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Effect of native mycorrhizal fungi inoculants on the growth and phosphorus uptake of tree legumes: *Erythryna brucei and Millettia ferruginea*

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The inoculation study was conducted in the greenhouse to investigate the effect of phosphorus (P) concentrations on growth and arbuscular mycorrhizal fungi (AMF) colonization of multipurpose tree legumes *Erythrina brucei and Millettia ferruginea*. Plant growth parameters (shoot length, dry weight) and P uptake increased significantly after inoculations with AM fungi, *Rhizophagus clarus, Rhizophagus intraradices* and the mixed species. Results on effect of P application on total Mycorrhizal Dependency(MD) of studied tree species showed that maximum MD values were recorded for *R. clarus* (34.87%) in *M. ferruginea* and (26.19%) in *E. brucei* respectively. For the mixed species was recorded, the next highest MD values 26% in *M. ferruginea* and 16.67% in *E. brucei.* The least MD values were recorded for treatments with *Rh. intraradices* in both trees under study. The optimum P concentrations for maximum benefits from the AM symbiosis in aforementioned tree species varied from 0.005 to 0.02 mg g^{-1} and corresponding peaks of arbuscules, vesicles, percent root colonization, and spore count per 50 cm^3 sand were noticed at similar concentrations. Thus, the results showed that the recorded plant growth peaks were due to AM colonization of the tree seedlings. Therefore, inoculating plants with a suitable AM inoculant could result in a benefit comparable to high P input and lead to a significant saving of inorganic P fertilizer.

Key words: Agroforestry, trees, *root* colonization, spore density.

INTRODUCTION

*Erythrina brucei (*Schweinf) and *Millettia ferruginea* (Hochst) Baker from the family *Fabacae* (Leguminosae) are the most common shade trees in agroforestry systems of Gedeo and Sidama of Southern Ethiopia. In addition to Gedeo and Sidama, *E. brucei* grows naturally in open places and along edges of upland forests or woodlands in Wello, Gojjam, Shewa, Bale and Hararge, and Keffa agroforestry systems at altitudes between 1400 and 2600 masl. It flowers from November up to January and at times of fruiting most of its leaves shed adding to

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the organic fertility of the soil.

M. ferruginea is also one of the most valuable multipurpose tree species of Ethiopia (Legesse, 1995; Tadesse et al., 2000). It is used to improve soil physical and chemical properties in agricultural activities, as fodder for ruminants, as a shade tree, building materials, and used as medicine (Legesse, 1995, 2002; Tadesse et al., 2000).

M. ferruginea commonly occurs between 1100–2500 m above sea level and is characterized as a component of upland forest (Thulin, 1989). *E. brucei* and *M. ferruginea* (Hochst) Baker (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1998, 2011; 2002) are good biological nitrogen fixers and can be used as organic sources of nitrogen in agroforestry systems.

These multipurpose shade trees play a vital role in the rural economy of the region. In order to meet the future demand of these shade trees, their growth and productivity has to be hastened from the nursery stage onwards and their requirements for major fertilizers like Phosphorus should be known. Furthermore inappropriate and untimely application of fertilizer in agricultural fields generated several environmental and soil problems (Tilman et al., 2002; Foley et al., 2005).

The perceived need to seek alternatives to current agricultural practices has resulted in an enhanced interest in agroforestry systems (Ingleby et al., 2007), which can conserve resources, improve environmental quality, rehabilitate degraded lands, and provide multiple outputs to meet the daily demands of the rural population (Pande and Tarafdar, 2004; Muleta et al., 2008).

Agroforestry, a land use system/technology in which trees are deliberately planted on the same unit of land with agricultural crops, has been recognized as one of the most promising strategy for rehabilitating degraded areas. Arbuscular mycorrhizal (AM) fungi can rehabilitate degraded lands subjected to agroforestry systems (Mutuo et al., 2005; Cardoso and Kuyper, 2006). The common mycorrhizal network may further enhance the benefits of agroforestry through vertical niche expansion of AMF (Cavagnaro et al., 2005; Theuerl and Buscot, 2010).

