Common Mycorrhizal Networks Asymmetrically Contribute to Increased N and P Acquisition by Millet/Chickpea Mixture

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Abstract

Aim Cereal/legume intercropping is known to increase yield, partly because of increased nitrogen (N) and phosphorus (P) acquisition. The aim of this paper was to investigate the role of common mycorrhizal networks (CMNs) in overyielding by the crop species mixture and to find out if the effect of a CMN depends on which of the two species was colonized by AM fungi.

Methods Microcosms with two compartments were used, separated by a 30- μ m nylon mesh. Both compartments contained either chickpea or millet, in monoculture or mixed. One or none of the two compartments was inoculated with the AMF species *Funneliformis mosseae*. The plant in the inoculated compartment was referred to as the AMF donor, and the plant in the neighboring, non-inoculated compartment as the AMF receiver.

Results Inoculation in one compartment resulted in mycorrhiza formation in the other compartment, providing evidence for the formation of CMNs. Inoculation of chickpea in the mixture increased N and P acquisition and biomass of both chickpea (AMF donor) and millet (AMF receiver), whereas inoculation of millet increased biomass of chickpea (AMF receiver) only, but did not increase N or P acquisition by any of the two species. Chickpea as AMF donor had higher numbers of phosphate-solubilizing bacteria in its rhizosphere compared to chickpea as receiver. The shoot N:P ratio of chickpea as AMF donor was lower than as receiver.

Conclusion Our study demonstrated asymmetry in nutrient gains by a mixture of cereal and a legume, dependent on which plant species was the AMF donor or receiver. This suggests that initiating mycorrhizal networks by legumes in intercropping could be an important factor contributing to the magnitude of the intercropping effect.

Introduction

Intercropping is the simultaneous cultivation of two or more crop species in close proximity in the same field (Willey 1990). It is widely practiced in low-input tropical agriculture, and is also resurgent in developed countries as a sustainable means for pest suppression (Zhang et al. 2019; Zhu et al. 2000) and nutrient management (Li et al. 2020b). Intercropping often produces greater combined biomass than their component crops when grown alone (Li et al. 2020b). The intercropping advantage is due to interspecific interactions aboveground and belowground, of which complementarity in (Brooker et al. 2015; Li et al. 2019), and facilitation of resource use (Li et al. 2014; Duchene et al. 2017) are most important. These positive interactions are often a result of the inclusion of a legume, which has the ability to fix N₂ and to release phosphorus (P)-mobilizing exudates (Faucon et al. 2015; Xue et al. 2016). Legumes are adapted to low temperature and sowing early in the growing season, whereas C₄-cereals are adapted to late season with high temperatures (e.g., maize, millet). This allows for temporal niche differentiation. Temporal complementarity in N and P acquisition contributes to overyielding in relay intercropping (Li et al. 2020a, b). Moreover, legumes can improve their own P acquisition and potentially

facilitate that of the neighboring species by releasing acid phosphatases or phytases, protons and/or carboxylates in the rhizosphere that hydrolyze organic P, desorb organic and inorganic P from metal (hydr-)oxides, and solubilize Ca phosphates (Betencourt et al. 2012; Li et al. 2016b; Wang et al. 2017; Li et al. 2004).

Inter- or intraspecific interactions between plant species in mixtures can be influenced by arbuscular mycorrhizal fungi (AMF). The roots of the majority of plant species are colonized by AMF, which are ubiquitous in natural and agricultural ecosystems (Smith and Read 2008). The fungal mycelium colonizes and links roots of the same or different plant species, thereby forming common mycorrhizal networks (CMNs) (Walder et al. 2012). CMNs can mediate species interactions among connected plants (Weremijewicz and Janos 2013; Wagg et al. 2011; Workman and Cruzan 2016). For instance, a complementarity effect was demonstrated in mixtures of mycorrhizal maize varieties whereas there was no complementarity effect in the non-mycorrhizal mixtures (Wang et al. 2020).

The extraradical mycelium is also a habitat for bacteria growing on exudates in the hyphosphere (Toljander et al. 2007; Gahan and Schmalenberger 2015). These bacteria can be beneficial to the AMF as well as to the mycorrhizal plant, for instance when they solubilize inorganic P that can be acquired by the fungus and transferred to the plant (Zhang et al. 2016; Zhang et al. 2018).

