Effect of cropping systems and arbuscular mycorrhizal fungi on soil microbial activity and root nodule nitrogenase

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Nitrogenase; Microbial activity; Soil enzymes; Mixed cropping; Glomus mosseae

Abstract Forage legumes are used to enhancement soil fertility of the agro ecosystem. Understanding effect of them on agro ecosystem soil status during when these legumes growing and after that is essential. In one experiment the effects of inoculation with the arbuscular mycorrhizal fungi (AMF), Glomus mosseae, and mixed cropping systems (MCS) on forage biomass yield, nitrogen production, nitrogenase activity and after harvesting on soil microbial activity were studied at various mixed cropping ratios of berseem clover (Trifolium alexandrinum L., B) to Persian clover (Trifolium resupinatum L., P) (B:P = 1:0, 3:1, 1:1, and 1:3). In the second experiment, the effect of treatments on soil microbial activity were studied by soil collection after clover harvesting and 8-week soil incubations in the laboratory. MCS had positive effects on root and shoot dry weight. The effects of AMF on plant yield were positive. AMF affected the fraction root and the vertical root distribution. Plants colonized by AMF showed shorter roots than control plants. At cut 1, with the AMF colonization, the greatest nitrogenase activity (79.61 μmol C2H4 g dwt−1 h−1) of root nodule was observed with B:P = 3:1. At cut 2, the Persian clover plants colonized by G. mosseae in the mixed crop (1:3) had a higher nitrogenase activity (77.38 μmol C2H4 g dwt−1 h−1). The greatest...
Soil enzymes are derived primarily from soil fungi, bacteria, plant roots, microbial cells, plant, and animal residues, etc. (Brown, 1973; Cao et al., 2003; Tarafdar and Marschner, 1994) and play a significant role in mediating biochemical transformations involving organic residue decomposition and nutrient cycling in soil (Martens et al., 1992; McLatchey and Reddy, 1998). Land management and utilization methods, crop species and cultivation systems, etc. can affect soil enzymatic activity. Thus, this parameter can be used as a sensitive index to reveal changes of soil quality due to land management, and to monitor soil microorganism activity related to soil nutrient transformation. Because all biochemical transformations in soil are dependent on, or related to the presence of enzymes, studying soil enzyme activities provides insight into biochemical processes in soil and may also reflect the differences in microbial dynamics and population.

AM-induced changes in plant physiology affect the microbial populations, both quantitatively and qualitatively, in either the rhizosphere and/or the rhizoplane. Therefore, the rhizosphere of a mycorrhizal plant can have features that differ from those of a non-mycorrhizal plant (Barea et al., 2002; Johansson et al., 2004). Soil microbial activity has been shown to depend on the presence of AM fungi and plant species (Wamberg et al., 2003; López-Gutierrez et al., 2004; Baligar et al., 2005; Marschner and Timonen, 2006). The AM symbiotic status changes the chemical composition of root exudates, while the development of an AM soil mycelium, which can act as a carbon source for microbial communities, introduces physical modifications into the environment surrounding the roots (Barea et al., 2005).

Berseem clover (Trifolium alexandrinum L., B) and Persian clover (Trifolium resupinatum L., P) are annual leguminous forage or cover crop species, well adapted to the semiarid conditions of 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 (Martiniello, 1999; Stringi et al., 1987). They can be grazed or cut for fodder 2–5 times a year, from late autumn to late spring in average seasons (if sown in early March), up to six times when irrigated, and twice as a summer crop (Zarea et al., 2008).

We report here the results of an investigation effect of AMF, Glomus mosseae, colonization and MCS on forage biomass yield, nitrogen production and root nodules nitrogenase activity and then, after harvesting the clovers, on soil microbial activity collected at various mixed cropping ratios of berseem clover to Persian clover. We have chosen an intercropping system including two N-fixing (legume) crops instead of a system based on a fixing (legume) crops instead of a system based on a fixing (legume), as the model in designing agricultural systems, to break down current intensive cropping system, wheat–wheat, that is prevalent under semi-arid areas. For better nutrient management in semi-arid areas, an increased use of the biological potential is important. It is
shown that different plant species can influence the composition and number of soil microorganisms due to variations in the quantity and quality of root exudates (Curl and Truelove, 1986; Bowen and Rovira, 1991). Bardgett et al. (1998) and Wardle et al. (1999) have reported that the abundance, activity, and composition of soil decomposer communities may vary markedly with different plant species, or specific functional plant groups such as legumes. Experiments of mixed versus monocultured plant species have demonstrated that plant mixtures can produce greater plant biomass (Forrester et al., 2004), and improve nutrient cycling and soil fertility (Montagnini, 2000). The increase in plant biomass in the mixture may have strong effects on soil microbial community. In most terrestrial ecosystems microbial biomass and growth have been shown to increase with increasing carbon input (Spehn et al., 2000).

