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Spore density and species diversity of arbuscular mycorrhizal fungi associated with rhizosphere of annual and perennial forage crops

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Abstract
Three perennial grass species and four annual fodder crops were taken to study root colonization, arbuscular mycorrhizal fungi (AMF) spore abundance, spore density and AMF species diversity in rhizosphere soil at two times of sampling. The objective of this study was to record the AMF population in natural condition for its better utilization for fodder yield enhancement through fodder seed bio-priming. Results of this study clearly indicated that perennial grasses had higher root colonization, spores density and diversity in the rhizosphere soil than annual fodder crops, which ranged between root colonization 42 to 88 percent in grasses and 32 to 79 percent in annual fodder crops. AMF spore density and diversity was also higher in perennial grasses than annual fodder crops. Among grasses, *C. ciliaris* had higher AMF spores in rhizosphere than other grasses and it was also higher than annual fodder crops. Four AMF genera were identified and *Glomus* had higher abundance and isolated from all the crops and grass rhizospheres. In annual fodder crops maize and sorghum rhizosphere had higher AMF population and least in oat.

Keywords: Arbuscular mycorrhiza, Forage crops, Rhizosphere, Species richness, Spore diversity

Introduction

Arbuscular mycorrhizal (AM) fungi are among the most ubiquitous soil microorganisms, forming mutualistic associations with 80 to 90% of vascular plant species in ecosystems throughout the world. Arbuscular mycorrhiza is the most ancient and wide-spread type of mycorrhiza and is more widely distributed than other types of mycorrhizal associations (Smith and Read, 2008). They are keystone organisms that form an interface between soils and plant roots and they are also sensitive to changes in soil and plant conditions. Studies have been undertaken on the distribution and diversity of AMF species in relation to individual plant species and plant commu-nities in farming systems (Jefwa *et al*., 2004) and recently, there is emerging interest in the role of mycorrhizae in ecosystem processes (Hu *et al*., 2013). Cavagnaro *et al.* (2015) showed that AM fungi had the ability to reduce nutrient loss from the soil by enlarging the nutrient interception zone and preventing nutrient loss after rain induced leaching events. AM fungal root colonization in host plants is nonspecific and more than one species of AM fungi have been found across multiple plant species (Tewari *et al*., 1993). AMF are widespread in different soils and associated with a wide range of plant species, including most commercial crops and trees (de Silva *et al*., 2013). Both the partner mutually benefited under scarcity, however, individual plant species and plant communities in natural and farming systems affect the distribution and diversity of AMF species (Beyene *et al*., 2016).

Therefore, according to the previous observations, AMF spore density, diversity and rates of root colonization are dependent on soil properties and plant species density and diversity (Allsopp and Stook ,1992). Though there are research reports that AMF has association with different tree and crop species but information is scarce on spore density and diversity of AMF in rhizospheric soil of fodder crops grown in different fodder production system. Therefore, the objective of the present investigation was to study AMF symbiosis and spore population in the rhizosphere soil of different annual and perennial fodder crops.

Materials and Methods

***Sample collection*:** The grass species (*C. ciliaris*, *H. Contortus* and *C. fulvus*) grew under natural environmental conditions at Research Farm of Grassland and Silvipasture Management Division of Indian Grassland and Fodder Research Institute, Jhansi located at 78^0 35' E longitude, 25° 26' N latitude were taken for observation as perennial fodder production. However, rhizospheric soil samples of annual fodder

crops (Maize, Sorghum, Pearl millet and Oat) were taken from fields of other divisions of Institute and farmers' fields during 2014-15 and 2015-16 cropping seasons. Three places from each field were randomly selected for the collection of rhizosphere soil and root samples. Two sampling times, *viz*., one at early growth *i.e*. 30-45 days after sowing and second at 60-70 days for annual fodder crops, while in grasses, soil samples were collected first at active growth period of grasses (July to September) and second at flowering stage (November and December). Rhizosphere soil sample of each grass and annual fodder crops were collected from 0-15 cm depth.

