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Interactions between phosphorus availability and an AM fungus (*Glomus intraradices*) and their effects on soil microbial respiration, biomass and enzyme activities in a calcareous soil

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P fertilization

Summary

The interactions between soil P availability and mycorrhizal fungi could potentially impact the activity of soil microorganisms and enzymes involved in nutrient turnover and cycling, and subsequent plant growth. However, much remains to be known of the possible interactions among phosphorus availability and mycorrhizal fungi in the rhizosphere of berseem clover (*Trifolium alexandrinum* L.) grown in calcareous soils deficient in available P. The primary purpose of this study was to look at the interaction between P availability and an arbuscular mycorrhizal (AM) fungus (*Glomus intraradices*) on the growth of berseem clover and on soil microbial activity associated with plant growth. Berseem clover was grown in P unfertilized soil (—P) and P fertilized soil (+P), inoculated (+M) and non-inoculated (—M) with the mycorrhizal fungus for 70 days under greenhouse conditions. We found an increased biomass production of shoot and root for AM fungus-inoculated berseem relative to uninoculated berseem grown at low P levels. AM fungus inoculation led to an improvement of P and N uptake. Soil respiration (SR) responded positively to P addition, but negatively to AM fungus inoculation, suggesting that P limitation may be responsible for stimulating effects on microbial activity by P fertilization. Results showed decreases in microbial respiration and biomass C in mycorrhizal treatments, implying that reduced availability of C may account for the suppressive effects of AM fungus inoculation on microbial activity. However, both AM fungus inoculation and P fertilization affected neither substrate-induced respiration (SIR) nor microbial metabolic quotients (qCO_2). So, both P and C availability may concurrently limit the microbial activity in these calcareous P-fixing soils. On the contrary, the activities of alkaline phosphatase (ALP) and acid phosphatase (ACP) enzymes responded negatively to P addition, but positively to AM fungus inoculation, indicating that

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AM fungus may only contribute to plant P nutrition without a significant contribution from the total microbial activity in the rhizosphere. Therefore, the contrasting effects of P and AM fungus on the soil microbial activity and biomass C and enzymes may have a positive or negative feedback to C dynamics and decomposition, and subsequently to nutrient cycling in these calcareous soils. In conclusion, soil microbial activity depended on the addition of P and/or the presence of AM fungus, which could affect either P or C availability.
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Introduction

Soil microbial activity including soil respiration (SR) and enzyme activities, and the size of microbial biomass, have been shown to depend on P fertilization and the presence of AM fungi in the soil–plant system (Amador and Jones, 1993; Wright and Reddy, 2001; Wamberg et al., 2003; López-Gutiérrez et al., 2004; Baligar et al., 2005; Marschner and Timonen, 2006). Phosphorus fertilization may affect soil microbial respiration and biomass, especially soil enzymes, with variable results depending on the soil P status. Phosphorus additions resulted in increased microbial respiration in soils with low P contents, but not in soils with high P contents (Amador and Jones, 1993; Smith, 2005). In contrast, P fertilization had an inhibitory effect on microbial respiration and substrate-induced respiration (SIR) in a pine forest floor, while no effects on microbial metabolic quotients (qCO_2) were detected (Thirukkumaran and Parkinson, 2000). Increasing levels of soil applied P significantly reduced acid phosphatase (ACP) activities and resulted in lower arylsulfatase and urease activities in acidic infertile upland soils under white clover cover (Baligar et al., 2005). Similarly, application of phosphate decreased activities of phosphatase, sulfatase, and urease (Haynes and Swift, 1988). Higher P concentrations may also depress the activity of some soil enzymes under natural conditions. In a wetland soil, P loading negatively influenced only the activity of alkaline phosphatase (ALP) while other soil enzymes remained unaffected (Wright and Reddy, 2001). The association of plants with mycorrhizal fungi can have strong influences on the responses of microbial activities to P fertilization, due to the fact that these fungi are able to enhance P availability to and uptake by plants (Smith and Read, 1997). Following inoculation, AM fungi may further influence microbial population and activity, and consequently nutrient dynamics in the soil through the release of organic compounds. There are many positive or negative interactions between AM fungus and soil microorganisms (see reviews by Bonkowski et al., 2000; Hodge, 2000; Johansson et al., 2004; Jones et al., 2004). Various studies indicated that AM fungus may alter the population composition (Bansal and Mukerji, 1994; Andrade et al., 1997; Vázquez et al., 2000) and activity of soil microorganism (Wamberg et al., 2003; Langley et al., 2005), likely due to quantitative and qualitative changes in root exudation of colonized plants occurring in the rhizosphere (Hodge, 2000; Barea et al., 2002). For example, root colonization by AM fungi reduced the exudation of sugars, amino acids and other organic compounds from the roots (Graham et al., 1981; Schwab et al., 1984; Bansal and Mukerji, 1994).