Few studies have been conducted on the effects of CMNs on interspecific interactions in intercropping. In most experiments, both intercropped species were inoculated with AMF at the same time and compared to non-mycorrhizal mixtures. For instance, without AMF, faba bean was inferior to wheat in a pot experiment, but inoculation with AMF reduced competitive inequality between wheat and faba bean such that the latter benefitted (Qiao et al. 2015). Colonization by AMF in maize/soybean (Meng et al. 2015) and rice/mung bean mixtures (Li et al. 2009) improved N₂ fixation of soybean and mung bean, and promoted N transfer from legume to cereal. The presence of AM- enhanced crop biomass (while reducing weed biomass) and N and P uptake by plants in a maize/faba bean/foxtail mixture (Qiao et al. 2016). However, it is unclear whether the order in which species are colonized, matters for the effect. In CMNs one plant species (the AMF receiver) can be dependent on previous colonization of a neighboring plant species (the AMF donor), which may influence source-sink relationships. This question could be important because in many current high-yielding intercropping systems, there is substantial temporal niche differentiation between both crops, implying that the crop that is planted later is likely colonized by the mycelial network supported by the AMF that colonized the previous crop.

Plants do not become mycorrhizal randomly: There is bidirectional partner selection in CMNs (Werner and Kiers 2015). Selective colonization by the fungus seems to depend on factors like order of arrival at the roots or resource abundance as provided by the plant. In the first case one would expect differences in colonization rate depending on which plant species is the AMF donor or receiver. In the latter case differences between a plant being AMF donor or receiver would be less important than plant properties like rate of photosynthesis. Compared to non-N₂-fixing species, N₂-fixing species may exhibit higher photosynthesis rates (Li et al. 2004) which could allow for greater investment in the AM or nodulation.

Legumes appear to promote CMNs with neighboring cereals (Duchene et al. 2017). Given differences in resource availability between legumes and cereals, responses to AM colonization and resource allocation by CMNs could be asymmetrical between intercropped species (Wang et al. 2019; Hu et al. 2019). It is unknown how plant selection by AMF that initiated CMNs affect nutrient acquisition and biomass production in cereal/legume intercropping.

We aimed to investigate the role of AM in overyielding with a specific focus on the role of CMNs in a mixture of a cereal and a legume. We therefore studied both P acquisition (as assessed by plant P content) and its underlying mechanisms (acid phosphatase and carboxylate production and phosphate-solubilizing bacteria), as well as N acquisition (assessed by plant N content and the contribution of N_2 fixation). We used the millet/chickpea combination, which has shown overyielding in earlier pot and field experiments (Li et al. 2019, 2021b). We hypothesized that 1) AM contribute to enhanced P and N acquisition, and overyielding by mixtures compared to monocultures because they promote nutrient transfer between the mixed species; 2) the order of host species that initiated the CMNs in species mixtures differentially affect the nutrient acquisition and overyielding.

Materials And Methods

Soil substrate

The soil used in this study was collected from Changping district, Beijing, China (39°59'N, 116°17'E). It was a silt loam soil with pH (soil:water 1:5) 8.2, organic matter 4.54 g kg⁻¹, mineral nitrogen 13.88 mg kg⁻¹, Olsen-P 2.55 mg kg⁻¹, and NH₄OAc-K 56 mg kg⁻¹. The soil was air-dried, sieved (2 mm) and then irradiated to eliminate indigenous microorganisms (γ -radiation (> 25 k Gray), Beijing Radiation Application Research Center). Macronutrient and micronutrient elements were added at the following concentrations (in mg kg⁻¹ dry soil): 40 P as KH₂PO₄ (to ensure the initial growth of plants), 200 N as NH₄NO₃ (to ensure the growth of millet and provide the same N levels for millet and chickpea), 100 K as K₂SO₄, 50 Mg as MgSO₄·7H₂O, 200 Ca as CaCl₂·2H₂O, 5 Fe as EDTAFe-Na, 5 Mn as MnSO₄·H₂O, 5 Zn as ZnSO₄·7H₂O, 5 Cu as CuSO₄·5H₂O, 0.68 B as H₃BO₃, 0.12 Mo as (NH₄)₆Mo₇O₂₄·4H₂O.

