The effect of inoculation of pea plants with mycorrhizal fungi and *Rhizobium* on nitrogen and phosphorus assimilation

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ABSTRACT

The study evaluated the response of pea (*Pisum sativum* cv. Avola) to arbuscular mycorrhizal fungi (AM) species *Glomus mosseae* and *Glomus intraradices* and *Rhizobium leguminosarum* bv. *viceae*, strain D 293, regarding the growth, photosynthesis, nodulation and nitrogen fixation activity. Pea plants were grown in a glasshouse until the flowering stage (35 days), in 4 kg plastic pots using leached cinnamonic forest soil (Chromic Luvisols – FAO) at P levels 13.2 (P1) and 39.8 (P2) mg P/kg soil. The obtained results demonstrated that the dual inoculation of pea plants significantly increased the plant biomass, photosynthetic rate, nodulation, and nitrogen fixation activity in comparison with single inoculation with *Rhizobium leguminosarum* bv. *viceae* strain D 293. On the other hand, coinoculation significantly increased the total phosphorus content in plant tissue, acid phosphatase activity and percentage of root colonization. The effectiveness of coinoculation with *Rhizobium leguminosarum* and *Glomus mosseae* was higher at the low phosphorus level while the coinoculation with *Glomus intraradices* appeared to be the most effective at higher phosphorus level.

Keywords: *Pisum sativum*; *Glomus mosseae*; *Glomus intraradices*; *Rhizobium leguminosarum*

The endomycorrhizal fungi produce a highly branched hyphal structure within the plant cell. This infection creates an absorptive structure with a very high surface area of transfer for nutrients between the plant and the fungus. Mycorrhizal fungi hyphae secrete acid and alkaline phosphatases (APA and ALP) into the rhizosphere. It was established that APA activity increases in roots growing under P stress (Woolhouse 1975). Therefore, the regulation of these enzymes is critical to a plant's survival in soils with limited P resources (Duff et al. 1991). There is extensive evidence for a decrease in the number of arbuscules under high external P (Bethlenfalvay et al. 1990, Smith and Smith 1996).

Phosphorus has a key role in the energy metabolism of all plant cells, and particularly in nitrogen fixation (Dilworth 1974). It was established that nodulating legumes require more P than legumes growing on mineral nitrogen (Al-Niemi et al. 1997). The AM fungi associated with legumes are an essential link for effective phosphorus nutrition, leading to enhanced nitrogen fixation that in turn promotes root and mycorrhizal growth. Synergistic effect of dual colonization of roots with AM fungi and *Rhizobium* on growth, nutrient uptake and nitrogen fixation in soybean (Bethlenfalvay et al. 1990) and pea (Xavier and Germida 2003) were reported. The effectiveness of the tripartite symbiosis – AM fungi, *Rhizobium* and plant, depends on the competition of the three symbionts for carbon (Jakobsen and Rosendahl 1990). Roots with AM fungi receive about 4–20% more photosynthates than comparable non-mycorrhizal roots (Smith and Reed 1997). Jakobsen and Rosendahl (1990) estimated that AM fungi could use up

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to 20% of the total fixed $^{14}CO_2$ in young plants. Concomitant development of mature functional nodules and effective AM infection depended on microsymbiont species and strains (Saxena et al. 1997).

The synchronization between the two symbiotic systems needs an optimal P level in the nutrient medium to stimulate the nodulation and nitrogen fixation and not to slow down the formation of effective mycorrhizal associates.

As the information presented in the studies in this area is insufficient, the objective of our study was to evaluate the effectiveness of triple symbioses – pea plants, AM fungi (*Glomus mosseae* and *Glomus intraradices*) and *Rhizobium leguminosarum* bv. *viceae* on the nitrogen and phosphorus assimilation at low and higher soil phosphorus levels.