The hypotheses of this research were that: (1) the agro ecosystem will more affected by intercropping (Persian and berseem clover); (2) AMF will have different effects in each intercropping system; and (3) effect of intercropping after harvesting may have different effects on soil properties.

2. Materials and methods

2.1. First experiment

2.1.1. Site description

Field experiments were conducted at the research farm of the Seed and Plant Improvement Institute, Karaj (54°50’N, 55°35’W, and 1312 m above sea level), Iran, during the dry season. The experimental sites were located in a semiarid region. The rainfall is restricted to five and a half months a year, from November to February, with negligible rainfall during spring and no rainfall in summer (May–August). The average maximum temperature from May to July is very high (26.5–44 °C), with a mean of 27 °C. In October, temperature reaches a maximum of 48 °C in August, and falls to a minimum of 12 °C. The soil at the site is classified as medium black, clayey, and shallow (15–20 cm in depth). Table 1 shows the physical and chemical analysis of soil across the locations before the experiment.

2.1.2. Treatments

The experimental design was a split-plot design with three randomized complete blocks, with the main plot treatments with or without AMF (G. mosseae) inocula, in three replications. The subplot treatments included five cropping systems (CS): stand ratios of 1:0 (84:0 plants m⁻²), 3:1 (63:21 plants m⁻²), 1:1 (42:42 plants m⁻²), and 1:3 (21:63 plants m⁻²) 0:1 (0:84 plants m⁻²) of berseem clover to Persian clover (B:P). The clover density was 84 plants m⁻². Plots with AM inoculum received 60 g m⁻² of mycorrhizal inoculum, (an average of 115 spores g⁻¹ soil and root fragments with 85% of colonized roots length), applied and incorporated below the soil surface (seed bed).

Berseem and Persian clovers were hand seeded at a depth of 1.0–1.5 cm by surface broadcasting and the seed was incorporated by raking. The spring conditions were very dry, so the plots were irrigated before and after seeding. The plots were hand weeded. Our previous research (unpublished) examined the correlations between the seeding densities and plant stands in both field and greenhouse environments for 2 years. The field establishment rates for the Persian and berseem clover seeds were 40% and 70% of the seed sowing rate, respectively. The clover seeding rates were based on these data, but ultimately, the seedlings were thinned to 84 plants m⁻² in the appropriate ratios. The plots, 3 m × 6 m in size, were separated by heavy gauge polyethylene barriers buried to a depth of 0.5 m into the soil, which stood (initially) 10–15 cm above the ground. The sowing date was May 18. The necessary plant protection, irrigation, and other management practices were followed during crop growth. No pesticides were applied. The clovers were grown with irrigation, given at 10-day intervals, when water to a height of 65 mm was applied. No serious incidence of insects or diseases was observed.

The AMF inoculum of G. mosseae was purchased from the Agricultural and Biotechnology Research Institute, Iran, and was a pure culture of G. mosseae isolated from Karaj (Iran). It was selected because it was commercially available in Iran and had been reported to increase the growth of some clover species. The mycorrhizal fungus inocula consisted of spores and hyphal root fragments from a stock culture of G. mosseae. Mycorrhizal inoculum was multiplied in an open pot culture of maize and consisted of soil, spores, hyphae, and AM root fragments. The field of experiment was fallow for 2 years before the experiment, and we also used a wet sieving technique to extract the spores at the initiate the experiment. We also used the most probable number (MPN) test to determine the number of propagules (mL⁻¹) in the original sample. Because the mycorrhizal spore propagules extracted (1–2 per kg) with the wet sieving technique and the MPN of the propagules (0.018 cm⁻³) from the native soil were extremely low, no attempt was made to fumigate the soil. The mycorrhizal colonization rate (%) of both clover species was assayed before the first and second cuttings.

