Response of Pasture Grasses to Inoculation With Mycorrhizal Fungi and N-Fixing Bacteria

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Abstract

The effect of inoculation with "Mycosym TRI-TON" biofertilizer applied singly or in combination with symbiotic and non-symbiotic N-fixing bacteria on the plant biomass, N and P content of some pasture grasses and soil and roots acid phopsphatase activity was studied in a pot experiment. Active and effective symbiotrophic associations pasture grasses – AMF – N-fixing bacteria, suitable for agricultural practices in the creation of meadows and pastures were selected. The best synergetic interactions were established for the combinations AMF + *Rh. melliloti* 116 for lucerne, AMF + *Rh. trifolii* 325 for red clover and for gramineous grasses – AMF + *Az. brasilense* 107.

Key words: acid phosphatase activity, AM (Arbuscular mycorrhizal) fungi, forage grass, N-fixing bacteria, N and P content, plant biomass

Introduction

Arbuscular mycorrhizal fungi (AMF) form trophic root associations with plants, improving the assimilation and transportation of nutrients from the soil to host plants (Barea, 1991; Grogan and Chapin, 2000; Atkinson et al., 2002; Lopez-Gutierrez et al., 2004). This is due to their capacity to form a lot of branched extracellular structures, which strongly increase the absorbing surface. At the same time the fungal hyphae produce acid and alkaline phosphatases, which dissolve insoluble phosphates, making them available to the plants (Smith and Smith, 1996; Sato et al., 2015). Besides that, the legumes form symbiothrophic associations with symbiotic N-fixing bacteria. In this three-parted association plant-AMF-Rhizobium N and P are the limiting elements. The plant roots and fungal exudates also stimulate the development of free-living N-fixing bacteria. In this trophical associations the microsymbionts mutually support themselves with the main biogenic elements (N, C and P). The positive effect of double and

triple inoculation on the quantity and quality of plant biomass of great deal of forage legumes and grasses is proven by many authors (Saini et al, 2004; Miyanchi et al., 2008; Stancheva et al., 2008; Djonova et al., 2011).

Plant species have different capacity to form symbiotrophic associations, which determines the necessity of studies for selection of proper participants in the synergetic interaction between plants, AMF and N-fixing bacteria. The application of plant-microbes associations in the pastures and meadows formation can contribute to an increase of soil fertility and hay production and protect the soil from erosion processes. The utilization of biological methods for improving the plant nutrition ensures receiving of clean plant production on one hand and on the other – protection of groundwater from fertilizers contamination.

The aim of the study was to determine the effect of inoculation with mycorrhizal biofertilizer "Mycosym TRI-TON" applied singly or in combination with N-fixing bacteria on the growth of pasture legumes and gramineous grasses, the N and P content of plant biomass and phosphatase activity.

Materials and Methods

The study was carried out under the conditions of pot experiment with orchard grass (*Dactylus glomerata*), lucerne (*Medicago sativa,* L.*)*, red clover (*Trifolium pretense* L.*)*, tall fescue (*Festuca arundina* Schreber) and timothy grass (*Phleum pretense* L.*)*, grown on Grey forest soils (Orthic Luvisols) (IUSS Working Group WRB, 2006) from the city of Troyan. Pots containing 1 kg of soil were used in the experiment. The agrochemical properties of soil were as follows: humus – 2.07%; NH_4N – 10.0 mg/100 g; NO_3-N – 7.0 mg/100 g; P (P_20_5) – 2.3 mg/100 g; K (K_2O) – 17.8 mg/100 g; pH $_{(KCL)}$ – 5.5. The experimental scheme included inoculation of the plants with mycorrhizal granular "Mycosym TRI-TON" biofertilizer, applied singly or in combination with N-fixing bacteria. The biofertilizer contained mycorrhizal fungus *Glomus intraradices* consisted of more than 50 spores/g and more than 200 IMP (infective mycorrhizal propagules). It was added to the soil (at 2 cm depth under soil surface) before sowing in quantity 0.5 g/pot. The seeds were previously treated with suspension of N-fixing microorganisms (1.10^8 cells/ml) as follows: red clover – *Rhizobium melliloti* 166 or 116; lucerne - *Rhizobium trifolii* 294 or 325 and gramineous grasses – *Azospirillum brasilense* 107 or *Azospirillum lipoferum* 14. The strains tested are from the collection of Soil Microbiology Department of Nikola Poushkarov Institute of Soil Science, Agrotechnologies and Plant Protection. The plants were cultivated up to beginning of flowering. The dry shoot and root biomass were determined at the end of the study.

