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Shedding light onto nutrient responses of arbuscular mycorrhizal plants: Nutrient interactions may lead to unpredicted outcomes of the symbiosis

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ABSTRACT

The role and importance of arbuscular mycorrhizae (AM) in plant nitrogen (N) nutrition is uncertain. We propose that this be clarified by using more integrative experimental designs, with the use of a gradient of N supply and the quantification of an extensive array of plant nutrient contents. Using such an experimental design, we investigated AM effects on plant N nutrition, whether the mycorrhizal N response (MNR) determines the mycorrhizal growth response (MGR), and how MNR influences plants' C economy.

Oryza sativa plants were inoculated with *Rhizophagus irregularis* or *Funneliformis mossae*. AM effects were studied along a gradient of N supplies. Biomass, photosynthesis, nutrient and starch contents, mycorrhizal colonization and *OsPT11* gene expression were measured. C investment in fungal growth was estimated. Results showed that, in rice, MGR was dependent on AM nutrient uptake effects, namely on the synergy between N and Zn, and not on C expenditure. The supply of C to the fungus was dependent on the plant's nutrient demand, indicated by high shoot C/N or low %N. We conclude that one of the real reasons for the negative MGR of rice, Zn deficiency of AMF plants, would have remained hidden without an experimental design allowing the observation of plants' response to AM along gradients of nutrient concentrations. Adopting more integrative and comprehensive experimental approaches in mycorrhizal studies seems therefore essential if we are to achieve a true understanding of AM function, namely of the mechanisms of C/N exchange regulation in AM.

1. Introduction

Arbuscular mycorrhizae (AM) are widespread symbiotic associations formed between plants and fungi, and are arguably one of the most important symbioses between living organisms. AM are generally considered to be mutualisms. Although they can have multiple non-nutritional effects on the host plant that may determine increased survival and fitness (e.g. protection against pathogens or toxic minerals, or resistance to drought) [1,2], the main benefit to the plant from mycorrhizae is generally considered to be improved nutrition, in exchange for which the plant provides the fungal partner with C.

Numerous works have established that AM transfers N to the host plant, originating from both inorganic and organic N sources [3–5]. However, the role and importance of AM on plant N nutrition, and on N dynamics in AM remains doubtful. Published reports have resulted in a group of very different, and often contradictory, set of results, difficult to paste together in a coherent common framework.

AM can have a large variety of effects on N uptake by the plant. The effect of mycorrhization on N uptake (mycorrhizal N response-MNR) has been found positive in many cases, including in field studies [6–10], but in others it was found negative or there was no AM effect [11–15]. It is not clear under which conditions will AM be beneficial for N uptake. It is been repeatedly assumed that AM will have a positive effect on nutrient uptake at low nutrient availabilities and negative at high, and this was observed in some studies [4,5,16–18]. However, in others increasing N supply has resulted in increased N uptake and N transport by AMF hyphae to the plant [16,19–22].

Abbreviations: AM, arbuscular mycorrhizal; NM, non-mycorrhizal; N, nitrogen; C, carbon; MNR, mycorrhizal N response; MGR, mycorrhizal growth response; MR, mycorrhizal response; MPR, mycorrhizal P response; MZnR, mycorrhizal Zn response; MKR, mycorrhizal K response; MMnR, mycorrhizal Mn response; ERM, extra-radical mycelia; NP, N productivity.

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Effects on plant growth (mycorrhizal growth response-MGR) also vary from positive to negative, and this may be connected to effects on plant nutrition, and namely to MNR. It is accepted that positive MGR will be a consequence of higher nutrient uptake by AM plants, and relief of nutrient limitation to plant growth. In agreement with this, positive MGR seems to be always accompanied by positive effects on N, P, or both nutrition [6,8,10,17,23,24]. The causes for negative MGR are less clear. It has been classically interpreted as resulting from excessive C drain [25–27], but there are increasingly more evidences that this is not always, if ever, the case [25,28–30].

Nutrient retention is the possible alternative cause for negative MGR [25,30–33]. Some evidence of fungal N retention by AM fungi has been obtained [34,35]. However negative MGR are not always connected to negative MNR, making it unclear whether negative effects of AM on N uptake are responsible for negative MGR [14,36–38].

There is also great uncertainty about the regulation of the C/N exchange between partners. Some evidence indicating reciprocity in C/N exchange has been obtained using monoxenic cultures [39], although the regulation of C/N exchange may be more complex than merely increased supply of N being rewarded with increased supply of C [15,37,40]. Data obtained in pot cultures also does not support a clear reciprocity in C:N exchange between partners. Higher mycorrhizal colonization rates, indicating higher plant C investment in fungal growth, were sometimes accompanied by higher MNR [24,41], but in others by negative or lower MNR [16,37,41,42]. Furthermore, different amounts of sucrose reaching roots did not result in different nutrient (N, P, Zn, Cu) uptake by AM plants, indicating a lack of direct connection between amount of C invested in fungal growth and nutrients received through the fungal partner [15]. It is therefore not clear whether increased C allocation toward the fungus will be rewarded with increased N uptake. However, mycorrhization degree and extra-radical mycelia (ERM) growth, or C allocation toward the fungus, have been repeatedly reported to respond to N supply, often being reduced at increasing N supplies [12,17,19,41,43–46] indicating that the host plant will change C investment in fungal growth according to N availability and plant needs. Cases of no response of colonization or ERM growth to N were, however, also reported [19,21,45,47], as were positive responses [18,20,48,49], again making unclear the importance of AM for N nutrition.

