RESPONSE OF ALFALFA (*MEDICAGO SATIVA* L) GROWTH AT LOW ACCESSIBLE PHOSPHORUS SOURCE TO THE DUAL INOCULATION WITH MYCORRHIZAL FUNGI AND NITROGEN FIXING BACTERIA

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Summary. The study evaluated the response of alfalfa (*Medicago sativa* L) to arbuscular mycorrhizal fungi (AM) species *Glomus intraradices* and *Sinorhizobium meliloti*, strain 1021 regarding the dry biomass accumulation, mycorrhizal fungi colonization, nodulation and nitrogen fixation activity. Alfalfa plants were grown in a glasshouse until the flowering stage (58 days), in 4 kg plastic pots using leached cinnamonic forest soil (Chromic luvisols – FAO) at P levels 42 mg P_2O_5 kg^{-1} soil (applied as 133 mg kg $soil^{-1}$ tunisian phosphorite). The obtained results demonstrated that the dual inoculation of alfalfa plants significantly increased the percent of root colonization and acid phosphatase activities in the root tissue and in the soil in comparison with a single inoculation with *Glomus intraradices*. Coinoculation also significantly increased the total phosphorus and nitrogen content in plant tissues. Under conditions of dual inoculation high nitrogenase activity was established, especially in the floral budding stage as compared to the single inoculation

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with the *Sinorhizobium meliloti*, strain 1021. The effectiveness of coinoculation with *Sinorhizobium meliloti* and *Glomus intraradices* was established regarding maximal plant biomass accumulation as a result of enhanced nitrogen and phosphorus assimilation.

Key words**:** acid phosphatase, alfalfa (*Medicago sativa* L)*, Glomus intraradices,* nitrogenase, root colonization, *Sinorhizobium meliloti*.

INTRODUCTION

Most land plants are symbiotic with arbuscular mycorrhizal fungi (AMF) and N_2 fixing bacteria, which taken up mineral nutrients from the soil and exchange them with plants photosinthetically fixed carbon. AMF can enhance nutrient acquisition by plant via their extraradical hyphae which increase the surface area for absorption. AMF are particularly effective in utilizing insoluble rock phosphorus ($Ca_3(PO_4)_2$) that cannot be used by plants (Marchner, 1995). The information about studies in this area is insufficient. AMF hyphae secrete acid phosphatases (APA) into the rhizosphere, and the root enzyme activity was found to increase in plants growing under P stress (Woolhouse, 1975). It was established that N_2 fixing symbioses are strongly P-limited (Al-Niemi et al., 1997). The AMF associated with legumes are an essential link for effective P nutrition, leading to enhanced N_2 fixation that in turn promotes root and mycorrhizal growth. Synergistic effect of dual colonization of roots with AMF and *Rhizobium* on growth, nutrient uptake and N_2 fixation in many legume plants have been reported (Xavier and Germida, 2003).

The present study examined the symbiotic interactions of *Medicago sativa-Glomus intraradices-Sinorhizobium meliloti* at a hardly accessible P source as Tunisian phosphorite. Our objective was to determine the effectiveness of triple symbioses on the N_2 fixing activity and P uptake and assimilation.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L) plants were grown in a glasshouse in 4 kg plastic pots using leached cinnamonic forest soil. Tunisian phosphorite was applied to the all experimental variants at 133 mg kg soil^{-1}. Inoculation with *Glomus intraradices* was done by the layering method (Jackson et al. 1972). The seeds were inoculated with the bacterial suspension of *Sinorhizobium meliloti*, strain 1021 TLS (the strain is provided by prof. F. de Brjiun, LIPM, Toulouse) at approximately 10^8 cells per cm^3.

Two formulations of foliar fertilizers (Agroleaf®, Scotts Company, USA) were applied: (1) Agroleaf® total - N:P:K=20:20:20 + microelements, was applied twice during the vegetative growth stage; (2) Agroleaf® with high K - N:P:K=15:10:31 + microelements, was applied four times during the floral budding and flowering stage.

The following scheme was used: control plants (C); foliar fed plants (F); foliar fed plants inoculated with *S. meliloti (*F+Rh); foliar fed plants inoculated with *G. intraradices* (F+G); foliar fed plants inoculated with *S. meliloti*+*G. intraradices* (F+Rh+G).

