Bukovská et al., 2018).

Los HMA suministran nutrientes minerales a la planta hospedera y obtienen a cambio compuestos de C reducidos derivados de la fotosíntesis de las plantas. Se estima que la asignación de C por

parte de las plantas a los HMA asociados oscila entre el 4% y el 20% del total asimilado fotosintéticamente (Jakobsen y Rosendahl, 1990; Graham, 2000). En este sentido, tienen una gran importancia desde los aspectos agrícolas y ecológicos, por lo que la explotación de estas asociaciones en entornos naturales y agronómicos se considera de alto valor ambiental y económico (Bonfante y Anca, 2009).

1.2 Redes micorrízicas comunes a varias plantas (Common Mycorrhizal Networks, CMN)

La gran mayoría de los HMA no son específicos de una planta hospedera, ya que una sola especie de HMA pueden conectar varias plantas de la misma o diferentes especies por redes de hifas fúngicas, estableciendo una red micorrízica común (CMN) en la que se pueden intercambiar nutrientes y compuestos de señalización entre las plantas conectadas (Newman, 1988; Weremijewicz et al., 2016; Figueiredo et al., 2021). Esto ocurre porque las hifas extraradicales de los HMA pueden extenderse en el suelo a grandes distancias entre una planta y otra; además se ha demostrado que los micelios individuales de HMA de la misma especie pueden fusionarse (mediante anastomosis), lo que resulta en la formación de grandes CMNs entre diferentes plantas (Jansa et al., 2003a; Giovannetti et al., 2004).

Un aspecto primordial de las CMNs es la transferencia de nutrientes entre plantas; razón por la que se prevé un ciclo de nutrientes más eficaz cuando las plantas se unen en una red micorrízica compartida por la extensión de exploración del suelo y porque los nutrientes de las raíces muertas serían absorbidos rápidamente por la red de micelio común (Mikkelsen et al., 2008). Además de su importante papel en la transferencia de nutrientes, las CMNs también pueden mediar interacciones entre las plantas, ya que en una CMN dos plantas pueden proporcionar C de manera desigual a un hongo compartido y/o adquirir nutrientes de manera desigual de un hongo que ambas sustentan con C, lo que puede implicar un beneficio para una especie y un perjuicio para la otra (Selosse et al., 2006).

La forma en que las CMN afectan las interacciones de las plantas puede depender de factores como las especies de hospederos y hongos involucrados, la fertilidad del suelo y la edad de las plantas (van der Heijden, 2004; Umbanhowar y McCann, 2005; Weremijewicz et al., 2016). Si los recursos

se pueden trasladar a través de conexiones miceliales en cantidades significativas que podrían afectar la aptitud de las plantas, las redes micorrízicas tienen entonces el potencial de influir en los patrones de establecimiento de plántulas, en la competencia entre plantas, la diversidad de plantas y la dinámica de comunidades de plantas, por lo que es probable que tales retroalimentaciones puedan dar forma a la estructura de la comunidad vegetal a escala local (Simard y Durall, 2004; Selosse et al., 2006; Figueiredo et al., 2021).

1.3 Importancia de los HMA en el contexto de los ecosistemas agrícolas y los naturales

Las plantas silvestres generalmente dependen más de los HMA en comparación de las plantas cultivadas, por lo que los resultados simbióticos pueden ser más pronunciados (positivos o negativos) para la planta (Hetrick et al., 1993; Xing et al., 2012). Así mismo, los cultivos semiperennes y perennes de mayor duración en general tienen una mayor dependencia y respuesta a los HMA (Boerner, 1992; Wilson y Hartnett, 1998); además, estos cultivos y la vegetación natural están asociados a una menor alteración mecánica del suelo, ya que se replantan en períodos más largos, y tienen menor requerimiento de insumos agrícolas (Rooney et al., 2009), por lo que es probable que promuevan el funcionamiento de los HMA y el mantenimiento de las redes de micelio en el suelo. Esto, aunado al mayor rango de respuesta y dependencia, podría permitirles beneficiarse de las asociaciones con HMA, lo que a su vez podría promover la mayor producción de biomasa en comparación con los sistemas convencionales de cultivos anuales.

De esta manera la preservación de las redes de micelio en sitios no manejados o de manera menos intensiva puede contribuir al reciclaje de nutrientes, sin necesidad de mayores aportes de nutrientes, y representa un potencial significativo para el secuestro de C en el suelo al no alterarse el suelo en períodos largos (Gottshall et al., 2017; Fall et al., 2022). Los cultivos anuales como ya se mencionó, generalmente se encuentran bajo un manejo más intensivo y constante que puede afectar a los HMA, por lo que la preservación del micelio contribuye a un enfoque de agricultura más sustentable que reduzca el uso de insumos agrícolas. Ambientalmente, el estudio y preservación del micelio micorrízico en ambos escenarios presenta un potencial significativo para el secuestro de C en el suelo, mejoramiento de la calidad del suelo, ya que hay un mayor almacenamiento de nutrientes y agregación y estabilidad del suelo, y un decremento en las emisiones de gases de efecto

invernadero y lixiviación de nutrientes (Solaiman, 2014; Fall et al., 2022).

Debido a las diferencias de ciclo de vida, fisiología y manejo, es probable que existan diferencias en la composición de la comunidad de HMA y otros microorganismos debido a la promoción de grupos específicos, por lo que si se incorporan cultivos diferentes es posible que diferentes especies de HMA o microorganismos asociados con una planta dada puedan ofrecer complementariedad funcional, lo que puede resultar beneficioso para la planta. Por ejemplo, es probable que una comunidad de plantas más diversa proporcione una gama más amplia de entornos radiculares y en la cantidad y calidad de sustancias orgánicas que liberan al suelo (Allen et al., 1995; Jansa et al., 2008; Walder et al., 2012) y en consecuencia, influir en la comunidad de microorganismos, estimulando rasgos funcionales específicos y abriéndose a potenciales nuevas interacciones benéficas para la planta como se ha visto en diferentes estudios (Zak et al., 2003; Sun et al., 2009; Duchene et al., 2017).

Particularmente para los HMA, se pueden promover la riqueza de especies, actividad y producción de propágulos (Burrows y Pfleger, 2002), y se ha reportado que la diversidad de las comunidades de HMA en las raíces se correlaciona positivamente con las concentraciones de P y N en las plantas (Johnson et al., 2004). Dado que los HMA pueden vincular plantas vecinas formando redes de micelio comunes, se podrían utilizar más eficientemente los recursos disponibles en el suelo y proporcionar beneficios a las especies cultivadas (Walder et al., 2012). Sin embargo, la contribución de los socios que conforman esta relación puede variar en los beneficios que brindan, por ejemplo, la transferencia de P puede ser mayor a plantas grandes que proporcionan más cantidad de C, y suprimir el crecimiento de los individuos más pequeños por una deficiencia de P (Weiner, 1990). Por lo tanto, diferentes plantas cultivadas juntas se pueden beneficiar o no de manera distinta, dependiendo de las especies de plantas y HMA involucrados y su compatibilidad, de manera que esto podría afectar significativamente el rendimiento de las plantas. Estas interacciones con potencial efecto benéfico, son determinantes del crecimiento y desarrollo de las plantas (Cano, 2011). Todas estas funciones son de gran importancia, sin embargo, los microorganismos y, por lo tanto, sus servicios ecosistémicos pueden verse afectados por diversos factores externos de origen antropogénico asociadas por ejemplo al empleo de diferentes prácticas agrícolas.

1.4 Efectos de las prácticas agrícolas sobre los HMA

Las condiciones del suelo y de las plantas para que se desarrollen ambos simbiontes pueden disminuir o impedir que uno de ellos o ambos puedan realizar un intercambio favorable a su máxima capacidad. Los sistemas agrícolas son particularmente alterados por las diferentes prácticas que se usan para facilitar el trabajo y promover el desarrollo del cultivo pero que también pueden afectar a las comunidades de HMA y en consecuencia afectar los beneficios que confieren a las plantas.

1.4.1 Plaguicidas

El uso de plaguicidas (incluyendo fungicidas, herbicidas, insecticidas y otros) juega un papel importante en las prácticas agrícolas actuales como el principal método utilizado para el manejo de organismos plaga (Alatorre et al., 2000; Aktar et al., 2009). Sin embargo, debido a su uso extensivo y a que menos del 0.1% de los plaguicidas que se aplican llegan al organismo objetivo, se ha dado lugar a efectos negativos como la contaminación inmediata de la atmósfera, aguas superficiales y subterráneas y el suelo, inducción de resistencia de los patógenos y el impacto en otros organismos no objetivo que habitan en el suelo (Pimentel y Levitan, 1986; Topp et al., 1997; Arias-Estévez et al., 2008; Aktar et al., 2009). Las investigaciones de los efectos de plaguicidas sobre los HMA han sido diversos, pero en general estos hongos se han mostrado sensibles a la adición de plaguicidas. Las estructuras intra- y extraradicales de los HMA pueden exponerse a sustancias activas a través de la absorción de raíces o hifas desde el suelo o cuando las sustancias se transportan sistémicamente desde las partes aéreas de las plantas a las raíces; numerosos reportes han mostrado que diferentes plaguicidas afectan la colonización y el desarrollo de los HMA en el suelo y, por tanto, sus actividades funcionales como la toma y transporte de P hacia la planta (Trappe et al., 1984; Abd-Alla et al., 2000; Jansa et al., 2006; Hage-Ahmed et al., 2019).

Los fungicidas generalmente tienen mayor impacto sobre los microorganismos del suelo incluyendo a los HMA, en comparación con los herbicidas e insecticidas (Bünemann y Condron, 2007). Por ejemplo, es bien reconocido el efecto del Benomil y Carbendazim para inhibir la

colonización de plantas y la toma de P (Larsen et al., 1996; Kling y Jakobsen, 1997; Schweiger y Jakobsen, 1998). En algunas de estas investigaciones se ha visto además que las estructuras fúngicas internas no se vieron afectadas, lo que podría sugerir un requerimiento de C para la planta hospedera pero con una función deficiente del micelio extraradical que puede derivar en depresiones del crecimiento de la planta. Además, algunas investigaciones han reflejado que los plaguicidas, particularmente los fungicidas, pueden suprimir diferencialmente especies de HMA y disminuir o no su abundancia, ya que la inhibición de ciertas especies puede resultar en la proliferación de otras en ausencia de competencia (Jansa et al., 2006; Ipsilantis et al., 2012; Rivera-Becerril et al., 2017; Hage-Ahmed et al., 2019). Hasta los alcances de mi investigación, no existen investigaciones adicionales acerca de si los cambios en la comunidad de HMA están relacionados con funciones reducidas (como la absorción de P). Esto es importante, ya que estos cambios pueden resultar en asociaciones simbióticas menos eficientes.

1.4.2 Fertilización

La fertilización es una práctica común en los sistemas de producción agrícola que permite estimular rápidamente el crecimiento de las plantas con el objetivo de maximizar la productividad y los rendimientos económicos. Estos insumos incluyen fertilizantes minerales como urea, nitrato de amonio, sulfatos y fosfatos, y fertilizantes orgánicos como el estiércol animal, compostas y biosólidos. Los fertilizantes minerales pueden tener efectos adversos como el dominio de malezas, la lixiviación de nutrientes del suelo que conlleva a pérdidas económicas y fomenta procesos de contaminación ambiental como la eutrofización y la acidificación de sistemas terrestres y acuáticos e incrementa las emisiones de gases de efecto invernadero (Tilman et al., 2002). Estos insumos pueden afectar a los microorganismos del suelo a través de efectos directos o indirectos.

La disponibilidad de nutrientes (P principalmente y N) en el suelo es uno de los factores ambientales más importantes que afectan la actividad de los HMA. Esto es esperado ya que, al suplir la limitación de recursos, las plantas pueden asignar el C a otras funciones y no al mantenimiento de la asociación micorrízica (Galvez et al., 2001; Treseder y Allen, 2002). Por esta razón, la fertilización mineral puede tener efectos especialmente negativos sobre el desarrollo y función de los HMA. Además, los efectos sobre la colonización intraradical varían, en algunos

casos ocurre una gran disminución en la colonización y en otros hay poco o ningún efecto. Si esta colonización no disminuye los costos para la planta tampoco disminuirán, lo que puede resultar en depresiones del crecimiento vegetal cuando el costo en términos de fotosintatos para mantener la simbiosis excede los beneficios de toma de nutrientes de los HMA (Johnson et al., 1997; Johnson y Graham, 2013).

Por el contrario, se ha reportado que la fertilización orgánica, que representa una liberación lenta de nutrientes, favorece la formación de asociaciones micorrízicas y, que en general, los HMA son estimulados en suelos con aplicación de materia orgánica, especialmente en el crecimiento del micelio extraradical (Albertsen et al., 2006; Gryndler et al., 2006; Gosling et al., 2006; Yu et al., 2013). Aunque esto puede depender de diferentes factores como la naturaleza del fertilizante orgánico y las dosis aplicadas, por ejemplo, ciertos tipos de abonos verdes, estiércol o biosólidos pueden reducir la colonización con HMA lo que probablemente está atribuido a la liberación de sustancias inhibidoras durante el proceso de descomposición (Sáinz et al., 1998; Larkin, 2013; Aguilar et al., 2017).

De igual manera, la fertilización mineral y orgánica puede modificar la proliferación de especies de HMA a largo plazo. En estudios a largo plazo se mostró la disminución en la diversidad de la comunidad de HMA debido a la aplicación de fertilizantes minerales asociada con el aumento de especies de HMA del género *Glomus* y la reducción de otros como *Acaulospora* y *Scutellospora* (Johnson, 1993; Oehl et al., 2004). En este sentido, además de reducir la colonización y el número de propágulos, la fertilización mineral también podría seleccionar especies de HMA que toleran altos niveles de nutrientes, pero con un funcionamiento reducido en términos de proporcionar un beneficio al hospedero (Johnson, 1993; Kiers et al., 2002).

1.4.3 Labranza

La labranza convencional es un método de preparación del suelo que tiene como objetivo mejorar las condiciones óptimas del lugar de siembra, como lo son promover la descomposición de residuos de los cultivos a través de la degradación física y su incorporación al suelo, la nivelación del suelo, la preparación de una cama orgánica para las semillas, la incorporación de fertilizantes y

plaguicidas, con el fin de lograr un establecimiento y crecimiento rápido de los cultivos y para el control de malezas (Rieger 2001; Kabir, 2005). Esta práctica combina la ruptura del suelo y la mezcla de la capa superior del suelo (Arshad et al. 1999). Se sabe que muchos organismos del suelo son sensibles a los cambios en la estructura del suelo que conllevan estas prácticas. La labranza afecta el entorno físico y químico del suelo en el que viven los microorganismos del suelo, lo que afecta su número, diversidad y actividad. En particular, la labranza convencional afecta la distribución y viabilidad de los propágulos de los HMA en el suelo, reduce la colonización de raíces y se ha relacionado a la interrupción de la red de micelio extraradical que queda del cultivo anterior, afectando la absorción temprana de P de la planta en desarrollo, afectando la interación planta-HMA lo que a menudo va acompañados de disminuciones en la biomasa de las plantas (O'Halloran et al., 1986; Evans y Miller, 1990; Vivekanandan y Fixen, 1991; Miller et al., 1995).

En contraste, prácticas alternativas de labranza, como la labranza reducida o la labranza cero, se han desarrollado principalmente para minimizar la erosión de las tierras agrícolas y se sugiere que la reducción de la labranza estimula la actividad micorrízica y de ese modo también la absorción de nutrientes por las plantas (Miller et al., 1995; Boddington y Dodd, 2000; Mozafar et al., 2000). En diversos estudios, atribuido a un establecimiento más rápido de la colonización, se observó una mayor concentración de P en el tejido vegetal y una mayor captación de P en etapas tempranas en sistemas sin labranza en comparación a los sistemas con labranza convencional, debido a una simbiosis micorrízica arbuscular (MA) más efectiva cuando el suelo no está perturbado, lo que puede eventualmente podría traducirse en un mayor rendimiento de las plantas (Miller, 2000a; Vivekanandan y Fixen, 1991; McGonigle y Miller, 1993; Mozafar et al., 2000; Miller 2000b; Grant et al, 2001; Jansa et al., 2006). Debido a que el crecimiento del maíz se ve afectado por la concentración de P en la etapa temprana, un aumento en la efectividad de la relación micorrízica puede ayudar a reducir la aplicación de fertilizantes en el suelo (Miller, 2000a) y la depresión temporal del crecimiento vegetal asociada al establecimiento de los HMA. Además, si bien el micelio extraradical es la parte de la simbiosis más íntimamente conectada con el suelo y la captación de nutrientes, (Smith y Read 1997; Leake et al., 2004).

La intensidad de la labranza también puede influir en la diversidad de los HMA a largo plazo. Existen estudios donde se observó una mayor diversidad, particularmente con una tendencia de aumento de la incidencia de especies de HMA como *Gigaspora, Scutellospora* y *Entrophospora*

bajo el manejo de labranza reducida, mientras que con labranza convencional se encontraron especies pertenecientes casi exclusivamente a *Glomus*. Esto podría deberse a las diferencias en la tolerancia a la alteración inducida por las prácticas de manejo entre las diferentes especies seguido por la proliferación por falta de competencia (Jansa et al., 2002, 2003b; Alguacil et al., 2008; Schnoor et al., 2011; Brito et al., 2012). Estos resultados sugieren que factores como la perturbación de la red de micelio y la disponibilidad de nutrientes juegan un papel importante en la supervivencia, colonización y funcionamiento de los HMA. Se ha sugerido que los HMA en condiciones de menos perturbación sean más competitivos y las prácticas de labranza convencional puedan promover especies menos competitivas (Mirás-Avalos et al., 2011), pero también es posible que se seleccionen especies de HMA con un funcionamiento reducido en términos de proporcionar un beneficio al huésped (Johnson, 1993; Kiers et al., 2002).

1.5 Costos metabólico para la manutención de los HMA

Los HMA dependen por completo de sus plantas hospedantes para obtener carbono (C) y pueden utilizar hasta un 20% del C fijado por la planta para su propio crecimiento y funcionamiento (Jakobsen y Rosendahl, 1990); esta demanda de C representa el costo de establecer la simbiosis. Sin embargo, existe una evidencia creciente de que el uso del C por parte de los HMA puede compensarse con un incremento en las tasas de intercambio gaseoso, ya que éstas son a menudo mayores en plantas micorrizadas que en plantas no-micorrizadas (Valentine et al., 2001; Amaya-Carpio et al., 2009; Zhu et al., 2012). Se ha sugerido que la mayor actividad fotosintética podría relacionarse al incremento de nutrientes en la planta, pero también se ha propuesto que puede deberse a la fuerza de la salida de C debido a que el acelerado metabolismo fúngico crea una fuerza de remoción de C del floema que evita la acumulación de fotosintatos y por lo mismo, la regulación negativa de la fotosíntesis por acumulación de productos (Pang y Paul, 1980; Harris et al., 1985;Wright et al., 1998a, 1998b; Mortimer et al., 2008; Kaschuck et al., 2009; Schweiger et al., 2014; Gavito et al., 2019). Por lo tanto, se fija más C por tiempo y por unidad de nutriente, lo que resulta en un mayor uso de nutrientes fotosintéticos (Brown y Bethlenfalvay, 1988, Fay et al., 1996). Algunos estudios han demostrado incluso que el aumento de la fuerza de salida de C al incrementar la biomasa o el número de simbiontes radicales (micorrízicos o fijadores simbióticos de N) conduce a una mejora adicional de la tasa fotosintética (Gavito et al., 2000, 2002; Mortimer

et al., 2008, 2009; Kaschuk et al., 2009; Bulgarelli et al., 2017). Gavito et al. (2019) reportaron evidencia directa de que la manipulación experimental de la salida de C al micelio micorrízico arbuscular afecta significativamente la tasa fotosintética de la planta, ya que la escisión de una parte importante del micelio extrarradical causó una reducción significativa en la tasa fotosintética.

Si bien existe la compensación del uso del C por los HMA con un incremento en la fotosíntesis, la perturbación del micelio de los HMA causada por prácticas agrícolas como la labranza convencional en el suelo podría exacerbar la demanda de C por parte de los HMA al tener que reconstruir la red miceliar en cada ciclo de cultivo. Podría crearse una salida de C fotosintético que es excesiva para ciertos cultivos y es poco probable que en todos los casos esté totalmente compensada por la estimulación de la fotosíntesis (Gavito et al., 2019). En esos casos, la salida excesiva de C fotosintético para reconstruir la red de micelio, sin un retorno de nutrientes o agua, podría llevar a una depresión temporal del crecimiento de la planta hasta el restablecimiento de la red de micelio en el suelo. Estas depresiones al inicio del proceso de colonización se han visto en algunas plantas pero el grado de depresión varía mucho ya que para algunas plantas la salida de C hacia los simbiontes es insignificante y para otras, es excesiva (Peng et al. 1993; Jifon et al. 2002).

1.6 Costo-beneficio de los HMA en los agroecosistemas

En múltiples ocasiones se ha demostrado en condiciones controladas el efecto promotor del crecimiento vegetal por parte de los HMA, por lo que han demostrado tener un gran potencial en los sistemas agrícolas. Sin embargo, las condiciones del suelo y ambiente en las que se desarrollan ambos simbiontes pueden impedir que, el hongo, la planta o ambos, puedan realizar un intercambio favorable a su máxima capacidad, por lo que la mejoría en la productividad de las plantas no es siempre el caso que se observa. Un mismo hongo pueda generar promociones o depresiones del crecimiento vegetal al asociarse con diferentes plantas; esta variación puede deberse a múltiples factores, desde la amplia variación de la capacidad de los HMA para proveer recursos y la compatibilidad funcional entre los simbiontes vegetales y los simbiontes fúngicos, hasta casos en que el micelio es consumido por fungívoros y no puede desempeñar su función. Por estas razones, los resultados en términos de biomasa vegetal de la interacción de diferentes hongos y plantas

resultan complejos e impredecibles (Johnson et al., 1997; McGonigle y Fitter, 1988; Klironomos, 2003).

Particularmente en los sistemas agrícolas convencionales, la implementación de distintas prácticas como la labranza, el monocultivo y la fertilización, al dañar el micelio, disminuir la diversidad y eliminar la limitación de nutrientes, pueden afectar el desarrollo y funcionamiento de los HMA e incluso, pueden llegar a promover la proliferación de especies que usan mucho carbono de las plantas sin transferirles nutrientes o agua, lo que podría conducir a esta asociación al parasitismo dado que el costo de la simbiosis excede los beneficios. Aunque se ha reportado que algunas plantas pueden asignar más C a los HMA benéficos que a los no benéficos (Bever et al., 2009; Kiers et al., 2011), este costo podría ser excesivo para algunos cultivos ya que en múltiples casos se han observado depresiones del crecimiento vegetal (p. ej., Koide, 1985; Peng et al., 1993). Esto ha originado que incluso se haya sugerido eliminar las asociaciones micorrízicas de la agricultura convencional, donde los recursos los facilita sin limitación el agricultor y los HMA se vuelven redundantes, para evitar depresiones de crecimiento y pérdidas económicas (Ryan y Graham, 2002).

No obstante, debido a la crisis ambiental, existe una urgencia por generar sistemas agrícolas más sustentables que buscan reducir los insumos en la agricultura aprovechando los mecanismos naturales, dentro de los que se encuentran las asociaciones micorrízicas (Rillig et al., 2019). Además, aunque el intercambio de nutrientes limitantes es la opción más obvia para el análisis costo-beneficio, otros efectos inducidos por los HMA pueden ser más importantes para la aptitud de la planta (Johnson et al., 1997), es decir, pueden tener otros efectos benéficos como la protección contra patógenos y estrés hídrico (Smith y Smith, 2011) que pueden resultar más importantes para el desarrollo de la planta que el intercambio de nutrientes en ciertos escenarios. Resulta primordial entonces estudiar el espectro completo de las respuestas de las plantas a la formación de micorrizas y cómo se pueden maximizar los beneficios de las asociaciones micorrízicas en los sistemas productivos.

1.7 Cultivos intercalados maíz-leguminosa

Dentro de la práctica de intercalado, los cultivos de maíz combinados con diferentes leguminosas son ampliamente utilizados. El uso de leguminosas se explica ampliamente por su capacidad de fijación de nitrógeno como resultado de la simbiosis con bacterias *Rhizobium* y *Bradyrhizobium* y, en consecuencia, la provisión de proteínas en forma de grano o forraje (Anil et al., 1998; Duchene et al., 2017). De este modo, las leguminosas poseen una simbiosis tripartita (micorriza-leguminosa-*Rhizobium/Bradyrhizobium*), demostrando en general una fuerte respuesta a los HMA probablemente debido a sus requisitos de nutrición vinculados a la actividad de sus nódulos de raíz, mejorando en consecuencia la abundancia y diversidad de los HMA en el suelo (Giller et al., 1991; Siqueira et al., 1991; Scheublin et al., 2004). En un intercalado con maíz esto puede representar una gran ventaja ya que existe evidencia de que las especies de HMA varían en su capacidad de proliferar en diferentes especies de cultivos y que el rendimiento de los cultivos en monocultivo generalmente disminuye con el tiempo porque las poblaciones de especies perjudiciales aumentan y las poblaciones de especies beneficiosas disminuyen en la comunidad de HMA (Rosendahl et al., 1990; Johnson et al., 1992). Por ello el intercalado, que permite el incremento de la diversidad, puede reducir la abundancia relativa de HMA perjudiciales y aumentar la abundancia relativa de los beneficiosos.

Por otro lado, también se han observado interacciones con las bacterias fijadoras de N asociadas a las leguminosas. Se ha reportado que su coexistencia no afecta la colonización por bacterias (Requena et al., 1997; Vázquez et al., 2000), y los HMA de hecho favorecen las condiciones para el desarrollo de ciertas cepas de bacterias ya que varias especies de leguminosas no logran nodularse exitosamente a menos que las raíces hayan sido previamente colonizadas por HMA (Hayman, 1986). Se han reportado aumentos significativos en la nodulación y la fijación de N_2 en leguminosas colonizadas con HMA (Cluett y Boucher, 1984). Además, la red de micelio proporciona la vía para la transferencia de N de la leguminosa al maíz, ya que durante el crecimiento de la leguminosa el N se deposita en la rizósfera como resultado del recambio continuo de raíces y nódulos y, tras la descomposición, la transferencia es mediada por el micelio (Giller et al., 1991; Van Kessel et al., 1985; Johansen y Jensen, 1996).

Sin embargo, también se han reportado casos en donde no se obtienen beneficios. Es posible que las características de crecimiento que permiten el establecimiento rápido de las plantas de maíz originen una interferencia temprana que provoque competencia por recursos que conducen al dominio del maíz cuando se intercala con leguminosas. Esto se puede ver intensificado ya que la asignación de recursos a través de la red de micelio de los HMA entre las plantas que conforman el cultivo intercalado se puede definir por la fuerza con la que cada especie conectada demanda y ofrece los recursos, y es posible que sea mayor para la planta de mayor tamaño o que se encuentra más desarrollada (Walder et al., 2012; Merrild et al., 2013). Estos autores también han reportado casos en los que un cultivo transfiere mucho C a la red micorrízica común, sin recibir beneficios de crecimiento o nutrición. Este es el caso de cultivos de cereales que mandan a las raíces, transfieren a HMA y liberan al suelo, grandes cantidades de C. Por estas razones, los beneficios de los HMA, así como los costos, varían entre las especies de plantas cultivadas y los HMA asociadas, así como el suministro de nutrientes en el suelo y las condiciones de crecimiento. Debido a lo anterior, es importante visualizar estrategias de manejo de esta práctica para maximizar el establecimiento y crecimiento de las plantas ya que cuando los cultivos son complementarios, como en la inclusión de leguminosas apropiadas en los sistemas de cultivo basados en cereales, se puede permitir una utilización más eficiente de los recursos disponibles en el suelo y se proporcionan beneficios a las especies cultivadas, lo que puede resultar en un rendimiento mayor en los cultivos, respecto a cuando se cultivan por separado.

Objetivo general de la tesis

Evaluar la eficiencia temprana de la simbiosis maíz-HMA y su relación con el desarrollo, nutrición y rendimiento de grano bajo las prácticas de labranza y diversificación del cultivo que afectan el funcionamiento de la red miceliar de los HMA para promover el manejo de las redes micorrízicas en los campos agrícolas.

Objetivos particulares

a) Examinar la influencia de las prácticas de fertilización, labranza y aplicación de fungicidas en el funcionamiento de la simbiosis establecida entre el maíz, cultivo que se beneficia de una mayor capacidad de absorción de P en etapas tempranas de desarrollo, y las comunidades nativas de HMA en condiciones de campo.

b) Analizar el efecto de la disrupción de la red de micelio de los HMA provocado por la fertilización, labranza y aplicación de fungicidas sobre las tasas de intercambio de gases de las plantas de maíz y su relación con el desarrollo del maíz.

c) Analizar el efecto de la práctica de cultivo intercalado, cuando la planta acompañante se integra a la red de micelio en diferentes tiempos, sobre el desarrollo de la colonización micorrízica, la nutrición, el desarrollo y el rendimiento de los dos cultivos.