SR is one of the least studied microbial processes associated with AM fungi. Yet, the influence of mycorrhizal inoculation on SR is variable, and depends on experimental conditions and the methodology involved. In the rhizosphere of pea plants (*Pisum sativum*) inoculated with *Glomus intraradices*, SR was negatively or positively affected by fungi, depending on the growth stage of the plant (Wamberg et al., 2003). The negative effect was apparently due to the change in carbon flow from plant to fungal hyphae and therefore higher anabolism occurred by fungus (Wamberg et al., 2003). Mycorrhizal inoculation increased microbial SR in sunflower rhizosphere before plant maturity, due to the mycorrhizal stimulation of plant growth (Langley et al., 2005). However, when shoots were removed, mycorrihzal sunflower had lower SR than the corresponding non-mycorrhizal plants. AM fungi may also influence other microbial properties of the soil. Van Aarle et al. (2003) reported an increased microbial biomass and bacterial activity in the presence of AM fungal hyphae in a limestone soil. In contrast, Kim et al. (1998) showed that inoculation of tomato with *Glomus etunicatum* had no effects on total soil microbial biomass C (MBC). In the Entisols and Vertisols under savannas, lower P mineralization and higher microbial immobilization were associated with higher AM fungus colonization (López-Gutiérrez et al., 2004). Therefore, AM fungi may directly (Zhu and Miller, 2003) or indirectly (Langley

et al., 2005) contribute to soil C and N dynamics. It has been suggested that AM fungus could influence C dynamics by transferring a large quantity of plant assimilates to fungal hyphae (Bago et al., 2000), but its significant role depends on other factors such as the kinds of hyphae produced and the residence time of accumulated hyphal residues (Zhu and Miller, 2003). On the other hand, AM fungi produce glomalin (a glycoprotein), which could be a recalcitrant pool of soil C (Wright and Upadhyaya, 1996, 1998; Rillig et al., 2001). It shows resistance to microbial decomposition (Wright and Upadhyaya, 1998; Rillig et al., 2001), and could lower soil microbial activity. The activity of AM fungus in the mycorrhizosphere could be a source of different soil enzymes required for biochemical reactions. There are various reports indicating that soil enzyme activities, such as phosphatases, dehydrogenase, urease, protease and beta-glucosidase are increased by AM fungus inoculation (Kothari et al., 1990; Bolan, 1991; Kim et al., 1998; Vázquez et al., 2000; Caravaca et al., 2003). However, AM fungus had no effect on the activity of soil phosphatases (Van Aarle et al., 2003).

The dilemma is that while P fertilization generally stimulates soil microbial activities, AM fungus inconsistently depress microbial population and activities. Indeed, discrepancies may stem from differences in soil P availability and from variations in the extent of root colonization by the AM fungi, which would impose different carbon demands on the plants. However, relatively little is known of the interactive effects of P fertilization and AM fungus on microbial activity in calcareous soils low in available P (Raiesi, 2006).

It is hypothesized that AM fungus may counteract the stimulating effects of P fertilization on soil microbial processes including respiration, biomass and enzyme activities. The main objective of this study was: (1) to study the interaction between P fertilization and AM fungus on the growth of berseem clover; and (2) to identify the importance of P fertilization and AM fungus interactions on soil microbial biomass and enzymatic activity in the rhizosphere of berseem clover.

Materials and methods

The interaction between P fertilization and the AM fungus (*G. intraradices* Schenk and Smith) on berseem clover (*Trifolium alexandrinum* L.) growth (including biomass production, N and P uptake) and its importance for some soil microbial properties (including respiration, biomass and enzymes) in the highly P-fixing calcareous soils with low C contents is not well established. Hence, a greenhouse experiment was conducted to address the interactive effects of P fertilization and AM fungi on microbial activity in the rhizosphere of berseem clover. A 2×2 factorial experiment consisting of two levels of P fertilization and two treatments of the AM fungus arranged in a randomized complete block design with eight replicates. The P fertilization treatments were P unfertilized soil (−P) having 10 ppm available P and P fertilized soil (+P) having 30 ppm P achieved using KH_2PO_4 salt. The AM fungus treatments consisted of mycorrhizal plant (+M) and non-mycorrhizal plant (−M) as control. The soil selected for this study was taken from the surface layer (0–30 cm) of a clover field in the southern part of Shahrekord, the capital of Chaharmahal va Bakhtiari, Iran. The soil is calcareous developed in limestone with a clay loam texture and low in organic C content (0.39%) and in plant available P (10 ppm). A representative soil sample was air-dried, and passed through a 2-mm sieve for laboratory analysis. Chemical parameters of the 30-cm surface layer are reported in Table 1.

