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Role of clover species and AM Fungi *(Glomus mosseae)* on forage yield, nutrients uptake, nitrogenase activity and soil microbial biomass

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The effects of earthworms (Ew), arbuscular mycorrhizal (AM) fungi and mixed cropping systems on nitrogenase activity of rhizosphere free-living bacteria, soil microbial biomass C (MBC) and growth of clovers were studied in various mixed cropping ratios of 1:0, 3:1, 1:1 and 1:3 berseem clover (*Trifolium alexandrinum* L.) to Persian clover (*Trifolium resupinatum* L.). AMF *G. mosseae* and cropping system gave significantly affected on total forage yield. Mixed cropping gave a greater stability of yield over monoculture. Although *G. mosseae* application increased mycorrhiza colonization rate but there was no obvious effect of clover ratios on mycorrhiza colonization rate. The greatest P-uptake was in the berseem clover:Persian clover ration treatment of 3:1. N-uptake accumulated in above-ground biomass of 173.64 kg ha^{-1} obtained from mixed cropping BP (3:1) with the presence of *G. mosseae*. AM fungi *G. mosseae* increased microbial biomass from 260.2 to 459.2 mg C kg^{-1}. The greatest amounts of 404 mg kg^{-1} soil microbial biomass C was found in the 1:1 ratio of berseem clover to Persian clover. With AM *G. mosseae* inoculation, the greatest nitrogenase activity of rhizosphere free-living bacteria was a 1:1 ratio of berseem clover to Persian clover.

Key words: Mycorrhiza, mixed cropping, soil microbial biomass, C nutrient uptake

Introduction

Soil quality, being an intricate interaction of chemical, biological, and physical components of the soil system, is indicated by several key factors, which are influenced by soil management practices Suman *et al*., (2006). In Iran as some parts of world, tragically soil quality of farms declined by intensive agriculture. This study tried to measure potential soil biological indicators in different intercropping systems to improve soil quality as well as

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stability crop production. Berseem clover (*Trifolium alexandrinum* L.) and Persian clover *(Trifolium resupinatum* L.) where are annual leguminous forage or cover crop species well adapted to semi-arid conditions of the Mediterranean areas. They are high-yielding, nutritious, cool-season forage crops Knight, (1985) grown in pure stands or in mixtures with annual grass species for overwinter grazing and for harvested forage in spring (Marschner and Timonen, 2004; Martiniello, 1999; Stringi *et al*., 1987).

Intercropping gives a greater stability of yields over monoculture Willey and Reddy. (1981). Morever, mixed or intercropping is widely practiced by indian farmers because it often gives higher cash returns and the total production per hectare than monoculture and ensure greater resource use efficiency (Herrara and Harwood, 1974).

Providing a direct physical link between soil and plant roots, the arbuscular mycorrhiza (AM) fungi are important rhizospheric microorganisms. They can increase plant uptake of nutrients especially relatively immobile elements such as P, Zn and Cu (Tinker and Gildon, 1983) and consequently increase root and shoot biomass and improve plant growth. The effect of AM fungus on soil microbiological properties recorded in the literature is inconsistent (Hodge, 2000; Johansson *et al*., 2004). There are many positive (Langley *et al*., 2005; Van Aarle *et al*., 2003; Wamberg *et al*., 2003); negative Langley *et al*. (2005) and Lo´pez-Gutie´rrez *et al*. (2004) or Kim *et al*. (1998) effects interactions between AM fungus and soil microorganisms have been detected, depending on other factors such as AM inoculum type, plants species, the plant growth stage, the kinds of hyphae produced, the residence time of hyphal residues.

Recent research revealed a third interaction, mixed cropping (MC) system, that may significant in terms of overall mycorrhizal colonization rate, soil microbial biomass C, N-uptake and plants growth of different crops.

Materials and methods

Study area and soil property:Field experiments were conducted at the Seed and Plant Improvement Institute, Karaj in 2006. Karaj is located in the northern of Iran (54 º50_ N, 55 º35_ W and 1312 m above sea level). The mean monthly Temperature ranges from 24.7ºC in August to 5.1 C in January and the mean annual rainfall is 262 mm. Most rain falls from November to April. The properties of soil are shown in Table 1.

