

## INVESTIGATING THE DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI FROM *GYMNEMA SYLVESTRE* AND *CURCUMA LONGA* IN VIETNAM

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### SUMMARY

Arbuscular mycorrhizal (AM) fungi are soil eukaryotes that belong to phylum Glomeromycota and have symbiosis with the vast majority of higher plants' roots. AM fungi are believed to be coevolved with terrestrial plants, the abundance and diversity of AM fungal communities as a result are host plant dependent. A survey of AM fungi from the rhizospheres of medicinal plants in Northern Vietnam including gurma *Gymnema sylvestre* and turmeric *Curcuma longa* was carried out. From the extracted total DNAs of the medicinal plants' rhizosphere soil samples, 35 mycorrhizal fungal species were identified by analyzing small subunit rRNA gene sequences. Result revealed that genus *Glomus* is the most abundant in the AM communities of *G. sylvestre* and *C. longa*, followed by *Gigaspora* and *Acaulospora*. Besides, AM species belonging to genera *Scutellospora*, *Diversispora* and *Rhizophagus* were observed in almost all rhizosphere soil samples. The spore counting by wet sieving and decanting method uncovered a variation in AM spore density of gurma and turmeric rhizosphere. In general, AM species were found more abundantly and more diverse in collected rhizome soil samples of *C. longa* (27 species belonging to 10 genera) than of *G. sylvestre* (17 species found belonging to 7 genera). The observed difference in AM communities of *G. sylvestre* and *C. longa* supports evidence for the dependence of AM fungal species on host plants, and indicates that AM fungi may have relation to the host plants' secondary metabolite production.

**Keywords:** *Arbuscular mycorrhizal*, *AM fungal diversity*, *Gymnema sylvestre*, *Curcuma longa*

### INTRODUCTION

The symbiosis of arbuscular mycorrhizal fungi (AMF) with the majority of higher plants, i.e., over 90% of plant species, is considered a result of a coevolution process (Smith, Read, 2010). The symbiosis brings beneficial impacts to host plants, including the interplant transport of nutrients and the enhancement of stress tolerance. On the other hand, it provides a source of carbon and nutrients for symbiotic fungi. The abundance of AMF in soil environment therefore influences the growth and productivity of host plants, and depends on a number of inorganic and organic factors such as the amount of nutrient in the soil and the relative amount of nutrients transferred to the plant (Reddy, Saravanan, 2013).

The existence of AMF in relationship with medicinal plants was well studied. The mycorrhizal fungi were proved to have symbiosis with medicinal plants (Muthukumar *et al.*, 2006) and possess positive effects to its bioactive compounds content such as osthole and coumarins contents in *Angelica archangelica* L. (Zitterl-Eglseer *et al.*, 2015), hypericin and pseudohypericin in *Hypericum perforatum* L. (Zubek *et al.*, 2012), and essential oil yield of menthol mint *Mentha arvensis* (Gupta *et al.*, 2002).

Gurma *Gymnema sylvestre* (Asclepiadaceae) and turmeric *Curcuma longa* L. (Zingiberaceae) are among the most important herbs that have long been used in Vietnam and several Asia countries. *G. sylvestre* is one of the major botanicals used in

traditional medicine to treat conditions ranging from diabetes, malaria, to snakebites (Singh *et al.*, 2008). The major secondary metabolites of gurma include a group of nine closely related acidic glycosides that have been found in all parts of the plant (Khramov *et al.*, 2008). Turmeric *C. longa* in recent years has become an economically important medicinal plant for the production of biological and pharmaceutical active curcuminoids. These curcuminoids are the major active compounds with diverse biological and pharmacological properties, including anti-oxidant, anti-inflammatory and anti-cancer activities (Ruby *et al.*, 1995; Maheshwari *et al.*, 2006; Jurenka, 2009).

