RESEARCH ARTICLE Legume Research- An International Journal, Volume 43 Issue 5: 706-710 (October 2020)

Response of Microorganisms in Alfalfa Rhizosphere to Microbial Inoculation

Snežana Andjelković, Jasmina Radović, Snežana Babić,
Tanja Vasić, Simonida Djurić[1], Dragana Stamenov[1], Timea Hajnal Jafari[1]

10.18805/LR-514

ABSTRACT

Background: Various microorganisms that can have a positive or negative effect on plant development are present in the rhizospheric soil of alfalfa. The research aimed to investigate the impact of two nitrogen-fixing bacteria and two species of the phytopathogen fungus *Colletotrichum* on the abundance of aminoheterotrophs, nitrifying and denitrifying bacteria in the rhizosphere of three cultivars of alfalfa.

Methods: The experiment was carried in vegetation pots as three factorial, where the first factor was alfalfa cultivar (Affinity+Z, K-28 and Perry), the second was the isolate of phytopathogen fungus *Colletotrichum*: *C. trifolii* (isolate Coll 4) and *C. destructivum* (two isolates: Coll-11 and Coll 657); and the third was the variant of bacterial inoculation (*Azotobacter chroococcum* and *Sinorhizobium meliloti*). The number of microorganisms was determined by introducing a diluted soil suspension into proper media and counted per one gram of absolutely dry soil.

Result: According to the Fisher test applied inoculation microbial inoculation with nitrogen-fixing bacteria and phytopathogen fungus had different effects on the abundance of examined microorganisms in rizospheric soil of different alfalfa cultivars.

Key words: Alfalfa, Cultivar, Isolate, Microbial Inoculation.

INTRODUCTION

Alfalfa is a plant species characterized by intensive biological nitrogen fixation process and due to its nutrition value widely grown forage legume worldwide (Karayilanli and Ayhan, 2016, Liu *et al.,* 2016; Strbanovic *et al,,* 2015). Rhizospheric microorganisms have participation in the mineralization of organic compounds, maintaining the soil structure, suppressing pathogens, stimulating plant growth and suppress plant pathogens by indirect means (Hrynkiewic and Baum, 2011). In the rhizosphere harbors diverse and rich regime of beneficial microorganisms which directly affect plant health and soil fertility in which a significant number of bacterial and fungal species (Gul *et al*., 2019).

Rhizobia, the famous mutualistic symbiotic bacteria, lives in the root tissue and provide macrosymbionts with nitrogen and an inexpensive way to enhance soil fertility and agricultural productivity (Meena *et al.,* 2014). They also have properties of biocontrol agents and may be applied to promote the growth of plants (Girija *et al*., 2020; Kumar *et al.,* 2011).

The bacteria of the genus *Azotobacter*, make an important contribution to providing nitrogen to plants and to increased nitrogen content of soil. *A. chroococcum* of rhizosphere is the most active producer of biologically active substances: auxin, gibberellins, pyridoxine, biotin, nicotinic acid (Hayat *et al.,* 2010), antifungal compounds, antibiotics (Dey *et al.,* 2017). Positive plant-microbe interactions enable environmentally friendly strategies for conventional and organic agriculture (Berg, 2009).

Apart from useful microorganisms,such as nitrogen-fixing bacteria, in the soil found plant pathogenic microorganisms. These microorganisms cause weaker growth or death of plants and lower microbial activity in the rhizosphere of infected plants. Anthracnose of alfalfa is one of the most important diseases of this plant species. The presence of anthracnose inflicts great damage, to reduce forage yield, plant survival and contribute to reduced stand persistence. It is most commonly caused by *C. trifolii* Bain et Essary but also by *C. destructivum* O'Gara. (Vasić *et al.,* 2009). In addition to having a negative effect on plant health, these microorganisms influence microbial activity in the rhizosphere of infected plants.

Soil microorganisms are involved in many biochemical processes (Yadav, 2015). Thanks to them, huge amounts of organic and inorganic nitrogen undergo many complex processes of transformation in the soil, after which this nutrient becomes available to plants (Majumder *et al*., 2018).

Institute for forage crops Kruševac, 37251 Globoder, Republic of Serbia.
[1]Faculty of Agriculture, University of Novi Sad, Dositej Obradović Square 8, 21000 Novi Sad, Republic of Serbia.

Corresponding Author: Snežana Andjelković, Institute for forage crops Kruševac, 37251 Globoder, Republic of Serbia.
Email: snezana.andjelkovic@ikbks.com

How to cite this article: Andjelković, S., Radović, J., Babić, S., Vasić, T., Djurić, S., Stamenov, D. and Jafari, T.H. (2020). Response of Microorganisms in Alfalfa Rhizosphere to Microbial Inoculation. Legume Research. 43(5): 706-710.