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

pH	8.03
ECe	0.12 dS m^{-1}
CEC	24 cmol(+) kg^{-1}
Organic carbon	3.9 mg g^{-1}
Total N	0.28 mg g^{-1}
P (Olsen's)	10 mg kg^{-1}
$CaCO_3$	410 mg g^{-1}
Sand	240 mg g^{-1}
Silt	460 mg g^{-1}
Clay	300 mg g^{-1}

Experiment description

Plants were grown in plastic pots for 70 days in a greenhouse at Agricultural Research Station in Shahrekord. Before planting, seeds were surface-sterilized with sodium hypochlorite solution for 5 min and rinsed with sterile distilled water. In a growth chamber, seeds were placed on moist filter papers in Petri dishes and germinated in the dark at 25 °C. Five 3-day-old seedlings of uniform size (1.5–2 cm) were transferred into plastic pots (30-cm diameter × 30 cm depth) containing 4 kg of autoclaved field soil-washed sand mixture (1.5:1 ratio). After transplant, plants were inoculated with the AM fungus *G. intraradices*, in the form of a

mixture of spores, soil, external mycelium and root fragments obtained from pot cultures. Twenty grams of inoculum (ca. 250 g per pot) were placed in each planting hole about 1 cm below the roots. The plants were inoculated with an effective strain of *Rhizobium leguminosarum* bv. *trifolii* 7 days after transplanting and irrigated with distilled water. The average air temperature in the greenhouse was 25–30 °C. Plants were grown under natural light. At the end of experiment, plants were harvested and separated into roots and shoots. Shoot materials were dried at 60 °C for 2 days to determine dry weights. Roots were hand-washed to remove soil particles, then dried at 60 °C for 2 days and weighted. The ratio of shoot to root was calculated for each treatment. Roots were washed and preserved in 50% ethanol for determination of the extent of mycorrhizal root colonization. The percentages of root length colonized (RLC) by mycorrhizal fungi (% colonization) and plant RLC was estimated by the grid-line intersect method (Giovanetti and Mosse, 1980) after clearing the root systems with 10% (w/v) KOH at 70 °C for 4 h and staining with trypan blue (Phillips and Hayman, 1970; Koske and Gemma, 1989). Shoot biomass was analyzed for the P and N concentrations using the methods described by Baruah and Barthakur (1997). Finally, soil samples were collected and stored at 4 °C for the determination of soil microbiological properties. All measurements were started within 2 days of sample collection.

SR

SR was measured in a laboratory incubation experiment for 38 days using the method described by Anderson (1982) and Alef (1995). A moist sample of 50 g was placed in 0.750 l plastic jars, and moistened by adding distilled water to a soil moisture content corresponded to about 70% of water holding capacity. Five containers without soil were considered as blanks. All jars were kept overnight in the dark at 25 °C prior to incubation. A plastic vial containing 10 ml of 0.5 M NaOH, for CO_2 trap was placed in the jars, and replaced with a fresh NaOH 2, 4, 6, 8, 11, 13, 15, 18, 21, 24, 28, 32, 34 and 38 days after the start of the incubation. Upon replacing the NaOH solution, the jars were opened and samples were re-aerated to supply adequate oxygen. The evolved CO_2 was trapped in NaOH and the excess alkali was titrated with 0.25 M HCl after precipitating the carbonate with 15% $BaCl_2$ solution. SR was calculated as the accumulative CO_2-C evolved from the soil. All measurements were run in four replicates.

Microbial biomass C

Soil MBC was estimated using the chloroform-fumigation incubation method adapted from Jenkinson and Powlson (1976). Two 20-g subsamples from the respiration experiment were placed in 50-ml glass beakers. One beaker was fumigated with ethanol-free chloroform in a vacuum desiccator for 24 h at room temperature in the dark. The chloroform vapor in the desiccator was removed by three repeated evacuations. Both fumigated and unfumigated soils were re-inoculated with 0.1 g moist soil. All beakers were placed in 0.750 l plastic containers with a vial containing 10 ml of 0.5 M NaOH for CO_2 absorption and a vial containing 10 ml of distilled water to keep soil moisture constant. The containers were closed tightly and incubated at 25 °C in the dark for 10 days. The evolved CO_2 was determined as described above. The MBC was calculated as

$$\mathrm{MBC} = \frac{(C_F - C_U)}{k_c},$$

where MBC is the microbial biomass C, C_F is the evolved CO_2-C from fumigated soil, C_U is the evolved CO_2-C from unfumigated soil (the flush of CO_2) and k_c is recovery factor equivalent to 0.45 (Jenkinson and Ladd, 1981). All measurements were carried out in four replicates.

Basal and substrate-induced respiration

For the measurement of the soil basal respiration (BR), a moist soil sample equivalent to 100 g (oven-dry basis) was placed in a 0.750 l plastic jar, adjusted to 60% of its water holding capacity and pre-incubated for 24 h in the dark at 25 °C. The evolved CO_2 was measured every 6 h for 5 days as described above. The BR was expressed as mg CO_2-C produced per kg soil per day. The metabolic quotient, qCO_2, was calculated by dividing BR (the average CO_2-C respired during 48 h, mg CO_2-C h^{-1}) by microbial biomass C, and expressed as mg CO_2-C g^{-1} C_{mic} per hour (Anderson and Domsch, 1990). For measuring SIR, soil samples (100 g dry weight equivalent) were treated with 5 ml of 3% glucose solution and then incubated at 25 °C, and the evolved CO_2 was measured 6 h after glucose addition (Chen et al., 2002). The measurements were carried out in four replicates.

