(RESEARCH ARTICLE)

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Diversity of arbuscular mycorrhizal fungi (Glomeromycota) in adjacent areas of different land use in Nepal

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Abstract

Disturbances can affect the incidence of Arbuscular Mycorrhizal Fungi (AMF) in both agricultural and natural ecosystems. The present study is a first attempt for the qualitative assessment of AMF diversity in adjacent areas of a forest ecosystem with different land uses and assess levels of mycorrhizal colonization by these fungi. A total of five soil samples were taken randomly from each of the following areas situated within the same landscape: undisturbed coniferous forest (UF), degraded forest (DF) and cultivated land (CL). A total of 22 taxa of arbuscular mycorrhizal fungi belonging to eight genera were identified morphologically, *Glomus* and *Acaulospora* being the most common. Species richness ranged from 11-14 among land use areas, with 14 species in UF and 11 species in CL. *Acaulospora mellea*, *Gigaspora* sp. and two non-identified *Glomus* species were detected in all areas. While species richness did not differ significantly amongst areas, diversity at the family level was 43% less in CL than in UF. Mean mycorrhizal colonization was higher in DF (28%) than CL (20%). We concluded that land use mainly affected fungal diversity only at the family level and had no impact on mycorrhizal development in sampled roots. This work provides the first step to identify native AMF species in Nepal that might be cultured for further use by small farmers in a sustainable agriculture approach.

Keywords: Disturbance; Edaphic factors; Glomeromycota; Coniferous forest; *Avena sativa*; Soil properties.

1. Introduction

Arbuscular Mycorrhizal Fungi (AMF – phylum Glomeromycota) are known to have a key role in terrestrial ecosystems as they contribute to improve plant nutrition, particularly uptake of low mobility nutrients from soil solutions such as P, Zn, and ultimately Cu, to increase plant resistance to drought, salinity and tolerance to pathogens and to improve soil structure [1, 2]. However, disturbances can affect several aspects of AMF communities in both agricultural and natural ecosystems through the process of limiting organic inputs into the soil and biomass removal [3, 4]. AMF establish the arbuscular mycorrhizal symbiotic association with 72% of vascular plants [5] and therefore are ubiquitous soil organisms accounting for 5-36 % of the total biomass in the soil and 9-55 % of the biomass of soil microorganisms [6]. Establishing an interface between soil and plant roots, AMF are sensitive to changes in soil and plant environment.

The AMF community varies greatly in size and species composition in different habitats due to differences in edaphic, climatic and host factors that determine sporulation and root colonization in a particular place [7]. Composition of AMF community [8], fungal composition in plant roots [9], fungal phospholipids in soil [10], and amount of extra radical

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mycelium [11] are the AMF variables which are affected by agricultural practices. Although different host plants show differential affinities for distinct AMF species due to which wider range of host plants might promote greater AMF diversity compared with monoculture [12], these effects are context dependent and vary across soil types, ecosystems, and precipitation regimens, etc. [13]. Plant species distribution, land management practices and long-term application of phosphorous fertilizer impact distribution of AM fungal species [14, 15, 16, 17]. Land use change and/or disturbance of soil negatively affect the functionality of AM fungi reducing mycorrhizal colonization [18].

Exploiting of natural forests turning them in cultivable lands or degraded forests causes changes in which plant species, soil organic matter, soil nutrients, soil structure, and soil fungi may be affected [1]. The cultivable land is cleared of multi species of plants and normally planted with a single species of one age-class. This constitutes a drastic site disturbance which alters mycorrhizal abundance and species composition in the site. Agricultural management factors in cultivable land such as the intensity of cultivation, the quality and quantity of fertilizers applied and the plant protection strategies used in modern agriculture have severely affected AMF community structure and plant interactions [19]. After disturbance of some Australian sites, decrease in spore number as well as shift in species composition was occurred [20]. Similarly, AMF species composition and abundance are affected by vegetation cover [21, 22, 23]. They also noticed a change in species composition. The removal of host plants, the soil disturbance, and compaction associated with land clearing are detrimental to AM propagules [24].