The need to increase food, fibre, and fuel wood production to keep pace with the fast-growing population is crucial (Wrage et al., 2010). The low biomass production of agroforestry tree species such as in degraded areas can, therefore, be circumvented by the use of AM fungi (Shukla et al., 2009).

Phosphorus (P) is an essential nutrient for plant growth (Schroeder and Janos, 2005) and it is taken up by plants as phosphate (Landis and Fraser, 2008), which is unevenly distributed and relatively immobile in soils (Baird et al., 2010; Gianinazzi et al., 2010). The key function of AM fungi is the exploration of the soil beyond the range of roots for better plant growth and nutrition (Oehl et al., 2002; van der Heijden et al., 2006; Yadav et al., 2013a) AMF has the potential to make cultivation successful at a lower soil P level through more effective exploitation of the P sources (Jakobsen et al., 2005; Ma and Rengel, 2008).

The P level has been shown to significantly influence AM colonization (Covacevich et al., 2007). Addition of P fertilizers above optimum can delay or decrease colonization of roots (de Miranda et al., 1989; Hinsinger, 2001) and reduce chlamydospore production by the fungus (de Miranda and Harris, 1994). Agroforestry is not only concerned with beneficial effect of one component on another, but also involves the manipulations of negative effects to minimize their influence on the productivity of the overall system. At the tree-crop interface of an agroforestry system, trees and crops compete inevitably for light, water, and nutrients and AMF play an important role in P uptake. Therefore, the present study was conducted to identify the effect of AMF inoculation and application of different rates of phosphorus on growth and P uptake of leguminous tree species *E. brucei and M. ferruginea* that grow in Sidama and Gedeo agroforestry.

MATERIALS AND METHODS

In this study, seeds of the selected shade trees in Sidama and Gedeo agroforestry were used. Three native species of AM fungi isolated and purified from the rhizosphere of trees and crops from Sidama agroforestry were used as AM inoculants. Taxonomic identification of spores was checked to be matched with the description provided by the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM, 2006). Inoculum used in this study was consisted of soil along with chopped root bits of *Sorghum bicolor*, spores, and extrametrical mycelia from trap culture pots.

To study the effect of P concentrations on tree growth and P uptake after inoculation with AMF, separate experiments were carried out for the two multipurpose shade trees in the agroforestry. The trials consisted of six P concentrations (0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg/g) and three mycorrhizal treatments (*Rhizophagus clarus, Rhizophagus intraradices* and mixture of the two) and un-inoculated plants (control). Thus, a total of 24 treatments were carried out per each plant species, and each treatment was replicated three times. Seeds were surface sterilized with Sodium hypochlorite, washed several (five to six) times with sterilized distilled water and germinated on sterilized river sand at 30°C. In this study plastic pots filled with 2 kg sterile sand were used.

At the time of sowing, 50 g of mycorrhizal inocula was applied to the hole where pre-germinated seedlings were individually transplanted. Phosphorus was applied to the pots at 0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg/g as KH_2PO_4. Plants were grown under greenhouse conditions and watered daily. One seedling was maintained per pot and half-strength Hoagland's solution in deionized water was applied at weekly intervals. The composition of the Hoagland's solution was (0.51 g/L KNO_3, 0.246 g/L $Ca(NO_3)_2$, 0.245 g/L $MgSO_4.7H_2O$, 1.43 g/L H_3BO_3, 0.91 g/L $MnCl_2.7H_2O$, 0.11 g/L $ZnSO_4.5H_2O$, 0.04 g/L $CuSO_4.5H_2O$, and 0.04 g/L $H_2MoO_4.H_2O$). To reduce the risks of cross contamination, pots were kept on separate benches, with a space of 40 cm between each treatment.

Seedlings were harvested after three months and analyzed for shoot length and dry weight by standard methods. Phosphorus uptake was recorded using Molybdenum blue method according to Jackson (1973). Mycorrhizal dependency was calculated according to Plenchette et al. (1983): [(M-NM)/M] × 100, where: M is the total dry biomass of mycorrhizal plant; NM is the total dry biomass of

non-mycorrhizal plant.

