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RESEARCH ARTICLE

How interacting fungal species and mineral nitrogen inputs affect transfer of nitrogen from litter via arbuscular mycorrhizal mycelium

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Abstract In the karst landscape, widespread in the world including southern China, soil nutrient supply is strongly constrained. In such environments, arbuscular mycorrhizal (AM) fungi may facilitate plant nutrient uptake. However, the possible role of different AM fungal species, and their interactions, especially in transferring nitrogen (N) from litter to plant, is poorly understood. We conducted two microcosm experiments to investigate the role that two karst soil AM fungi, *Glomus etunicatum* and *Glomus mosseae*, play in the transfer of N from decomposing litter to the host plant and to determine how N availability influences these processes. In experiment 1, *Cinnamomum camphora* tree seedlings were grown in compartments inoculated with *G. etunicatum*. *Lolium perenne* leaf litter labeled with $\delta^{15}N$ was added to the soil in unplanted compartments. Compartments containing the $\delta^{15}N$ labeled litter were either accessible to hyphae but not to seedling roots or were not accessible to hyphae or roots. The addition of mineral N to one of the host compartments at the start of the experiment significantly increased the biomass of the *C. camphora* seedlings, N content and N:P ratio, AM mycelium length, and soil microbial biomass carbon and N. However, significantly, more $\delta^{15}N$ was acquired, from the leaf litter by the AM hyphae and transferred to the host when mineral N was not added to the soil. In experiment 2, in which *C. camphora* seedlings were inoculated with both *G. etunicatum* and *G. mosseae* rather than with *G. mosseae* alone, there was a significant increase in mycelial growth (50.21%), in soil microbial biomass carbon (417.73%) in the rhizosphere, and in the amount of $\delta^{15}N$ that was transferred to the host. These findings suggest that maintaining AM fungal diversity in karst soils could be important for mediating N transfer from organic material to host plants in N-poor soils.

Keywords Arbuscular mycorrhizae · Nitrogen · Transfer · Litter

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Introduction

Arbuscular mycorrhizas (AMs) are a symbiosis between arbuscular mycorrhizal fungi (AMFs) and roots and occur in most plant species in terrestrial ecosystems (Brundrett 2002; Smith and Read 2010; Wang and Qiu 2006). AMs are considered to be a mutualistic association, involving carbon transfer from the host plant to the fungus and nutrients from the soil to the plant via fungal hyphae (Smith and Read 1996; Smith and Read 2010). This mutualism has been viewed as a fundamental driver of the evolution of land plants (Hodge and Fitter 2010). A

high diversity of plant species has been shown to be beneficial to AM fungal species diversity (Kernaghan 2005) and vice versa (van der Heijden et al. 1998), which is consistent with habitat heterogeneity promoting diversity (Yu et al. 2001). In many limestone regions of the world, including the widespread karst landscape of China, even more so in areas subject to land degradation and soil erosion. Here, soil nutrient supply is strongly constrained and AM fungi are likely to play an important role in plant performance. How do different species of AM fungi contribute to vegetation performance in the karst habitat under such nutrient shortage? Nutrients released from organic matter as a result of microbial decomposition can generally be taken up by plants; however, there has been disagreement over whether or not AM fungi are able to supply their host with minerals as a result of saprotrophic nutrition by decomposing leaf litter (Hawkins et al. 2000; Hodge et al. 2001; Tisdall et al. 1997; Tu et al. 2006; Wright and Upadhyaya 1998).

Although mycorrhizal fungi have been shown to absorb and transfer nitrogen (N) and phosphorus (P) from soil to plants (Ames et al. 1983; Bago et al. 1996; He et al. 2006; He et al. 2009; Hodge and Fitter 2010; Mader et al. 2000), it is still unclear what proportion of the N and P transported by AM originates from litter rather than from mineral nutrient sources. Read and Perez-Moreno (2003) found that ericoid and ectomycorrhizal systems have developed strong saprotrophic capabilities to derive N and P from organic complexes in soil, but the abilities of AM fungi were unclear. AM fungi were previously thought to have little or no saprotrophic capability because they are unable to breakdown stable biopolymers in organic matter (Tisdall et al. 1997; Wright and Upadhyaya 1998). However, St. John et al. (1983) proposed that the proliferation of AM mycelium into litter supported the idea that AM fungi are able to exhibit saprophytic behavior. An experiment performed to investigate the ability of the AM fungi *Glomus hoi* showed that arbuscular mycorrhizal symbiosis promoted decomposition and increased N transfer released from decomposition products of litter (Hodge et al. 2001). Tu et al. (2006) found that low-level mineral N inputs significantly enhanced plant N capture and residue decomposition by an experiment including single *Glomus etunicatum* and mixed AM species. However, direct evidence about AM decomposing organic material is absent by far. In addition, Thirkell et al. (2015) found that organic matter fertilization of AM plants can foster mutually beneficial symbiosis based on N transfer under *Glomus intraradices* inoculation. However, these results do not necessarily carry universality for natural ecosystems in other parts of the world, especially in karst areas where multiple species of AM fungi may coexist (Wei et al. 2011).

