Почвознание агрохимия и екология, 55, 2/2021
Bulgarian Journal of Soil Science Agrochemisty and Ecology, 55, 2/2021

Response of Alfalfa (*Medicago sativa* L.) to microbial inoculations with *Rhizobium meliloti, Pseudomonas fluorescens* and *Glomus intraradices*

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Citation: Mitova, I., & Tomova, T. (2021). Response of Alfalfa (*Medicago sativa* L.) to microbial inoculations with *Rhizobium meliloti, Pseudomonas fluorescens* and *Glomus intraradices*. *Bulgarian Journal of Soil Science, Agrochemistry and Ecology, 55*(2), 21-27.

Abstract

The paper is aimed at establishing the effect of inoculation of alfalfa (*Medicago sativa*, L.) with N-fixing bacteria *Rhizobium meliloti*, mycorrhizal fungus *Glomus intraradices* and phosphate solubilizing bacterium *Ps. fluorescens* on the yield and N and P content of aboveground biomass. The plants were grown on Chromic Luvisol in a two-year field experiment under irrigation regime. Two cuts were harvested in the first year and four - in the second year. Positive effect of single and double microbial inoculations with *Rh. meliloti, Ps. fluorescens* and *Gl. intraradices* was established in both years. The largest increase in plant biomass yield and N uptake by the shoots was obtained with the single inoculation with *Rh. meliloti* 225 and mixed inoculation with *Rh. meliloti* 116 + *Ps. fluorescens*. The increases of alfalfa yield for these treatments were 29 and 34% for the first year and 32 and 27% for the second year respectively. The P uptake of aboveground biomass was the highest in the treatments with mixed inoculations of *Rh. meliloti* 116 with *Ps. fluorescens* or Gl. intraradices. The results obtained demonstrate the positive effect of microbial inoculations on the yield and nutrient uptake of alfalfa and could be of practical benefit for sustainable agricultural practices.

Key words: alfalfa, symbiotic N-fixing bacteria, AM (Arbuscular mycorrhizal) fungus, phosphate solubilizing bacteria, yield, nitrogen (N) and phosphorus (P) uptake

Introduction

The increase in the prices of mineral fertilizers during recent decades and the risk of soil and water contamination in intensive fertilization necessitates the utilization of alternative nutritional sources for plants. In this aspect, development of biological systems of agriculture acquires special relevance. Biofertilizers play a crucial role in these systems. They are created on the basis of various useful soil microorganisms, which assist plant nutrition (Subba Rao, 1993; Anthony & Kloepper, 2009; Singh et al., 2011). The first and the most widely used biofertilizer was created from nodule-forming bacteria (belonging to genus *Rhizobium*), which assimilate atmospheric nitrogen in symbiosis with leguminous plants. Biofertilizers for many leguminous plants have been utilized in agricultural practice of many countries for about a century.

The effectiveness of symbiotic system plant – nodule-forming bacteria depends on the availability of phosphorus (P) resources, necessary for plant growth. During the last few decades some studies proved that mixed inoculation of leguminous plants with nodule-forming bacteria and microorganisms, improving P nutrition of plants is more effective than single one (Staikovic` et al., 2011; Hungria et al., 2013; Kravchenko et al., 2013; Djonova & Petkova, 2014).

Alfalfa (*Medicago sativa* L.) is one of the most important forage crop in many countries. The rich contents of proteins, vitamins, calcium, high biomass yield, high digestibility of biomass and relatively low demand for soil conditions determine its importance for agriculture, especially for areas with developed livestock. It is suitable for both green eating and preparation of hay and silage and is one of the best precursors for almost all cereals and technical crops. It is established that alfalfa can be supplied up to 90% nitrogen from the atmosphere through plant-Rhizobium symbiosis. Comparing to the other leguminous crops alfalfa assimilate the largest amount of atmospheric nitrogen (Kirilov, 2016). In this way alfalfa contributes to the accumulation of atmospheric N in the soil, helping for decrease of mineral fertilizers application.

The importance of alfalfa as a fodder crop determines the increased interest in exploring the possibilities for augmentation of its productivity by microbial inoculants. Favorable effect of double inoculation of alfalfa with nodule-forming bacteria and AM fungi on plant growth was established by Stancheva et al. (2008), Djonova & Petkova (2014) and Jafari et al. (2018). Stajkovic`- Srbinovic`et al. (2017) reported that in a field experiment double inoculation of alfalfa with *Sinorhizobium meliloti* and rhizobacteria (representatives of genera's *Azotobacter, Bacillus, Pseudomonas* and *Enterobacter*) increased yield and nutrient uptake of alfalfa, indicating positive interactions between Rhizobium and rhizobacterial strains. The effect was expressed stronger in the first year of the experiment.

