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Full Length Research Paper

Arbuscular mycorrhizal fungi associated with Huangshan Magnolia (*Magnolia cylindrica*)

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Root and rhizosphere soil samples of medicinal plant Huangshan Magnolia (*Magnolia cylindrica*) from Chinese famous national forest park of Huangshan (Yellow Mountain) were studied to determine the root colonization and the diversity of spore populations of arbuscular mycorrhizal (AM) fungi. The results showed that AM fungal colonization structures including hyphae, hyphal coils and vesicles were present in all root samples. Paris-type AM were identified in the roots according to the morphological structure. Seventeen species of AM fungi were isolated and identified from the rhizosphere soil samples. The species were of the genera *Acaulospora* (6 species), *Glomus* (8 species), *Gigaspora* (1 species) and *Scutellospora* (2 species). Based on importance value, 3 species from *Acaulospora* and 3 from *Glomus* were dominant. The AM fungi spore density ranged from 157 to 448 (average 315) per 100 g soil and the species richness ranged from 4 to 8 (average 6.5) per soil sample. Shannon-Wiener index and Evenness were calculated to evaluate the diversity of the AM fungi community associated with *M. cylindrica*.

Key words: *Magnolia cylindrica*, arbuscular mycorrhizal fungi, diversity.

INTRODUCTION

Huangshan Magnolia (*Magnolia cylindrica*), belonged to Family Magnoliaceae, is an endangered native medicinal and famous ornamental plant species that was first found in Huangshan (Yellow Mountain) of Anhui Province, East-Central China. The flower buds of *M. cylindrica* are used as medicine for diuresis and facilitating expectoration (Li, 2006). Preservation and cultivation of *M. cylindrica* have been justified for its restricted distribution and rare wild population in East-Central China and for its economic value as traditional Chinese medicine. Medicinal plants are important for medical therapy, pharmacological research and drug development. Due to an rapidly increasing demand for medicinal plants (Muthukumar et al., 2006), tourism disturbance (Yang et al., 2011) and to a loss and fragmentation of natural habitats (Radhika and Rodrigues, 2010), lots of medicinal plants including *M. cylindrica* have been so far assessed as under threat in the wild (IUCN, 2010; Li, 2006). The growth of medicinal plants is mutually related to their rhizosphere ecosystem where soil microbes are closely together with plant roots. While there is little attention has been paid to the soil microbes that associated with the medicinal plants, though large number of reports showed that soil microbes play a vital role in plant growth (Amerian and Stewart, 2001; Karagiannidis et al., 2002; Ravikumar et al., 1997; Sohn et al., 2003). To preserve the precious wild medicinal plants, it is urgent to know the microbial community in their rhizosphere soil. Such information would be valuable in future work for preserving wild medicinal plants population and for establishing cultivated populations as the source of medicinal products to meet the great market demand.

Arbuscular mycorrhizal (AM) fungi are perhaps the most common, likely forming associations with the majority of plant species (Bever et al., 2001). These two partners are usually regarded as a mutual aid group because the fungi promote plant growth, improve mineral and water uptake into the plant (Augé, 2001; Dutra et al., 1996; Feng et al., 2003; Schubert and Lubraco, 2000; Tawaraya et al., 2003). On the other hand, the plants supplied the fungi with the photosynthate as their nutrients (Douds et al., 2000). The symbiont of AM formed between plant roots and AM fungi become the bridge for their material and energy flux. Thus, much attention should be paid to the AM fungi community that associated with *M. cylindrica*