MATERIAL AND METHODS

Pea plants (*Pisum sativum* L.) cv. Avola were grown in a glasshouse until the flowering stage (35 days), in 4 kg plastic pots (3 plants per pot) using leached cinnamonic forest soil (Chromic Luvisols – FAO) with the following agrochemical characteristics: pH(H_2O) = 6.2, 8 mg/kg soil total mobile nitrogen (N-NO_3^- + N-NH_4^+), 13.2 mg P/kg soil, 100 mg K/kg soil. Mineral nitrogen was determined spectrophotometrically after Kjeldahl digestion; assimilated P and K by the acetate – lactate method (Ivanov 1984).

Fertilizers were applied prior to the seeding as follows: 50 and 133 mg $Ca(NO_3)_2$/kg soil for the inoculated and non-inoculated variants, respectively; and 200 mg $MgSO_4$/kg soil. The low P1 level (13.2 mg P/kg soil) is a natural soil P reserve. The P2 level (33.6 mg P/kg soil) is achieved by adding 260 mg KH_2PO_4/kg soil. Water was added to make up about 60% of water-holding capacity (WHC). Two cultures of arbuscular mycorrhizal (AM) fungi, *Glomus mosseae* and *Glomus intraradices* were used. Inoculation with AM fungi was done at seeding by the layering method (Jackson et al. 1972). The seeds were inoculated with the bacterial suspension of *Rhizobium leguminosarum* bv. *viceae* strain D 293 at approximately 10^8 cells per cm^3.

The following scheme was used: I. At low P1 level: 1. Control plants; 2. Plants inoculated with *Rh. leguminosarum*; 3. Plants inoculated with *G. mosseae*; 4. Plants inoculated with *Rh. leguminosarum* + *G. mosseae*; 5. Plants inoculated with *G. intraradices*; 6. Plants inoculated with *Rh. leguminosarum* + *G. intraradices*; II. At higher P2 level the variants 7–12 are the same as the variants at P1 level.

Nitrogen fixation activity of root nodules was assayed by the acetylene reduction assay (ARA, EC 1.7.99.2) immediately after harvesting according to Hardy et al. (1973). Acid phosphatase activity (APA, EC 3.1.3.2) was measured with a modification of the method of Tabatabai and Bremner (1969). Root tissue was homogenized with 0.1M sodium acetate buffer (pH 5.0). After centrifugation the supernatant was assayed for the enzyme activity by incubation in 5mM p-nitrophenyl phosphate and 0.1M sodium acetate buffer (pH 5.0). The reaction was stopped by the addition of 0.2M NaOH, and absorption was measured at 405 nm.