Poly Vinyl Chloride containers (length 9 cm × depth 9 cm × width 6 cm), with a 30- μ m nylon mesh in the middle (to create two compartments), were used. The mesh allowed transport of water and nutrients between both compartments and also allowed penetration by AMF hyphae but not by plant roots. The sterilized soil (0.5 kg) was placed in each compartment. Inoculum of *Funneliformis mosseae* BEG 119 (T.H. Nicolson & Gerdemann) C. Walker & Schluessler consisted of a mixture of spores, mycelium, fine root segments and growth medium of soil and river sand. The inoculum was propagated in sterilized mixture medium of soil and river sand with maize (*Zea mays* L. cv. Zhengdan 958) grown for 4 months. Mycorrhizal compartments received 20 g of inoculum (approximately 10,000 spores kg⁻¹ soil) placed 2 cm under the seeds. The non-mycorrhizal compartments were amended with an equal amount of sterilized (121°C for 2 h) inoculum. To minimize differences in microbial communities in mycorrhizal and

non-mycorrhizal soils, 10 mL of AMF-free soil filtrate was added to each compartment. The filtrate was prepared as follows: 500 g of mycorrhizal fungal inoculum was added to 1 L of sterilized water. Then the soil suspension was filtered through five layers of filter paper (diameter 11 cm, Shuangquan Brand) to remove mycorrhizal fungal hyphae and spores.

Experimental design

Chickpea (*Cicer arietinum* L. cv. Longying-1) and millet (*Setaria italica* L. cv. Longgu-11, supplied by Gansu Academy of Agricultural Science) were grown in monoculture (two plants of the same species in each compartment of a container), or in mixture. AMF or sterilized inoculum was added to one of the two compartments, resulting in seven treatments with 12 experimental units (Fig. 1): chickpea monoculture with or without one compartment inoculated with AMF; millet monoculture with or without one compartment inoculated; millet/chickpea mixture, with only chickpea or only millet inoculated, and the same mixture without inoculation. The compartment inoculated with AMF was labeled as + AMF, the compartment without AMF (AMF receiver) with an inoculated plant in the neighboring compartment (AMF donor) was labeled as + AMFN. If both compartments of a container were not inoculated, the plants were labeled as -AMF. The containers were arranged in a randomized block design in the greenhouse. Each treatment was replicated four times to give a total of 28 containers.

Seeds were surface-sterilized with 10% H₂O₂ for 30 min, rinsed thoroughly in sterile distilled water and pre-germinated on filter paper. The sterilized soil and the corresponding inoculum were filled into the millet compartments 2 weeks after sowing of chickpea and the germinated seeds of millet were sown 2 weeks later than chickpea, similar to what is commonly done under field conditions (Li 2020; Li et al. 2021a). This later sowing prevented competition for light between the two species caused by fast growth of millet. All containers were thinned to two plants per compartment 1 week after sowing. All chickpea plants were inoculated with *Mesorhizobium muleiense* (provided by the Culture Collection of Beijing Agricultural University) by seed inoculation. The germinated chickpea seeds were soaked in the rhizobium suspension for 30 min prior to sowing and liquid rhizobium inoculum was also added when transferring the chickpea.

The experiment was conducted for a period of 8 weeks from October till December 2016 in a greenhouse at China Agricultural University, Beijing, China. The light intensity was approximately 700–1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with 60–80% relative humidity. Heating was supplied such that the temperature ranged from 13°C at night to 25°C during the day. All containers were irrigated with deionized water daily to maintain soil moisture content at about 75 % of field capacity.

Harvest and sample analysis

All plants were harvested 8 weeks after chickpea sowing, *i.e.* 6 weeks after millet sowing. The two plants in each compartment were harvested and analyzed separately. The shoots were cut at the soil surface, and roots were collected from the soil carefully. The roots of one of the plants with tightly adhering rhizosphere soil were immersed in 50 mL 0.2 mM CaCl₂ solution and shaken carefully to extract

rhizosphere soil solution. A subsample of this extract was stored at -20°C for HPLC analysis in which six carboxylates were identified (tartrate, malate, citrate, succinate, fumarate, and trans-aconitate) (Li et al. 2010). The other subsample was stored at 4°C for determination of acid phosphatase (APase) activity.

Because the solution: rhizosphere soil ratios differed depending on root size and the amounts of rhizosphere soil, we could not directly compare the absolute carboxylate concentrations in the extracts among different treatments. We therefore calculated exudate ratios between different treatments under two extreme assumptions (Li et al. 2021): 1) The soil solid phase is inert and does not buffer the carboxylate concentration. Under this assumption the carboxylate concentration in the extract is a dilution of the rhizosphere solution concentration, and the carboxylate concentration can be expressed in $\mu\text{mol g}^{-1}$ rhizosphere soil dw; 2) The soil solid phase completely buffers the carboxylate concentration, such that the carboxylate concentration in the extract is the same as the concentration in the rhizosphere soil solution (expressed in $\mu\text{mol L}^{-1}$). We then tested whether the ratio, calculated under both assumptions, showed a significant difference (if both ratios $\pm 2 \times \text{SE} > 1$ or if both ratios $\pm 2 \times \text{SE} < 1$) between two treatments.