***Estimation of AMF colonization*:** Percentage root colonization of AM fungi was determined from root samples collected from the rhizosphere soil of each grass and fodder crops separately. Root samples along with rhizosphere soil were collected in polythene bags for each randomly selected site separately, sealed and brought to the laboratory. Study of the root samples for AM colonization was performed on the very next day of the collection. Roots were kept in running tap water for half an hour and washed thrice. To determine root percentage colonization, the roots were cleared in 10% KOH and stained with Tryphan blue 0.1 % (Phillips and Hayman, 1970). The presence of fungal structures within the roots was verified by a method that used root segments mounted on slides and observed under the microscope (400 X). Five slides were mounted for each sampled, containing five 1 cm-long root segments each. The percentage of root colonization was calculated using the following formula: root colonization (%) = (Number of arbuscular mycorrhiza positive segments/Total number of segments studied) × 100%. The number of AMF spores was determined by wet sieving method based on a 10 g soil aliquot for each sample followed by centrifugation in sucrose. The spores were counted under a stereoscopic microscope (40X), and identification was made using an optical microscope (100 to 400X), with the descriptions provided by the site of International Collection of Vesicular and Arbuscular Mycorrhizal Fungi (*http://invam.caf.wvu.edu*) and the original species descriptions.

***Diversity studies*:** Ecological measures of AMF diversity, including spore density (SD) per 100 g rhizosphere soil and species diversity Shannon-Wiener index (5H) were used to describe the structure of AM fungi communities. Diversity studies of AM fungi were carried out from rhizospheric soil of each grass/fodder crop species separately. The following data were collected and analyzed in order to evaluate the composition and the diversity of AM fungi. Spore density (SD) was the number of all AM fungal spores per 100 g air-dried soil and species diversity (*H*) was calculated by Shannon-Weiner index. All the statistical analysis was conducted by online software (Sheoran *et al*., 1998).

Results and Discussion

***AMF association and species in rhizosphere soil of forage crops*:** Rhizosphere soil samples from three perennial and four annual fodder crops were collected at two growth stages for AMF association and species richness. All the selected perennial grasses and annual fodder crops were found having association of AMF. The degree of association varied among grasses as well as annual fodder crops. A total of 15 AMF species belonging from four genera, *Glomus, Gigaspora, Acaulospora* and *Scutellospora* and some unidentified were isolated from rhizosphere soil of perennial grass species and annual fodder crops. In rhizosphere soils of perennial grasses, higher species richness and spore abundance of AMF were observed (Table 1). Out of two soil sampling timing, first (I) sampling clearly showed higher species richness of AMF associated with perennial grasses. Among three grass higher AMF species richness recorded with *C. ciliaris* (14) followed by *H. contortus* (10) and least with *C. fulvus* (7) at July to August sampling time which was decreased at second time of sampling (November to December). AMF symbiosis formed with as many plant species as 250,000 and only 150 - 200 species of AMF have so far been distinguished on the basis of morphology. AMF are more widely distributed than other types of mycorrhizal associations (Smith and Read, 2008; Geoffrey and Mark, 2014). Wang and Qiu (2006) reported that majority of angiosperms are associated with symbiotic fungi forming AM. In the *Poaceae*, 99.6% of the species studied were AM symbionts. Lugo *et al.* (2012) also reported that native grasses were colonized by AMF. Torrecillas *et al.* (2012) isolated and identified thirty-six AMF phylotypes from six plant species studied and all are hosted different AMF communities. Yuan *et al*. (2011) reported from China that five plant species were showed the seasonal change and preference of arbuscular mycorrhizal colonization and community composition. The AM root length colonization rates were different among the five plant species and were generally high in early (May and June) and late (September) growth seasons and low in August. A total of 18 AM fungal species belongs to five genera were isolated and most AM fungi had no host specificity. *Glomus* species were the domi-