2.1.3. Measurements

Crops were harvested twice by sickle at ground level, once before corn were sown and twice before wheat were sown in this area. The first cut, regrowth harvesting (cut 2), and total aboveground biomass yields were recorded. At cut 2, the observations on root dry matter development were extended by taking 10 soil cores of 30 cm deep with a diameter of 5 cm to assess root dry matter. The soil cores were cut in three

<table>
<thead>
<tr>
<th>Soil property</th>
<th>Caution exchange capacity (CEC)</th>
<th>pH (CaCl₂)</th>
<th>Organic matter</th>
<th>Soil chemical</th>
</tr>
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<tr>
<td></td>
<td>8.4–11.0</td>
<td>7.1–7.8</td>
<td>1.08–1.09%</td>
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<td>P (mg kg⁻¹)</td>
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<td>K (kg ha⁻¹)</td>
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<td>Fe (mg kg⁻¹)</td>
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<td>270</td>
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<td>Mycorrhizal spore propagules (kg⁻¹ soil)</td>
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<td>2–3.7</td>
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parts of 10 cm each and pooled layer wise. Soil was washed out until clean roots remained. The roots were dried for 24 h at 70 °C. Samples were cut from an inner plant area of 2 m² at 5–7.5 cm above the soil level and the clover biomasses were separated by species. Shoot samples were oven dried at 70 °C until daily checks indicated no further reduction in weight. The dried samples were weighed and allowed to remain as green manure on each plot. The dried herbage was ground, passed through a 0.5 mm screen, and analyzed for N concentration (Barua and Barthakur, 1997).

The mycorrhizal colonization assessment was performed with the method described by Brundrett et al. (1996). We sampled 100:0, 75:25, 50:50, 25:75, and 0:100 intersections per root cluster, for a total of 100 intersections per sample of B:P plants from each treatment. The samples were washed in distilled water, stained with trypan blue, and the mycorrhizal colonization levels determined using the gridline intersect method of Giovanetti and Mosse (1980).

2.1.4. Acetylene reduction assay (ARA)
Acetylene reduction assay (ARA) assessment was performed with the method described by Alef and Kleiner (1995). NA on roots nodules was studied by the acetylene reduction technique at cuts 1 and 2. Briefly, 100:0, 75:25, 50:50, 75:25, and 0:100 number of root nodule of the B:P was placed in vessels stopped with Suba-Seals and placed in the incubation system. Thirty nodulated root samples of both clover species, each with 3–4 mature nodules, were used. Each nodulated root sample was excised from lateral roots by carefully cutting with sharp scissors late in the afternoon, leaving a sufficient length of root to avoid damage to nodules. The nodulated root samples were washed in distilled water, dried, and placed in three separate gas-tight vials each containing 10% acetylene, whilst a third nodulated root sample was placed in a vial containing no acetylene to account for root ethylene production. In addition, control samples were taken, containing only roots without nodules in order to detect any non-nodule related acetylene-reduction activity by the roots. Vials containing only air and ethylene were also used to ensure the absence of ethylene in the acetylene gas. Samples were assayed after 24 h 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 root nodules. These observations were replicated three times in three weekly samplings. Nodule dry weight was determined after oven-drying for 24 h at 70 °C. Moles of ethylene produced per gram of nodules per hour of incubation period was calculated as the measure of NA.

2.2. Second experiment

2.2.1. Treatments and soil sampling
After harvesting the clovers, effect of MCS and AMF inocula on soil enzymatic was assayed. To investigate the effects of first experiment treatments on soil biological activity, soil samples of all plots were obtained from the same 0–30 cm depth. Bulk densities were not significantly different (1.16 ± 0.035) among the different plots. Therefore, treatments consisted of split-plot design with three randomized complete blocks, with the main plot treatments with or without G. mosseae inocula. Samples of soil (5 kg) were kept separately in 750 ml plastic containers in the laboratory. Soil water content of treatments soil samples was at 30% (dry weight basis). Soils were not air-dried and stored with this moisture content. Soils stored for about 8 weeks before being sieved through a 2 mm mesh, to remove organic debris and gravel, and to destroy large aggregates. For each soil, there were three replicate containers for the various MCS.