In Nepal, global climate change and human activities such as overgrazing, harvesting of fodder, and unsustainable agricultural practices are causing dynamic change in the land use pattern. The land use practices affect chemical, physical and biological properties of soil [25], however the biological properties of soil are rarely considered for agriculture. Soil in Nepal is deficient in N, P, and K due to shortage of organic matter in the soil [26]. However, farmers who have been using chemical fertilizer in Kathmandu valley and some of the Terai districts have started to experience its adverse effects on soil quality [26]. Therefore, for the mitigation of these effects, farmers started using compost manures, green manures, integration of legumes in the cropping system, use of farm yard manure (FYM), homemade botanical pesticides, among other practices in their agricultural crops. Up to now, no investigation has been carried out in Nepal to elucidate how AMF communities and mycorrhizal colonization varies according to changes among land use systems within the same landscape and how these changes relate with edaphic factors. Moreover, no surveys of AMF species have been done in Nepal, despite its territory spans biomes like tropical grasslands, tropical moist forests, tropical and temperate conifer forest and montane grasslands. These studies are important to understand whether AMF communities are being affected by changes from native to agricultural systems and to provide subsidies to include AMF communities as important components to assess the effects of land use changes and as another biological tool for farmers to improve production. Our aim in this study was to determine the variation of AMF communities and levels of mycorrhizal colonization along distinct land use areas occupying the same landscape.

2. Material and methods

2.1. Study area

The study sites were located in Bhaktapur district, the smallest of seventy-seven districts of Nepal, with an area of 119 km^2 located in the eastern part of Kathmandu valley. The average annual temperature in Bhaktapur is 17.9°C and rainfall is about 1,583 mm. June is warmest with temperatures averaging 23.2°C and January is the coldest. Main economic activities in the district are animal husbandry and agriculture which is accelerating land use, a special concern as Bhaktapur is one of the top ten districts of Nepal with only 25 km^2 of forest.

Three areas impacted to different degrees by human activities were selected to assess AMF community composition: undisturbed forest (UF), degraded forest (DF), and cultivated land (CL). All of these sites were located adjacent to each other in same landscape (Figure 1). UF is a dense coniferous forest situated at the top of the landscape where grazing and cutting fodders for cattle were prohibited while the DF site is a disturbed forest located adjacent to the UF and impacted by local people's activities (*e.g.* extraction of timber, animal husbandry, agriculture).
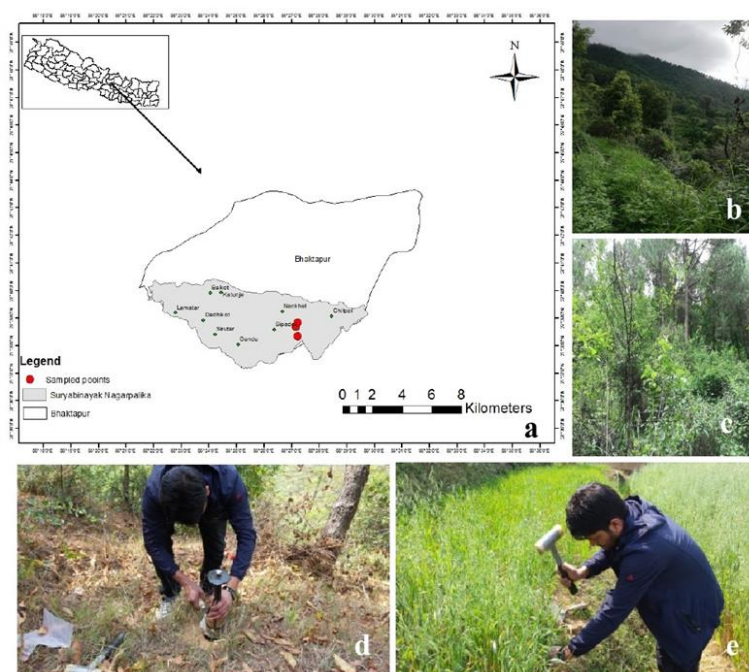
Figure 1 Sampling sites
a: Map of sampling site; b: Landscape view; c: Undisturbed forest; d: Degraded forest; e: Cultivated land

Pinus roxburghii Sarg. *Alnus nepalensis* D. Don, *Toona ciliata* M. Roem., *Schima wallichii* (DC.) Korth., *Rhododendron arboreum* Sm., *Nephrolepis exaltata* (Linn.) Schott, *Eupotorium adenophorum* Spreng. and *Arundinella nepalensis* Trin. were dominant on UF while *Pinus roxburghii* Sarg., *Alnus nepalensis* D. Don, *Ilex opaca* Soland. ex Ait., *Arundinella nepalensis* Trin., *Digitaria longiflora* (Retz.) Pers, and *Eupotorium adenophorum* Spreng. were on DF. CL is cultivable land located at base of the landscape which is planted with different crops according to season. Oats (*Avena sativa* L) were being cropped at the sampling time. Geographical information and soil properties for each site are summarized in Table 1.