The experimental design was a factorial experiment in complete randomize design with 3×6 in three replications. The treatments were factor A include: with or without AM *G. mosseae* and the factor B include 5 cropping system, stand ratios of 1:0, 3:1, 1:1, and 1:3, 0:1 of berseem clover to Persian clover (BP). Each stand ratio treatment contained 84 plants. The mixed cropping design was based on the replacement principle.

Our previous research (Unpublished) examined correlations between seeding densities and plant stands in both field and greenhouse environments for two years. Plant stand establishment was recorded approximately 70 and 40% of the seeding rate for Persian and berseem, respectively. Clover seeding rates were based on this research, but ultimately seedlings were thinned to 84 plants m^{-2} in the appropriate ratio.

Mycorrhiza inoculum

Mycorrhizal fungus inoculums consisted of spore, hyphae and jowar root fragment from a stock culture of *Glomus mosseae*. The inoculated (115 spores cm^{-3} of inoculum) dosage was 60 g of inoculums m^{-2}. Since mycorrhizal spore propagules extracted from the native soil were extremely low (1–2 per kg), no attempt was made to fumigate the soil. The mycorrhiza colonization rate of both clovers species was assayed before the first cutting. The mycorrhiza colonization assessment was carried out using the method described by Brundrett *et al*. (1996). Root were stained in trypanblue, and mycorrhiza colonization levels determined using the gridline intersect method of Giovanetti and Moss (1980).

Plant sampling

Plant samples were cut from an inner plant area of 2 m^2 by hand at 5 to 7.5 cm above soil level. Shoot samples were oven dried at 70 ˚C until daily checks indicated no further decreases in weight. Dried samples were weighed, and allowed to remaining as green manure on each plot. Dried herbage was ground to pass throw a 0.5-mm screen and analyzed for N concentration Brundrett *et al*. (1996).

Acetylene reduction assay (ARA)

The nitrogenase activity of rhizosphere of free-living bacteria in soils was studied using the acetylene reduction technique. Whole-rhizosphere soil of three to eight plants from each treatment were sampled late in the afternoon, and placed in vessels stopped with Suba-Seals. The vessels were evacuated to 40 mmHg (ca. 5.3 kPa) and flushed four times with argon; 10% of this gas mixture was then removed and replaced with acetylene. The vessels were incubated at 28 to 30°C, and 1.0-ml samples were assayed after 48 hours for ethylene by injection into a Hewlett-Packard gas chromatograph with a Poropak R column (Shimadzu, GC-148B, Japan). Ethylene production per hour was finally related to the dry weight of the soil.

Microbial biomass determination

The method of Jenkinson (1988) was used to MBC after regrowth harvesting. Briefly, each soil was weighed into duplicate beakers and fumigated with ethanol-free chloroform at 25 C for 24 hours. After removal of chloroform vapour by repeated evacuation, the soils were inoculated with 1% unfumigated soils and placed in 1.5-L Mason jars. This was undertaken following the method of Vance *et al*. (1987). The soils were then extracted with 0.5 M K_2SO_4. Controls were prepared by extracting soil without fumigation. The soil suspension was filtered through aWhatman no. 42 filter paper (Whatman Ltd., UK). Total organic C content in the soil extracts was measured with a dichromate digestion method. Microbial biomass C was calculated from the difference in extractable organic C between the fumigated and unfumigated soil, as follows:

MBC - 2.64 *F*EC [Amora-Lazcano *et al*., (1998)]

Where FEC refers to the difference in extractable organic C between fumigated and unfumigated treatments; and 2.64 is the proportionality factor of MBC released by fumigation extract- radiation (Vance *et al*., 1987).

Data analysis

All measured variables were assumed to be normally distributed and statistical analyses by ANOVA were performed using SAS software (SAS, 1990). The significance of difference between treatments was estimated using the LSD range test with a 0.05 if a main effect or interaction was significant.

Results

Mycorrhiza colonization rate and plant biomass

Analyses variance of various treatments on plant and soil characters are shown in Table 2. Clover plants were colonized in all treatments that were inoculated with AM fungi *G. mosseae* (43%). Non-inoculated treatments (control) registered only 5–6% colonization. There was no obvious effect of clover ratios on mycorrhiza colonization rate.