Estructura de la tesis

Para abordar a los objetivos planteados, la presente tesis se estructuró, además de este primer capítulo de introducción general, de tres capítulos experimentales (Capítulos 2 al 4) y un capítulo de conclusiones generales. El alcance de cada capítulo experimental se describe a continuación:

Capítulo II. En este capítulo se presenta un estudio que tuvo como objetivo perturbar el funcionamiento de la simbiosis establecida entre el maíz y las comunidades de HMA nativos en condiciones de campo con prácticas de fertilización, fungicida y labranza para medir su efecto en la nutrición, crecimiento y rendimiento del maíz. Se estableció un experimento en campo donde se utilizó una combinación de fertilización, labranza y aplicación de fungicidas para modular el desarrollo y funcionamiento de las redes miceliares de HMA en las primeras etapas de desarrollo de la planta. Se analizó la colonización de raíces y suelo, biomasa, y la nutrición vegetal en tres etapas tempranas del desarrollo de la planta, y el rendimiento y calidad nutricional del grano, y se establecieron relaciones funcionales entre estas variables.

Capítulo III. En este capítulo se presenta un estudio que tuvo como objetivo evaluar cómo influye la interrupción de la integridad y el funcionamiento de la red de HMA por efecto de labranza y aplicación de fungicida y fertilización, en el intercambio de gases en las primeras etapas del desarrollo del maíz. Para cumplir este objetivo se midieron diferentes parámetros de intercambio gaseoso (conductividad estomática, transpiración, tasa instantánea de asimilación de CO_2, tasa de asimilación de CO_2 estimada para toda la planta, así como curvas A/Ci para estimar las principales limitantes bioquímicas de la fotosíntesis en plantas C4: J_{max} y V_{cmax}) en tres etapas tempranas del desarrollo de las plantas establecidas en el experimento de campo mencionado en el capítulo I. Así mismo, los parámetros de intercambio gaseoso se relacionaron con las variables del desarrollo, nutrición y el rendimiento del maíz obtenidos en el capítulo I.

Capítulo IV. En este capítulo se presenta un estudio que tuvo como objetivo investigar si el tiempo de siembra afecta la competencia/facilitación temprana mediada por las redes micorrízicas comunes en un cultivo intercalado de maíz y frijol y si beneficia a uno o ambos cultivos o a ninguno, en términos de nutrición, crecimiento y rendimiento. Se estableció un experimento en campo donde se utilizó la labranza para modular el desarrollo y funcionamiento de las redes de micorrízicas en las primeras etapas de desarrollo de la planta y se evaluaron tres tiempos de siembra (frijol solo, maíz solo, frijol sembrado antes que el maíz, siembra simultánea y frijol sembrado después que el maíz). Se analizó la colonización de raíces y suelo, biomasa, y la nutrición vegetal en diferentes etapas tempranas del desarrollo de la planta. Así mismo, se evaluó el rendimiento y calidad nutricional del grano y se establecieron relaciones funcionales entre las variables analizadas.

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CAPÍTULO II

Agronomic practices and mycorrhizal development and function in maize: Root fungal interactions may affect early nutrition and yield

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Agronomic practices and mycorrhizal development and function in maize: Root fungal interactions may affect early nutrition and yield

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ABSTRACT

Efficient management of soil biota and fertilization to reduce environmental impacts of agriculture requires understanding of functional soil biota-crop relationships and how they interact with management practices. An experiment was conducted to examine functional interactions between native arbuscular mycorrhizal (AM) fungi and maize nutrition, development and yield, for understanding and capturing most mycorrhizal benefits in the field. Soil tillage, fertilization and fungicide treatments were used to manipulate early AM development and functioning and test the hypothesis that the rapid root colonization and preservation of AM networks in soil would increase early P uptake and biomass, and later increase the yield and nutritional quality of the grain in comparison with treatments reducing early mycorrhizal development and function. The treatments succeeded in modulating mycorrhizal development and achieved up to 95% reductions in early root colonization. Tillage generally had few significant effects on most variables. Furthermore, the fungicide improved early nutrition and yield, and fertilization increased the nutrition, biomass, yield and grain quality. Early functional AM-maize relationships were positive at the third leaf (V3) stage but became negative at the sixth (V6) and tenth (V10) leaf stages, when root colonization by AM fungi and by other fungi increased, especially in treatments without fungicide. The abundance of AM fungi and other fungi in roots was negatively related to early shoot N and P, and some treatments did not reach the critical concentrations of N or P at V6. The shoot N and P reductions did not significantly affect the biomass during vegetative growth, but the yield was reduced by 10% in treatments without fungicide. Fertilization, which increased the yield by 30%, compensated for yield depression in treatments without fungicide. Close monitoring of early events in the development and functioning of AM-maize symbiosis suggested that early reductions in maize shoot N and P, and later the yield, were related to the combined effects of root colonization by mycorrhizal and non-mycorrhizal fungi. These results provide new evidence for the involvement of other root fungi in maize's early nutrition and yield depressions already reported in low fertility soils, which had been overlooked and attributed solely to AM fungi.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are a main component of the rhizosphere microbiota in most agricultural fields that with conservation and adequate management may represent a sustainable alternative to conventional fertilization practices (Richardson et al., 2011). The AM association is established by numerous crops and is characterized by a bilateral exchange of nutrients via the formation of an extensive mycelial network that explores the soil and transfers nutrients (Mikkelsen et al., 2008; Fitter et al., 2011). This symbiosis is, therefore, particularly relevant in crop nutrition. Soil and plant conditions for the optimal development and/or functioning of the symbionts, however, may not always be present and prevent them from reaching their maximum exchange capacity. Fertilization and tillage are widespread practices that affect the composition and functioning of soil communities in the field (Feng and Balkcom, 2017; de Souza and Freitas, 2018; De Graaff et al., 2019) and may also affect crop development and yield (Ren et al., 2020; Hu et al., 2021; Dincă et al., 2022). Those conditions affecting symbiotic efficiency and balance become important for managing agricultural fields as sustainable practices are encouraged to reduce the negative effects of conventional practices that still rely on intensive use of tillage and fast-release fertilizers (Drinkwater et al., 2017).

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Phosphorus fertilization is an important factor influencing AM fungi because high levels of mineral P fertilization usually reduce the diversity of AM fungal communities and root colonization (Treseder, 2004; Liu et al., 2012; Sheng et al., 2013). Fertilization might also promote the proliferation of opportunistic AM fungal species that take carbon from plants without transferring nutrients to them, thereby affecting their functional relations with plants (Johnson et al., 2015). The alteration of soil conditions by tillage is another factor, which through the disturbance of surface layers in the soil profile, may significantly alter the development and function of AM extraradical hyphal networks and, consequently, soil and crop nutrient dynamics (Jansa et al., 2006; Bowles et al., 2016). Tillage disturbance may have important consequences on mycorrhizal development and functioning in crops, especially for those crops with root systems with low nutrient uptake capacity at early developmental stages (e.g. maize) and benefiting from nutrient transfer by AM fungi (Gavito and Miller, 1998; Yang et al., 2015). Maintaining the integrity of the mycorrhizal networks in the soil, through appropriate agricultural practices, may have different consequences for the functioning of mycorrhizal associations formed by different crops and AM fungal communities (Kabir, 2005; Jansa et al., 2006). In maize, the fast development of the AM intraradical and external mycelium has been linked to better early nutrition, which may eventually translate into higher yield (Miller et al., 1995; Mozafar et al., 2000; Grant et al., 2001). Nonetheless, this effect has been inconsistent as there are also reports of negative effects of AM colonization on maize nutrition that have been attributed to plant-AM fungi competition for nutrients, the AM fungi failure to deliver nutrients to plants, and/or an excessive photosynthate demand from AM fungi (Smith and Smith, 2012; Wang et al., 2018). To date, few studies have shown that managing mycorrhizal associations leads to yield improvements in field studies or that management to promote mycorrhizal associations does not compromise yields (Ryan and Graham 2018). In maize, the lack of AM benefits on yield improvement is puzzling because it has been repeatedly shown that rapid AM early colonization and preservation of extraradical networks improve early P nutrition and have lower carbon cost than other nutrient uptake mechanisms (Verlinden et al., 2018).

The objective of this work was to explore in detail the functioning of the symbiosis established between maize, a crop that benefits from increased P uptake capacity at early developmental stages, and the native AM fungal communities of a conventionally managed agricultural field. Our goal was to identify agronomic practices that preserve and maximize the AM contribution to maize yield by exploring if functional AM fungi-maize relationships were maintained as expected throughout the crop cycle. A combination of fertilization, tillage and fungicide application was used to manipulate in-situ the development of mycorrhizal colonization in roots and the connection to preserved or disturbed networks at early stages. We hypothesized that the application of fertilization, tillage and fungicide would have additive, negative effects on AM fungal development and the nutrient transfer to plants. Thus, we expected that reducing AM root colonization with fungicide and fertilization, and disturbing the mycelium integrity and nutrient transfer function of AM fungal networks with tillage, would lead to lower early uptake of N and P, biomass, yield and the nutritional quality of the grain. In contrast, maintaining high root colonization without fertilizer or fungicide, and preserving AM fungal networks with no tillage, would improve functional AM fungi-maize relationships and result in improved nutrition, growth and yield.

2. Methods

2.1. Study site

This experiment was performed in an agricultural plot (19° 50′ 57″ N, 101° 8′ 54″ W, altitude 1930 masl) in the municipality of Tarímbaro, Michoacán, in Mexico. The soil type is vertisol (WRB), with clay texture, pH 8 (1:10 soil:water), 1.1% organic matter, 29 mg kg^{-1} available P (Mehlich, 1984) and 42 mg kg^{-1} available N (KCl extraction; Robertson et al., 1999). The plot had experienced high-input conventional tillage management supported by irrigation. At the end of each cropping cycle, all biomass was usually removed from the field, and the soil was cultivated. After the previous maize cycle in 2018, cultivation with a disc harrow was implemented at approximately 10–15 cm depth, and vetch (*Vicia sativa* L.) was established as green manure from December 2018 to April 2019 at a density of approximately 400,000 seeds ha^{-1}, with drip irrigation for 8 h every 12 days. No mineral fertilizers were added, and weed control was manual.

2.2. Experimental design

In April 2019, a factorial block design was established with three factors: tillage (conventional, CT, and no tillage, NT), the application of carbendazim fungicide to inhibit the development of AM fungi (without, -Carb, and with, +Carb), and NPK fertilization (without, -Fert, and with, +Fert), for a total of eight treatment combinations. To facilitate agronomic practices, tillage treatments were first assigned to four spatial replicate blocks, then the carbendazim treatments and finally the fertilization treatments. Each of the four blocks contained one experimental unit of the eight treatment combinations, resulting in 32 experimental units (Fig. S1).

The experimental units consisted of plots with four rows, 10 m long, with a 80-cm distance between rows. To avoid edge effects, six rows surrounding the experiment acted as a buffer zone. In addition, all sampling and harvests were conducted only in the two central rows within each experimental unit. In early April 2019, the vetch green manure was incorporated into the soil at cultivation with a rotavator at a 20-cm depth in conventional tillage treatments. In no tillage treatments, vetch was only cut at the base and left covering the soil. The fungicide carbendazim, known commercially as Tlaloc 50% P.H. ® (UPL Agro, Mexico), was used to establish controls with low development of AM fungi. This fungicide is not specific for AM fungi and may affect other organisms, but is affordable and easy to apply at the field scale (Kahiluoto et al., 2000; O'Connor et al., 2009). The concentration applied in this study was 75 kg ha^{-1}, approximately 100 times the recommended dose of the product, as suggested by Schweiger and Jakobsen (1998), for effectively reducing AM root colonization. It was sprayed in solution over the soil until saturation 2 days before planting. Half of this dose was applied again two weeks after the first application to reinforce the effect of the fungicide. Treatments without carbendazim, were sprayed with sterile water. On May 29, H-318 (Milpal®) hybrid maize seeds were sown at a density of 80,000 ha^{-1}, with a 4-row disc planter. A chemical fertilizer mixture containing 118 (N), 95 (P), 47 (K), 5 (Ca), 8 (Zn), 4 (Fe), 3 (B), Cu (1) kg ha^{-1} was added simultaneously to the corresponding treatments at planting time below the seed. Monoammonium phosphate (MAP), potassium sulfate (SOP), urea, ammonium sulfate, ferrous sulfate, zinc sulfate, boron, manganese sulfate and copper sulfate were used for the mixture. Fertilizer was applied again at stages V4 (fourth leaf's collar visible; 30% of the initial dose), V10 (tenth leaf's collar visible; 50%) and R1 (silking visible; 20%) to the fertilized treatments. Paraquat, tembotrione 4.4 herbicides were used before sowing maize and 15 days after, respectively, to control weed outbreaks. Pests were controlled with permethrin 9% at sowing. Products were used according to the dose indicated by the product. Maize emerged during the first week, and sampling began in the second week of June. Drip irrigation for 8 h every 12 days continued to complement rainfall as required.

2.3. Soil and plant sampling, processing and measurements

The five plants of each experimental unit harvested on days 15, 25, 48, and 79 after sowing, corresponding to stages V3, V6, V10 and R2, were stored in plastic bags and kept refrigerated until processing in the lab. Soil near the roots and the root samples were taken from the top 20

cm and composited in a sealed plastic bag that was stored in a cooler until processing. In the lab, the plants were dried at 70 °C to constant weight. From the second harvest, because of the large amount of biomass, the total fresh weight was recorded. All aerial parts were then cut into small pieces and mixed; a random subsample then was taken to determine moisture content and calculate the total dry weight. The rest was discarded. We followed the same subsampling procedure in the fourth harvest, at silking, to separate the vegetative and reproductive tissue samples.

Soil samples were stored immediately at −20 °C for fatty acid analysis and were freeze-dried. Roots were washed, and 1 to 2-g subsamples were stored in tubes with 50% alcohol solution to evaluate percentages of AM fungal root colonization. Root subsamples were washed, cleared and stained with trypan blue according to Phillips and Hayman (1970). One hundred root segments, randomly selected per sample, were mounted on slides and examined to determine root colonization by AM fungi. Colonization at vegetative stages V3, V6 and V10 was quantified using the magnified intersections method by McGonigle et al. (1990), which separately scores arbuscules, vesicles, hyphae only, and negative segments and assumes that arbuscules and vesicles form from hyphae. Simultaneously, since the presence of other fungi in roots was frequent, they were quantified as a separate category, scoring root intersections with melanized and septate hyphae, microsclerotia and spores. Their structures, however, were not counted separately. Colonization by organisms other than fungi was negligible, so it was not scored.

Although the AM fungal mycelium is different enough to not be confused with the other most common fungi observed in this study (with melanized and septate mycelium, described as dark septate endophyte-like fungi, or DSE-like), there may be other endophytic fungi in the roots that become stained. Other endophytic fungi with hyaline hyphae have also been recorded in the roots of various plant species, and they interact, overlap in function, and may be confused with AM fungi (Porras-Alfaro and Bayman, 2011). Therefore, arbuscular measurements are reported to quantify AM fungi development. Arbuscules are unique to AM fungi because similar hyphae or vesicles can be formed by other fungi. Furthermore, they are the main sites for nutrient exchange between symbionts (Smith and Read, 2008), thus reducing possible morphological and functional confusion with other organisms. Therefore, arbuscular colonization is provided as a conservative measure of the extent of functional interaction between the plant and AM fungi. In addition, total fungal colonization is presented as the sum of AM arbuscules, vesicles, and hyphae, and any fungal structures.

Shoot N and P concentrations were measured in shoots at stages V3, V6, V10 and R2, and in the grain at harvest (R6). Samples were ground in a Thomas Scientific mill, acid-digested by the semi-Kjeldahl method (Bremmer, 1996) and filtered. Additionally, N (Weatherburn, 1967) and P (Murphy and Riley, 1962) were measured by color development in a Bran-Luebbe III autoanalyzer. Soil available nutrients were measured from samples taken at stage V3 when all treatments had been implemented. Soil inorganic nitrogen (NH_4^+ and NO_3^-) was extracted from fresh subsamples (KCl extraction; Robertson et al., 1999). Soil available P was determined using the Mehlich-3 extraction (Mehlich, 1984). Both N and P forms extracted were measured in the aforementioned autoanalyzer.

Soil samples previously lyophilized and ground in a steel-ball mill were used for fatty-acid analysis to estimate the abundance of live external mycelium of AM fungi. Stage V6 samples were selected for this measurement based on root colonization measurements showing very low colonization at V3. Fatty acids were extracted from the soil, saponified, and fractioned into the following: neutral lipid fatty acids (NLFA), glycolipid fatty acids (GLFA), and phospholipid fatty acid (PLFA) fractions, as well as methylated and volatilized for gas chromatography according to Frostegård and Bååth (1996). A known amount of a standard 19:0 fatty acid methyl ester was added to each sample for quantifying the extracted fatty acids. The fatty acids in phospholipid fatty acids and neutral lipid fatty-acid fractions were identified and quantified by gas chromatography using a 50 m HP5 capillary fused-silica column (Hewlett Packard) with He as the carrier gas. The fatty acids were identified from their retention times in relation to the internal standard (fatty acid methyl ester 19:0) using the software package Sherlock version 6.0 (MIDI Inc.) Fatty acid analysis was performed with a gas chromatograph (Agilent 7890 B). The PLFA and NLFA fractions of the fatty acid 16:1ω5 were used to indicate the development of the external mycelium of AM fungi in the soil (Olsson, 1999).

For the final harvest, all plants in 10 m of the central rows within each experimental unit were harvested. The cobs were removed from all plants and weighed in fresh and afterwards a sample of 10 cobs was taken. The shoots of those plants were also weighed in fresh and afterwards a sample of 10 shoots was taken for moisture content measurement. The 10 shoots were chopped into 10–15 cm and mixed before taking a sample of approximately 0.5 kg to determine moisture content. Cob and stover subsamples were later dried in the lab at 70 °C to constant weight. The grain was separated and weighed; a subsample was finely ground to determine N and P concentration, as explained for shoot samples at earlier stages. The grain yield per plant was corrected with the measured moisture contents, and the mean plant density of 59 plants in 10 m was used to calculate total final grain yield and stover biomass. There were no significant differences in plant density between treatments.

2.4. Pot experiment

A complementary pot bioassay was performed to evaluate the influence of soil biota on early maize nutrition and growth because an unexpected drop in shoot N and the common presence of root-inhabiting microorganisms other than AM fungi had been observed at early developmental stages in the field experiment. Autoclaved and fresh field soil were used and combined with fertilization treatments to test if the native soil biota harmed the maize and if fertilization compensated for this. No filtrates or fungicides were used to separate the effects of native propagules of AM fungi and other biota in this bioassay because they cannot be completely separated through physical or chemical methods without altering other non-target groups. A randomized factorial design with three main factors was used: 1) soil disinfection (autoclaved and fresh field soil), 2) NPK fertilization (with and without), and 3) time of harvest (V3 and V6 development stage), with four replicates in each treatment combination. Soil from the agricultural plot where the field experiment was performed was either autoclaved for 2 h at 121 °C to remove most native organisms or used fresh as collected. One kg of autoclaved or fresh soil, as required, was placed in pots, and H-318 maize seeds were sown. The plants in fertilized treatments received 150 mg of N, 40 mg of P and 52 mg of K per kg soil as NPK fertilizer, mixed into the soil at the beginning of the bioassay. The plants were grown under greenhouse conditions and watered on a daily basis to maintain approximately 80% of the water holding capacity. The temperature was approximately 15 °C at night and 25–30 °C during daytime. Four replicates per treatment were randomly taken and harvested at the V3 and V6 development stages. Fungal root colonization, shoot dry weight and N and P concentrations were evaluated as explained above for the field experiment.

2.5. Statistical analysis

Data were analyzed with linear mixed models (LMMs) including tillage, fertilization, carbendazim and time as fixed effects, with block as a random effect. Models were fitted using the 'lmer' function in the 'lme4' package for software R (Bates et al., 2015). For identifying the relevant predictors, model reduction was performed by fitting all the possible nested models and selecting the best models, being those with the lowest Akaike Information Criterion (AIC) values. When relevant, post-hoc Tukey tests assessed the differences in mean values across

treatments using the lsmeans function in the 'lsmeans' package (Lenth, 2016). Log and logit transformations were used whenever necessary to meet test assumptions. Tillage, fertilization and carbendazim effects on dry weights on five sampling dates were evaluated with an LMM, using time as a continuous variable. Time was log-transformed, and its effect was modeled with a polynomial function that included linear, quadratic and cubic terms to account for the nonlinear observed trends. Tillage, fertilization and carbendazim effects on fungal root colonization and shoot nutrients measured on three or four dates were examined with the LMMs, with time included as a categorical variable (stage of development). AM fungal mycelium colonization in soil, grain yield and nutrients in maize grain, measured only once, were evaluated with LMMs not including time. The hypothesized relationships between relevant variables were examined with Pearson correlations. Models were fitted using the "lm" function, and the coefficients were calculated using the "cor.test" function in the "stats" library for R. Growth and nutrient concentration response ratios to carbendazim application treatments were calculated as described in Johnson (2010). Because AM fungi were not added in our study but inhibited by the addition of fungicide, response ratios were expressed as responses to carbendazim application. The proportional response of the different variables was compared to the inhibition of AM root colonization and possibly other root-inhabiting fungi because, as mentioned above, carbendazim is not AM fungi-specific. The carbendazim response ratio was then calculated as follows: CR = $\log_e$ (AC/NCmean), where AC was the value of each replicate of the total dry weight or nutrient concentration measured in the treatments with carbendazim. Furthermore, NC was the mean of dry weight or nutrient concentration of plants in treatments under the same conditions but without carbendazim ($n = 4$). A positive CR value suggests that carbendazim application was beneficial, and a negative value means it was detrimental. Generalized linear models (GLMs) were used for the analysis of bioassay plants testing the effect of sterilization, fertilization and developmental stage on shoot dry weight, as well as nitrogen and phosphorus shoot concentrations using 'glm' function in the 'lme4' package for software R (Bates et al., 2015). Significant differences were tested with post-hoc Tukey tests using the least square means package lsmeans (Lenth, 2016). Model residuals were always inspected for normality, homoscedasticity, and outliers using plot (model) statement. All tests were performed in R (R Core Team, 2020).

3. Results

Overall, fertilization most significantly affected plant variables, followed by carbendazim application, whereas tillage had minor effects (Tables 1 and 2). On the other hand, most significant effects on AM fungal development were found in carbendazim application and tillage, with fertilization effects as minor (Tables 1 and 2). There were few two-factor interactions (mostly fertilization x developmental stage (S)), 2 three-factor interactions (T x F x C on total fungal colonization and T x C x S on AM arbuscular colonization), and no four-factor interactions. Therefore, only single factor and two-factor interaction effects are presented in Table 1, and three-factor interactions are explained in the text. All interactions are presented in supplementary materials (Table S1).

3.1. AM fungal development

All factors had significant main and interacting effects on arbuscular colonization at the V3 and V6 stages (Table 1, S1). Among them, fertilization had the smallest and least consistent effects on AM colonization. Arbuscular colonization generally increased with time until V10 and remained low (around 10%), but carbendazim, tillage and fertilization succeeded at delaying colonization in some treatment combinations at V3 (Table 1, Table 3). Those differences diminished at V6 and disappeared at V10. Thus, the delay achieved in those treatments lasted only 4–6 weeks. Carbendazim negatively affected AM arbuscular colonization, but the reduction became significant only in treatments with no tillage and only at V3 (T x C x S). Conventional tillage treatments showed very low AM arbuscular colonization at V3, and carbendazim application had no additional effect on these treatments. Root colonization by other organisms, mostly other fungi, became evident already at V3 (Table 3). Colonization by other fungi had a slight but significant increase from V3 to V6 but no further at V10, was also significantly higher with no tillage as compared with conventional tillage, and decreased with carbendazim addition. As in AM arbuscular colonization, total fungal colonization (any fungal structures in roots) in treatments with carbendazim was significantly lower than in most treatments with carbendazim, regardless of tillage and fertilization. In treatments without carbendazim, tillage and fertilization had additive detrimental effects on total fungal colonization (T x C x F). AM fungal development in soil, measured at V6 with PLFA and NLFA 16:1 ω5, differed only in the PLFA fraction and was slightly higher with no tillage than with conventional tillage treatments (Table 2, Table S2).

3.2. Plant nutrition

Soil available N and P at V3 were affected only by fertilization, which resulted in a two-fold increase in available N and about a six-fold increase in available P (Table 2, S2). Fertilization significantly increased shoot N concentration at all stages and shoot N content at V3, V6 and R2 (Table 1) but shoot P concentration and shoot P content only at V3 and V6 (Tables 1 and 3). Shoot N concentration and shoot N content were also generally higher in treatments with, than without, carbendazim. At V3, only treatments without fertilization and with carbendazim did not reach the critical shoot P concentration of 4 mg g^{-1} (Table 3), but all treatments were above the critical shoot N concentration of 25–30 mg g^{-1} (Zhao et al., 2018). At V6, this changed, and the NT-Fert-Carb and

Table 1
P values and significance levels for the main effects of tillage (T), fertilization (F), carbendazim (C) and stage of development (S) factors and their two-factor interactions on all variables measured at three, four or five stages of maize development. Three- and four-factor interactions are presented in Table S1. Stage column indicates the range of plant development stages evaluated for each variable. Significance levels: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Parameter	Stages	T	F	C	S	T*F	T*C	F*C	T*S	F*S	C*S
Root colonization											
AM arbuscular	V3–V10	***	*	***	***	*	***	*	**	0.9	***
Other fungi	V3–V10	0.7	0.5	***	***	*	***	0.2	0.5	1	0.1
Total fungal	V3–V10	***	*	***	***	*	***	***	**	0.9	***
Shoot DW	V3-R6	0.7	***	0.1	**	0.8	0.9	0.2	0.6	***	0.5
Shoot nutrients											
P concentration	V3-R2	0.5	***	0.1	***	0.8	0.4	0.7	0.7	***	0.2
Total P uptake	V3-R2	1	***	0.1	***	0.9	0.7	0.4	0.9	***	0.1
N concentration	V3-R2	0.1	***	*	***	0.9	1	0.8	0.6	0.1	0.4
Total N uptake	V3-R2	0.1	***	*	***	0.7	1	0.3	0.5	**	0.1
Carbendazim responses											
P concentration	V3-R2	0.8	0.1		**	0.2	–	–	0.9	0.1	–
N concentration	V3-R2	0.9	0.8	–	0.1	0.5	–	–	0.4	0.1	–

Table 2
P values and significance for the main effects of tillage (T), fertilization (F) and carbendazim (C) factors and their two- and three-factor interactions for all variables measured at only one stage of plant development. Significance levels: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Stage column indicates the plant development stage when the measurement was made.

Parameter	Stage	T	F	C	T*F	T*C	F*C	T*F*C
Soil available nutrients								
N concentration	V3	0.44	***	0.92	0.70	0.78	0.74	0.18
P concentration	V3	0.06	***	0.76	0.69	0.72	0.33	0.46
AM fungal biomarkers								
NLFA 16:1 ω5	V6	0.23	0.84	0.37	0.77	0.82	0.1	0.12
PLFA 16:1 ω5	V6	*	0.79	0.44	0.42	0.22	0.22	0.39
Yield parameters								
Grain yield	R6	0.17	***	**	0.71	0.91	0.46	0.86
Grain P concentration	R6	0.87	***	0.36	0.56	0.89	0.16	0.19
Grain N concentration	R6	0.87	***	0.07	0.74	0.55	0.97	0.59

Table 3
Means (±SE, n = 4) of root colonization (%) and shoot nutrient concentration (mg g^{-1} DW) at three early developmental stages in the tillage, fertilization and carbendazim treatment combinations. Total fungal colonization percentage includes root intersections with any fungal structures in roots. CT: Conventional Tillage; NT: No Tillage; Fert: Fertilization; Carb: Carbendazim.