Barea et al. (1989) proposed that the interaction of mycorrhiza and nutrients affected on N transfer and N uptake in soil by a mycorrhizal mediating experiment in legume-grass plants inoculated with *Glomus mosseae*. Interaction of mycorrhizal-mediated N transfer may be different from the mycorrhizal fungi species and its composition and nutrient addition by organic or inorganic form. Different plants and fungi bring independent characteristics to the symbiosis (Allen et al. 2003); for example, Mensah et al. (2015) proposed that functional diversity within species of arbuscular mycorrhizal fungi was associated with differences in nitrogen uptake and fungal phosphate metabolism by experiment inoculated by 31 different isolates from 10 AM fungal morphospecies on the P and N nutrition. Therefore, suitable plant and mycorrhizal fungi were important to improve N transfer for host plant mediated by mycorrhizal symbiosis. Exogenous N input and increasing richness of mycorrhizal fungi species interactively affected N transport and N uptake; i.e., the mineral N input significantly increased N acquisition of host plants and N transfer from soil litter in mixture inoculation with multiple AM species documented by Tu et al. (2006). Adding N as organic form additionally promoted N uptake of increasing 15–20% (Barrett et al. 2014; Leigh et al. 2009); however, additional N inputs negatively affected the abundance and the function of AM fungal species reported by Buwalda and Goh (1982), Corkidi et al. (2002), and Egerton-Warburton and Allen (2000). Addition of N- to P-limited soils increased AM fungal biomass, which was proposed that nutrient limitation was an evolutionary force that generates site-specific coadaptation among plant genotypes and AM fungi (Johnson et al. 2015). However, the exact mechanism of N transfer and, more importantly, the amounts of N transferred via the AMF compared to the N requirements of the plant was still indistinct (Smith and Smith 2011). In addition, mycorrhizal-mediating N transfer has important ecological significance in karst habitat with heterogeneity, as resources are depleted in one patch; the mycorrhizal fungi have the capacity to explore a neighboring patch (Cui and Caldwell 1996). Thus, increasing mycorrhizal fungal species richness could improve ecosystem function (Hart et al. 2001) in N transfer balancing nutrition.

N inputs to terrestrial ecosystems have been increased by human activities through atmospheric N deposition and mineral N fertilization, changing the global N cycle (Chapin et al. 2011). Elevated N inputs may change plant primary productivity and species composition (Gough et al. 2000; Wedin and Tilman 1996). In general, increased N inputs are thought to negatively affect the functioning and abundance of AM fungi (Buwalda and Goh 1982; Treseder 2004). However, Treseder and Allen (2002) showed that AM fungal biomass was enhanced by adding N to soil that had strong N limitation. Thus, it is unclear whether and how different host plants acquire N via its transfer from litter through AM mycelium and whether and how these processes depend on the AM species or on interactions between cooccurring AM species. To increase our understanding of the mechanisms involved in N cycling in karst ecosystems, we investigated (1) how AM fungi in the karst habitat transport N from litter to their host plants by N

input treatment and (2) how single versus mixed species of AM fungi from karst soil habitats mediate N transfer to the host plant with and without exogenous N input.

Materials and methods

Experimental design

Two microcosm experiments were performed; experiment 1 examined the possible decomposing and transfer functions of AM fungi by tracking N using $\delta^{15}N$ labeled litter, and experiment 2 investigated whether and how N transport in a mixed inoculation treatment with two species of AM fungi contrasted with that of single species treatment, adding $\delta^{15}N$ directly in mineral form rather than via labeled litter. The microcosm experiments (modified from Hodge et al. (2001) and Tu et al. (2006) were conducted in a transparent plastic greenhouse at Southwest China University, Chongqing, People's Republic of China (106° 22′ E, 29° 49′ N; altitude 300 m). This region has a subtropical climate with mild and humid winters and hot monsoon summers with periodic drought. Five plexiglass microcosms were used as replicates for each experiment. Each microcosm was divided into six compartments each measuring 13 × 14 × 15 cm (width × depth × height) (Fig. 1). Three compartments in a row were designated as the "HOST" compartmental group, in which host plants were grown and inoculated with AM fungi. The other three compartments were designated as the "TEST" group to examine mycorrhizal fungal function. The HOST and TEST compartments were adjacent to one another and separated by two layers of 20- or 0.45-μm nylon mesh (Amersham Hybond, USA); the 20-μm mesh could be penetrated by hyphae but not by roots, whereas the 0.45-μm mesh could neither be penetrated by hyphae nor by roots. Each compartment was filled with a mixed substrate containing 3.5 kg of limestone soil, sand, and quartz (2:1:1 by mass) that had been autoclaved at 0.14 MPa at 126 °C for 1 h, so as to kill all microbes. The limestone soil was collected from a typical karst habitat on Jigong Mountain near Chongqing. The growth substrate had a pH of 6.81 and contained 6.1 g/kg of soil organic carbon (SOC), 463.5 mg/kg of total N, 33.3 mg/kg of available N, 158 mg/kg of total phosphorus, and 1.81 mg/kg of available phosphorus.