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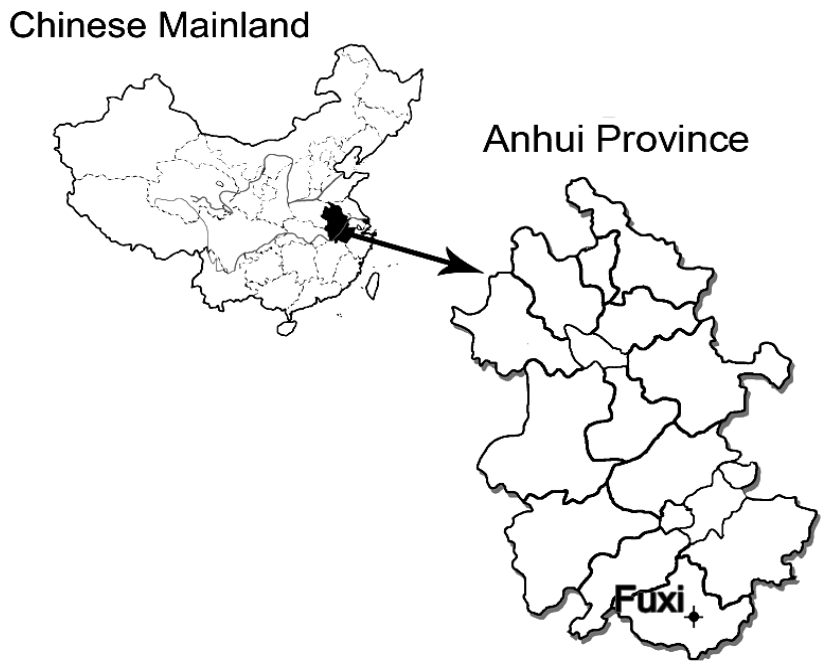

Figure 1. The geographical locality of the study site.

under the ground to facilitate the preservation and cultivation of this precious species. Magnoliaceae species have lots of primary characters and are classified as one of the ancient groups in Engler, Hutchinson and Angiosperm Phylogeny Group II (The Angiosperm Phylogeny Group, 2003) classification of angiosperm plants. Similarly, results of fossil and molecular studies reveal that AM fungi have existed in the earth for more than 4 billion years and are kinds of the most ancient fungi (Berbee and Taylor, 1993; Redecker et al., 2000; Simon et al., 1993; Taylor et al., 1995). As Magnoliaceae spp were proved to be AM plants (Smith and Smith, 1997), an interesting hypothesis arise that the pattern of symbiosis between Magnoliaceae species and AM fungi may be original during the co-evolution of fungi and host plants, and deserved to be well studied.

In this study, root and rhizosphere soil samples of twelve *M. cylindrica* from Huangshan were studied to determine the root colonization and the diversity AM of spore populations of AM fungi. The work aims at understanding the AM fungi community associated with *M. cylindrica* for preservation and cultivation of this precious species. Moreover, we hope to learn the pattern of symbiosis between *M. cylindrica* and AM fungi, which would be valuable for knowing more about Magnoliaceae plants and AM fungi.

MATERIALS AND METHODS

Study site

The field work was carried out in Fuxi of Zhaixi Village (30°05.760′N, 118°22.169′E, mean elevation of 883 m), which is in the south of Huangshan, Anhui Province, East-Central China (Figure 1). Huangshan is one of the 'World Natural and Cultural Heritage' sites and an international well-known tourist destination. This area has a subtropical monsoon climate with mean annual temperature of 16.3°C and mean annual rainfall of about 2057 mm. In recently years, rapidly increasing visitors and developing tourism have brought much pressure on preserving the biodiversity in Huangshan (Yang et al., 2011).

Sampling collection and treatments

The sampling work was done in April, 2007. Young roots (with root tips) connected to each sampled *M. cylindrica* (n = 12) were collected for quantification of AM colonization status. Root samples were dipped in 50% FAA (formalin, glacial acetic acid and 70% ethanol in 1:1:18 v/v) and stored at 4°C 10% (w/v) of KOH was used to clear the roots by heating to 90°C in a water bath for 90 to 120 min. The time of heating depended on the root structures and their pigmentation. The cooled root samples were washed several times in distilled water and stained with 0.5% acid fuchsin according to Berch and Kendruck (1982). Fungal colonization was quantified using the magnified intersection method (McGonigle et al., 1990) under a light microscope (Olympus CX31). Three repetitions were detected for each root sample and the presence of AM structures was recorded.