Treatment			Stage	AM arbuscular colonization	Other fungi colonization	Total fungal colonization	Shoot P	Shoot N
CT	-Fert	-Carb	V3	1.3 ± 0.63	11 ± 2.3	29 ± 5.2	4.3 ± 0.33	38 ± 1.8
		+Carb		0.50 ± 0.29	3.0 ± 1.2	11 ± 1.5	3.3 ± 0.32	32 ± 1.8
	+Fert	-Carb		2.3 ± 1.3	9.2 ± 1.2	21 ± 0.90	7.2 ± 0.03	47 ± 2.5
		+Carb		2.0 ± 0.71	7.5 ± 1.0	16 ± 0.40	8.3 ± 0.93	49 ± 2.1
NT	-Fert	-Carb		11 ± 1.3	17 ± 4.4	47 ± 7.1	4.2 ± 0.65	36 ± 1.8
		+Carb		1.0 ± 0.71	5.5 ± 2.5	16 ± 2.7	3.7 ± 0.28	37 ± 1.8
	+Fert	-Carb		7.5 ± 1.3	18 ± 3.9	40 ± 2.6	7.4 ± 0.26	45 ± 3.0
		+Carb		2.0 ± 0.91	2.8 ± 1.7	11 ± 1.4	6.4 ± 0.18	45 ± 2.4
CT	-Fert	-Carb	V6	4.8 ± 0.85	17 ± 3.3	41 ± 3.3	4.2 ± 0.76	35 ± 2.7
		+Carb		3.8 ± 0.48	6.7 ± 0.95	19 ± 1.7	4.8 ± 0.37	38 ± 1.6
	+Fert	-Carb		5.8 ± 1.5	13 ± 0.91	29 ± 2.0	5.8 ± 0.44	35 ± 1.3
		+Carb		5.8 ± 0.63	12 ± 1.2	25 ± 1.0	6.8 ± 2.08	39 ± 5.9
NT	-Fert	-Carb		9.3 ± 1.3	20 ± 2.7	52 ± 1.9	3.2 ± 0.44	28 ± 2.4
		+Carb		4.5 ± 0.65	7.5 ± 1.7	21 ± 1.4	4.8 ± 0.35	34 ± 2.2
	+Fert	-Carb		8.3 ± 1.1	18 ± 2.1	45 ± 0.60	6.4 ± 0.83	33 ± 2.0
		+Carb		6.0 ± 0.91	8.3 ± 2.3	21 ± 2.3	6.2 ± 0.24	37 ± 1.8
CT	-Fert	-Carb	V10	6.3 ± 1.4	13 ± 3.3	43 ± 0.90	2.6 ± 0.30	21 ± 1.3
		+Carb		7.5 ± 1.7	8.8 ± 1.5	32 ± 3.9	3.2 ± 0.53	23 ± 2.0
	+Fert	-Carb		8.3 ± 2.1	15 ± 0.85	40 ± 1.8	3.6 ± 0.35	23 ± 0.91
		+Carb		12 ± 0.85	11 ± 0.85	38 ± 2.8	3.9 ± 0.19	27 ± 1.9
NT	-Fert	-Carb		8.8 ± 1.1	17 ± 1.8	47 ± 0.50	2.8 ± 0.50	22 ± 1.4
		+Carb		10 ± 1.8	7.5 ± 2.3	37 ± 3.1	3.3 ± 0.50	22 ± 3.0
	+Fert	-Carb		9.3 ± 1.3	14 ± 1.3	43 ± 2.1	4.0 ± 0.10	22 ± 1.6
		+Carb		11 ± 1.66	6.8 ± 0.63	28 ± 2.1	3.8 ± 0.24	25 ± 1.9

NT + Fert-Carb treatments were below the critical shoot N concentration of 34 mg g^{-1} (Plénet and Lemaire, 1999). Additionally, all unfertilized treatments were below 5 mg P g^{-1} (Barry and Miller, 1989), which is the critical shoot P concentration for V6. At V10, only the treatments with fertilization and carbendazim were clearly above the critical N concentration (22), and only treatments with fertilization were above 3.5 mg P g^{-1}. Thus, the P deficiency at V3–V6 evolved into N deficiency at V6–V10, in treatments without fertilization and, to a lesser extent, in treatments with fertilization but without carbendazim (Table 3). At stage R2, the nutritional differences were minor, but the treatments with early N and P deficiencies continued having low values. Accordingly, the shoot N response and shoot P response to carbendazim application were negative at V3 but became positive at V6 and V10 (data not shown).

3.3. Biomass, yield and nutritional quality of the grain

Shoot dry weight throughout the entire crop cycle was only affected by fertilization and mainly at early stages; V3 and V6 (F x S; Fig. 1). Fertilization was also the most important factor for grain quantity and quality (Table 2). Grain yield (33.6%), grain N concentration (49%) and grain P concentration (23%) increased with fertilizers (Table 4). Carbendazim application significantly increased grain yield (13%) but had no effect on grain N and P concentration. Tillage did not affect harvest parameters.

3.4. Relation between AM development, early nutrition, growth and yield

Overall, the mycorrhizal development variables evaluated did not affect plant nutrition as predicted (Table 5, S3) but actually seemed to negatively affect shoot N and P concentration. Because root colonization by other fungi developed synchronously and became as high or even higher than arbuscular colonization, a relation between colonization by other fungi and all root fungi (any fungal structures in roots) and maize nutrition was also examined. The same negative trends for N and P at the three vegetative stages for other fungi and total fungal colonization were observed, but only the negative relationship of N concentration with total fungal colonization was significant at the V6 stage (Fig. 2). Shoot dry weight was positively correlated with shoot P concentration at V3, V6 and R2 development stages, and with shoot N concentrations only at V3 and R2 (Table 5).

Maize grain yield was negatively correlated with the total fungal colonization of roots at V3 and V6 (Table 5, Fig. 3). The negative trend was similar for root colonization by other fungi but not significant. The shoot P concentration at all developmental stages was positively related

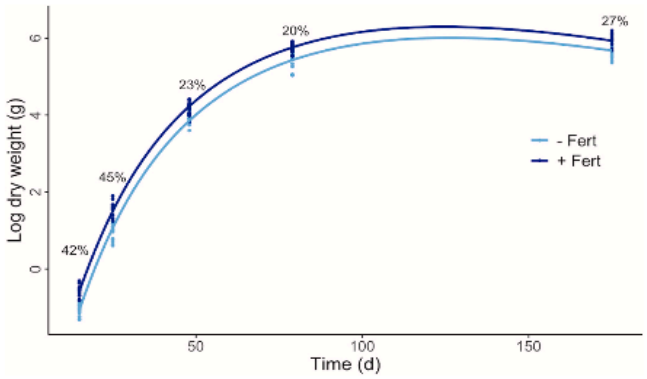

Fig. 1. Effect of fertilization on shoot biomass of maize at V3 (15 d), V6 (25 d), V10 (48 d), R2 (75 d) and R6 (175 d) stages of plant development. The lines represent model predictions for the relationship between biomass and time for each fertilization level. Both points and lines are conditioned on values of carbendazim and tillage, which were also included in the model as predictors. Percent difference between fertilization treatments in shoot dry weights at each stage are shown. Fert: Fertilization.

Table 4
Effects of fertilization and carbendazim on maize grain yield, grain N and P concentrations. Values are means of 16 replicates ± standard errors. Different capital letters indicate significant differences ($p < 0.05$) between means in fertilization treatments and different lowercase letters indicate significant differences between means in carbendazim treatments according to Tukey's Test.

	- Fertilization	+ Fertilization	- Carbendazim	+ Carbendazim
Grain yield (ton ha^{-1})	8.07 ± 0.41^{B}	12.17 ± 0.47^{A}	9.41 ± 0.73^{b}	10.83 ± 0.59^{a}
Grain N (mg g^{-1} DW)	13.66 ± 0.45^{B}	19.45 ± 0.39^{A}	16.10 ± 0.91^{a}	17.00 ± 0.79^{a}
Grain P (mg g^{-1} DW)	5.65 ± 0.27^{B}	7.38 ± 0.26^{A}	6.26 ± 0.41^{a}	6.76 ± 0.26^{a}

to yield, but for shoot N this positive relationship was lost at V6 (Table 5, Fig. 3).

3.5. Pot experiment

The pot bioassay for measuring the effect of soil biota and fertilization on early maize growth and nutrition showed that AM arbuscular colonization (V3: non-autoclaved 8.3 ± 0.82, autoclaved 0.5 ± 0.3; V6: non-autoclaved 17.6 ± 2.1, autoclaved 0.25 ± 0.16) and by other fungi (V3: non-autoclaved 10.8 ± 1.3, autoclaved 0.25 ± 0.16; V6: non-autoclaved 16 ± 1.86, autoclaved 0.38 ± 0.18) was minimal in the autoclaved treatments at both stages. In non-autoclaved soil treatments, root colonization by other fungi was close to the AM fungi colonization and increased from stage V3 to V6, as observed in the field experiment. At V3, already the soil disinfection positively affected maize biomass, but no effect of fertilization was observed (Fig. 4a). At V6, the differences increased, and the disinfection increased biomass by 53% in the absence of fertilization and by 247% combined with fertilization, as compared with the non-autoclaved soil (Fig. 4a). Shoot P and N concentrations were higher in plants growing in the non-autoclaved than in the autoclaved soil at V3, and no differences were observed at V6. At V3, the critical P concentration was reached in all treatments (Fig. 4b). In contrast, treatments in non-autoclaved soil and without fertilization did not reach the critical shoot N (20–30 mg g^{-1}) concentration (Fig. 4c).

4. Discussion

The results presented here revealed that the hypothesized functional relationship was disrupted a few days after plant emergence. Our prediction was that early AM fungal establishment and intact extraradical networks would lead to high early N and P uptake and biomass, which would eventually result in improved yield and better nutritional quality of the grain. Fungicide application reduced early fungal colonization in

Table 5
Pearson correlation coefficients between selected plant and microbial predictor and response variables measured. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Only the NLFA 16:1ω5 is used as an estimator of the AM fungal abundance, as the PLFA fraction may also reflect bacterial abundance.

Parameter	Stage	Shoot N	Shoot P	Shoot DW	Yield
AM arbuscular colonization	V3	−0.01 ns	0.07 ns	0.04 ns	−0.22 ns
	V6	−0.33 ns	−0.32 ns	0.10 ns	−0.07 ns
	V10	0.09 ns	−0.26 ns	0.18 ns	0.26 ns
Other fungi colonization	V3	0.09 ns	0.08 ns	0.06 ns	−0.22 ns
	V6	−0.33 ns	−0.14 ns	−0.13 ns	−0.22 ns
	V10	−0.16 ns	−0.05 ns	0.22 ns	−0.07 ns
Total fungal colonization	V3	−0.06 ns	−0.04 ns	−0.08 ns	−0.40 *
	V6	−0.43 *	−0.27 ns	−0.26 ns	−0.42 *
	V10	−0.27 ns	−0.21 ns	0.06 ns	−0.30 ns
Soil NLFA 16:1 ω5	V6	0.03 ns	−0.27 ns	−0.1 ns	−0.26 ns
Shoot P concentration	V3	–	–	0.79 ***	0.71 ***
	V6	–	–	0.50 **	0.70 ***
	V10	–	–	0.05 ns	0.69 ***
	R2	–	–	0.60 ***	0.66 ***
Shoot N concentration	V3	–	–	0.60 ***	0.47 **
	V6	–	–	0.23 ns	0.31 ns
	V10	–	–	−0.34 ns	0.44 *
	R2	–	–	0.43 *	0.54 **

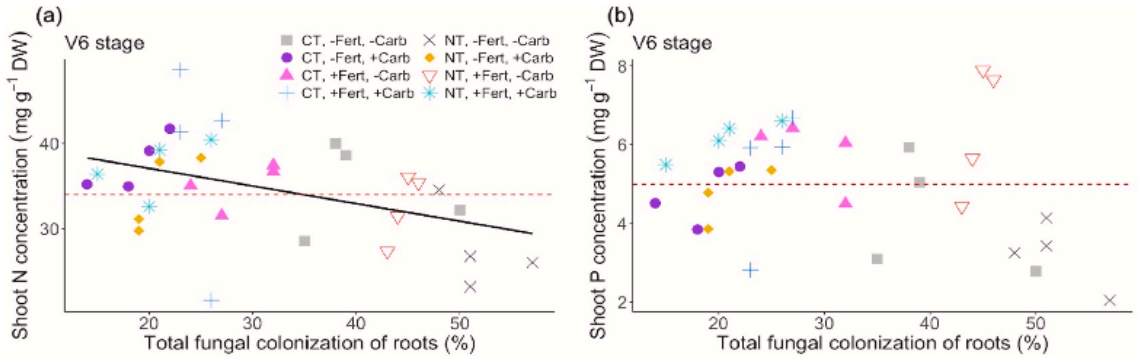

Fig. 2. Relationship between (a) shoot N ($R^2 = 0.16, p = 0.01$), (b) shoot P ($R^2 = 0.04, p = 0.14$) concentration and total fungal colonization (any fungal structures in roots)) at stage V6. Each point represents the mean value (n = 5 plants) obtained from a single replicate plot of each treatment combination. NT: No Tillage; CT: Conventional Tillage; Carb: Carbendazim; Fert: Fertilization. The dotted lines represent the critical shoot N or P concentration for this stage.

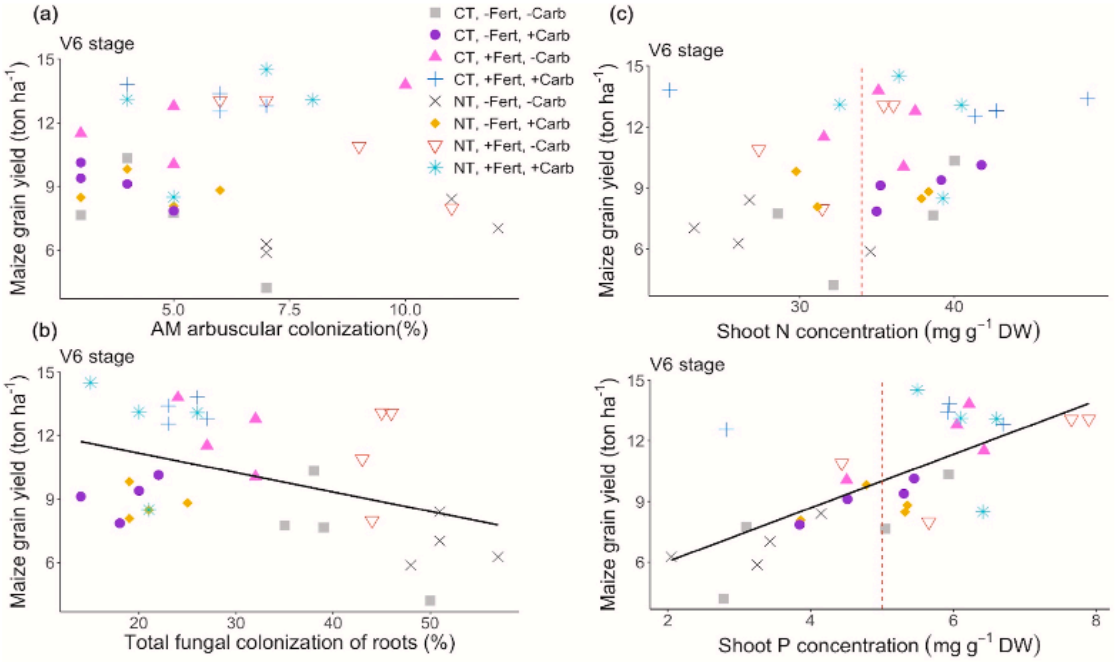

Fig. 3. Relationship between maize grain yield and (a) AM arbuscular colonization ($R^2 = 0.03$, $p = 0.72$), (b) total fungal colonization (any fungal structures in roots) ($R^2 = 0.15$, $p = 0.02$), (c) shoot N concentration ($R^2 = 0.07$, $p = 0.09$), (d) shoot P concentration ($R^2 = 0.47$, $p < 0.001$) at V6 stage. Each point represents the mean value (n = 5 plants) obtained from a single replicate plot per treatment. NT: No Tillage; CT: Conventional Tillage; Carb: Carbendazim; Fert: Fertilization. The dotted lines represent the critical shoot N or P concentration for this stage.

roots but, instead of decreasing nutrient uptake, increased it. Root fungal colonization, which correlated with apparently ephemeral negative effects on maize early N nutrition (and to a lesser extent P) and did not significantly affect maize early growth, was also related to a reduction in final yield. Although a similar negative relation between mycorrhizal colonization, early shoot N and yield reduction has already been reported in maize (Wang et al., 2018), this study's detailed following of early events in root fungal colonization development and plant nutrition showed that the early shoot N and yield reductions were positively related to colonization by AM fungi, other fungi, and both types of colonization.

4.1. Did tillage, carbendazim and fertilization affect early mycorrhizal development and function?

The initial AM fungal development was reduced, at least at the V3 and V6 stage, by tillage and carbendazim, as in other studies (Jansa et al., 2006; O'Connor et al., 2009). Despite the very high dose of fungicide applied, AM colonization recovered rapidly after the initial inhibition thus achieving our goal of delaying it instead of entirely suppressing it.

Surprisingly, after V3 abundant root colonization by other fungi than AM fungi was observed. The other fungi seemed to be predominantly ascomycetes, and most of them resembled dark septate endophytic (DSE) fungi (Fig S2). They were not classified, however, or their pathogenicity, because this was beyond the scope of our study. DSE fungi commonly overlap and interact with mycorrhizal fungi, and some meta-analyses emphasize the variability in host responses to DSE, showing context-dependent scenarios ranging from mutualism to parasitism (Kageyama et al., 2008; Mandyam and Jumpponen, 2015). In addition to this potential overlap in functional interaction with the host plant, the early development of the other fungi was also reduced by carbendazim application, which hindered separating the role of each group of fungi in maize nutrition and development.

Maize N and P nutrition at V3 were, as hypothesized, better in the treatments without fungicide, and the only treatments that did not reach the critical shoot N and P concentrations were those with fungicide or tillage, and without fertilizer. This supported our hypothesis that tillage and carbendazim applications would reduce early nutrient absorption by maize. From V6, though, this changed and the treatments without fertilizer and without fungicide had the lowest N and P concentrations. In addition, the treatments with fungicide and fertilizer became the best nourished.

4.2. Did preserving AM mycelial networks lead to a positive relationship with maize development and yield parameters?

At the V3 stage, no tillage and no fungicide were related to the greater absorption of N and P. From V6, however, this functional relationship was unsupported. Some of the other predicted functional relationships, such as higher early nutrition (V3–V6) would increase the yield, occurred as predicted.

The wide range of nutritional differences generated by the combination of fertilization, tillage and fungicide treatments had surprisingly minor effects on maize biomass until silking. Fertilization effects were consistently dominant throughout the crop cycle. Conversely, tillage did not affect biomass accumulation or yield, and the small magnitude but positive carbendazim effects were concentrated in the yield. Reports where the fast establishment of AM fungi and early plant nutrition benefits did not translate into higher yields have been explained by other factors probably hiding or eliminating differences in grain yield that otherwise would have been observed (Miller, 2000; Galvez et al., 2001). This study provides evidence for one of those unknown factors that disrupted the expected functional relationships: the interactions between root-inhabiting fungi at low or moderate fertility. After V3, the low to medium soil N and P availability generated a negative relation between root fungal colonization and maize N and P nutrition. In some treatments, plants became nutrient deficient, which resulted in a

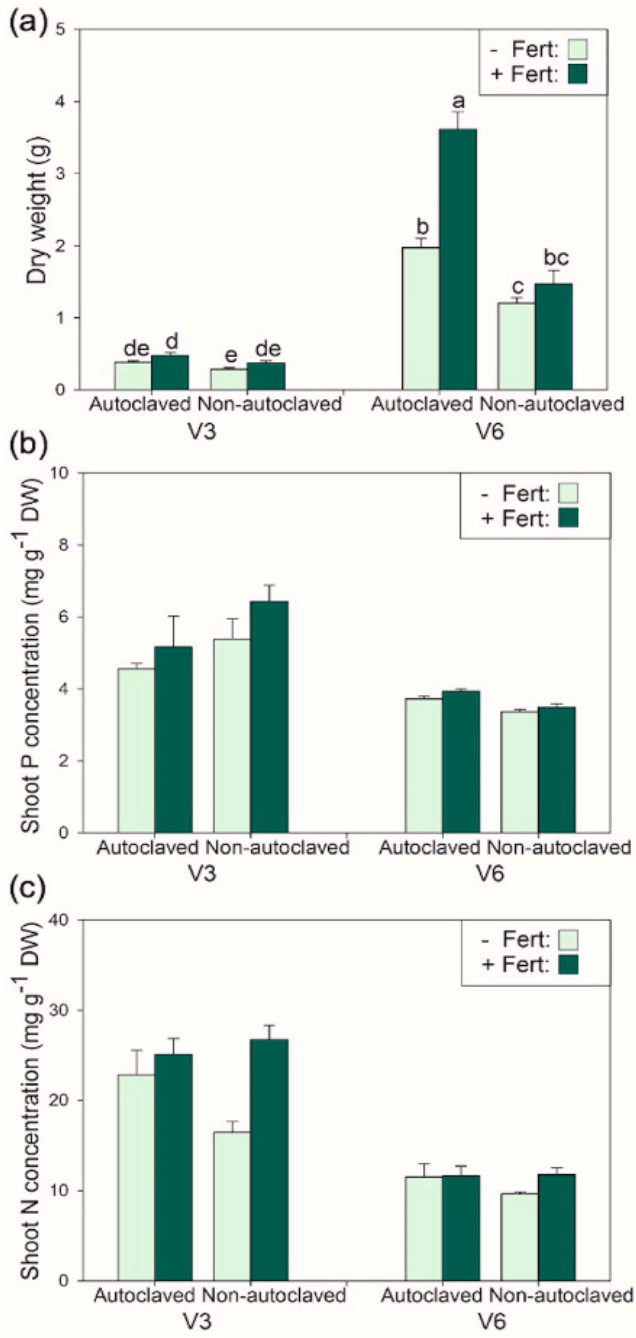

Fig. 4. Effects of fertilization and soil autoclaving treatments at V3 and V6 stages of plant development on (a) shoot dry weight, (b) shoot P concentration and (c) shoot N concentration. Different letters indicate significant differences ($p < 0.05$) between means according to Tukey's Test. Fert: Fertilization.

negative relation between the fungal colonization of maize roots and yield parameters.

4.3. Mycorrhizal and other root-inhabiting fungi are seemingly involved in maize yield loss

We considered the possibility that carbendazim affected plant N nutrition directly through microbial N degradation. Soil available N 17 days after the addition of carbendazim (close to V3), however, showed no significant differences between the treatments without and with carbendazim (-Carb: 42.1 ± 1.1; +Carb: 42.9 ± 1.3 mg soil inorganic N kg^{-1} soil). Thus, the possibility that carbendazim directly contributed to early plant N nutrition was discarded. Several studies (Van Gestel et al., 2004; Wang et al., 2018) have reported that even at high concentrations carbendazim fungicide did not contribute N to the soil. Although it has been suggested that AM fungi and roots may compete for N in low N soils (Reynolds et al., 2005; Püschel et al., 2016; Wang et al., 2018), this seems unlikely because even when soil N and P contents become very low for plants, they are sufficiently high for AM fungi to supply their own metabolic demands (Gavito and Olsson, 2008a,b). The present study was not conducted at extremely low N conditions where N availability could be a major driver of plant-AM fungi competition at early plant developmental stages. This may, however, not be the case for root endophytic fungi that do not form mycelium outside the roots (Mandyam and Jumpponen, 2005; Brundrett, 2006).

The complementary pot experiment, which included a soil autoclaving treatment, supports the interpretation in this current work that the N and yield reductions seem more related to a soil health problem than to a nutrient limitation inducing N competition. In the absence of fertilization, plants growing in fresh field soil were undernourished and small, but those growing in autoclaved soil were not. Additionally, the fertilizer applied could not be used for growth when the soil was not autoclaved and could not fully compensate for soil biota effects. Furthermore, the maize biomass was much higher when autoclaving and nutrients were provided. Unfortunately, the experimental design used here does not allow either separating the effects of AM fungi and other root biota but provides further evidence that other root-inhabiting fungi might be involved in maize nutrition and yield depressions.

As previously suggested, a final possibility is that excessive fertilization has led to a community of opportunistic AM fungi (Verbruggen and Kiers, 2010) and other fungi that do not cause visible damage but drain C and mineral nutrients and affect yield the most. Dissecting whether AM fungi, other root-inhabiting fungi, or both are involved in the nutrient reductions in shoots and the yield depressions observed in some field studies requires conducting controlled and field experiments with relevant measurements to identify and clearly separate the effects of the different groups of root-inhabiting fungi.

5. Conclusions

Results revealed a disruption in the hypothesized functional relationship between AM fungi and maize a few days after plant emergence which changed the course of the functional relationships expected. The early interplay between nutrient availability, AM fungi, other root fungi, and maize nutrition reduced yield without causing disease symptoms or growth depression. The evidence reported here indicates unnoticed interactions of common agronomic practices and nutrient availability that seemingly exacerbate the cost of root-inhabiting microorganisms for maize plants and inhibit mycorrhizal contributions to nutrition and yield. These interactions should be further investigated regarding the efficient management of mycorrhizal associations and capturing their benefits in biodiverse and sustainable agroecosystems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100525.

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Supplemental material

Table S1. *P* values and significance for the main effects of Tillage (T), Fertilization (F), Carbendazim (C) and Stage of development (S) factors and their factor interactions on all variables measured at three, four or five stages of maize development. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

	Stage	T	F	C	S	T*F	T*C	F*C	T*S	F*S	C*S	T*F*C	T*F*S	T*C*S	F*C*S	T*F*C*S
Root colonization																
Arbuscular	V3,V6,V10	***	*	***	***	*	***	*	**	0.9	***	0.7	0.8	**	0.6	0.6
Other fungi	V3,V6,V10	0.7	0.5	***	***	*	***	0.2	0.5	1	0.1	0.1	0.7	0.6	0.7	0.1
Total	V3,V6,V10	***	*	***	***	*	***	***	**	0.9	***	***	0.5	0.07	0.2	0.7
Plant growth parameters																
Shoot DW	V3,V6,V10,R2,R6	0.7	***	0.1	**	0.8	0.9	0.2	0.6	***	0.5	0.4	0.3	0.3	0.7	0.8
Plant Phosphorus																
Shoot P concentration	V3,V6,V10,R2	0.5	***	0.1	***	0.8	0.4	0.7	0.7	***	0.2	0.1	0.4	0.8	0.4	0.3
Total shoot P	V3,V6,V10,R2	1	***	0.1	***	0.9	0.7	0.4	0.9	***	0.1	0.5	0.7	0.8	0.9	0.5
Plant Nitrogen																
Shoot N concentration	V3,V6,V10,R2	0.1	***	*	***	0.9	1	0.8	0.6	0.1	0.4	0.6	0.5	0.5	0.2	0.8
Total shoot N	V3,V6,V10,R2	0.1	***	0.1	***	0.7	0.1	0.3	0.5	**	0.1	0.4	0.2	0.1	1	0.7
Carbendazim responses																
Shoot P concentration	V3,V6,V10,R2	0.8	0.1		**	0.2	-	-	0.9	0.1	-	-	0.2	-	-	-
Total shoot P	V3,V6,V10,R2	0.7	0.1	-	**	0.2	-	-	0.6	0.7	-	-	0.1	-	-	-
Shoot N concentration	V3,V6,V10,R2	0.9	0.8	-	0.1	0.5	-	-	0.4	0.1	-	-	0.1	-	-	-
Total shoot N	V3,V6,V10,R2	0.8	0.6	-	**	0.2	-	-	*	0.9	-	-	0.4	-	-	-

Table S2. Means (± SE, n=4) for variables measured at different developmental stages in the tillage, fertilization and carbendazim treatment combinations. Soil N and P concentrations: mg kg^{-1} DW, AM fungi biomarkers: nmol g^{-1} soil, shoot DW: (g $plant^{-1}$), total shoot N and P: mg $plant^{-1}$ DW, yield: ton ha^{-1}, grain N and P concentrations: mg g^{-1} DW.

