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Diversity and Species Composition of Arbuscular Mycorrhizal Fungi in *Citrus* Species

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Abstract Two Citrus species viz., *Citrus sinensis* and *Citrus limon* were studied for its associated arbuscular mycorrhizal fungi (AMF). A rhizosphere soil was found to be acidic and soil phosphorus was low for both the plant species. AMF colonization in the form of arbuscules, vesicles and hyphae were observed. The percent of AMF colonization in *C. sinensis* was 39.11%, whereas in *C. limon* it was 48.12%. However, AMF colonization in trap culture from *C. sinensis* was found to be higher (17.97%) as compared to *C. limon* (13.64%). A total of 19 AMF species belonging to two genera viz., *Acaulospora* and *Glomus* (14 AMF species from *C. sinensis* rhizosphere soil and 15 AMF species from *C. limon* rhizosphere soil) were isolated and identified on the basis of their morphological characteristics. From trap culture, 11 AMF species were isolated; 6 species from *C. sinensis* derived inoculum and 8 from C. limon derived inoculum. Two additional AMF species i.e., *G. mosseae* and *G. manihotis* were recovered from *C. limon* derived inoculum, and another two species i.e., *A. mellea* and *A. dilatata* were recovered from *C. limon* derived inoculum which otherwise were not recovered in the original field soils. This study gives the gist of AMF status of two Citrus species and it revealed that the AMF composition and diversity varies in the two Citrus species.

Keywords: Arbuscular mycorrhizal fungi, Citrus, colonization, diversity, *Acaulospora*, *Glomus*

Introduction

Arbuscular mycorrhizal fungi (AMF) are associated with the roots of approximately 80% of all terrestrial plants species (Smith and Read, 1997). Benefits derived by plants from AMF include a higher nutrient uptake, especially of phosphorus, an increased drought-stress tolerance, and an improved tolerance to some pathogens (Koide and Mosse, 2004). These beneficial effects of mycorrhiza are mainly attributed to the fungal hyphae spreading through the soil beyond the rhizosphere, which enables more efficient soil exploitation for nutrients (Li *et al*., 2006). Colonization of AMF increased in low soil nutrient conditions, in which plant nutrient demand increased (Smith and Read, 1997).

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Some plant roots are also colonized by a diverse group of pigmented, septate and thick-walled fungi called dark septate endophyte (DSE). It refers to a miscellaneous group of ascomycetous and anamorphic fungi that form a characterized inter-and intracellular structures including superficial net of hyphae, penetrating into cortical layer, microsclerotia and occasionally a partial mantle (Jumpponen and Trappe, 1998). DSE are abundant in many plant genera and many habitats worldwide (Jumpponen and Trappe, 1998), and have been reported to confer a positive effect on plant growth (Read and Haselwandter, 1981).

Citrus species that belong to Rutaceae family is one of the most important commercial horticultural plants grown in Meghalaya, Northeast India. A vast reservoir of *Citrus* diversity is found in this region in both wild and cultivated forms (Singh *et al*., 2006). Natural undisturbed populations of *Citrus* genepool observed in Northeast India 60-70 years back provided strong evidence that most of the *Citrus* species originated in this region and claimed to be the epicentre of *Citrus* biodiversity (Malik *et al*., 2006). Northeast India, being the home of several *Citrus* species, rich genetic diversity occurs in the region. A recent study on genetic resources of *Citrus* in northeast India indicated the presence of 23 species, 1 subspecies and 68 varieties, thus according this area has a special status as a treasure house of *Citrus* germplasm (Sharma *et al*., 2004).

Most commonly found *Citrus* species in this region are *Citrus sinensis* and *Citrus limon*. Although cultivation of these plants species are done extensively in many parts of Meghalaya, no work has been done on the association of AMF in *Citrus* species from this region. Therefore, the aim of this investigation was to study the AMF diversity associated with two *Citrus* species viz. *C. sinensis* and *C. limon* from East Khasi Hills, Meghalaya.

Materials and methods

Study Site and Sampling

Plant samples were collected during the month of October, 2011 from the nursery plantation of Divisional forest office, Polo, Shillong, Meghalaya. The roots and rhizosphere soils of C. sinensis and C. limon (ten replicates of each plant species) were collected in sterilized plastic bags and transported to the laboratory for analysis.

Estimation of AMF colonization

The root samples were washed thoroughly in tap water, cut it to approximately 1 cm and cleared in 10 % KOH for 1 h at 90 ℃, acidified with 1 % HCL and stained with trypan blue (Philips and Hayman, 1970).

