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Study on status of arbuscular mycorrhizal fungal populations in some pastures of Turkey

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Abstract The status of arbuscular mycorrhizal fungal populations in soil and root samples collected from different pastures of Turkey was studied. Soil physicochemical parameters as well as AM fungal population parameters including spore density (SD), frequency of mycorrhization (F%) and intensity of mycorrhization (M%) were measured and compared in soil and root samples. The correlation between these parameters was also determined. The fungal spores from soils with the highest population indices were propagated, purified and morphologically identified. The significant differences were observed on physicochemical parameters as well as AM fungal population indices among different soil samples and regions. The maximum and minimum averages of fungal spore density, mycorrhizal frequency and intensity of mycorrhization were observed in Sinop and Aydın soils, respectively. Also, a positive correlation was observed between fungal spore density and intensity of mycorrhization in different sampling regions. The results showed that there is a negative correlation between AM fungal indices (spore density and mycorrhizal colonization intensity) with soil EC, pH, available P, and available K. On the other hand, a positive correlation could be observed between AM fungal indices and soil organic matter. Based on morphological methods, 4 definite and 2 indefinite AMF species were identified after fungal propagation. The definite fungal species were *Funneliformis caledonius, Rhizophagus fasciculatus, Rhizophagus intraradices* and *Glomus versiforme*. The indefinite species were *Glomus* sp. and *Paraglomus* sp.

Keywords: Arbuscular mycorrhizal fungi, Colonization, Identification, Spore density

Introduction

The human population in the world is increasing very rapidly. So, it will be necessary to increase agricultural production to the year 2050 (FAO, 2017). Due to the increasing needs, people are trying to obtain more products from the resources. In Turkey, the misuse, overgrazing, destruction, use of excessively

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composed fertilizers disrupt the ecological balance, causing hazardous effects on soil and ecosystems characteristics especially macro and microorganisms. Although synthetic fertilizers have important role in vegetation of pastures, they pollute the water and soil at the same time (Palta *et al.,* 2010). Meadows and pastures cover nearly 18.8% of the total land area with 14.6 million hectares in Turkey (TÜİK, 2017), which reported almost 61.5% decrease in natural meadows and pastures in the last 70 years due to excessive animals grazing. The incorrect exploitation of pastures as well as lack of protection and improvement strategies are one of the most important challenges in Turkey and resulted in decreasing pastures ecological and production efficiency (Ünal *et al.*, 2012; Gökkuş *et al.,* 2018). The rehabilitation of these areas should be done without polluting the soil and water as well as without disturbing the ecological balance. AM fungi form symbioses with ~80% of terrestrial plant species. These fungi play important role especially improving nutrient absorbance of host plants through root colonization (Wilson and Hartnett, 1998; Šmilauer *et al.,* 2020). AM fungi have several benefits to host plant including phosphorus absorption, resistance against some soil-borne pathogens, promote root regeneration, accelerate plant growth and reducing the chemical fertilizers (Demir and Onoğur, 1999; Kara and Tilki, 2001). The response of plants to AM fungi is related to root morphology (hair roots length and density) as well as susceptibility to mycorrhizal colonization (Wilson and Hartnett, 1998; Derelle *et al.,* 2012). The use of AM fungi is increasing worldwide. In this way, the soil and water are not polluted while the pasture areas are rehabilitated and the ecological balance is not destroyed (Palta *et al.,* 2010). The activity of Mycorrhizal fungi in the soil has been determined to depend on many factors, including soil texture, chemistry, temperature, pH, moisture, organic matter content, as well as applications of lime, fertilizer, and chemical pesticides (Trappe *et al.,* 1984; Dodd and Jeffries, 1989). So, determination of the status of these fungi populations in pasture rhizospheres is very important to efficient use of them for future purposes. In this study, the status of arbuscular mycorrhizal fungi population as well as its correlation with different soil physico-chemical parameters was determined in some pasture areas in in Turkey.

Materials and methods

Study sites and soil sampling

Soil samples containing host plant roots randomly collected from different pasture regions in Turkey including Ankara, Aydın, Aksaray and

Sinop (Figure 1). It was tried to collect samples from different locations as well as different host plants to compare the fungal population diversity in different locations. Soil samples collected as composite samples from different places in followed by mixing and selection of one sample (approximately 5kg). The coordinates of all samples were recorded by GPS system. Samples were kept in cooled conditions up to transferring to the laboratory. Totally 47 soil samples were collected from different sampling regions.