Treatment	Stage	Soil available P	Soil available N	NLFA	PLFA	Shoot Dry Weight	Total shoot P	Total shoot N	Grain yield	Grain P conc.	Grain N conc.
CT, -Fert, -Carb	V3	27 ±4.2	42 ±2.3	-	-	0.34 ±0.04	1.5 ±0.27	13 ±1.4	-	-	-
NT, -Fert, -Carb	V3	31 ±1.7	42 ±0.80	-	-	0.34 ±0.03	1.5 ±0.35	13 ±1.6	-	-	-
CT, +Fert, -Carb	V3	177 ±14	83 ±6.4	-	-	0.63 ±0.07	4.6 ±0.51	29 ±2.5	-	-	-
NT, +Fert, -Carb	V3	199 ±14	88 ±7.6	-	-	0.65 ±0.07	4.7 ±0.38	29 ±4.5	-	-	-
CT, -Fert, +Carb	V3	31 ±2.8	41 ±1.7	-	-	0.37 ±0.02	1.2 ±0.11	12 ±0.44	-	-	-
NT, -Fert, +Carb	V3	34 ±2.3	44 ±1.8	-	-	0.36 ±0.03	1.4 ±0.23	13 ±1.8	-	-	-
CT, +Fert, +Carb	V3	164 ±41	85 ±3.3	-	-	0.57 ±0.04	4.6 ±0.25	28 ±1.9	-	-	-
NT, +Fert, +Carb	V3	215 ±34	82 ±3.8	-	-	0.58 ±0.06	3.8 ±0.44	26 ±2.9	-	-	-
CT, -Fert, -Carb	V6	-	-	3.4 ±0.70	2.5 ±0.30	2.7 ±0.36	13 ±3.4	97 ±20	-	-	-
NT, -Fert, -Carb	V6	-	-	4.9 ±2.1	2.7 ±0.31	1.7 ±0.17	8.8 ±1.7	47 ±3.3	-	-	-
CT, +Fert, -Carb	V6	-	-	3.0 ±0.40	2.7 ±0.40	4.1 ±0.33	24 ±3.0	144 ±15	-	-	-
NT, +Fert, -Carb	V6	-	-	2.8 ±1.0	2.9 ±0.22	4.2 ±0.40	26 ±1.1	133 ±4.4	-	-	-
CT, -Fert, +Carb	V6	-	-	3.8 ±0.41	2.5 ±0.20	2.5 ±0.30	12 ±1.6	94 ±12	-	-	-
NT, -Fert, +Carb	V6	-	-	3.5 ±0.70	3.6 ±0.10	2.6 ±0.42	12 ±2.4	87 ±16	-	-	-
CT, +Fert, +Carb	V6	-	-	3.5 ±1.7	2.5 ±0.30	4.5 ±0.40	29 ±3.9	169 ±26	-	-	-
NT, +Fert, +Carb	V6	-	-	5.8 ±1.4	2.9 ±0.23	4.5 ±0.18	28 ±1.5	169 ±13	-	-	-
CT, -Fert, -Carb	V10	-	-	-	-	66 ±15	175 ±48	1330 ±288	-	-	-
NT, -Fert, -Carb	V10	-	-	-	-	60 ±8.5	171 ±39	1288 ±178	-	-	-
CT, +Fert, -Carb	V10	-	-	-	-	87 ±14	307 ±47	1946 ±280	-	-	-
NT, +Fert, -Carb	V10	-	-	-	-	85 ±17	335 ±70	1854 ±396	-	-	-
CT, -Fert, +Carb	V10	-	-	-	-	56 ±6.0	175 ±22	1259 ±81	-	-	-
NT, -Fert, +Carb	V10	-	-	-	-	66 ±9.1	210 ±30	1411 ±166	-	-	-
CT, +Fert, +Carb	V10	-	-	-	-	79 ±12	308 ±47	2043 ±172	-	-	-
NT, +Fert, +Carb	V10	-	-	-	-	71 ±12	269 ±45	1740 ±192	-	-	-
CT, -Fert, -Carb	R2	-	-	-	-	172 ±32	363 ±118	2479 ±917	-	-	-
NT, -Fert, -Carb	R2	-	-	-	-	223 ±18	430 ±127	2519 ±561	-	-	-
CT, +Fert, -Carb	R2	-	-	-	-	220 ±16	544 ±32	3570 ±295	-	-	-
NT, +Fert, -Carb	R2	-	-	-	-	253 ±9.8	614 ±38	4016 ±361	-	-	-
CT, -Fert, +Carb	R2	-	-	-	-	218 ±11	567 ±66	3351 ±387	-	-	-
NT, -Fert, +Carb	R2	-	-	-	-	207 ±20	477 ±77	2566 ±422	-	-	-
CT, +Fert, +Carb	R2	-	-	-	-	276 ±8.5	707 ±33	4771 ±428	-	-	-
NT, +Fert, +Carb	R2	-	-	-	-	281 ±7	827 ±88	4167 ±818	-	-	-
CT, -Fert, -Carb	R6	-	-	-	-	271 ±37	-	-	7.5 ±1.3	5.5 ±0.86	13 ±1.4
NT, -Fert, -Carb	R6	-	-	-	-	253 ±18	-	-	6.9 ±0.56	5.0 ±0.59	13 ±1.1
CT, +Fert, -Carb	R6	-	-	-	-	372 ±17	-	-	12 ±0.81	7.1 ±0.44	19 ±1.3
NT, +Fert, -Carb	R6	-	-	-	-	387 ±60	-	-	11 ±1.2	7.5 ±0.73	19 ±0.82
CT, -Fert, +Carb	R6	-	-	-	-	291 ±9.4	-	-	9.1 ±0.47	6.0 ±0.05	14 ±0.37
NT, -Fert, +Carb	R6	-	-	-	-	289 ±12	-	-	8.8 ±0.37	6.1 ±0.28	14 ±0.68

CT, +Fert, +Carb	R6	-	-	-	-	394 ±3.7	-	-	13 ±0.29	7.6 ±0.36	20 ±0.30
NT, +Fert, +Carb	R6	-	-	-	-	367 ±42	-	-	12 ±1.3	7.3 ±0.70	20 ±0.55

Table S3. Pearson correlation coefficients between selected plant and microbial predictors and response variables measured. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Only the NLFA 16:1ω5 is shown as an estimator of the AM fungal abundance, as the PLFA fraction can also reflect bacterial abundance.

		Plant growth		Nutrition			
Parameter	Stage	Shoot DW	Yield	Shoot P conc.	Total P	Shoot N conc.	Total N
AM arbuscular	V3	0.04 ns	-0.22 ns	0.07 ns	0.06 ns	-0.01 ns	0.02 ns
colonization	V6	0.10 ns	-0.07 ns	-0.32 ns	0.09 ns	-0.33 ns	0.01 *
	V10	0.18 ns	0.26 ns	-0.26 ns	0.28 ns	0.09 ns	0.26 ns
Other Fungi	V3	0.06 ns	-0.22 ns	0.08 ns	0.09 ns	0.09 ns	0.09 ns
colonization	V6	-0.13 ns	-0.22 ns	-0.14 ns	-0.01 ns	-0.33 ns	-0.22 ns
	V10	0.22 ns	-0.07 ns	-0.05 ns	0.17 ns	-0.16 ns	0.17 ns
Total root	V3	-0.08 ns	-0.4 *	-0.04 ns	-0.06 ns	-0.06 ns	-0.08 ns
colonization	V6	-0.26 ns	-0.42 *	-0.27 ns	-0.19 ns	-0.43 *	-0.36 *
	V10	0.06 ns	-0.30 ns	-0.21 ns	-0.03 ns	-0.27 ns	-0.04 ns
NLFA 16:1 ω5	V6	-0.10 ns	-0.26 ns	-0.27 ns	-0.12 ns	0.03 ns	-0.07 ns
Shoot P concentration.	V3	0.79 ***	0.71 ***	-	-	-	-
	V6	0.50 **	0.70 ***	-	-	-	-
	V10	0.05 ns	0.69 ***	-	-	-	-
	R2	0.60 ***	0.66 ***	-	-	-	-
Shoot N concentration	V3	0.60 ***	0.47 **	-	-	-	-
	V6	0.23 ns	0.31 ns	-	-	-	-
	V10	-0.34 ns	0.44 *	-	-	-	-
	R2	0.43 *	0.54 **	-	-	-	-

Grain P concentration	R6	-	0.73 ***	-	-	-	-
Grain N concentration	R6	-	0.85 ***	-	-	-	-

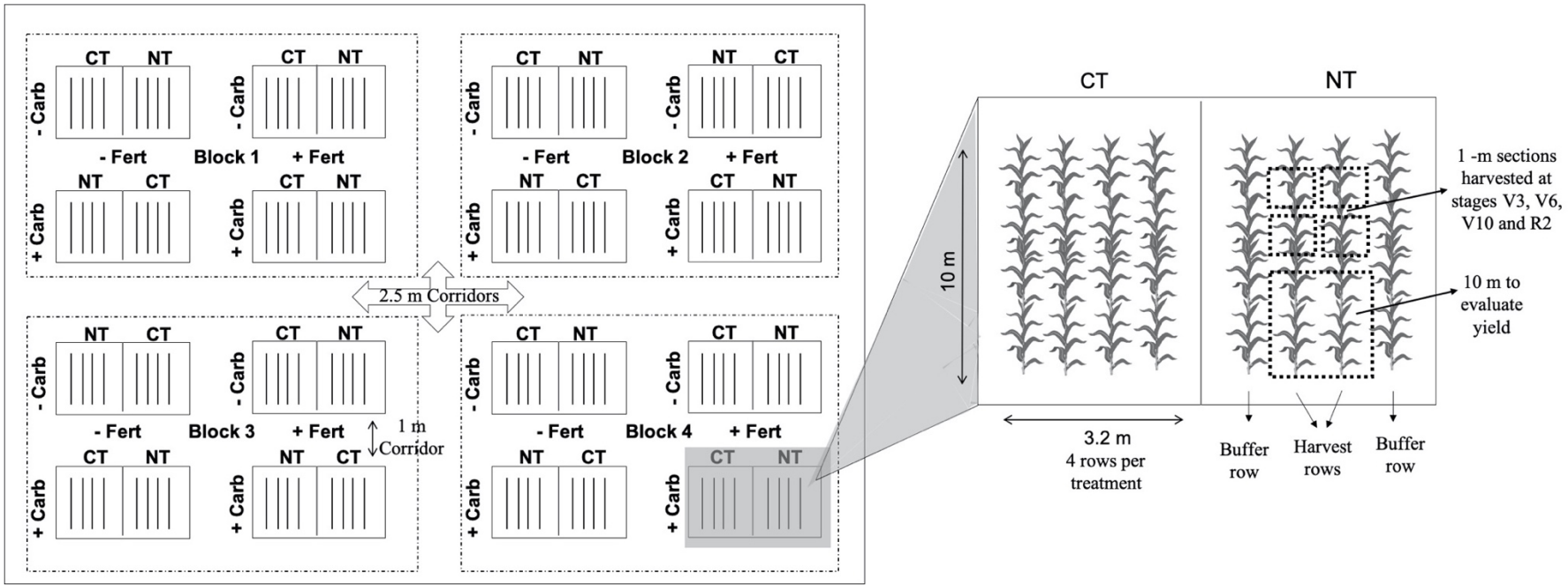

Figure S1. Diagram of the experimental plot showing soil tillage, fertilization and Carbendazim treatments used to modulate early AM development and functioning. Each block contained one experimental unit of the eight treatment combinations.

CAPÍTULO III

The interaction of agronomic practices and mycorrhizal networks on gas exchange of maize at early developmental stages

Abstract

Arbuscular mycorrhizal (AM) fungi may stimulate host plant gas exchange by supplying mineral nutrients and by constituting a carbon sink. Conventional tillage and fertilization in agricultural systems could influence crop gas exchange through their effects on AM fungi. However, there is scarce field evidence for the effects of mycorrhizae on crop gas exchange and how they are affected by agronomic practices. We used tillage, fertilization, and fungicide, to modulate early (stages V3, V6, and V10; third, sixth and tenth leaf fully emerged with visible collar) mycorrhizal root colonization and extraradical network integrity and study functional interactions between native AM fungi and maize gas-exchange parameters in a field experiment. We expected maximum influence of mycorrhizal root colonization and network preservation on gas-exchange at V6 when both symbionts were on active growth and exchange. Tillage reduced the transpiration rate (E). Fertilization increased E, photosynthetic rates (Asat) and whole plant C assimilation at different stages. At V6, fungicide and tillage reduced the estimated photosynthetic capacity parameters of Jmax and Vcmax. At V6, high root colonization by AM fungi and other fungi was related to reduced maize N nutrition and lead to negative relations with photosynthesis. At V10, most negative relations turned positive again and differences in instantaneous gas-exchange rates disappeared. Our results provide evidence for early interactions between tillage, AM fungi, other root-inhabiting fungi and maize gas-exchange measurements, and for the interaction between AM root colonization and network integrity and photosynthetic capacity of maize.

Keywords: arbuscular mycorrhizal fungi; gas exchange; transpiration; stomatal conductance; *Zea mays*

1. Introduction

Photosynthetic efficiency over the growing season is a key determinant of the productivity of crops (Nowicka et al. 2018; Simkin et al. 2019) and plants in general. There is wide evidence suggesting that arbuscular mycorrhizal (AM) fungi colonizing plant roots stimulate their host plants' gas-exchange relationships by improving nutrition and hydration (Augé et al. 2015; Bulgarelli et al. 2017; Khan et al., 2022). It has also been suggested that the C-sink strength generated by AM fungal metabolism rapidly removes C from the phloem and prevents down-regulation of photosynthesis by photosynthate accumulation (Kaschuk et al. 2009; Schweiger et al. 2014; Řezáčová et al. 2018; Bhantana et al., 2021). In these two ways, acting as C sink and contributing to plant nutrition, AM fungi might to some extent compensate their use of plant C by stimulating C assimilation rate and assimilation capacity. There is, however, still scarce evidence for the relevance of these mechanisms in field conditions as usually the complex C assimilation studies are carried out under highly controlled conditions (Gavito et al. 2019). As a result, the efficiency of the mycorrhizal symbiosis under agronomic management where human interventions disturb natural biotic and abiotic conditions is still poorly understood and field evidence supporting the selection of management practices that maximize crop benefits or minimize crop costs of hosting AM fungi is urgently needed (Rillig et al. 2019).

Alteration of soil conditions by tillage is a factor that, through the disturbance of surface layers in the soil profile, may significantly alter the development and function of AM associations and, consequently, soil and crop nutrient dynamics (Jansa et al. 2006; Bowles et al. 2016; De la Cruz-Ortiz et al., 2020; Gutiérrez-Núñez et al., 2022). Conventional tillage may exacerbate the plant carbon drain by AM fungi having to rebuild their fragmented mycelial networks at the beginning of each growing cycle, delay nutrient and water transfer,

and cause a nutritional and/or growth depression. Tillage might, on the other hand, alleviate early C drainage for a low-C budget plant that becomes connected to an extensive mycelial network by delaying the time span to establish the network until the plant has reached enough biomass to support it (Jansa 2002; Valentine et al. 2013). Tillage disturbance may thus have important consequences on mycorrhizal development and functioning in crops, especially for those crops with root systems having low nutrient uptake capacity at early developmental stages like maize.

In maize, fast development of the intraradical mycelium and integrity of the extraradical mycelium of AM fungi connected to an extensive soil mycelial network, has been linked to better early nutrition, which may eventually translate into higher yield (Miller et al. 1995; Mozafar et al. 2000; Grant et al. 2001). Although some studies under controlled conditions have examined maize nutritional and gas-exchange responses to AM fungal colonization (e.g. Zhu et al. 2012, 2015; Mathur et al. 2018; Huang et al., 2020), few studies have demonstrated how they work under field conditions. Improved early nutrition by forming AM associations should lead to improved C assimilation, growth and eventually yield and grain quality but most field evidence is so far found only for improved early nutrition and yield benefits remain scarcely reported and inconclusive. This study aimed to contribute to the understanding of mycorrhizal functioning under agricultural management by investigating how the disruption of AM network integrity and function by tillage influenced gas exchange, and particularly C assimilation, and how these variables were related to maize nutrition at early stages of maize development. Fertilization and fungicide treatments were also included to further modulate development and function of the associations formed by the native AM fungal communities in an agricultural field.

We hypothesized that maintenance of the integrity and function of AM fungal networks would lead to stimulated gas exchange by improving early maize nutrition and providing a carbon sink strength that increased photosynthesis (Figure 1). However, we expected that physiological plant-fungal coupling would change along early development of both symbionts, as shown by Schweiger et al. (2014) under controlled conditions. We proposed a conceptual framework for photosynthetic capacity stimulation and predicted responses at each developmental stage, as follows:

a) Predicted A/Ci responses in mycorrhizal and non-mycorrhizal plants (Fig. 1a). Besides instantaneous photosynthetic rates, the most commonly measured parameter, key photosynthetic capacity parameters allow evaluating how the biochemical and biophysical components of photosynthesis affect the net assimilation of C (Wullschleger 1993; Sharkey et al. 2007). Main limitations of photosynthesis can be explored from C assimilation/CO_2 internal concentration (A/Ci) fitted curves: 1) the maximum carboxylation rate (Vcmax) estimates if the reaction is limited by substrate for the regeneration of RuBP in the Calvin cycle, 2) the rate of electron transport (Jmax) estimates if the reaction is limited by energy in molecules with P, and 3) the triose-phosphate utilization (TPU) that estimates the use of triose phosphate for the synthesis and export of sugars through the phloem and indicates if photosynthates produced are continuously removed. Unfortunately, it is not possible to determine whether C4 plants have a TPU-limited state, since the gas exchange characteristic that is used to diagnose TPU limitation (the CO_2 and O_2 dependence that results from the variation in the ratio of carboxylation to oxygenation) is not observed in C4 photosynthesis (McClain and Sharkey, 2019), and this parameter is not included in our hypothetical scenarios (following points b, c and d). The mycorrhizal stimulation of photosynthetic capacity is expected to arise, in a summarized way, from improved N and P (Vcmax), and P

(Jmax) nutrition (Kaschuk et al. 2009; Bulgarelli et al. 2017; Gavito et al. 2019; Yang et al., 2022). At early and intermediate plant developmental stages, photosynthesis may be mainly limited by N and P controlling Rubisco activity, electron transport rates, and consequently ribulose-1,5-biphosphate regeneration processes, and AM symbioses may reduce these limitations through improved plant nutrition. At later stages, plants heading towards reproduction reduce C supply to AM fungi as C is preferentially allocated to reproductive structures, mycorrhizal stimulation of gas exchange and photosynthetic capacity declines.

b) Initial stage (V3, Fig. 1b), early root development and AM colonization establishment when plants begin to need nutrients after exhausting seed reserves, shortly after emergence. Mycelium disruption caused by tillage was expected to exacerbate the C demand of AM fungi to rebuild the network while root colonization and nutrient transfer were expected to be delayed by tillage and additionally by fungicide application. The preserved mycelium network with no-tillage was predicted to increase early N and P transfer to the plant and thereby also gas exchange and photosynthetic capacity (mainly as Vcmax and Jmax). AM root colonization was predicted to be positively related to nutrition, Asat, Jmax and Vcmax. Plants with no-tillage, without fungicide, would show higher colonization, nutrition, gas-exchange and photosynthetic capacity than plants with conventional tillage and fungicide application. Fertilization was predicted to be important at this stage and fungicide application was predicted to have a negative effect on most variables.

c) Root proliferation and intraradical colonization well established (V6, Fig. 1c): Peak of mycorrhizal benefit. Improved nutrition and overall physiology allow maintaining high gas exchange and growth. We expected the AM symbiosis to improve gas-exchange relations and photosynthetic capacity mainly by increasing P nutrition (most limiting nutrient) and consequently Jmax as plants accelerated growth. Plants with no-tillage would have higher

AM colonization, better nutrition, and higher gas exchange and photosynthetic capacity (thus higher Asat, stomatal conductance, transpiration, and particularly Jmax) than plants with conventional tillage. Fungicide might still have a negative on colonization, nutrition and gas exchange, but less pronounced than at V3. At this stage, we expected fertilization to become highly relevant to meet critical nutrient concentrations and the highest mycorrhizal benefits in plants with higher mycorrhizal colonization depending on the extent of nutrient limitation. If high, fertilized plants would be those achieving the highest values.

d) Rapid vegetative growth approaching the reproductive stage (V10, Fig. 1d). Mycorrhizal nutritional benefits were expected to decrease as roots proliferated and could scavenge more nutrients and water. Sink-strength benefits might increase depending on plant nutrition. Tillage and fungicide effects were expected to decrease and fertilization effects could still be important depending on reaching critical nutrient concentrations. The fungal C-sink strength was expected to be beneficial in best nourished plants and neutral to negative with low plant nutrition. As plants approached reproduction, C assimilation differences were expected to reduce or disappear at this point.

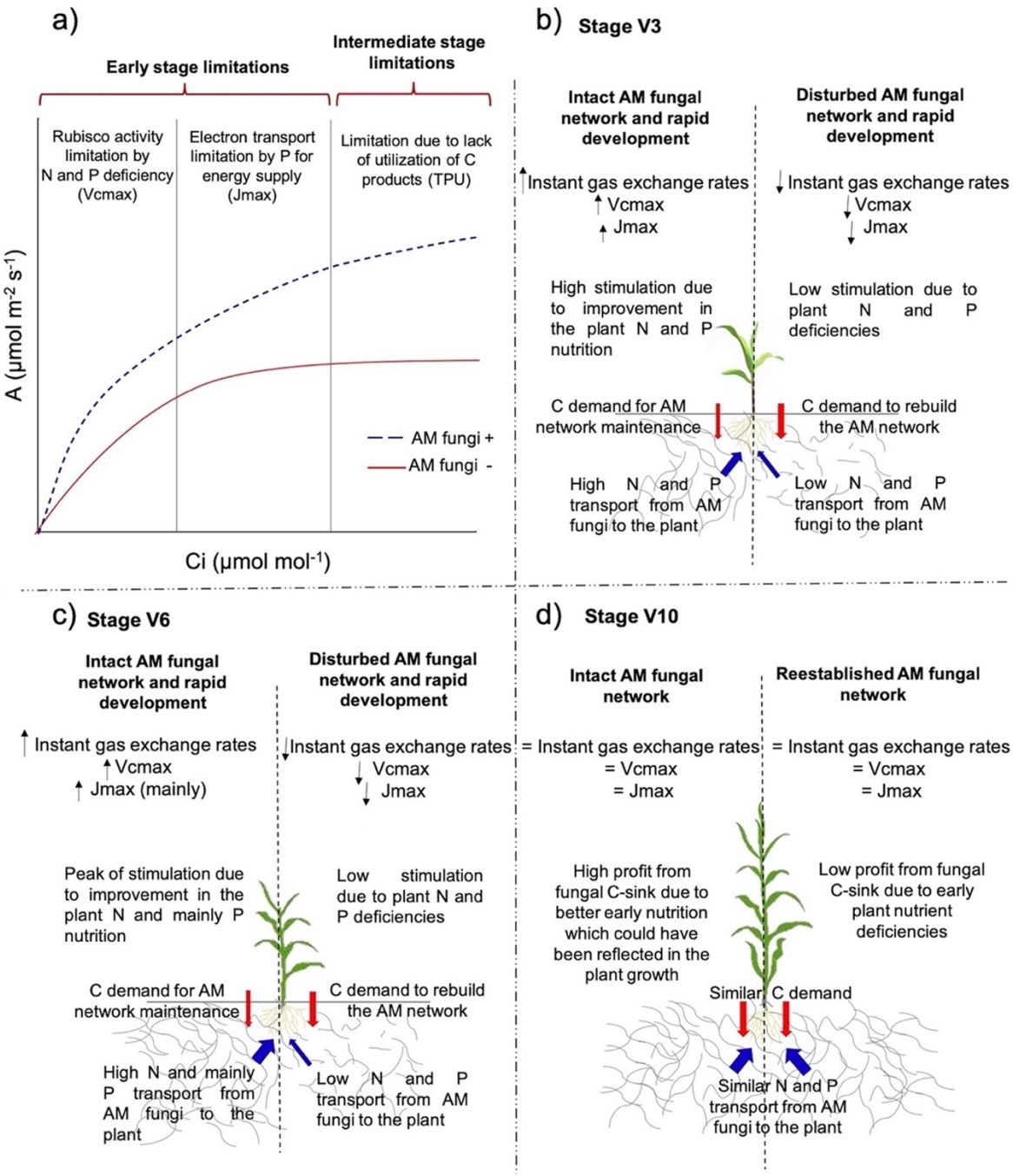

Figure 1. Conceptual framework depicting our hypotheses and predictions for photosynthetic capacity parameters (a) and mycorrhizal nutritional and gas-exchange benefits at the three developmental stages of maize examined, V3 (b), V6 (c) and V10 (d). The arrows indicate

the direction of the response of the gas exchange parameters and the magnitude of the nutrient transfer.

2.Methods

2.1 Study site

This experiment was carried out in an agricultural plot (19° 50′ 57″ N, 101° 8′ 54″ W, altitude 1930 masl) in the municipality of Tarímbaro, Michoacán, in Mexico. The soil type is Vertisol (WRB), with clay texture, pH 8.0 (1:10 soil:water), 1.1% organic matter, 29 mg kg^{-1} soil available P and 42 mg kg^{-1} soil available N. The temperature varies from 8 to 29°C and the average annual precipitation is 625 mm. The plot had experienced high-inut conventional tillage management supported by irrigation. At the end of each cropping cycle, all biomass was usually removed from the field, and the soil was cultivated. After the previous maize cycle in 2018, plowing with disc harrow was implemented at approximately 10-15 cm depth and vetch (*Vicia sativa* L.) was established as green manure from December 2018 to April 2019, with drip irrigation for 8 hours every 12 days. No mineral fertilizers were added and weed control was manual.

2.2. Experimental design

In April 2019, a factorial block design with three main factors was established. Tillage was used to disrupt AM mycelial networks (conventional tillage, CT, and no-tillage, NT). NPK fertilization (without, -Fert, and with, +Fert) was used to examine nutritional benefits and potential growth depression in well-nourished plants. Carbendazim fungicide application was used to delay the early development of AM fungi (without, -Carb, and with, +Carb), as an

additional measure to further inhibit mycorrhizal growth and function. Each of the four blocks contained one experimental unit of the eight treatment combinations, resulting in 32 experimental units (Fig. S1). The experimental units consisted of plots with four rows, 10-m long, with 80 cm of distance between rows. To avoid edge effects, six rows surrounding all the experiment acted as a buffer zone and, in addition, all sampling and harvests were conducted only in the two central rows within each experimental unit.

In early April 2019, green manure from a previous vetch cover crop was incorporated to the soil at cultivation with a rotavator at 20 cm depth in conventional tillage treatments. In no-tillage treatments, vetch was only cut at the base and shoots were left covering the soil. The fungicide carbendazim, as the commercial product Tlaloc 50% P.H. ® (UPL Agro Mexico), was applied afterwards in mid-May. Although it is well known that it cannot completely inhibit AM fungi development and that it may also affect other fungi (e.g. Schweiger and Jakobsen 1998; Kahiluoto et al. 2000; O'Connor et al. 2009), carbendazim is the most practical approach to manipulate AM fungi development in large-scale field experiments and phytotoxic effects have been negligible on plant growth (Fitter and Nichols 1988; O'Connor et al. 2002; Shi 2010; Wang et al. 2018). The concentration applied in this study was 75 kg ha^{-1}, approximately 100 times the recommended dose of the product, as suggested by Schweiger and Jakobsen (1998) to almost completely inhibit the AMF activity in the field. Half of this dose was applied again two weeks after the first application to reinforce the effect of the fungicide. It was sprayed in solution over the soil until saturation 2 days before planting as it tends to stay on the soil surface. Treatments without carbendazim, were sprayed with sterile water. On May 29, H-318 (Milpal®) hybrid maize seeds were sown at a density of 80,000 ha^{-1}, with a 4-row disc planter. Simultaneously, a chemical fertilizer mixture containing 118 (N), 95 (P), 47 (K), 5 (Ca), 8 (Zn), 4 (Fe), 3 (B), Cu (1) kg ha^{-1} was

applied to the corresponding treatments below the seed. Fertilizer was applied again at V4 (30% of the initial dose), V10 (50%) and VR1 (20%) to fertilized treatments. Paraquat, tembotrione 4.4 and permethrin 9% products were used to control outbreaks of weeds and pests. Drip irrigation for 8 hours every 12 days continued to complement rainfall as required.

2.3. Gas exchange measurements

Gas-exchange measurements were made through instantaneous net CO_2 assimilation rates (Asat), transpiration rates (E), stomatal conductance (gs) and at stages V3, V6 and V10 (15, 25 and 48 d after sowing) to explore potential differences in C assimilation and water relations. Measurements of gs, E and Asat were carried out on the same day between 9:00 and 11:00, in five neighboring plants from one of the middle rows within each experimental unit and always on clear sky days. Measurements were made by blocks, on the youngest fully-developed leaf using a portable gas exchange system (Ciras-3, PP Systems, Amesbury, MA, USA) attached to a Parkinson leaf cuvette (PLC3) with a 4.5 cm^2 leaf chamber. CO_2 concentration inside the chamber was controlled by the CIRAS-3 at 400 μmol mol^{-1}, and measurements were made at light saturation and ambient temperature. The selected leaves were placed in the leaf chamber and were allowed to reach a stable state (approx. 2 min) before recording the Asat, gs and E rates. The five plants measured from each experimental unit were harvested, stored in plastic bags and kept refrigerated until processing in the laboratory. The five plants measured within each experimental unit were averaged to obtain a single value per replicate of each treatment.

Besides instantaneous (|) rates, A/Ci response curves were used to explore limitations in the photosynthetic capacity of maize plants. A/Ci curves were performed 1-2 days after the V3 and V6 instantaneous Asat measurements, using rapid assimilation-internal CO_2

concentration A/Ci response curves (RACiRs) (Stinziano et al. 2017), in one plant adjacent to those previously harvested in each experimental unit. Because of the overall small plant size complicating leaf clamping, and the long time required to obtain A/Ci curves even using the rapid method, photosynthetic capacity parameters were evaluated only in the treatments without fertilization at stage V3. We assumed nutritional differences at this stage were minor given that plants were just exhausting seed reserves. Thus, fertilization could not be tested as factor for photosynthetic capacity parameters measured at V3. At V6 all treatments were evaluated, and at V10 equipment failure prevented us from finishing enough measurements before plants reached the reproductive stage, so only V3 and V6 curves were analyzed. In A/Ci curves, CO_2 increased linearly from 50 to 1000 μmol mol-^{1} min^{-1} following an initial 2 min period of constant concentration of 50 μmol mol^{-1} with light saturation (photosynthetic photon flux density of 1800 μmol m^{-2} s^{-1}). Data were stored approximately every 2 s during the increase in reference CO_2. All curves were corrected with an empty chamber curve measurement made under the same conditions within each day of measurement. The data extracted for A/Ci curve were further processed for analysis using a curve-fitting program in Excel (Zhou et al., 2019) to determine Vcmax and Jmax parameters. This method is based on the fit of intensive A/Ci curves (with 6-8 more sampling points than the commonly used for C_3 species, as the A/Ci slope is very steep and the assimilation rate saturates quickly, and C_4 species have more photosynthetic parameters as the carbon concentration mechanism adds complexity) to a C_4 photosynthesis model (von Caemmerer 2000). The model uses non-linear fitting to obtain solutions that minimize the squared difference between the observed and predicted A, by varying five parameters (maximum phosphoenol pyruvate carboxylation (Vpmax), maximum rubisco carboxylation rate (Vcmax), the rate of electron transport for

Ribulose biphosphate regeneration (J), day respiration (Rd) and mesophyll conductance (gm)).