2.2.2. Soil analysis
Substrate-induced respiration (SIR) was determined by adding 5 ml of glucose solution (32 mg/ml) to 25 g subsamples of soil had been air-dried and placing them in 1 l jars for 6 h. The carbon dioxide (CO₂) respired during this period, was trapped in 20 ml 0.02 M NaOH, and subsequently measured by titration with HCl to a phenolphthalein endpoint after adding excess BaCl₂ (Anderson, 1982). The activity of the ALP and ACP enzymes were measured by the methods described by Tabatabai (1994). Briefly, a fresh soil sample of 1 g was placed in 50 ml test tube, to which one drop of tolune and 4 ml of modified universal buffer (pH 11 for ALP and pH 6.5 for ACP) was added. The samples were incubated with p-nitrophenyl phosphate at 37 °C for 60 min. After filtration, the yellow color intensity was measured by spectrophotometer at 420 nm. The enzyme activities were expressed as mg p-nitrophenol (PNP) released per gram soil within 1 h. The measurements were carried out in three replicates and the activity of enzymes was averaged for all three replicates. Dehydrogenase activity was determined according to Garcia et al. (1997). For this, 1 g of soil was exposed to 0.2 ml of 0.4% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 °C in darkness. The INTF (iodo-nitrotetrazolium-umformazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtering though a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

2.3. Data analysis
The analysis of variance (ANOVA) for both experiments was based on the common mixed model for a split-plot randomized complete blocks design. All measured variables were assumed to be normally distributed and statistical analysis by ANOVA was performed using SAS software (1990). The normal distribution of the data was determined using the Shapiro–Wilk W test. The significance of the differences between treatments was estimated using the LSD range test, and a main effect or interaction was deemed significant at P ≤ 0.05. Linear regressions were carried out using Spearman correlation coefficient.

3. Results

3.1. Mycorrhiza colonization value
Clover plants were colonized after all treatments involving inoculation with AMF. The percentage mycorrhizal root colonization was significantly (P ≤ 0.01) greater in all the mycorrhizal treatments than in the non-inoculated controls. Non-inoculated treatments (control) had only 4–7% colonization from indigenous mycorrhizal fungus. However by harvesting-time, results showed that both clovers species colonized by G. mosseae had significantly better performances on growth parameter when compared to the control treatment colonized
than the berseem monoculture (Table 2). Biomass production by indigenous mycorrhizal fungus which may relate to the extremely lower of spore propagules indigenous mycorrhizal fungus. Inoculated treatments \((F = 2366.8, P < 0.0001)\) had higher mycorrhizal colonization. The MCS had no effect on the mycorrhizal colonization value. Mycorrhizal colonization values of 40.9%, 45.3%, 45.6%, 44.43%, and 42% were observed for ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 intersections root of the BP plants (Fig. 1).

### 3.2. Shoot growth

MCS and AMF had significant positive effects on forage yield (Table 2). There were no direct interactive effects between MCS and AMF on plant yield (Table 2). At cuts 1 and 2, the berseem clover was roughly taller than the Persian clover, regardless of the stand ratio. The average rates of plant height were 15–54 cm (cut 1) and 50–54 cm (cut 2) for Persian clover and berseem clover, respectively. At cuts 1 and 2, the berseem clover and Persian clover monocultures had the highest yields, and the berseem clover monoculture had the highest yield at cut 1 (Table 2). The dry matter yields of Persian clover in mixed crops were lower than the berseem clover yields in monocultures in cut 1, but the total yields of the mixtures were higher than those of the berseem clover monoculture. The total mixed cropping forage yield was highest in MCS, at 3:1 kg ha\(^{-1}\).

At cut 2, the Persian clover monoculture had a higher yield than the berseem monoculture (Table 2). Biomass production of Persian clover was the faster developing at cut 2, mainly due to its relatively lower temperature and higher root accumulation. For the species represented in this study, no clear relation between total dry matter accumulation and maximum height was observed. Persian clover, the species with the higher amount of accumulated dry matter at cut 2, was the shorter species. The berseem clover forage yield was also significantly higher in the monoculture crop (1:0) than in the mixed crops in the second cut (Table 2). The maximum dry matter yield for berseem clover under MCS was obtained for 3:1, followed by 1:1. Among the Persian clover intercrop yields, the dry matter yield of the Persian clover increased with reductions in the berseem clover plants but was significantly lower than that of the monoculture crop (Table 2). The same statement is true for berseem clover since dry matter yield of berseem clover increases with reductions in Persian clover but is lower than of

by indigenous mycorrhizal fungus which may relate to the extremely lower of spore propagules indigenous mycorrhizal fungus. Inoculated treatments \((F = 2366.8, P < 0.0001)\) had higher mycorrhizal colonization. The MCS had no effect on the mycorrhizal colonization value. Mycorrhizal colonization values of 40.9%, 45.3%, 45.6%, 44.43%, and 42% were observed for ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 intersections root of the BP plants (Fig. 1).