The yield of clovers was expressed as dry weight for various treatments at each (Anil *et al*., 1998) cutting as well as the sum of total dry weight. Clover ratio and AM fungi had significant ($P \leq 0.05$) effect on total forage yield (Table 2). Total forage yield was greatest with a 1:1 ratio of berseem to Persian clover (Table 3).

Nutrient uptake

The main effects of AMF treatment and CS on P uptake were statistically significant (*P≤0.01*) (Table 2). Shoot P uptake in treatments with AMF gave significantly higher than that in control. AMF *G. mosseae* increased P-uptake of 116.4 to 117.8 µ g g^{-1}. The main effect of CS on P uptake was significant. P uptake by ratio of 3:1 berseem clover: Persian clover was the highest among treatments (Table 3).

The main effects of AMF treatment and its interactions with CS on N uptake were statistically significant (*P≤0.01*) (Table 2). With present of AMF *G. mosseae*, Clovers in treatments with ratio of 3:1 and 1:1 gave the highest N accumulation (Fig. 1).

Nitrogenase activity

The main effect of AMF and CS and their interactions on NA of free-living bacteria were statistically significant (*P≤0.05*). NA of free-living bacteria was increased by AM and CS ratio of 1:1 berseem clover to Persian clover (Fig. 2).

Microbial biomass

Results indicated main treatment effects of AM fungus and MC on MBC was significant. MBC in mycorrhizal treatments was significantly higher than that in non-mycorrhizal treatments (Table 2). Various ratios of mixed cropping increased MBC.

Discussion

Plant biomass

In agreement with other studies (Anil *et al*., 1998; Evans, 1960; Grimes *et al*., 1983; Kurata, 1986) intercropping improved the growth of both berseem and Persian clovers. Both clover species had a greater herbage dry weight m^{-2}, which may be made both clovers more competitive for light, water and nutrients when grown in the mixture with another clover species. The crops did not experience inter-specific competition. Superiority of fodder yields of berseem clover over Persian clover was perhaps due to a higher number of tillers and branches (data not shown). Despite tiller formation, higher fodder yield obtained in berseem clover than Persian clover was possibly due to more rapid dry matter accumulation in berseem clover. There was not tried before finding yield of Persian/berseem clover as summer crop from this area to give opportunity to compare this result with the other results from literature. But in other area such in Michigan, Shrestha *et al*. (1998) reported total annual forage yield of 5400 Kg ha^{-1} when berseem clover was harvested in two cuttings in the year following spring seeding and In Montana, Westcott *et al.* (1995) reported forage yields from a two cutting system of 7700 Kg ha^{-1}. Clovers exhibit various yield in response to climate and cropping date. However in another study, ratio of 3:1 Persian clover to berseem clover had the highest forage dry matter than the other mixed ratios (Zarea *et al.*, 2008). But in this study there was not significant different among mixed cropping ratios on dry forage yield.

The synergistic symbiosis between plants and arbescular mycorrhizal fungi has been subjected of intensive researches (Clark and Zeto, 2000; Hodge, 2000; Huat *et al.*, 2002; Marschner and Timonen, 2004; Smith and Read, 1997; Van Aarle *et al.*, 2003 and Yu and Cheng, 2003).

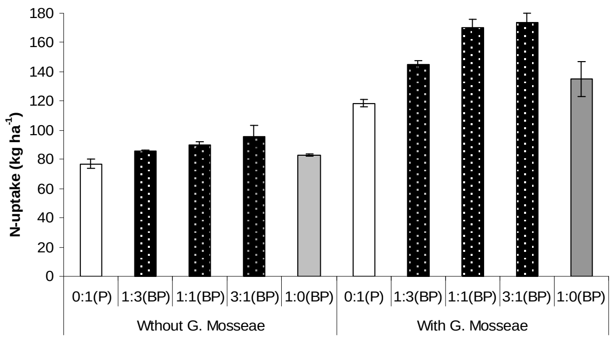

Fig. 1. N-uptake accumulated in above-ground biomass (Kg ha^{-1}) of single or mixed cropping clovers (P and/or B) inoculated or not with *G. mosseae.* Means±s.e., $p<0.05$.