Twelve soil samples (about 500 g for each) were also collected at a depth of 5 to 30 cm from each plant rhizosphere. The soil samples were air-dried for about 2 weeks and stored at 4°C for up to 2 months until they could be treated. AM fungi spores from the rhizosphere soil samples were isolated through the wet-sieving method described by An et al. (1990). For each soil sample, 20 g soil was independently suspended in 200 ml water, stirred with a magnetic stirrer for 10 min and the suspension was sieved with tap water. Spores were collected by 70, 100, 150 and 900 µm sieves, filtered onto a filter paper and placed in a Petri dish for examination

Table 1. Ecological parameters for evaluating AM fungal community structure.

Parameters	Formula and definition
Isolation frequency (IF)	The percentage of soil samples where a species or a genus occurred
Relative abundance (RA)	The percentage of the spore number of a species or a genus
Importance value (IV)	(IF + RA) /2
Spore density (SD)	Spore number per 100 g air-dried soil
Species richness (SR)	Species number per soil sample
Shannon-Wiener index (*H'*)	$H' = -\sum P_i \ln P_i$
Evenness (*E*)	$E = H'/ H'_{max}$

$P_i = n_i/N$, where n_i is the spore numbers of a species and N is the total number of identified spore samples. $H'_{max} = \ln S$, where S is the total number of identified species.

under a binocular stereomicroscope. The intact healthy spores were sorted into groups and counted. AM spores were mounted in polyvinyl lactic acid (PVA) and PVA mixed 1:1 (v/v) with Meltzer's reagent (Morton, 1988; Morton and Benny, 1990) for identification. The identification was based on morphological descriptions provided by the international collection of vesicular and AM fungi (http://invam.caf.wvu.edu) and originally published species descriptions. AM fungi species were identified using a light microscope (Olympus CX31). Typical taxonomic characters of AM fungi spores were imaged with Olympus BX51 digital camera.

Statistical analyses

The AM fungi colonization status was evaluated by calculating the colonization rate of AM structures. The structure of the AM fungal community was evaluated by some ecological parameters of diversity such as isolation frequency, relative abundance, importance value, spore density, species richness, Shannon-Wiener index and evenness (Table 1). Isolation frequency reflects the distribution status of an AM fungal species according to its occurring percentage of samples. Different relative abundance can reveal the strong or weak sporulation ability of different AM fungi in an ecosystem. In our study, these two parameters were integrated when analyzing the dominance of AM fungi species and genus according to importance value (IV), that is, the dominant species and genus (IV ≥ 25). Spore density was calculated from direct counts of AM fungal spores under a binocular stereomicroscope and all isolated spores from soil samples were counted including some spores that lacked distinguishable morphological characters. Only spore specimens identified to species (total 385 spores) were included in the statistical analysis of isolation frequency, relative abundance and species richness. AM fungal diversity was evaluated by Shannon-Wiener index and Evenness. Pearson correlation analysis and curve estimation of regression analysis by employing SPSS 10.0 were used to reveal the relationship between the parameters.

RESULTS

AM fungal colonization status

AM fungal colonization structures including hyphae, hyphal coils and vesicles were present in all root samples. Paris-type AM were identified in the roots of *M. cylindrica* according to the morphological AM structure. After infecting the roots, one ends of the AM fungal hyphae were free on the root surface, while the other ends colonized the cortex and spread from cell to cell to form dense continuous intracellular hyphal coils (Figure 2, Section 1, 2, 3). Few vesicles were developed in and between the cortical cells. The structure of arbuscules that may associate with hyphal coils in described *Paris*-type AM was not found. The average colonization rate of hyphae, hyphal coils and vesicles were 49.01 ± 4.07% (range from 34.93 to 74.55%), 28.34 ± 2.46% (16.07 to 47.73%) and 4.47 ± 0.73% (1.15 to 8.62%), respectively (mean ± SE, n = 12). There is significant positive correlation between colonization rate of hyphae and hyphal coils ($r = 0.947$, $P<0.001$). The curve estimated was shown in Figure 3.

