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Full Length Research Paper

Arbuscular mycorrhizas and dark septate endophytes colonization status in medicinal plant *Lycium barbarum* L. in arid Northwestern China

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***Lycium barbarum* L. is an oriental medicinal plant. The arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) fungal associations of this plant species are completely unknown. In this study, the AM and DSE fungal colonization status in three *L. barbarum* cultivars in arid Northwestern China were investigated. The results showed that the three cultivars were simultaneously colonized by *Paris*-type AM and DSE fungal associations. The highest colonization by AM was found in *L. barbarum* Ningqi No.1. The significant "month" and "cultivar" indicates that the AM colonization changed among the months within the cultivar. Meanwhile, roots of the three cultivars were heavily colonized by DSE fungi. Melanized hyphae were frequently observed in the three cultivars. Microsclerotia of varied shapes were also found in the cortex cells of *L. barbarum*. Hyaline hyphae were most abundant in August but their occurrence decreased in December. Lipid contents were abundant in hyaline hyphae, and changed with month. Lipid may act as energy material reserves to sustain the survival of DSE-host symbioses under drought environmental conditions.**

Key words: *Lycium barbarum*, fungal symbionts, hyaline hyphae, lipid, melanized septate hyphae.

INTRODUCTION

Lycium barbarum L., an economically important traditional medicinal plant, belongs to Solanaceae family (Lu and Wang, 2003). It is a deciduous perennial shrub that is widely distributed in arid and semi-arid regions of Northwestern China, Southeastern Europe and the Mediterranean areas (Yin and Dang, 2008). In the past decades, investigation of the bioactivities of chemical constituents in fruit extracts of *L. barbarum* has received much attention (Gan et al., 2003; Gao et al., 2008), because the chemical constituents were demonstrated to cure several chronic diseases such as cancer, diabetes, hyperlipidemia, etc (Gan et al., 2004; Li, 2001; Li et al., 2007). In this context, *L. barbarum* seedlings were widely cultivated in China. It is interesting that *L. barbarum* can grow particularly well in arid areas in which other tree species do not survive. *L. barbarum* must develop and maintain several favorable adaptive mechanisms, among which developing root mutualistic symbioses may be an important one under drought environmental conditions. Unfortunately, to date, little attention has been paid to investigate the symbiotic status between *L. barbarum* and root associated fungi.

Plants can be colonized by various fungal symbionts, including ectomycorrhizal (ECM) fungi, arbuscular mycorrhizal (AM) fungi and dark septate endophytic (DSE) fungi. Among these symbionts, arbuscular mycorrhiza is divided into *Arum*-types, *Paris*-types and *Intermediate*-types according to the presence or absence of intercellular hyphal growth in the root cortex of host plant (Dichson, 2004; Smith and Smith, 1997; Bedini et al., 2000). DSE fungus, classified as ascomycetous dark septate endophytes, can form melanized septate hyphae and microsclerotia (Stoyke and Currah, 1991; Jumpponen

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and Trappe, 1998). DSE fungi have some times been reported to be equally or more abundant than AM fungi in the living roots of host plant (Mandyam and Jumpponen, 2008). Kohn and Stasovski (1990) also suggested that DSE fungi have better adaptive ability under stressful conditions than AM fungi. However, compared with the various mycorrhizal associations, the DSE symbioses are poorly understood. It has been suggested that DSE symbioses are “mycorrhizal” (Jumpponen, 2001), but the functional ability of DSE fungi is current subject of debate in the scientific community (Mandyam and Jumpponen, 2005; Grünig et al., 2008).

To understand the function of DSE fungi it requires a clear description of the structures of DSE fungi formed in roots of host plants, in order to integrative appreciate the DSE-host symbioses. The microscopic work conducted by Barrow and Aaltonen (2001) and Barrow (2003) in arid Southwestern USA rangelands showed the DSE colonization status in the roots of *Atriplex canescens* and *Bouteloua* sp. DSE fungi also colonized the endangered Chinese medicinal plant *Saussurea involucrata* Kar. et Kir. ex Maxim (Wu and Guo, 2008). Interestingly, co-occurrence status of AM and DSE fungi was also observed in the root of several other host plants (Trowbridge and Jumpponen, 2004; Muthukumar et al., 2006; Tan et al., 2006; Schmidt et al., 2008; Fernandez et al., 2008; Lugo et al., 2009; de Marins et al., 2009). However, there is no published work on the morphology of the AM-DSE-host symbioses in *L. barbarum*. Accordingly, the objective of the present study was to investigate the AM-DSE-host symbioses in the three cultivars of *L. barbarum* growing in arid Northwestern China.