Submitted: 03-07-2019 **Accepted:** 02-09-2020 **Published:** 12-10-2020

Aminoheterotrophs comprise a large group of bacteria and fungi that transform proteins and other organic nitrogen compounds. They have the ability to produce extracellular proteolytic enzymes that cause the hydrolytic degradation of large protein chains to smaller units that the cell can absorb. The ammonifiers get nutrients and energy due to the decomposition of fresh organic matter in the root system of plants (Doolotkeldieva *et al.,* 2015). The diversity and non-specificity of aminoheterotrophs is the reason that this group of microorganisms in the soil is numerous.

In contrast to aminoheterotrophs, nitrifying bacteria are less abundant in the soil. They are free living organisms, widespread in soil and influence soil fertility and a part of the nitrogen cycle. The soil matrix, abundance of nutrients, pH, temperature, oxygen, moisture are factors that affect changes in the population of nitrifying organisms (Sahravat, 2008).

Denitrifying bacteria are one of the most important groups of bacteria involved in the nitrogen cycle and able to adapt to many abiotic factors in soil (Stres *et al.,* 2008). Microorganisms capable of denitrification are widely distributed in the nature and 10-15% of the bacterial population in soil are denitrifiers (Huang *et al*., 2011).

The objective of the study was to investigate the effect of inoculating nitrogen-fixing bacteria (*A. chroococcum* and *S. meliloti*) and two species of the phytopathogenic fungus *Colletotrichum* (*C. trifolii* and *C. destructivum*) on the number of aminoheterotrophs, nitrifying bacteria and denitrifying bacteria in rhizosphere of three cultivars of alfalfa (Affinity+Z, K-28 and Perry).

MATERIALS AND METHODS

The experiment was carried out in 8 dm^3 volume vegetation pots in covered space at the Institute for forage crops in Kruševac during spring and summer of 2016. For the purposes of research soil with pHKCl 5.90, total nitrogen 0.138%, humus 2.62%, P_2O_5 6.6 mg 100 g^{-1}, K_2O 24.05 mg 100 g^{-1} was used. Three alfalfa cultivars (Affinity+Z, Perry and K-28) with different resistance to anthracnose were used. The experiment was a three-factorial (cultivar of alfalfa, phytopathogenic fungi and microbial inoculation).

Before sowing, the seed was inoculated with *S. meliloti* and *A. chroococcum* (10 ml of inoculum per pot with 108 cells in 1 ml). The *S. meliloti* cultures were grown on YM substrate. *A. chroococcum* cultures were raised on the liquid substrate. The plants were treated with *Colletotrichum* conidia (4–6×10^4 ml^{-1}) after 42 days after sown. The number of conidia was determined using of cell counting chamber. The inoculation was done using the following inocula:
1) *C. destructivum* (Coll-11) + *S. meliloti*, 2) *C. destructivum* (Coll-11) + *A. chroococcum*, 3) *C. destructivum* (Coll-11), 4) *C. destructivum* (CC 657) + *S. meliloti*, 5) *C. destructivum* (CC 657) + *A. chroococcum*, 6) *C. destructivum* (CC 657), 7) *C. trifolii* (Coll-4) + *S. meliloti*, 8) *C. trifolii* (Coll-4) + *A. chroococcum* and 9) *C. trifolii* (Coll-4).

The effect of inoculation was determined at the onset of flowering. Microbiological research included investigating the number of aminoheterotrophs, nitrifying bacteria and denitrifying bacteria in the rhizosphere. The number of aminoheterotrophs (soil suspension had a dilution 10^{-6}) was determined on MPA medium (Pochon and Tardieux, 1962); the number of denitrifying bacteria (soil suspension had a dilution 10^{-4}) determined on medium by Giljtaj (Koleško, 1981).

The number of nitrifying bacteria (nitrite and nitrate bacteria) was determined on liquid substrates with the dilution of 10^{-3} (Jarak and Djurić, 2006). All microbial analyses were performed in three replications and the average number of microorganisms was calculated per gram of absolutely dry soil and the data were logarithmic.

The data were statistically processed by programme *Statistics 8.0*. The significance of the difference between the applied treatments was determined using Fisher's LSD test.

RESULTS AND DISCUSSION

The results obtained in this research showed that applied inoculation had different effects on the abundance of examined microorganisms in rizospheric soil of on different alfalfa cultivars (Table 1).

The number of aminoheterotrophs in rhizospheric soil without inoculation (control) of the cultivars Affinity + Z (7.107) and K-28 (7.109) was statistically significantly lower than the number in cultivar Perry (7.364).