The N content of the roots and stems was determined by Kjieldahl digestion (Bremner and Mulvaney, 1982). The P content of the same plant parts was analyses by the molybdenum-vanadate method (Olsen and Sommers, 1982). The percentage of mycorrhiza infection of the roots was determined microscopically (Giovanetti and Mosse, 1980). To visualize the AMF colonization, roots were cleared by boiling for 4 min in 10% KON, rinsed three times with tap

water, and acidified with 0.1% HCl. Roots were stained boiling in 0.05% Trypan blue for 10 min. Following staining, the roots were rinsed with tap water, mounted on slides, and observed directly in lactic acid. The acid phosphatase (APA, E.C. 3.1.3.2) activity in both rhizosphere soil and roots of control variants and of those, treated with "Mycosym TRI-TON" was measured according to the method of Schneider et al. (2000), based on the original one of Tabatabai and Bremner (1969). Root tissue was homogenized with 0.1 M sodium acetate buffer (pH 5.0). After centrifugation, the supernatant was assayed for the enzyme activity by incubation in 5mM p-nitrophenyl phosphate and 0.1 M sodium acetate buffer (pH 5.0). The reaction was stopped by the addition of 0.2 M NaOH, and absorption was measured at 405 nm. The acid phosphatase activity was determined by colorimetric estimation of the *p*-nitrophenol released by phosphatase activity. Soil sample (1 g) was incubated at 37 C for 1 h with buffered (pH 6.5) sodium *p*-nitrophenyl phosphate solution and toluene. After incubation, the flask was immediately placed on ice and then 1 ml of 2 M $CaCl_2$ and 4 ml of 0.2 M NaOH were added to terminate the reaction and extract the *p*-nitrophenol formed. Absorbance of released *p*-nitrophenol was determined spectrophotometrically at 405 nm. For the blank, *p*-nitrophenyl phosphate was added after the incubation. The acid phosphatase activity was expressed as μmol *p*-nitrophenol per gram dry matter and incubation time 1h (μmol *p*-nitrophenol g^{-1} dry matter h^{-1}).

The statistical processing of the data on plant dry biomass included determination of the least significant differences (LSD) ($P \leq 0.05$) among the treatments. A statistical software package (StatGraphics Plus, version 5.1 for Windows, USA) was used.

Results and Discussion

The tested biopreparation "Mycosym TRI-TON" exerted positive influence on the plant biomass weight in all experimental cultures (Table 1). The different plants demonstrated specificity in their interaction with the microsymbionts. In lucerne, the dual inoculation with "Mycosym TRI-TON" and *Rh. meliloti* 166 showed the highest and significant effect on the dry shoot weight compared with the variant with single mycorrhiza inoculation. The differences among the treated variants were not established in red clover. The effect of the treatments on the dry root biomass compared with the control was significant but the differences among the treatments were not statistically proven. These data are in accordance with the higher percentage of root mycorrhization, obtained for the treated plants. In orchard grass and timothy grass the highest values of dry shoot biomass were established by the variants with dual inoculation with Mycosym and *Azospirillum brasilense* 107. The symbiotrophic association in timothy grass showed significant effect compared with the single inoculation with Mycosym for both dry shoot and root biomass. Similar data for the pointed out treatment were established also for the percentage of root mycorrhization, which was increased about 20% compared with the control and 10% compared with the variants with inoculation. The physiological influence on the tall fescue root biomass was observed in the dual inoculation *Azospirillum lipoferum* 14 + Mycosym.