The stained root samples were mounted on microscope slides in lactoglycerol and examined for AMF colonization under light microscope. Root lengths with mycorrhizal colonization in the form of arbuscules, vesicles and hyphae in 100 root segments from each plant species were estimated using the magnified intersection method of McGonigle *et al.*, (1990).

AMF spore isolation, enumeration and identification

AMF spores were extracted by wet sieving and decanting method of Uma *et al.* (2010). Suspension of 25 g rhizosphere soil sample in water was decanted through a series of 710 to 37 µm sieves. The residues on the sieves were washed into beaker with water and filtered through filter papers. Each filter paper was spread on petri dish and spores were counted using a dissection microscope at 40 × magnification. Sporocarps and spore clusters were considered as one unit. AMF spores were picked up using a needle, mounted in polyvinyl alcohol-lactoglycerol with Meltzer's reagent. AMF spores were identified based on morphological characteristics such as shape, size, colour, wall ornamentation, etc. using identification keys of International culture collection of vesicular and arbuscular mycorrhizal fungi, i.e. INVAM (http://www.invam.caf.wvu.edu) and AMF phylogeny (www.amf-phylogeny.com). Spore density and species richness were expressed as number of AMF spores and numbers of AMF species in 25 g soil sample.

Trap culture

The methods of AMF trap culture were followed from INVAM (http://invam.caf.wvu.edu). Trap cultures were established using *Paspalum notatum* Flügge as a host plant. Rhizosphere soils and roots of *C. sinensis* and *C. limon* were collected in a separate plastic bag. Roots were chopped into small fragments and mixed thoroughly with the associated soil that serves as inoculum. This chopped blend is mixed 1:1 (v/v) with autoclaved coarse sand. Seeds of *P. notatum* were evenly sown on 25 cm diameter plastic pots containing the AMF inoculum and monitored in green house for five months. It was watered whenever required. After five months, the roots of the trap plants were evaluated for AMF colonization and spores were isolated and analyzed as described above.

Statistical analysis

Relative abundance, isolation frequency, Shannon-Wiener index of diversity (H') and Simpson index of dominance were calculated (Dandan and Zhiwei, 2007).

Soil 866honol-chemical analysis

Soil pH was determined using a digital pH meter. Soil moisture was determined by drying 10 g fresh soil at 105℃ for 24 h in a hot-air oven. Organic carbon was analyzed by colorimetric method (Anderson and Ingram, 1993) and available phosphorus by molybdenum blue method (Allen *et al*., 1974). The soil 866honol-chemical properties are presented in Table 1.

Table 1. Physico-chemical properties of *Citrus* rhizosphere soils

Plant species	pH	Moisture Content	Organic Carbon	Phosphorus
C. sinensis	5.69 ± 0.03	30.27 ± 0.08	1.12 ± 0.01	0.24 ± 0.01
C. limon	5.44 ± 0.01	25.13 ± 0.05	1.0 ± 0.03	0.12 ± 0.01

Results

AMF colonization

AMF colonization in the form of hyphae, arbuscules and vesicles were detected in both *Citrus* species (Table 2). Total AMF colonization was higher in *C. limon* (48.12%) as compared to *C. sinensis* (39.11%) as shown in Fig.1. In the trap cultures, AMF as well as DSE colonization were detected (Table 3). AMF colonization in trap culture for *C. sinensis* was found to be higher (17.97%) as compared to *C. limon* (13.64%) as shown in Fig. 2. The AMF structures found in the two plant species such as hyphae, arbuscules and vesicles are given in Fig. 3.

Table 2. AMF structural colonization (%) in two *Citrus* species

	Mycorrhizal structure		
Plant species	**Arbuscules**	**Vesicles**	**Hyphae**
Citrus sinensis	10.00 ±0.02	10.57 ±0.01	18.54 ±0.03
Citrus limon	36.65 ±0.03	4.68 ±0.01	6.79 ±0.01

Figure 1. Mycorrhizal colonization in two *Citrus* species.

Table 3. AMF structural colonization (%) in trap plants (*Paspalum notatum*) with inoculum source from two *Citrus* species

	Mycorrhizal structure			
Plant species	**Arbuscules**	**Vesicles**	**Hyphae**	**DSE**
P. notatum (C.s)	6.82 ±0.01	2.26 ±0.01	8.89 ±0.01	1.02 ±0.01
P. notatum (C.l)	3.28 ±0.01	3.58 ±0.01	6.78 ±0.01	2.58 ±0.00

Figure 2. Mycorrhizal colonization in trap plants (*Paspalum notatum*) with inoculum source from two *Citrus* species.