Assay of soil enzymes

The activity of the ALP and ACP enzymes were measured by the methods described by Tabatabai

(1994). A fresh soil sample of 1 g was placed in 50 ml test tube, to which one drop of toluene 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 phosphatase 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 μg *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.

Statistical analysis

The effect of treatments was determined by factorial two-way analysis of variance (SAS Institute Inc., 1999). Mean values were separated by protected Fisher's least significant difference (LSD). Differences were considered significant only when *P* values were lower than 0.05, unless stated otherwise. The relative change (%) of a variable was calculated from $(C-T)/C \times 100$, where T is the measured value in the treated soils and C is the measured value in the control (unfertilized or uninoculated) soil.

Results

Plant growth and nutrient uptake

There was no AM fungus colonization in roots of uninoculated clover. The results of analysis of variance (Table 2) indicated that the treatments affected most growth and nutritional parameters of the clover. Most plant variables were influenced by the interactions between the two factors, P fertilization and mycorrhizal inoculation. AM fungus colonization tended to be significantly lower (44%) in mycorrhizal plants grown at high P level (Table 3). Similarly, P additions decreased the length of the colonized roots by 48% (Table 3). AM fungus inoculation and P fertilization had a significant effect on shoot and root biomass (Table 2). Shoot and root dry weights were generally higher for mycorrhizal than non-mycorrhizal plants, but shoot weight did not differ significantly between mycorrhizal and non-mycorrhizal plants grown at high P levels (Table 3). Phosphorus addition led to enhanced root and shoot biomass production only in uninoculated treatments. The ratio of shoot to root (S/R) tended to increase with inoculating clover with AM fungus at low P level, while this effect was not observed at high P level (Table 3). The main effects of AM fungus treatment and its interactions with P level on N and P uptake were statistically significant (Table 2). Shoot N uptake in treatments without P and AM fungus was significantly lower than that in other treatments. In addition, the main effect of AM fungus on N uptake was significant only in the treatments without P additions. Phosphorus fertilization and AM fungus inoculation resulted in a significant N uptake by berseem clover. AM fungus inoculation increased shoot P uptake at both low and high P levels, whereas the added P increased shoot P uptake only in treatments without AM fungus.

Table 2. Significance of the main treatment effects and their interactions based on factorial ANOVA (*F*-values) with two factors (phosphorus and mycorrhizae) on berseem clover responses

Plant response	Effect		
	Phosphorus (P)	Mycorrhizae (M)	P × M
Colonization (Colon.)	11.72^{**}	146.2^{***}	11.72^{**}
Root length colonized (RLC)	5.07^{*}	49.1^{***}	5.06^{*}
Shoot dry weight (shoot d.w.)	29.41^{***}	98.1^{***}	67.56^{***}
Root dry weight (root d.w.)	36.51^{***}	94.0^{***}	33.88^{***}
Shoot/root (S/R)	4.0^{ns}	5.77^{*}	12.49^{**}
N uptake	3.86^{ns}	21.01^{***}	4.73^{*}
P uptake	3.34^{ns}	110.1^{***}	15.01^{**}

$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant

Microbial activity and biomass

The cumulative microbial respiration from the incubated soils of mycorrhizal and non-mycorrhizal plants without P addition and fertilized with P are shown in Fig. 1. SR during the first 7 days of the incubation was similar for all treatments. However, a significant ($P<0.05$) difference in SR was observed among the treatments afterwards (Fig. 1). Generally, SR decreased in mycorrhizal treatments, but it increased in P fertilization treatments. Results of analysis of variance showing the significance of the main treatment effects and their interactions on microbial soil properties are presented in Table 4. AM fungus treatments significantly lowered the rate of soil respiration while P fertilization increased SR rates ($P<0.05$, Fig. 2a). However, there was no interaction between the two factors on this parameter (Table 4). Neither AM fungus nor P fertilization affected SIR (Table 4).