To study the effect of P application on AM colonization of the two trees, aforesaid treatments (24) were replicated four times and six plants were maintained per replicate/pot. Two plants per pot were harvested after 1, 2, and 3 months of sowing, for observations. Formation of arbuscules and vesicles were monitored and then, was calculated the colonization index and spore count per 50 cm^3 sand. Fine roots were cleared with 10% KOH and stained with acid fuchsin (0.01% in lactoglycerol) as reported by Phillips and Hayman (1970) and then was recorded colonization rates of arbuscules and vesicles. Colonization percentage was determined by gridline intersection method of Giovannetti and Mosse (1980). Sporocarp and spores were isolated according to Gerdemann and Nicolson (1963) and counted.

Statistical analysis

All the data on plant growth were subjected to a one-way analysis of variance for testing the effects of AM inoculation and P application, and their interactions. The means were compared and ranked using Duncan's Multiple range test (P<0.05). The mean of experiments were analyzed statistically using a general linear model for analysis of variance of completely randomized designs. Analysis of variance (ANOVA) and correlation analysis were carried out with the SPSS software package (version 20.0). (SAS, 1982).

RESULTS

Plant growth, shoot dry biomass and P uptake

The results on effect of AM inoculation (*Rh. clarus, Rh. intraracices and* the mixed species) and P (0, 0.005, 0.01, 0.02, 0.05, and 0.1 mg g^{-1}) application on growth and P uptake by *E. brucei* and *M. ferruginea are* presented in Figure 1. Most of the peaks of shoot length, and dry weight, by these trees occurred from 0.005 to 0.02 mg g^{-1}. In un-inoculated plants, such peaks were inclined towards increasing P concentration. For the three AM fungi studied, these peaks indicate that the optimum P concentrations for maximum benefits from the AM symbiosis in plant species lied mostly from 0.005 to 0.02 mg g^{-1} P concentration. For shoot length the optimum P concentration for most effective AM inoculants, *Rh. clarus*, *Rh. intraradices* and the mixed species in *E. brucei* and *M. ferruginea* was 0.02 mg P g^{-1}. In both *E. brucei* and *M. ferruginea* inoculated with the three AM species, plant height increased with increasing P concentration until P=0.02 mg g^{-1} and shoot dry weight has increased with increasing P concentration until P=0.01 mg/g. However, the increase both in shoot length and dry weight of both *E. brucei* and *M. ferruginea* has decreased with increasing P concentration above p= 0.02 and P=0.01 mg g^{-1} respectively. Thus, the two tree species inoculated with the three AM species has positively reacted with increasing P concentration and inoculating above-mentioned trees, with a suitable AM inoculant (at lower P concentration) can be effective as high inputs of recommended P fertilizers.

Therefore, the optimum P concentration for the two selected agroforestry shade trees studied with different AM fungi for maximum benefit from the symbiosis was low (0.005–0.02 mg P g^{-1} substrate). Since different AM fungi can transport different amounts of P to plants, their effects on plant growth can also be different. Despite this fact however, in the current study the two species from *Glomeromycota* and the mixture of the two has produced similar results in the green house as compared to the un-inoculated which has been given similar P concentration with other treatments. In both trees studied, AM inoculants used; *Rh. clarus, Rh. intraradices* and the mixed species has significantly increased shoot length, dry weight, and P uptake at P<0.05% level.

Mycorhizal dependency (MD) of seedlings of the trees

Total results on mycorrhizal dependency of *E. brucei and M. ferruginea* seedlings are presented in Table 1. In both trees, the three AM inoculants: *Rh. clarus, Rh. intraradices* and the mixed species significantly (p<0.005) increased shoot length and total shoot dry biomass. Maximum MD values were recorded for *Rh. clarus* (34.87%) in *M. ferruginea* and (26.19%) in *E. brucei* respectively. For the mixed species was recorded, the next highest MD values 26% in *M. ferruginea* and 16.67% in *E. brucei.* The least MD values were recorded for treatments with *Rh. intraradices* in both trees under test.