AM fungal diversity

Three hundred and eighty five spores of AM fungi were wet-sieved from 12 rhizosphere soil samples, from which 17 species were identified (Table 2). Six of these species were in *Acaulospora*, 8 in *Glomus*, 1 in *Gigaspora* and 2 in *Scutellospora*. Some identified spores of AM fungi species were illustrated in Figure 2. Based on importance value shown in Table 2, *Acaulospora* (78.96%) and *Glomus* (68.57%) were the dominant genera in the rhizosphere soil of *M. cylindrica*. There were 3 dominant species (*Acaulospora denticulate*, *Acaulospora spinosa* and *Acaulospora tuberculata*) in *Acaulospora* and 3 dominant species (*Glomus claroideum*, *Glomus macrocarpum* and *Glomus monosporum*) in *Glomus,* respectively because of their high importance value (Table 2). Sporulation and distribution of AM fungi were reflected as a whole according to significant positive correlation between relative abundance and isolation frequency of AM fungi ($r = 0.841$, $P<0.001$). The curve estimated was shown in Figure 4. The average spore density was 315 ± 41 spores (range from 157 to 448) per 100 g soil and average species richness was 6.5 ± 0.4 species (range from 4 to 8) per soil sample. There was no correlation

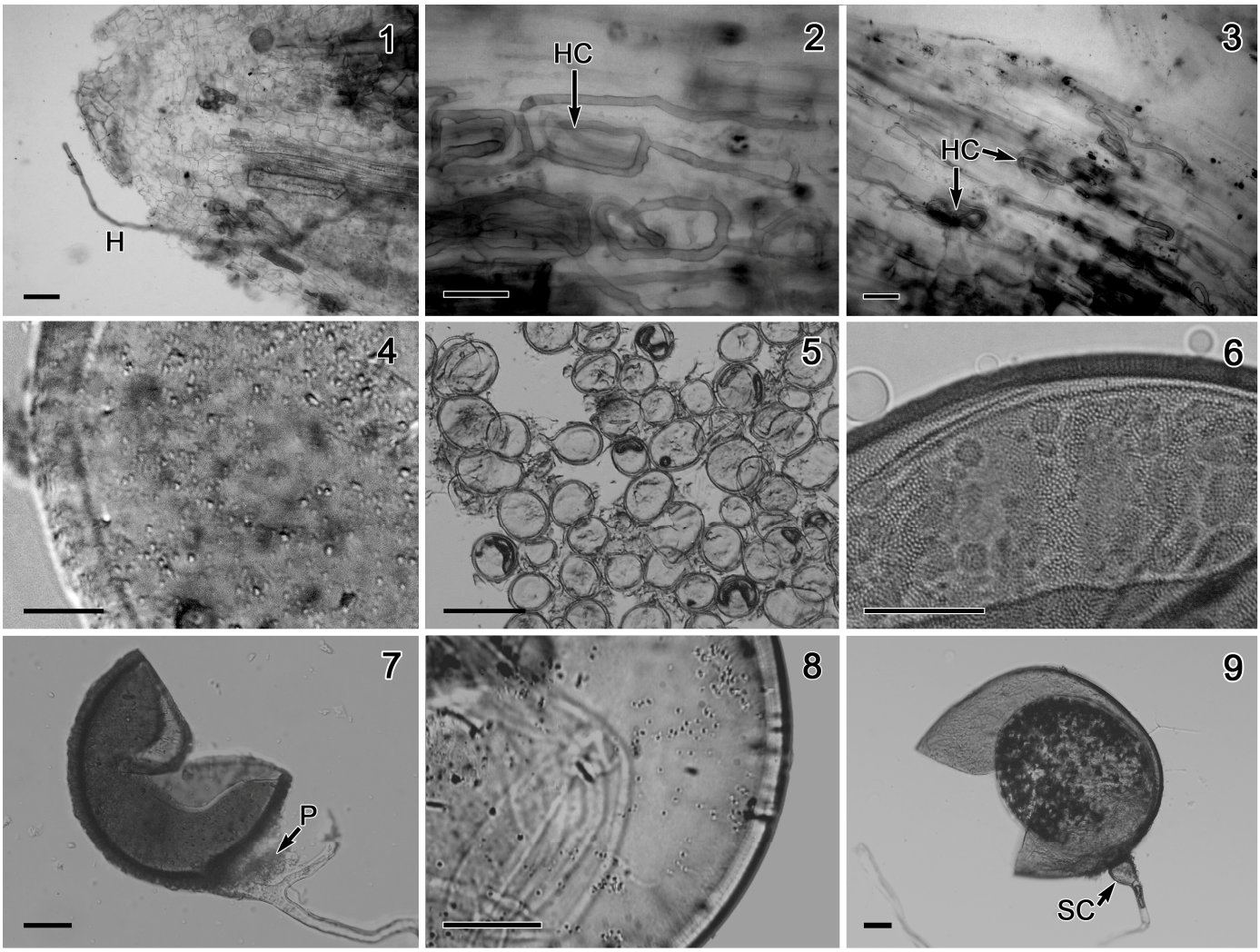

Figure 2. Morphological structures of AM and some AM fungal spores isolated from the rhizosphere soil of *Magnolia cylindrica*. (1) Free hyphae (H) on the root surface, while the other end of the hyphae colonizing the cortical cells, (2 and 3) plenty of continuous hyphal coils (HC) formed in the cortical cells, (4) *A. denticulate*, "denticulate" ornamentations on the spore wall, (5) *G. microaggregatum*, spores in cluster, (6) *A. tuberculata*, the tubercles on the spore surface, (7) *G. monosporum*, the remains of a sporocarp peridium (P) on the spore surface, (8) *A. spinosa*, closely packed spines on the spore wall, (9) *S. verrucosa* with a sporogenous cell (SC), bars line = 30 µm.