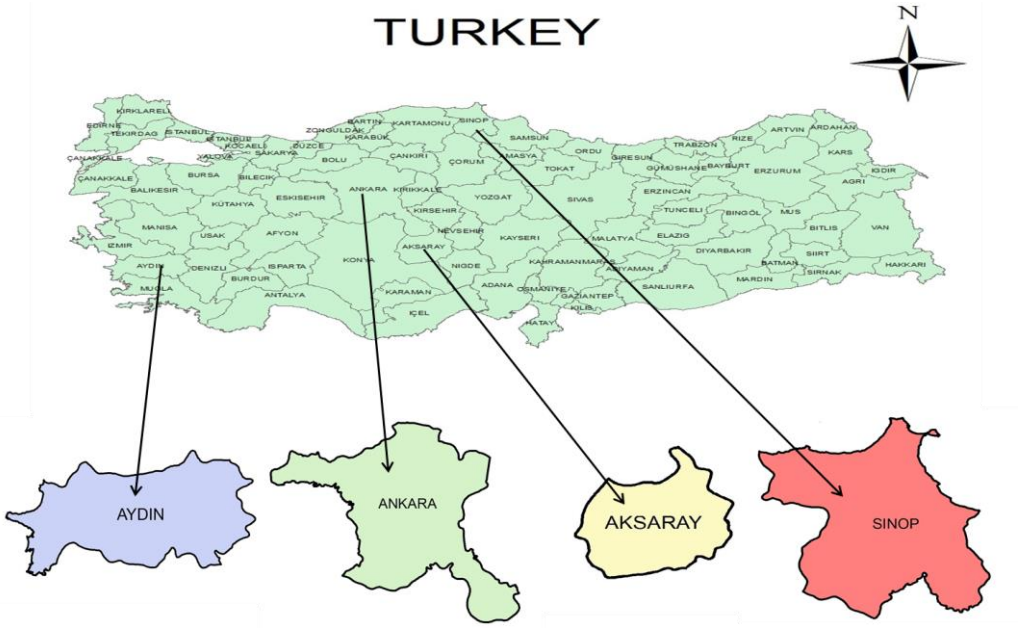

Figure 1. Different sampling areas for collecting soil and root samples

Soil physicochemical analysis

Different soil physicochemical parameters including EC, pH, available phosphorus, available potassium and organic material content were measured in all collected soil samples. The maximum, minimum, maximum average, minimum average as well as total average of each parameter were recorded for further analysis. Soil pH and EC were measured following saturated paste methods (Porta *et al.*, 1986) using a pH meter and conductivity meter; available P was determined using Olsen method (Olsen *et al.*, 1954). Organic material content was also measured using modified method proposed by Richards (1954).

Fungal spores population assay

One the most important indices in AMF biodiversity assays is measuring the fungal populations in soils. For this purpose, it is needed to isolate fungal spores from the soil and measuring their population by counting number of spores in a defined volume of soil. So, the AMF spores and sporocarps extracted from 1g each soil sample (Kept at 4℃) from all of the sampling regions in three replicates using standard wet sieving and centrifugation in 50% sucrose solution method with some modifications (Dalpé, 1993). These modifications include using of ultrasonic for dispersing of soil particles from the spores and only centrifugation with 50% sucrose solution. By using these modifications, spore population could be measured easily only in 1g soil sample. The number of spores recorded in each replicate followed by measuring the average numbers per soil sample. The minimum, maximum, maximum average, minimum average as well as total average of spore numbers were recorded for further analysis. The soils with the maximum number of spores selected as starter inoculum for further propagation.

Measurement of fungal colonization ındices in host plant roots

The other important index in AMF biodiversity assays is measuring the fungal colonization patterns in host plant roots. For this purpose, it is needed to clearing and staining the host plant roots collected from different sampling regions. So, 0.5g root from each collected sample (kept at -20℃) washed carefully in distilled water to remove all soil particles. Root clearing and staining was carried out based on standard method (Phillips and Hayman, 1970) with some modifications. With this modification, the time will be reduced and the volume of chemicals used also decreased. For this purpose, roots cut to small pieces, 3ml KOH 10% added and heated 3-4 times each 20-30 seconds in microwave to completely clearing the roots. Then, washing carefully the root segments and adding 5 ml Trypan Blue or Cotton Blue (0.05%) for staining. Stained roots kept at room temperature overnight followed by washing them 3 to 4 times with Lactoglycerol. Root pieces cut in to equal segments (each 0.5 cm) and 9 segments put in slides and observed under light microscope (10X, 40X, 100X) for observation AM fungal structres including extra and intra radical mycelia, vesicles, spores as well as arbuscules. Two AMF colonization indices were also measured in each slide in 9 root segments. These two indices were mycorrhizal frequency (F%) and intensity of mycorrhization (M%) which were calculated based on method proposed by Trouvelot *et al.* (1986). For each root sample, 3 replicates were considered, indices were measured in all

replicates and the maximum, minimum, maximum average, minimum average as well as total average of these indices were measured. The soils and host plant roots with maximum colonization indices selected as starter inoculum for further propagation.