The association of AM fungi with turmeric *C. longa* was assessed and characterized in different Indian turmeric genotypes previously (Rreddy *et al.*, 2003; Muthukumar *et al.*, 2006). The predominant mycorrhizal species with turmeric were found to belong to genera of *Glomus*, *Gigaspora* and *Sclerocystis* (Rreddy *et al.*, 2003). Previous study in the effect of AM fungi on *G. sylvestre* and the gymnemic acid production (Zimare *et al.*, 2013) proved an obvious enhancement in shoot, root length and biomass, as well as gymnemic acid content after inoculating with AM fungi *Glomus fasciculatum* and *G. mosseae*.

In recent decade, with advances in molecular techniques, studies in AMF have turned to directly identification from soil samples by polymerase chain reaction (PCR) methods (Husband *et al.*, 2002). In the present investigation, the diversity of AMF on medicinal plants *G. sylvestre* and *C. longa* in Vietnam was studied using PCR based method for specific AM fungal ribosomal RNA (rDNA) sequences. We aimed to use molecular approaches to determine and assess the distribution of AM fungi among different medicinal plant hosts.

## MATERIALS AND METHODS

### **Sampling**

Samples were collected in the Botanical Garden of Ministry of Health, Thanhtri, Hanoi, Vietnam (21°2' N, 105°51' E) from rhizome soils (5-15 cm depth from the top soil) at three randomly sites of cultivating plots (about 30 m<sup>2</sup> each) in September 2017. After sampling, rhizosphere soil samples were then stored at -20°C prior to DNA extraction.

### **AMF spore counts**

AM spores from rhizosphere soil samples of *G.*

*sylvestre* and *C. longa* were counted using wet sieving and decanting method (Gerdemann, Nicolson, 1963). Accordingly, 100 g of soil samples were suspended in sterile water and passed through 200 and 30 µm sieves, followed by sucrose gradient centrifugation (Furlan *et al.*, 1980). After centrifugation, spores and sporocarps were transferred into Petri dishes and observed under microscope. Spore abundances in samples were determined as the number of AMF spores per 100 g soil.

### **Total DNA extraction**

Rhizome's surface soil samples were stored at -20°C before being extracted by PowerSoil® DNA Isolation Kit (Thermo Fisher Scientific, USA) following manufacturer's protocol. The resulting genomic DNA was checked for purity on 0.8% agarose gels and quantified using Nanodrop ND-1000 Spectrophotometer (Invitrogen, USA).

### **PCR amplification**

Fragments of small subunit (SSU) rRNA gene from extracted genomic DNA samples were amplified using universal eukaryotic forward primer NS31 (5'-TTG GAG GGC AAG TCT GGT GCC-3') (Simon *et al.*, 1992) and reverse primers mixture AM containing AM1 (5'-GTT TCC CGT AAG GCG CCG AA-3') (Helgason *et al.*, 1998), AM2 (5'-GTT TCC CGT AAG GTG CCA AA-3') and AM3 (5'-GTT TCC CGT AAG GTG CCG AA-3') (Santos-González *et al.*, 2007) to amplify AM fungal SSU sequences. PCR reactions were carried out using PCR Master Mix (New England Biolabs, USA) composed of 2.5 mM dNTP, 2.5 mM MgCl<sub>2</sub>, 1 µl Taq polymerase and reaction buffer in the total volume of 25 µl with the following cycle conditions: 95°C for 3 min, followed by 25 cycles of 94°C for 4 min, 54°C for 30 s and 72°C for 1 min and a final extension of 72°C for 10 min.

### **Cloning and sequence determination**

PCR products were cloned using pBT vector system (Promega) and transformed into *Escherichia coli* (DH5α). Putative positive clones were screened using a second amplification with primers M13f (5'-GTAAAACGACGGCCAGT-3') and M13r (5'-AACAGCTATGACCATG-3'). (PCR conditions: 24 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 1 min). At least 30 positive clones from each sample were tested for the PCR amplicon on agarose gels. The clones were sequenced on an ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems)

sequencer using the dye terminator cycle sequencing kit with AmpliTaqFS DNA polymerase (Applied Biosystems).