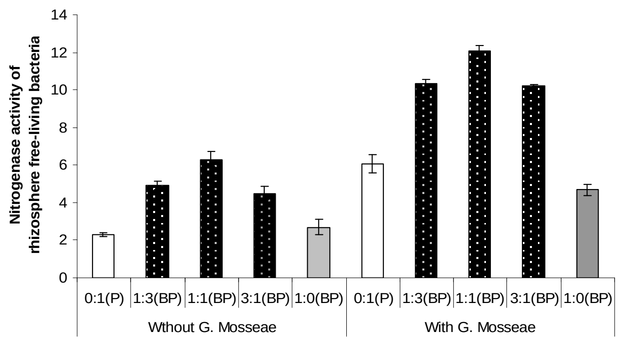

Fig. 2. Nitrogenase activity of free-nitrogen fixer bacteria of single or mixed cropping clovers (P and/or B) inoculated or not with *G. mosseae.* Means±s.e., $p<0.05$.

Shoot N and P uptake

There are few reports on advantage of N uptake by cereal/cereal intercropping system over sole cropping. The explanation of N increase of both clovers species intercropping was not applicable to none legume/ none legume intercropping because that associated with no N fixation. There are closed relationships between yield advantage and nutrient uptake by intercropping species (Morris and Garrity, 1993).

Barea *et al*. (1987) showed that N uptake in plant inoculation with AM was significantly higher than for uninoculation with AM roots. Azco´n-Aguilar *et al*. (1993) reported that mycorrhizal plants have access to forms of N that was unavailable to non-mycorrhizal plants. Benefit effect of AMF on enhancement P uptake reported elsewhere.

Nitrogenase activity

Mycorrhizal treatment clearly led to distinctive increase in the activities of NA. AM fungus could influence microbial activity and biomass by affecting root exudates quantitatively and qualitatively in the hyphosphere. Our findings are in agreement with other studies indicating a higher activity of various soil enzymes in the presence of AM fungus (Kim *et al*., 1998; Rao and Tak, 2001; Wang, 2006). Va´zquez *et al.* (2000) reported higher enzyme activities in the rhizosphere of mycorrhizal plants that may be due to increase in C and nutrient exudation from infected roots. Amora-Lazcano *et al*. (1998) studied the response of N-transforming microorganisms to two different *Glomus* species. The occurrence of autotrophic nitrifying bacteria in pot cultures of sweet corn colonised by the AM fungi *G. mosseae* and *G. fasciculatum* was significantly higher than in nonmycorrhizal cultures, whereas ammonifying and denitrifying bacterial populations significantly decreased in pot cultures of mycorrhizal plants. Evidently, the presence of AM fungi can modify populations of N-transforming microorganisms and these interactions may affect nutrient availability in soils (Amora-Lazcano *et al*., 1998).

Mixed cropping increased NA of free-living bacteria. Potential benefits of intercropping increased in yields, protein and forage quality, N contributions from legumes, greater yield stability, and reduced diseases Anil (1998). Intercropping of berseem clover with Persian clover may affect root exudates quantitatively and qualitatively in the rhizosphere and in return activity of free-living bacteria.

Appears that clover species revealed different mechanisms to interact with nitrogenase activity and nutrient uptake to the cropping system and AM Fungi. This study has shown that cropping system in corporation with AM.

Fungi were able to stimulate shoot growth, P-uptake, N-uptake and NA and this effect depend on clover species and the way both clover species are grown, the cropping system.

Table 3. Mycorrhiza colonization rate(%), Forage yield, P-uptake, nitrogenase activity and Soil biomass C of single or mixed cropping clovers (P and/or B).

Treatments		Mycorhiza Colonization	Forage dry matter Kg ha^{-1}	P-uptake µg g^{-1}	Soil microbial biomass C Mg C kg^{-1} soil
Arbuscular mycorrhiza	+AM	43.50 a	6224.70a	117.8a	459.20a
	-AM	5.12 b	6200.50b	116.40b	260.27b
LSD 0.05%		1.77	23.294	0.68	29.833
Cropping system	B(1:0)	22.80b	6227.60d	117.6ab	334.67b
	B (3:1)	24.8a	6375.60b	118.6a	362.50ab
	BP(1:1)	24.8a	6617.60a	116.2c	404.00a
	BP(1:3)	25.1a	6289.60c	116.6bc	361.67ab
	P(0:1)	24.0b	5552.60e	116.45c	335.83b
LSD 0.05%		2.8	36.83	1.07	47.17
Arbuscular mycorrhiza× Cropping system		NS	NS	NS	NS