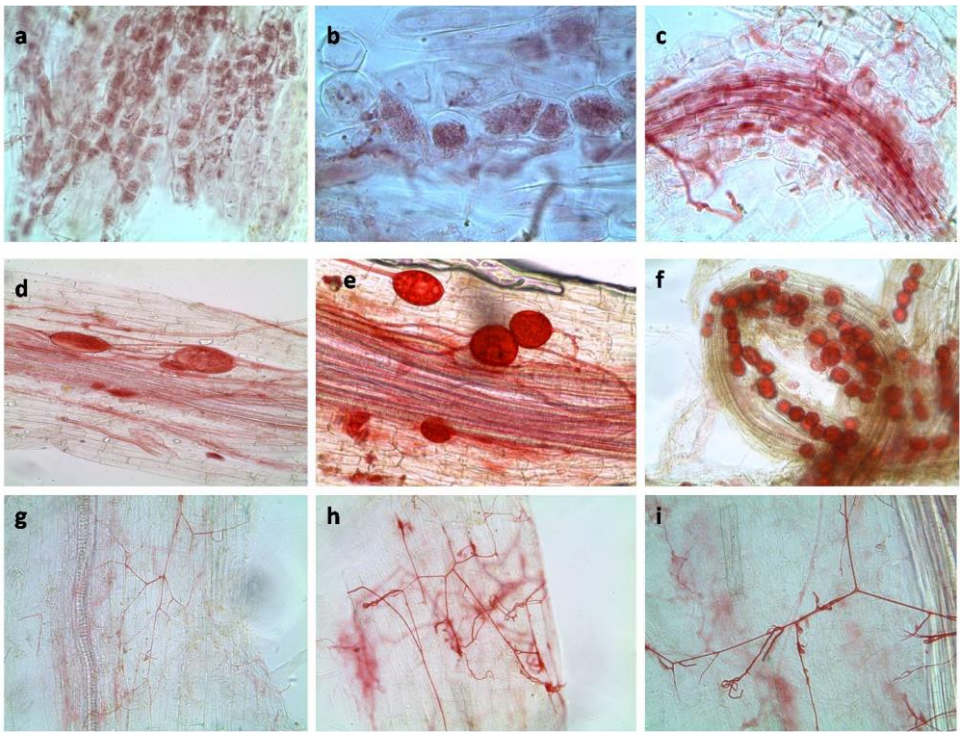

Figure 3. AMF colonization in the form of (a-c) arbuscules, (d-f) vesicles, (g) hyphae and (h-j) DSE in *Citrus* species.

AMF species composition and diversity

A total of 19 AMF species belonging to two genera viz., *Acaulospora* and *Glomus* (14 AMF species from *C. sinensis* rhizosphere soil and 15 AMF species from *C. limon* rhizosphere soil) were isolated and identified on the basis of their morphological characteristics (Table 4). Nine AMF species were found to be common in both the *Citrus* species. It was observed that most dominant AMF species was *Acaulospora koskei* in both *Citrus* species. 11 AMF species were isolated from trap cultures belonging to *Acaulospora* and *Glomus* species where 3 AMF species were found to be common in both the *Citrus* species (Table 5). *Glomus rubiforme* was the dominant AMF species isolated from trap culture with inoculum source from *C. sinensis*, whereas, it was *Acaulospora koskei* in case of *C. limon.* The AMF spore density in 25 g dry soil sample each was 987 in *C. sinensis* and 438 in *C. limon*, whereas in trap culture it was 45 in *C. sinensis* derived trap culture and 53 in *C. limon* derived trap culture. Micrographs of some of the isolated AMF species from two *Citrus* species are given in Fig. 4.

Table 4. Isolated AMF species with their relative abundance and isolation frequency from two *Citrus* species