#### Sequence analyses and phylogenetic inference

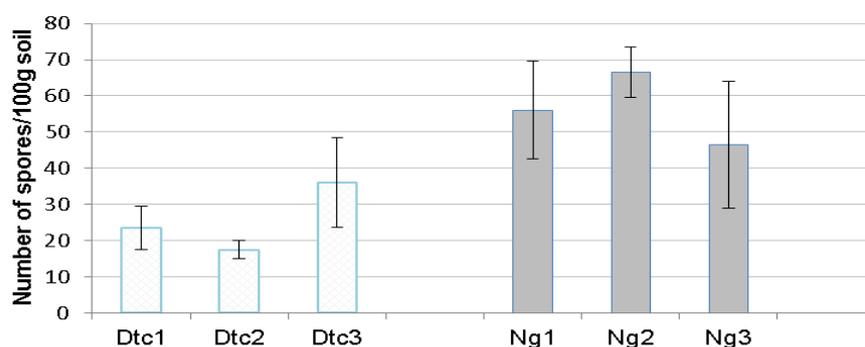
All the sequences were analyzed using SeqMan Pro (DNASTAR, USA). Phylogenetic analysis was performed on representative sequences retrieved from the database of NCBI (<https://www.ncbi.nlm.nih.gov/nucleotide/>). Sequences with more than 96% identity were assigned to a taxonomical unit. Sequences were aligned using the

multiple sequence comparison alignment tools in MEGA 6.0 (Tamura *et al.*, 2013).

#### RESULTS AND DISCUSSION

##### AMF spore abundance in the rhizospheres of *G. sylvestre* and *C. longa*

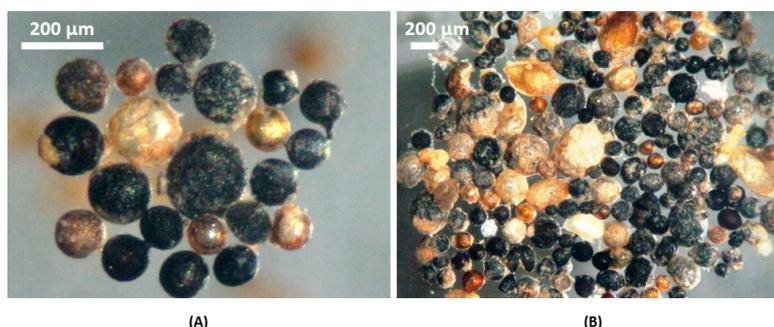
By spore counts assessment, AMF spores were found to be harboured in all rhizosphere soil samples of *G. sylvestre* and *C. longa*. The counting result is depicted in Fig. 1 and the isolated AMF spores from rhizosphere soil samples are shown in fig. 2.



**Figure 1.** AMF spore abundance in the rhizosphere soil samples of *G. sylvestre* (Dtc1, Dtc2 and Dtc3) and *C. longa* (Ng1, Ng2, Ng3). The average values of three replicates were used to represent the spore abundance in each soil sample. Error bars represent standard deviation (n=3).

Interestingly, the AM spore counts were recorded with the highest number in *C. longa*'s rhizosphere soil sample Ng2 with  $66.5 \pm 7$  spores per 100 g soil and the lowest in *G. sylvestre*'s soil sample Dtc2 with  $17.5 \pm 2.5$  spores per 100 g soil. The difference in rhizosphere soil mycorrhizal abundance between the two host plants is considerably significant (Figure 1). That is, the spore abundance in *C. longa*'s rhizosphere soil samples

(ranging from 38 to 74 spores.100 g<sup>-1</sup> soil) was higher than that one in *G. sylvestre*'s (ranging from 15 to 49 spores.100 g<sup>-1</sup> soil). The result is important to preliminarily assess AM abundance in the rhizome of two medicinal plants and with that of Panwar and Tarafdar (2006) and Thapa and others (2015) comparable, contributing to better understanding in the mycorrhizal community of medicinal plants in different environmental conditions in general.



**Figure 2.** Microscopic images (x40) of isolated AMF spores in rhizosphere soil samples of *G. sylvestre* (A) and *C. longa* (B) using wet sieving method. The images showed different types of spores ranging from approximately 60 to 200 µm in the rhizosphere soil sample of *G. sylvestre* (A) and 50 to 350 µm in the rhizosphere soil sample of *C. longa* (B).