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Table 1. Main characteristics of the soil used for the pot experiment.

pH (0.01 M CaCl2)	Organic matter (%)	total N (%)	available P(0.5 mol/L NaHCO3) mg/kg	Bacteria (CFU) (g^{-1} soil× 10^5)	MPN of N_2 fixers (g^{-1} soil×10^3 cells)	Populations of *Azotobacter* (g^{-1} soil×10^{-2} cells)	No of earthworms (depth in 5 to 35 cm m^{-2})	Mycorrhizal spore propagules (no. kg^{-1} soil)
7.1	1.08	0.027	12.21	21.9	18.7	5.2	2.7	2

Table 2. Analyses variance of mycorrhiza colonization rate%, forage dry matter yield, nitrogenase activity, shoot N-P uptake and Soil biomass C of single or mixed cropping clovers (P and/or B).

Treatments	df	Mean Square					
		Mycorhiza Colonization	Forage dry matter	P-uptake	Nitrogenase activity of free- N fixation bacteria	N-uptake	Soil microbia biomass C
Block	2	7.39	10876.2**	2.71*	2.4	178.1*	3469.23
Arbescular mycorrhiza	1	11078.4**	4392.2*	16.13**	155.04**	28280.02**	296808.53**
Cropping system	4	5.56	948521.9**	6.32**	33.71**	1446.9**	4755.71*
Arbescular mycorrhiza× Cropping system	4	12.7	500.0	1.15	4.07*	445.83**	100.78
Error	1	5.33	921.9	0.79	1.31	30.13	1512.27
C.V.	-	9.48	0.48	0.75	7.8	4.69	10.8

- significantly ($P \leq 0.05$)
- ** significantly ($P \leq 0.01$)

Nitrogenase; Microbial activity; Soil enzymes; Mixed cropping; Glomus mosseae. Abstract Forage legumes are used to enhancement soil fertility of the agro ecosystem.Â Inter-specific facilitation of P-uptake by intercropped species has also been reported (Inal et al., 2007). These effects on P-utilization are related to the release or activation of enzymes (e.g. like acid phosphates) and root exudation of carboxylates, phosphatases, and phytase under P deficiency which improve solubility and uptake of P in rhizosphere (Neumann and Romheld, 1999; Inal et al., 2007).Â Soil enzymes are derived primarily from soil fungi, bacteria, plant roots, microbial cells, plant, and animal residues, etc. Plant and Soil. 233, 269-281.Â AM fungi ameliorates growth, yield and nutrient uptake in Cicer arietinum (L.) under salt stress. Russ. Agri.Â Arbuscular mycorrhizal fungi increased growth, nutrient uptake and tolerance to salinity in olive trees under nursery conditions. J. Plant Physiol DOI: 10.1016/j.jplph.2009.02.010. Quilambo, O.A., (2000). Functioning of peanut (Arachis hypogaea L.) under nutrient deficiency and drought stress in relation to symbiotic associations. PhD thesis. University of Groningen, the Netherlands.Van Denderen B.V., Groningen. ISBN 903671284X. Rabie, G.H., (2005). Influence of arbuscular mycorrhizal fungi and kinetin on the response of mungbean plants to irrigation with seawater. Mycorrhiza. 15, 225-230. The AM inoculated plants showed positive effects on plant growth, dry biomass production, chlorophyll content, mineral uptake, electrolyte leakage, proline, protein content and yield of mungbean plants in comparison to non-mycorrhizal ones but the extent of response varied with the increasing level of salinity.Â Role of mycorrhizal symbiosis in growth and salt Avoidance of pistachio plants.Â In the light of the above, the present study was carried out to study the efficiency of dual inoculation of AM fungus (Glomus mosseae and Acaulospora laevis) with Bradyrhizobium japonicum in alleviating the adverse effect of salinity stress of mungbean.Â Both AM fungi were mass multiplied in sterilized soil and sand (3:1) substrate using maize as a suitable host in polyhouse conditions.