MATERIALS AND METHODS

Sample collection

A field experiment was performed in a natural habitat at the experimental farm of Ningxia Wolfberry Engineering and Technology Research Center (NWE-TRC), Ningxia Hui Autonomous Region, Northwestern China (106°12′E, 38°28′N, and 1200 m above sea level). Water shortage restrains the agriculture and forestry in this region. Rainfall is erratic, and mainly occurs between June and September with an annual average precipitation 127 mm (Gu et al., 2007). The soil is classified as sierozem. The vegetation was manually removed before plantation establishment. The soil was tilled down to 15 cm at the beginning. Holes of 30 × 30 cm^2 and 30 cm deep were dug manually and *L. barbarum* seedlings were planted at least 1 m apart, one in each hole, with 3 m between plots.

There were at least 50 seedlings per treatment. The seedlings were free of root associated fungi before planted in soil. In March, 2005, a field experiment was carried out as a randomized complete block design. A 1 m wide buffer zone was established between plots. Three cultivated cultivars were conducted, viz. *L. barbarum* L. cv Ningqi No.1, *L. barbarum* L. cv Ningqi No.5 and *L. barbarum* L. cv Ningqicai No.1 (hereinafter abbreviated as NQ-1, NQ-5 and NQC-1, respectively), were planted manually. NQ-1, a cultivated variety of *L. barbarum*, is widely planted in Northwestern China (Zheng et al., 2010). NQ-5 is a local variety. NQC-1 was only planted in Ningxia Hui Autonomous Region by local medicinal herb researcher. After four years, in August, October and December of 2009, root samples from ten plants of each cultivar were collected, put into sterile envelopes, transported to the laboratory and processed within 48 h.

AM and DSE structural status determination

Root samples were cleared and stained by the methods described by Phillips and Hayman (1970), Barrow and Aaltonen (2001), Barrow (2003), Liu and Chen (2007) and modified for microscopic observation. Briefly, roots of each cultivar were carefully washed with distilled water for three times in order to remove soil particles, and then cut into 1 cm long segments. Subsequently, 1 cm long root segments (Diameter < 2 mm) were randomly selected and cleared in 2.5% KOH solution in a water-bath at 90°C for 60 min and rinsed three times with distilled water. Then segments were immersed in 2% HCl for 5 min, bleached in 10% alkaline H_2O_2 for 10 min and rinsed three times with distilled water. All root segments were divided into two equal parts for staining. Firstly, 1 cm long roots were immersed in 0.3% (w/v) Sudan IV solution at room temperature, containing 740 ml 95% ethanol, 3.0 g Sudan IV (Tianjin, China), and added distilled water to 1000 ml. A second batch of 1 cm long roots was soaked for two hours in 0.05% (w/v) trypan blue solution at room temperature, containing 300 g carbolic acid, 0.5 g trypan blue, 500 ml (lactic acid : glycerol, v=1:1), and 300 ml distilled water. All stained roots were transferred into acidified glycerol and incubated for 12 h at room temperature. All the stained root segments were transferred cautiously to the glass plate filled with acidified glycerol. Morphology of the endophytic mycelia of DSE fungi was observed at magnifications of × 400 and × 1000 with a microscope equipped with differential interference contrast optics (Olympus BX51, Japan). AM structures such as arbuscule, vesicle and hyphae were examined. The location and distribution of DSE structures, including melanized septate hyphae, microsclerotia, hyaline hyphae, were also detected (Mandyam and Jumpponen, 2008). The percentage of AM and DSE colonization was estimated by the method of Zhang et al. (2010). Root length colonized (RLC) by DSE and AM colonization was also calculated. Typical structures of AM and DSE were captured with a digital CCD DP71 sensor and the meaningful pictures were managed by Image-Pro Express 6.0 analyses software (Olympus, Japan).