The determination of rhizosphere APase activity was performed as described by Neumann (2006). The analysis involved colorimetric estimation of the p-nitrophenol released by phosphatase activity after incubation of soil with 4 mL of 0.04 M sodium maleate buffer (pH 5.3) at 28°C for 30 minutes. The reaction was stopped by 0.5 M NaOH, and the absorbance was measured spectrophotometrically at 405 nm. One unit of APase activity was defined as the activity per gram soil that produced 1 μmol p-nitrophenol per hour. A similar procedure as for carboxylates was followed for APase activity to decide whether there was a significant difference in acid phosphatase activity between two treatments.

Rhizosphere soil was also collected by brushing the roots from the second plant in each compartment. That soil was stored at 4°C for isolation of bacteria that could solubilize tricalcium phosphate (referred to as PSB). Five gram of rhizosphere soil was aseptically transferred to an Erlenmeyer flask with 50 mL of sterile water and shaken at 180 rpm for 20 min. 100 μL of a 1000-fold dilution of the suspension was plated on agar medium consisting of (per liter) glucose 10 g, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g, CaCl_2 2.22 g, MgSO_4 0.1 g, FeSO_4 0.002 g, MnSO_4 0.002 g, KCl 0.2 g, NaCl 0.2 g, 1% bromocresol purple, agar 1.5%, and $\text{Ca}_3(\text{PO}_4)_2$ 5 g as insoluble P source. The number of colonies was counted after 2 days of incubation in the dark at 28°C and expressed as colony-forming units (CFU) per gram soil (Xing et al. 2016).

Shoots and roots (roots were collected as many as possible from the soil and washed) were oven-dried at 76°C for 72 h before weighing. The nodule number of chickpea roots were counted after root washing. A small amount of roots (approx. 0.5 g fresh roots) was selected for AMF colonization assessment. The plant samples (both shoot and roots) were ground and digested with $\text{HNO}_3\text{-H}_2\text{O}_2$ in a microwave accelerated reaction system (CEM, Matthews, NC, USA). The P concentration in the digested solutions was determined by inductively coupled plasma optical emission spectroscopy (ICP-AES, OPTIMA 7300 DV, Perkin–Elmer, USA).

The percentage root length colonized by AMF was estimated after clearing washed roots in 10 % KOH and staining with 0.05 % (v/v) trypan blue in lactic acid according to Phillips and Hayman (1970). Thirty pieces of root from each sample were randomly selected and each piece of root was intersected and scored three times, resulting 90 root scores for each sample. The number of colonized roots divided by total number of roots examined was calculated as the colonization rate.

Total P uptake per plant was calculated by subtracting seed P content (chickpea: 0.34 mg per seed; millet: 0.0034 mg per seed) from the sum of shoot P content and root P content (Eq. 1).

$$TotalPuptake = (ShootPcontent + RootPcontent) - SeedPcontent$$

1

A subsample of the dried shoot samples was ground in a ball mill to a fine powder and the N concentration and the atom% ^{15}N values were determined using a mass spectrometer (DELTA^{plus} XP, Thermo Finnigan Electron Corporation, Germany). The percentage of N derived from atmosphere (%Ndfa) was calculated according to the following equation (Fan et al. 2006):

2

Where $\delta^{15}N_{millet}$ and $\delta^{15}N_{chickpea}$ are the $\delta^{15}N$ value of millet and chickpea, respectively, and $B = 1.8$ (Lopez-Bellido et al. 2010). $\delta^{15}N$ value of sole millet was used for both sole and mixture treatments, as we could not exclude the possibility that N, which was fixed by chickpea, was transferred to millet.

The amount of N derived from atmosphere (Ndfa) was calculated as:

$$Ndfa = \%Ndfa \times N_{chickpea}$$

3

The observed biomass (or P or N content) of species mixtures was compared with the expected biomass (or P or N content). For the + AMFN millet/chickpea mixture with chickpea as AMF donor (compartments 6 + 10 in Fig. 1), the expected biomass (or P or N content) was the sum of biomass (or P or N content) of sole chickpea as AMF donor (comp. 9) and sole millet as AMF receiver (comp. 5). For the millet/chickpea mixture with millet as AMF donor (compartments 4 + 12), the expected biomass (or P or N content) was the sum of biomass (or P or N content) of sole chickpea as AMF receiver (comp. 11) and sole millet as AMF donor (comp. 3). For the millet/chickpea mixture without AMF, the expected biomass (or P or N content) was the average biomass (or P or N content) of sole species in both compartments without AMF.