Plant species, as well as varieties of the same plant species, exert different capacity to participate in symbiotrophic relationships with microbial inoculants. This necessitates studies for different inoculants and plant varieties to be carried out in order to select proper participants in the plant-microbes synergetic interactions.

The objective of the study is to evaluate the effect of inoculation of alfalfa with N-fixing bacteria *Rhizobium meliloti*, phosphate solubilizing bacteria *Pseudomonas fluorescen*s and AM fungus *Glomus intraradices* on the yield and N and P assimilation of plant biomass in a two-year field experiment.

Materials and Methods

The study was carried out under field conditions on Chromic Luvisol (IUSS Working Group WRB, 2006) in the experimental field of Chelopechene village (Sofia district). The soil had a heavy sandy-clayey mechanical composition and low to medium availability of total forms of phosphorus, which is essential element for alfalfa growth. In the autumn of 2018 triple superphosphate (15 kg/da) was applied with main soil treatment. Starting dose of mineral N (4 kg/da) was applied before sowing of the seeds in the form of ammonium nitrate. Before the beginning of the experiment the agrochemical parameters of the soil were as follows: humus – 1.61%; mineral N – 8.6 mg/100 g; available P (P_20_5) – 13.5 mg/100 g; available K (K_2O) – 26.4 mg/100 g; $pH_{(H2O)}$ – 6.7. The experimental design was completely randomized and included plots of 2 m^2 in three replicates. In the past 7-8 years legumes have not been grown on the experimental field. The sowing of alfalfa, variety "Pleven 6" was done at rate of 4 kg seeds/da. Before sowing, the seeds were treated with suspensions of N-fixing and phosphate solubilizing bacteria (1.108 cells/ml) according to the scheme below. The strains tested were from the microbiological collection of Nikola Poushkarov Institute of Soil Science, Agrotechnologies and Plant Protection. The inoculum of *Glomus intraradices* EEZ 01, obtained as a two-member culture with oats was added to the soil (at 5 cm depth under the soil surface) before sowing in quantity of 150 g per m^2. The mycorrhizal strain was provided from the AMF collection of Estacion Experimental del Zaidin (CSIC Granada, Spain). Our previous investigations in pot conditions (Petkova et al., 2018) showed that *Rhizobium meliloti* 116 had better capacity for synergetic interactions with *Glomus intraradices* and *Ps. fluorescens* than *Rhizobium meliloti* 225. On the basis of these results the following experimental scheme was used in the experiment:

1. Control;
2. *Rhizobium meliloti* 116;
3. *Pseudomonas fluorescens*;
4. *Glomus intraradices*;
5. *Glomus intraradices* + *Rh. meliloti* 116;
6. *Rhizobium meliloti* 225;
7. *Ps. fluorescens* + *Rhizobium meliloti* 116.

The experiment was set in April 2018. Plants were cultivated up to beginning of flowering and then shoot biomass was cut. In the first year, two cuts were harvested until September and in the second year four cuts were done in the period May-September. The area for determination of hay yield from each plot was 0.25 m^2.

The plants were cultivated in irrigation conditions. During 2018 the maintenance of soil moisture in the range between 70 - 80% of WHC in the one-meter soil layer was carried with 9 irrigations at rate of 270 m^3/da. In 2019 nineteen irrigations were carried out at rate of 750 m^3/da. Each time the plants were harvested the dry weight of shoots was determined. Plant shoots were dried in an oven at 600 C to constant weight and the average dry weight per treatment was calculated. The N and P contents of the aboveground biomass from both cuts harvested in 2018 and second and fourth cuts in 2019 were analyzed. The N contents was determined by the method of Kieldal (Bremner & Milvaney, 1982) and P contents - by molybdenum-vanadate method (Mincheva & Brashnarova, 1975). On the basis of the data on the quantity of shoots biomass and their N and P content, the uptake of these elements by plants in aboveground biomass was calculated.

The statistical processing of data included determination of the least significant differences (LSD) ($P \leq 0.05$) among the treatments (STATGRAPHICS. Plus 2.1).