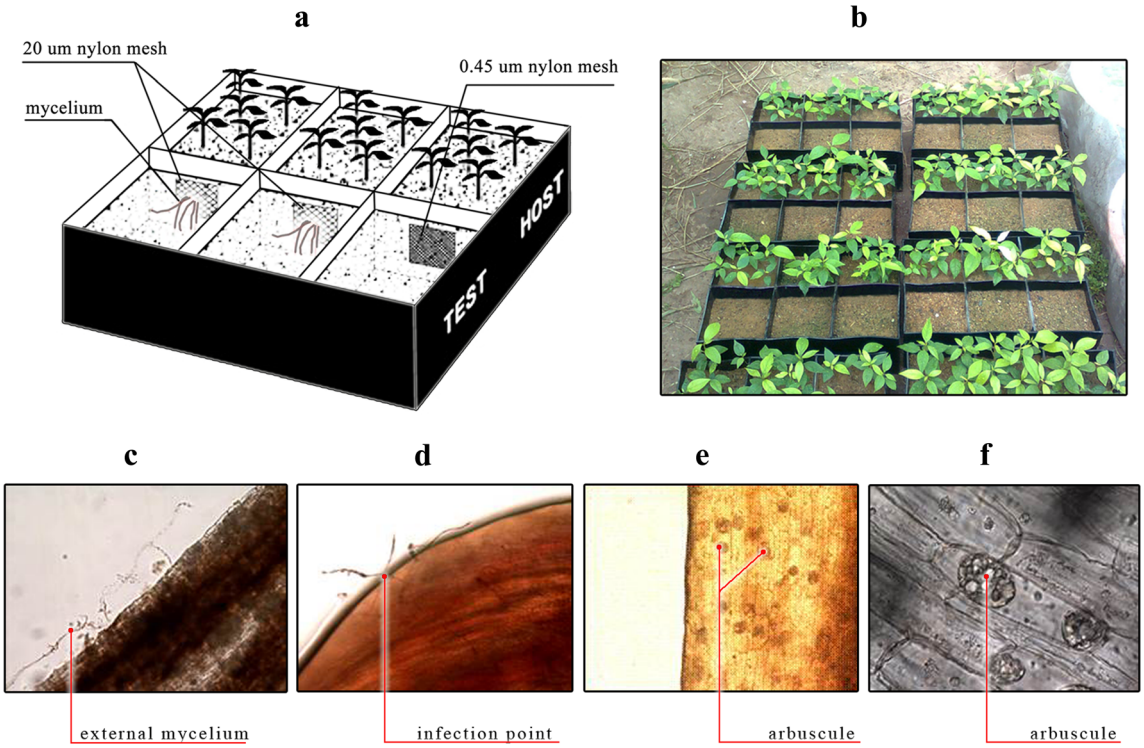

Fig. 1 Experimental microcosm unit, seedling culture, and microscopic images of mycorrhizal formation. The experimental microcosm unit was composed of six equal-sized compartments (**a**). Three compartments were designated the "HOST" section, in which *C. camphora* seedlings inoculated with AM fungi were planted. Two of the HOST compartments were separated from the adjacent compartments, designated the "TEST" section, by two layers of 20-μm nylon mesh (penetrable by hyphae but not by plant roots), and one HOST compartment was separated from the adjacent TEST compartment by two layers of 0.45-μm mesh (impenetrable by hyphae or plant roots). All compartments were filled with sterilized soil. The experimental seedlings were cultured for 5 weeks after the germination of the *C. camphora* in greenhouse (**b**). The microscopic images of mycorrhizal formation (**c**–**f**) were taken after the harvest of *C. camphora* seedlings at the end of the experiments, including the external mycelium of root (**c**), infection point of root (**d**), the arbuscules of root cells (**e**) under 20 times, and arbuscules of root cells (**e**) of 100 times

Experiment 1: effects of mineral N inputs on AM-mediated litter decomposition and nutrient transfer

Experiment 1 involved three treatments and a single AM fungal species, *G. etunicatum* Becker and Gerdemann: (1) without N addition (AMF − N) and using a two-layer, 20-μm nylon mesh screen that prevented plant roots but allowed AM fungal hyphae to penetrate the TEST compartment in each microcosm unit; (2) with N addition to the host plants (AMF + N) and a two-layer, 20-μm mesh screen as above; and (3) without N addition and using a two-layer, 0.45-μm nylon mesh screen that prevented both AM fungal hyphae and plant roots from accessing the TEST compartment (AMF − NH). There was a 2-mm atmospherical interlayer in the two-layer mesh to minimize the effects of nutrient diffusion between the HOST and TEST compartments. A single 50-g inoculum of *G. etunicatum*, containing probable 500 spores as well as hyphae and colonized root pieces, was applied to the soil medium in the each HOST compartment. The *G. etunicatum* inoculum used in this experiment was purchased from the Institute of Plant and Natural Resources, Beijing Academy of Agriculture and Forestry Science of China (BGCAM 0046). The *G. etunicatum* inoculum had been isolated from a karst site in Guizhou Province in southwest China and identified and characterized according to the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.caf.wvu.edu). Five seeds of *Cinnamomum camphora* (L.) Presl, a common forestry tree species in China, were sterilized with 10% H_2O_2 for 10 min and then germinated in the HOST compartment soil. To assess the effect of N inputs on litter decomposition, 30.0 g of dried and chopped (<1-cm length) shoot tissue litter of the widespread grass *Lolium perenne* Linn. was well mixed with the soil substrate in the TEST compartments of each treatment replicate at the beginning of the experiment. The shoots of *L. perenne* had been cultivated in the laboratory from seeds obtained from Jigong Mountain, Chongqing, and labeled with $\delta^{15}N$, and the leaf litter that formed was collected. The surface of *L. perenne* were sprayed with 20% $^{15}NH_4SO_4$ solution ($\delta^{15}N = 99.4\%$; Shanghai Engineering Research Center of Stable Isotope, People's Republic of China) after 30 days of the seedlings' growth for labeling with ^{15}N. Plant leaves were collected, washed in distilled water three times, chopped to 0.5–1-cm lengths, and then dried at 80 °C as litter in this experiment 1. The $\delta^{15}N$ value of the added litter was 18,960‰. To minimize the effect of mineral N on the initial colonization of mycorrhizal fungi, in the AMF + N treatment, 857 mg of additional NH_4NO_3 equaling to 300 mg of N was applied to all HOST compartments by solution at 6 weeks after seedling emergence, and each soil was equivalent to 60 mg of N. The plants were allowed to grow for 15 weeks in total after the germination, and then harvested, dried in an oven at 105 °C for 48 h, and analyzed for biomass and concentrations of N and P and $\delta^{15}N$ value (see below). Soil samples from each compartment were collected for determination of N, P, $\delta^{15}N$, soil microbial biomass carbon (SMBC), soil microbial biomass nitrogen (SMBN), and SOC. While we could not follow the actual transport of P between litter and plant via AM in this study, we still compared its concentration in plant and soil in the different treatments to get an indication of such transport.