Nitrogen fixation activity of root nodules was assayed by the acetylene reduction assay (ARA, EC 1.7.99.2) according to Hardy et al. (1973). Soil and root acid phosphatase activity (APA, EC 3.1.3.2) were measured according to the method of Tabatabai and Bremner (1969) with modification. Plant total N was analyzed after Kjeldhal digestion on nitrogen analyzer Contiflo (Hungary). Plant total P was determined spectrophotometrically (Lowry and Lopez, 1946). Total K was quantified by flame spectrometry. The rate of mycorrhiza infection of the roots was determined microscopically (Giovaneti 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 ANOVA analysis. The STASTICA (version 6.0) package was used for statistical analysis.

RESULTS AND DISCUSSION

Coinoculation with *S. meliloti* and *G. intraradices* of alfalfa plants

growth at low accessible phosphorus source resulted in the statistically proved highest levels of plant DW (Fig. 1), as compared to the variants with single inoculation. Our results indicated that foliar supplied nutrients did not suppress the effect of N_2 fixing bacteria and AMF on the DW. Jia et al. (2004) reported that inoculation with AMF promoted biomass production

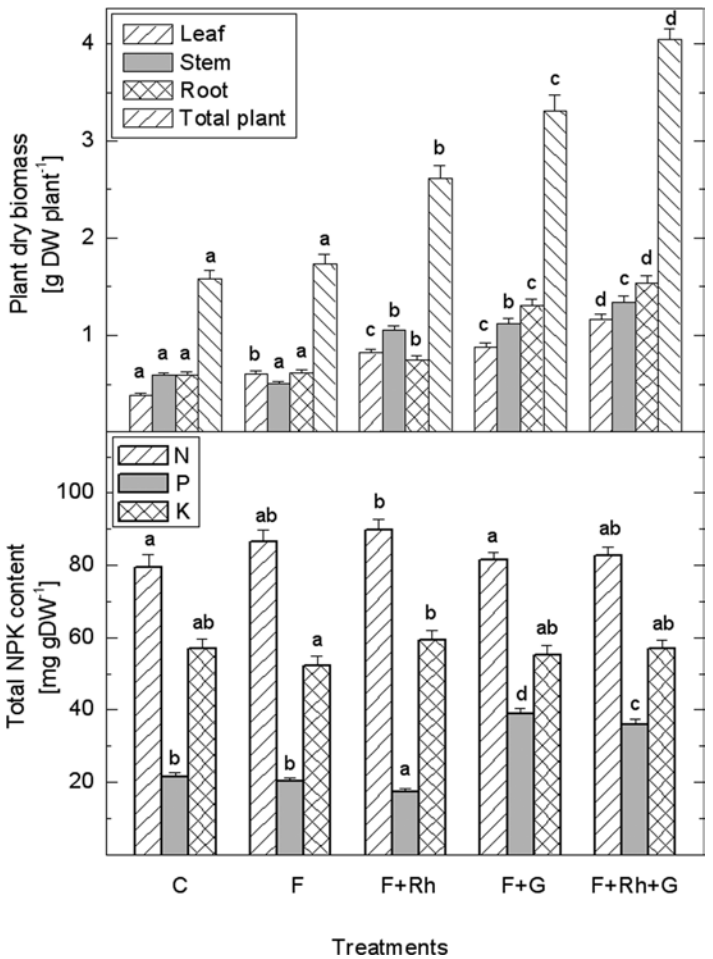

Fig 1. Nitrogen, phosphorus and potassium total content in leaves and plant biomass accumulation in alfalfa plants inoculated with *Sinorhizobium meliloti* and *Glomus intraradices*, as a result of insoluble Tunisian phosphorite application. Values are means ± SE, n = 7; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