Like other fitting models, this model assumes that each of the limiting biochemical processes of photosynthesis can be described mathematically, and is expressed in different Ci values (von Caemmerer 2000). It also makes the assumption that light and dark reactions optimally co-limit the rate of assimilation and assume that electron transport is limited by ATP production and that there is a similar ATP cost for different subtypes of C4, and that the parameters, Kc, Ko, γ*, Kp, α and gbs are the same for different species. As with other C4 estimation methods (Yin et al. 2011; Ubierna et al. 2013; Bellasio et al. 2015), this model does not estimate triose phosphate (TPU) utilization.

There were four A/Ci curves per treatment, one in each of the four blocks and the measurements were made by blocks containing one replicate of each treatment. Every measured response curve was fitted separately.

2.4. Fungal colonization in roots and maize nutrition and biomass measurements

After photosynthetic and measurements, plants were harvested and processed to evaluate leaf area, shoot dry weight, shoot N and P concentrations, root colonization by AM fungi and other root-inhabiting fungi, as described and reported by Gutiérrez-Núñez et al. (2022). Leaf area was used to estimate the total potential CO_2 assimilation per plant (Asat * leaf area). We used this whole-plant photosynthesis measurement in addition to CO_2 assimilation rates since both P and N nutrition and AMF colonization can increase leaf area per unit of plant biomass and thus also plant C assimilation on a whole plant basis (Jia and Gray, 2004; Grimoldi et al., 2005).

N was determined by the semi-Kjeldahl method (Bremmer 1996) and P by the colorimetric molybdate method after ascorbic acid reduction (Murphy and Riley 1962). Root colonization by AM fungi and other microorganisms was determined using the line-intercept method described by McGonigle et al. (1990). The NLFA fractions of the fatty acid 16:1ω5 was used as indicator of the abundance of the external mycelium of AM fungi in soil (Olsson 1999). These root and soil colonization data were used to establish relations between the development of AM fungi and stomatal conductance, transpiration, instantaneous gas exchange rates and photosynthetic capacity.

2.5. Statistical analysis

Data were analyzed with linear mixed models (LMMs) including tillage, fertilization, carbendazim and time as fixed effects and block as a random effect. Models were fitted using 'lmer' function in the 'lme4' package for software R (Bates et al. 2015). In order to identify the relevant predictors, model reduction was performed by fitting all the possible nested models and selecting the best models as those with the lowest Akaike Information Criterion (AIC) values. When relevant, post-hoc Tukey tests were used to assess differences in mean values across treatments using the lsmeans function in the 'lsmeans' package (Lenth 2016). Log transformations were used whenever necessary to meet test assumptions. Tillage, fertilization and carbendazim effects on gas-exchange variables measured at three dates were examined with the LMMs including time as a categorical variable (stage of development). Photosynthetic capacity variables, measured only once, were evaluated with LMMs not including time. The hypothesized relationships between relevant maize mycorrhizal development and early nutrition variables measured and reported by Gutiérrez-Núñez et al. (2022) and the gas-exchange parameters were examined with Pearson correlations. Models

were fitted using the ¨lm¨ function and the coefficients were calculated using the "cor.test" function in ¨stats¨ library for RModel residuals were always inspected for normality, homoscedasticity, and outliers using plot (model) statement. All tests were performed in R (R Core Team 2020).

3. Results

Tillage and fertilization had minor, and Carbendazim fungicide none, effects on instantaneous gas-exchange measurements (Table 1). Tillage and fungicide, but not fertilization, increased photosynthetic capacity parameters (Table 2).

Table 1. *P*-values and significance for the main effects of tillage (T), fertilization (F), carbendazim (C) and stage of plant development (S) factors and for their two-factor interactions on photosynthetic variables measured at three stages of maize development * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Three- and four-factor interactions were not significant for any of the evaluated parameters, so *p* values are not shown. Stages V3, V6, and V10: third, sixth and tenth leaf fully emerged with visible collar.

Parameter	Stages	T	F	C	S	T*F	T*C	F*C	T*S	F*S	C*S
Asat	V3-V10	0.3	0.5	0.1	***	0.7	0.2	0.4	0.8	**	0.1
Whole plant A	V3-V10	0.8	***	0.2	***	0.9	0.9	0.4	0.3	**	0.1
gs	V3-V10	0.6	0.3	0.3	***	0.4	0.6	0.6	0.6	0.1	0.5
E	V3-V10	*	0.2	0.7	*	0.5	0.2	0.2	0.7	*	0.3

Table 2. *P*-values and significance for the main effects of tillage (T), fertilization (F), carbendazim (C) and stage of plant development (S) factors and their two-factor interactions

on photosynthetic variables measured at V3 and V6 stages of plant development. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Photosynthetic capacity parameters were not evaluated in the treatments with fertilization at stage V3. T*C, F*C and T*F*C interactions were not significant for any of the evaluated parameters, so p values are not shown. Stages V3 and V6: third and sixth leaf fully emerged with visible collar.

Parameter	Stage	T	F	C	T*F
Vcmax	V3	0.93	-	0.34	-
Jmax	V3	0.15	-	0.20	-
Vcmax	V6	0.17	0.54	**	0.26
Jmax	V6	*	0.72	***	0.22

3.1. Gas-exchange instantaneous measurements

Tillage affected only E, which was 7% higher in plants with no-tillage than in plants with conventional tillage (Table 1). There was a significant fertilization x developmental stage interaction for transpiration (E) and photosynthetic rates (Asat) and for the estimated total assimilation per plant (whole plant A) (Table 1; Fig. 2 a, b, c). Fertilization increased photosynthetic rate Asat (13%) at V3, and whole plant A between V3 (41%) and V6 (25%) but no longer at V10. Fertilized plants had higher E (17%) than non-fertilized plants only at V3 and stomatal conductance (gs) changed only in time. At V10 there were no differences.

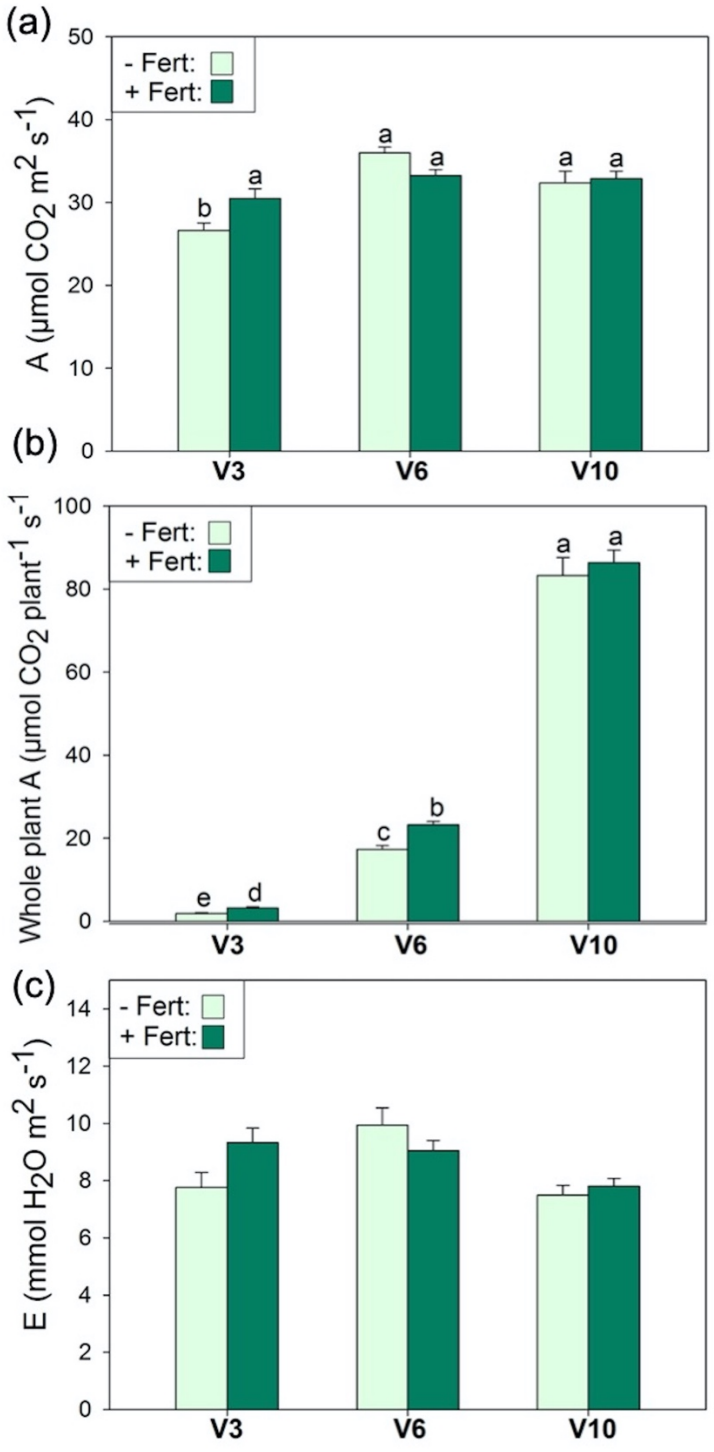

Figure 2. Effects of fertilization treatments at different stages of plant development on (a) Net-assimilation rate (Asat), (b) whole plant A and (c) transpiration (E). Different letters indicate significant differences ($p < 0.05$) according to Tukey′s Test.

3.2. Photosynthetic capacity parameters *Vcmax* and *Jmax*

Photosynthetic capacity parameters differed only at V6 (Table 2). Carbendazim increased Jmax by 30% and Vcmax by 23%. Jmax increased by 19% in CT plants compared to RT plants and the same trend was observed in Vcmax (Tables 2 and 3).

Table 3. Vcmax and Jmax (μmol CO_2 m^2 s^{-1}) measured at V6 showing the effects of tillage and carbendazim. Values are means of eight replicates ± standard errors. Different capital letters indicate significant differences ($p < 0.05$) between means in tillage treatments and different lowercase letters indicate significant differences between means in carbendazim treatments according to Tukey′s Test. NT: no-tillage; CT: conventional tillage.

	NT	CT	+Carbendazim	-Carbendazim
Vcmax	74.0 ± 4.4	84.5 ± 7.4	89.7 ± 6.0^{a}	68.8 ± 4.8^{b}
Jmax	410.9 ± 28.4^{B}	506.8 ± 47.6^{A}	539.6 ± 36.0^{a}	378.1 ± 31.8^{b}

3.3. Relation between AM fungi development and early maize nutrition, C assimilation, and growth

Many of the explored functional relations have shown a surprising change of direction from positive to negative from stage V3 to stage V6 that in some cases returned to positive at V10 (Table 4). At V6, plants with high mycorrhizal colonization showed also high colonization by other root-inhabiting fungi (resembling dark-septate endophytes) and switched from

having high shoot N and P concentrations to low concentrations, mainly in shoot N. Photosynthetic rates and whole plant photosynthesis had not relation with the development of AM fungi, other fungi and total fungal colonization in roots, with the exception of one negative correlation between photosynthetic rate and AM colonization at V10 (Table 4). However, the relationship between Asat and AM colonization showed a negative trend already from stage V6. The gs was positively correlated with shoot P concentrations at V3 and V10, but the relation was also negative at V6. The same trend was observed for shoot N and gs, but this relation became significant only at stage V10. The E correlated positively with shoot P concentration at V3 and with root colonization by other fungi at V10 (Table 4). Shoot dry weight was positively correlated with net photosynthetic rate at V3, but negatively correlated at V6 and V10 (Table 4). As a result of this, shoot N correlated positively with photosynthetic rates and whole plant photosynthesis at V3 and V10, but not at V6 (Table 4, Fig. 3). Photosynthetic rates were positively correlated with shoot P concentration only at V3, but the estimated whole plant photosynthesis was positively related with shoot P at all stages. In accordance, Jmax and Vcmax were negatively correlated with total fungal colonization at V6 (Fig. 4 a,b). The same trend was observed for AM hyphal and arbuscular colonization, and other fungi colonization. Jmax was positively correlated with N concentration at V6 and it is shown that treatments with less fungal colonization have lower N concentration and Jmax rate; the same trend was observed for Vcmax (Fig. 4c, d).

Table 4. Pearson correlation coefficients between selected plant and microbial predictor and response variables measured. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Correlations that include photosynthetic capacity parameters (Jmax and Vcmax) in stage V3 do not include the treatments with fertilization.

		Instantaneous gas exchange rates				Photosynthetic capacity	
Parameter	Stage	Asat	Whole plant A	gs	E	Vcmax	Jmax
AM hyphal	V3	0.04 ns	0.04 ns	0.14 ns	-0.09 ns	0.37 ns	0.40 ns
Colonization (%)	V6	-0.02 ns	-0.33 ns	-0.05 ns	0.13 ns	-0.41 *	-0.43 *
	V10	0.15 ns	-0.06 ns	-0.13 ns	0.02 ns	-	-
AM arbuscular	V3	0.27 ns	0.22 ns	0.26 ns	0.07 ns	0.23 ns	0.33 ns
Colonization (%)	V6	-0.13 ns	-0.09 ns	-0.21 ns	0.06 ns	-0.37 ns	-0.39 ns
	V10	-0.4 *	-0.17 ns	-0.21 ns	-0.08 ns	-	-
Other Fungi	V3	0.07 ns	0.19 ns	0.08 ns	-0.18 ns	-0.09 ns	0.08 ns
Colonization (%)	V6	-0.11 ns	-0.14 ns	0.07 ns	-0.10 ns	-0.38 ns	-0.36 ns
	V10	0.28 ns	-0.22 ns	0.18 ns	0.37 *	-	-
Total fungal	V3	0.06 ns	0.12 ns	0.13 ns	-0.14 ns	0.21 ns	0.30 ns
Colonization (%)	V6	0.01 ns	-0.3 ns	0.03 ns	0.14 ns	-0.43 *	-0.44 *
	V10	0.28 ns	-0.18 ns	0.03 ns	0.25 ns	-	-
NLFA 16:1 ω5 (nmol g^{-1} soil)	V6	-0.24 ns	-0.24 ns	0.11 ns	0.05 ns	-0.11 ns	-0.08 ns
Shoot P conc. (mg g^{-1})	V3	0.47 **	0.56 ***	0.37 *	0.42 *	0.34 ns	0.19 ns
	V6	-0.2 ns	0.43 *	-0.36 *	-0.17 ns	-0.03 ns	-0.08 ns
	V10	0.31 ns	0.42 *	0.37 *	0.22 ns	-	-
Shoot N conc. (mg g^{-1})	V3	0.39 *	0.47 **	0.31 ns	0.17 ns	0.13 ns	0.13 ns
	V6	-0.32 ns	0.20 ns	-0.31 ns	-0.28 ns	0.39 ns	0.56 **
	V10	0.42 *	0.68 ***	0.47 **	0.21 ns	-	-
Shoot DW (g $plant^{-1}$)	V3	0.7 ns	-	0.54 **	0.32	-	-
	V6	-0.36 *	-	-0.08 ns	-0.16 ns	-	-
	V10	-0.37 *	-	-0.21 ns	0.26 ns	-	-

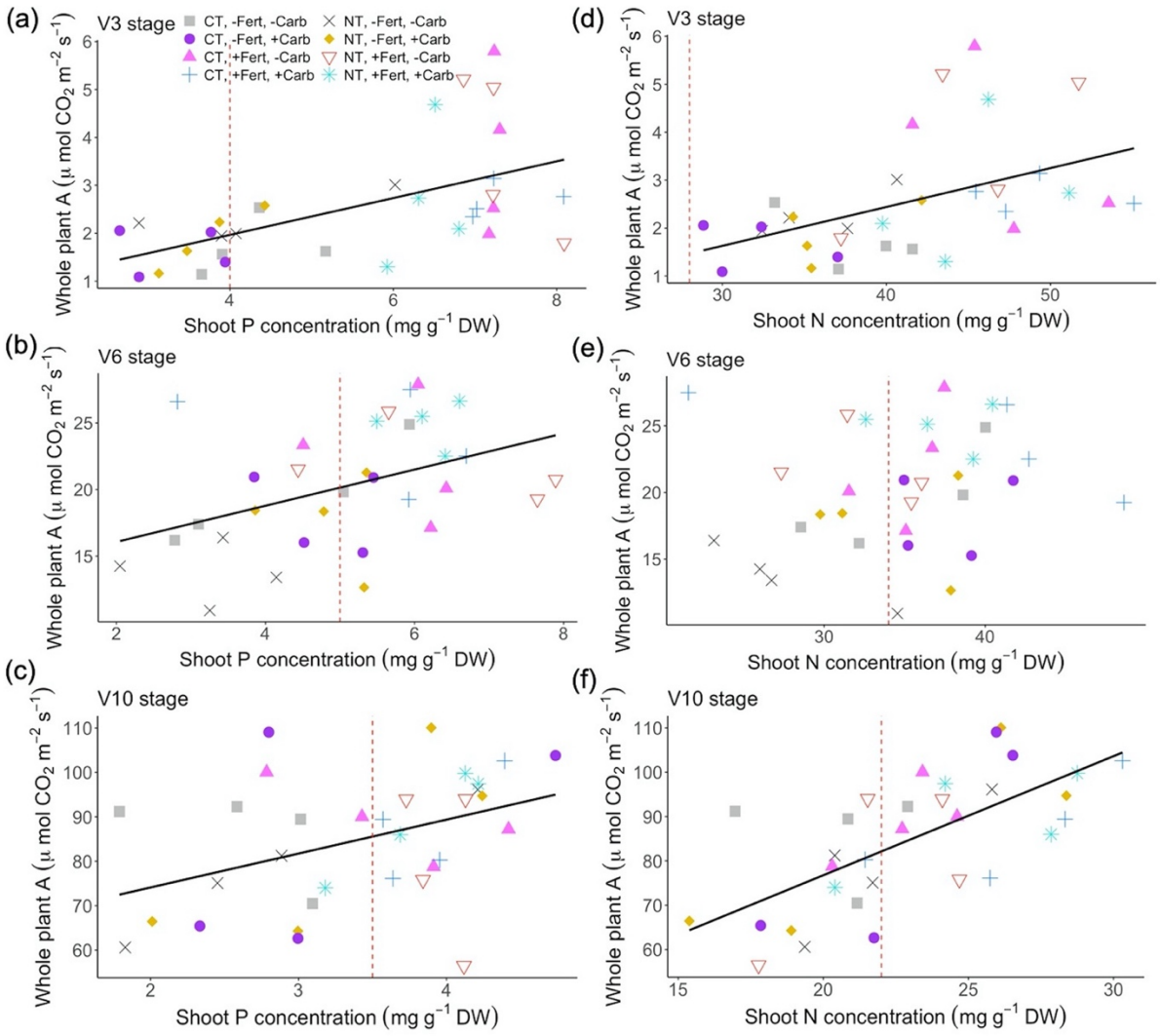

Figure 3. Relationship between Whole plant Assimilation and Shoot P concentration at (a) V3 (R2=0.29, p= <0.001), (b) V6 (R2=0.15, p=0.015) and (c) V10 (R2=0.15, p=0.018) , and with Shoot N concentration at (d) V3 (R2=0.19, p=0.007), (e) V6 (R2=0.01, p=0.26), and (f) V10 (R2=0.45, p= <0.001). Each point represents a single replicate of each treatment. The dotted lines represent the critical shoot N or P concentration for this stage.

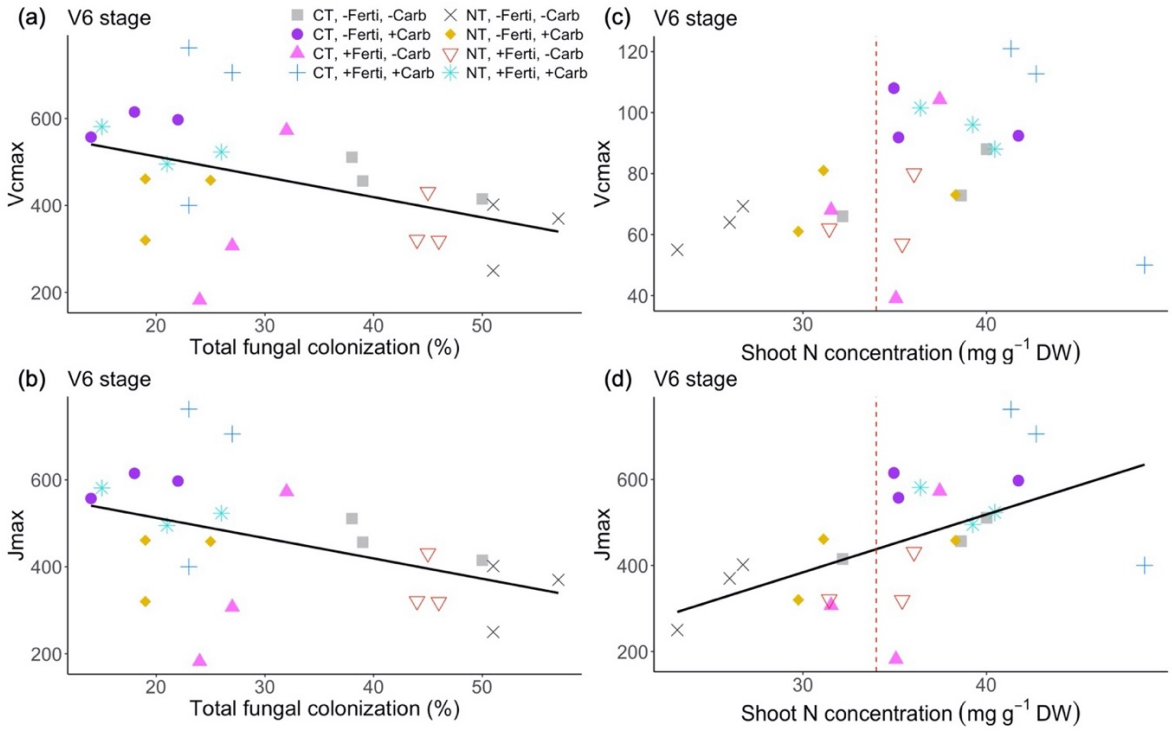

Figure 4. Relationship between Vcmax, Jmax and total fungal colonization (a (R2=0.15, p= 0.035), b (R2=0.15, p= 0.034)) at V6, and shoot N concentration (c (R2=0.12, p= 0.06), d (R2=0.28, p= 0.005)) at V6. Each point represents a single replicate of each treatment.

4. Discussion

The predictions of higher stomatal conductance, transpiration, photosynthetic rates and photosynthetic capacity with preserved than with disturbed AM fungal networks and in plants with rapid AM root colonization than in plants with delayed AM root colonization were partially supported by our results. We observed, as expected, improved P nutrition and a slight stimulation of stomatal conductance, transpiration, photosynthetic rate and electron transport rate in treatments with high AM colonization at the earliest stage of maize development (V3), just a few days after plant emergence. However, the results of this study

also revealed a period of disruption of these positive functional relationships at the V6 stage, affecting mainly the treatments without fertilization. Most surprisingly, many variables and functional relations explored, switched from no response or positive response to AM colonization at the V3 stage, to negative at the V6 stage, and in some cases returned to positive or became less negative at V10. The reversion period lasted only a few weeks and had significant effects on plant nutrition and C assimilation at V6 but had no effects on plant biomass. However, the most affected treatments by this reversion period showed reduced yield and grain quality at harvest (Gutiérrez-Núñez et al. 2022). One possible reason for such transient disruption were unexpected interactions between root colonizing fungi. Colonization by other fungi became in some cases as high as colonization by AM fungi and was associated to reduced maize N and P nutrition in highly colonized plants. Consequently, root fungal colonization also correlated to reduced photosynthetic rates and photosynthetic capacity parameters Jmax and Vcmax. As both AM and other fungal colonization developed synchronously in roots and were likely temporarily inhibited by carbendazim, it is not possible to separate the effects of each group and both types of colonization which were negatively related to plant nutrition, gas exchange and photosynthetic capacity at V6. However, dissecting if AM fungi, the other fungi, other not-so-evident root biota, or their interactions, caused this ephemeral functional reversion is a complex and long task beyond the scope of the study which will be addressed in future experiments.

Maize photosynthetic rates and transpiration were clearly increased by fertilization. Photosynthesis is usually positively related to plant nutrition, which depends on the soil nutrient availability (Cakmak and Engels 1999; Pasquini and Santiago 2011). As a consequence of the increase in photosynthesis, plants quickly lose water via transpiration and at the same time, transpiration may facilitate plant nutrient uptake and translocation

(McDonald et al. 2002; Novak and Vidovic 2003; Beerling and Franks 2010). This stimulation was reflected in both the net photosynthetic rate and the estimated whole plant photosynthesis (Asat x Biomass) at V3, suggesting that soil initial nutrient availability was low for plants still starting to form roots and mycorrhizal associations and fertilization improved gas exchange, since photosynthesis was not limited by nutrients. AM plants often show higher stomatal conductance and transpiration rate than non-mycorrhizal plants (Augé 2000; Ruiz-Lozano and Aroca 2010; Augé et al. 2015; Zhu et al. 2018). This in turn could increase net photosynthesis by enhancing water use efficiency and diffusion of CO^2 within the mesophyll (Ruiz-Lozano 2003; Augé 2000; Boldt et al. 2011). The lack of differences in stomatal conductance and minor differences in transpirations is likely explained by the irrigation system in the experimental plot. In our study, the plants were all mycorrhizal but were not colonized in the same way and time, and gas-exchange responses maintained the expected relation with AM colonization. Soil AM fungal colonization and AM network integrity are very difficult to measure under field conditions. In this regard, our NLFA 16:1ω5 measurements did not show any relation to C assimilation measurements. This biomarker measures only AM fungal abundance in soil but cannot inform on network integrity; so far no other measurement can quantify AM network integrity which is usually inferred from its function, nutrient transfer and using tracers (Selosse et al. 2006; Mikkelsen et al. 2008; Figueiredo et al. 2021).

Fertilization results in an increased leaf nutrient content, thus leaf photosynthesis will not be limited by nutrient-dependent processes (e.g. ATP availability or rubisco activation), and also results in an increased leaf area and plant growth (Jia and Gray 2004; Kaschuk et al. 2009; Wang et al. 2012). The stimulation of whole plant photosynthesis was greater and more

prolonged in time than the stimulation of Asat, which suggests that mineral fertilization increased photosynthesis by enhancing the leaf biomass.

Although treatments with fertilization were always above critical N and P concentrations, some treatment combinations without fertilization were slightly below or close to the required nutritional levels. Plant photosynthesis rates varies considerably with leaf nutrient content, especially N and P content, and plants require minimum concentrations of nutrients to carry out cellular processes to grow (Reich and Schoettle 1988; Knecht and Göransson 2004; Domingues et al. 2010). For instance, N plays a significant role during light harvesting and CO_2 fixation (Evans 1983; Wang et al. 2012), and P is the main component of ATP and NADPH that are produced in the light reaction of photosynthesis to provide the energy for the regeneration of ribulose-1,5-bisphosphate (RuBP) in the Calvin cycle (Farquhar et al. 1980; Reich et al. 2009).

Maize is a crop that benefits from mycorrhizal associations mainly at early plant developmental stages, when its root system is not large enough to find low-mobility mineral nutrients (Gavito and Miller 1998; Miller 2000) and mycorrhizae may represent a low C investment to acquire them and compensate the fungal use of plant C (Verlinden et al. 2018). When nutrients are not limiting, early maize development may also benefit from a dynamic C sink that positively feedbacks the photosynthesis reaction (Gavito et al. 2019). This is, therefore, an important point for the establishment and management of the mycorrhizal association under field conditions (Polcyn et al. 2019). As we predicted, at V10 differences in instantaneous gas-exchange measurements were minor and, although we could not measure photosynthetic capacity, we believe we would have observed the same. The close following of early events in maize development, gas-exchange relations and photosynthetic capacity as influenced by preservation of mycorrhizal networks (tillage), nutrient availability

(fertilization) and the extent of colonization (carbendazim), allowed us to identify critical steps where mycorrhizal functioning may turn as expected or interact with other factors, apparently other root biota in this case, and deviate from the expected functional relationships. To our knowledge, we are reporting the first data from A/Ci curves performed in field conditions on a crop colonized by native AM fungi, where AM colonization and network integrity were manipulated *in situ* and comparisons were made between plants more or less colonized and connected to preserved or disturbed extraradical mycelium networks, instead of comparing between nonmycorrhizal and mycorrhizal plants. Despite the transient disruption period observed and other limitations, these may be the most realistic measurements to date of gas exchange and photosynthetic capacity testing manipulation of the mycorrhizal symbiosis.

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CAPÍTULO IV

Timing of connection to mycorrhizal networks matters: nutrition, N fixation, and transfer of fixed N in maize-bean intercropping

Abstract

The mycorrhiza-mediated transfer of nutrients to plants connected through mycorrhizal networks may be managed through the timing at which plants integrate to the networks and the integrity of those networks. This is particularly relevant for intercropping agrosystems. We used soil tillage to disrupt mycorrhizal networks and sowing time treatments to manipulate the connection time of maize and bean in a field experiment. We hypothesized that 1) the crop connecting first to undisturbed networks under reduced tillage would benefit most by acquiring more nutrients, and 2) tillage disruption delaying colonization and connection to preserved mycorrhizal networks would reduce mycorrhizal colonization in roots and soil, crop nutrition, N fixation by beans, and transfer of fixed N to maize.