Statistical analysis

Results were statistically tested by one- and two-way analyses of variance (ANOVAs) using SAS version 8.1 software package (SAS Institute Inc., Cary, NC, USA). Comparisons between means were carried out using Duncan’s multiple range tests. Graphical work was carried out using Sigma Plot for Windows version 10.0 software packages.

RESULTS

Microscopic examination of stained root segments revealed that all tested *L. barbarum* cultivars were colonized by AM and DSE fungi. AM colonization varied both month and cultivar as indicated by MANOVA (Table 1). The significant “month” and “cultivar” indicates that the AM colonization varied among the months within the cultivar. However, there were no significant two-way interactions. In three cultivars, the month variation in AM total colonization, hyphae, vesicles, and arbuscules were

Table 1. F-values for two-way ANOVAs of the variables for AM and DSE colonization.

Indices		Month (F value)	Cultivar (F value)	Month × Cultivar (F value)
AM status	Total colonization	18.81***	12.34***	0.70 *ns*
	Hyphae	100.59***	16.93***	1.89 *ns*
	Vesicle	41.73***	11.39***	1.61 *ns*
	Arbuscule	64.17***	18.87***	0.26 *ns*
DSE status	Total colonization	1.88 *ns*	2.04 *ns*	0.03 *ns*
	Melanized hyphae	7.76 **	1.86 *ns*	1.68 *ns*
	Microsclerotia	33.48***	5.01*	1.66 *ns*
	Hyaline hyphae	77.96***	1.61 *ns*	1.37 *ns*

ns non-significant P > 0.05, *P < 0.05, ** P < 0.01,*** P < 0.001.