Effect on AMF structural colonization and spore density

Arbuscular mycorrhizal fungi structural colonization (Arbuscules & Vesicles) of the trees after inoculation with *Rh. clarus, Rh. intraracices* and the mixed species are presented in Table 2. In the current study, formation of arbuscules by *Rh. clarus and Rh. intraradices* and the mixed species was more favored at lower P concentrations (0.05 to 0.02 mg P g^{-1} substrate). However, there were also some rates of colonization below and above 0.05 and 0.02 mg g-1 p concentration in all inoculated tree species (Table 2). The result also indicates that arbuscule formation was the earlier during the 1st month of inoculation and that of formation of vesicles was intensive during the 2nd and 3rd months of the inoculation.

Finally, the tree species inoculated with AM fungi showed mycorrhizal colonization that was characterized by the presence of arbuscules and vesicles (Table 2). However, mycorrhizal colonization, arbuscule and vesicle formation decreased significantly with the increase in P concentration. Also similar trend was observed with mycorrhizal spore number (Table 3), and positive correlation was recorded between mycorrhizal spore number and percentage root colonization.

Maximum root colonization and spore count per 50 cm^3

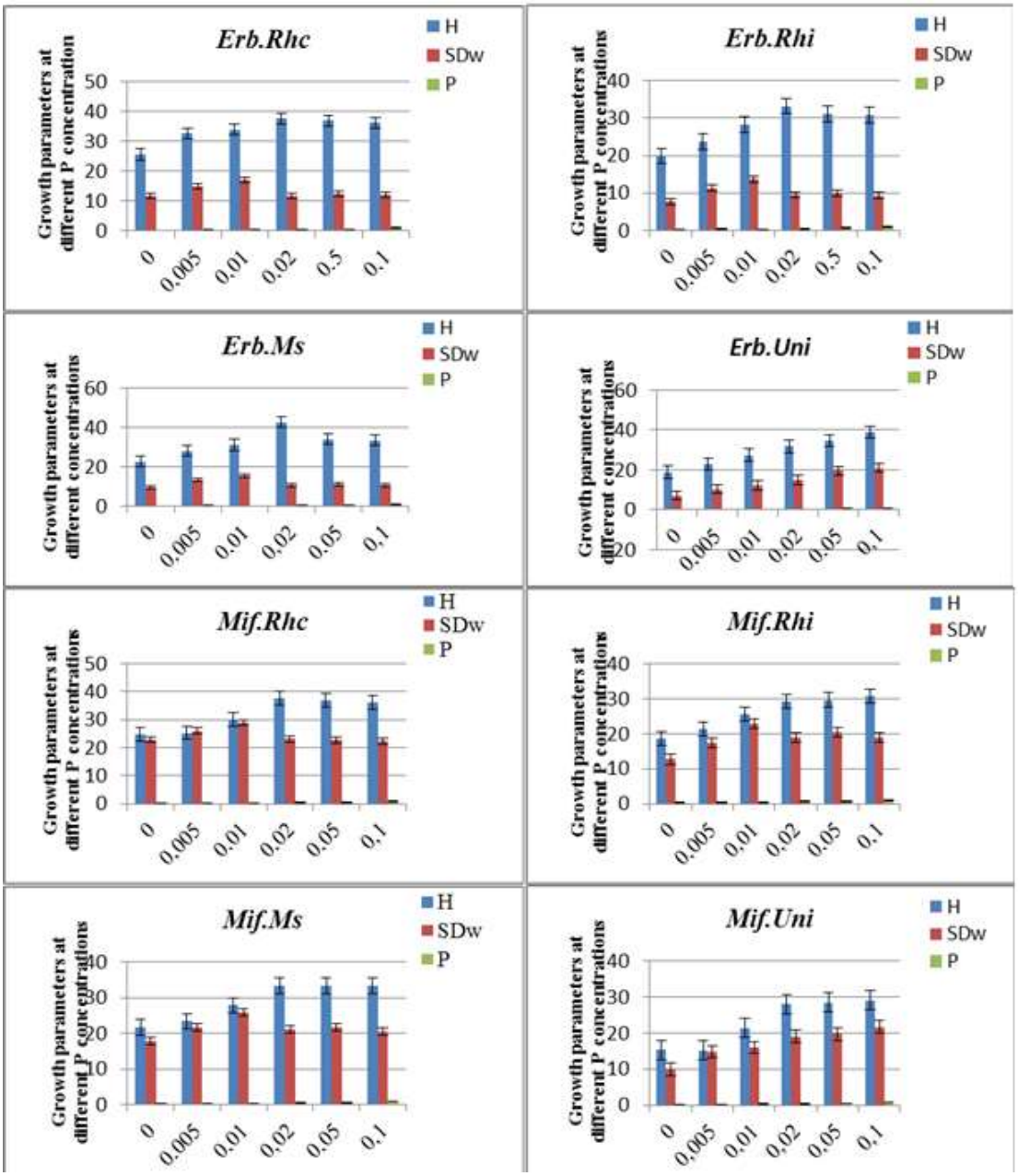

Figure 1. Plant height, shoot dry weight and P uptake at different P concentrations (mg/g) in AMF inoculated and un-inoculated treatments. Key: H, plant height; SDW, shoot dry weight; P, phosphorus; Erb., *Erythrina brucei*; Mif, *Millettia ferruginea;* Rhi., *Rhisophagus intraradices;* Rhc., *Rhizophagus clarus;* Ms., mixed species*; uni.,* un-inoculated.