Statistical analysis

For millet and chickpea separately, two-way ANOVA was performed on biomass, N and P content, CFU, Ndfa, nodule number, and AMF fractional colonization. Independent variables were cropping system (two levels: monoculture and mixture) and AMF inoculation (three levels: -AMF, +AMF, +AMFN). This ANOVA tested for the general effect of AMF on N and P acquisition and the underlying drivers through rhizosphere modification (see results in Supplementary Information). Subsequently we tested for specific

effects of CMNs through a second two-way ANOVA with cropping system (two levels: monoculture and mixture) and mycorrhiza (two levels: +AMF, +AMFN) (results in the following section). Data on P content of chickpea were log-transformed. Data on CFU of PSB were log-transformed for both plant species. A t-test was applied when comparing the observed biomass (N or P content) and the expected biomass (N or P content).

We used ANOVA, Tukey's HSD and t-test in the R programming language (R version 3.1.2, R Core Team, 2014).

Results

All non-inoculated plants remained free of mycorrhizal colonization. AMF receiver plants were all colonized (Fig. S1) indicating that the AMF fungus was compatible with both hosts and that CMNs had actually been formed. Sole millet biomass and N content did not benefit from mycorrhiza (bar 1 vs 3 & 5, Fig. S2a; Fig. S3a; Table S1), whereas chickpea did (bar 7 vs 9 & 11, Fig. S2b; Fig. S3b). For sole millet and sole chickpea, P uptake was increased by AM-colonized plants (+ AMF) or by plants with a colonized neighbor (AMFN) compared to that nonmycorrhizal plants (- AMF; bars 1 vs 3 & 5; bars 7 vs 9 & 11, Fig. S2c, d). In mixtures, millet biomass did not profit from AM colonization when it was the AMF donor (bar 2 vs 4), but was increased when it was the AMF receiver (bar 2 vs 6; Fig. S2a), whereas the mycorrhizal benefit of chickpea in mixtures was evident for biomass (Fig. S2b) and even more strongly so for P uptake Fig. S2d) regardless of whether it was a donor (bar 8 vs 10) or receiver (bar 8 vs 12). The same trends were noted for N content in response to AM colonization (Fig. S3a, b). N:P ratios on non-mycorrhizal millet and chickpea were higher than 20 and were significantly lower in mycorrhizal plants, especially in chickpea where N:P ratios decline from 26 to 9 (Fig. S5). There was no overyielding in biomass, P uptake or N content when plants were non-mycorrhizal (Fig. S4).

There was a significant interaction effect ($P < 0.001$ in both cases, Table S2) between cropping system and AMF inoculation on millet biomass (Fig. 2a) and P uptake (Fig. 2c). Mixing increased millet biomass and P uptake when chickpea was the AMF donor (bar 5 vs 6) but not when millet itself was the AMF donor (bar 3 vs 4). Contrary to millet, there were no significant interactions between AMF and cropping systems for biomass ($P = 0.92$) and P uptake ($P = 0.12$) in chickpea (Table S2). Chickpea biomass increased irrespective whether chickpea was AMF donor or receiver, but P uptake increased only when chickpea was AMF donor (bar 9 vs 10, Fig. 2b, 2d).

Mixing decreased shoot N content of mixed millet compared to sole millet when millet was the AMF donor (bar 3 vs 4), but increased millet shoot N content compared to sole millet when millet was the AMF receiver (bar 5 vs 6, Fig. 3a). The effect of mixing and inoculation on shoot N content of chickpea was contrary to that of millet and similar to the effects for P content. Mixing increased shoot N content of chickpea when chickpea was the AMF donor (bar 9 vs 10, Fig. 3b), but not when chickpea was the AMF receiver (bar 11 vs 12, Fig. 3b). In summary, inoculation of chickpea in the mixture increased shoot N

content of both millet and chickpea, but inoculation of millet in the mixture decreased millet shoot N content and did not affect that of chickpea.

N content of mixed chickpea derived from N_2 fixation was higher than that of sole chickpea regardless of which plant was AMF donor (Fig. 3c).

Mixing did not affect the nodule number of chickpea. But the nodule number of sole chickpea was higher when chickpea was the AMF donor than that when chickpea was the AMF receiver (bar 9 vs 11 & 12, Fig. 3d).