### 3.3. Root growth

Total root dry weight, the fraction root and the vertical root distribution in the upper 30 cm of the soil are presented in Tables 3 and 4. Total root dry weight of Persian clover was significantly higher than the total root dry weight of berseem clover (Table 3).

MCS had significant positive effects on root weight production (Table 3). At cuts 1 and 2, the Persian clover monocultures had the highest root weight than berseem clover monoculture (Table 3). At cuts 1 and 2, the dry matter roots of berseem clover in mixed crops were higher than those of that in monoculture (Table 3). At cut 1, the total mixed cropping dry matter roots was highest in MCS, at 1:3 g plant\(^{-1}\). At cut 2, the maximum root dry weight was obtained for 0:1, 1:3, 1:1, and 3:1. The total root dry matters of the mixtures (cuts 1 plus 2) were affected by various ratios of mixtures (Table 3). The B.P ratio of 1:3 had the highest total root dry mass intercrop yield (Table 3). AM fungi had no effect on root dry mass (Table 3). There were no direct interactive effects between MCS and AMF on root biomass production (Table 3). At cuts 1 and 2, the maximum ratio of shoot to root (S/R) was obtained for 1:0 and 1:3, respectively. The B.P ratio of 1:3 had the highest total S/R. The ratio of S/R tended to increase with inculcating clover with AM fungus (Table 3).

The differences in vertical root distribution were observed (Table 4). Persian clover had a higher fraction (0.8) of its root material in the top soil layer (0–10 cm) than berseem clover (0.5). Berseem clover in turn had a higher fraction root dry matter in the lower zone (Table 4). Result showed the differences in vertical root distribution affected by MCS (Table 4). AMF limited vertical root distribution in the upper 10 cm of the soil (Table 4). Non-mycorrhizal plants had higher root distribution in the downer 10 cm of the soil. There were no direct interactive effects between MCS and AMF on root distribution (Table 4).

### 3.4. Nitrogenase activity

The main effects of AM and MCS on NA were statistically significant at both cuts 1 and 2 (Table 5). The NA of root nodule was increased by AM and MCS at B:P = 3:1 and B:P = 1:3 at first cut and second cut, respectively. There was no significant difference between the ratios 1:3 and 1:1 at cut 2 (Table 5). The AMF colonization was correlated with nitrogenase activity (Fig. 2). There was synergism between the two clovers on nitrogenase activity (Table 5). The nitrogenase activity in berseem clover at cut 1 is increased by the presence of a relatively small amount of Persian clover, while at cut 2, nitrogenase activity of Persian clover is increased by a small amount of berseem clover.

### 3.5. Shoot N concentration

The main effects of AMF and MCS treatments and their interactions on N concentration were statistically significant.
Shoot N concentration (23.5 mg g\(^{-1}\)) after treatment with AMF at culture ratios of B:P = 1:1 was significantly higher than that after other treatments (Fig. 3). The clear relationship between N concentration and nitrogenase activity is illustrated in Fig. 4. The nodules on roots systems that containing the higher NA activity was clearly much shoot N concentration than those of nodules on root systems with less-abundant NA activity.

### 3.6. Microbial activity

The main effects of AMF and MCS treatments on organic matter were statistically significant (Table 6). The percent organic matter varied with the MCS and AMF inoculant. The highest percent organic matter (1.43 was found in the %) was found at BP 1:3 and was further increased at AMF inoculant plots (Table 6).

The microbial respiration (SIR), DHA, ALP, and ACP enzymes from the incubated soils of mycorrhizal and non-mycorrhizal plants with varies cropping systems are shown in Table 6. Results indicated main treatment effects of MCS and AM fungus, and their interaction on SIR, DHA, ALP, and ACP enzymes microbial metabolic (Table 6).

The soil obtained from B:P = 1:3 had a much greater SIR (Table 6). There was no significant difference between the ratios 1:3 and 1:1. AMF had significant positive effects on SIR...
There were no direct interactive effects between MCS and AMF on SIR (Table 6). There was higher ALP activity in mycorrhizal treatments regardless of MCS. However, B:P = 1:3 increased the activity of ALP in both non-mycorrhizal and mycorrhizal treatments. The activity of this enzyme was not affected by an interaction of MCS and AMF. The main treatment effects of MCS and AMF and their interaction on the activity of ACP was statistically significant (Table 6). AM fungus increased the activity of ACP, whereas MCS at B:P = 3:1 increased it regardless of AM fungal treatments (Table 6). Results also showed that AMF effect on ALP activity is more striking than on ACP activity (Table 6). There was a beneficial effect of both mixture cropping at B:P = 1:1 and B:P = 1:3 and AMF inoculation on DHA (Table 6).