**PCR amplification results**

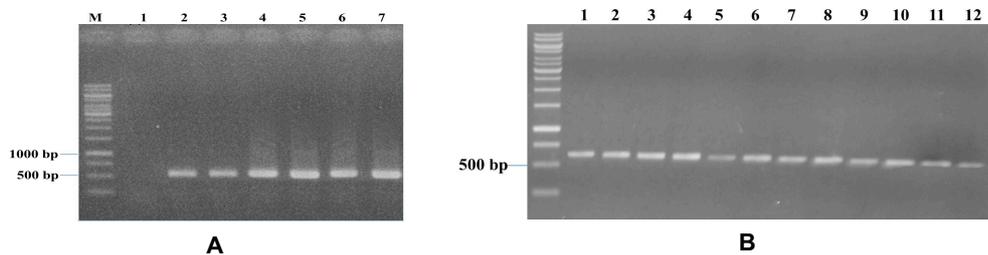
Products from the PCR amplification using primers NS31 and reverse primers-mixture AM with the length of about 550 bp (Figure 3A) were diluted and cloned in *E. coli* DH5 $\alpha$ . After the second PCR amplification using primers M13f and M13r for the selected colonies, the target DNA fragments were about 614 bp (Figure 3B).

**Diversity of AMF in *G. sylvestre*'s and *C. longa*'s rhizome**

The species numbers of AMF in rhizome of

gurma *G. sylvestre* and turmeric *C. longa* were determined by analyzing ribosomal SSU nucleotide sequences of the total DNAs extracted from soil samples of two host plants (namely Dtc1, Dtc2, Dtc3 and Ng1, Ng2, Ng3).

Result (Table 3) reveals that AM fungal communities differ between host plants and among sampling sites. In detail, while the numbers of AM species found in samples Dtc1, Dtc2, Dtc3 of *G. sylvestre* were 7, 6 and 8 (Table 1), these of three *C. longa*'s rhizome samples (Ng1, Ng2, Ng3) were 17, 12 and 15, respectively (Table 2).



**Figure 3.** PCR amplification results from rhizosphere soil samples of two host plants *G. sylvestre* and *C. longa* using primers NS31 and reverse primers mixture AM (A) and from selected colonies after cloning (B). M: Marker; 1(A): negative control; 2–4 (A): soil samples Dtc1, Dtc2 and Dtc3; 5–7 (A): soil samples Ng1, Ng2 and Ng3.; 1-12 (B): white colonies after cloning from sample Ng2.

**Table 1.** Number of AMF species found in three rhizome samples Dtc 1, Dtc2 and Dtc3 of gurma *G. sylvestre*.

No.	AM order	AM family	AM genera	Number of species found		
				Dtc1	Dtc2	Dtc3
1	Diversisporales	Acaulosporaceae	<i>Acaulospora</i>	2	1	2
2		Diversisporaceae	<i>Diversispora</i>	1	0	1
3		Gigasporaceae	<i>Gigaspora</i>	1	2	1
4		Scutellospora	<i>Scutellospora</i>	1	0	1
5	Glomerales	Glomeraceae	<i>Glomus</i>	2	2	2
6			<i>Rhizophagus</i>	0	1	0
7			<i>Funneliformis</i>	0	0	1
<b>Total</b>				<b>7</b>	<b>6</b>	<b>8</b>

**Table 2.** Number of AMF species found in three rhizome samples Ng1, Ng2 and Ng3 of turmeric *C. longa*.

No.	AM order	AM family	AM genera	Number of species found			
				Ng1	Ng2	Ng3	
1	Diversisporales	Acaulosporaceae	<i>Acaulospora</i>	1	2	2	
2			<i>Entrophospora</i>	0	0	1	
3		Diversisporaceae	<i>Diversispora</i>	2	1	1	
4		Gigasporaceae	<i>Gigaspora</i>	3	2	2	
5		<i>Scutellospora</i>	<i>Scutellospora</i>	1	1	1	
6		Glomerales	Glomeraceae	<i>Glomus</i>	5	4	5
7				<i>Rhizophagus</i>	1	1	2
8				<i>Funneliformis</i>	2	0	1
9				Claroideoglomeraceae	<i>Claroideoglomus</i>	1	1
10		Paraglomerales	Paraglomeraceae	<i>Paraglomus</i>	1	0	0
<b>Total</b>				<b>17</b>	<b>12</b>	<b>15</b>	