between spore density and species richness. Shannon-Wiener index of diversity (*H'*) and Evenness (*E*) were 2.22 and 0.78, respectively.

DISCUSSION

This work showed that all root samples of *M. cylindrica* were colonized by AM fungi. The AM fungi extend their waved mycelial network on a large scale, which provides a greater absorptive surface than root hairs and thus is helpful for *M. cylindrica* to absorb nutrition in soil. Also the mycelial network combines *M. cylindrica* and other plants to make a large functional organism under the ground. The morphology of AM structures can mainly be divided into two types, *Arum and Paris*, first described by Gallaud (1905). The *Arum*-type of colonization is defined by hyphae of AM fungi growing intercellularly in the root cortex and penetrating the cortical cells to produce arbuscules as terminal structures on "Trunk hyphae" (Sannazzaro et al., 2004). By contrast, in the Paris-type, colonization spreads directly from cell to cell in the root. This is further characterized by the absence of intercellular hyphae, and the development of intracellular hyphal coils that frequently associated with few intercalary arbuscules.

In the root of *M. cylindrica*, the plentiful supply of hyphal coils provide for the nutrition exchange between cortical cells and fungi (Dickson et al., 2007). Smith and Smith (1997) pointed out that, *Paris*-type AM followed the original pattern of symbiosis and was dominant in lower plants. In this type, AM fungi do not form the functional