Table 1. AMF species in rhizosphere soil of perennial grass species

AMF Species	*C. ciliaris*		*H. contortus*		*C. fulvus*	
Sampling time	**I**	**II**	**I**	**II**	**I**	**II**
Glomus fasciculatum	+	+	+	+	+	+
Glomus aggregatum	+	+	+	-	+	-
Glomus intraradices	+	+	-	+	-	-
Glomus mosseae	+	+	+	+	+	-
Glomus macrocarpum	+	-	+	+	-	
Gigaspora margarita	+	+	+	+	-	-
Gigaspora gigantea	+	+	-	-	+	+
Acaulospora scrobiculata	-	+	+	+	-	-
Acaulospora longula	+	+	-	+	+	-
Acaulospora sp	+	-	-	-	-	-
Scutellospora biornata	+	+	+	+		-
Scutellospora nigra	+	-	+	-	+	+
Unidentified 1	+	-	+	-	-	-
Unidentified 2	+	+	-	+	-	-
Unidentified 3	+	+	+	-	+	+
Species richness	14	11	10	9	7	4

Table 2. AMF species in rhizosphere soil of annual fodder species

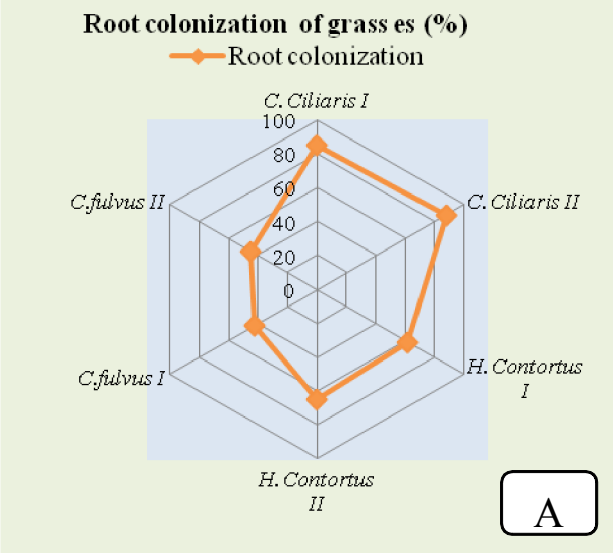
AMF species	*Z. mays*		*S. bicolor*		*P. glaucum*		*A. sativa*	
Sampling time	**I**	**II**	**I**		**II**	**I**	**II**	**I**
Glomus fasciculatum	+	+	+	+	-	+	+	+
Glomus aggregatum	+	+	+	+	+	+	-	-
Glomus intraradices	-	+	-	-	-	+	-	+
Glomus mosseae	-	+	-	+	-	-	-	-
Glomus macrocarpum	+	-	+	+	-	+	-	+
Gigaspora margarita	-	+	-	+	-	+	-	-
Gigaspora gigantea	-	+	-	+	-	-	-	-
Acaulospora scrobiculata	-	-	-	+	-	-	-	-
Acaulospora longula	+	-	-	-	-	+	-	+
Acaulospora sp	-	+	-	+	+	-	-	+
Scutellospora biornata	+	+	+	-	-	+	+	-
Scutellospora nigra	-	+	-	+	-	-	-	-
Unidentified 1	-	+	-	+	-	-	-	+
Unidentified 2	+	+	+	-	-	+	-	-
Unidentified 3	+	-	+	-	+	-	-	+
Species richness	7	11	6	10	3	8	2	7

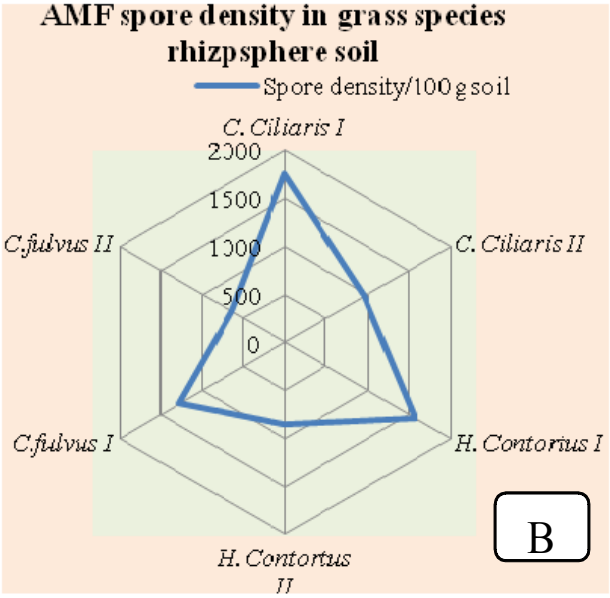
-nant species and showed various sporulation patterns in the five plants during the growth seasons. The AM fungal spore densities and species richness increased from May to September and decreased in October and were different in the same month in the five plants.