Sl.No.	AMF species	Relative abundance (%)		IF (%)
		C. sinensis	*C. lemon*	
1.	*Acaulospora capsiculata* Blaszk.	10.81	9.52	100
2.	*Acaulospora 868honolog* Walker, Pfeiffer & Bloss	—	2.38	50
3.	*Acaulospora 868honologica* Sieverding & Toro	2.70	2.38	100
4.	*Acaulospora foveata* Trappe & Janos	2.70	—	50
5.	*Acaulospora koskei* Blaszk.	43.24	30.95	100
6.	*Acaulospora 868honolog* Morton	2.70	2.38	100
7.	*Acaulospora laevis* Gerd. & Trappe	—	2.38	50
8.	*Acaulospora mellea* Spain & Schenck	2.70	—	50
9.	*Acaulospora rehmii* Sieverd. & Toro	2.70	—	50
10.	*Acaulospora rugosa* Morton	2.70	—	50
11.	*Acaulospora tuberculata* Janos & Trappe	—	2.38	50
12.	*Glomus caledonium* Nicolson & Gerdemann	—	2.38	50
13.	*Glomus etunicatum* Becker & Gerdemann	5.41	7.14	100
14.	*Glomus intraradices* Schenck & Smith	2.70	2.38	100
15.	*Glomus luteum* Kenn., Stutz & Morton	2.70	19.05	100
16.	*Glomus rubiforme* Gerdemann & Trappe	10.81	4.76	100
17.	*Glomus tortuosum* Schenck & Smith	2.70	4.76	100
18.	*Glomus verruculosum* Blaszkowski & Tadych	5.41	2.38	100
19.	*Glomus versiforme* (Karsten) Berch	—	4.76	50

Table 5. AMF species isolated from trap plants (*Paspalum notatum*) with inoculum source from two *Citrus* species

Sl.No.	AMF species	Relative abundance (%)		IF (%)
		P.n (C.c)	*P.n (C.l)*	
1.	*Acaulospora capsiculata* Blaszk.	6.67	11.11	100
2.	*Acaulospora 869honolog* Walker, Pfeiffer & Bloss	—	5.56	50
3.	*Acaulospora 869honologica* Sieverding & Toro	—	5.56	50
4.	*Acaulospora dilatata* Morton	—	11.11	50
5.	*Acaulospora koskei* Blaszk.	20	33.33	100
6.	*Acaulospora 869honolog* Morton	—	11.11	50
7.	*Acaulospora mellea* Spain & Schenck	—	11.11	50
8.	*Acaulospora rugosa* Morton	13.33	—	50
9.	*Glomus manihotis* Howeler, Sieverding & Schenck	6.67	—	50
10.	*Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe	6.67	—	50
11.	*Glomus rubiforme* Gerdemann & Trappe	46.67	11.11	100

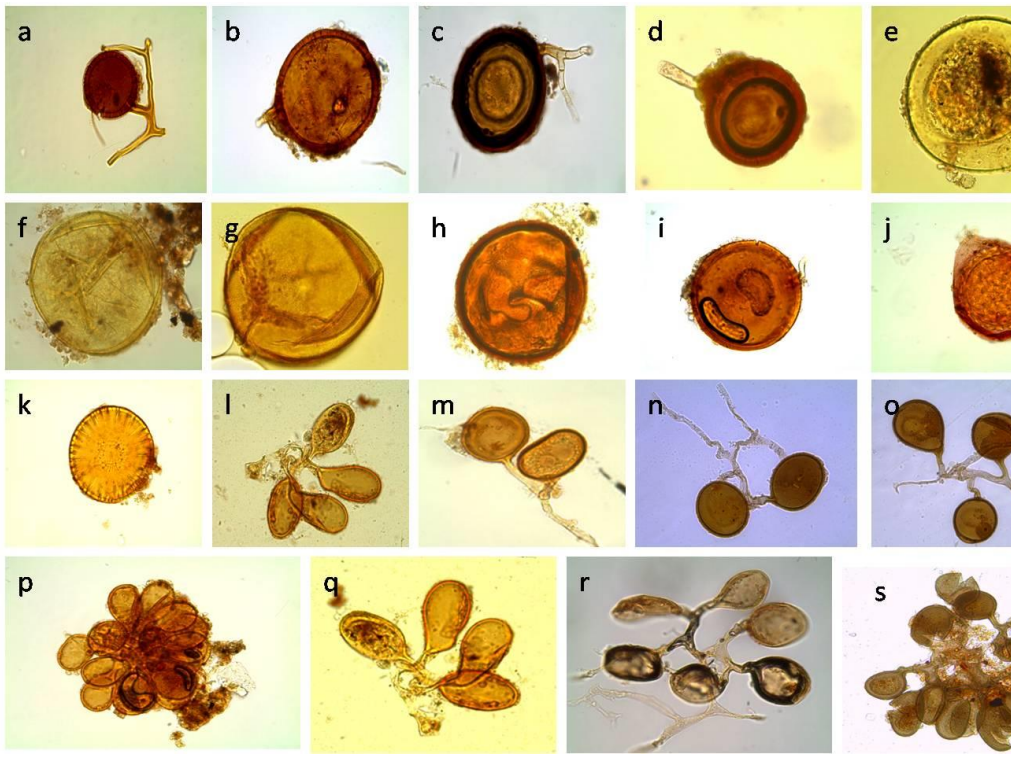

Figure 4. Some of the AMF spores isolated from *Citrus* rhizosphere soils; (a-d) *Glomus* spp., (e-k) *Acaulospora* spp., (l-s) Sporocarps.