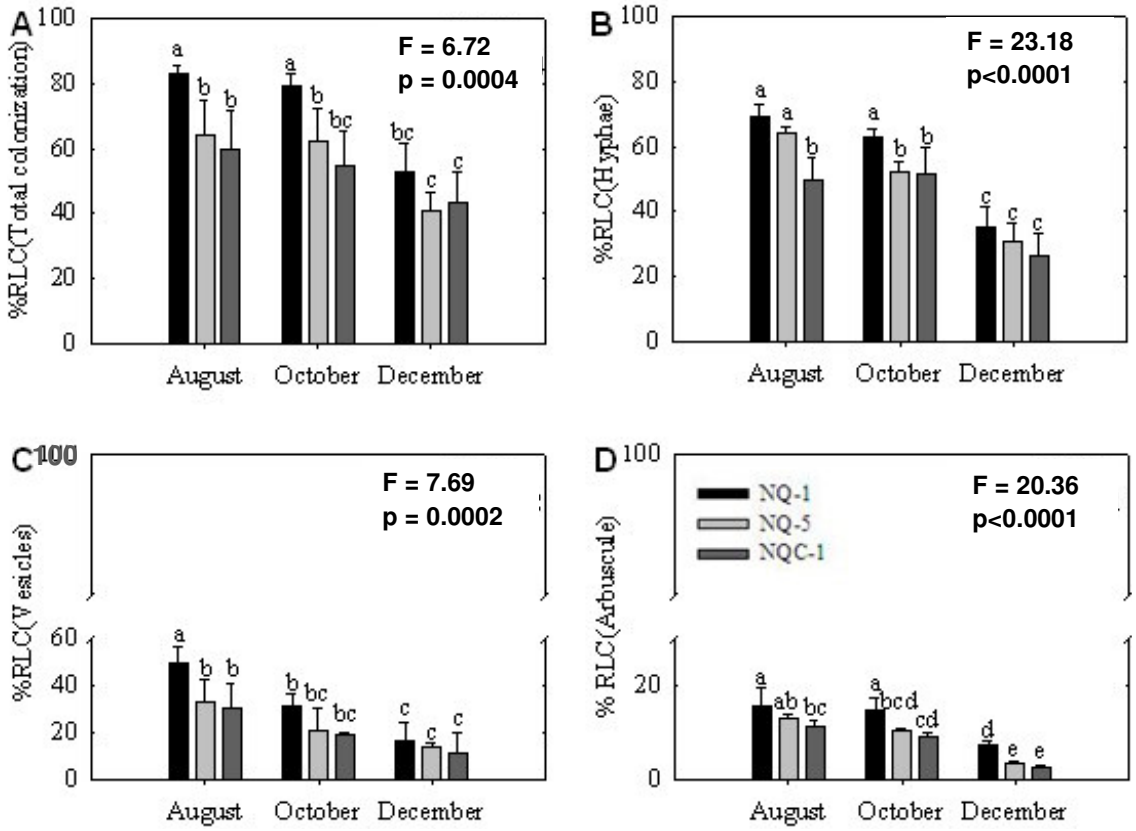

Figure 1. Arbuscular mycorrhizal colonization status in three *L. barbarum* cultivars. Roots of the three *L. barbarum* cultivars were sampled in August, October and December of 2009 respectively. %RLC by total colonization (A), hyphae (B), vesicle (C) and arbuscule (D) were showed. The data shown are the means and standard deviation. The same letter indicates no significant difference by Duncan's test.

observed (Table 1 and Figures 1A to D). The %RLC by AM hyphae varied significantly and was highest in August, and lowest in December. The %RLC by DSE microsclerotia varied monthly as indicated by MANOVA (Table 1). Hyaline hyphae (HH) were most abundant in August but their occurrence decreased in December. However, microsclerotia showed lowest colonization in August (Figures 2A to D). Univariate analyses were performed for significant effects in multivariate analysis (Table 1). The morphological AM type observed in *L.*

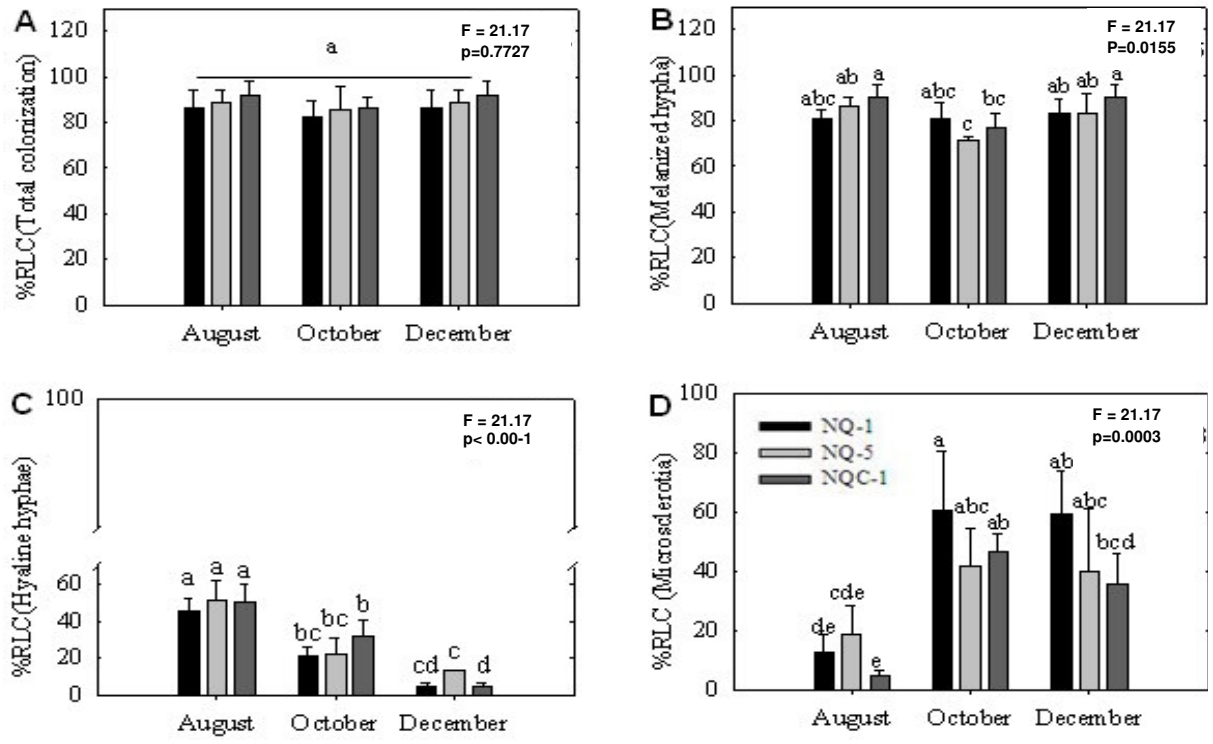

Figure 2. Dark septate endophytes colonization status in three *L. barbarum* cultivars. Roots of the three *L. barbarum* cultivars were sampled in August, October and December of 2009, respectively. %RLC by total colonization (A), melanized septate hyphae (B), hyaline hyphae (C) and microsclerotia (D) were showed. The data shown are the means and standard deviation. The same letter indicates no significant difference by Duncan's test.