Mycorrhizal network disruption with tillage reduced early mycorrhizal development in roots and soil, and N and P nutrition of both crops. In general, sowing time had no effect in maize but tillage increased plant density, growth, and the yield of maize by alleviating soil compaction. Reduced tillage and early sowing increased nutrition, development, N fixation and yield of bean. N derived from symbiotic fixation in bean and transferred to maize were higher with bean sown before maize under reduced tillage. Early connection to mycorrhizal networks favored mostly bean and made little difference to maize regardless of sowing time, contrary to our expectations. The greatest overall benefit for both crops was obtained when bean was sown before maize and connected early to preserved mycorrhizal networks under reduced tillage.

Keywords: nitrogen, phosphorus, sowing, symbiosis, tillage

1. Introduction

There is a global concern to develop a productive, sustainable agriculture as well as to mitigate the negative environmental impacts of conventional agriculture (Brooker et al. 2015). Intercropping is a suitable ancient management alternative that provides the opportunity to increase plant diversity, the effective use of land and agricultural inputs, and crop yield (Macheru-Muna et al. 2010; Li et al. 2020a). Intercropping represents a sustainable intensification practice, especially for smallholder farms, as an alternative to monoculture (Seran and Brintha, 2010; Nassary et al. 2020). The association of a cereals and legumes that forms symbiosis with N-fixing bacteria, is and old favorite combination for improving nutrition, yield and grain quality (Raseduzzaman and Jensen, 2017; Li et al. 2020a). Intercropping maize (*Zea mays* L.) with common bean (*Phaseolus vulgaris* L.) is a common practice that has resulted in higher yields for both crops when compared to sole cropping (Li et al. 2020b). The benefit for bean is often regarded as of second importance. Despite being used for millennia, there is still poor understanding about how maize-bean intercropping components, including their root symbionts, interact with each other and how they may be optimized by management seeking more sustainable practices.

Maize and bean plants are generally sown simultaneously for practical reasons, as it facilitates sowing and fertilizer application under conventional agriculture. In traditional and alternative agricultural systems practiced worldwide; however, sowing in intercropping may vary both in time and space as farmers use several sowing schemes (Seran and Brintha 2010; Lithourgidis et al. 2011). Recently, the need for sustainable intensification in food production has encouraged research exploring multiple versions of intercropping arrangements and sustainable management practices (Li et al. 2020b). Sowing time becomes relevant to understand under the light of recent evidence showing the strength of mycorrhizal networks

to mobilize resources belowground and affect the development of interconnected plants (Bell et al. 2021). Plants of the same or different species and sizes may be connected through extensive functional mycelial networks of arbuscular mycorrhizal fungi (AMF), linking them belowground and forming common mycorrhizal networks (CMNs) (Giovannetti et al. 2004; Mikkelsen et al. 2008; Lekberg et al. 2010). In CMNs the nutrients are transported and carbon is drained from the different associated plants at different rates; moreover, the symbiotic costs and benefits for the partners establishing this relationship may vary, thereby affecting the coexistence of plants (Hart et al. 2012; Weremijewicz and Janos, 2013; Řezáčová et al., 2018). Some studies (e.g., Merrild et al. 2013; Fellbaum et al. 2014; Weremijewicz et al. 2016) have shown that the transfer of nutrients may be greater to large plants that can supply greater amounts of C and suppress the growth of smaller individuals. This suggests that size and age asymmetry between individuals may be an important factor for increased competition between plants in a CMN. However, other studies have shown cases where this preferential allocation does not occur (Moora and Zobel, 1996; Walder and van der Heijden, 2015). Walder et al. (2012) showed that the plant with the highest biomass consistently provided most of the carbon to the networks but the plant with the lowest biomass acquired the highest amount of nutrients (N and P) and benefited most from connecting to the CMNs.

Plants may thus benefit differentially from the connection to CMNs, and factors such as size, the plant and AM fungal species involved, and their functional compatibility, may be involved in the allocation of nutrients among the symbionts connected (van der Heijden and Horton, 2009). There are functional characteristics of plant groups that may allow them to take greater advantage and trade different resources in the CMN (Walder et al. 2012). This is the case of N fixation in the legume-rhizobia symbiotic relationship that may provide additional N for the CMN, or gramineous species with large C budgets, releasing C. Still

little is known about how the combined forces and resources shared by the different symbionts connected interact and affect each other's development.

Sowing time of the intercropped plants has shown significant impacts on the yields of the involved crops, as temporal niche differentiation can greatly reduce competition for scarce resources (Hu et al. 2016; Li et al. 2020b). An advantage could be given to the species becoming first planted and connected to the mycelium network, thereby mobilizing mineral nutrients and water to support its initial growth without competition. Recently, timing of mycorrhizal colonization and of plant connection to mycorrhizal networks were reported to cause asymmetry in crop nutrition and overyielding in a millet-chickpea mixture under controlled conditions (Li et al. 2022). On the other hand, the rapid connection to an extensive CMNs may represent a substantial C drain for certain crops that outweighs other nutritional benefits (Walder et al. 2012; Werner and Kiers, 2015). A better understanding of the complementary interaction of crops coupled through mycorrhizal networks can thus provide an effective approach to manage the efficiency of nutrient use and the productivity of both crops, compared to sole cropping.

Our objective was to investigate whether sowing time and integration to CMNs could be managed to benefit one or both crops in terms of nutrition, growth and yield in maize-bean intercropping. We expected that the allocation of resources to intercropped plants through the AM mycelium networks would be defined by the strength with which each connected species demands and offers resources. The crop having a head start would benefit more by acquiring more N and P from CMNs, growing faster, and reducing the growth of the crop planted later. In this sense, the intact CMNs would amplify these competition/facilitation relations between the intercropped plants compared to CMNs disrupted by soil tillage. We hypothesized that 1) the benefits from connecting to mycorrhizal

networks would be greater for the crop that is established first and retrieved resources from the network without competition, and 2) tillage disruption delaying colonization and connection to preserved mycorrhizal networks would reduce mycorrhizal colonization in roots and soil, crop nutrition, N fixation by beans, and transfer of fixed N to maize.

2. Methods

2.1. Study site

This experiment was carried out in an agricultural field in the municipality of Charo, Michoacán, Mexico (19° 41' 14" N, 101° 03' 24" W). The soil is a Vertisol (WRB) clay with pH 6.2, 3.5% organic matter, 40 mg kg^{-1} soil nitrate, 19 mg kg^{-1} soil ammonium and 17 mg kg^{-1} soil available phosphorus measured just before starting the experiment. The temperature varies from 11 to 29°C and the average annual precipitation is 754 mm. The plot had been under conventional management for maize and sorghum production, but it had been under a fallow period of 3 years. Prior to the establishment of the experiment the shoots of remaining sorghum and weeds were cut and Paraquat herbicide was applied to control their growth.

2.2. Experimental design

A field experiment was established in June 2020 combining sowing time of maize and bean in intercropping and tillage treatments in a factorial design with five blocks. The tillage factor had two levels (conventional (CT) and reduced (RT)), and the sowing time factor had five levels (bean alone (BA), maize alone (MA), bean sown before maize (BSB), simultaneous sowing of maize and bean (SS), and bean sown after maize (BSA)). Maize sowing time was fixed, and bean sowing time was either advanced or delayed to achieve the treatments. Maize alone and bean alone were included to compare the performance of each crop without

interaction with the other crop and the land equivalent ratio. The experimental plot was first split into two and the tillage treatments were randomly assigned to the resulting two subplots to facilitate agronomic practices and reduce soil disturbance by tractor traffic in RT treatments (Fig.1). Then each subplot was further divided into five blocks that were considered as five replicates. The sowing treatments were randomly assigned to five sub-subplots within each of the five blocks. Each sub-subplot consisted of eight 5-m long rows that were 80 cm apart. Each block thus contained one replicate of the treatment combinations. Maize and bean plants were alternated every two rows (strip intercropping). To avoid edge effects, two rows on each side of the sub-subplots acted as a buffer zone surrounding it (Fig. 1).

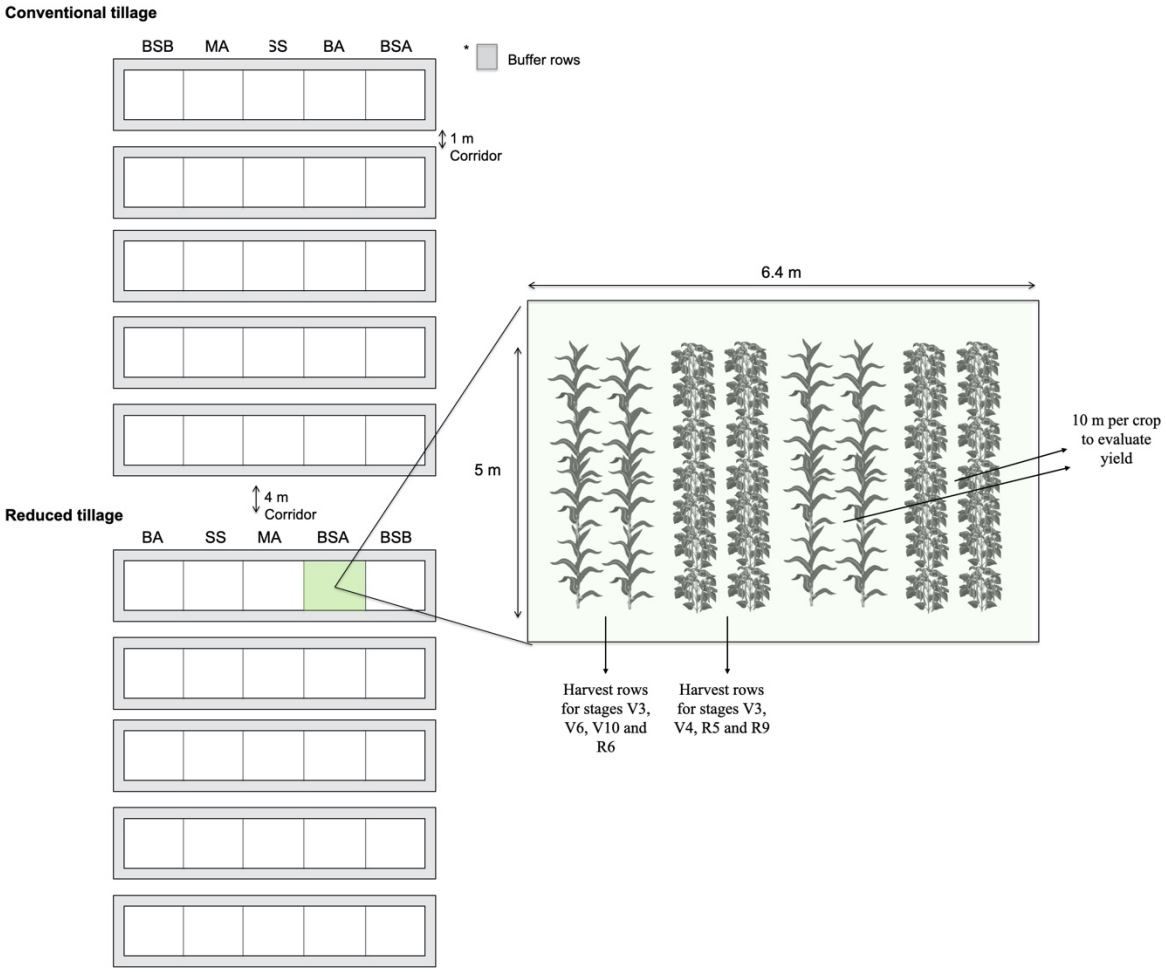


Fig. 1. Diagram of the experimental plot showing tillage and sowing time treatments. Each of the blocks contained one experimental unit of the 5 treatment combinations. For each crop (maize and bean), harvests were carried out at random in two sampling rows and two rows were reserved to evaluate the yield.

Plowing was carried out in the conventional tillage subplot with an 18-disc harrow at a depth of 10-15 cm, followed by chiseling. On June 18, black bean seeds cv. San Blas obtained from CIMMYT were sown in the "bean sown before" treatment plots with a disc planter for minimum tillage of 2 rows (Model SD-2009-DSF-2). Twelve days later, maize H-318 (Milpal®) and black bean were sown in the "maize alone", "bean alone" and

"simultaneous sowing" treatments with the same planter. Both crops were sown at a density of 100,000 plants ha-1 when sown alone, and at a density of 50,000 plants ha-1 for each crop when in combination treatment to reduce competition for space (Dawo et al., 2009). Three weeks later, bean was sown in the "bean sown after" treatment plots. Sowing two rows of each crop alternately in space (strip intercropping) allowed independent cultivation practices but in close proximity of plants to warranty intra and interspecific interactions (Li et al., 2020a).

Both N and P were supplied in two applications and in different amounts to promote the establishment of symbioses with native arbuscular mycorrhizal fungi and rhizobial bacteria, which is known to be reduced by high N and P availability (Treseder, 2004; Bonilla and Bolaños, 2009). At the time of sowing, we added chemical fertilizer in a side band as 33-3-0 and 18-46-0 to provide 100 kg ha^{-1} of N and 9 kg ha^{-1} of P when maize was sown alone; 50 kg ha^{-1} of N and 4.5 kg ha^{-1} of P to maize when maize was intercropped with bean; 25 kg ha^{-1} of N and 4.5 kg ha^{-1} of P in bean, considering the soil available nutrients and that maize has higher requirements than bean. At V9 and R5 (pre-flowering) stages for maize and bean respectively, we applied manually the second dose to the soil surrounding plant shoots as 100 kg ha^{-1} of N and 9 kg ha^{-1} of P for maize and 9 kg ha^{-1} of N and 23 kg ha^{-1} of P for bean. Besides manual control, pesticides Paraquat and Cypermethrin were applied at recommended doses by manufacturer for weed and pest control.

2.3. Soil and plant sampling, processing, and measurements

Soil available nutrients were measured from samples taken at stage V3, when all treatments had been implemented, approximately twenty days after sowing. Soil inorganic nitrogen (NH_4^+ and NO_3^-) was extracted from fresh sub-samples by KCl extraction (Robertson et al.

1999). Soil available P was determined using the Mehlich-3 extraction protocol (Mehlich, 1984). Both N (Weatherburn, 1967) and P (Murphy and Riley, 1962) were measured by colorimetrical reading in a Bran-Luebbe III autoanalyzer.

Plant growth and nutrition were followed through four harvests carried out when plants reached the defined stage for maize (Abendroth et al., 2011) and bean (Cavalcante et al., 2020). Given the different sowing times for bean, the stages were reached on different dates. At the first harvest plants were at V3 for maize (17 days after sowing -das-) and V3 for bean (19-29 das), the second harvest was at V6 for maize (31 das) and V4 (32-44 das) for bean, the third harvest was at V10 for maize (51 das) and R5 (54-56 das, pre-flowering) for bean, and the fourth harvest at maturity (R6, 150 das) for maize and R9 (100-122 das) for bean. The shoots of three plants from each of the five blocks were cut and stored in plastic bags. Soil near the roots and root samples of the plants harvested were collected from the top 20 cm and composited per block in a sealed plastic bag that was stored inside coolers until processing in the lab. Shoots were washed to remove soil residues and dried at 70 °C to constant weight. Roots were removed manually from a 20 g subsample and the root-free soil samples were stored immediately at -20°C for fatty acid analysis. Roots were washed, and 1-2 g subsamples were stored in tubes with 50% alcohol solution to evaluate arbuscular mycorrhizal (AM) root colonization.

Root subsamples were washed, cleared, and stained with trypan blue according to Phillips and Hayman (1970). One hundred root segments, randomly selected per sample, were mounted on slides and examined under the microscope to determine root colonization by AM fungi and other microorganisms. Roots from V3 and V6 for maize and V3 and V4 for bean were examined using the line-intercept method described by McGonigle et al.

(1990). We report the percentage of root intersections that were not negative and thus contained any AM structure.

Soil samples, previously freeze-dried and pulverized in a steel-ball mill, were used to estimate the abundance of live external mycelium of AM fungi in the different treatments. We selected samples from V6 in maize and V4 in bean for fatty-acid analyses given that root colonization measurements showed very low intraradical colonization at the first harvest. Whole cell fatty acids were extracted from one-gram soil subsamples, saponified, methylated and volatilized for gas chromatography according to Sasser (1990). A known amount of a standard 19:0 fatty acid methyl ester was added to each sample to allow quantification of the extracted fatty acids. The fatty acids were identified and quantified using a 50 m HP5 capillary fused silica column (Hewlett Packard) with He as carrier gas in a gas chromatograph (Agilent 7890B). The fatty acids were identified from their retention times and quantified in relation to the internal standard (fatty acid methyl ester 19:0) with Sherlock software package version 6.0 (MIDI software, MIDI Inc.). The fatty acid 16:1ω5 was used as indicator of the abundance of the external mycelium of AM fungi in soil (Olsson, 1999), a biomarker not specific for AM fungi but representing mostly AM fungi in field soil samples (Olsson 1999).

Gas-exchange measurements were made through instantaneous rates of CO_2 assimilation at light saturation (Asat), stomatal conductance (gs), and transpiration (E), when maize was at V10 and bean was at R6 to explore potential differences in C assimilation and water relations. Measurements were carried out on the same day of the third harvest during the morning and with clear sky. Measurements were made by blocks, on the youngest fully developed leaf of two of the same plants that were later harvested (these two values were averaged to obtain a single value per block of each treatment), using a portable gas exchange system (Ciras-3, PP Systems, Amesbury, MA, USA) attached to a Parkinson leaf cuvette

(PLC3) with a 4.5 cm^2 leaf chamber. CO_2 concentration inside the chamber was controlled by the CIRAS-3 at 400 μmol mol^{-1}, at light saturation and 27 °C (set from ambient temperature). The selected leaves were placed in the leaf chamber and were allowed to reach a stable state (approx. 2 min) before recording the net photosynthetic rate. Blocks containing one replicate of each treatment were completed within one hour and all blocks accomplished within the same day. Due to the large amount of biomass at this harvest, the shoots were cut, weighed in fresh and then cut into 10-15 cm pieces, mixed and a random subsample was taken to determine moisture content and calculate the total dry weight. The rest was discarded. No soil or root samples were collected at V10.

For the final harvest, all the plants of maize or bean present in the 10 m reserved for yield evaluation within each experimental unit were harvested. Beans finished their cycle after three months and maize after five months. For bean, grains were separated from the pod, weighed in fresh and a sample was taken to determine dry weight. For maize, the cobs were removed from plants, weighed in fresh and a subsample of 5 cobs was taken to separate the grain and calculate the yield. The shoots were cut and the same weighing in fresh and sampling procedure to determine moisture content of the V10 harvest was followed. Subsamples of stover and grain of maize, and pod and grain of bean, were dried at 70 °C to constant weight and weighed. Moisture contents were used to calculate total grain final yield and stover biomass for maize and bean per block. The Land Equivalent Ratio (LER), defined as the area of sole crops that would be required to obtain the same yield or biomass of the component crops as a unit area of intercrop (Mead and Willey, 1980) was calculated. The Land Equivalent Ratio (LER), was used for the evaluation of the intercropping system as it is often used as an indicator to determine the efficacy of intercropping compared to sole crop (Brintha and Seran, 2009; Yu et al., 2015). It was calculated as $LER = Y_1/M_1 + Y_2/M_2$, where

Y_1 and Y_2 are the yields (per unit of total area of the intercrop) of species 1 and 2 in the intercrop, and M_1 and M_2 are the yields of the species in sole crops (per unit area of the respective sole crop). A LER value < 1.0 indicates that intercropping is disadvantageous, a value of 1.0 indicates no difference in yield between the intercrop and the sole crops, any value > 1.0 indicates a yield advantage for intercropping.

N and P concentrations were measured in shoot samples taken at vegetative stages, and in the grain at final harvest. Samples were ground in a Thomas Scientific mill, acid digested by the semi-Kjeldahl method (Bremmer, 1996), filtered, and both N and P forms were measured by colorimetrical reading as explained above for soil measurements. N and P content in the shoots (mg per plant) was calculated by multiplying the N or P concentrations by the shoot dry weight. Five to six mg of ground plant samples (V4 and grain for bean, V6 and grain for maize) were weighed and packed into tin capsules. Samples were analyzed by mass spectrometry with an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA, USA) coupled to a stable isotope ratio mass spectrometer (Delta PlusXP, Thermo Finnigan, Bremen) to determine % C, % N, $\delta^{15}N$ (‰, per mil) and $\delta^{13}C$ (‰,).

$\delta^{13}C$ was used as an indicator of water use efficiency (WUE), that refers to the ability of plants to limit water loss while maintaining net carbon uptake in the leaves and thus leads to higher yield (Ruiz-Lozano and Aroca, 2010), where less negative (higher) values indicate higher WUE (Condon et al., 2002; Iqbal et al., 2005). $\delta^{15}N$ was used to determine the isotopic fractionation and percentage of N derived from the atmosphere (%Ndfa) for both shoot and grain samples of bean plants using the ^{15}N natural abundance method (Shearer and Kohl, 1986). Values obtained for maize sown alone under conventional tillage treatment was used as a non-fixing reference plant with minimal chances to obtain N derived from symbiotic fixation ($\delta^{15}N$ values of 4.1 and 3.3 for vegetative and grain stages, respectively).

$$\%Ndfa = (\delta^{15}N_{non\ fixing\ reference\ plant} - \delta^{15}N_{N2\ fixing\ legume} / \delta^{15}N_{non\ fixing\ reference\ plant} - \beta) \times 100$$

Where β is the $\delta^{15}N$ value from the nitrogen fixing plant grown in N free medium (obtaining all N from N_2 fixation). Different β values were used for shoot biomass and seed. We used the β value -2.6 for shoots as a mean of the values obtained by Polania et al. (2016) and Pacheco et al. (2017) for common bean varieties with the growth habit and seed size corresponding to the variety used here. For the grain, the β value used was -2.2 as a mean of the values obtained by Polania et al. (2016) and Farid (2015) for common bean grain. A timeline diagram of sample collection and measured variables is provided in figure 2.

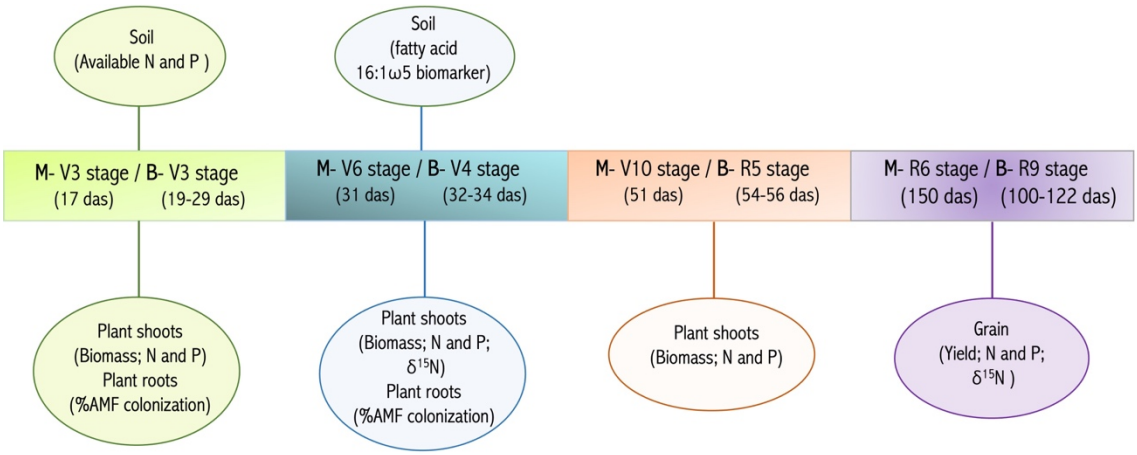


Figure 2. Timeline of sample collection and measured variables in the experiment. M- and B- initial letters indicate bean (B) and maize (M) and stage indicates the plant development stage(s) and the days after sowing (das) passed when the measurements were made.

2.4. Pot experiment

We conducted a pot bioassay to evaluate the influence of soil biota on nutrition and early growth of maize and bean plants and to explore a potential soil health problem given the low emergence and early growth of maize. We used a randomized factorial design with two main factors, soil disinfection (disinfected and non-disinfected soil) and time of harvest (V3, V6, V10 development stage for maize and V3, V4, and R6 for bean). There were four replicates for each treatment combination and the same design was used for maize and bean plants, but they were grown separately as two independent bioassays. Fresh soil was collected from the top 20 cm of the agricultural plot where the field experiment was carried out but just outside the area of the experiment. Half of the soil was disinfected by autoclaving for 2 h at 121 °C to remove native organisms and the other half was used as collected. The soil was then placed in 2 kg pots and H-318 maize or San Blas bean seeds were sown in each pot. We added 200 mg of N and 40 mg of P for maize, and 60 mg of N and 40 mg of P for bean divided in two applications, the first at sowing and the second at V3. Four plants per treatment were harvested at V3, V6 and V10. Root colonization by AM fungi and other organisms, shoot dry weight and N and P concentrations were evaluated as explained above for the field experiment.

2.5. Statistical analysis

Data were analyzed with linear mixed models (LMMs) including tillage, time of sowing and plant developmental stage as fixed effects and block as a random effect. Models were fitted using ‘lmer’ function in the ‘lme4’ package for software R (Bates et al., 2015). To identify the relevant predictors, model reduction was performed by fitting all the possible nested models and selecting the best models as those with the lowest Akaike Information Criterion

(AIC) values. When relevant, post-hoc Tukey tests were used to assess differences in mean values across treatments using the lsmeans function in the 'lsmeans' package (Lenth, 2016). Log and Logit transformations were used whenever necessary to meet test assumptions. Photosynthesis variables, AM fungal abundance in soil, %N, $\delta^{15}N$, $\delta^{13}C$, grain yield, nutrients in maize grain and LER were evaluated with LMMs not including plant developmental stage as a factor. Generalized linear models (GLM) were used for the analysis of bioassay plants testing the effect of disinfection and developmental stage on shoot dry weight, N and P shoot concentrations using 'glm' function in the 'lme4' package for software R (Bates et al., 2015). Significant differences were tested with post-hoc Tukey tests using a least square means package lsmeans (Lenth, 2016). Model residuals were always inspected for normality, homoscedasticity, and outliers using plot (model) statement. Relationships between relevant response variables and plant and microbial properties were tested using linear mixed effects models. All the models included block as a random effect on the intercept. All the variables (predictors and responses) were standardized before model fitting. Models were fitted using the ¨lmer¨ function in the 'lme4' package for software R (Bates et al., 2015). The R function "corrplot" was used to plot the graph of the correlation matrix. All tests were performed in R (R Core Team, 2020).

3. Results

Maize emergence was low in general in this clay soil and some plants that emerged became stunted afterwards, especially under RT. At harvest the plant count was below 50% of the planting density and was significantly lower under RT than under CT (CT: 33.2 ±1.7a; RT: 22.9 ±1.4b). Bean emergence was much higher than maize emergence, with no effect of tillage treatments, and was significantly higher in treatments where bean was sown

simultaneously with maize than in the other sowing treatments (BA: 49.1 ±1.4b; BSB: 52.7 ±1.8b; SS: 59.5 ±1.5a; BSA: 49.0 ±1.7b).

For maize development, tillage and developmental stage had consistent effects on mycorrhizal colonization, biomass, and nutrition parameters, but sowing treatment affected only few parameters. There were several two-factor interactions and no three-factor interactions in maize (Table 1a). For bean development, both tillage and sowing treatment effects on most parameters varied with the developmental stage (Table 1b).

Table 1. *P* values and significance for the main effects of Tillage (T), Sowing (S), and Developmental Stage (DS) factors and their interactions on all variables measured at several harvests in a) Maize plants and b) Bean plants * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

a)

Parameters	Stages	T	S	DS	T*S	T*DS	S*DS	T*S*DS
Plant growth parameters								
Shoot DW	V3-R6	***	0.35	***	0.07	0.4	*	0.07
Root colonization								
AMF	V3,V6	***	*	***	0.7	***	***	0.64
Shoot nutrients								
P conc.	V3-V10	***	0.11	***	**	***	0.44	0.37
P content	V3-V10	*	*	***	0.34	*	0.67	0.39
N conc.	V3-V10	*	**	***	0.06	0.05	***	0.45
N content	V3-V10	***	0.38	***	0.7	**	**	0.44

b)

Parameters	Stages	T	S	DS	T*S	T*DS	S*DS	T*S*DS
Plant growth parameters								
Shoot DW	V3-R6	0.07	***	***	0.73	0.24	***	***
Root colonization								
AM fungi	V3,V6	***	***	***	**	***	***	0.58
Shoot nutrients								

P conc.	V3-V10	***	***	***	0.27	***	***	**
P content	V3-V10	*	***	***	0.74	***	***	***
N conc.	V3-V10	***	***	***	***	**	***	*
N content	V3-V10	**	***	***	*	0.18	***	**

3.1. *Soil available nutrients and AM fungal development*

Despite the different fertilization doses used for maize and bean to prevent inhibition of the establishment of mycorrhizal and rhizobial symbioses, soil around roots sampled at the V3 stage of maize, three weeks after sowing, did not differ in available P and NH_4 between tillage or sowing time treatments. Soil nitrate differed although not in relation with the fertilizer doses applied but with the crop planted. In maize rows, soil nitrate was higher in the treatments with RT than with CT (CT: 45.6 $\pm 5.8^{b}$ mg g^{-1}; RT: 68.9 $\pm 11.7^{a}$). For bean rows, treatments bean sown before (BSB) maize and bean alone (BA) had higher nitrate concentration than treatments sown simultaneously (SS) or sown after maize (BA: 43.2 $\pm 3.7^{ab}$; BSB: 56.0 $\pm 8.3^{a}$; SS: 23.6 $\pm 3.5^{b}$; BSA: 29.2 $\pm 10.5^{b}$).