There was significant overyielding in mixtures with respect to biomass ($P = 0.03$), P uptake ($P = 0.003$) and shoot N content ($P = 0.01$) when chickpea was the AMF donor (Fig. 4). However, there was no difference between observed and expected biomass (and P uptake, shoot N content) of millet/chickpea mixture when millet was the AMF donor. When millet was the AMF donor, chickpeas biomass, P uptake and shoot N content increased at the expense of millet, suggesting that the fungus provided its nutrients to the (larger) plant that rewarded its more.

The N:P ratio of plants differed depending on the identity of the AMF donor (Table S2). Mixing increased the N:P ratio of millet (bar 3 vs 4) but not chickpea (bar 11 vs 12) when millet was the AMF donor (Fig. 5a). However, mixing decreased the N:P ratio of chickpea (bar 9 vs 10) but not millet (bar 5 vs 6) when chickpea was the AMF donor (Fig. 5b).

Both acid phosphatase activity and carboxylate concentration of millet were not affected by planting pattern and identity of the AMF donor (Table S3).

Acid phosphatase activity of chickpea was similar among treatments. The carboxylate concentration of mixed chickpea was lower when chickpea was AMF donor compared to that when chickpea was AMF receiver (Table S3).

Mixing decreased the CFU of PSB in both millet and chickpea rhizosphere when millet was AMF donor (bar 3 vs 4; bar 11 vs 12, Fig. 6a, b). However, mixing did not affect the CFU of PSB in both millet and chickpea rhizosphere when chickpea was AMF donor (bar 5 vs 6; bar 9 vs 10, Fig. 6a, b). The CFU of PSB in mixed chickpea was higher when chickpea was AMF donor compared to that of chickpea as AMF receiver (bar 10 vs 12, Fig. 6b).

There was no relationship between the CFU of PSB in millet rhizosphere and the carboxylate concentration under the assumption of no buffering or strong buffering capacity of the rhizosphere soil (Fig. 6c, e), but the CFU of PSB in chickpea rhizosphere decreased in response to the increasing carboxylate concentration of chickpea under both assumptions ($P < 0.001$, Fig. 6d, f).

There was no difference in AM colonization of millet or chickpea between different treatments (Fig. S1).

Our study demonstrated that the presence of AMF substantially increased both P and N uptake by millet/chickpea mixture and caused overyielding (Fig. S2-4). More importantly, our results demonstrated that the contribution of AMF to overyielding of mixtures depended on the identity of the AMF donor that initiated the mycorrhizal networks (Fig. 4). Both biomass and N and P uptake by millet and chickpea were increased compared to sole species when chickpea was the AMF donor and millet AMF receiver. When chickpea was AMF receiver and millet the AMF donor, only chickpea benefitted in biomass and there was no overyielding. Mixing decreased the N:P ratio of chickpea but not millet when chickpea was AMF donor. These results suggest plant selection by AM. The N:P ratio of chickpea without AMF was larger than 20 (N:P = 26, Fig. S5), indicating P limitation (Güsewell 2004). The N:P ratio of chickpea as AMF donor was lower than 10 (N:P = 7.7, Fig. 5), indicating N limitation (Güsewell 2004). AM released plants from P limitation, but when chickpea was AMF donor plants became N-limited, which promoted N_2 fixation. The enhanced N_2 fixation of chickpea could be beneficial to the growth of millet and microbes since the colonies of PSB in the chickpea rhizosphere were higher when chickpea was AMF donor compared to chickpea as AMF receiver (Fig. 6b).

We found that AM caused enhanced both P and N acquisition (Fig. S2, S3), and hence overyielding in millet/chickpea mixture (Fig. 4 vs. Fig. S4). Our results were consistent with previous studies that the presence of AMF contributed to positive root interactions between intercropped species (Qiao et al. 2016; Li et al. 2009), while there was no positive effect of species mixtures (Qiao et al. 2016) or variety mixtures (Wang et al. 2020) on the growth of plants in the absence of AMF.