AM fungal propagation

For this purpose, each fungal spore isolated carefully from the candidate soil samples (soils with highest fungal spore density and colonization indices) and put on small sterile filter paper pieces. The filter paper contatining one fungal spore put inside the sterile falkon tubes (50 ml) filled with sterilized sand and cultivated by clover seeds. The tubes kept in favourable growth chamber for about 2 months. Then, the soil and roots of the falcon tubes transferred in big size pots (400gr) with clover seeds and kept at the same conditions for 3 months more. This procedure repeated 3-4 times at the same manner for propagation of AM fungal spores. During this time, tubes and pots irrigiated with 30% strength Hoagland's nutrient solution without phosphorus. Finally, followed by 4-5 times propagation, the shoots will be removed and pots kept 1 month more without any irrigation for fungal stimulation to sporulation. At harvest time, the roots will be carefully removed from the substrate, removed from soil particles and kept at -20 ℃ for further analysis.

AM fungal species morphological ıdentification

For morphological identification of AM species, we used of propagated AM fungal spores. For this purpose, after harvesting culture pots, fungal spores were isolated from 50 g air-dried soil samples. Then, spores were separated based on their morphotypes and mounted on microscopic slides using PVLG (Polyvinyl Lactic Acid Glycerol) and a mixture of PVLG and Melzer reagent (1:1; V:V) and kept overnight at room temperature to detect dextrinoid sugar spore reaction (Brundrett *et al.*, 1996; Sasvári *et al*., 2012; Belay *et al*., 2013). AM fungal spore identification was carrried out using spore characteristics under compound light microscope (40X, 100X). Spores identification were carried out up to species level using standard manuals (Morton and Benny, 1990; Schenck and Perez, 1990; Redecker *et al*., 2000; Morton and Redecker, 2001; Blaszkowski, 2012).

Results

Totally, 47 soil samples were collected from different regions in Turkey including Ankara, Aydın, Sinop and Aksaray. The analysis of variance on soil physicochemical parameters showed that there are significant differences on the parameters among different soil samples at $P < 0.01$ and $P < 0.001$. The results (Table 1) showed that the maximum averages of EC (2.91 ± 2.18), available P (6.03 ± 0.93) as well as available K (230.25 ± 15.8) were observed in soils collected from Aydın region. Also, the minimum averages of EC (0.17 ± 0.01), available P (2.9 ± 0.7) and available K (22 ± 10) were obtained in the soils collected from Sinop region. The maximum and minimum averages of pH (7.88 ± 0.07, 5.87 ± 0.22; respectively) observed in the soils collected from Aydin and Ankara regions. The maximum and minimum averages of organic materials (5.16 ± 0.24, 2.37 ± 0.34; respectively) were also obtained from the Sinop and Aksaray soil samples.

Table 1. Soil physicochemical parameters assay in different sampling soils

Parameter	Max	Min	Max Average	Min Average	Total Average
EC (ds/m^2)	29	0.16	2.91 ± 2.18**	0.17 ± 0.01**	1.04 ± 0.35***
PH	8.32	5.65	7.88 ± 0.07**	5.87 ± 0.22**	7.19 ± 0.29***
Available P (mg/kg)	16.2	0.1	6.03 ± 0.93**	2.9 ± 0.7**	4.92 ± 0.45***
Avaialable K (mg/kg)	603	12	230.25 ± 15.8**	22 ± 10**	119.99 ± 27.97***
OM (%)	11.31	3.02	5.16 ± 0.24**	2.37 ± 0.34**	3.44 ± 0.42***

The data in the table are mean ± SE
**: Significant at $P < 0.01$
***: Significant at $P < 0.001$