Table 1. *Shoot and root dry biomass and root mycorrhization*

Treatments	Shoot dry biomass (g/pot)	Root dry biomass(g/pot)	Root mycorrhization(%)
Lucerne			
Control	1.00a	1.23a	38.97
+ Mycosym	1.25b	1.70b	42.70
+Myc.+Rh. Melliloti 116	1.45bc	1.60b	47.08
+ Myc. +Rh. melliloti 166	1.57c	1.60b	42.93
LSD P ≤ 0.05	0.25	0.20	
Red clover			
Control	1.32a	1.00a	41.88
+ Mycosym	2.03b	1.27b	49.41
+ Myc. +Rh.trifolii 294	2.05bc	1.29b	43.00
+ Myc. +Rh. trifolii 325	1.95c	1.16b	47.65
LSD P ≤ 0.05	0.31	0.12	
Orchard grass			
Control	1.15a	1.39a	24.33
+ Mycosym	1.85b	1.72b	36.88
+ Myc. + Az. brasilense 107	1.90b	1.63ab	45.95
Myc. + Az. lipoferum 14	1.43a	1.52ab	40.96
LSD P ≤ 0.05	0.31	0.25	
Timothy grass			
Control	1.30a	0.80a	23.92
+ Mycosym	1.75b	1.24b	37.15
+ Myc. + Az. brasilense 107	2.13c	1.63c	46.14
Myc. + Az. lipoferum 14	1.73b	0.97a	43.82
LSD P ≤ 0.05	0.37	0.18	
Tall fescue			
Control	1.30a	1.07a	25.01
+ Mycosym	1.85b	1.22a	34.96
+ Myc. + Az. brasilense 107	1.95b	1.28a	45.85
Myc. + Az. lipoferum 14	1.78b	1.53b	39.93
LSD P ≤ 0.05	0.25	0.24	
Values in the same column, followed by different letters are significantly different at P ≤ 0.05			

The combined treatment Mycosym + *Azospirillum brasilense* 107 lead to the highest percentage of root mycorrhization but the symbiotrophic association of the two mycrosimbionts influenced only the weight of shoot biomass without that of the roots.