4. Discussion

4.1. Shoot growth

When plant species are intercropped, it is likely that yield advantages occur as a result of the complementary use of resources by the component crops (Evans, 1960; Grimes et al., 1983; Anil et al., 1998). These results show that the productions of forage berseem clover and Persian clover monocultures were higher than that in the mixed crop cultures. This was partly the result of the greater plant populations in the monocrops. Moreover, these crops were not subjected to inter-specific competition. At the first cut, the superiority of the forage yields of berseem clover over those of Persian clover was perhaps attributable to a higher number of tillers and branches (data not shown). Despite tiller formation, the higher forage yield of the berseem clover relative to that of the Persian clover was possibly because of the more rapid accumulation of dry matter in the berseem clover. Compared with berseem clover, Persian clover generally has limited growth, especially when subjected to high temperatures. The higher temperatures during cut 1 reduced the growth rate of Persian clover compared with that of berseem clover, indicating that berseem clover better tolerates high temperatures. Furthermore, during cut 1, photosynthesis assimilation in Persian clover compared to berseem clover more served in root (data not shown) that help Persian clover regrowth.

At cut 2, the crop experienced inter-specific competition. The yield of the berseem clover was affected by the Persian clover. Although the Persian clover reduced the yield of the berseem clover, an overall benefit was observed when the yields of both crops were considered together (1:3). The aboveground dry matter of the Persian clover regrowth increased more during the growth season because of a higher number of tillers and branches compared with those at cut 1 (data not shown).

<table>
<thead>
<tr>
<th>Table 5</th>
<th>The vertical root distribution (fraction) over the upper 30 cm of the soil.</th>
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<tbody>
<tr>
<td>Treatments</td>
<td>Root distribution</td>
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<tr>
<td></td>
<td>0–10 cm</td>
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<tr>
<td>+ AM</td>
<td>0.79a</td>
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<tr>
<td>– AM</td>
<td>0.74b</td>
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<td>LSD (0.05)</td>
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<tr>
<td>F</td>
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<td>B(1:0)</td>
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<td>B(3:1)</td>
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<td>F</td>
<td>663***</td>
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</tbody>
</table>

Different letters (a–c) within a column indicate significant differences at the 0.05 level; NS: not significant.

* Least significant differences between treatments at P = 0.05.

** Interaction between treatment.

*** F values significant at P < 0.01.

**** F values significant at P < 0.001.

<table>
<thead>
<tr>
<th>Table 6</th>
<th>The activity of nitrogenase from single or mixed cropping clovers (P and/or B) colonized or not with mycorrhiza.</th>
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</tr>
<tr>
<td>– AM</td>
<td>55.84b</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>1.5656</td>
</tr>
<tr>
<td>F</td>
<td>590.31***</td>
</tr>
<tr>
<td>B(1:0)</td>
<td>47.01c</td>
</tr>
<tr>
<td>B(3:1)</td>
<td>79.61a</td>
</tr>
<tr>
<td>BP(1:1)</td>
<td>71.05b</td>
</tr>
<tr>
<td>BP(1:3)</td>
<td>71.58b</td>
</tr>
<tr>
<td>P(0:1)</td>
<td>38.28d</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.01</td>
</tr>
<tr>
<td>F</td>
<td>663***</td>
</tr>
</tbody>
</table>

Different letters (a–d) within a column indicate significant differences at the 0.05 level; NS: not significant.

* Least significant differences between treatments at P = 0.05.

** Interaction between treatment.

*** F values significant at P < 0.01.

**** F values significant at P < 0.001.
The total intercropping yield (cuts 1 and 2) gives an accurate assessment of the greater biological efficiency of the intercropping system. The total intercropping yield values indicate that berseem clover recorded a yield advantage at B:P ratios of 1:3 and 1:1 in the intercropping system, attributed to crop complementarities. This advantage is probably the result of different above- and below-ground growth habits and the morphological characteristics of the intercrop components, allowing their more efficient utilization of plant growth resources, i.e., water, nutrients, and radiant energy (Fukai and Trenbath, 1993). There has been no previous attempt to determine the yield of Persian/berseem clovers as a summer crop in this area, so we cannot compare this result with those of other research in the literature. However, in other areas, such as Michigan, Shrestha et al. (1998) reported a total annual forage yield of 5400 kg ha$^{-1}$ when berseem clover was harvested in two cuttings in a 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 yields in response to climate and cropping dates. However, in another

**Figure 2** Correlation between (a) plant involving inoculation with AMF and nitrogenase activity, (b) plant non-inoculated with AMF and nitrogenase activity at cuts 1 and 2, mycorrhiza colonization rate is square root transformed. Pearson correlation coefficient and its significance is given.