Experiment 2: effects of inoculating plants with two species of AM fungi on nutrient transfer to the host plant

Experiment 2 involved three treatments: (1) a single AM fungal species with hyphal access to the TEST soil via a two-layer, 20-μm nylon mesh (AMF + H); (2) two AM fungal species with hyphal access to the TEST soil via a two-layer, 20-μm nylon mesh (AMFs + H); and (3) a single AM fungal species without hyphal access to the TEST soil owing to the use of a two-layer, 0.45-μm nylon mesh screen (AMF − H). The host plant treatments and the unfertilized soil medium were otherwise identical to those described in experiment 1. The two AM fungal species used in the experiment were *G. etunicatum* (BGCAM 0046) and *G. mosseae* Nicol. & Gerd (BGCAM 0012), which had been isolated from a karst site in Guizhou Province (see above). The single species treatments involved *G. mosseae* (AMF + H and AMF − H). A mixed 50 × 25-g inoculum of *G. etunicatum* containing probable 250 spores and 25-g inoculum of *G. mosseae* containing probable 200 spores was applied to the soil medium in the each HOST compartment. The procedure for inoculating seeds of *C. camphora* was similar to that used in experiment 1. To assess the effects of dual AM fungal species on nutrient transport by fungal hyphae, *G. etunicatum* and *G. mosseae* (AMFs + H) were applied to the soil in the HOST compartment at the beginning of the experiment. After 6 weeks, a solution of 100 ml of sterilized water containing 85 mg $(NH_4)_2SO_4$ with a $\delta^{15}N$ value 99.4% (Shanghai Engineering Research Center of Stable Isotope, People's Republic of China) was injected into the soil of each TEST compartment. No litter was added to the soil in the TEST compartment; however, 10 seeds of *L. perenne* were planted in the soil of each TEST compartment 12 weeks after seedling emergence of *C. camphora* in the HOST compartments to determine the balance of nutrients in the microcosm by testing $\delta^{15}N$. Five plants of *L. perenne* seedlings were retained for growing in each TEST compartment at the third weeks after the germination of *L. perenne* seedlings. All plants and soil materials were harvested 15 weeks after the germination of the *C. camphora* seedlings. Samples of soil and plants were collected in the same way as those collected in experiment 1 to determine concentrations of N, P, $\delta^{15}N$, SMBC, SMBN, and SOC.

Mycorrhizal root colonization and plant biomass

Mycorrhizal infection in the two experiments was measured following the methods described by Kormanik et al. (1980). Fresh roots of *C. camphora* were washed and then chopped into lengths

of about 1 cm. All samples were immersed in 5% (*w*/*v*) KOH at 95 °C for 40 min to clear the roots and then stained by placing the samples in 0.05% magenta at 95 °C for 40 min, followed by a 2% lactic solution for 3 min for neutralization. The stained roots were placed on a Petri dish with gridlines and examined under the microscope at ×40 magnification (Kormanik et al. 1980). The mycorrhizal colonization was expressed as the proportion of roots that were colonized by the AM fungus at all the root–grid intersection points (Brundrett et al. 1984; Giovannetti and Mosse 1980). Harvested plants were dried at 105 °C for 48 h and were then weighed to obtain the dry masses of the roots, leaves, stems, and total biomass.

Nitrogen and phosphorus determinations

Total N concentrations were determined by Kjeldahl measurement after digestion with sulfuric acid using a Büchi Distillation Unit B-324, Switzerland. Samples of dried plant and soil were milled and then passed through a mesh sieve of 0.25-mm diameter to N measure the $\delta^{15}N$ value by mass spectrometry using a Thermal Finnigan TC/EA-IRMS, DELTA V Advantage, Germany. Total P concentrations were determined using the molybdenum-antimony anti-spectrophotometric method described by Bao (2000). The concentrations of N and P were, respectively, determined by a detached root, stem, and leaf for a plant, which were dried at 105 °C for 48 h, and were converted to total biomass N and biomass P pools by multiplying, respectively, by the dry biomass of the root, stem, and leaf. Total plant biomass N and P were the sum of root, stem, and leaf biomass N and P.

Hyphal length

A fresh soil sample (0.5 g) was placed into a 600-ml beaker, to which 250 ml deionized water was added, and the beaker was then left to stand for 30 min. The sample solution was then stirred vigorously for 1 min, using a glass rod to break up soil aggregates before placing on a magnetic stirrer and stirring at high speed. The soil solution was transferred to a 300-ml beaker and left to stand for 40 min. The supernatant solution was vacuum filtered using 0.45-μm nylon mesh. The mesh was then placed in a plastic vial; 5 ml trypan blue stain was added to the vial and then left to stand for 30 s to stain the hyphae at the mesh. This treatment was repeated eight times per soil sample until essentially no material pelleted out anymore. The line intersect method was used to estimate the length of hyphae present on the membrane filter as described by Brundrett et al. (Hodge 2003).

Determinations of SMBC, SMBN, and SOC

SOC concentration was measured using the $K_2Cr_2O_7$-H_2SO_4 calefaction method (Nelson and Sommers 1982). SMBC and SMBN were determined using the chloroform fumigation extraction method (Vance et al. 1987). Subsamples of soil were fumigated with ethanol-free $CHCl_3$ for 24 h and then extracted with 0.5 M K_2SO_4 solution. The extracted solution was analyzed for SOC, SMBC, and SMBN using a TOC Multi N/C 3100, Jena, Germany.

Statistical analyses

Each of the two experiments had a randomized block design with five replicates in each treatment. Microcosm units were treated as blocks. The effects of treatments on dependent variables were statistically analyzed using one-way ANOVA using SPSS 13.0 software. The least significant difference (LSD) test was applied to compare HOST and TEST compartment data obtained for individual treatments at the 5% significance level. The data about the $\delta^{15}N$ value, the mycelium length, the mycorrhizal colonization, biomass, the SMBC, the SMBN, the SOC, and the concentrations of N and P were tested by normal distribution and homogeneity of variances before running the ANOVA.

Results

Experiment 1: effects of mineral N inputs on AM-mediated nutrient transfer

Host plant infection, biomass, and nutrients

There was no significant difference in the proportion of *C. camphora* seedling roots that were colonized by the AM fungus between the AMF + N treatment and the AMF − N treatment. The dry biomass and biomass N of the *C. camphora* seedlings that received the additional N inputs in the AMF + N treatment were 16.3 and 17.8% higher, respectively, than those of the seedlings that received the AMF − N treatment (see Table 1). Biomass P was not affected significantly by the input treatment. As a consequence, additional N inputs resulted in a higher N:P ratio in the AMF + N treatment. The dry biomass, biomass N, biomass P, and the N:P ratio of seedlings that received the AMF − NH treatment were significantly lower than that of seedlings that received the AMF − N treatment, indicating that AM fungal hyphae were responsible for the greater *C. camphora* seedling biomass in the AMF − N treatment.