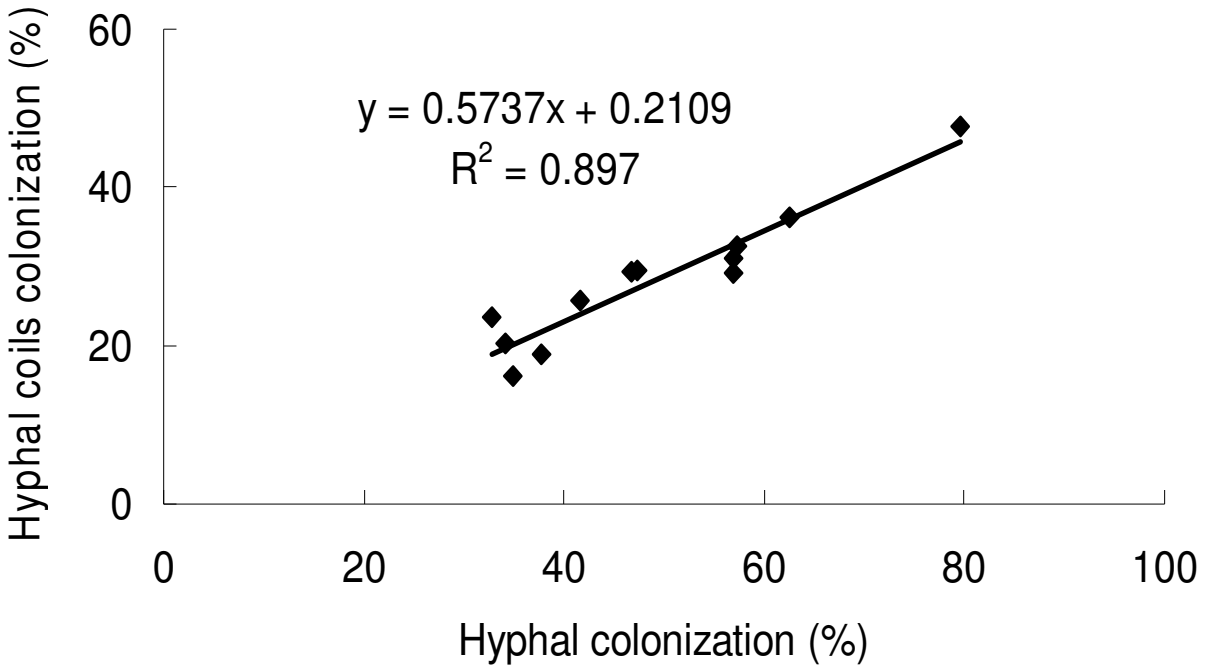

Figure 3. Relationship of AM fungi hyphal and hyphal coil colonization in roots of *Magnolia cylindrical.*

Table 2. Isolation frequency (IF), relative abundance (RA) and importance value (IV) of AM fungi species associated with *Magnolia cylindrica*.

Species No.	AM fungi species	Spore No.	IF (%)	RA (%)	IV (%)
	Acaulospora	223	100	57.92	78.96
1	*A. denticulata* Sieverding and Toro	99	91.67	25.71	58.69
2	*A. laevis* Gerd. and Trappe	6	41.67	1.56	21.62
3	*A. mellea* Spain and Schenck	4	16.67	1.04	8.86
4	*A. scrobiculata* Trappe	5	25.00	1.30	13.15
5	*A. spinosa* Walker and Trappe	75	58.33	19.48	38.91
6	*A. tuberculata* Janos and Trappe	34	66.67	8.83	37.75
	Glomus	143	100	37.14	68.57
7	*G. claroideum* Schenck and Smith	20	66.67	5.19	35.93
8	*G. clarum* Nicol. and Schenck	8	25.00	2.08	13.54
9	*G. constrictum* Trappe	2	8.33	0.52	4.43
10	*G. geosporum* (Nicol. and Gerd.) Walker	9	33.33	2.34	17.84
11	*G. macrocarpum* (Tul. and Tul.) Thaxter	62	66.67	16.10	41.39
12	*G. microaggregatum* Koske, Gemma and Olexia	8	16.67	2.08	9.38
13	*G. monosporum* Gerd. and Trappe	28	58.33	7.27	32.80
14	*G. verruculosum* Blaszkowski and Tadych	6	16.67	1.56	9.12
	Gigaspora	6	25.00	1.56	13.28
15	*Gigaspora gigantea* (Nicol. and Gerd.) Gerdmann and Trappe	6	25.00	1.56	13.28
	Scutellospora	13	25.00	3.38	14.19
16	*S. heterogana* (Nicol. and Gerd.) Walker and Sanders	2	8.33	0.52	4.43
17	*S. verrucosa* (Koske and Walker) Walker and Sanders	11	25.00	2.86	13.93
Total	AMF = 17 Species	385		100	