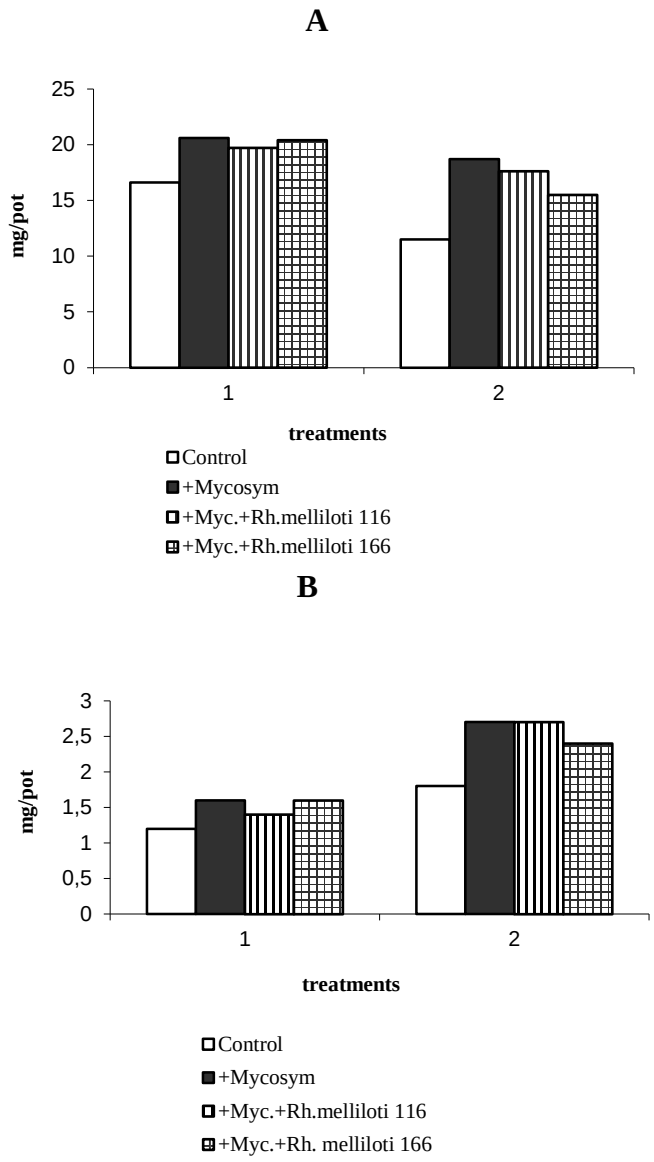


Figure 1. (A) *Nitrogen and (B) phosphorus content of shoots (1) and roots (2) of lucerne*

The data presented in Figures 1-5 show the N and P uptake in plant biomass at the end of the experimental time (beginning of flowering). Specificity of their distribution in shoot and root biomass for different plant species was observed. For both leguminous grasses root and shoot N content was higher in the treated plants compared with the control (Fig.1 and 2). The N content in the shoots was higher than in the roots. This was clearly expressed in red clover, especially in the shoots, where the N content in the variant with dual inoculation AMF + *Rh. trifolii* 325 had the

highest value. There were not significant differences in the N content in the variants with single and mixed treatment for lucerne, which indicated that in these variants the mineral nutrition was mainly improved due to the applied treatments. It was established that the AMF-application improved the P nutrition of both plant species tested. The P content of lucerne was higher in the roots than in the shoots while in the red clover the phosphates were exported in the shoots. Again the effect of the dual inoculation AMF + *Rh. trifolii* 325 was the highest concerning the shoot P content.