Hyphal length and soil organic carbon and nutrients

Hyphal length in the HOST compartments did not differ significantly among the three treatments. SMBC, SMBN, and SOC were significantly increased in the HOST and TEST compartments by the exogenous input of N to the soil of the

Table 1 Additional N input effects on mycorrhizal colonization, dry biomass, N and P, and N:P ratio of individual *C. camphora* plants

Treatments	Root colonization (%)	Dry biomass (g IP^{-1})	Biomass N (mg IP^{-1})	Biomass P (mg IP^{-1})	Biomass N:P
AMF − N	62.7 ± 2.1 a	0.61 ± 0.03 b	2.58 ± 0.14 b	0.41 ± 0.02 a	6.25 ± 0.02 b
AMF + N	61.2 ± 2.3 a	0.71 ± 0.03 a	3.04 ± 0.29 a	0.37 ± 0.03 ab	8.08 ± 0.03 a
AMF − NH	63.5 ± 3.6 a	0.40 ± 0.02 c	1.85 ± 0.13 c	0.30 ± 0.02 b	6.13 ± 0.02 c

Values are means ± SE. Different letters within a column indicate a significant difference at the $P < 0.05$ level using LSD analysis

AMF − N treatment no input of exogenous N to the HOST compartment, TEST compartment accessible to AM fungal hyphae via 20-μm nylon mesh; *AMF + N treatment* input of exogenous N to the HOST compartment, TEST compartment accessible to AM fungal hyphae via 20-μm nylon mesh; *AMF − NH treatment* no input of exogenous N to the HOST compartment, TEST compartment inaccessible to AM fungal hyphae or roots via 0.45-μm nylon mesh; *IP* individual *C. camphora* seedling

HOST compartment in the AMF + N treatment compared with the AMF − N treatment. Hyphal length was also significantly greater in the TEST compartment of the AMF + N treatment compared with the hyphal length in the TEST compartment of the AMF − N treatment. No hyphae were found in the TEST compartment of the AMF − NH treatment. Soil in the HOST and TEST compartments that received the AMF − NH treatment contained significantly higher levels of organic N and C than soils that received the AMF − N treatment, except for SMBC and SOC in the soils of the TEST compartment, which were not significantly different to that of the soil in the AMF − N treatment (see Table 2).

$\delta^{15}N$ of soil and plant leaves

The $\delta^{15}N$ value of *C. camphora* leaves after the AMF + N treatment was significantly lower than that of leaves after the AMF − N treatment or the AMF − NH treatment (Fig. 2a). Soil $\delta^{15}N$ in the TEST compartment that received the AMF − N treatment was significantly higher than that in AMF + N treatment but lower than that in AMF − NH treatment (Fig. 2b), whereas the opposite results were obtained for the soil in the HOST compartments. The additional mineral N available in the AMF + N treatment facilitated N absorption by the host plant (see Table 1) but decreased the acquisition of $\delta^{15}N$ from the litter in the adjacent TEST compartment.

Fig. 2 N input effects on $\delta^{15}N$ value of *C. camphora* seedling leaves (**a**) and of soil in HOST and TEST compartments (**b**). For explanation of the three treatments (AMF − N, AMF + N, AMF − NH), see Table 1. The $\delta^{15}N$ value of the natural soil was 16.34‰, and the value of natural leaves of *C. camphora* seedling was 11.10‰. *Different English letters* (*a*, *b*, and *c*) *above the bars* indicate a significant difference in the $\delta^{15}N$ value of *C. camphora* seedling leaves (**a**) or in the $\delta^{15}N$ value in the HOST soil compartment (**b**). *Different Greek letters* (α, β, and γ) *above the bars* indicate a significant difference in the $\delta^{15}N$ value in the TEST soil compartment (**b**) between treatments

Experiment 2: effects of inoculating plants with two species of AM fungi on nutrient transfer to host plant

Host plant colonization, dry biomass, and nutrients

There was no significant difference in the proportion of *C. camphora* roots colonized by mycorrhiza among the three treatments (Tables 3). The dry biomass, biomass N, and the N:P ratio of individual *C. camphora* seedlings that had

Table 2 Additional N input effects on mycelium length and soil microbial biomass carbon and nitrogen

Treatments	Mycelium length (m g^{-1})	SMBC (mg kg^{-1})	SMBN (mg kg^{-1})	SOC (g kg^{-1})
HOST				
AMF − N	15.38 ± 2.14 a	68.34 ± 4.91 b	21.95 ± 1.57 b	10.65 ± 0.06 c
AMF + N	14.14 ± 1.86 a	146.11 ± 30.55 a	37.23 ± 3.57 a	12.59 ± 0.05 a
AMF − NH	17.13 ± 1.94 a	109.32 ± 22.44 ab	43.01 ± 2.18 a	12.22 ± 0.03 b
TEST				
AMF − N	10.13 ± 1.27 b	77.32 ± 15.01 b	24.29 ± 2.37 b	10.65 ± 0.06 b
AMF + N	34.58 ± 4.69 a	196.03 ± 14.35 a	42.37 ± 2.25 a	11.55 ± 0.05 a
AMF − NH	0 c	96.10 ± 25.35 b	48.14 ± 4.88 a	10.69 ± 0.03 b

Values are means ± SE. Different letters within the same column indicate the significant differences between treatments in the HOST or TEST compartments at the $P < 0.05$ level using LSD analysis

See Table 1 for an explanation of the AMF − N, AMF + N, and AMF − NH treatments; *SMBC* soil microbial biomass carbon; *SMBN* soil microbial biomass nitrogen; *SOC* soil organic carbon