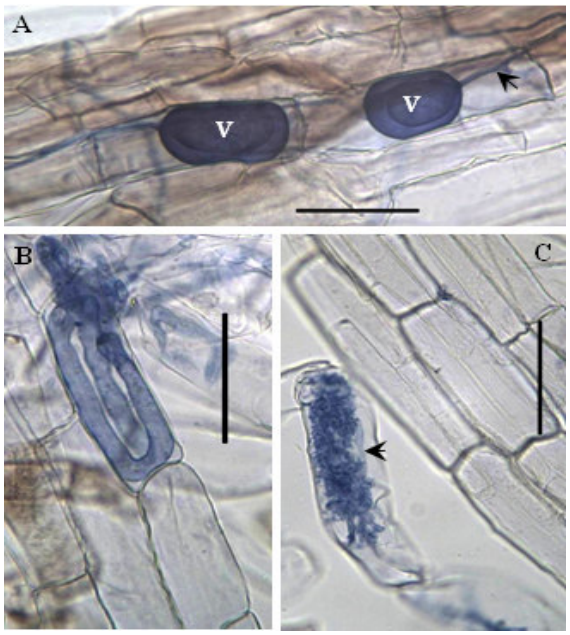

Figure 3. (A) Intercellular hypha (arrowed) and vesicle (V) formed in the root cortical cells of *L. barbarum* L. cv Ningqi No.1. (B) Typical *Paris*-type coils formed in the root cortical cells of *L. barbarum* L. cv Ningqi No.1. (C) Arbuscules (arrowed) formed in the root cortical cells of *L. barbarum* L. cv Ningqi No.1. Scale *bars* are 20 µm in all pictures.

barbarum was considered as typical *Paris*-type (Figure 3B). Abundance of vesicle and arbuscule was also observed (Figures 3A and C). Numerous red lipid droplets were distributed in the hyaline hyphae of DSE fungi (Figures 4A and E). An intercellular septate coil stained positively with trypan blue was observed (Figure 4B). Intercellular melanized hyphae and microsclerotia were also observed in the cortex cells of *L. barbarum* Ningqicai No.1 (Figures 4C, D, and F). Hypha penetrated into the cortex and formed melanized microsclerotia. Microsclerotia with lipids were painted red with Sudan IV (Figure 4D). Abundant lipids were also found in microsclerotia (Figure 4D). Intracellular hyaline hyphae with lipid-filled with no distinguishable or stained fungal wall were also observed (Figure 4E).

DISCUSSION

It has been believed that plant harbors several mutualistic symbionts, among which arbuscular mycorrhiza fungi have the widest range of locations, and can improve host plant growth, particularly in drought environmental conditions (Yamato et al., 2009; Mena-Violante et al., 2006). DSE fungi are limitedly studied or are often left out of consideration, although, these fungal species occur abundantly in living roots of plant species (Ahlich and