AMF species richness in rhizosphere soil of annual fodder crops differed from perennial grasses. Early collected soil sample showed less AMF species richness in all fodder crop tested and which were 7 in *Z. mays* followed by 6 in *S. bicolor*, 3 in *P. glaucum* and only 2 in rhizosphere soil of *A. sativa*. However, the AMF species richness increased at second time soil sampling and it was 11, 10, 8 and 7 in rhizosphere soil of *Z. mays, S. bicolor, P. glaucum* and *A. sativa* respectively. It clearly indicated that annual fodder crops had less and delayed root colonization and spore abundance of AMF than perennial grasses (Table 2). Gaur and Adholeya (2002) observed the variation in dependence on mycorrhiza among the fodder crops. *T. alexandrinum* showed a maximum dependence of 72% in contrast to 5.7% dependency in *S. vulgare*. Plant species showed differences in percentage AM colonization, with a high root infection recorded in *Z. mays* (76%). Spore produc-

-tion and infectious propagules (IP) were as high as 78 spores/IP g^{-1} and 103 spores/IP g^{-1} in *S. vulgare*. Nakmee *et al.* (2016) found that ten species of native arbuscular mycorrhizal fungi belong to *Glomus* sp., *Acaulospora* sp. and *Scutellospora* sp. had association with sorghum roots.

Root colonization and AMF spore density in rhizosphere soil: Root colonization study in perennial grasses and annual fodder crops clearly indicated that AMF colonized almost all grass species as well as annual fodder crops. Root colonization percentage ranged between 42 to 88 percent in grass root, highest in *C. ciliaris* and lowest in *C. fulvus* (Fig 1A). However, root colonization percent variation in annual fodder crops was 32 to 79 percent (Fig 1C). It was also observed that grasses had little variation in different time of observation, while fodder crops had much variation in root colonization at first and second time of observation. Root colonization varied from 32 to 70 percent in annual fodder crops at first soil sampling which reached to 41 to 79 percent at second observation (Fig 1C). Moreover perennial grasses had little variation in root colonization at different time of observation. Similarly, AMF spore density in rhizosphere soil of perennial grasses and annual fodder crops had also much variation. Variation in AMF spore density in perennial grasses ranges between 648 to 1751 spores per 100 g rhizosphere soil (Fig 1B). Higher (1751.2) AMF spores recorded in rhizosphere soil of *C. ciliaris* folowed by *H. contortus* (1569) and *C. fulvus* (1271) at first observation (July to August; Fig 1B). At second sampling AMF spores density varied from 645 to 975 spores per 100 g soil. In annual fodder crops AMF spore density varid from 402 to 627 and maximum was recorded in rhizosphere soil of *Z. mays* (627 spore/100 g soil) and lowest in *A. sativa* (402 spore/100 g) (Fig 1D). Panja *et al.* (2014) reported that mean AMF spore population densities in the rhizosphere of forage crops varied from 72.2-113.2 per 30 g dry soil. Out of total fifteen AMF spore types eleven AMF spore types belonged to four genera *viz.*, *Glomus*, *Gigaspora*, *Sclerocystis* and *Acaulopsora* and four spore types belonged to unidentified category. Kumar *et al.* (2016) also recovered 1-22 spores/g soil from litchi plantation. Kumar *et al.* (2012) reported from agricultural fields of Mysore, that genus *Glomus* was dominant by exhibiting 19 species followed by *Acaulospora* of about 10 species. Spore count and species richness was found higher in host plants like maize and sorghum. AMF spore count was 479 to 824/100 g soil. Moreira *et al.* (2006) observed from Brazil that root percent colonization rates at first collection date were relatively low and did not differ amongst the ecosystems. At the second period, native forest had higher colonization than the other two areas, with much higher figures than during the first period, for all areas. Alguacil *et al.* (2012) observed that perennial plant species had higher AMF spore diversity than annual. Difference in root colonization percent and spore density of AMF in rhizosphere soil of different perennial grasses and annual fodder crops might be due to their root architecture and root metabolites which provide much space in perennial grasses than annual. Perennial grasses grow vigorously and fine root density in soil is much higher than annual fodder crops. The other reason might be rhizosphere soil of perennial grasses had less or no disturbance while soil of annual fodder crops had much disturbance due to tillage and other intercultural practices.