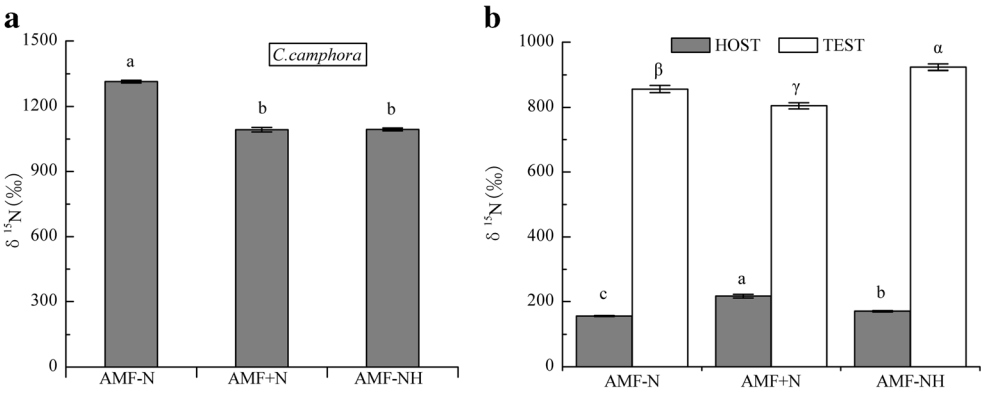

Fig. 2 The seedling culture photos in microcosms and microscopic mycorrhizal images of roots of *C. camphora* plants. a, *C. camphora* seedlings were planted in microcosms; b and c, hyphae and infection point at 20 times magnification; c and d, the arbuscular or vesicular structure in cells of roots of *C. camphora* seedling at 20 and 100 times magnification

received the AMFs + H treatment were significantly higher than that after the AMF + H treatment. Plant dry biomass, biomass N, biomass P, and the N:P ratio were significantly lower in *C. camphora* seedlings after the AMF − H treatment (i.e., seedlings that had no exchange with the *L. perenne* plants in the TEST compartment) compared with seedlings that had received the AMF + H treatment (i.e., *C. camphora* seedlings that had possible exchange with the *L. perenne* plants in the TEST compartment).

Hyphal length and soil organic carbon and nutrients

The hyphal length in the HOST compartment, SMBC, and SOC were significantly greater in soils inoculated with the two AM fungi (AMFs + H treatment) than in soils inoculated with only one AM fungus (AMF + H treatment) (Table 4). However, it did not make a significant difference to the hyphal length or to the SOC in the HOST compartment of soils inoculated with one fungus (AMF + H and AMF − H treatments). SMBC and SOC were significantly higher in the AMFs + H treatment compared with the AMF + H treatment; however, the SMBN was not significantly different. SMBC in the AMF − H treatment was significantly higher than that in the AMF + H treatment, whereas SMBN was significantly lower in the AMF − H treatment compared with the AMF + H treatment. No hyphae were present in the TEST compartment that had received the AMF − H treatment. The hyphal length in the TEST compartment was not significantly different between the AMF + H and AMFs + H treatments; the SMBC was significantly lower in the TEST compartment for the AMFs + H treatment compared with that of the AMF + H treatment, whereas SOC was higher. Significantly lower levels of SMBC, SMBN, and SOC were found in the TEST compartment of the AMF − H treatment compared with the other treatments.

$\delta^{15}N$ of soil and plant leaves

The $\delta^{15}N$ values were significantly different between the three treatments. The $\delta^{15}N$ value of *C. camphora* leaves was significantly higher among seedlings that had been inoculated with two AM fungi (AMFs + H treatment) compared with those inoculated with only one AM fungus. The $\delta^{15}N$ value of *C. camphora* leaves in the AMF − H treatment (i.e., the $\delta^{15}N$ in soil or taken up by *L. perenne* plants in the TEST compartment was inaccessible to the AM fungal hyphae) was significantly lower than that of leaves in the AMF + H treatment (Fig. 3a). By contrast, the highest $\delta^{15}N$ values of *L. perenne* plants were found in the AMF − H treatment and the lowest in AMFs + H treatment (Fig. 3b). This suggests that *C. camphora* seedlings acquired $\delta^{15}N$ from the TEST soil via mycorrhizal hyphae and that the *L. perenne* plants utilized the surplus $\delta^{15}N$ in the TEST soil. The $\delta^{15}N$ values of the soil in the TEST compartments were significantly different among

Table 3 Effect of AM fungal species composition on colonization, dry biomass, N and P, and N:P ratio of individual *C. camphora* plants

Treatments	Colonization of roots (%)	Dry biomass (mg IP^{-1})	Biomass N (mg IP^{-1})	Biomass P (mg IP^{-1})	Biomass N:P
AMF + H	61.7 ± 3.1 a	0.49 ± 0.02 b	2.31 ± 0.12 b	0.38 ± 0.02 a	6.09 ± 0.04 b
AMFs + H	62.2 ± 7.3 a	0.65 ± 0.02 a	2.70 ± 0.07 a	0.41 ± 0.01 a	6.50 ± 0.02 a
AMF − H	60.5 ± 4.3 a	0.41 ± 0.02 c	1.84 ± 0.08 c	0.31 ± 0.01 b	5.94 ± 0.01 c

Values are means ± SE. Different letters within a column indicate a significant difference at the $P < 0.05$ level using LSD analysis

AMF + H treatment seedlings in HOST compartment inoculated with *G. mosseae* only, TEST compartment accessible to AM fungal hyphae via 20-μm nylon mesh; *AMFs + H treatment* seedlings in HOST compartment inoculated with *G. mosseae* and *G. etunicatum*, TEST compartment accessible to AM fungal hyphae via 20-μm nylon mesh; *AMF − H treatment* seedlings in HOST compartment inoculated with *G. mosseae* only, TEST compartment inaccessible to AM fungal hyphae or roots via 0.45-μm nylon mesh; *IP* individual *C. camphora* seedling

Table 4 Effect of AM fungal species composition on mycelium length and soil microbial biomass carbon and nitrogen