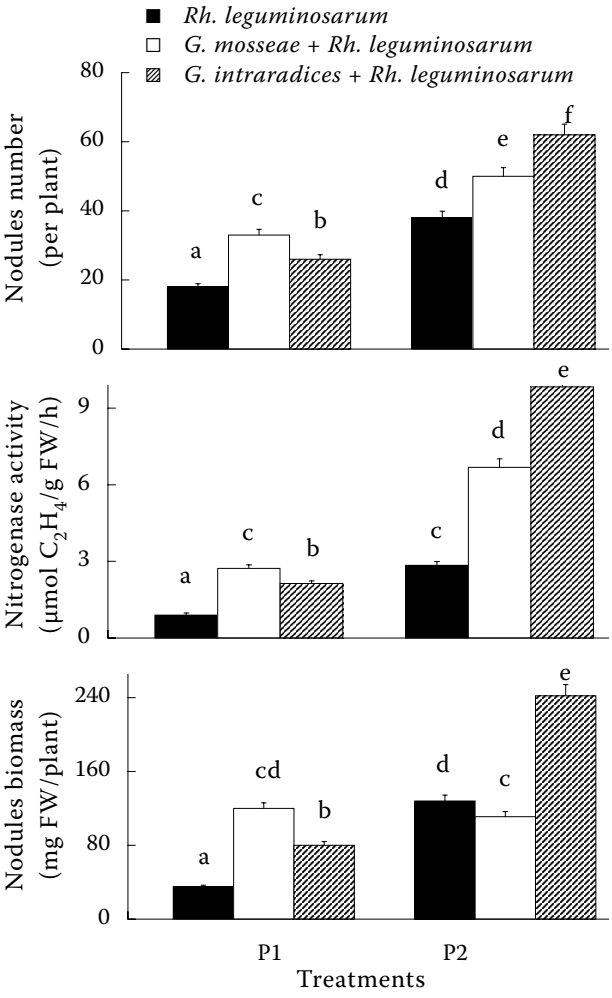

Figure 1. Nodules number, biomass and nitrogenase activity in inoculated pea plants at low (P1) and higher (P2) phosphorus levels; FW – fresh weight; values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

The rate of photosynthesis was measured under conditions of natural irradiance with portable photosynthetic equipment Li-Cor 6000 (USA), equipped with a leaf chamber. The total N content was measured after Kjeldahl digestion. Total tissue P content was determined according to Lowry and Lopez (1946). The rate of mycorrhiza infection of the roots was determined microscopically (Giovanetti and Mosse 1980).

Data are expressed as means ± SE where $n = 3$. Comparison of means was performed by the Fisher LSD test ($P \leq 0.05$) after performing multifactor ANOVA analysis. The STATISTICA (version 6.0) package was used for statistical analysis.

RESULTS AND DISCUSSION

Pea plants inoculated only with *Rh. leguminosarum* and supplied with elevated P levels exhibited a significant increase in the nodule number and fresh biomass and its nitrogen fixing activity, respectively (Figure 1). Moreover, promotion in plant dry biomass and photosynthetic rate at elevated phosphorus level was observed (Table 1). At low P1 level without additional P application, coinoculation with *Rhizobium* and *Glomus mosseae* resulted in the statistically highest levels of nodule number, nodule fresh weight and ARA (Figure 1), plant dry biomass and photosynthesis (Table 1) as compared to the variants with single *Rhizobium* inoculation and with dual inoculation with *Rhizobium* and *Glomus intraradices*. At the higher P2 level supplied a change of priority was observed: the statistically most significant and highest nodule number, maximal nodule fresh biomass and ARA values (Figure 1), plant dry biomass and photosynthesis (Table 1) were obtained for treatments inoculated with *Rh. leguminosarum* and *G. intraradices*. Therefore a better compatibility between *Rh. leguminosarum* and *G. mosseae* was obtained at variants with P1 level while *Rh. leguminosarum* and *G. intraradices* appeared to be more compatible pairings at variants with P2 level. An increase of parameters that quantified the N_2-fixing organ – nodule number and dry weight, as well as specific ARA activity with an increase of P applied was observed also in common bean by Olivera et al. (2004). Jia et al. (2004) reported that inoculation with AM fungi promoted biomass production and photosynthetic rates in *Vicia faba* because of the enhanced P supply due to AM fungi inoculation. Some authors (Saxena et al. 1997) reported that tripartite symbiosis be-

Table 1. Total N and P content, plant dry biomass and photosynthetic rate in inoculated pea plants