Our results show that the order of species that initiated the mycorrhizal networks differentially affect the overyielding of N and P acquisition. Previous studies (Wang et al. 2020; Qiao et al. 2016) did not address issues of mycorrhizal networks. Even when both plants are inoculated with the same fungal species, it does not inevitably imply that a fungal individual connects both plants. Our study specifically addressed situations where a plant can only be colonized by an AMF that was already on the roots of a neighbor (so when certain plants act as donor or receiver of AMF inoculum). The possible explanation for the overyielding when the CMNs initiated by chickpea rather than by millet could be different functional-trait differences of both plant species that affected the plant-microorganism interactions (Legay et al. 2016). The trade of carbon for P and N within a CMN in the species mixture was not even. Hyphae preferentially allocate P to plants providing more C (Kiers et al. 2011). However, Walder et al. (2012), in an experiment where both plant species were inoculated at the same time, found a strong asymmetry in terms of trade: sorghum invested twice as much into the CMNs as flax, but flax acquired most of the total N and P provided by the CMNs. In our study, mixed millet obtained less P than monoculture millet when millet was the AMF donor from which CMNs were initiated (Fig. 2c, comp. 4 vs.3), but gained P when chickpea was the AMF donor (comp. 5 vs 6). That asymmetry suggests that if the first AMF donor was a legume then the fungus could further exploit opportunities to improve the nutrient acquisition by both legume and its neighbour. This differential behavior reflects strategies by AMF to differential host plant species, depending on the optimal cost-benefit ratio from the fungal perspective. Size and biomass of chickpea were larger than those of millet in the current study, and AMF preferentially allocated P to chickpea. The

larger benefit that the legume derived than the cereal, resulted in an AMF-mediated reduction of the competitive inequality between both crop species (see also Wagg et al. 2011).

Our results show that mycorrhizal chickpea acquired P via AMF mycelia rather than by its own P mobilization through carboxylates when it was an AMF donor. When chickpea was the AMF donor, chickpea reduced carboxylate exudation (Table S3) but increased the CFU of PSB (Fig. 6) compared to the treatment where chickpea was the AMF receiver. Moreover, the CFU of PSB in the chickpea rhizosphere negatively correlated with chickpea's carboxylate concentration (Fig. 6). However, with millet as AMF donor chickpea upregulated carboxylate production and lowered the CFU of PSB. These data suggest a tradeoff between alternative strategies, either through exudates or to PSB, which are possibly associated with the hyphosphere (Zhang et al. 2018; Zhang et al. 2016; Ryan et al. 2012).

In the present study, AM fungal colonization was higher in millet than chickpea (Fig. S1). Higher fractional colonization of the cereal than the legume was contrary to a previous study showing higher AM fungal colonization in chickpea than pearl millet (*Pennisetum americanum* L.), possibly related to the more coarser root architecture of the former (Wen et al. 2019). However, the latter study also indicated higher fractional colonization of both sorghum and maize than chickpea. P uptake was lower for millet as AMF donor than as AMF receiver (Fig. 2b) although the AM fungal colonization of millet was similar in both treatments. The reason could be related to a more efficient or larger network with chickpea as AMF donor, as millet biomass and N content were also smaller when it was AMF than AMF receiver. The N:P ratio of millet was higher, and indicated P-limitation, when millet was AMF donor than when chickpea was AMF donor. In that condition chickpea was N-limited, which could have resulted in more P being delivered to millet, resulting in a lower N:P ratio. Further studies are needed to address underlying mechanisms on how C-N-P trade affects differential allocation of nutrients to the plants and differential allocation of C to the fungus depending on whether a plant is a AMF donor or receiver. Further studies are also required to address how the exudates of PSB could potentially mediate the interactions between mycorrhizal hyphae and PSB with respect to P mobilization.

Our results also showed that the increased P uptake by millet could also be due to the increased abundance of PSB in the treatments of mixed millet with chickpea as AMF donor. The increased CFU of PSB in the rhizosphere of chickpea as AMF donor compared to that of chickpea as AMF receiver reflects the increased amount of microbiota of the mycorrhizosphere. The CFU of PSB in the rhizosphere of millet as AMF receiver was slightly higher than that of millet as AMF donor. The reason could be that AMF hyphal exudates support the growth of the PSB, and the PSB benefit the AM by improving P availability, which has an indirect benefit for P uptake by plants (Zhang et al. 2016).