Results and Discussion

The data on alfalfa yield showed a positive effect of applied inoculations in respect to the control (Tables 1 and 2). For the first cut in 2018 the yield of all inoculated treatments was higher than that of the control (Table 1). The increase was significant for Treatment 6 (single inoculation with *Rh. meliloti* 225 – 29%) and Tr. 7 (mixed inoculation with *Rh. meliloti* 116 and *Ps. fluorescens* - 34%). There were no significant differences among treatments for the second cut of the same year. The highest and significant increase of alfalfa yield for the second year of the experiment for the second cut was registered for Tr. 4 (single inoculation with *Gl. intraradices* – 28% as comparing to the control) and Tr. 6 (single inoculation with *Rh. meliloti* 225 – 26%). At the end of the experiment the yield increase was obtained again for the latter treatment and also for Tr.7 (mixed inoculation with *Rh. meliloti* 116 and *Ps. fluorescens*). The increase was 32% and 27% respectively. The difference between two treatments mentioned above was not significant. Between mixed inoculations, the one of *Rh. meliloti* 116 with *Ps. fluorescens* (Treatment 7) showed better effect on the yield in comparison to that of *Rh. meliloti* 116 with *Gl. intraradices* (Treatment 5) for the first cut in the beginning of the experiment. This result can be explained by the capacity of *Ps. fluorescens* to stimulate the formation of additional infection sites that than are occupied by *Rhizobium* (Plazinski & Rolfe, 1985). At later stages of the study, no statistically proven differences in yield between these treatments were registered. When comparing the yields from two years of the experiment in absolute values between the respective identical treatment options, it is seen that the yields in the second year are higher than that in the first year. This is due to the better development of the root system and adaptation of alfalfa to soil and climatic conditions.

Table 1. Microbial inoculation effects on alfalfa yield (kg/m^2) in the first year of the field experiment

Treatments	Yield (kg/m^2) First cut	Yield (kg/m^2) Second cut
1. Control	0.38a	0.37a
2. *Rh. meliloti* 116	0.46abc	0.47a
3. *Ps. fluorescens*	0.45abc	0.41a
4. *Glomus intraradices*	0.41ab	0.38a
5. *Glomus intraradices* + *Rh. meliloti* 116	0.39a	0.45a
6. *Rh. meliloti* 225	0.49bc	0.43a
7. *Ps. fluorescens* + *Rh. meliloti* 116	0.51c	0.36a
LSD P ≤ 0.05	0.10	0.12

*Values in the same column, followed by different letters are significantly different at P≤0.05

Table 2. Microbial inoculation effects on alfalfa yield (kg/m^2) in the second year of the field experiment

Treatments	Yield (kg/m^2)			
	Number of the cut			
	First	Second	Third	Fourth
1. Control	0.47a	0.46a	0.72a	0.41a
2. *Rh. meliloti* 116	0.53a	0.53ab	0.79a	0.36a
3. *Ps. fluorescens*	0.47a	0.51ab	0.83a	0.42a
4. *Glomus intraradices*	0.60a	0.59b	0.66a	0.43a
5. *Glomus intraradices* +*Rh. meliloti* 116	0.52a	0.55ab	0.56a	0.44ab
6. *Rh. meliloti* 225	0.51a	0.58b	0.70a	0.54c
7. *Ps. fluorescens* + *Rh. meliloti* 116	0.60a	0.49a	0.71a	0.52bc
LSD P ≤ 0.05	0.16	0.10	0.28	0.086

*Values in the same column followed by different letters are significantly different at P≤0.05

The data on N and P contents of aboveground biomass confirmed the favorable effect of inoculations, especially with respect to N. In the first year of plant vegetation the increase of N uptake from the shoots was expressed more clearly for the first cut. At this stage of the study the N uptake from aboveground biomass was significantly higher in comparison to the control for all inoculated treatments (Fig. 1). Its values were the highest for Tr. 7 (double inoculation with *Rh. meliloti* 116 and *Ps. fluorescence*). Significant increase of N uptake of the shoots was registered for Tr. 2 (single inoculation with *Rh. meliloti* 116) and Tr. 5 (double inoculation with *Gl. intraradices* and *Rh. meliloti* 116) for the second cut in 2018. In the second year of the study the highest and significant values of N uptake for the second cut were established for treatments 4 (single inoculation with *Gl. intraradices*), 5 (mixed inoculation with *Gl. intraradices* and *Rh. meliloti* 116), 6 (single inoculation with *Rh. meliloti* 225) and 7 (double inoculation with *Rh. meliloti* 116 and *Ps. fluorescens*) (Fig. 2). At the end of the experiment Tr. 6 and Tr. 7 were characterized by the highest N uptake by the shoots (Fig. 2). The data for the last two mentioned treatments are in accordance with the data on alfalfa yield, represented on Tables 1 and 2.

As regard to the P uptake of aboveground biomass the effect of inoculations was weaker than that of N assimilation (Fig. 3 and 4). Figure 3 indicated that for both cuts in 2018 in all inoculated treatments the values were higher than the control but the difference was significant only for Tr.7 (mixed inoculation with *Rh. meliloti* 116 and *Ps. fluorescens* – first cut) and Tr. 5 (double inoculation with *Rh. meliloti* 116 and *Glomus intraradices* – second cut). In 2019 significant increases of P uptake by the shoots for the second cut, were registered in single and mixed inoculations with *Gl. intraradices* (Treatments 4 and 5) and Tr. 7 (double inoculation with *Rh. meliloti* 116 and *Ps. fluorescence*). These results indicated the positive effect of the activity of microbial inoculants known to improve P uptake, i.e. *Ps. fluorescens* and *Gl. intraradices*. At the end of the experiment the P uptake of the shoots for all inoculated treatments did not differ significantly from that of the control.