In maize and bean, AM fungal root colonization in CT treatments was significantly lower than in RT treatments at V3. Also, the BSB treatment resulted in the highest root colonization by AM fungi at V3 (Fig. 3a, c). At V6, differences disappeared, and bean colonization was only lower in the treatments where bean was sown after maize (Fig. 3c). There was a sowing treatment x tillage interaction in bean, with a similar trend in maize, suggesting that the differences between the BSB treatment and the other treatments were more marked in CT than in RT treatments (Fig. 3d). The abundance of 16:1ω5 in soil, as a proxy for AMF abundance, measured at the second harvest showed in general small differences between treatments. There was a sowing treatment x tillage interaction in both

maize and bean samples but this interaction did not show a consistent direction of tillage effects on the abundance of 16:1ω 5 in the sowing treatments (Fig. 4 a, b).

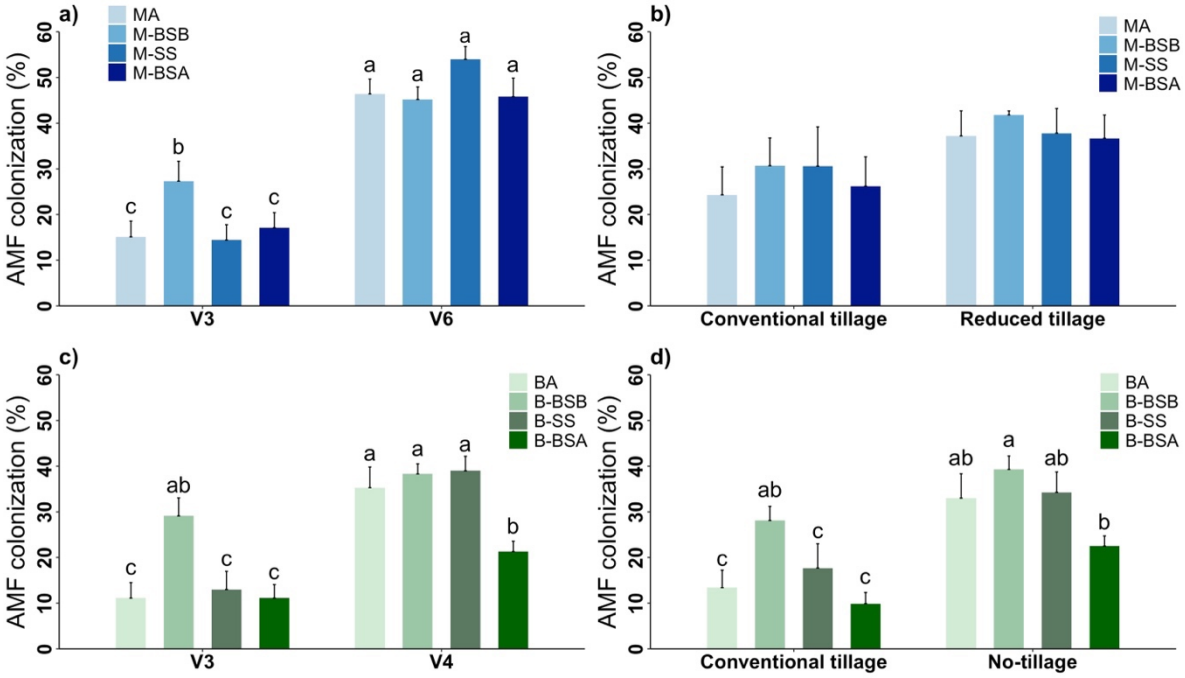


Fig. 3. AM root colonization. a) maize (sowing treatment x stage interaction), b) (sowing treatment x tillage-not significant), c) bean (sowing treatment x stage), d) bean (sowing treatment x tillage). Means + S.E., n=5. Different letters indicate significant differences ($p < 0.05$) between means according to Tukey's Test.

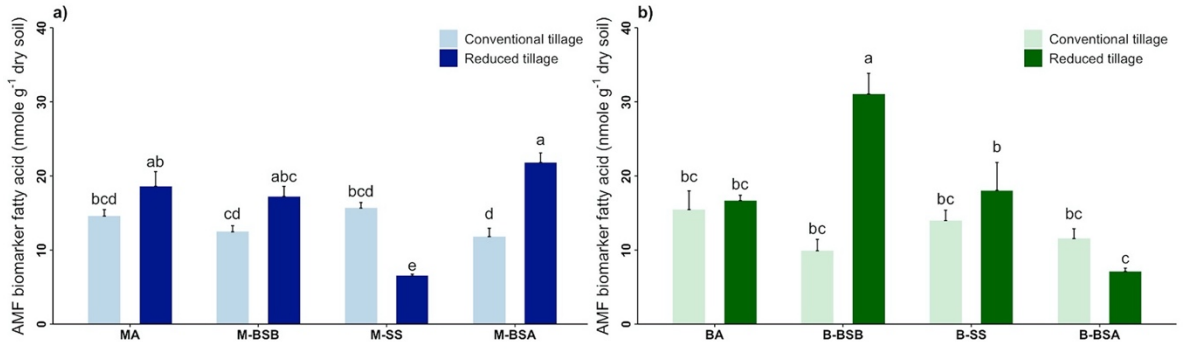
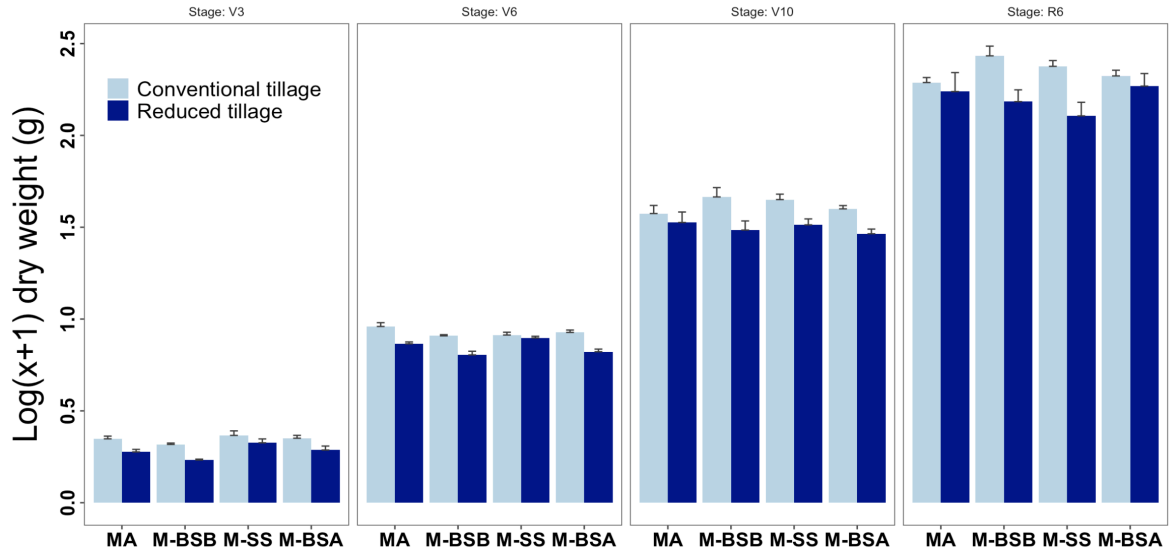


Fig. 4. Tillage and sowing time interactions on AM fungal abundance in soil measured at the maize V6 and bean V4 stages as indicated by the 16:1ω5 biomarker in soil collected near roots of the plants harvested. a) soil from maize plants and b) soil from bean plants. Means + S.E., n=5. Different letters indicate significant differences ($p < 0.05$) between means according to Tukey's Test.

3.2. *Plant biomass, nutrition, and gas exchange*

Maize biomass was mainly affected by tillage (Table 1). CT treatments had overall significantly higher biomass than RT treatments (CT 70 ±11 g plant^{-1}; RT 52 ±9). Except for minor differences at V3, maize biomass was very similar in the sowing treatments along the crop cycle (Fig. 5a). Despite small variations with tillage or developmental stage, bean in the BSA treatments had in general the lowest, and the BSB treatments the highest, biomass (Fig. 5b).

a)



b)

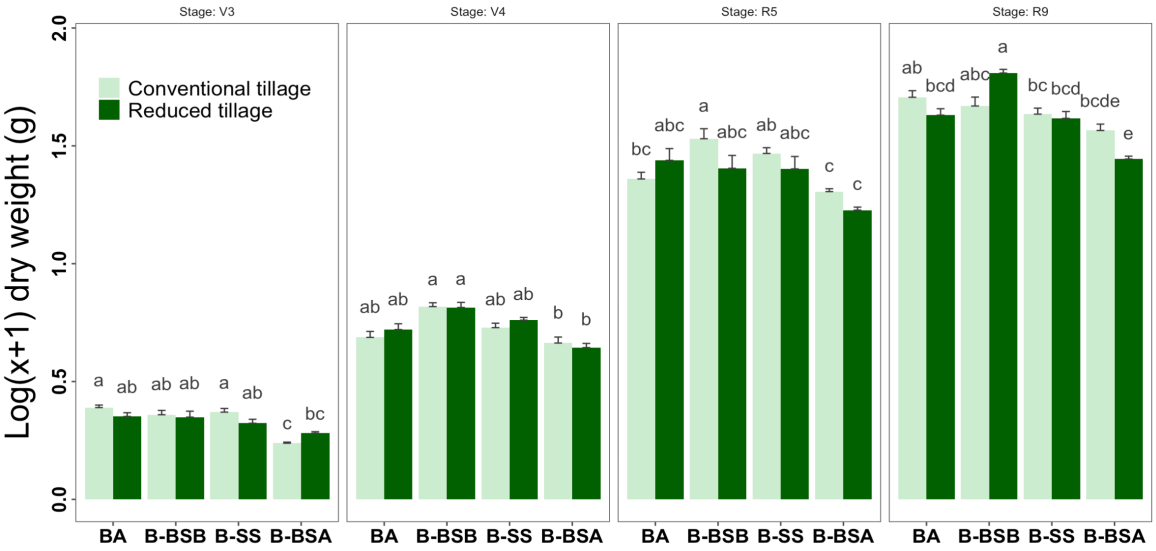


Figure 5. Shoot dry weight (g) of a) maize and b) bean at different stages of plant development in the different sowing time treatments and conventional and reduced tillage (Sowing time*tillage*developmental stage). Means ± S.E., n=5. Different letters indicate significant differences ($p < 0.05$) between means within each stage of plant development according to Tukey's Test.

All maize treatments reached the critical N concentrations: 25-30 mg g^{-1} for V3, 34 mg g^{-1} for V6 and 22 mg g^{-1} for V10 (Plénet and Lemaire, 1999; Zhao *et al*., 2018; Fig. 6). In general, maize N concentration was higher with RT than with CT. At V3, only the treatment with simultaneous sowing under CT was clearly not reaching the critical P concentration (4 mg g^{-1}), but at V6 no treatment reached the critical concentration of 5 mg g^{-1} suggested by Barry and Miller (1989) for the V6 stage. At V10, only RT treatments were above the 3.5 mg P g^{-1} required as critical P concentration for this stage (Fig. 6g-i). For bean, shoot nutrient concentrations reached the optimal range considered for common bean crops at V3 and V4 (Ambrosano et al., 1997; Araújo et al., 2000; Fageria, 2004; Fig. 6 d-f and j-l), except in the treatment when bean was sown after maize and with CT. This treatment had markedly low N and P concentrations at V4 (Fig. 6e and k). Shoot N and P content (mg $plant^{-1}$) differences were more marked in maize than in bean because of the early biomass reduction in RT treatments (data not shown). Maize shoot N content, and to a lower extent P content, were lower in RT than in CT treatments, particularly when bean was sown after maize. Bean shoot N and P content, on the other hand, were higher in RT than in CT treatments and lower when bean was sown after maize.

There were few differences in the gas-exchange rates of maize and bean plants at V10. There were no differences in photosynthetic rates of maize plants, transpiration rates were slightly higher only in the SS treatment (MA 2.8 $\pm 0.59^{b}$; BSB 2.9 $\pm 0.53^{b}$; SS 3.9 $\pm 0.60^{a}$; BSA 2.7 $\pm 0.67^{b}$) and stomatal conductance was higher in RT than in CT (RT 518 $\pm 44^{a}$, CT 354 $\pm 40^{b}$; mmol m^{-2} s^{-1}). For bean, the only treatment with significantly lower Asat was BSA under RT (T x S, $p < 0.05$), the BA treatment under CT was the only treatment with lower evapotranspiration than the others (T x S, $p < 0.001$) and there were no differences in stomatal conductance (Table 2).

Table 2. Means (± SE, n=5) of Net-assimilation rate (Asat) and transpiration (E) at V10 developmental stage of bean plants in the tillage and sowing treatment combinations. Different letters within columns indicate significant differences ($p < 0.05$) according to Tukey′s Test.

Treatments		Asat (μmol CO_2 m^{-2} s^{-1})	E (mmol H_2O m^2 s^{-1})
CT	BA	24.9 ±1.1^{a}	1.4 ±0.86^{b}
	BSB	25.9 ±0.96^{a}	2.8 ±1.4^{ab}
	SS	20.9 ±1.6^{ab}	3.1 ±0.79^{ab}
	BSA	24.4 ±0.88^{ab}	4.7 ±1.1^{a}
RT	BA	25.5 ±0.77^{a}	4.7 ±0.84^{a}
	BSB	25.5 ±1.4^{a}	3.3 ±1.1^{ab}
	SS	22.7 ±1.5^{ab}	4.3 ±0.96^{ab}
	BSA	19.5 ±0.95^{b}	3.4 ±0.61^{a}

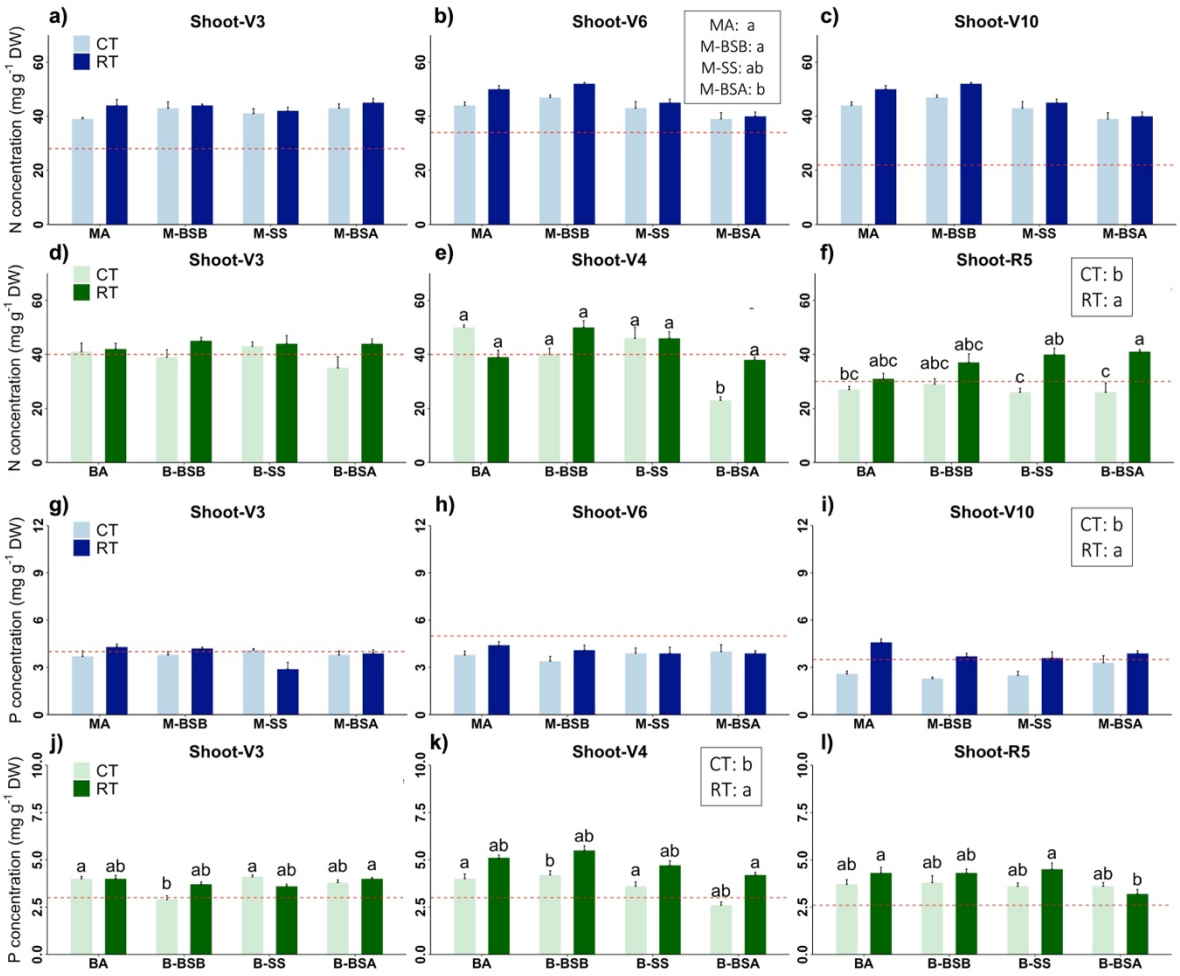


Fig. 6. Shoot N concentrations and shoot P concentrations (mg g^{-1} DW) of maize (a-c and g-i) and bean (d-f and j-l) at different plant developmental stages in the tillage and sowing time treatment combinations. Blue bars (maize), green bars (bean). Red dotted lines represent the critical/adequate shoot N or P concentration for each developmental stage. Means $\pm$ S.E., n=5. Different letters in bars and boxes indicate significant differences ($p < 0.05$) between means according to Tukey′s Test.

3.3. *Yield and nutritional quality of the grain*

Overall maize grain yield, here expressed per plant given the large differences in plant density between the tillage treatments, seemed lower with RT than with CT, but differences became significant only for BSB and SS treatments (Fig. 7a). In contrast, bean grain yield was little affected by tillage and was only higher in the BSB treatment with RT (Fig. 7b).

Tillage was the most important factor on grain nutrient quality (Table 3 and 4). Maize grain N concentration (11%), and bean grain N concentration (5%) and grain P concentration (20%), were higher with RT than with CT (Table 3 and 4). Maize and bean in treatments where bean was sown before maize generally had the highest concentrations of N and P in grain. BSB treatments recorded the highest LER values with both conventional and no tillage, but LER values were higher than 1 only for BSB (marginally also for SS) under CT indicating higher LER than sole crops (Fig. 7c).

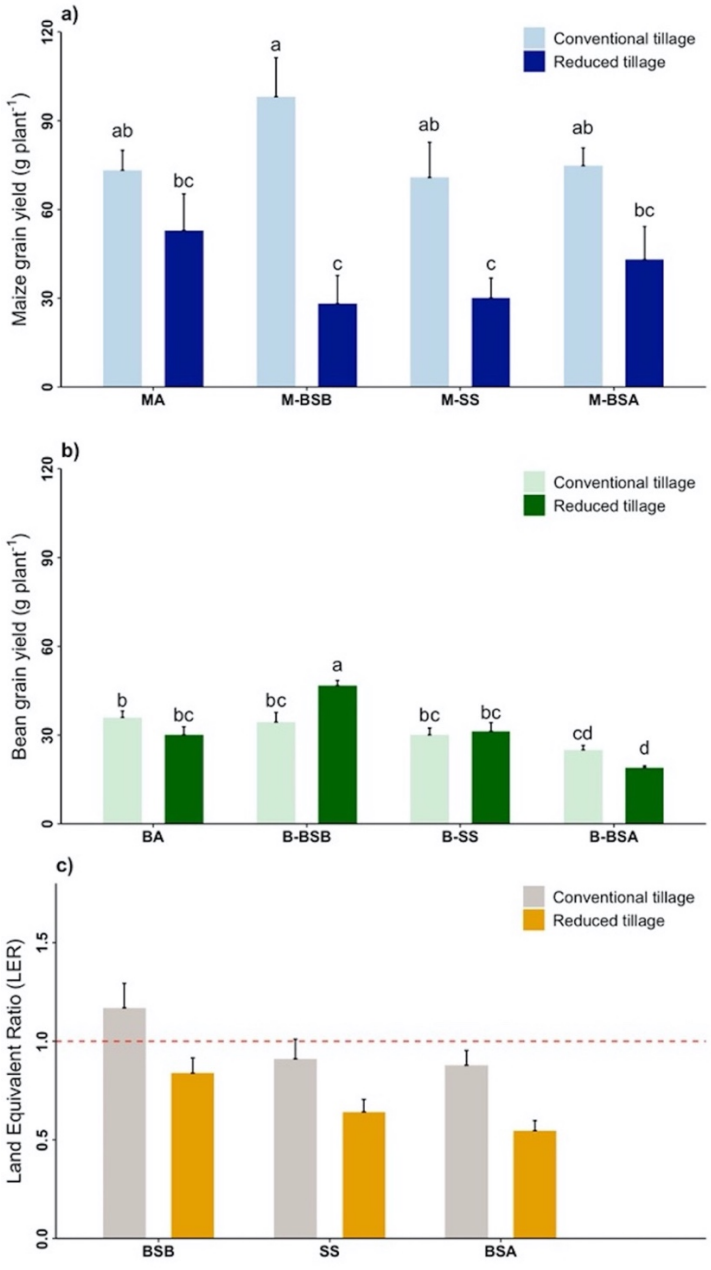


Fig. 7. Yield (g plant^{-1}) of individual maize (a) and bean plants and (c) LER ratio. Means ± S.E., n=5. Different letters indicate significant differences ($p < 0.05$) between means within each stage of plant development according to Tukey′s Test. A LER value < 1.0 indicates that intercropping is disadvantageous, a value of 1.0 indicates no difference in yield between the intercrop and the sole crops, any value > 1.0 indicates a yield advantage for intercropping.

Table 3. *P* values and significance for the main effects of Tillage (T) and Sowing (S) and their two-factor interactions on variables measured at harvest in a) Maize plants and b) Bean plants. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Stage column indicates the plant development stage where the parameter measurement was made.

a)

Parameter	Plant stage	T	S	T*S
Grain yield	R6	***	0.34	*
Grain N	R6	***	**	*
Grain P	R6	0.34	0.05	0.82
Grain δ13C	R6	**	0.05	0.53
Grain δ15N	R6	0.38	*	0.62

b)

Parameter	Plant stage	T	S	T*S
Yield	R9	0.81	***	***
Grain N	R9	*	**	**
Grain P	R9	***	0.19	0.06
Grain δ13C	R9	***	***	***
Grain δ15N	R9	***	*	***
Grain Ndfa	R9	***	*	***

Table 4. Means (± SE, n=5) and *p* values for the main effects of Tillage and Sowing and their two-factor interaction on variables measured in grain at harvest of a) maize and b) bean plants in the tillage and sowing treatment combinations. Different letters indicate significant differences ($p < 0.05$) between means according to Tukey's Test.

a) Maize

Treatment		Grain $\delta^{15}N$	Grain N concentration (mg g^{-1} DW)	Grain P concentration (mg g^{-1} DW)
CT	MA	3.26 ±0.23	18.8 ±0.67^{bc}	7.54 ±0.66
	BSB	2.74 ±0.28	17.3 ±0.89^{c}	7.39 ±0.33
	SS	3.19 ±0.18	18.2 ±0.30^{bc}	7.43 ±0.22
	BSA	3.15 ±0.05	17.3 ±0.15^{c}	6.23 ±0.66
RT	MA	3.59 ±0.15	20.9 ±0.90^{ab}	7.60 ±0.71
	BSB	3.00 ±0.10	22.3 ±0.92^{a}	8.42 ±0.92
	SS	3.34 ±0.26	19.5 ±0.36^{abc}	7.64 ±0.19
	BSA	2.84 ±0.10	17.9 ±0.54^{bc}	6.74 ±0.47
Tillage *p* value		0.38	**<0.001**	0.34
Sowing *p* value		**0.04** (MA^{a} BSB^{b} BSS^{ab} BSA^{ab})	**0.005**	**0.05** (MA^{ab} BSB^{a} SS^{ab} BSA^{b})
Interaction *p* value		0.62	**0.002**	0.822

b) Bean

Treatment		Grain $\delta^{15}N$	N concentration (mg g^{-1} DW)	P concentration (mg g^{-1} DW)
CT	BA	1.8 ±0.25^{ab}	31.6 ±0.68^{c}	5.49 ±0.31
	BSB	1.6 ±0.09^{ab}	34.5 ±1.05^{abc}	4.90 ±0.11
	SS	1.1 ±0.43^{bc}	31.3 ±0.51^{c}	5.90 ±0.45
	BSA	2.6 ±0.04^{a}	35.3 ±1.38^{abc}	5.39 ±0.34
RT	BA	0.38 ±0.18^{c}	32.7 ±1.04^{bc}	6.73 ±0.09
	BSB	0.95 ±0.22^{bc}	36.3 ±1.03^{ab}	7.12 ±0.31
	SS	0.71 ±0.40^{bc}	37.3 ±0.99^{a}	6.89 ±0.10
	BSA	0.91 ±0.07^{bc}	33.5 ±0.96^{abc}	6.21 ±0.28
Tillage *p* value		**<0.001**	**0.011**	**<0.001 (**CT ^{b} RT ^{a})
Sowing *p* value		**0.044**	**0.009**	0.195
Interaction *p* value		**<0.001**	**0.001**	0.06

3.4. *Water use efficiency, N fixation and N transfer to maize plants*

In maize, at V6, the BSB treatment had higher water use efficiency (WUE, less negative $\delta^{13}C$) than the other treatments regardless of tillage. In maize grain, the CT treatments had higher WUE than RT treatments (Table 5). In bean at V4 (data not shown) and grain, the only treatment with lower WUE was the BSA treatment (Table 5).

At V6, regardless of tillage, BSB treatments presented the lowest $\delta^{15}N$ value in maize plants, suggesting transfer of fixed N from bean to maize, and a higher transfer in BSB (Fig 8a; Sowing, $p<0.001$). Simultaneous sowing showed also lower $\delta^{15}N$ values, but less clearly than the BSB treatment. MA and BSA, had higher and very similar values in maize. Similar trends were found in maize grain and the BSB treatments had the lowest δ15N value (Table 4). Bean at stage V4 and grain, had lower $\delta^{15}N$ values in RT than in CT treatments in all sowing treatments (Fig. 8b, Tillage, $p<0.001$; Table 4).

For bean, at V4 and in grain, RT treatments had overall higher percentage of N derived from atmospheric fixation (Ndfa) than CT treatments and plants in the different sowing treatments obtained different proportions of N from fixation (T x S, $p<0.001$, Fig. 9a). The treatment BSB with RT had the highest (38%) Ndfa at V4, and the lowest (almost zero) in BA and BSB with CT. At harvest, all RT treatments obtained close to 40% Ndfa, whereas CT treatments obtained 30% less, and less than 10% in the BSA treatment (T x S, $p<0.001$, Fig. 9b).

Table 5. Means (± SE, n=5) $\delta^{13}C$ of maize and bean plants measured in grain at harvest in the tillage and sowing treatment combinations.

Treatments		δ13C	δ13C
CT	MA/BA	-12.6 ±0.06	-27.9 ±0.03
	BSB	-12.4 ±0.03	-28.3 ±0.08
	SS	-12.5 ±0.03	-28.4 ±0.08
	BSA	-12.4 ±0.04	-36.2 ±0.60
RT	MA/BA	-12.6 ±0.08	-28.1 ±0.07
	BSB	-12.5 ±0.10	-27.6 ±0.03
	SS	-12.6 ±0.03	-28.2 ±0.08
	BSA	-12.6 ±0.08	-31.5 ±2.09

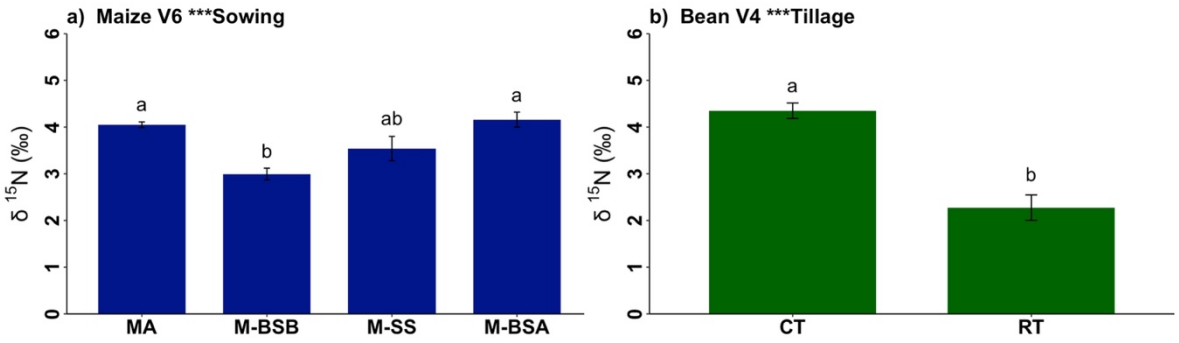


Figure 8. Means (± SE, n=5) of $\delta^{15}N$ values at early stages of a) maize (V6) and b) bean (V4) plants in the sowing and tillage treatments. Different letters indicate significant differences ($p < 0.05$) between means according to Tukey′s Test.