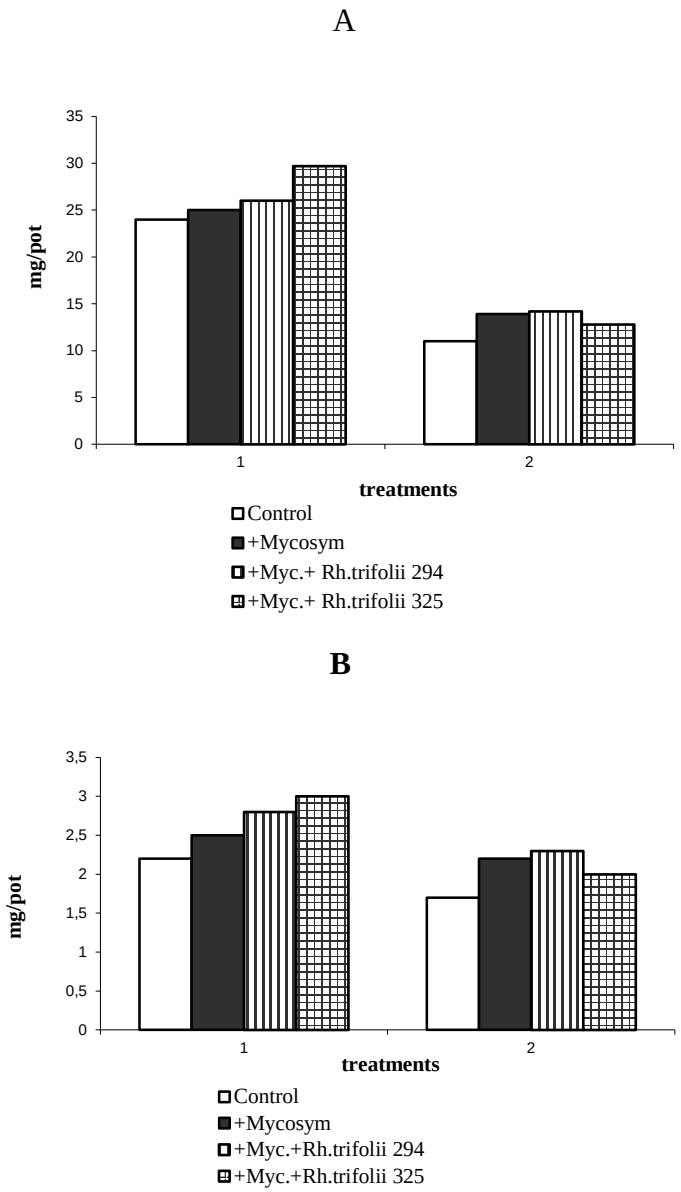


Figure 2. *(A) Nitrogen and (B) phosphorus content of shoots (1) and roots (2) of red clover*

In the gramineous grasses in all treated variants the values of biomass N and P content were higher in comparison with the control and these elements were mostly concentrated in the shoots than in the roots (Figures 3, 4 and 5). The highest values of N and P shoot content were obtained in the variant with dual inoculation, included AMF + *Az. brasilense* 107 for the three pasture grasses.

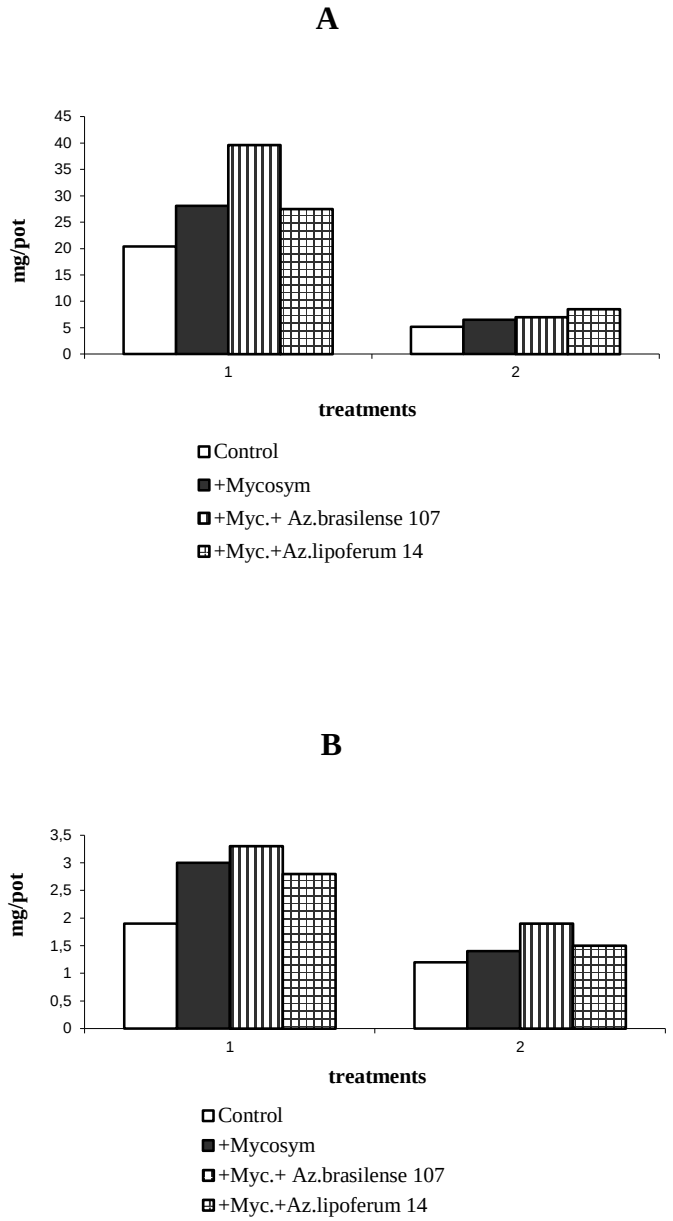
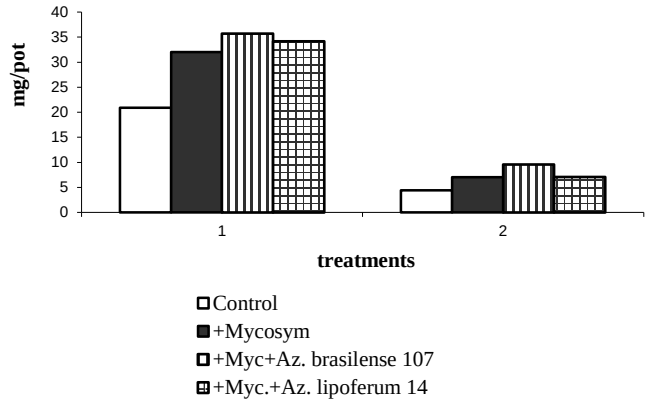