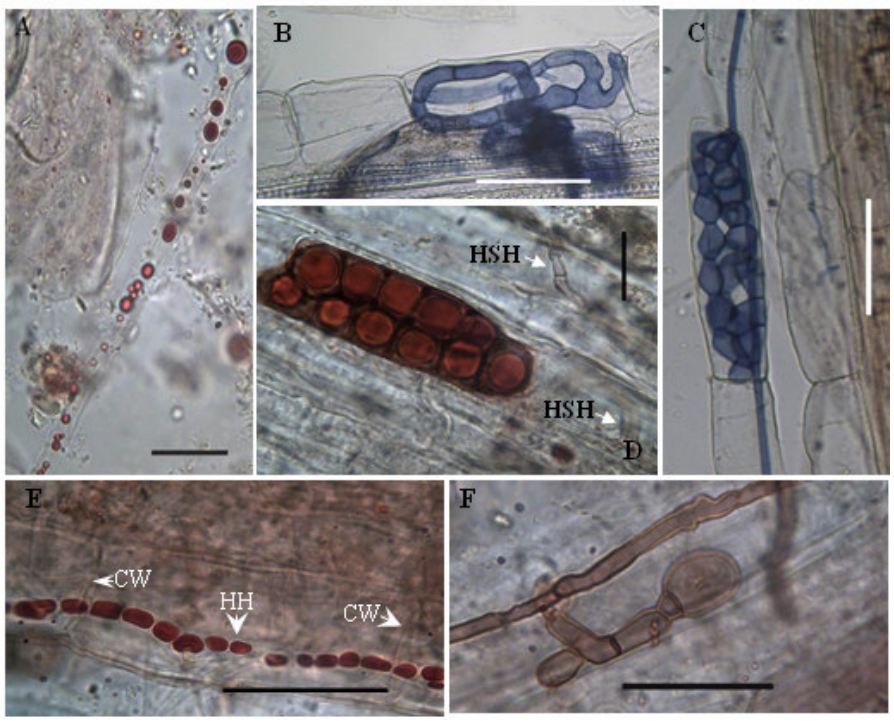

Figure 4. (A) Hyaline hyphae with lipid vacuoles formed in root of cortical cells of *L. barbarum* L. cv Ningqicai No.1. (B) Septate coils stained positively with trypan blue in root of cortical cells of *L. barbarum* L. cv Ningqi No.1. (C) Microsclerotia in the cortex of *L. barbarum* L. cv Ningqi No.1 roots. (D) Melanized microsclerotia developing from hyaline septate hypha (HSH) in the cortex of *L. barbarum* L. cv Ningqi No.5 roots. DSE microsclerotia with lipid body stained red with Sudan IV (D) in the cortex of *L. barbarum* L. cv Ningqi No.5 roots. (E) Hyaline hyphae (HH) with lipid body stained red with Sudan IV penetrate through the cell wall (CW) in the cortex of *L. barbarum* L. cv Ningqi No.5 roots. (F) Intercellular melanized septate hyphae fromed in the cortex of *L. barbarum* L. cv Ningqicai No.1. Scale *bars* are 20 µm in all pictures.

Sieber, 1996). Recently, laboratory studies have been conducted by Wu and Guo (2008) and Wu et al. (2010), which showed that DSE fungus can promote the growth of the endangered Chinese medicinal plant *Saussurea involucrata*, and increase its rutin content. These facts lead us to hypothesize that AM and DSE fungi might be valuable to improve the growth of medicinal plant *L. barbarum*, and to increase the quality and quantity of *L. barbarum* fruit chemical components. However, the ecological roles of DSE fungi remain enigmatic (Jumpponen and Trappe, 1998; Grünig et al., 2002, 2008). It has been demonstrated that structure determines function. Therefore, investigation of the DSE structures formed in host plant is undoubtedly the crucial gateway to open the veil. In the present study, AM and DSE fungal colonization status were studied, the following statements need to be discussed.

The current study shown that AM and DSE fungi had a good symbiotic association with the roots of *L. barbarum*. Co-occurrence of these two associations was detected microscopically. DSE associations were recorded for the first time in *L. barbarum*. Previous studies have shown that morphological types of AM depend on the characteristic of plants. Yamato and Iwasaki (2002) suggested that AM morphological types are mostly discriminated in a family level except for Rosaceae. In the present study, *L. barbarum* belonged to Solanaceae, in other Solanaceae plants such as tomato, also formed *Paris*-type AM, and Cavagnaro et al. (2001) suggested that morphology of arbuscular mycorrhizas is influenced by fungal identity. In this study, all tested root samples of *L. barbarum* formed *Paris*-type AM. It might be *Paris*-type AM that is favored by host plant grown in arid conditions. Similarly, Yamato and Iwasaki (2002) indicated that *Paris*-type AM is advantageous for the host plants grown in the soils with a low level of nutrient availability. Brundrett and Kendrick (1990) discussed that *Paris*-type AM might be suitable for woodland plants growing slowly in a woodland environment. Our speculation is also consistent with that of McGee (1986), who found that *Paris*-type morphology has been more often seen in plants in arid systems. However, the work by O'Connor et al. (2001) suggested that *Paris*-type colonization is rare among AM plants in the Southern Simpson Desert, Australia. Bedini et al.