In the present study, AM genera of *Glomus*, *Gigaspora* and *Acaulospora* were more predominant than other AM groups. Contrastingly, *Entrophospora*, *Claroideoglossum*, *Funneliformis* and *Paraglossum* were amongst the least distributed genera. At species level, taxonomical units such as *Diversispora* sp., *Gigaspora gigantea*, *Glomus etunicatum* and *Glomus indicum* were found in rhizosphere soil samples of both plants (Table 3). The diversity of species belonging to genus *Glomus*

suggests that the species have a mechanism of adaptation with different host plants. The variation from samples to samples in distribution of almost all sequenced AMF species, for example *Acaulospora minuta*, *A. rogusa*, *Diversispora* sp. and *Funneliformis* sp. might due to the difference in sampling sites within each host plant's rhizosphere. As the annotation of sequences from clones was merely dependent on the partial SSU fragments, the result is thus not absolutely accurate at species level.

**Table 3.** Distribution of AM fungal species in rhizome samples of *C. longa* (Ng1, Ng2, Ng3) and *G. sylvestre* (Dtc1, Dtc2, Dtc3).

No.	AMF species	Appearance in rhizosphere samples <sup>(*)</sup>					
		Ng1	Ng2	Ng3	Dtc1	Dtc2	Dtc3
1	<i>Acaulospora longula</i> (#AJ306439)				+		+
2	<i>Acaulospora minuta</i> (#FR869690)	+					
3	<i>Acaulospora rogusa</i> (#LN881566)		+				
4	<i>Acaulospora spinosa</i> (#JX461237)				+	+	
5	<i>Acaulospora spinose</i> (#KC193264)			+			
6	<i>Entrophospora infrequens</i> (#U94713)		+	+			
7	<i>Diversispora</i> sp. (#MH286006)	+					
8	<i>Diversispora</i> sp. (#MH286031)	+					
9	<i>Diversispora</i> sp. (#MH286014)			+			
10	<i>Diversispora</i> sp. (#KP756538)		+				+
11	<i>Diversispora</i> sp. (#KP 756476.1)				+		
12	<i>Gigaspora albida</i> (#AF004705)	+	+				
13	<i>Gigaspora gigantea</i> (#AJ539242)	+			+		+
14	<i>Gigaspora</i> sp. (#MF599215)			+		+	
15	<i>Gigaspora</i> sp. (#AF396820)		+				
16	<i>Gigaspora</i> sp. (#MF599209)	+					
17	<i>Scutellospora calospora</i> (#KU136421)					+	+
18	<i>Scutellospora heterogama</i> (#AF004692.1)		+				
19	<i>Scutellospora pellucia</i> (#AY035663.1)				+		
20	<i>Scutellospora</i> sp. (#AF396813)	+		+			
21	<i>Glomus claroideum</i> (#AJ567810)				+		+
22	<i>Glomus cubense</i> (#JF692725)	+	+	+			
23	<i>Glomus etunicatum</i> (#AJ239125)		+		+	+	
24	<i>Glomus geosporum</i> (#AJ319786)	+		+			
25	<i>Glomus indicum</i> (#GU059543)	+		+		+	
26	<i>Glomus microaggregatum</i> (#HG425991)		+				
27	<i>Glomus mosseae</i> (#AM423117)	+	+	+			
28	<i>Glomus occultum</i> (#AF005481.1)	+		+			
29	<i>Glomus</i> sp. (#MF614120)						+
30	<i>Rhizophagus intraradices</i> (#FM865586)			+		+	
31	<i>Rhizophagus</i> sp. (#KY416592)	+	+	+			
32	<i>Funneliformis mosseae</i> (#FR750031)	+		+			
33	<i>Funneliformis</i> sp. (#MG008538)	+					+
34	<i>Claroideoglossum luteum</i> (#KP144302)	+	+				
35	<i>Paraglossum</i> sp. (#MG076805)	+					

Note: Symbols: + indicates appearance of mycorrhizal species in sample.