Figure 3. *(A) Nitrogen and (B) phosphorus content of shoots (1) and roots (2) of orchard grass*

The data on soil and roots acid phosphatase activity are presented in Figures 6 and 7. In the gramineous grasses the values of acid phosphatase activity in the roots were considerably lower compared with the leguminous plants. These values were comparatively higher in tall fescue and orchard grass. From both leguminous pastures higher values were obtained in the red clover. These data are in accordance with the higher percentage of the red clover root mycorrhization and considerably higher increase of shoot biomass (53 %) (Table 1). Similar data were obtained in our previous study with the same plant species, grown on slightly eroded Leached Cinnamonic soil (Djonova et al., 2014). Considerable differences in the values of soil acid phosphatase activity among plant species tested were not established but in all treated variants they were higher in comparison with the untreated plants.

A



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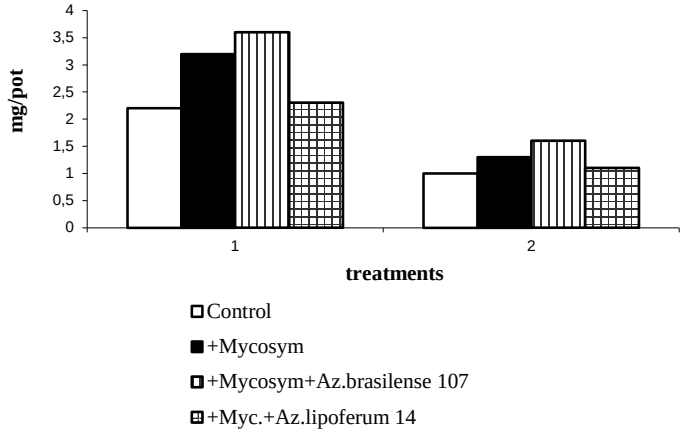
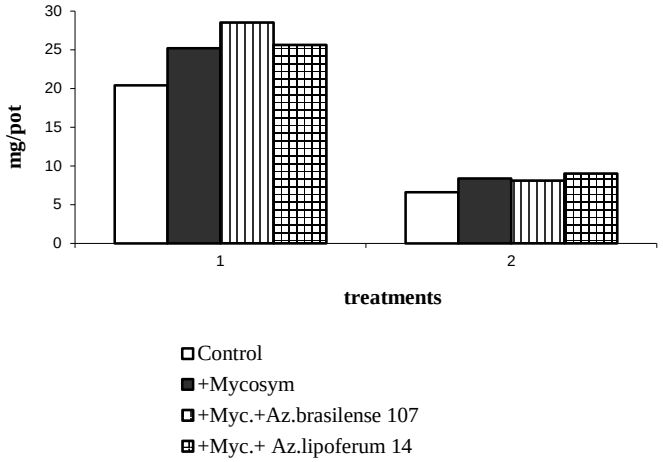