Table 1. Total shoots dry weight and mycorrhizal dependency (MD) of the trees.

Plant species	MD%						Un-inoculated
	Rh. clarus		*Rh. intraradices*		Mixed species		
	Total SDW	MD	Total SDW	MD	Total SDW	MD	SDW
Erythrina brucei	79.7^{b}	26.19^{a}	61.5^{b}	4.34^{ab}	70.6^{b}	16.67^{b}	58.83^{b}
Millettia ferruginea	146.03^{e}	34.87^{c}	111^{e}	14.32^{e}	128.52^{e}	26.0^{cd}	95.11^{c}

SDW, shoot dry weight; MD, mycorrhizal dependency. Means in the same column followed by different letter(s) are significantly different by ANOVA and Duncan's Multiple Range Test at P<0.05 level.

Table 2. Effects of different phosphorus concentrations (milligrams per gram) on AMF structural colonization (after 1st, 2nd and 3rd months of growth).

Plants	P (mg/g)	*Rhizophagus clsarus*						*Rhizophagus intraradices*						Mixed					
		AC%			VC%			AC%			VC%			AC%			VC%		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Erythrina brucei	0	−	-	+	−	−	+	-	+	+	−	+	+	−	+	+	−	+	+
	0.005	−	+	++	−	+	+	+	+	+	-	+	+	+	+	++	-	+	+
	0.01	+	++	++	−	+	++	+	+	++	-	+	++	+	+	++	-	++	++
	0.02	+	++	++	−	++	+++	+	+	+	-	++	++	+	+	++	-	++	+++
	0.05	−	−	++	+	+	+	−	+	+	−	+	+	−	+	+	−	++	+
	0.1	−	−	+	−	−	+	−	−	+	−	+	+	−	−	+	−	−	+
Millettia ferruginea	0	−	-	+	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+
	0.005	+	++	++	−	+	++	+	+	+	+	+	++	+	+	++	-	++	++
	0.01	+	++	++	+	++	+++	+	+	++	−	+	++	+	+	++	-	++	++
	0.02	+	+++	+++	−	++	++	+	+	+	+	++	+++	+	+	++	-	++	+++
	0.05	−	−	++	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+
	0.1	−	−	+	−	−	+	−	−	+	−	+	+	−	−	+	−	−	+

1, 2, 3, number of months of plant growth; A, arbuscuales; V, vesicles; -, absent, +, fair; ++, Moderate; +++, high.