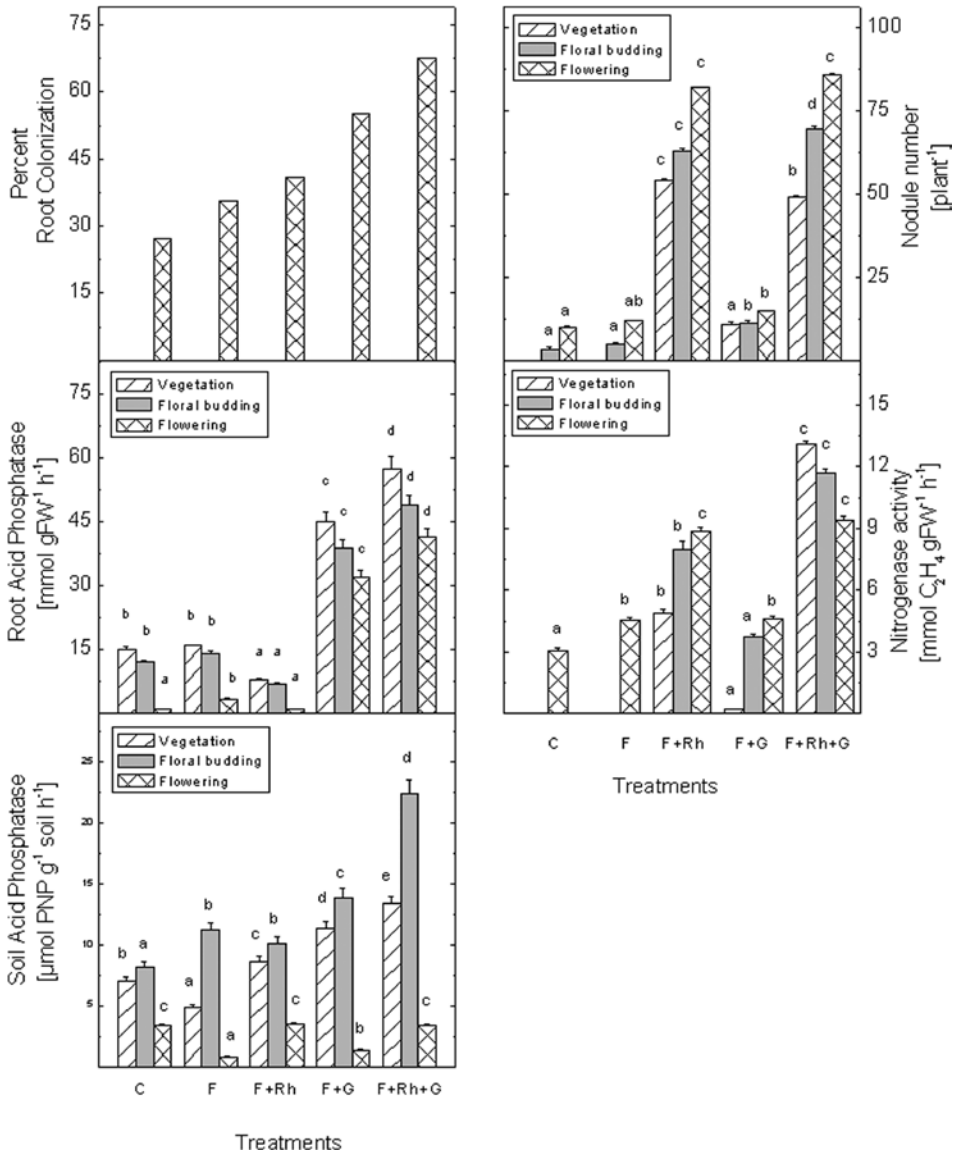

Fig 2. Percentage of root AMF colonization, soil and root acid phosphatase activity, nodules number and nitrogenase activity in alfalfa plants inoculated with *Sinorhizobium meliloti* and *Glomus intraradices* as a result of insoluble Tunisian phosphorite application. Values are means ± SE, n = 3; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

and photosynthetic rates in *Vicia faba* because of the enhanced P supply due to AMF inoculation.

Our data indicated that plant N content increased with the addition of foliar supplied nutrients (Fig. 1), but the highest values were observed in the treatments inoculated with *S. meliloti*. Phosphorus increased significantly as a result of AMF inoculation, while the content of K remained unchanged in the all experimental treatments. The total plant growth positively correlated with the total P concentration in the plant tissues (Fig. 1). Similarly, the P absorption ability was reported to be strongly connected with dry matter production (Lynch et al., 1991).

APA (root and soil) changed from vegetative to flowering stage (Fig. 2). A reduction of both phosphatases at flowering stage was observed. Root APA revealed mainly as a result of inoculation with fungi and dual inoculation as well and gradually decreased from vegetation to the flowering. Root APA was found to be maximal at alfalfa vegetative period when the plant requirements for P are high. In contrast with the root APA, maximal values of soil APA were established at floral budding stage. Dodd et al. (1987) reported that root colonization of wheat and onion with *Glomus* species resulted in significant root surface enlargement and root APA. Plant roots, fungi and bacteria separately possess APA (Abd-Ala, 1994), and that is

Table 1. Initial and residual mobile phosphorus content in the soil in experiments with alfalfa inoculated with *Sinorhizobium meliloti* and *Glomus intraradices* as a result of insoluble Tunisian phosphorite application. 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.