Table 3. Effects of P fertilization (−P = unfertilized; +P = fertilized) and AMF inoculation (−M = without inoculation; +M = with inoculation) on root colonization; shoot and root biomass production and shoot N and P uptake by clover after 70 days under greenhouse conditions

Treatment	Colon (%)	RLC (m)	Shoot d.w. ($g\,pot^{-1}$)	Root d.w. ($g\,pot^{-1}$)	Shoot/root ratio	Shoot nutrient uptake	
						N ($mg\,pot^{-1}$)	P ($\mu g\,pot^{-1}$)
−P−M	0.00 (0.00) c	0.00 (0.00) c	0.283 (0.17) c	0.290 (0.04) c	0.98 (0.17) b	11.4 (1.05) b	26.0 (7.57) c
−P+M	33.5 (4.20) a	9.68 (2.07) a	3.264 (0.28) a	2.042 (0.17) a	1.61 (0.17) a	55.7 (1.49) a	698 (79.9) a
+P−M	0.00 (0.00) c	0.00 (0.00) c	2.530 (0.31) b	1.629 (0.12) b	1.57 (0.11) a	35.8 (9.80) a	292 (32.8) b
+P+M	18.7 (0.94) b	4.97 (0.27) b	2.804 (0.17) ab	2.067 (0.09) a	1.37 (0.05) a	54.5 (5.51) a	602 (35.0) a
$LSD_{0.05}$	6.64	3.22	0.507	0.348	0.27	20.3	144

Means ± SE ($n = 8$) in columns followed by the same letter are not significantly different at 5% level.

Soil microbial biomass C (MBC) in non-mycorrhizal treatments was significantly higher than that in mycorrhizal treatments at high P level (Fig. 2c). In contrast, P fertilization had no significant effect on soil microbial biomass C (Table 4). Results indicated no main treatment effects of P fertilization and AM fungus, and their interaction on microbial metabolic quotients (Table 4 and Fig. 3a). As a general trend, there was higher ALP activity in mycorrhizal treatments regardless of soil P conditions. However, P fertilization depressed the activity of ALP in both non-mycorrhizal and mycorrhizal treatments. Surprisingly, the activity of this enzyme was not affected by an interaction of P fertilization and AM fungus. The main treatment effects of P fertilization and AM fungus, and their interaction on the activity of ACP was statistically significant (Table 4). AM fungus increased the activity of ACP only at low P level, whereas P fertilization decreased it regardless of AM fungal treatments (Table 4). Results also showed that AM fungus effect on ALP activity is more striking than on ACP activity (Fig. 3b).

Discussion

Phosphorus and AM fungus effects on berseem growth and nutrient uptake

Phosphorus addition clearly resulted in the enhancement of plant growth and nutrient uptake in the absence of AM fungal inoculation in these P-deficient soils. This is in agreement with previous results obtained from most experiments (Azcón and El-Atrash, 1997; Graham and Abbott, 2000). In calcareous soils, a large proportion of P is found as precipitated calcium-phosphate minerals, which are insoluble and unavailable to plants in the short-term (Ström et al., 2005). Consequently, P fertilization may frequently lead to increased crop growth and production. Mycorrhizal colonization and the improved P and N uptake increased growth of both roots and shoots. Berseem clover inoculated with *G. intraradices* had higher shoot and root dry weights than non-mycorrhizal clover, particularly at low P levels. The beneficial effects of AM fungus inoculation of most natural and agricultural plants grown under field and controlled conditions are widely recorded throughout the literature (see Smith and Read, 1997; Rao and Tak, 2001; Azcón et al., 2003; Ryan and Angus, 2003; Martin and Stutz, 2004; Duponnois et al., 2005; Li et al., 2005). Most studies showed that mycorrhizal plants grown under low P conditions

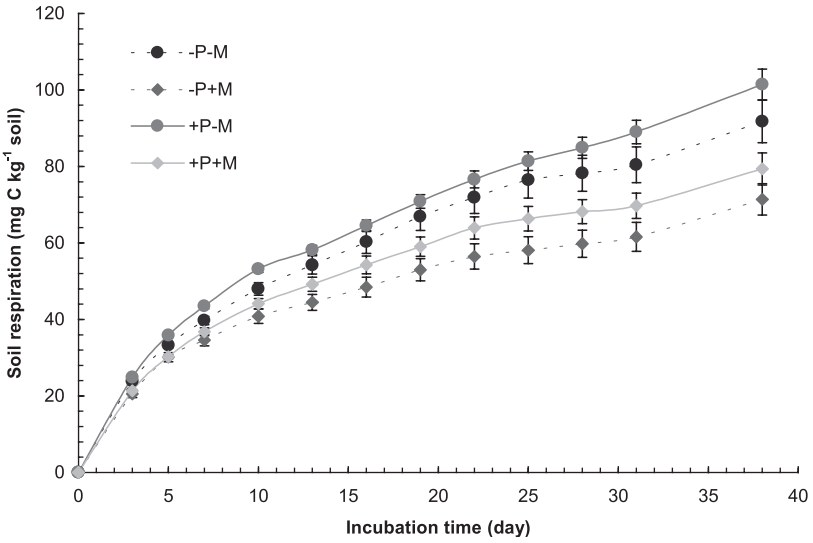

Figure 1. Effects of P fertilization (−P = unfertilized; +P = fertilized) and AM fungus inoculation (−M = without inoculation; +M = with inoculation) on SR in the rhizosphere of clover after 70 days under greenhouse conditions. Each point represents mean ($n = 8$) and bars indicate SD.