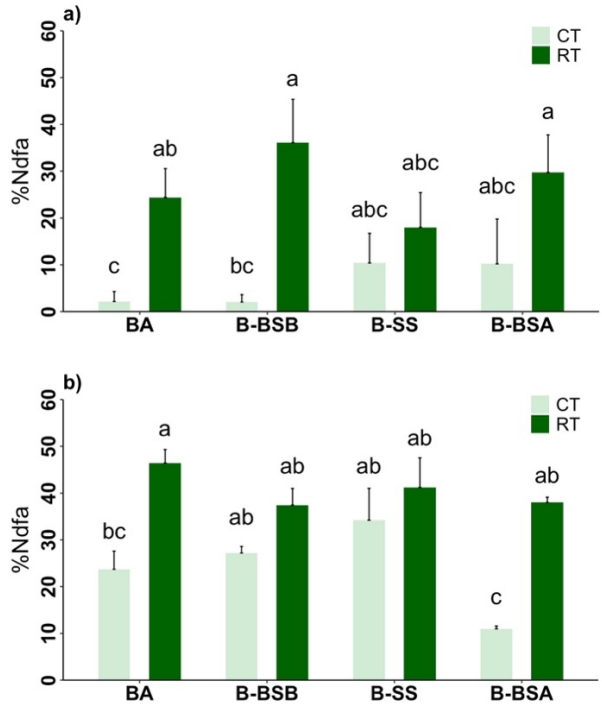


Figure 9. Means (± SE, n=5) of percent of N derived from N fixation in bean at V4 (a) and harvest (b) in the tillage and sowing treatment combinations.

3.5. *Relationships among AM fungal development, early nutrition, growth and yield*

Most relations between early mycorrhizal development, nutrition, growth, and yield were either neutral or negative for maize, and positive for beans (Fig. 10). For maize, mycorrhizal development variables had negative relations with shoot dry weight and grain yield but neutral or positive with shoot and grain N and grain P concentration, especially grain N (Fig. 10). For bean, mycorrhizal development had positive relationships with shoot and grain P and $\delta^{15}N$.

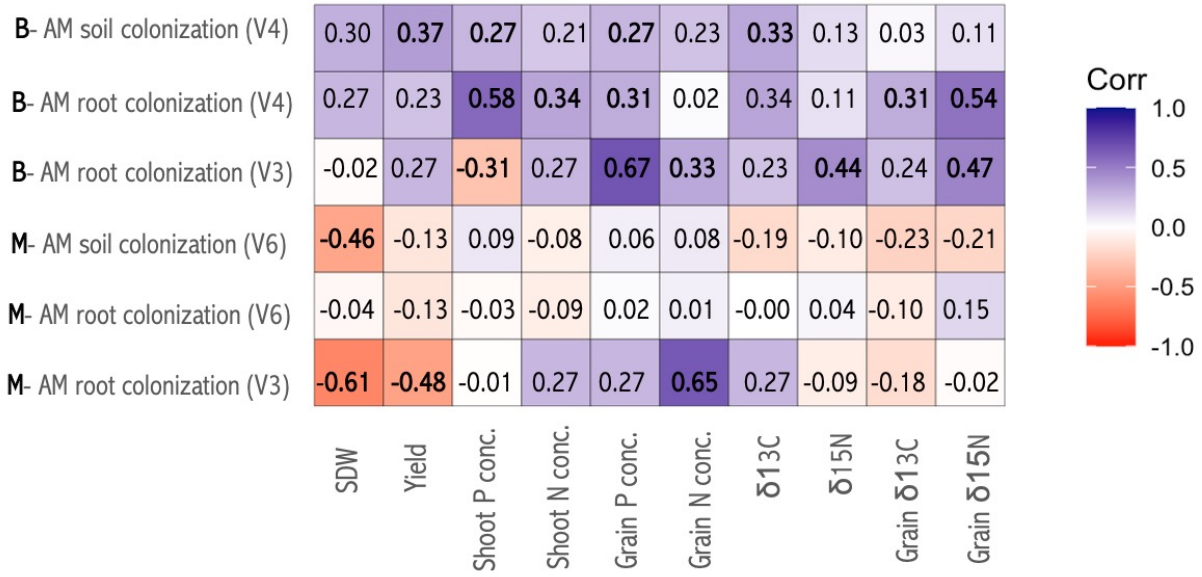


Fig. 10. Correlogram showing the relationships between the variables of AM colonization and the variables related to the development and nutrition of maize and bean plants at different stages of plant development (V3/V4 for bean and V3/V6 for maize). Bold initial letters indicate the relationships between variables for bean (**B**) and maize (**M**). Color of squares indicate the strength and direction of correlation. Standardized effect estimates of multiple linear regression are presented inside each square and bold values indicate correlations with a P value <0.05.

Early P nutrition in maize was negatively related to shoot biomass and grain yield but was positively related to N allocation to grain (Table 6a). Early N nutrition of maize was related to higher transfer of fixed N (lower values of $\delta^{15}N$), higher WUE (lower values of $\delta^{13}C$) and grain N concentration. Higher transfer of fixed N to maize at V6 was related to higher N allocation to grain. In bean, early P nutrition was positively related to the yield and nutritional quality of the grain, including grain N from fixation. N nutrition of bean at V4 was positively related to WUE in grain (Table 6b).

Table 6. Standardized effect estimates of relationships between N and P concentrations and plant properties at several plant developmental stages in a) maize plants and b) bean plants. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

a)

Parameter	Stage	SDW	Yield	δ13C	δ15N	Grain P conc	Grain N conc	Grain δ13C	Grain δ15N
Shoot P conc.	V3	-0.23ns	0.24ns	-	-	-0.03ns	0.02ns	0.11ns	-0.09ns
	V6	-0.23ns	-0.08ns	-0.27ns	0.16ns	-0.32ns	0.11ns	-0.03ns	0.25ns
	V10	-0.54***	-0.38**	-	-	0.06ns	0.39*	-0.37*	0.16ns
Shoot N conc.	V3	-0.08ns	-0.03ns	-	-	-0.19ns	-0.02ns	-0.04ns	-0.18ns
	V6	-0.11ns	-0.27ns	0.55***	-0.34ns	0.31ns	0.45*	-0.28ns	0.25ns
	V10	0.08ns	-0.07ns	-	-	0.27ns	0.01ns	-0.03ns	-0.13ns

b)

Parameter	Stage	SDW	Yield	δ13C	%Ndfa	Grain P conc	Grain N conc	Grain δ13C	Grain %Ndfa
Shoot P conc.	V3	0.04ns	-0.26ns	-	-	0.53***	-0.20ns	-0.03ns	0.18ns
	V4	-0.08ns	0.34ns	-0.21ns	0.31ns	0.09ns	0.35ns	0.09ns	0.14ns
	R5	0.31*	0.38*	-	-	0.34*	0.34*	0.00ns	0.25ns
Shoot N conc.	V3	0.19ns	0.04ns	-	-	0.01ns	-0.02ns	0.23ns	0.33*
	V4	0.11ns	0.18ns	0.24ns	0.11ns	-0.02ns	-0.18ns	0.48***	0.02ns
	R5	-0.28ns	-0.05ns	-	-	0.36**	0.20ns	-0.41**	0.41**

3.6. Pot experiment

The pot experiment to explore if there was a soil health problem regardless of the treatments applied, given the observed low emergence and stunting of some maize plants, showed that soil disinfection inhibited AM colonization but had no significant effects on shoot dry weight for both crops (Tables 7 and 8). Also, maize and bean reached adequate shoot N and P concentration in all treatments (Table 9). Thus, we discarded a major soil health problem confounding our results.

Table 7. *P* values and significance for the main effects of Sterilization (S) and Development Stage (DS) factors and their interactions on all variables measured in more than one stage of maize development in a) Maize plants and b) Bean plants * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Plant stages column indicates the plant development stages where the parameter measurement was made.

a)

Parameter	Plant stages	Sterilization	DS	S*DS
DW	V3-V10	0.18	***	0.98
AMF	V3-V6	***	***	***
Others	V3-V6	*	0.45	0.75
N conc	V6-V10	***	**	0.14
Total N	V6-V10	0.07	***	0.6
P conc	V6-V10	0.36	0.43	0.21
Total P	V6-V10	0.18	***	0.47

b)

Parameter	Plant stages	Sterilization	DS	S*DS
DW	V3-V10	0.37	***	0.73
AMF	V3-V6	***	***	***
Others	V3-V6	*	0.53	0.53
N conc	V6-V10	0.36	***	0.71
Total N	V6-V10	0.3	***	0.68
P conc	V6-V10	0.51	***	0.46

Total P	V6-V10	0.42	**	0.95

Table 8. AM fungi root colonization (%) at different developmental stages of maize and bean plants in the sterilization and plant developmental stage treatment combinations in maize and bean plants.

	Maize		Bean	
Treatment	Plant stage	AMF root colonization	Plant stage	AMF root colonization
Autoclaved	V3	0 ±0[c]	V3	0 ±0[c]
Non-autoclaved		24 ±1.5[b]		22 ±1.4[b]
Autoclaved	V6	0 ±0[c]	V4	0 ±0[c]
Non-autoclaved		46 ±5.0[a]		44 ±3.7[a]

Table 9. Means (± SE, n=3) shoot nutrient concentration (mg g^{-1} DW) and total shoot nutrient concentration (mg plant DW) at different developmental stages of maize and bean plants in the sterilization and plant developmental stage treatment combinations.

	Maize			Bean		
Treatment	Plant stage	N conc	P conc	Plant stage	N conc	P conc
Autoclaved	V6	46 ±0.25	2.9 ±0.24	V4	58 ±4.1	7.9 ±0.49
Non-autoclaved		38 ±2.3	3.1 ±0.84		56 ±0.86	7.1 ±0.35
Autoclaved	V10	41 ±0.87	3.2 ±0.38	R5	47 ±2.1	5.2 ±0.28
Non-autoclaved		36 ±0.50	2.1 ±0.20		44 ±1.0	5.2 ±0.95

4. Discussion

Sowing time in maize-bean intercropping matters, according to our results, but not equally for both crops. The sowing order was overall more important for bean than for maize, which is relevant knowledge given that crop relay is gaining popularity in intercropping. Tillage, in turn, affected the nutrition of both crops and the development of maize. Additionally, reducing tillage to preserve mycorrhizal networks increased bean N fixation and yield, and sowing bean before maize increased the transfer of fixed N to maize.

4.1. Soil disturbance effects on early AM fungal development and function

As we predicted, soil disturbance due to conventional tillage resulted in a delay in mycorrhizal development at the early growth stages of maize and bean development. Root colonization recovered quickly and at V6 it was almost uniform, and soil colonization was still lower only in BSA (for maize) and BSB (for bean) treatments. This is consistent with previous reports which described that the initial AM colonization was affected by tillage (e.g. Jansa et al., 2006; O'Connor et al., 2009) and with the fact that intra-radical colonization occurs first and the mycelial network develops in the soil afterwards (Tikhonovich and Provorov, 2007). Early AM fungal root colonization and preserved extraradical networks lead to better early plant nutrition at least at one stage of development in both maize and bean, and most of the treatments that did not reach the critical shoot N and P concentrations were those with conventional tillage. Higher early N fixation by bean plants under RT than under CT treatments and the fact that under RT maize received a greater transfer of fixed N from the BSB bean treatment, as early as the V6 stage, suggest that maize and bean became rapidly connected through CMN. Preservation of CMN with RT was important to enhance N fixation in bean and its transfer to maize, as evidenced when combining the head start for

early N fixation in bean and the rapid transfer of fixed N to maize facilitated by CMN. This alteration of early development and functioning of mycorrhizal networks at early stages by tillage allowed us to test our hypotheses.

4.2. Bean and maize nutrition

The prediction that maize would supply the network mainly with surplus C and bean with surplus N, and that the resource in competition for both crops would be P, seemed being supported by our results. Maize got ample supply of N in all treatments but had insufficient P in several sowing treatments and developmental stages, and especially with conventional tillage (Fig. 4). Bean could marginally maintain its critical N concentration and had insufficient N in some sowing treatments and developmental stages, especially with conventional tillage, but got enough P except in one case, BSB-CT at V4 (Fig. 6e). The preservation of CMNs with RT likely increased %Ndfa in bean through adequate P nutrition supporting N fixation. This suggests that bean N and P nutrition benefited more from the preserved CMNs than maize nutrition. Still, maize obtained significantly more fixed N from preserved CMNs when a time advantage was given to bean, a benefit not reflected in growth or yield but with relevant implications for nutrient use efficiency in intercropping. Non-fixing plants in intercrop with fixing plants can also benefit from the transfer of N released from dead tissues and root exudates that are taken up from the soil solution via roots or CMNs (Laberge et al., 2011; Meng et al., 2015). It has been reported that nodulation and nitrogenase activity in common bean begins 10-11 days after emergence, and that the onset of active N_2 fixation that can supply plant demand occurs after up to 25 days (Hungria et al., 1991; Zoffoli et al., 2021). Therefore, sowing bean plants before maize could allow time for the establishment of a successful colonization of legume roots by AM fungi and rhizobia, which

combined with preserved CMNs resulted in the greater transfer of fixed N to maize. This can be very important for intercropping in low-N soils or in fields under fertilizer reduction and optimization practices.

4.3. Were the benefits of connecting to mycorrhizal networks greater for the crop that was established first in the network?

Our expectation that the allocation of mineral nutrients would be greater to the most developed crop (in terms of biomass production) connected to the CMNs, was in general not supported. The head start and rapid connection to intact CMNs was an advantage for bean but not for maize, and BSB with RT turned out the overall best combination for growth and nutrition of bean. The resource that bean provided at plant early stages to the CMNs, most likely N, seemed to play a more important role than its biomass demanding resources from the CMNs. Maize at RT had higher N and P concentrations when bean was not present but also when bean developed earlier. However, the possible physical impedance, or unknown detrimental factor of soil with reduced tillage (not likely soil temperature in this region), negatively affected maize growth and yield parameters despite the slightly improved N and P nutrition. The differences meant being able to reach or not the critical concentrations in the case of maize. BSB with CT was the best combination for maize growth. Bean, not affected so badly by soil impedance, got more N, and marginally more P, with RT when maize developed simultaneously or earlier. These results suggest that bean benefited from the presence of maize but was a nutrient competitor for maize, and by sowing bean first this competition was reduced.

4.4 Did early connection to preserved CMNs lead to a positive relationship with crop nutrition, N fixation by bean, and transfer of fixed N to maize?

Although most relationships between growth parameters and AMF were neutral or negative for maize, growth, shoot nutrients and the yield of bean plants became positively related to AM fungal colonization in roots and soil. This is an encouraging result to promote early AMF-host interactions through preserved CMNs and resource use optimization in agricultural environments with low fertility, as shown by Zhu et al. (2023). Intercropping in various planting schemes have demonstrated to be beneficial for both crops in cereal-legume combinations (Qiao et al., 2016), although not always in the same proportion, depending on soil fertility and crop combinations (Li et al., 2020a, 2022). The improvement in rhizobial activity and biological N fixation in mycorrhizal plants has been reported (van der Heijden et al., 2016; Püschel et al., 2017) and is probably explained by the improvement in the P nutrition of the host plants, since the maintenance of rhizobial symbiosis has a great P demand (Jakobsen, 1985; Püschel et al., 2017).

Growth suppression of bean seedlings by maize has also been shown in other studies (Francis et al., 1982; Nurk et al., 2017), since well-established maize with high biomass can probably acquire an advantage over bean seedlings due to increased nutrient uptake. Reduced bean photosynthesis as a consequence of such reduction in nutrient supply has reduced light penetration due to shading and water availability (Kaschuck et al., 2009; Makoi et al., 2010). The C4 plant maize, the plant with the largest biomass and typically not C limited, as other C3 plants (Walder et al., 2012), likely provided most of the C to the AM fungal network without being affected (Řezáčová et al. 2018). Therefore, the greater growth response of bean to mycorrhizae seems related to mineral nutrients, whereas maize may have had a growth limitation by other soil factors. The plant density, growth and yield reductions observed in

maize in all RT treatments point out to physical growth constraints probably related to the high clay content in soil. Our pot experiment showed that, when soil was sieved to fill the pots, maize emergence, and early nutrition and development were optimal and equal in fresh and autoclaved soil. We discarded thereby a soil health problem in RT and reinforced that the RT soil in the field had physical constraints for maize development that were alleviated by conventional tillage. Our study should be replicated in the future, ideally in other sites with different soil and environmental conditions, and especially in soils with less clay that causes compaction problems for maize development.

In conclusion, our results provide more evidence supporting that CMNs can be managed to provide more benefits to crops and that soil conservation practices through reduced tillage and fertilization practices are essential to capture more benefits from CMNs, as suggested in recent reviews (Alaux et al., 2021; Brito et al., 2021). Our hypothesis that the allocation of resources was greater for the most developed plant was not supported and the results suggested instead that the resources mobilized by each species connecting to CMNs were more important than their biomass. Two important detected outcomes were the improvement of N fixation in bean and of the presumably transfer of fixed N to maize associated to an advanced connection to preserved CMNs. Phosphorus mobilization through CMNs seemed to be the resource giving the head advantage to bean when planted first and triggering the increase in symbiotic N fixation and transfer to maize. These results are encouraging to support sustainable practices aimed to reducing tillage and chemical fertilization to favor natural nutrient acquisition and mobilization mechanisms and symbiotic associations.

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CAPÍTULO V

Conclusiones

Existe una necesidad creciente por comprender de forma más profunda las interacciones biológicas dentro de los agroecosistemas modernos, particularmente, la respuesta de los HMA y su asociación con las plantas ante la implementación de diferentes prácticas agrícolas. En este estudio surgen algunas conclusiones derivadas de los resultados obtenidos en esta tesis que aportan al conocimiento existente.

1) Las prácticas de la agricultura convencional como la labranza convencional, la fertilización mineral y el uso de fungicidas disminuyó la biomasa, el grado de colonización y/o funcionalidad temprana de los HMA.

2) La fotosíntesis fue estimulada por la colonización por HMA en la etapa más temprana del desarrollo del maíz (etapa V3), lo cual parece relacionarse con una mejor nutrición mineral que estimuló la tasa de transporte de electrones e incrementó la eficiencia del uso del agua y la difusión del CO_2 dentro del mesófilo. En los tratamientos con menor micorrización arbuscular la falta de nutrientes pareció ser la limitación para incrementar las tasas de fotosíntesis.

3) La predicción de mayor intercambio de gases, mayor nutrición de las plantas, mayor biomasa y mayor rendimiento y calidad nutricional del grano de las plantas de maíz con redes micorrízicas preservadas que en suelos perturbados fue apoyada sólo parcialmente, en la etapa más temprana del desarrollo de la planta evaluada, posiblemente debido a la interrupción de la relación funcional entre los HMA y el maíz por las interacciones entre los organismos colonizadores de raíces y la disponibilidad de nutrientes que inhibieron las contribuciones de la simbiosis micorrízica hacia las plantas. Los datos obtenidos sugieren que otros hongos, además de los HMA, que habitan las raíces también estuvieron implicados en una interacción negativa entre la disponibilidad de nutrientes y la nutrición del maíz, que al parecer exacerbaron el costo de los microorganismos que habitan las raíces que derivaron en la reducción del rendimiento del maíz. Parece ser que las prácticas de la agricultura convencional provocaron cambios en la estructura de la comunidad, donde incluso pudieron seleccionarse especies menos eficientes para el beneficio de la planta,

causando depresiones del crecimiento. Esto muestra que el esquema actual predominante de manejo agrícola es perjudicial para los HMA, por lo que resulta evidente la necesidad de más investigación, especialmente en condiciones de campo y a largo plazo, para comprender cómo la asociación micorrízica arbuscular responde a la implementación de diferentes prácticas agrícolas y cómo se ve reflejado en el crecimiento y rendimiento de los cultivos.

Los resultados de los puntos 1-3 revelaron una interrupción de la relación funcional hipotética entre los HMA y el maíz unos días después de la emergencia de la planta, lo que cambió el curso de las relaciones funcionales esperadas. Los resultados de investigaciones acerca de los efectos/beneficios de las asociaciones planta-HMA no siempre han sido tan exitosos como se esperaba, dando como resultado una respuesta variada del crecimiento de las plantas a los HMA (Klironomos, 2003; Feddermann et al., 2010). Esta interacción planta-HMA puede estar influenciada por factores como la eficiencia simbiótica, disponibilidad de nutrientes y agua, pero también por las interacciones entre plantas, HMA y otros microorganismos del suelo que regulan la eficiencia de la relación simbiótica (Berendsen et al., 2012; Bennet y Groten, 2022). Aquí, se asumió que la magnitud de las respuestas de las plantas fue la consecuencia de las interacciones tempranas de la comunidad de HMA-otros hongos-disponibilidad de nutrientes-etapa de desarrollo de las plantas en suelos donde dominaban las prácticas agronómicas comunes. En consecuencia, es posible que ciertas prácticas agrícolas convencionales, como fertilización y uso de plaguicidas, pueden exacerbar el costo de los microorganismos que habitan en las raíces y hacer que estas simbiosis sean innecesarias para optimizar el rendimiento de los cultivos.

Dado que, especialmente en condiciones de campo no existen controles adecuados para evaluar las funciones de los HMA, otros hongos no micorrízicos y otros microorganismos están presentes simultáneamente en las raíces de las plantas. Es difícil entonces diseccionar el papel de los HMA y es probable que los resultados aquí obtenidos estuvieran asociados muy probablemente a la presencia de aumentos de población en ambos grupos de hongos en las raíces. Estas interacciones generalmente pasan desapercibidas, ya que los estudios sobre el papel de los HMA en relación con el desarrollo de las plantas normalmente no incluyen

las posibles influencias de los hongos no micorrízicos que colonizan las raíces en condiciones naturales de campo. En consecuencia, esta tesis contribuye a la investigación dirigida a comprender otros factores que influyen en la simbiosis micorrízica y a guiar el diseño de futuros experimentos que incluyan la evaluación de las interacciones entre micorrizas y otros organismos que habitan en las raíces para que los beneficios de las micorrizas puedan aprovecharse en el desarrollo de estrategias de manejo para agroecosistemas sostenibles (Johnson y Gibson, 2020). Además, los resultados de las depresiones del crecimiento causadas aparentemente por los HMA deben tener en cuenta el efecto del método para obtener tratamientos de control de HMA en organismos no objetivo.

4) Nuestra expectativa de que los beneficios de la micorriza serían mayores para el cultivo que se conectara primero a una red intacta funcional y recuperara recursos de ella más rápido y sin competencia se vio respaldada cuando se sembró frijol primero, lo cual se reflejó en su desarrollo, nutrición y rendimiento, pero no cuando se sembró primero maíz. El frijol, pero no el maíz, tuvo una ventaja competitiva en el cultivo intercalado si se conservaban las redes y era el primer cultivo que se conectaba a la red. Para el maíz, el recurso limitante fue P y para el frijol fue N, lo que sugiere que el intercambio de recursos favoreció la adquisición de N en el maíz y la adquisición de P en el frijol. El maíz no alteró significativamente su crecimiento derivado de una competencia con el frijol en ninguno de los escenarios evaluados, pero también obtuvo el mayor beneficio en rendimiento y en la transferencia de N derivado de la fijación de N cuando el frijol se sembró primero.

El sistema de cultivos intercalados de leguminosas-cereales tiene grandes perspectivas de desarrollo en los sistemas agrícolas futuros (Lai et al., 2022). Este cultivo intercalado a menudo aumenta el rendimiento, en parte debido a las interacciones interespecíficas por encima y por debajo del suelo y a la facilitación para el forrajeo y uso de recursos (Lv et al., 2014; Li et al., 2022). Sin embargo, la asignación y utilización de nutrientes en los sistemas de cultivo intercalado de leguminosas-cereales y el papel de los HMA aún no están claras.

En la mayoría de los experimentos, ambas especies de plantas intercaladas se inocularon con HMA al mismo tiempo y se compararon con mezclas sin micorrizas y no está claro si el orden en que se colonizan las especies es importante para los efectos de la asociación micorrízica (Werner y Kiers 2015; Li et al, 2022). Los resultados del punto 4 tienen una potencial importancia en la práctica agrícola de los cultivos intercalados ya que en muchos sistemas actuales de cultivos intercalados de alto rendimiento, existe una diferenciación de nicho temporal sustancial entre ambos cultivos (Li et al., 2022), lo que implica que el cultivo que se planta primero probablemente esté colonizado primero por los HMA, pero los efectos de esto han sido poco estudiados y sobre todo en condiciones de campo. Los resultados demostraron que la asignación de recursos y la estimulación del crecimiento fue mayor para el frijol cuando era la planta más desarrollada, en este caso cuando se sembró primero, reduciendo la desigualdad competitiva entre las especies asociadas en el cultivo y afectando el rendimiento general en campo. También de manera importante se demostró, en condiciones de campo, la transferencia de nitrógeno fijado del frijol al maíz y como esta fue mayor cuando el frijol fue sembrado antes que el maíz. Estos hallazgos resaltan que la red micorrízica juega un rol importante en la transferencia de N en los sistemas de cultivo intercalado, sumando la escasa evidencia en campo que sugiere la participación de los HMA en las funciones de transferencia de N fijado en los cultivos intercalados de leguminosas-cereales (Wahbi et al., 2016; Li et al., 2022; Zhang et al., 2022). Esta transferencia puede resultar muy importante en suelos deficientes de N, considerando que el maíz es un cultivo de alta demanda de N y su deficiencia conduce a la reducción de su crecimiento y desarrollo (Machado et al., 2001; Agba y Long, 2005). Estos beneficios observados podrían ser un factor importante que contribuya a los beneficios de los cultivos intercalados.

Son necesarios entonces más estudios futuros que corroboren los resultados observados en el frijol y donde se puedan ver claramente los efectos sobre el maíz, con diferentes especies de plantas, suelos y condiciones ambientales, bajo diferentes prácticas de manejo en condiciones de campo. Estos estudios pueden complementarse en lo posible con la evaluación directa de la transferencia de nutrientes entre los HMA y la planta a través de marcadores isotópicos, y con estudios en macetas que permitan examinar con más detalle/control de ciertos aspectos como la transferencia directa de C de la planta a los hongos, ya que es fundamental

comprender mejor los mecanismos de transferencia de N fijado y regulado por los HMA en sistemas de cultivo intercalado. Los mecanismos a través de los cuales los HMA alteran la relación competitiva entre las plantas asociadas aún no están claros (Ingraffia et al., 2019), una mejor comprensión del uso complementario de cultivos junto con el potencial biológico para la adquisición eficiente de recursos por parte de los cultivos puede proporcionar un enfoque eficaz para mejorar la eficiencia del uso de nutrientes y la productividad de ambos cultivos.

Es importante considerar que los dos sitios de campo evaluados en este estudio tuvieron tiempos breves de recuperación del suelo (1-3 años) la historia de las perturbaciones, producto de las prácticas de manejo convencional, las cuales afectan la estructura y actividad microbiana del suelo. Las implicaciones funcionales de los cambios impulsados en la comunidad de HMA por estas prácticas podrían cambiar la asociación HMA-planta hacia un mutualismo reducido o parasitismo (Johnson et al., 1997; Verbruggen y Kiers, 2010). Resulta importante entonces impulsar el proceso de recuperación gradual de los mecanismos naturales del suelo, a través de la implementación de prácticas más amigables, como evitar la fertilización excesiva, que permitan expresar toda la capacidad biológica del suelo y aprovechar de manera efectiva los beneficios de las micorrizas en los agroecosistemas.

Lo anterior podría conducir al futuro desarrollo/mejora de estrategias de manejo que permitan explotar al máximo la eficiencia simbiótica con las comunidades de HMA nativas en los campos y, por ende, aumentar el beneficio de la simbiosis micorrízica arbuscular en el rendimiento agrícola. Esto sin perder de vista la urgencia de generar sistemas agrícolas más sostenibles que promuevan la transición hacia modelos productivos que busquen maximizar los mecanismos naturales de mantenimiento de la fertilidad en el suelo a largo plazo. Por lo tanto, es fundamental entender cómo se pueden maximizar los beneficios de las asociaciones de micorrizas en los sistemas productivos mexicanos considerando el contexto actual donde aún prevalece el esquema convencional y aún falta mucho conocimiento para diseñar prácticas alternativas exitosas. La investigación de los HMA continúa basándose principalmente en experimentos de invernaderos, sin embargo, ante la necesidad de comprender el papel de las comunidades de HMA en entornos naturales se han mejorado

metodologías e incrementado la experimentación en campo que se aproxima más al entorno donde se aplicará el conocimiento. Más experimentos de campo a largo plazo también serán indispensables en el futuro.

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