Table 1 Description of the study sites.

Properties	Land use types		
	UF	DF	CL
Longitude	E 85°27'13.35"	E 85°27'9.78"	E 85°27'13.74"
Latitude	N 27°38'20.72"	N 27°38'41.76"	N 27°38'50.34"
Altitude	1708 m	1487 m	1409 m
Soil type	Clay loam	Clay loam	Clay loam
Soil moisture	22.13±2.49	14.56±3.78	12.76±1.54
Soil pH (H_2O)	5.07 ± 0.088	4.83 ± 0.033	4.9 ± 0.15
EC (μS)	277.33 ± 24.86	172.90 ± 6.76	166.67 ± 4.77
Carbon %	2.08 ± 0.24	1.99 ± 0.15	1.88 ± 0.14
Nitrogen %	0.10 ± 0.01	0.10 ± 0.0058	0.09 ± 0.0067
Phosphorous (mg/ kg)	14.69 ±0.53	0.46 ± 0.0	82.89 ± 36.9
Potassium (mg/kg)	121.15 ± 12.2	93.26 ± 20.10	183.9 ± 38.2

Data are reported as (mean ± SEM) for replicate samples; UF: undisturbed forest; DF: degraded forest; CL: cultivated land with Oat (*Avena sativa*); pH: percentage of hydrogen ion; EC: electrical conductivity

2.2. Sampling procedure

In each land area, five soil samples (*ca.* 1 kg) were randomly collected from January to September 2019 up to a depth of 20 cm using a soil corer (Moreira et al. 2015). Only few samples were taken in this study in spite of diverse landscape of UF and DF since it was a preliminary work to assess diversity of AMF. Soil samples were placed in zip locked polythene bags, labeled, transported to the laboratory and stored at 4℃ until they were processed.

Large soil aggregates were crushed gently and root pieces were picked separately using a forceps. Around half of each preserved sample was spread separately on paper trough to allow air dry for physical and chemical analysis of soil and other half was used for identification of AMF. Then, the samples were sieved separately through 2000 μm sieve to discard larger soil aggregates and organic debris.

2.3. Physical and chemical analysis of soil

An aliquot of 250 g of air-dried soil sample was used to determine carbon (Walkley and Black method), total nitrogen (Kjeldahl's method), available phosphorous (Olsen's method), and potassium (neutral normal ammonium acetate method). A soil suspension of 1:2.5 (soil-water mixture) was made through intermittent stirring for measurement of pH and electric conductivity of the soil samples [27]. Soil moisture was measured by weight after 20 g samples were incubated at 105℃ for 24 hours in hot air oven (Table 1). Soil moisture percentage was calculated using the following equation:

$$\text{Soil moisture (M)} = \frac{\text{mass of moist soil}}{\text{mass of dry soil}} \times 100\%$$

2.4. Assessment of the root colonization

Roots from each soil sample were cut into approximately 1 cm pieces and a 0.5 g subsample was placed in labeled perforated plastic cassettes and washed. Cassettes were incubated in 10% KOH solution and autoclaved at 15 psi and 121°C for 15 min. After rinsing with tap water, pigmented root pieces were immersed in alkaline H_2O_2 at room temperature for 10 to 20 min or until roots were bleached. Alkaline H_2O_2 was prepared by adding 3 ml of 0.5% NH_4OH to 30 ml of 10% H_2O_2 and 567 ml of tap water. Cassettes then were thoroughly rinsed and covered with 2% HCl for 3-4 min. Acidified roots were stained with 0.05% trypan blue in lactoglycerol solution by incubation in a water bath at 95°C for 20 minutes. Excess stain was removed using 50% glycerol.