Table 4. Significance of the main treatment effects and their interactions based on factorial ANOVA (*F*-values) with two factors (phosphorus and mycorrhizae) on soil microbial properties

Soil microbial variable	Effect		
	Phosphorus (P)	Mycorrhizae (M)	P × M
SR rate	4.52*	22.91**	0.02^{ns}
Substrate-induced respiration (SIR)	1.37^{ns}	0.01^{ns}	0.4^{ns}
Microbial biomass C (MBC)	0.66^{ns}	9.69**	0.04^{ns}
Metabolic quotient (qCO_2)	2.75^{ns}	2.72^{ns}	0.22^{ns}
Acid phosphatase (ACP)	262.6***	96.2***	44.63***
Alkaline phosphatase (ALP)	62.2***	6.72*	0.51^{ns}

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns—not significant.

had higher biomass production compared to plants grown under high P conditions (Habte and Manjunath, 1987; Graham and Abbott, 2000; Khaliq and Sanders, 2000). In contrast, it has been reported that application of high amounts of P was required for the beneficial effects of AM on wheat yield to be apparent in the highly P-fixing calcareous soils (Li et al., 2005). The higher shoot-to-root ratio in mycorrhizal plants could be due to the high colonization and presumably as a result of production of large quantity of extraradical mycelium. Elevated shoot-to-root ratio is a common influence of AM fungi on colonized plants (see Khalil et al., 1994, 1999; Smith and Read, 1997). This suggests that the AM colonized plants might allocate a large part of their photosynthates to the mycorrhizal fungus. The higher shoot-to-root ratio (or lower root-to-shoot ratio) may also indicate the improved plant nutrition with AM fungus association (Khalil et al., 1999). Results indicate that mycorrhizal inoculation enhanced plant P and N uptake, particularly under low P conditions, which led to increased shoot and root biomass. These data suggest that berseem clover may have a relatively high affinity (33.5%) to form a symbiotic association with *G. intraradices*. However, there is no published report about the extent of berseem's dependence of AM fungus in the literature.

Phosphorus effects on microbial biomass and activity

The interactions between P fertilization and arbuscular mycorrhizal (AM) fungi on soil microbial activity and biomass could be very important in natural and managed ecosystems. The main reason is that the contrasting effects of P and AM on the overall soil microbial activity and biomass may have a positive or negative feedback to nutrient cycling, which is essential for nutrient uptake and growth of the host plants in the long-term. Results of this study indicated the highest SR under high P

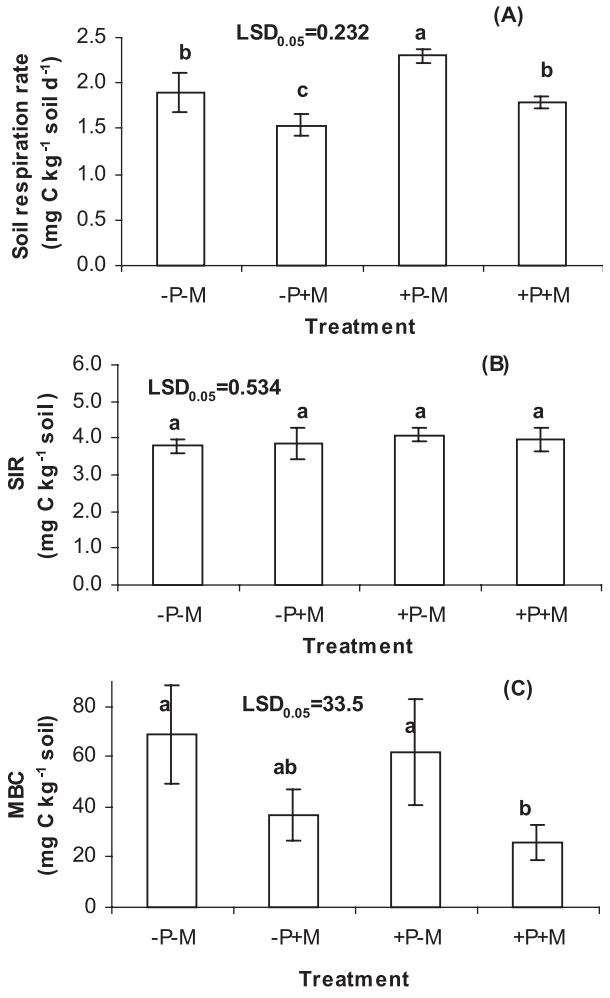

Figure 2. Effects of P fertilization (−P = unfertilized; +P = fertilized) and AM fungus inoculation (−M = without inoculation; +M = with inoculation) on (A) SR rate (mg C kg^{-1} soil d^{-1}), (B) substrate-induced respiration (SIR; mg C kg^{-1} soil) and (C) microbial biomass C (MBC; mg C kg^{-1} soil) in the rhizosphere of clover after 70 days under greenhouse conditions. Means ($n = 8$) ±SD followed by the same letter are not significantly different based on LSD test at 5% level.