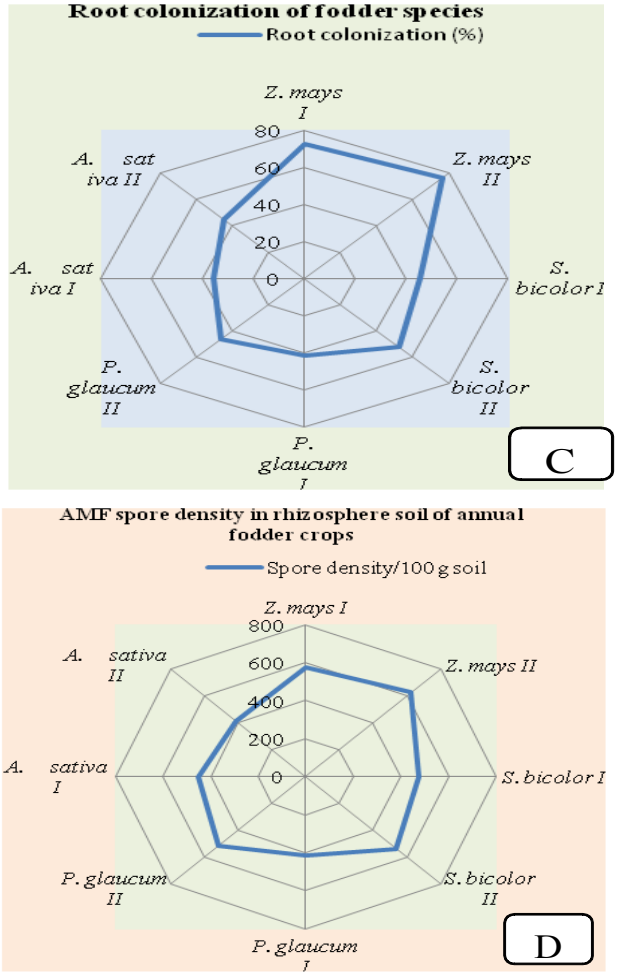


Fig 1. AMF root colonization (%) of grass species (A) and fodder crops (C) and AMF spore density in rhizosphere soil of perennial grasses (B) and annual fodder crops (D).

***AMF spore population structure in perennial grass species*:** Rhizosphere soil analysis for AMF spore identification and categorization for association of particular AMF with particular grass and fodder species revealed that genus *Glomus* was found abundance and frequently associated in all studied perennial and annual fodder crops however, the spore density of individual AMF also varied with grass and fodder crops. Among grasses, *C. ciliaris* had higher association with high spore density (1024/100 g soil). AMF genera *Scutellospora* had spore density next to *Glomus* except in *H. contortus* (Fig 2). However, in second growth stage, genus *Glomus* had first rank in spore density in all grass species. Lower AMF spore density in annual fodder crops than perennial grasses clearly indicated that less disturbed soil had more AMF spore population than disturbed soil. High root density in grasses also helped to have higher AMF spore population in rhizosphere soil. Luise *et al*. (2014) reported that agricultural practices influence the AMF colonization and resulted in variation in biomass. A study regarding abundance and richness of AMF in selected land use types, including restored pine stand, degraded grassland, *Paraserianthes* stand and natural forest patch at Central Province of Sri Lanka suggested that highest AMF spore abundance was observed in the natural forest patch. However, the AMF spore richness was higher in both natural forest patch and restored pine stand than in degraded grassland and *Paraserianthes* stand (Mafaziya and Madawala, 2015).