Treatments	Mycelium length (m g^{-1})	SMBC (mg kg^{-1})	SMBN (mg kg^{-1})	SOC (g kg^{-1})
HOST				
AMF + H	16.53 ± 1.67 b	26.57 ± 21.70 c	53.20 ± 3.65 a	11.57 ± 0.11 b
AMFs + H	24.83 ± 2.15 a	137.56 ± 12.82 a	48.12 ± 5.97 ab	14.27 ± 0.09 a
AMF − H	17.12 ± 1.38 b	58.14 ± 17.04 b	34.86 ± 3.64 b	11.45 ± 0.15 b
TEST				
AMF + H	13.19 ± 2.44 a	203.67 ± 18.80 a	31.35 ± 3.70 a	10.16 ± 0.12 b
AMFs + H	11.49 ± 2.17 a	138.78 ± 18.71 b	25.57 ± 1.78 ab	11.28 ± 0.09 a
AMF − H	0 b	79.21 ± 13.70 c	20.20 ± 1.34 b	9.28 ± 0.08 c

Values are means ± SE. Different letters within a column for either the HOST or TEST compartments indicate a significant difference between treatments at the $P < 0.05$ level using LSD analysis

See Table 3 for an explanation of the AMF + H, AMFs + H, and AMF − H treatments; *SMBC* soil microbial biomass carbon; *SMBN* soil microbial biomass nitrogen; *SOC* soil organic carbon

the three treatments (Fig. 3c); the highest values were found in the AMF − H treatment and the lowest values in the AMFs + H treatment. In the HOST compartments, the $\delta^{15}N$ value of the soil in AMFs + H treatment and AMF + H treatment were not significantly different but were significantly higher than the $\delta^{15}N$ value of the soil in the AMF − H treatment.

Discussion

Agricultural systems around the world are being intensified to meet the increasing demand for food, feed, and fiber. Mineral N inputs as a result of human activities, including the application of mineral N fertilizer and atmospheric N deposition,

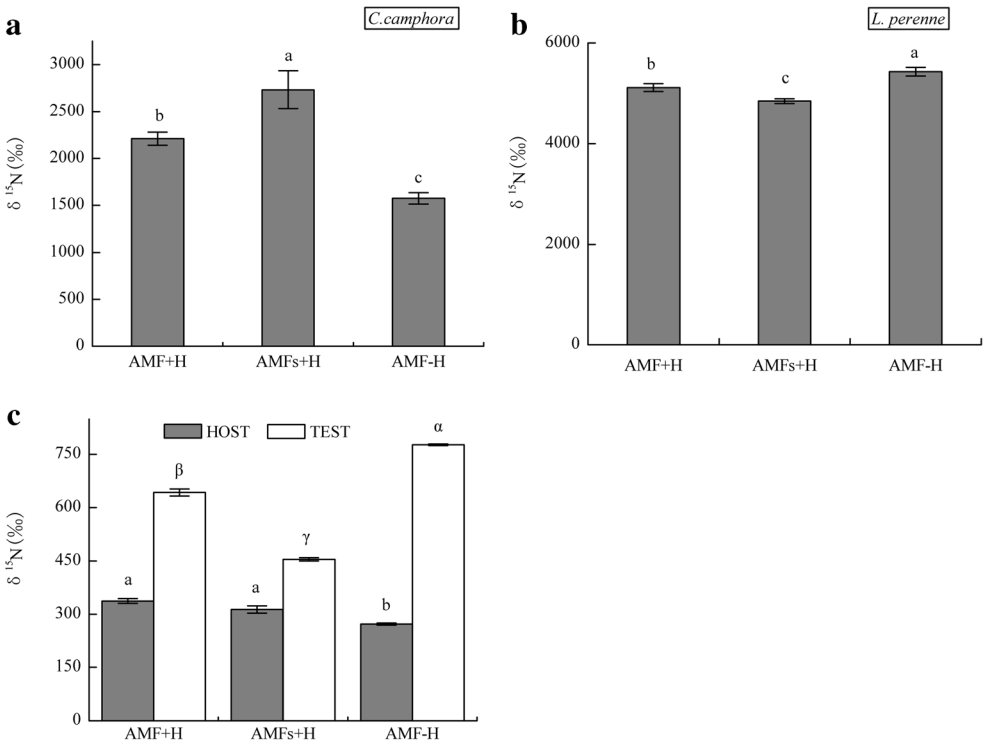

Fig. 3 Effect of AM fungal species composition on $\delta^{15}N$ values of *C. camphora* (**a**), *L. perenne* (**b**), and compartment soil (**c**). For explanation of the three treatments (AMF + H, AMFs + H, AMF − H), see Table 3. *Different letters above the bars* indicate a significant difference at 0.05 level in the $\delta^{15}N$ value of *C. camphora* seedling leaves (**a**), *L. perenne* (**b**), or in the $\delta^{15}N$ value in the TEST soil compartment or in the HOST soil compartment (**c**) between treatments using LSD analysis. Without ^{15}N supplement, the $\delta^{15}N$ value of the natural soil was 16.34‰, and the natural leaves of *C. camphora* seedling was 11.10‰, and the natural leaves of *L. perenne* plant was 8.75‰. *Different English letters* (*a*, *b*, and *c*) *above the bars* indicate a significant difference in the $\delta^{15}N$ value of plant leaves (**a**, **b**) or in the $\delta^{15}N$ value in the HOST soil compartment (**c**). *Different Greek letters* (α, β, and γ) *above the bars* indicate a significant difference in the $\delta^{15}N$ value in the TEST soil compartment (**c**) between treatments

are changing global N cycling. Previous studies have shown that N inputs profoundly affect AM symbiosis via the exchange of N and C between AM fungi and plants by mycelium (Nakano et al. 2001; Smith and Read 2010). Some studies have suggested that N inputs negatively affect the abundance and the function of AM fungal species (Buwalda and Goh 1982; Corkidi et al. 2002; Egerton-Warburton and Allen 2000), whereas others have shown that exogenous N inputs increased the growth of plants and hyphae when there was a shortage of N in soil (Hawkins and George 1999; Treseder and Allen 2002). Our results showed that *G. mosseae* and *G. etunicatum* were capable of acquiring N from leaf litter and transferring it to the host plant. However, in spite of careful prior sterilizing of soil and plants at the beginning of our experiment, because of our open system, we cannot exclude the possibility of contamination by another decomposing fungus or bacteria through the air into the soil that might have mineralized N and made it available for *G. etunicatum*. For all that, the miscellaneous bacteria similarly affected both the HOST and TEST compartments and eliminated the errors from the effect interference by the control treatment of mycorrhizal fungi in the experimental microcosm.