Treatments	Total N (mg/plant)	Total P (mg/plant)	Plant dry biomass (g/plant)	Photosynthesis (mg $CO_2/m^2/s$)
P1				
1. control plants	^{a}17.40 ± 090	^{a}5.87 ± 0.29	^{a}0.83 ± 0.04	^{b}13.08 ± 0.65
2. *Rh. leguminosarum*	^{a}22.15 ± 1.11	^{bc}12.39 ± 0.62	^{b}0.97 ± 0.05	^{a}11.57 ± 0.58
3. *G. mosseae*	^{a}27.81 ± 1.39	^{c}14.88 ± 0.74	^{de}1.27 ± 0.06	^{c}16.52 ± 0.83
4. *G. mosseae* + *Rh. leguminosarum*	^{b}35.90 ± 1.80	^{e}20.46 ± 1.03	^{e}1.34 ± 0.06	^{c}15.88 ± 0.79
5. *G. intraradices*	^{a}26.26 ± 1.31	^{b}11.05 ± 0.55	^{c}1.15 ± 0.06	^{a}10.92 ± 0.55
6. *G. intraradices* + *Rh. leguminosarum*	^{b}32.00 ± 1.60	^{d}16.96 ± 0.85	^{cd}1.20 ± 0.06	^{b}13.04 ± 0.65
P2				
7. control plants	^{a}28.80 ± 1.44	^{a}22.67 ± 1.13	^{a}1.19 ± 0.06	^{a}17.10 ± 0.86
8. *Rh. leguminosarum*	^{ab}34.18 ± 1.71	^{a}26.38 ± 1.32	^{b}1.33 ± 0.07	^{a}18.58 ± 0.93
9. *G. mosseae*	^{ab}35.47 ± 1.77	^{b}33.33 ± 1.67	^{bc}1.45 ± 0.07	^{b}21.29 ± 1.06
10. *G. mosseae* + *Rh. leguminosarum*	^{b}42.53 ± 2.13	^{d}43.91 ± 2.20	^{c}1.54 ± 0.08	^{c}27.46 ± 1.37
11. *G. intraradices*	^{b}40.92 ± 2.05	^{c}38.36 ± 1.92	^{c}1.52 ± 0.08	^{c}27.89 ± 1.39
12. *G. intraradices* + *Rh. leguminosarum*	^{c}52.42 ± 2.62	^{e}60.10 ± 3.02	^{d}1.80 ± 0.09	^{d}31.78 ± 1.59

Values are means ± SE, $n = 3$; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

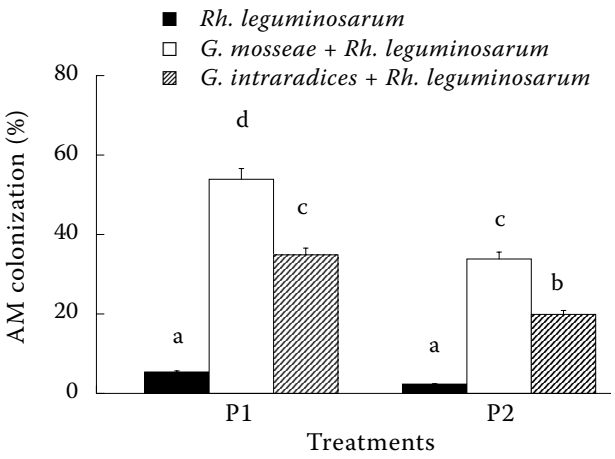

Figure 2. Arbuscular mycorrhizal (AM) colonization in inoculated pea plants at low (P1) and higher (P2) phosphorus levels; values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

tween green gram, *Bradyrhizobium japonicum* and several AM fungi cultures increased the growth, nodulation and ARA to varying levels for almost all pairs. The reduction of photosynthetic rate as the P concentration decreases is related to the increase in root carbohydrate content – feed back effect (Rychter and Randall 1994). A specific compatibility between AM fungi and *Rhizobium* was reported in pea plants (Xavier and Germida 2003).

A significant reduction of colonized root parts towards whole root (Figure 2) with additional P supply was observed in all experimental variants. Many authors claimed that mycorrhizal infection decreased with P supply (Jackson et al. 1972, Bethlenfalvay et al. 1990). The rate of mycorrhizal colonization in variants inoculated with *Rh. leguminosarum* only, may be due to the natural diversity in the soil of AM fungi different from species applied in the experiment. It could be suggested that the higher number of AM structures (as compared with the variant inoculated only with nitrogen fixing bacteria) was a result of colonization of used *G. mosseae* and *G. intraradices* separately and in combination with *Rh. leguminosarum*. The mycorrhizal status is best manifested in the variants with dual inoculation with *Rh. leguminosarum* and *G. mosseae* at both P levels, which is in correspondence with the values of the nitrogen fixing parameters only in plants grown without additional phosphorus.