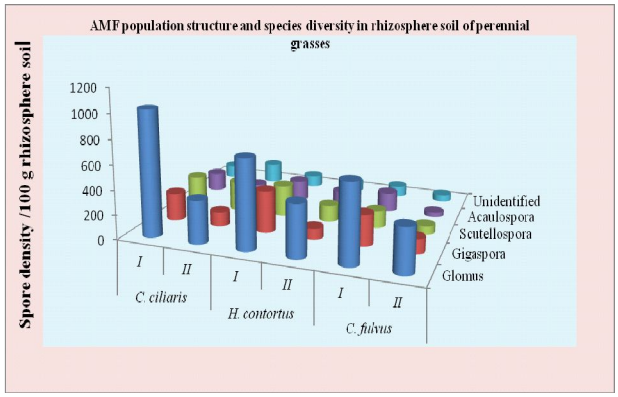


Fig 2. AMF species diversity in rhizosphere soil of perennial grasses at different growth stages

***AMF spore population structure in annual fodder crops*:** Observations on AMF spore density in rhizosphere soil of annual fodder crops revealed that all the annual fodder crops had strong association with AMF and genus, *Glomus* was found as more abundant AMF species followed by *Gigaspora*, however, genus, *Scutellospora* and *Acaulospora* association were scanty. Majority of AMF species were unidentified. In general annual fodder crops had less AMF spore density in their rhizosphere soil than perennial grasses. The AMF spore density was less at first observation and gradually increased and become higher at second observation. *Z. mays* had higher population of *Glomus* (356 spores/100 g soil) followed by *P. glaucum*, *S. bicolor* and least with *A. sativa,* which were 349, 347 and 109/100 g soil, respectively (Fig 3). Yang *et al*. (2010) observed genotypes in oat those showed a significant influence on the percentage of root length colonized and abundance of arbuscules and vesicles, and there was much greater colonization of naked oat than of husk oat and even oat varieties had differences in abundance and colonization. Saranya and Kumutha (2012) reported that maize plant required 5-6 AMF spores/g soil for effective 100 per cent root colonization.

In an experiment with five fodder crops, *Z. mays*, *M. sativa*, *T. alexandrinum*, A. *sativa,* and *S. vulgare* when inoculated with a consortia of AMF and it indicated that mycorrhizal inoculation increased yield in terms of shoot dry weight by 257% in *T. alexandrinum* followed by 50% in *A. sativa,* 28% in *Z. mays,* 20% in *M. sativa* and 6% in *S. vulgare*. Variation in dependence on mycorrhiza was also observed among the fodder crops. *T. alexandrinum* showed a maximum dependence of 72% in contrast to 5.7% dependency in *S. vulgare*. Plant species showed a difference in percentage AM colonization and a high root infection was recorded in *Z. mays* (76%). Spore production and infectious propagules (IP) were as high as 103 spores/ IP per g soil in *S. vulgare* (Gaur and Adholeya, 2002).

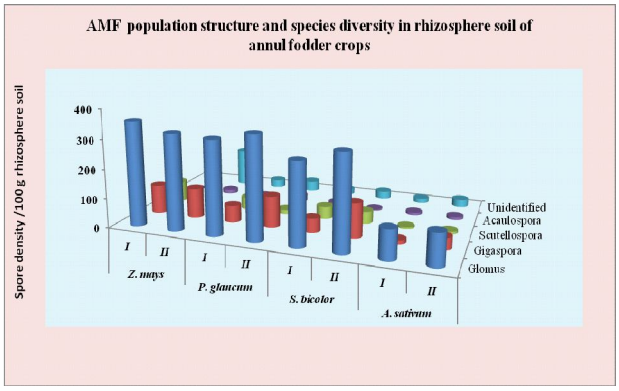


Fig 3. AMF species divesrity in rhizosphere soil of annual fodder crops at different time of isolation