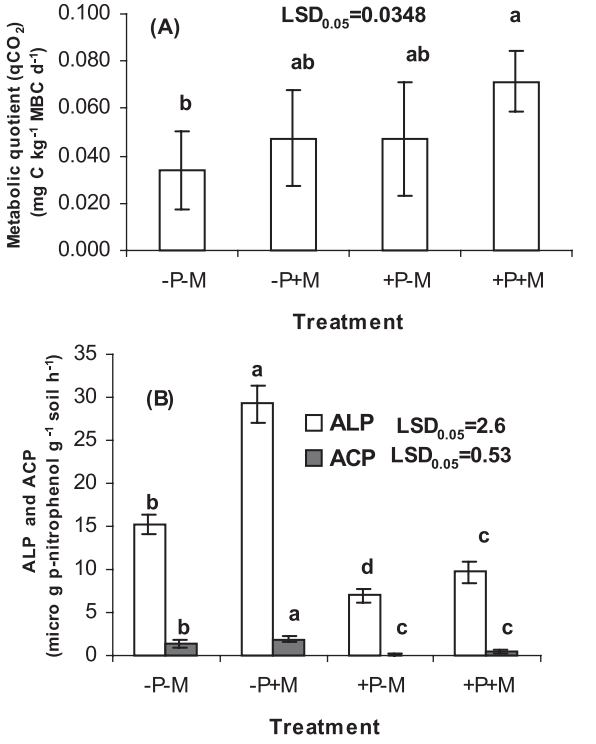

Figure 3. Effects of P fertilization (−P = unfertilized; +P = fertilized) and AM fungus inoculation (−M = without inoculation; +M = with inoculation) on (A) the microbial metabolic quotient (qCO_2 (mg C kg^{-1} MBC d^{-1}) and (B) the activity of acid (ACP) and alkaline (ALP) phosphatases (μg *p*-nitrophenol g^{-1} soil h^{-1}) in the rhizosphere of clover after 70 days under greenhouse conditions. Means ($n = 8$) ±SD followed by the same letter are not significantly different based on LSD test at 5% level.

conditions, and the lowest SR in AM fungus treatments. This suggests that P and C availability may concurrently limit the microbial respiration in these calcareous P-fixing soils. Several studies reported the stimulating effects of P fertilization on soil microbial respiration (Amador and Jones, 1993; Ilstedt and Singh, 2005; Smith, 2005), but Thirukkumaran and Parkinson (2000) observed that P additions had an inhibitory impact on SR, which has been ascribed to osmotic effects. There are two possible mechanisms by which soil P status affects microbial activity. The first mechanism is the direct influence of P on soil microorganisms, as a nutrient required for microbial growth and maintenance, which is always stimulatory. The second mechanism is the indirect effects through decreased root exudations under high P conditions, which is often inhibitory. Root exudates are energy-rich compounds readily available to microorganisms, and contribute to higher microbial activity in the rhizosphere (Grayston et al., 1996; Kuzyakov, 2002; Kuzyakov et al., 2003). Some studies showed that plants grown under low P conditions increased exudation of amino acids, reducing sugars and carboxylic acids compared to plants grown under high P conditions (Ratnayake et al., 1978; Graham et al., 1981; Schwab et al., 1983; Neumann and Romheld, 1999). Therefore, we could expect more root exudates under low P conditions, where we observed decreased SR. However, our results suggest that without C limitation soil microbial respiration is not affected by P availability, since P fertilization had no effects on SIR. This further indicates that substrate availability is far more

important than P availability for microbial activity. The present results indicate lower, but insignificant, microbial biomass in P fertilized soils. Obviously, high variation in microbial biomass contributed to lack of statistically significant differences. The small decline in microbial biomass C under high P conditions is probably due to the lower input of plant derived and easily available C. However, the microbial metabolic quotient (qCO_2) was not affected by P fertilization, suggesting microbial C efficiency would not depend on the soil P status.

The results of our study indicate that P addition substantially decreased the activities of the ACP and ALP enzymes. It was indicated that the activities of enzymes involved in P transformation are inversely related to P availability (Tadano et al., 1993) and under P limited conditions its high demand, resulting in an increase in phosphatase activity, as occurred in low P conditions of this study. It seems that any decrease in the available phosphate may cause an overall increase in phosphatase activity. When plants are subjected to P deficiency, secretion of ACP from roots is a regular reaction (Fox and Comerford, 1992; Gilbert et al., 1999; Richardson et al., 2001). However, it is unclear whether the release of ACPs into the rhizosphere improves P acquisition.