According to the Fisher test (Table 1), the response of aminoheterotrophs to inoculation with isolates of phytopathogen fungus and nitrogen-fixing bacteria mostly was negative in cultivar Affitnity + Z and Perry. However, interaction the cultivar K-28 and applied inoculation had a positive effect on the number of aminoheterotrophs in all variants, except in the variant with *C. destructivum* (CC 657). According to the results of Mrkovaèki *et al*. (2016) inoculation of sugar beet with strains of *Azotobacter chroococcum*, the number of ammonifiers was higher compared to the control in the first period, while it decreased in the second sampling period. Stamenov *et al*. (2017) reported that use of isolates *Pseudomonas* P12 and *Bacillus* B1 positively affected the number of aminoheterotrophs in rhizosphere of English ryegrass.

The number of nitrifiers in the soil samples analyzed was between 0.100 and 3.143 (Table1). In the control treatment of cultivar K-28 the number of these microorganisms (2.322) was significantly lower than cultivars of alfalfa Affinity + Z (2.926) and Perry (2.966). All of the inoculation variants in cultivar Affitnity+Z achieved a statistically significant difference in a negative sense compared to the control. In the cultivar K-28, the number of nitrifiers in some treatments was statistically significantly decrased compared to control, while in other treatments number was at level of control (no statistically significant difference). In the cultivar Perry, application of nitrogen-fixing bacteria and phytopathogen fungus didn't show a clear trend

Table 1: The effect of inoculation on abundance of microorganisms in rhizosphere of alfalfa (log of number).

Cultivar of alfalfa	Izolate of phytopathogen fungus	Nitrogen-fixing bacteria	The number of amino heterotrophs	The number of nitrifying bacteria	The number of denitrifying bacteria
Affinity +Z	*C. destructivum* (Coll-11)	*S. meliloti*	7.130^{m}	2.775^{c}	3.203^{j}
		A. chroococcum	6.995^{s}	2.346^{e}	3.215^{j}
		Ø	6.951^{u}	$0,133^{k}$	$2.908^{k.l}$
	C. destructivum (CC 657)	*S. meliloti*	6.946^{v}	1.857^{h}	3.378^{i}
		A. chroococcum	6.614^{y}	0.318^{j}	$2.912^{k.l}$
		Ø	7.261^{i}	0.492^{i}	$2,919^{k}$
	C. trifolii (Coll-4)	*S. meliloti*	6.985^{t}	0.100^{k}	$3,509^{h}$
		A. chroococcum	6.824^{x}	2.352^{e}	1.100^{o}
		Ø	6.586^{z}	$1,978^{h}$	2.916^{k}
	Control	Ø	7.107^{n}	2.926^{b}	1.301^{n}
K-28	*C. destructivum* (Coll-11)	*S. meliloti*	7.727^{b}	$2.318^{e.f.g}$	3.728^{e}
		A. chroococcum	7.808^{a}	$2.326^{e.f.g}$	3.799^{d}
		Ø	7.163^{l}	1.987^{h}	3.210^{j}
	C. destructivum (CC 657)	*S. meliloti*	7.552^{e}	1.973^{h}	3.978^{b}
		A. chroococcum	7.669^{c}	2.199^{g}	3.877^{c}
		Ø	$6.942^{v.w}$	$0.200^{j.k}$	3.942^{b}
	C. trifolii (Coll-4)	*S. meliloti*	7.601^{d}	$2.342^{e.f}$	3.367^{i}
		A. chroococcum	7.500^{f}	1.978^{h}	3.591^{g}
		Ø	7.171^{k}	$2.212^{f.g}$	4.123^{a}
	Control	Ø	7.109^{n}	$2.322^{e.f.g}$	3.663^{f}
Perry	*C. destructivum* (Coll-11)	*S. meliloti*	7.032^{r}	2.786^{c}	3.332^{i}
		A. chroococcum	7.204^{j}	2.983^{b}	3.203^{j}
		Ø	6.865^{w}	$3.143^{a.b}$	3.167^{j}
	C. destructivum (CC 657)	S. meliloti	7.057^{q}	0.100^{k}	$2.878^{k.l}$
		A. chroococcum	7.037^{r}	$0.218^{j.k}$	2.854^{l}
		Ø	7.077^{p}	$3.031^{a.b}$	$2.901^{k.l}$
	C. trifolii (Coll-4)	*S. meliloti*	7.130^{m}	2.639^{d}	2.342^{m}
		A. chroococcum	7.090^{o}	0.100^{k}	$2.878^{k.l}$
		Ø	7.316^{h}	2.958^{b}	2.342^{m}
	Control	Ø	7.364^{g}	2.966^{b}	3.188^{j}

Note: Mean values with the same superscript(s) are not significantly different according to Fisher's LSD test ($p < 0.05$).

in the change in the number of nitrifiers compared to the control. The interaction between plants and nitrifiers is complex (Skiba *et al.,* 2011). Subbarao *et al*. (2007) suggest that most legumes evaluated showed negative biological nitrification inhibition activity in root exudates, indicating that they are likely to stimulate nitrification.