A possible solution to the contradictory data obtained so far may be that mycorrhizal responses may not be linear, but curvilinear, and more positive at intermediate N, or other factor, levels [50–52]. Differences in results could therefore be a consequence of where in the gradient of AM effects the observations are being made. Another hypothesis is that mycorrhizal benefit, rather than being based on need/availability of one nutrient, be it N, P or other, depends on the stoichiometry of nutrients in need, i.e., on the relative availability of C, N and P [26,53]. On the other hand, the possibility that nutrients other than N, P and C can also be crucial cannot be discarded, namely due to synergies or antagonisms between the uptake of different nutrients.

The most common experimental designs are unable to address these hypotheses. On the one hand, experimental designs tend to be of the type high nutrient/low nutrient, and are unable to detect possible curvilinearity, or to access where in the gradient of AM effects the observations are being made. On the other hand, attention is almost exclusively given to the nutrient being manipulated, and more often than not other nutrients are not quantified, and/or their possible effects on the results are not considered. In this study we propose that a more integrative experimental design, with the use of gradients of the tested variable, namely the supply of N, together with the quantification of several nutrients and their interactions, could be essential in clarifying the conflicting information obtained so far. By studying a gradient of N supplies, not only N availability is manipulated but also N:P and N:C stoichiometry, and nutrient limitations: as N becomes less growth limiting, the possibility that P or C become growth limiting increases. With the use of such an experimental design, we aimed at investigating (i) if AM benefit for plant N nutrition changes with N availability; (ii) if MNR determines MGR, (iii) whether MGR depends solely on MNR, or on the relative availability of N and other nutrients, and (iv) how does MNR influences plant C economy, namely C:N exchange reciprocity, and how does this change with N availability.

Rice (*Oryza sativa* L.) was chosen as plant symbiont for several reasons: Mycorrhizal rice has been observed to receive more than 70% of its overall Pi via the symbiotic pathway, and therefore to be highly dependent on the AM symbiosis [54]. However, effects of AM on rice growth and N uptake have been reported that were positive [46,55–59], others null or negative [9,58,60–62].

In natural conditions rice is mostly N, and not P, limited. NH_4^+ may represent the more important form of available N in rice cultivation, and it's been repeatedly reported to be the preferred N species taken up by rice [63–65]. Rice also lives in what amounts to a natural semi-hydroponical system, where flooding potentially eliminates patchiness and low NH_4^+ mobility.

Two fungal partners were tested, *Rhizophagus irregularis*, which was tested for the whole N supply gradient established, and *Funneliformis mossae*, which was tested at only two of the N supplies (one growth limiting and one non-growth limiting), in order to check whether the responses were fungal species dependent.

2. Materials and methods

2.1. Plant and fungal material

Rice seeds (*Oryza sativa* L. cv. niponbare) were surface sterilized with 30% sodium hypochlorite for 15 min, 7% hydrogen peroxide for 15 min, rinsed in several changes of distilled water, and sown in autoclaved vermiculite. Seedlings were transplanted to 300 ml pots when the first true leaf was fully expanded (three plantlets per pot), with a sand/vermiculite (v/v) mixture as substrate, sterilized at 120 °C for 20 min.

2.2. Experimental design

Three mycorrhizal treatments were established: non-mycorrhizal, and *Rhizophagus irregularis* (DAOM 197198) or *Funneliformis mossae* (BEG 12) inoculated. The plants were inoculated at the time of transfer from the sowing beds into the pots. For the inoculation, 10% (v/v) of a sepiolite-vermiculite-based inoculum, containing spores, hyphae and fragments of mycorrhizal roots, were mixed with the substrate.

Six different nitrogen (N) treatments were established for non-mycorrhizal and *R. irregularis* inoculated plants. Plants were watered with a nutrient solution containing either 0.15, 0.23, 0.45, 0.79, 1.88 or 3 mM $(NH_4)_2SO_4$ as N source. The plants were watered 3 times a week with nutrient solution, and with distilled watered as needed. At the end of the experiment, the plants had received a total of 4, 6, 12, 21, 50 or 80 mg N. *F. mossae* inoculated plants were only established for two of the N treatments, 6 and 50 mg N. All nutrient solutions also contained 0.63 mM K_2SO_4, 0.94 mM $CaCl_2$, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4{\cdot}7H_2O$ as macronutrients, and 9.1 μM $MnCl_2$, 46.2 μM H_3BO_3, 0.32 μM $CuSO_4$, 0.76 μM $ZnSO_4$, 0.02 μM Na_2MoO_4 and 180 μM NaFeEDTA as micronutrients.

The experiment was performed in a growth chamber under a 16-h light/8-h dark photoperiod at 24/18 °C, 60% relative humidity, and a light intensity of 320 $\mu mol\ m^{-2}\ s^{-1}$ at plant height. The position of pots within the growth chamber was changed 3 times a week.