AM fungus effects on microbial biomass and activity

Mycorrhizas can affect the composition and activity of soil microorganisms by changes in root exudation patterns or fungal exudates (Marschner et al. 1997; Marschner and Timonen, 2006). Mycorrhizal colonization resulted in a significant decline in SR. Similarly, the amount of soil microbial biomass C was lower in AM fungus treatments compared to treatments without AM fungus inoculation. However, we observed no effects of AM fungus inoculation on SIR and the microbial metabolic quotient (qCO_2). The effect of AM fungus on soil microbiological properties recorded in the literature are inconsistent and complex, where negative (Christensen and Jakobsen, 1993; Wamberg et al., 2003; López-Gutiérrez et al., 2004; Langley et al., 2005); positive (Van Aarle et al., 2003; Wamberg et al., 2003; Langley et al., 2005) or no (Kim et al., 1998) effects 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. AM fungus could influence microbial activity and biomass, most likely by affecting root exudates quantitatively and qualitatively in the hyphosphere. Changes in root exudation and rhizodeposition following AM colonization have been reported, and include a reduction in total sugars, reducing sugars and amino acids (Schwab et al., 1983, 1984; Bansal and Mukerji, 1994). AM fungus may reduce the leakage of root metabolites via decreased membrane permeability, especially under high P levels (Graham et al., 1981). We observed that SIR remained unaffected by the presence of mycorrhizal fungi, further supporting that substrate availability in mycorrhizal treatments could be lower than in non-mycorrhizal treatments.

It is also likely that the releases of glomalin by AM fungus could alternatively contribute to the decreased microbial respiration and biomass C, as it appears to be relatively recalcitrant (Wright and Upadhyaya, 1998; Steinberg and Rillig, 2003). Since glomalin was not measured, it is not valid to make any conclusive comments on its effect on microbial activity.

Mycorrhizal treatment clearly led to distinctive increases in the activities of enzymes involved in P dynamics. Inoculation with AM fungus increased the activity of ALP enzyme by 193% and 140% in low and high P levels, respectively. However, increases in the activity ACP enzyme (143%) were significant only at low soil P level. 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 et al., 2006). There was a positive correlation between ACP activity and P uptake in low-P soils (Khalil et al., 1994), but Gianinazzi-Pearson et al. (1981) found no effect of mycorrhiza on ACP activity. In contrast, Vázquez et al. (2000) reported higher enzyme activities in the rhizosphere of mycorrhizal plants that may be due to the increases in C and nutrient exudation from infected roots. However, the results of our study do not support it, since the microbial respiration did not increase in the rhizosphere of mycorrhizal berseem. Phosphatase activity in soil originates from many sources including plant roots (Dodd et al., 1987; Dinkelaker and Marschner, 1992), mycorrhizal fungi (Tarafdar and Marschner, 1994; Joner et al., 2000) and bacteria (Tarafdar and Claassen, 1988; Ezawa and Yoshida, 1994). Our data suggest that an AM fungus symbiosis contributed to the increase in the activities of phosphatases. This may be a consequence of a direct contribution from the external mycelium and an indirect effect of improved host plant P status. Joner et al. (2000) proposed that higher acid phosphate activity in mycorrhizal treatments might be a result of a direct fungal

leakage or an induced leakage by the plant roots. Additionally, decreased microbial activity in AM fungus treatments may suggest that soil microorganisms do not contribute to the increase in the activity of phosphatases. Therefore, AM fungus may only contribute to plant P nutrition without a significant contribution from rhizospheric microorganisms.

Conclusions

Overall, we found several main treatment effects of P fertilization and AM fungal inoculation and their interactions on berseem clover and soil microbial activities. An increased biomass production of shoot and root was evident, especially for AM fungus-inoculated berseem grown under low P conditions. Phosphorus addition and AM fungus inoculation substantially improved P and N uptake by the plant. It was also shown that P additions stimulated soil microbial respiration, whereas AM fungus inoculation had a depressive impact on this microbial property. Increased microbial respiration and small decreases in biomass C in response to P fertilization may indicate that P is a limiting factor for microbial activity in the studied soil–plant system. On the other hand, decreased microbial respiration and biomass C in mycorrhizal treatments may imply that C availability could be a limiting factor for microbial activity and size. Our data suggest that P and C availability may simultaneously limit the microbial respiration in these calcareous soils, but it seems that C availability is much more important than P availability. The activity of ALP enzyme with AM fungus increased by 193% and 140% in low and high P levels, respectively. However, increases in the activity ACP enzyme (143%) were significant only at low soil P level. Our results suggest that while P fertilization generally stimulates soil microbial activities, AM fungus could depress microbial activities. Consequently, this contrasting effect may impose a positive or negative feedback to soil C turnover, and thus to microbial transformations of essential nutrients in these calcareous soils. The net response is yet unclear and needs further elucidation. We propose that a greater knowledge of interactions in the mycorrhizosphere and their effects on soil microbial activity and subsequent nutrient availability is even now desirable. This would enable the development of agricultural practices to optimize plant growth and production in calcareous soils with low P, especially in naturally mycorrhizal plants fertilized with surplus P.

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