Table 2. AM fungal indices in different soils and roots samples

Index	Max	Min	Max Average	Min Average	Total Average
SD	180	17	99.27 ± 9.29**	28.67 ± 4.07**	68.144 ± 8.38***
F	100	33.33	96.29 ± 1.58**	81.48 ± 8.45**	90.08 ± 2.29***
M	73.8	0.7	34 ± 2.58**	22.87 ± 3.56**	28.52 ± 1.67***

SD: Spore Density (spores/1g soil); F: Mycorrhizal Frequency (%); M: Intensity of Mycorrhization (%)
The data in the table are mean ± SE
**: Significant at $P < 0.01$
***: Significant at $P < 0.001$

The analysis of variance on AM fungal population assay as well as fungal colonization indices showed that there are significant differences on the studied parameters among different soil and root samples at $P < 0.01$ and $P < 0.001$. The results (Table 2) showed that the maximum averages of fungal spore density (99.27 ± 9.29), mycorrhizal frequency (96.29 ± 1.58) and intensity of

mycorrhization (34 ± 2.58) were observed in soils collected from Sinop region. Also, the minimum averages of these parameters (28.67 ± 4.07; 81.48 ± 8.45; 22.87 ± 3.56, respectively) were obtained in the soils collected from Aydin region.

For better conclusion, the relationship between AM fungal spore density and intensity of mycorrhization index (M%) was determined by Pearson correlation analysis. The results (Table 3) showed that there is positive correlation (r= 0.953**, P<0.01) between these two parameters in different sampling regions.

Table 3. Correlation between spore density and intensity of mycorrhization index in different sampling regions

SD/M%	**r**	0.953**
Sig. (2-tailed)		0.000

**: Correlation is significant at P<0.01 (2-tailed)

For confirmation effects of sampling regions and soil parameters on intensity of mycorrhization index, the two ways ANOVA was carried out on collected data. Results (Table 4) showed that all of these parameters as well as their interactions had significant effects at P<0.01 and P<0.001 which confirmed that fungal colonization intensity affected by different factors such as sampling location as well as soil physicochemical parameters.

Table 4. Effects of soil parameters and sampling region on intensity of mycorrhization index

	Soil	**Region**	**Soil ×Region**
F	75.05	28.55	2.57
(M%)	**	***	***

Significant differences: ***: 0.001; **: 0.01

The correlation among soil physicochemical parameters with AMF biodiversity parameters including spore density (SD) and Mycorrhizal intensity (M%) were studied. Results (Table 5) showed that there is negative correlation between fungal spore density and soil EC, PH, available P, and available K (r= -0.375**; r= -0.327; r= -0.210**; r= -0.287**, P<0.01, respectively). Also, the positive correlation could be observed between fungal spore density and soil organic matter (OM%) (r= 0.158, P<0.01). There is negative correlation between mycorrhizal colonization intensity (M%) and soil EC, PH, available P and available K (r= -0.425**; r= -0.023; r= -0.187**; r= -0.382**, P<0.01, respectively). Also, the positive correlation could be observed between M% index and soil OM (r= 0.002).

Table 5. Correlation between AMF diversity indices and important soil parameters

Index	EC	PH	AP	OM	AK
SD	-0.375**	-0.327	-0.210**	0.158	-0.287**
M	-0.425**	-0.023	-0.187**	0.002	-0.382**

AP: available P; AK: available K; OM: organic matter; SD: spore density; M: mycorrhizal intensity; **: corellation is significant at P<0.01.

Considering above mentioned results, it is concluded that soil EC, PH, available P and K had negative effects on AMF biodiversity indices. Soil organic matter had positive effects on fungal indices in different sampling regions. If we compared the results obtained in table 5 with the results indicated in tables 1 and 2, it was concluded that the soils with the highest total averages of EC, pH, available P and available K (Collected from Aydin region) had the least values on total averages of fungal spore density as well as mycorrhizal intensity.

After propagation of single fungal spores in trap pot cultures in growth chamber conditions, we studied the host plant roots for possibility of fungal colonization as well as observation of fungal structures. AM fungal structure could be observed in root segments including mycelia, vesicles, spores as well as arbuscules (Figure 2). The intensity of fungal structures was varied among different soils. It was observed that in samples with high EC values the intensity of vesicles are more than other structures.