Shannon-Weiner index of AMF diversity was found to be 2.04 in *Citrus sinensis* and 2.24 in *Citrus limon*, and Simpson's index of AMF was 0.22 in *Citrus sinensis* and 0.16 in *C. limon.* In the trap culture Shannon's index of AMF in *C. sinensis* is 1.49 and for *Citrus limon* is 1.91. Simpson's index was 0.29 for *C. sinensis* and 0.18 for *C. limon*.

Discussion

In this study, AMF colonization in *Citrus sinensis* and *Citrus limon* are low to moderate. Espeleta *et al*. (1999) reported AMF colonization in about 20 % of the fine root segments in *Citrus volkameriana*. *Citrus* rootstocks vary widely in their mycorrhizal dependency (Graham and Syvertsen, 1985). The extent of mycorrhizal infection in root systems is known to be influenced by environmental conditions and the physiological conditions of the plants; the most important being the age of the plants, the level of phosphate in the soil relative to the requirements of the plant and the capacity of the population of mycorrhiza propagules in the soil to form mycorrhiza (Yago *et al.,* 2009). The seedlings (four to five months) could have been still young to record a higher colonization percentage since the root system infected normally is influenced by 870honological stages of plants.

AMF species belonging to *Acaulospora* and *Glomus* species were isolated from *C. sinensis* and *C. limon*. Camprubí and Calvet (1996) suggested that *Glomus* species were the most common AMF found in *Citrus* soils. Klironomos and Hart (2002) found differences among AMF genera in their life history characteristics and suggested that the mycelium is of major importance as propagule for some *Glomus* species. Spores belonging to other genera were not observed in *Citrus* soil. The absence of other genus is not surprising as they are not always detected in surveys of AMF. Occurrence of only 2 genera may be related to their high competitive interaction and adaptability thus, allowing them to establish better than the others, supporting the view of Singh *et al.* (2008). Individual AMF species compete for resources through a combination of strategies resulting in the maintenance of a diverse AMF community.

In trap culture set up with *Paspalum notatum* as a host plants, rate of AMF colonization was higher in *C. sinensis* derived inoculum than those of *C. limon* derived inoculum. However, AMF spore density was higher in trap culture with *C. limon* derived inoculum as compared to *C. sinensis* derived inoculum. This indicates that spore density does not exactly reflect the AMF community that is actually colonizing the plant roots, and thus, variation in spore production could not be explained by mycorrhizal colonization level (Brundrett *et al*., 1999). The abundance and distribution of AMF in the plant root is often poorly related to their sporulation capacity in the soil (Boddington and Dodd, 2000). Evaluation of AMF spores as well as

colonization is therefore important to know the level of its association, as observation of spore populations alone may not provide adequate information about AMF community structure, because of the differences in growth and sporulation among AMF species (Land and Schönbeck, 1991).

A total of 11 AMF species were isolated from trap cultures; 6 species from *C. sinensis* derived inoculum and 8 from *C. limon* derived inoculum. Two additional AMF species i.e., *G. mosseae* and *G. manihotis* were recovered from *C. limon* derived inoculum, and another two species i.e., *A. mellea* and *A. dilatata* were recovered from *C. limon* derived inoculum which otherwise were not recovered in the original field soils. The discrepancy in the AMF species composition in field soils and trap cultures has been attributed to different growth conditions, including the time period of cultivation (Oehl *et al*., 2003). Depending on the different environmental conditions, some of the AMF rarely sporulating in the field soil might start forming spores in the trap culture. On the other hand, some AMF species frequently forming spores in the field soil may not be detected in the traps either because the conditions in the pots are less favourable for their sporulation or because those species are outcompeted by others (Brundrett *et al*., 1999). Therefore, it is necessary to study from both field soils and trap culture for complete AMF analysis.

Citrus is one of the most important fruit crop in India (Anonymous, 2002). There are many studies trying to improve the yield of *Citrus* plantations including tests of increased resistance against pathogens using fungal inoculants (Graham and Lindermann, 1986). Mass production of indigenous AMF and selection of appropriate mycorrhizal strain can be utilized in *Citrus* plantation for better growth and yield.

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