The results obtained proved the positive effect of applied single and mixed inoculations on alfalfa growth and they are in accordance with the data reported by Guinazi et al. (2010). The authors cited established that double inoculation of alfalfa with *Sinorhizobium meliloti* B399 and *Ps. fluorescens* sp. FM7d caused a significant increase in root and shoot dry weight, length and surface area of roots and symbiotic properties of alfalfa plants.

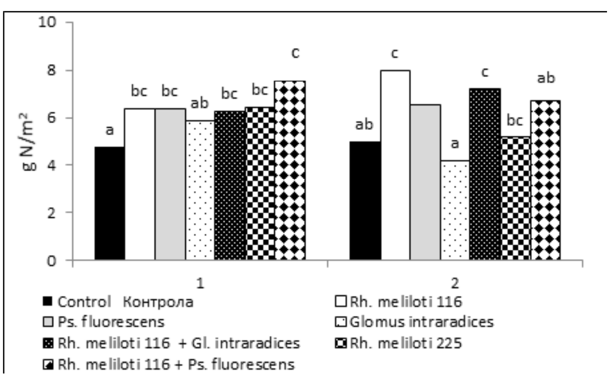

Fig. 1. Nitrogen (N) uptake by the shoots (first year). 1 - first cut; 2 - second cut

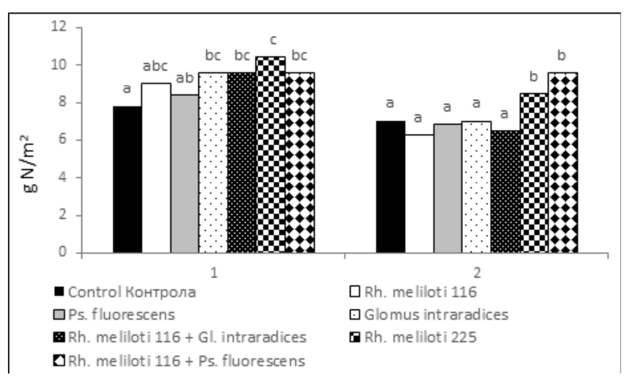

Fig. 2. Nitrogen (N) uptake by the shoots (second year). 1 - second cut; 2 - fourth cut

*Values in the same column followed by different letters are significantly different at $P \leq 0.05$

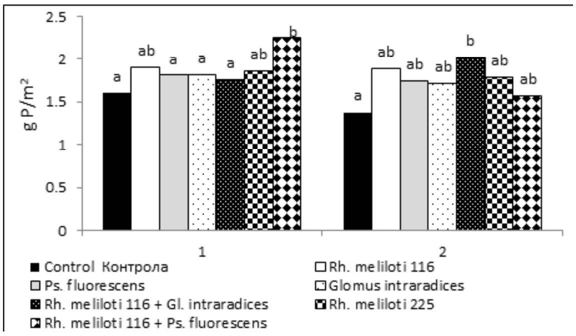

Fig. 3. Phosphorus (P) uptake by the shoots (first year); 1 - first cut; 2 - second cut

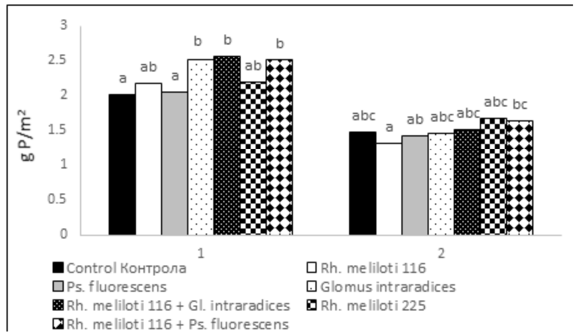

Fig. 4. Phosphorus (P) uptake by the shoots (second year); 1- second cut; 2 - fourth cut
*Values in the same column followed by different letters are significantly different at P≤0.05

Conclusions

In the conditions of two-year field experiment with alfalfa, grown on Chromic Luvisol under irrigation regime, a positive effect of single and double microbial inoculations with *Rh. meliloti, Ps. fluorescens* and *Gl. intraradices* was established in both years. The largest increase in plant biomass yield and N uptake by the shoots was obtained with the single inoculation with *Rh. meliloti* 225 and mixed inoculation with *Rh. meliloti* 116 + *Ps. fluorescens*. The increases of alfalfa yield for these treatments were 29 and 34% for the first year and 32 and 27% for the second year respectively. The highest P uptake was registered in the mixed inoculations of *Rh. meliloti* 116 with *Ps. fluorescens* or *Gl. intraradices*. The results obtained demonstrate that single and mixed inoculation of alfalfa seeds could be of practical benefit in sustainable agricultural practices.

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