Table 3. Effects of different phosphorus concentrations (milligrams per gram) on root colonization and spore density (after three months of growth).

Plant species	P mg/g	*Rhizophagus clarus*		*Rhizophagus intraradices*		Mixed species	
		RLC (%)	SD/50 cm^3 soil	RLC (%)	SD/50 cm^3 soil	RLC (%)	SD/50 cm^3 soil
Erythrina brucei	0	12.33^{ab}	25.33^{b}	16.33^{bc}	32.67^{b}	20.00^{bc}	26.17^{b}
	0.005	17.50^{bc}	30.67^{b}	22.67^{cd}	35.67^{b}	23.33^{c}	36.67^{cd}
	0.01	32.67^{d}	57.33^{c}	33.33^{e}	56.67^{c}	33.33^{d}	39.33^{d}
	0.02	21.33^{c}	49.67^{c}	25.67^{de}	42.33^{b}	20.50^{bc}	32.33^{c}
	0.05	10.33^{ab}	6.67^{a}	9.00^{ab}	13.33^{a}	14.67^{b}	9.00^{a}
	0.1	5.17^{a}	3.33^{a}	4.17^{a}	6.67^{a}	3.00^{a}	7.50^{a}
Millettia ferruginea	0	13.33^{b}	26.67^{b}	16.50^{bc}	36.67^{b}	19.33^{bc}	26.67^{ab}
	0.005	17.73^{c}	34.00^{b}	23.33^{c}	40.67^{b}	26.33^{bc}	47.67^{c}
	0.01	28.67^{d}	62.33^{c}	36.33^{d}	69.33^{c}	39.00^{d}	69.33^{d}
	0.02	20.00^{c}	51.00^{c}	24.00^{c}	60.00^{c}	30.33^{cd}	39.67^{bc}
	0.05	11.67^{b}	10.67^{a}	7.67^{ab}	15.67^{a}	17.33^{b}	14.33^{a}
	0.1	6.00^{a}	6.00^{a}	5.33^{a}	10.67^{a}	2.67^{a}	11.00^{a}

RLC, root length colonization; SD, spore density. For each plant species means in the same column followed by different letter(s) are significantly different by ANOVA and Duncan's Multiple Range Test at P<0.05 level.

sand was observed at P concentrations ranging from 0.005 to 0.02 mg g^{-1} in tree seedlings infected by AM fungi (Table 3). In this study, results showed that the P optimum for maximum benefit from AM symbiosis for inoculated agroforestry tree seedlings was in between 0.005 and 0.02 mg g^{-1} and the seedling growth reduced with increasing P concentration.

Therefore, inoculating trees with a suitable AM inoculants could result in a benefit comparable to high P input. However, extrapolation of the results to the real conditions of agroforestry systems should be done with precaution because of differences in the substrate used, that is, sand in the present study. The information on P optimum can form the basis of further pot/field experiments involving integration of chemical fertilizers with AM fungi.

DISCUSSION

Previous studies, in field conditions have shown that

agricultural management practices, such as tillage, fertilization and cropping systems, have a negative impact on the AMF associated with temperate and tropical agronomic plant species (Cardoso and Kuyper, 2006). Fertilization is an important abiotic factor influencing growth, colonization, sporulation, composition and distribution of AMF (Wang et al., 2009).

Other studies conducted in green house conditions (Habte and Manjunath, 1991) have demonstrated that AM fungi usually have their maximum effect on host plant growth when the level of P in the growth medium is optimum. According to Habte and Manjunath (1991), when the soil solution P concentration is at or near 0.002 mg/l, most plant species will respond dramatically to mycorrhizal colonization.

Results of the current study on agroforestry trees and crops revealed the pick for maximum benefit at 0.02 mg P g^{-1} growth medium (sand) and that, as P concentration is increased from 0.005 to 0.02 mg/g, the reliance of plants on AM fungi for P uptake increased and diminished progressively as P concentration increased (from 0.05 to 0.1 mg/g) after which only the very highly mycorrhizal-dependent species respond significantly to mycorrhizal colonization.