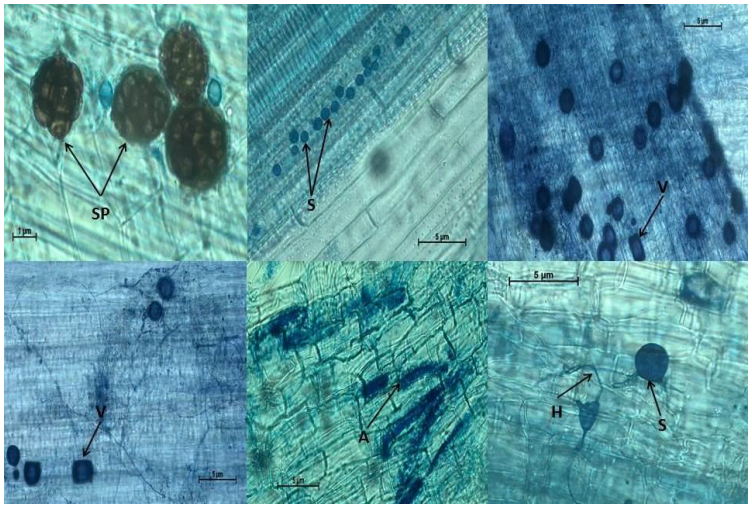

Figure 2. Different fungal structures observed after clearing and staining of plant roots followed by single spore propagation. SP: Sporocarp; S: Spore; V: Vesicle; A: Arbuscule; H: Hyphae. Photos captured with magnification 40X (5 µm) and 100X (1 µm)

Propagated spores were also isolated for morphological identification. Based on morphological methods, we identified 4 definite and 2 indefinite AMF species in samples. These species belonged to 4 genera including *Glomus, Rhizophagus, Funneliformis* and *Paraglomus*. The definite fungal species were *Funneliformis caledonius, Rhizophagus fasciculatus, Rhizophagus intraradices* and *Glomus versiforme*. The indefinite species were *Glomus* sp. and *Paraglomus* sp. (Figure 3). The present study showed that *Glomus* and *Rhizophagus* were the predominant genera which could be propagated widely among all samples.

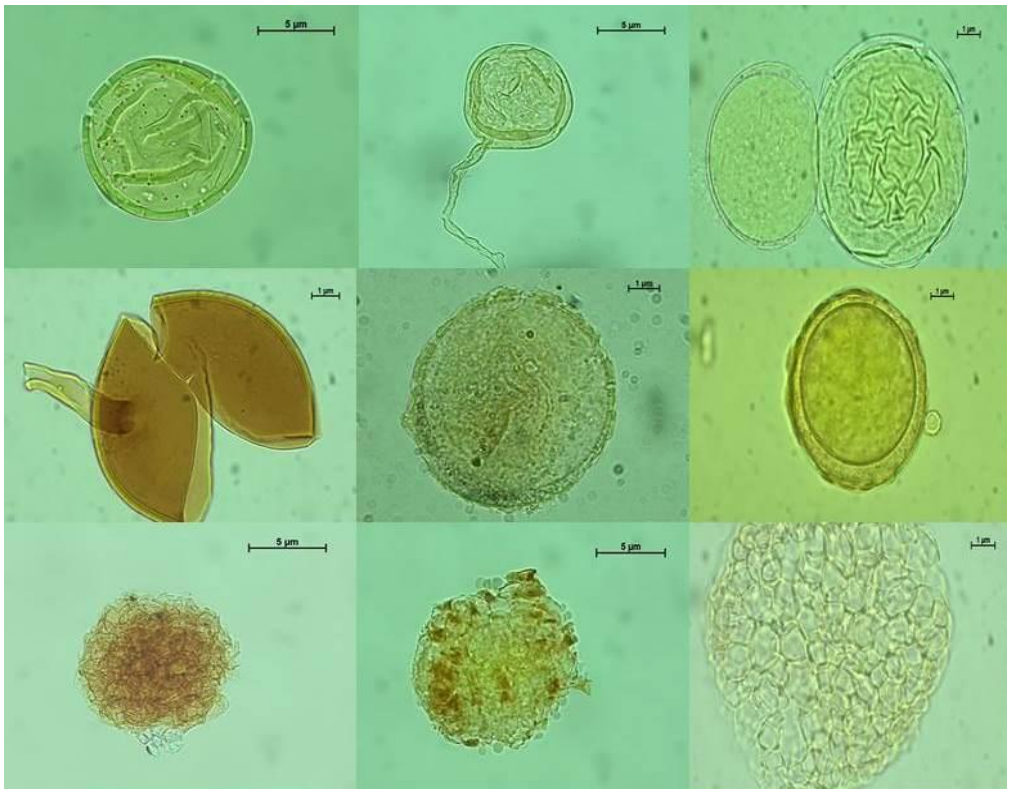

Figure 3. Fungal species identified using morphological methods from single spore propagated. Photos captured by light compound microscope with magnification 40X (5µm) and 100X (1µm). First row up from left to right: *F. caledonius; R. fasciculatus; Glomus* sp.; Middle row from left to right: *R. intraradices; Paraglomus* sp.*; Glomus versiforme;* Third row below: Sporocarps