Treatments	Mobile phosphorus (P_2O_5)
	mg kg^{-1}
Initial	84±4.0
C	37±1.5
F	41±1.3
F+Rh	40±1.2
F+G	54±1.6
F+Rh+G	51±1.4

why maximum APA values of the coinoculated variants may due to the commutative effect of the three phosphatase activities. The mycorrhizal status is best manifested in the variants with dual inoculation with *S. meliloti*, which is in correspondence with the values of the N_2 fixing parameters as nodule number and ARA (Fig. 2). Number of nodules is significantly higher in the treatments when inoculation with *S. meliloti* was applied and gradually increased from vegetative to the flowering period. ARA is best manifested in the treatments with dual inoculation but decreased from the vegetative to the flowering period.

The content of available P in the soil after the harvest markedly increased after the inoculation of alfalfa with *G. intraradices* (Table 1). It can be supposed that AMF have the ability to decompose the Tunisian phosphorite. Therefore they are involved in the process of conversion content of P_2O_5 in soluble and accessible form. That is why P levels in plant tissues are maximal in the plants inoculated with *G. intraradices* due to their ability to dissolve rock phosphates and to increase root area surface.

In conclusion, the dual inoculation of alfalfa plants grown at a low accessible phosphorus source increased DW, N_2 fixing activity, AM root colonization, APA and P content in plants to different levels when compared with the single inoculation with *G. intraradices* or *S. meliloty.* Foliar application of nutrients did not suppress the effectiveness of created dual and triple symbiotic systems.

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References

Abd-Alla, M.H., 1994. Use of organic phosphorus by *Rhizobium leguminosarum biovar Viceae* phosphatases. Biol. Fertil. Soils, 18, 216-218.

Al-Niemi, T.S., M.L. Kahn, T.R. McDermott, 1997. P metabolism in the

bean-*Rhizobium* tropici symbiosis. Plant Physiol., 113, 1233-1242.

Dodd, J.C., C.C. Burton, R.G. Burns, P.J. Jeffries, 1987. Phosphatase activity associated with the roots and the rhizosphere of plants infected with vesicular-arbuscular mycorrhizal fungi. New Phytol., 107, 163-172.

Giovanetti, M., B. Mosse, 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytopatol., 84, 489-500.

Hardy, R.W. F., R. C. Burns, R. D. Holsten, 1973. Applications of the acetylene-ethylene assay for measurement of N_2-fixation. Soil Biol. Biochem., 5, 47-81.

Jackson, N.E., R.E. Franklin, R.H. Miller, 1972. Effects of vesicular-arbuscular mycorrhizae on growth and phosphorus content of three agronomic crops. Soil Sci. Soc. Am. Proc., 36, 64-67.

Jia, Y. S., V.M. Gray, C.J. Straker, 2004. The influence of *Rhizobium* and AMF on nitrogen and phosphorus accumulation by *Vicia faba*. Annals Bot., 94, 251-258.

Lowry, O., A. Lopez, 1946. Determination of inorganic phosphate in the presents of labile phosphate esters. J. Biol. Chem., 162, 421-426.

Lynch, J., A. Lauchli, E. Epstein, 1991. Vegetative growth of the common bean in response to phosphorus nutrition. Crop Sci., 31, 380-387.

Marschner, H., 1995. Mineral nutrition of higher plants, Academic Press, London.

Tabatabai, M.A., J.M. Bremner, 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem., 1, 301-307.

Woolhouse, H.W., 1975. Membrane structures and transports problems considered in relation to phosphorus and carbohydrate movements and regulation of endotrophic mycorrhizal associacions. In: Sanders F.E., Mosse B., Tinker P.B. (ed.): Endomycorrhizas, London, Academic Press, 209-239.

Xavier, L.J.C., J.J. Germida, 2003. Selective interaction between arbuscular mycorrizal fungi and *Rhizobium leguminosarum bv. Viceae* enhance pea yield and nutrition. Biol. Fertil. Soils, 37, 262-267.