In the present study, nodule numbers of chickpea when mycorrhizal were higher than when non-mycorrhizal (Fig. S3d). There was no additional effect of the order of network formation on nodule numbers. The results were consistent with previous studies that showed the formation of mycorrhiza is beneficial to formation of nodules (Li et al. 2009; Ding et al. 2012), because legumes require P for N₂ fixation (Miteuskov et al. 2002). However, the N₂ fixation content of chickpea was affected by both AMF

inoculation and intercropping. Mixing millet with chickpea increased the N_2 fixation of chickpea (Fig. 3c). That was consistent with previous studies, which showed that maize/faba bean intercropping increased nodulation and N_2 fixation of intercropped faba bean (Li et al. 2016a), and intercropping wheat/faba bean increased nodule number and weight, and N_2 fixation of faba bean (Liu et al. 2017). That could be associated with the positive effect of root exudation of flavonoids in cereal rhizosphere on legume nodulation (Li et al. 2016a). Moreover, the differential decrease of N:P ratio of legume in response to the order of network formation suggests that AM colonization released P limitation but lead to N limitation, which could further promote N_2 fixation and nutrient transfer through the CMNs in the mixture. Millet also benefitted in N content from mixing with chickpea when millet was the AMF receiver, indicating potential N transfer via CMNs. Moreover, while our experiment was not set up to investigate N-transfers through CMNs, we observed lower $\delta^{15}N$ in intercropped millet when mycorrhizal, consistent with observations that part of the fixed N_2 was transported through the CMNs to millet.

The outcomes of species interactions such as P and N content and biomass were affected by CMNs and hence by the question which plant species was AMF donor or receiver. The presence of AMF facilitated N_2 fixation of chickpea and increased PSB in the rhizosphere, which benefitted N and P acquisition by both millet and chickpea, leading to overyielding. The fungus used in the present study is a common species in arable soils. The results may be also applicable to other fungal species as arable soils are dominated by members of the *Glomeraceae* to which this fungus also belongs (Daniell et al. 2001). CMNs initially formed by legumes could be a mechanism for overyielding of P uptake by cereal/legume intercropping in the field. Considering that AMF are essentially ubiquitous in the field, it is likely that hyphal networks between cereal and legume intercrops could balance the interspecific competition through preferentially colonizing the legume and differentially allocating the acquired nutrients to both plants. Under field conditions, for simultaneous intercropping systems, both species growing in an intercropping system may act both as AMF donor and receiver at the same time. However, in the practice of intercropping in China, legumes are normally sown earlier than cereals in relay strip intercropping (Li et al. 2020b; Li et al. 2020a). Through temporal niche differentiation the legume becomes the AMF donor and its hyphae can then connect to the cereal through CMNs, improving P and N acquisition by both species in such relay strip intercropping systems. This CMNs initiated by legumes could be an important factor contributing to the benefits of intercropping. Therefore field studies are needed for understanding the resource sharing between different crops species and also among same species simultaneously and harnessing such CMN for overyielding.

Declarations

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on a reasonable request.

Not relevant to the study.

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Figures

Figure 1

Design of the experiment, with seven treatments. + or – refers to plants with or without AMF inoculation. The numbers refer to the bar numbers in Figures 1, 2, 4, 5, 6. Numbers 1-6 represent millet, numbers 7-12

represent chickpea.

Figure 2

Plant biomass (shoot and root) (a, b) and P uptake (c, d) of millet (a, c) and chickpea (b, d) in the sole and mixture treatments, in the mycorrhizal treatment where it served as donor (+AMF) and the mycorrhizal treatment where it served as receiver (+AMFN). The same labels apply to all other figures. Bars represent means $\pm$ SE (n = 4). Bars with the same letter within a panel are not significantly different (P > 0.05). The numbers in the bars refer to the plant compartments as presented in Fig. 1.

Figure 3

Shoot nitrogen (N) content of millet (a) and chickpea (b) in sole (Sole) and mixture (Mixed), and N fixation content (c), nodule number of chickpea in sole and mixture (d). The numbers in the bars refer to the plant compartments as presented in Fig. 1.

Figure 4

Comparison of expected (Exp) and observed (Obs) biomass (shoot and root) (a), P uptake (b), shoot N content (c) of millet/chickpea mixture per container. +AMFC represents the millet/chickpea mixture with chickpea as AMF donor; +AMFM represents the millet/chickpea mixture with millet as AMF donor. Bars represent means $\pm$ SE. Asterisks refer to significant differences between observed and expected biomass (P uptake, N content) ** $P < 0.01$, * $P < 0.05$.

Figure 5

N:P ratio of millet (a) and chickpea (b) in sole (Sole) and mixture (Mixed).

Figure 6

CFU of phosphate solubilizing bacteria in the rhizosphere soil of millet (a) and chickpea (b) in sole (Sole) and mixture (Mixed). The correlations between CFU and carboxylate concentration are shown under the assumptions of no buffering (c, d) and strong buffering (e, f) in the rhizosphere soil.

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