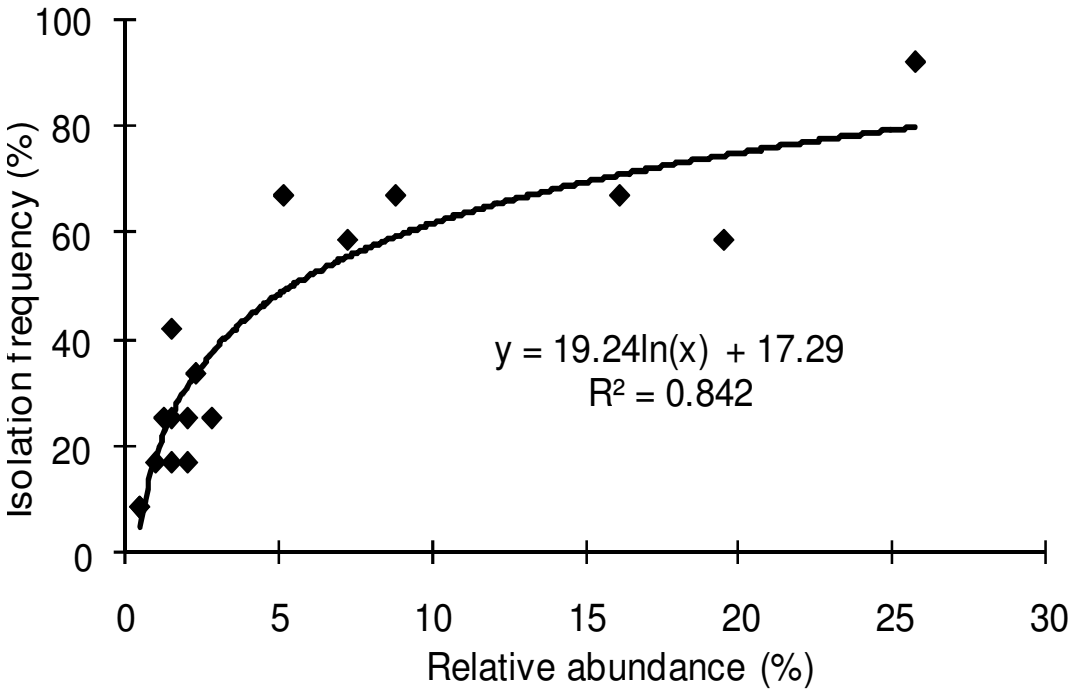

Figure 4. Relationship of relative abundance and isolation frequency of AM fungi species in the rhizosphere soil of *Magnolia cylindrica*

structure of arbuscules as exchange interface with larger areas of surface than hyphal coils for enhancing the nutrition interchange in Arum-type AM. Moreover, in this study, the structure of intercalary arbuscules that may have developed from hyphal coils as described with Paris-type AM was absent in the root of *M. cylindrica*. This may indicate that the pattern of symbiosis between AM fungi and *M. cylindrica* was in the early phases of the evolution of Paris-type AM.

The dominance of AM fungal genera in Huangshan may be related to their sporogenous characteristics. *Glomus* and *Acaulospora* possess the smallest size spores in AM fungi taxa. Small spores are easy to distribute and produce a large number of spores in a short time (Hepper, 1984). The dominant species of *A. denticulate*, *A. spinosa*, *A. tuberculata*, *G. claroideum*, *G. macrocarpum* and *G. monosporum* may play important roles in constructing the stable symbiotic relationship between AM fungi and *M. cylindrica*. The significant positive correlation between relative abundance and isolation frequency of AM fungi showed that a strong capacity of sporulation and wide distribution usually existed concurrently (Moreira-Souza et al., 2003; Zhao and Zhao, 2007).

The species distribution patterns within a community, expressed as diversity indexes, vary remarkably (Öpik, 2004). In earlier reports, the Shannon-Wiener index of AM fungi community associated with *Paeonia suffruticosa* (Paeoniaceae) was 1.45 in a city garden (Guo et al., 2007), 0.81 with *Olea europaea* (Oleaceae) and 1.08 with *Pistacia lentiscus* (Anacardiaceae) in Mediterranean shrublands (Azcón-Aguilar et al., 2003). In this study, the index was 2.22 with *M. cylindrica* in the wild of subtropical forest. Different AM species composition and disparity in AM fungal relative abundance cause the differences between diversity indexes (Yang et al., 2011). The diversity of AM fungal community associated with *M. cylindrica* may be the result of mutual selection between AM fungi and the plants in specific environment.

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