**Figure 3** Shoot N-concentration (mg g$^{-1}$) of single or mixed cropping clovers (P and/or B) inoculated or not with and mycorrhiza. Means ± S.E., $P < 0.05$.

**Figure 4** Correlation between shoot N concentration and nitrogenase activity. Pearson correlation coefficient and its significance is given.
study, a ratio of Persian clover to berseem clover of 3:1 had a higher forage dry matter yield than those at other mixed cropping ratios (Zarea et al., 2008). However, in this study, there was no significant difference in the dry forage yields among the various mixed cropping ratios.

AMF fungi had a positive effect on the forage dry matter. The synergistic symbiosis between plants and AMF has been the subject of intensive research (Smith and Read, 1997; Clark and Zeto, 2000; Hodge, 2000; Huat et al., 2002; Yu and Cheng, 2003; Zarea et al., 2010). This advantage is probably the result of the greater uptake of nutrients, especially N and P or higher shoot-to-root ratio. Negative effects of AM fungi on root growth have been previously observed (Marschner, 1995; Marschner and Crowley, 1996; Khalil et al., 1994, 1999; Smith and Zeto, 2000; Hodge, 2000; Huat et al., 2002; Yu and Cheng, 2003). This advantage is probably attributable to the more efficient competition with weeds for N (data not shown). Although, many reports have indicated better nutrient uptake in intercropping systems, in such studies, one cropping partner is always a legume and the other a non-legume crop. There have been few reports of an improvement in N uptake achieved with a cereal/cereal intercropping system relative to the N uptake in sole cropping. There is a close relationship between yield advantage and the nutrient uptake by the intercropped species (Morris and Garrity, 1993). These results confirm that clover mixtures acquire large amounts of N from atmospheric fixation or the soil when in symbiosis with AMF. This is probably attributable to the more successful competition with weeds for N (data not shown) and the better use of the resources because the AMF symbiosis enhances plant growth and increases the plants’ access to forms of N that are unavailable to non-mycorrhizal plants. This corresponds to the observations of Barea et al. (1987) and Azcón-Aguilar et al. (1993), who found greater N uptake in plants inoculated with AMF.

### 4.3. Shoot N concentration

The better uptake of N in the mixed system is attributed to the low levels of competition for nutrients between berseem clover and Persian clover because the duration of and variations in their rooting and shooting habits differ, especially during cut 1. The competition for resources between berseem and Persian clovers is very low, probably because Persian clover has shallower roots and a lower growth rate at cut 1 (data not shown). Although, many reports have indicated better nutrient uptake in intercropping systems, in such studies, one cropping partner is always a legume and the other a non-legume crop. There have been few reports of an improvement in N uptake achieved with a cereal/cereal intercropping system relative to the N uptake in sole cropping. There is a close relationship between yield advantage and the nutrient uptake by the intercropped species (Morris and Garrity, 1993). These results confirm that clover mixtures acquire large amounts of N from atmospheric fixation or the soil when in symbiosis with AMF. This is probably attributable to the more successful competition with weeds for N (data not shown) and the better use of the resources because the AMF symbiosis enhances plant growth and increases the plants’ access to forms of N that are unavailable to non-mycorrhizal plants. This corresponds to the observations of Barea et al. (1987) and Azcón-Aguilar et al. (1993), who found greater N uptake in plants inoculated with AMF.