(2000) proposed that host plant genotype affects AM morphology. Cavagnaro et al. (2001) also speculated that the formation of AM morphological types depends on the AM fungal species as the differences in enzymatic characteristics of different AM fungal species might influence the AM types.

In the present study, it is shown that DSE colonization exceeds AM colonization in December. Our observations on the DSE and AM colonization agree to some extent with Mandyam and Jumpponen (2008). Recently, Lugo et al. (2009) examined the AM and DSE status of five epiphytic and four terrestrial bromeliaceae from an arid area of central Argentina, and the results showed that terrestrial bromeliaceae forms AM-DSE associations, but epiphytes are only DSE fungi colonized. In this report, *L. barbarum* formed AM-DSE associations. Typan blue and Sudan IV solutions were used to stain DSE fungal structure in this work. This method, first used by Barrow and Aaltonen (2001), suggested that estimation of the degree of root colonization by DSE tends to be inaccurate as a result of abundant hyaline hyphae that are difficult to visualize. And duel-staining with Sudan IV and Typan blue was successfully used to detect hyaline hyphae by staining lipids body red and cell walls of hyaline hyphae blue.

L. barbarum is abundant in arid and semi-arid areas of Northwestern China. It can grow well under heavy drought stress areas (Gu et al., 2007). Meanwhile, DSE fungi are ubiquitous (Jumpponen and Trappe, 1998), being especially common in stressful environments like arid area (Barrow, 2003). Recently, Lugo et al. (2009) reported that endosymbiosis of DSE in bromeliaceaen species was from an arid area in Central Argentina. These facts induce us to hypothesize that there might be DSE fungi in the root of *L. barbarum*, and DSE fungi might generate protective mechanism (e.g. particularly structures and metabolites) for mycelial cell and host plant survival under drought stress. For example, the observed lipids body might help association to survive under drought periods. It might be that lipid bodies are accumulated in wet conditions, and used as carbon sources when the host is exposed to drought stress (Barrow, 2003). Similarly, microsclerotia serve to outlast drought stressful conditions. The study conducted by Yu et al. (2001) showed that concentration of phosphorus (P) is significantly higher in microsclerotia than in the mycelium of DSE fungi and those microsclerotia accumulate high level protein, glycogen and poly-phosphate. It remains to test whether NQ-5, in which microsclerotia are most abundant, is better adapted to adverse conditions than other cultivars. In this study, abundant lipid bodies and melanins were also found in microsclerotia. Drought stress may enhance melanin accumulation in cell wall of mycelium of DSE fungi. Melanins act as darkly pigmented polymers that can protect organisms against environmental stress (Henson et al., 1999). Fungal melanin is likely needed for survival in the stressful environment. DSE fungi are abundant in higher elevation or Polar Regions (Schmidt et al., 2008). The solar ultraviolet radiation is stronger. Maybe melanin in fungi is an adaptive trait against solar ultraviolet radiation (Singaravelan et al., 2008). However, the specific mechanism is still not clear. Therefore, further research is needed. Unfortunately, research about the function of DSE fungus is just beginning.

AM and DSE fungi were not identified to species in the present study. Further study is required to understand the AM fungal identity in the rhizosphere soils of *L. barbarum*. Moreover, isolation and identification of DSE using molecular methods will be a future challenge. Identification could occur according to the approach proposed by Grunig et al. (2008). Once some of the most abundant AM and DSE fungi are isolated, they could be used to inoculate *L. barbarum* to promote the quality and quantity of chemical constituents in *L. barbarum* fruit. In our experiment, a DSE fungal named *Phoma chrysanthemicola* M. Tang and H. H. Zhang sp. nov., inoculation can improve the growth of *L. barbarum* seedlings (unpublished data). Because AM symbiosis can frequently confer increased host resistance to drought stress, and DSE colonization may improve root water dynamics in arid areas (Cho et al., 2006; Mandyam and Jumpponen, 2005).

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