The stained pieces of roots (around 50 to 100) from each sample were scattered on a petri plate and observed under stereomicroscope (OLYMPUS SZ2-ILST) at 10×10 magnification. The root pieces were counted as colonized and non-colonized and results expresses in percentage of mycorrhizal colonization.

2.5. Spore extraction, purification and identification

Spores were extracted from 100 g of air-dried soil sample by wet sieving and decanting [28] using two nested sieves of 100 μm and 45 μm openings. Material retained in these sieves were transferred to a 60% sucrose solution and centrifuged. The layer containing spores was decanted into a petri dish. Spores were separated based on shape, size and color under a dissecting microscope (OLYMPUS SZ2-ILST). Spores of similar phenotypes were grouped and mounted in slides with polyvinyl lacto-glycerol (PVLG) and PVLG with Melzer's reagent (1:1). Mounted spores were observed under compound trinocular microscope (OPTIKA B-383PLi) and photographs were taken in different magnifications with an OPTIKA 4083.13E HDMI Easy camera. Spores were identified by comparison with descriptions presented on webpages of the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) (www.invam.wvu.edu) and by comparison with published descriptions. The classification of Redecker et al. [29] was followed for this analysis.

3. Results

A total of 22 spore morphotypes belonging to eight genera were extracted from the soil samples of different land use systems, of which nine were identifiable only to genus (Table 2, Figure 2). Species richness ranged from 11-14 in different land use systems, with 14 species registered in UF and 11 species in CL (Table 2). *Acaulospora mellea*, *Gigaspora* sp1, *Glomus* sp1, and *Glomus* sp2 were recovered from all land use systems. *Ambispora* cf. *leptoticha, Pacispora* sp1, and *Rhizophagus intraradices* were found exclusively in UF, *Ambispora* cf. *gerdemannii, Ambispora* cf. *reticulata, Acaulospora* sp1, *Acaulospora* sp2, and *Paraglomus* cf. *occultum* were detected exclusively in DF while *Acaulospora morrowiae* was identified only from the CL (Table 2).
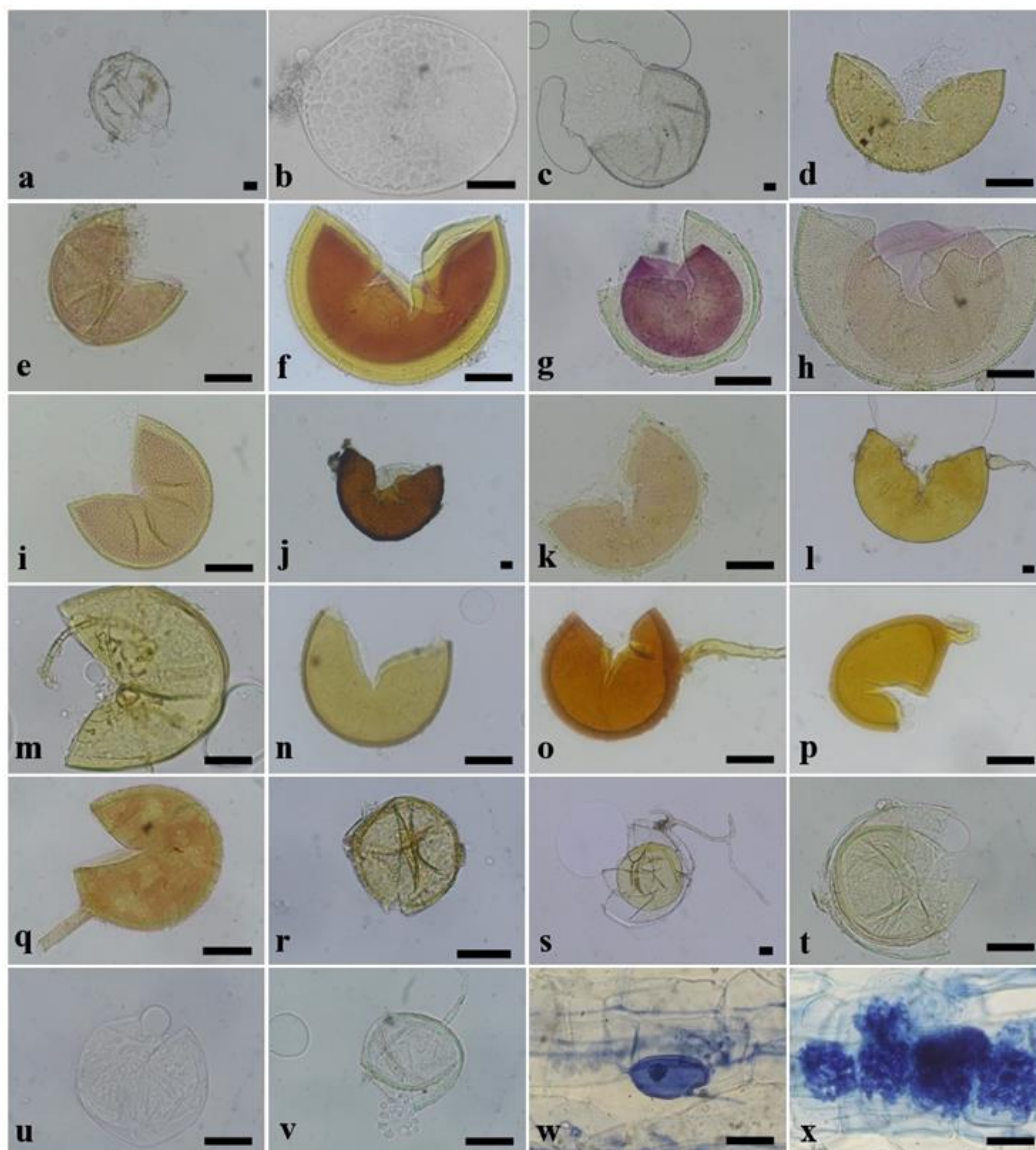
Figure 2 Spores of arbuscular mycorrhizal fungal species identified in three land use systems in Bhaktapur district, Nepal, and mycorrhizal structures