Figure 4. *(A) Nitrogen and (B) phosphorus content of shoots (1) and roots (2) of timothy grass*

A



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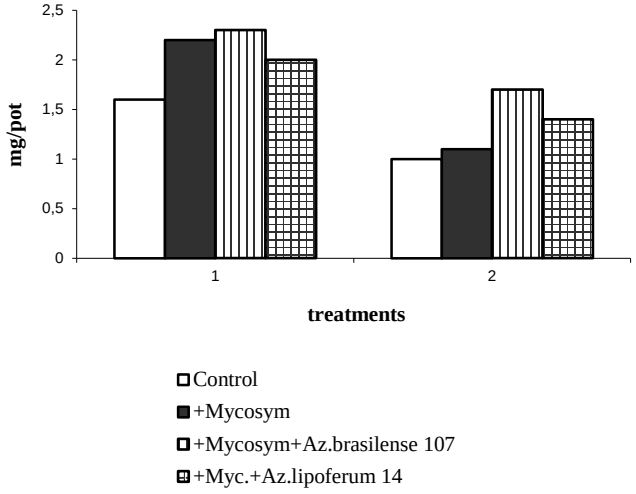

Figure 5. *(A) Nitrogen and (B) phosphorus content of shoots (1) and roots (2) of tall fescue*

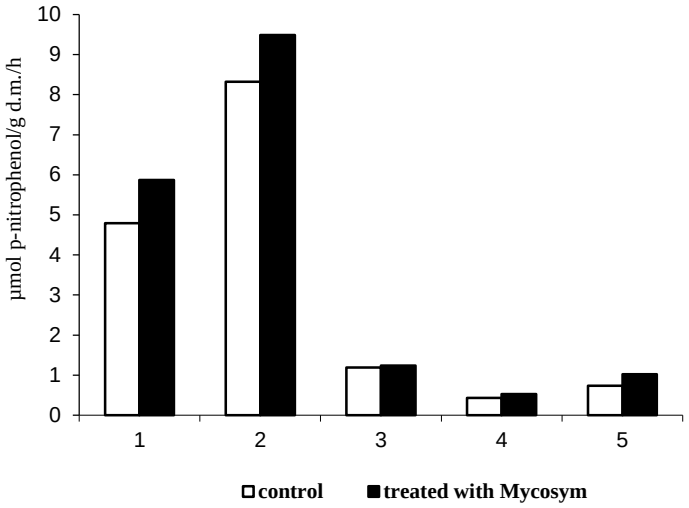

Figure 6. *Acid phosphatase activity in the roots of pasture grasses: 1-lucerne; 2-red clover; 3-orchard grass; 4-timothy grass; 5-tall fescue*

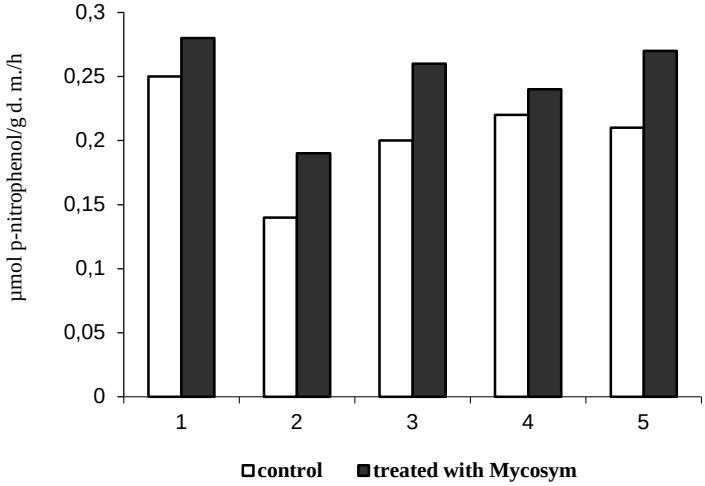

Figure 7. *Acid phosphatase activity in the rhizosphere soil of pasture grasses: 1-lucerne; 2-red clover; 3-orchard grass; 4-timothy grass; 5-tall fescue*