Discussion

This study results showed that AM fungal population assay as well as fungal colonization indices were different among soil and root samples. These differences were expected in fungal spore numbers as well as M% index since the soil physicochemical properties were different among soil samples in each region and also the soil samples collected from different host plants rhizosphere (Becerra *et al.*, 2014). The positive and significant correlation (r= 0.953**, P<0.01) between AM fungal spore density and intensity of mycorrhization index (M%) in different sampling regions can be attributed to the fact that spore numbers reflect the colonization potential and they are related to the rate and the extent of mycorrhizal formation. Our results are in agreement with the findings of Wu *et al*. (2006). Also, correlation analysis among soil physico-chemical parameters and AM fungal biodiversity indices showed that soil EC, PH, available P and K had negative effects on AMF biodiversity indices, while, the soil organic matter had positive effects in different sampling regions which were in agreement of previous findings (Carrenho *et al.*, 2007; Khade and Rodrigues, 2008; He *et al*., 2010; Khakpour and Khara, 2012; Amballa and Reddy, 2016). The distribution of AM fungal species is affected by various factors including both biotic and abiotic factors (Hayman, 1982). Some studies indicated that abiotic factors may be more important than biotic factors for establishing population patterns (Panwar and Tarafdar, 2006). However, other studies suggested that abiotic factors alone are not sufficient in explaining AM species distribution, and some biotic factors have to be taken into account (Uhlmann *et al.*, 2004). If we compared the results obtained in table 5 with the results indicated in tables 1 and 2, it was concluded that the soils with the highest total averages of EC, pH, available P and available K (Collected from Aydin region) had the least values on total averages of fungal spore density as well as mycorrhizal intensity.

Study on AM fungal structure in root segments showed that the intensity of vesicles in samples with high EC values is more than other structures. It is expected since vesicles have storage or propagule functions and can support the regrowth of intercellular hyphae when appropriate conditions occur (Smith and Read, 1995). In stressful environments, AM fungi may have a tendency to invest more energy in storage structures needed for their survival (Turnau *et al.*, 1996). So, it is expected that in soils with high EC values these stuructures produced intensively. Trap cultures, using host plants grown in soil diluted with sterile sand, are most commonly used to propagation AM fungi (Morton *et al.*, 1993). This pot culturing method usually results in the isolation of more species than other methods and provides additional information on fungal diversity that

complements spore occurrence data obtained using the same soil samples and may provide valuable new information about the biology of AM fungi (Liu and Wang, 2003). Clover, sorghum, maize and sudangrass are commonly used as trap plants (Liu and Wang, 2003; Moreira *et al.*, 2007; Yao *et al.*, 2010). Liu and Wang (2003) have evaluated the appropriateness of four plant species as trap plants based on the morphological classification of AM fungi, and found that clover was the best, in terms of spore and species numbers. In this study we used clover as host trap plant.

The present study showed that *Glomus* and *Rhizophagus* were the predominant genera which could be propagated widely among all samples. They are considered as widespread fungi in many ecosystems and predominant occurrence of them in the rhizosphere soils of different plants was also reported earlier by several other authors (Reddy *et al.*, 2007; Snoeck *et al.*, 2010; Hindumathi and Reddy, 2011; Abdelhalim *et al.*, 2013; Satya Vani *et al.*, 2014). This might be explained by development, since the cycle of genus *Glomus* not to be affected by nutrient rich environment and tillage and the potential to produce relatively high number of spores within a very short period of time (Oehl *et al*., 2009) but other genera such as *Gigaspora* need more time to produce spores (Wang and Jiang, 2015). However, the morphological method used in the present study, which is based on the AM fungus spores, has limitations in that spore production is highly dependent on physiological parameters of the AMF and on environmental conditions. In recent years, molecular identification techniques have been used increasingly in studies on the ecology of arbuscular mycorrhizae because they offer the opportunity to identify AMF in any given root sample without the need for spores (Redecker *et al.*, 2003). In combination with the classic morphological analyses of spores, molecular identification of AMF by ribosomal sequences is highly promising and should provide a workable strategy to better characterize AMF communities.

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