a. *Ambispora* cf. *gerdemann*ii; b. *A.* cf. *reticulata*; c. *A.* cf. *leptoticha*; d. *Acaulospora* cf. *alpina*; e. *Ac. delicata*; f. *Ac. mellea*; g. *Ac. morrowiae*; h. *Ac. scrobiculata*; i. *Acaulospora* sp1; j. *Acaulospora* sp2; k. *Pacispora* sp1; l. *Gigaspora* sp1; m. *Gigaspora* sp2; n. *Claroideoglomus etunicatum*; o. *Glomus* cf. *ambisporum*; p. *Glomus* sp1; q. *Glomus* sp2; r. *Glomus* sp3; s. *Rhizophagus clarus*; t. *R. intraradices*; u. *Paraglomus* cf. *occultum*; v. *Paraglomus* sp1; w. Root cells with vesicle; x. Root cells with arbuscules. Bar 30 μm

The family with the highest number of species recorded was *Acaulosporaceae* (7) followed by *Glomeraceae* (6), *Ambisporaceae* (3), *Gigasporaceae* (2), and *Paraglomeraceae* (2). *Claroideoglomeraceae* and *Pacisporaceae* were represented by only one species each (Table 2). Considering the number of species per family in each land use system, *Glomeraceae* predominates in UF with 27.27% while *Acaulosporaceae* predominated in DF and CL with 22.73%.

3.1. AM fungal root colonization

Root pieces from soil samples of all land use showed evidence of arbuscular mycorrhizal colonization by the presence of typical structures like arbuscules, hyphae, and vesicles (Figure 2). Colonization levels were similar in DF and UF (28%), and slightly lower in CL (20%). Mycorrhizal colonization did not differ significantly ($p > 0.05$) amongst the land use areas.

Table 2 Families and species of arbuscular mycorrhizal fungi recovered from undisturbed forest (UF), degraded forest (DF), cultivated land (CL) in Bhaktapur district, Nepal.