AMF species diversity in the rhizosphere soil: AMF species diversity analysis in the rhizosphere soil of perennial and annual fodder crops clearly indicated that perennial grasses had more or less similar AMF diversity at early date of observation but at second stage, perennial grasses had higher species diversity than annual fodder crops. AMF species diversity at first and second stages ranged between 2.16 to 2.26 and 2.29 to 2.81, respectively in rhizosphere soil of perennial grasses, while it was 2.13 to 2.27 and 2.23 to 2.29 in annual fodder crops (Table 3). Similarly species evenness index ranged between 0.90- 0.91 and 0.81- 0.91 in perennial and 0.82-0.86 and 0.84-0.87 in annual fodder crops at first and second time of sampling, respectively. Katsunori *et al*. (2008) reported that spore density in the soybean and maize fields markedly differed with the sampling site. The density of AM fungal spores in the soybean field was negatively correlated with the available phosphorus content and showed a positive correlation with the phosphate adsorption. Beyene *et al*. (2016) observed from agroforestry land use system that diversity of AMF wasbased on soil properties. At moderate to low P and N concentrations the rate of AMF root colonization and spore density was high in comparison to the rhizosphere soils with high P and N concentrations. The highest percentage of total AMF colonization was recorded in shade trees *Millettia ferruginea* (84%) and *Erythrina brucei* (80%) followed by intercropped perennial crops *Ensete ventricosum* (86%), *Catha edulis* (85%) and *Coffea arabica* (80%) and the lowest percentage AMF colonization was recorded in *Rhamnus prinoides* (53%) and *Colocasia esculenta* (52%). The highest number of AM spore population was recorded in rhizosphere soils of *Croton macrostachyus* (1066) and the lowest spore density was recorded in *Dioscorea alata* (100) spore per 100 g of dry soil. In a greenhouse experiment for AMF trapping with four types of host plants, namely kudzu (*Pueraria javanica*), sorghum (*Sorghum bicolor*), corn (*Zea mays*) and soybean (*Glycine max*), highest (56.40%) root colonization was found in corn and sorghum when compared to the colonization from other host plants. *Zea mays* had advantages on several parameters such as abundance of spores, root colonization, inoculums weight, root fresh weight and root dry weight. Indeed, *Zea mays* was found as the best host according to variables of spore abundance, root colonization, inoculums weight, root fresh and dry weight (Ridwan *et al.,* 2016). The highest diversity with 53 plant species was found in the evergreen forest, 77.4% of them were AM, while in the grassland with 22 plant species, 91% of them were AM. The deciduous forest had 11 plant species only with the lowest proportion of AM plant species (55%). Amongst the AMF species, 13 species were of *Acaulospora* genus, 10 of *Glomus*, 4 species each of *Scutellospora* and *Archaeospora*, 3 species each of *Pacispora* and *Entrophospora*, and one species each of *Paraglomus* and *Diversispora*. AMF species were more abundant in the grassland (29 spp.) than in the evergreen forest (20 spp.) which was likely related to a higher relative proportion of AM plant species in the grassland (Castillo *et al*., 2006).

Table 3. AMF species diversity in the rhizosphere soil of perennial grass and annual fodder crops

Perennial and annual crops	AMF species diversity (H)		AMF species evenness (E)	
Sampling time	I	II	I	II
C. ciliaris	2.16	2.37	0.91	0.89
H. contortus	2.19	2.81	0.90	0.81
C. fulvus	2.26	2.29	0.91	0.91
Z. mays	2.13	2.23	0.86	0.84
S. bicolor	2.17	2.23	0.86	0.87
P. glaucum	2.14	2.26	0.85	0.87
A. sativa	2.27	2.29	0.82	0.87

Conclusion

Arbuscular Mycorrhizal fungi (AMF) constitute a group of root obligate biotrophs that exchange mutual benefits with about 80% of plants and considered natural biofertilizer. Fodder crops (both annual and perennial) are usually grown in poor soil with less input. Thus available knowledge on AMF diversity and species richness associated with particular fodder crops could be applied at field level as biofertilizers to enhance fodder productivity. Present finding clearly indicated that perennial grasses had higher root colonization (42 to 88%) than annual fodder crops (32 to 79%). *Glomus* had higher abundance and isolated from all the crops and grass rhizospheres. Hence, grass species of *C. ciliaris* could be used as host of AMF multiplication and *Glomus* species may be applied as seed bioprimng of fodder crops in enhancing fodder productivity in poor soil.

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