The $\delta^{15}N$ value of *C. camphora* seedling leaves growing in soil that did not receive exogenous N at the start of experiment 1 (AMF − N treatment) was significantly higher than that in the leaves of plants that did receive exogenous N (AMF + N treatment) and of that in the leaves of seedlings that did not have access to the *L. perenne* litter in the TEST compartment; this indicates that in soil with low levels of available mineral N, *G. etunicatum* hyphae preferred to acquire and transport ^{15}N from the *L. perenne* litter to the host plant. These results are consistent with the findings of Hodge et al. (2001) and Tu et al. (2006). The increasing transfer $\delta^{15}N$ in AMF − N treatment compared with AMF + N treatment even no difference of dry biomass indicated that the AM symbiosis formed by *C. camphora* roots and *G. etunicatum* fungi enhanced N capture and transfer, since decomposing litters were crucial N sources, which are most likely provided to AM mycelium for host plant; namely, AM symbiosis must have used the products of decomposition, although the mechanism remains unknown. When mineral N was readily available in the soil of the HOST compartment (AMF + N), the $\delta^{15}N$ value of the *C. camphora* leaves was not significantly different from those of seedlings with no access to the *L. perenne* litter in the TEST compartment, which suggests that when mineral N was readily available, the seedlings preferentially obtained N from the rhizosphere soil even though the hyphal length in the TEST compartment was significantly greater and more of the *L. perenne* litter was transferred to the HOST compartment soil. The increase in dry biomass, biomass N, and the N:P ratio of *C. camphora* seedlings of AMF + N treatment in the HOST compartment as a result of the addition of mineral N to the soil supports previous reports that exogenous N inputs increase the growth of plants and hyphae when there is a shortage of N in the soil and that saprotrophic activity by AM fungi is unlikely to be significant in agricultural environments where fertilizer is applied (Hawkins and George 1999; Treseder and Allen 2002).

Microbes are key players in soil C and N dynamics (Nannipieri et al. 1990), and they represent an active source of nutrients (Mazzarino et al. 1998). The addition of N to the soil has previously been shown to increase SMBC, SMBN, and SOC of soil and to enhance mycelial growth and decomposition of added residues in soil (Tu et al. 2006). The proliferation of mycorrhizal hyphae may facilitate litter decomposition in areas of active decomposition by increasing the availability of nutrients (Nakano et al. 2001; Zak et al. 2000). Hodge et al. (2001) demonstrated mycorrhizal enhancement of litter decomposition by labeling organic material with ^{15}N and ^{13}C. In our study, decomposition of the *L. perenne* litter increased SMBC and SOC, which accorded with the results of Hodge et al. (2001).

Not only ericoid and ectomycorrhizal fungi (Read and Perez-Moreno 2003) but also arbuscular mycorrhizal fungi can decompose litter and transfer its nutrients (Hodge et al. 2001; Tu et al. 2006), even the mechanism remains unknown. This is of ecological importance for understanding global changes in C and nutrient cycling, which are induced by atmospheric N deposition and the elevation of CO_2 concentration (Treseder 2004). AM fungal growth directly increases C inputs into soil (Miller and Jastrow 1990; Tisdall et al. 1997), and it has been suggested that this will promote C storage in the ecosystem (Rillig 2004). Our results also highlight the need for long-term field experiments to assess the decomposition effects of mycorrhizae on carbon and nutrient dynamics in (semi-)natural terrestrial ecosystems. Plant litter is the basic nutrient carrier in nutrient cycling. Karst soil has a naturally low availability of N and P, and in southern China, this low availability is aggregated owing to land degradation leading to soil and water loss. If AM fungi can decompose litter and supply their host plant with nutrients in the field, this would be of significant importance for maintaining nutrient availability in karst areas.

Previous studies have shown that multiple AM fungal species affect the nutrient supply (van der Heijden et al. 1998; Van Der Heijden et al. 2003) and mediate N transfer and residue decomposition (Tu et al. 2006) and that there is an additive effect of AMF species coexistence. In our study, the dual inoculation with *G. etunicatum* and *G. mosseae* compared with the single inoculation with *G. mosseae* did not change the proportion of the seedling roots that were colonized or biomass P significantly but significantly increased seedling dry biomass, biomass N, and the N:P ratio, as well as SMBC, SOC, and the hyphal length. There was also a significant stimulatory effect of dual inoculation of AM fungi in terms of the amount of $\delta^{15}N$ that was acquired from the

TEST compartment and transferred to the *C. camphora* seedlings in the HOST compartment (Fig. 3). These results indicated that combinations of more than one AM fungal species can be important in nutrient transfer to host plant in karst areas. However, field situations are more complex than our controlled environment, and follow-up experiments incorporating key natural elements (e.g., interaction with other microbes and plants, dynamic water regimes) are much needed.

AM fungi are probably known to have a saprotrophic ability (Hodge et al. 2001; Tu et al. 2006) that stimulates litter decomposition. However, whether AM fungi in karst soil are directly involved in the decomposition of organic matter or if other soil microorganisms could enter and were stimulated to decompose the organic matter for the AM fungi has still to be determined. The stimulatory effects of inoculating two instead of a single AM fungal species on nutrient uptake by host plants suggest that mycorrhizae have an impact on the nutritional balance of the ecosystem that is influenced not only by soil N availability but also by AMF species composition. Therefore, conducting long-term field experiments incorporating more key microbial species, and plant species, will be essential for understanding the roles of AM fungi and the net impact of hyphae on soil nutrient dynamics in karst ecosystems.

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Compliance with ethical standards

Ethics statement The work described has not been published before. The work is not under consideration for publication anywhere else, and its publication has been approved by all coauthors.

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