The activity of acid phosphatase in the roots infected with *G. mosseae* was significantly higher than the enzyme activity values in the roots infected with *G. intraradices* (Figure 3). However, statistically proved maximal values were observed in the variants with tripartite symbiosis between pea, *Rh. leguminosarum* and *G. mosseae* AM fungi. Plant roots, fungi and bacteria separately possess APA (Abd-Ala 1994), and that is why maximum APA values of the coinoculated variants may be caused by the commutative effect of the three phosphatase activities. Olivera et al. (2004) suggested that acid phosphatase activity in common bean roots increased with the P level in the culture medium.

Our data indicate that plant phosphorus content increased with P addition to the soil substrate (Table 1). The total plant growth positively correlated with the total P concentration in the plant tissues. Similarly, the P absorption ability was reported to be strongly connected with dry matter production (Lynch et al. 1991). However, the plant total N was not significantly influenced at different P levels applied into the soil substrate (Table 1). In the variants without P supply, the total N content was slightly lower. Kolawole and Kang (1997) have reported that the P application increased the N content in legume seeds. The

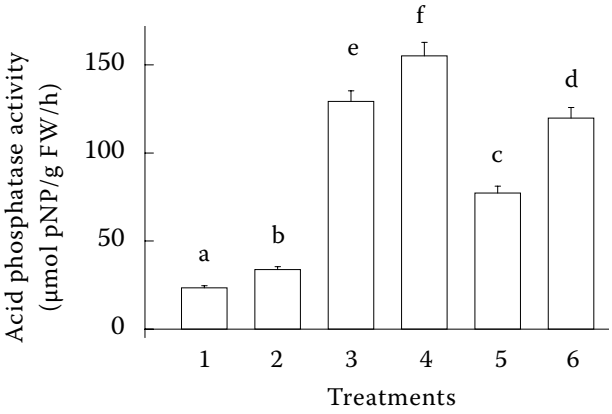

Figure 3. Root acid phosphatase activity in inoculated pea plants at P2 phosphorus level

Treatments: 1. control plants; 2. plants inoculated with *Rh. leguminosarum*; 3. plants inoculated with *G. mosseae*; 4. plants inoculated with *Rh. leguminosarum* + *G. mosseae*; 5. plants inoculated with *G. intraradices*; 6. plants inoculated with *Rh. leguminosarum* + *G. intraradices*. FW – fresh weight; values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis

absence of the relationship between shoot N content and elevated P levels in the nutrient medium was established by Olivera et al. (2004), which is in agreement with our results. The obtained results indicated that the highest total P content at 39.6 mg P/kg soil was observed in the variants with dual inoculation with *Rh. leguminosarum* and *G. mosseae*. The maximal P concentration in the variants with phosphates application is a result of the enhanced P uptake despite the reduction in mycorrhizal colonization and acid phosphatase activity.

The present study demonstrated that the dual inoculation of pea plants increased growth, photosynthetic rate, nodulation parameters, nitrogen fixation activity and phosphorus content to different levels over single inoculation with *Rh. leguminosarum* and depended on the level of phosphorus in the soil. The efficiency of coinoculation with *Rhizobium* and *G. mosseae* was higher at 13.2 mg P/kg soil phosphorus level. A higher efficiency of coinoculation with *G. intraradices* at 39.6 mg P/kg soil appeared to reveal parameters connected with nitrogen fixation, photosynthesis and total dry biomass, despite the lower percent of mycorrhizal colonization and lower phosphatase activity. The obtained results allow us to suggest that the dual inoculation of pea with *G. mosseae* in combination with *Rh. leguminosarum* is more competitive at low phosphorus level while coinoculation with *G. intraradices* and *Rh. leguminosarum* is more effective at higher P content. Therefore the usage of *G. intraradices* is more appropriate at higher phosphorus levels, because of its favourable effect on the biological nitrogen fixation and plant dry biomass.

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