Nitrogen-fixing bacteria (symbiotic, free-living and associative) and AM fungi are plant beneficial microorganisms having the potential to improve biomass production. Their utilization for plant inoculation contributes to soil fertility conservation, reduction of mineral fertilization and ecological safe plant production. They are used as bioinoculants in many countries (Xavier and Germida, 2003; Barea et al., 2004; Perfanova, 2011; Singh et al., 2011; Hungria et al., 2013). The combined inoculation with AM fungi and N-fixing bacteria could have different effect on plant growth depending on the type of microsymbiontic interactions. Mycorrhizal colonization can affect root exudation and hence the composition and function of rhizobacterial community. Some authors reported for competition between AM fungi and Rhizobial bacteria for sites of root penetration (Barea, 1987; Jakobsen et al., 1992). Catford et al. (2003) showed that an established symbiosis in alfalfa could systematically exerted an inhibition on the second one. This inhibition could be explained by the common regulating signals between plant and both AM-fungi and Rhizobial bacteria. It has been established that root flavonoids are the molecular signals involved in the general recognizing process between plant and microorganisms (Juge et al., 2012).

On the other hand there is much information confirming that AM-fungi improve growth and N-fixation in symbiotrophic association plant – N-fixing microorganisms (Valdenegro et al., 2001; Saini et al., 2004; Djonova et al., 2014). This effect is due to the fact that N-fixation depends on steady adequate supply of phosphates to roots. AM fungi are able to take up phosphates from soil solutions with low phosphate concentrations more efficiently than simple roots. They take up, accumulate and transfer large amounts of phosphates to the plant by releasing the nutrients in root cells containing arbuscules. From there phosphates are transferred to cortical root cells, ready to be used by plants. The interactions in symbiotic system AM fungi – N-fixing microorganisms – plant are complex and that is why empirical studies should be carried out for each concrete symbiotrophic association.

In our study an increase of root and shoot biomass and their N and P content was established, which demonstrated synergetic interaction between tested AM fungi and Rhizobial strains. Obviously, plants recognized inoculants as beneficial partners. The effect of mixed application of "Mycosym TRY-TON" showed specificity for the tested plant species and depended on the type of interaction among microsymbionts. The best synergetic interations were established for alfalfa, treated with AMF + *Rh. melliloti* 116 and for gramineous grasses – AMF + *Az. brasilense* 107.

Many authors reported that AM-fungi increase the acid phosphatase activity in roots and rhizosphere zone (Bolan et al., 1987; Tarafdar and Marschner, 1994; Sato et al., 2015). The data presented in Figures 6 and 7 confirm the stimulative effect of mycorrhizal inoculation on acid phosphatase activity. The higher values of root acid phosphatase activity by the pasture legumes corresponded to their higher mycorrhization and better supply of the plants with biologically fixed nitrogen. The level of soil phosphatase activity is influenced by the root exudates, which from the other hand, also influence on the activity of the free-living soil phosphate-decomposing

bacteria. There were no considerable differences of the values of soil acid phosphatase activity among plant species tested, which could be due to the vegetation stage of the plants at the end of the experimental time (Figure 7). Probably, in later stages they may occur depending on the specificity of the composition and quantity of the exudates of the tested plants.

The obtained results outline active and effective symbiotrophic associations pasture grasses – AMF – N-fixing bacteria, suitable for agricultural practices in creating of meadows and pastures. These areas are situated mostly in mountains on eroded soils in drink water supplying zones. The application of such biological methods ensures not only higher quality and quantity of plant production but also protects the soil from erosion and contamination.

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