In general, the results have contributed to depict the abundance and diversity of AM communities in medicinal plants' rhizospheres, and are comparable to the reported ones in AMF of medicinal plants (Muthukumar *et al.*, 2006, Thapa *et al.*, 2015). In addition, the results proposed a difference in biodiversity of arbuscular mycorrhizal fungi in different host plants under almost similar climate and environmental conditions. The difference in the rhizosphere fungal communities between two host plants could be due to root exudates that were previously proved to depend on plant's cultivar, species and developmental stage (Badri & Vivanco, 2009). Admittedly, statistical aspect of AM fungal abundances between two host plants turmeric and gurma is contemplating to be analyzed.

## CONCLUSION

In conclusion, our study contributes to understandings on the rhizosphere microbial communities of selected medicinal plants in Vietnam, including gurma *G. sylvestre* and turmeric *C. longa*. The data show variations in the abundance and diversity between two medicinal plants' AMF. In nearly similar environmental conditions, AM community of *G. sylvestre* appeared to be less abundant and also less diverse than that of *C. longa*. Further research should focus on the variation of AM fungal communities of selected medicinal plants under biotic and abiotic factors, as well as effects of isolated AM fungal species on the bioactive secondary metabolites production of the plants.

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## NGHIÊN CỨU ĐA DẠNG KHU HỆ NẤM RỄ CỦA CÂY DÂY THÌA CANH (*GYMNEMA SYLVESTRE*) VÀ CÂY NGHỆ VÀNG (*CURCUMA LONGA*) TẠI VIỆT NAM

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### TÓM TẮT

Nấm rễ (Arbuscular mycorrhiza, AM) là các cơ thể nhân chuẩn thuộc ngành Glomeromycota và có mối quan hệ cộng sinh với rễ của phần lớn các thực vật bậc cao. Nấm rễ được cho là đồng tiến hóa với các loài thực vật trên cạn, vì vậy mà mức độ đa dạng và sự phân bố của quần xã nấm rễ AM phụ thuộc vào loài thực vật chủ. Nghiên cứu được tiến hành để khảo sát khu hệ nấm rễ AM của một số cây được liệu tại miền Bắc Việt Nam bao gồm cây dây thìa canh *Gymnema sylvestre* và cây nghệ vàng *Curcuma longa*. Từ các mẫu đất vùng rễ của hai loài nghiên cứu, bằng phương pháp phân tích đoạn trình tự gen mã hóa RNA của tiểu phần bé ribosome trong DNA tổng số của các mẫu đất, 35 loài nấm AM đã được phát hiện. Kết quả cho thấy, chi *Glomus*, tiếp theo là các chi *Gigaspora* và *Acaulospora*, là các nhóm phổ biến nhất trong khu hệ nấm rễ của cả hai cây chủ *G. sylvestre* và *C. longa*. Ngoài ra, nấm rễ thuộc các chi như *Scutellospora*, *Diversispora* và *Rhizophagus* cũng được phát hiện ở gần như tất cả các mẫu. Kết quả đếm bào tử bằng phương pháp lọc ướm cho thấy sự khác biệt trong mật độ bào tử AM của nhóm các mẫu đất thu từ vùng rễ cây dây thìa canh và nhóm các mẫu đất thu từ vùng rễ cây nghệ. Nhìn chung khu hệ nấm AM của cây nghệ vàng có mật độ cao hơn và đa dạng hơn (27 loài thuộc 10 chi) so với khu hệ này ở cây dây thìa canh (17 loài thuộc 7 chi). Sự khác biệt trong khu hệ nấm rễ của cây *G. sylvestre* và *C. longa* góp phần làm rõ cho sự phụ thuộc của các loài nấm AM với cây chủ, và gợi ý về mối liên hệ của chúng với các hợp chất trao đổi thứ cấp tạo ra bởi cây chủ.

**Từ khóa:** Nấm rễ AM, đa dạng nấm rễ, *Gymnema sylvestre*, *Curcuma longa*