Family/ AMF species	UF	DF	CL
Ambisporaceae			
Ambispora cf. *gerdemannii* (Rose, Daniels & Trappe) Walker, Vestberg & Schüßler		×	
Ambispora cf. *reticulata* Oehl & Sieverd.		×	
Ambispora cf. *leptoticha* (Schenck & Sm.) Walker, Vestberg & Schüßler	×		
Acaulosporaceae			
Acaulospora cf. *alpina* Oehl, Sýkorová & Sieverd.	×		×
Acaulospora delicata Walker, Pfeiffer & Trappe		×	×
Acaulospora mellea Spain & N.C. Schenck	×	×	×
Acaulospora morrowiae Spain & Schenck			×
Acaulospora scrobiculata Trappe		×	×
Acaulospora sp1		×	
Acaulospora sp2		×	
Pacisporaceae			
Pacispora sp1	×		
Gigasporaceae			
Gigaspora sp1	×	×	×
Gigaspora sp2	×	×	
Claroideoglomeraceae			
Claroideoglomus etunicatum (Becker & Gerd.) Walker & Schüßler	×	×	
Glomeraceae			
Glomus cf. *ambisporum* Sm. & Schenck	×	×	
Glomus sp1	×	×	×
Glomus sp2	×	×	×
Glomus sp3	×		×
Rhizophagus clarus	×		×
R. intraradices (Schenck & Sm.) Walker & Schüßler	×		
Paraglomeraceae			
Paraglomus cf. *occultum* (Walker) Morton & Redecker		×	
Paraglomus sp1	×		×
Number of species	**14**	**13**	**11**

4. Discussion

In this study, we report species of arbuscular mycorrhizal fungi (AMF) based on spore morphology in different land uses in Nepal. *Acaulosporaceae* and *Glomeraceae* in all land uses is not surprising since it was previously shown that

Glomus and *Acaulospora* species are most abundant AMF in tropical areas [30, 31, 32]. Presence of these genera also was reported from high elevation regions with harsh climatic condition and soil with pH lower than 5 [33]. Dominance of *Acaulosporaceae* could be in all land use systems, as members of this family tend to be more prevalent in lower pH soils [< 5.0; 34]. The distribution of *Glomeraceae* in all land use systems could be partly the result of high tolerance to environmental factors and strong mycelial networks within plant communities hypothesized to be traits exhibited by members of this family [35, 36].

In spite of some non-endomycorrhizal plants on UF and DF, the higher species richness in the forests than in cultivated land may be associated with the presence of plant diversity in the undisturbed forest compared to DF and CL. According to [37] a positive relationship between AMF and plant diversity was occurred. Higher diversity of rhizospheric AMF may occur in natural habitats [38, 39] compared to that in agricultural ecosystems [40, 41, 42]. The decline of species richness in CL compared to that in UF supports such pattern.

Sporulation is not likely representative of total AMF diversity in each community. Diversity of host plants impacts on timing and abundance of sporulation, and the environment at the time of sampling is a huge factor. Potential drivers of AMF diversity and community structure may include plant community composition [43] and environmental factors such as soil organic carbon [44], soil phosphate [45], and soil texture [46]. Apart from these specific environmental factors, direct land use-related circumstances such as land-use intensity and tillage have also been suggested as primary determinants of AMF community composition [8, 42, 47]. Land use conversion and farming practices may impact the occurrence of AMF in multiple ways, such as changing above-ground vegetation, altering soil properties and increasing soil disturbance [48].

Composition of AM fungal community and mycorrhizal root colonization largely depends on content of organic matter, pH of the soil, and water content in the soil [49]. The soil properties can have a significant effect on the population of AMF spores in the soil, irrespective of geographical location, soil cultivation system, and plant density [50], as we found in this study despite the small sample size (15 from three land areas). The DF lies on a steep part of the landscape, which is favorable for erosion of the soil layer with higher phosphorous status so amount of available phosphorous in DF may be too low compared to other land uses.

This study contributed to our knowledge on the biogeography of arbuscular mycorrhizal fungi from a country that has not been previously surveyed for these fungi. Moreover, considering the role of AMF in improving plant growth and nutrition and impacting soil properties, this inventory is a first step to identify potential species that can be isolated and further used by local farmers. Using locally adapted fungi has a high potential for local farmers to reduce their dependency on imported chemical fertilizers and pesticides, turning agriculture more sustainable and environmental friendly.

5. Conclusion

A relatively high number of AM species were found in different land use system in Nepal. AMF species richness varied only slightly among the different land uses, with the greatest differences at the family level. As would be expected from the limited sampling regimen, land use or edaphic variables impacted on variation in mycorrhizal colonization.

Compliance with ethical standards

Disclosure of conflict of interest

The authors disclose that there is no conflict of interest.

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