Our results also confirm previous results (Ravnskov and Jakobsen, 1995). The mechanism underlying the reduction in plant growth just above optimum P probably includes both effects of P on root growth and direct effects on the fungi (Cardoso et al., 2006). Increase in P supply may decrease the availability of organic substrates from roots to fungi. Azcon et al. (2003) reported that low P concentration in lettuce plants allowed the maximum colonization and occurrence of AM fungi. Koide (1991) showed that P levels influenced AM colonization. Addition of P fertilizers above optimum delayed and/or inhibited AM infection (de Miranda et al*.,* 1989; Baon et al., 1992).

Several other authors have reported that mycorrhizal roots are able to absorb several times more phosphate than non-inoculated roots from soils and from solutions (Nielsen, 1983; Fitter, 1988). Increased efficiency of phosphorus uptake by mycorrhizal plants could have led to higher concentrations of P in the plant tissues. The greater phosphate absorption by AMF has been suggested to have arisen due to superior efficiency of uptake from labile forms of soil phosphate, which is not attributable to a capacity to mobilize phosphate sources unavailable to non mycorrhizal roots (Pearson and Gianinaazzi, 1983). Mycorrhizal roots are known to have not only a considerably greater phosphate inflow rates, but also to possess a pathway of phosphate uptake with a much higher affinity for phosphate than non mycorrhizal roots.

In our study, maximum root colonization and spore count per 50 cm^3 sand was observed at P concentrations ranging from 0.005 to 0.02 mg g^{-1} in plants infected by AM fungi and effectiveness decreased with increasing P concentration. Our results support reports by Kahiluoto et al. (2000) who observed that with increasing P supply, there was a decrease in the colonization and the effectiveness of mycorrhizal colonization. The results are also in agreement with many reports which suggest that addition of phosphate fertilizers above optimum levels results in a delay in infection and reduced chlamydospore production by AM fungi (Koide, 1991; Thingstrup et al., 1998).

In general, the trees studied are fast growing plants, requires more nutrients during the initial stage of seedling establishment. During this period, the root system is not well developed and the AM fungal symbiosis might play a vital role by supplying the nutrients to the host plant (Muthukumar and Udaiyan, 2006). The results of present study showed that mycorrhizal inoculations increased the plant growth and P uptake in different treatments with a few exceptions. This can be due to increase in the sand volume explored for nutrient and water uptake by the mycorrhizal plants from the medium as compared to non-mycorrhizal plants. Our results support previous studies.

The high rate of P fertilizer application, that is, 0.05 and 0.1 mg g^{-1} lead to antagonistic inhibition of mycorrhizal colonization whereas in lower dose with application of the vigorous AM fungi *Rh. intraradices*, was able to increase the root colonization and spore density significantly. However, increased P supply increased some growth parameters connected to plant height, shoot and root dry weight. Thus, soil amendment with AM fungi have the potential to possibly reduce the application of phosphorus fertilizer for crop improvement, growth, yield and nutritional value of the perennial crops and shade trees in Sidama agroforestry.

Our results indicated that inoculating plants with a suitable AM inoculant could result in a benefit comparable to high P input. However, extrapolation of the results to the real conditions of agroforestry systems should be done with precaution because of differences in the substrate used, i.e., sand in the present study. The information on minimum P concentration for better performance of AMF in the agroforestry can form the basis for further pot/field experiments involving integration of chemical fertilizers with AM fungi.

Conclusion

The present study demonstrated that inoculation of multipurpose shade trees with *Rh. intraradices, Rh. clarus* and mixture inoculums of the two, increased all plant growth parameters, but at the same time decreased percentage of mycorrhizal colonization and spore density as the concentration of P increased. Thus, soil amendments with AM fungi have the potential to possibly reduce the application of phosphorus fertilizer for tree and crop growth and improvement in agroforestry. However, in order to come up with more accurate and

reliable information on functional efficiency of the AMF species applied, further pot and field experiment should be carried out.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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