### 4.4. Microbial activity

Higher activities of enzymes in soil incubated for 8 weeks from various cropping systems may be related to generally higher organic matter provided through root and external AMF mycelial growth. This is a food source that creates an ideal soil environment for microbial populations and their associated activities; and some of the highest respiration and enzyme activities were noted in these plots. The higher organic matter levels in the BP 1:3 plots also support the beneficial effects of mixture cropping or AMF colonization. An explanation for

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**Table 6** Effects of cropping systems and mycorrhiza on OM, SIR, DHA, phosphatases enzyme activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OM%</th>
<th>SIR (mg C kg⁻¹ soil)</th>
<th>Phosphatase (mg p-nitrophenol g⁻¹ soil h⁻¹)</th>
<th>DHA (µg INTF/g h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ AM</td>
<td>1.5a</td>
<td>5.7a</td>
<td>437.6a</td>
<td>156.5</td>
</tr>
<tr>
<td>− AM</td>
<td>1.1b</td>
<td>4.8b</td>
<td>292.8b</td>
<td>125.0b</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.3</td>
<td>0.2</td>
<td>139.0</td>
<td>23.0</td>
</tr>
<tr>
<td>F</td>
<td>163.39***</td>
<td>114.82***</td>
<td>499.40***</td>
<td>24.24***</td>
</tr>
<tr>
<td>B(1:0)</td>
<td>1.2c</td>
<td>5.0c</td>
<td>326.6c</td>
<td>120.1c</td>
</tr>
<tr>
<td>B(3:1)</td>
<td>1.2c</td>
<td>5.14bc</td>
<td>364.5b</td>
<td>129.4bc</td>
</tr>
<tr>
<td>BP(1:1)</td>
<td>1.3c</td>
<td>5.43a</td>
<td>379.8ab</td>
<td>141.4bc</td>
</tr>
<tr>
<td>BP(1:3)</td>
<td>1.4a</td>
<td>5.62a</td>
<td>391.8a</td>
<td>164.1a</td>
</tr>
<tr>
<td>P(0:1)</td>
<td>1.3ab</td>
<td>5.3ab</td>
<td>363.1b</td>
<td>148.8ab</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.1</td>
<td>0.2</td>
<td>21.7</td>
<td>21.4</td>
</tr>
<tr>
<td>F</td>
<td>1.1b</td>
<td>4.8b</td>
<td>326.6c</td>
<td>120.1c</td>
</tr>
<tr>
<td>Cropping systems vs. AM</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Different letters (a–c) within a column indicate significant differences at the 0.05 level; NS: not significant.

** F values significant at P < 0.01.

* Least significant differences between treatments at P = 0.05.

** Interaction between treatment.

*** F values significant at P < 0.001.
these mixed cropping effects in enzyme activities may involve the different root growth parameters of berseem and Persian clover plants. Previous reports by other workers have suggested root growth differences can be positive regulators of soil microbial biomass (Lynch and Panting, 1980). Dry matter accumulations of Persian clover roots were linear with time. At cut 1, photosynthesis assimilation in Persian clover was greater in roots compared to berseem clover. Root dry matter accumulations were 4–6 times greater for Persian than berseem clover, especially at cut 2 (data not shown). This huge dry matter accumulation of Persian clover roots may increase organic matter levels and subsequently microbial activity. When both clover species were present, different qualitative exudation from roots may activate different groups of microbes from those activated when one clover species is present.

Soil enzymatic activity can reflect the strength of biochemical process in soil, and the rate of decomposition and transformation of organic manure in soil was mainly controlled by soil microbes and enzyme activities (Yang et al., 2008). When soil microbes and enzymes activities were higher, organic manure decomposed and transformed more quickly, and then released more nutrients per unit time. The nutrients from the decomposition and transformation of organic manure can be maintained in an available form in soil, and thus can supply plant growth and development needs (Yang et al., 2008). For example, phosphatase can hydrolyze some kind of organic compounds containing P into inorganic P, which can be absorbed and utilized by plants, thus soil enzyme activity was positively correlated with soil available nutrients. Soil enzymatic activities can indicate the function of soil ecosystem because they can reflect the intensity and direction of various biochemical processes occurred in soil, and also correlate closely with soil fertility (Yang and Wang, 2002; He et al., 2002).

The results of these investigations indicate that MCS and AMF stimulated soil microbial activities, depending on the MCS ratio. The combined effects of MCS and AMF were beneficial to plant growth, N uptake, NA of root nodules, and microbial activity. These values were affected by the ratio of berseem to Persian clover in the mixture and time (8-week soil incubations). In our previous report, the greatest NA of free-living rhizosphere and soil microbial biomass was observed with B:P = 3:1 at cut 2 (Zarea et al., 2009). Therefore, the present research has revealed another factor, time, that appears to be significant in overall effects of AMF and MCS on soil biology, and to agriculture that uses the model in designing agricultural systems.

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References


Effect of cropping systems and arbuscular mycorrhizal fungi on soil microbial activity