Data in Table 1 show that the control variants of the examined alfalfa cultivars differed statistically significantly in the number of denitrifiers. Using *C. trifolii* (Coll 4) and *A. chroococcum* in the cultivar Affinity + Z the lowest number of denitrifying bacteria (1,100) was recorded. In all other variants of inoculation in the cultivar of alfalfa, the number of denitrifying bacteria is statistically significant increased compared to control.

In all most treatments inoculation cultivar K-28 had higher number of denitrifying bacteria compared to control. However, interaction of cultivar K-28 and C. *destructivum* (Coll-11), as well as interaction of this cultivar with *C. trifolii* (Coll-4) and *S. meliloti*, the number of denitrifying bacteria was lower than in the control. The response of denitrifying bacteria to inoculation cultivar Perry was negative or there was no difference compared to the control. Only the application of *C. destructivum* (Coll-11) and *S. meliloti* in this cultivar showed a statistically significantly higher number of denitrifying bacteria. Achouak *et al.* (2019) demonstrated that plant species shape denitrification activity and modulate the diversity of the active microbiota through root exudation.

The number of investigated groups microorganisms varied in the rhizosphere of three cultivars of alfalfa, which means that the composition root secretions also depend of the cultivar. Diverse microbial communities in the rhizosphere respond to the abundance and great diversity of root exudates that specific for plant species (Nath *et al.,* 2015). The plant genotype drives the diversity of root microbial community structure and function, thus demonstrating that plants are able to filter their root microbiomes in a defined environment (Reinhold-Hurek and Hurek, 2011). Apart from the plant, microbiological inoculation also affect microbiological activity in soil (Vasić *et al.,* 2014).

CONCLUSION

These results indicated that the abundance of amino- -heterotrophs, nitrifying and denitrifying bacteria were dependant upon alfalfa cultivar and the applied inocula. The use microbial inoculation on three cultivars of alfalfa showed different effect on the number of investigated groups microorganisms which indicates that probably each cultivar had a specific composition of root secretions and different conditions in the rhizosphere.

ACKNOWLEDGEMENT

Research was financed by the Ministry of Education, Science and Tehnological Development, Republic of Serbia.

REFERENCES

Achouak, W., Abrouk, D., Guyonnet, J., Barakat, M., Ortet, P., Simon, l., Lerondelle, C., Heulin, T. and Haichar, F.Z. (2019). Plant hosts control microbial denitrification activity. FEMS Microbiology Ecology. 95(3): fiz021.

Berg, G, (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology. 84 (1): 11-8.

Doolotkeldieva, T., Bobusheva, S. and Konurbaeva, M (2015). Effects of *Streptomyces* biofertilizer to soil fertility and rhizosphere's functional biodiversity of agricultural plants. Advances in Microbiology. 5: 555-571

Dey, R., Sarkar, K., Dutta, S., Murmu, S. and Mandal, N. (2017). Role of *Azotobacter* sp. isolates as a plant growth promoting agent and their antagonistic potentiality against soil borne pathogen (*Rhizoctonia solani*) under *in vitro* condition. Int. J. Curr. Microbiol. App. Sci. 6 (11): 2830-2836.

Girija, D., Panchami, P.S., Jose, P.E. Saeed T. and Nair, S.S. (2020). Isolation and characterization of native cowpea rhizobia from Wayanad India. Legume Research.(43): 126-133.

Gul, A., Salam, A., Afridi, M.S., Bangash, N.k., Ali, F., Ali, M.Y., Khan, S. and Mubeeen, R. (2019). Effect of urea, bio-fertilizers and their interaction on the growth, yield and yield attributes of *Cyamopsis tetragonoloba*. Indian Journal of Agricultural Research. 53(4): 423-428 .

Hayat, R., Ali. S., Amara, U., Khalid, R. and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. Ann. Microbiol. 60: 579-598.

Hrynkiewicz, K. and Baum, C. (2011). The Potential of Rhizosphere Microorganisms to Promote the Plant Growth in Disturbed Soils. In: Environmental Protection Strategies for Sustainable Development, Strategies for Sustainability. [Malik A and Grohmann E (eds.)], Springer: 35-64.

Huang, S., Chen, C., Yang, X., Wu, Q. and Zhang, R. (2011). Distribution of typical denitrifying functional genes and diversity of the nirS-encoding bacterial community related to environmental characteristics of river sediments. Biogeosciences. 8: 3041-3051.

Jarak, M. and Djurić S. (2006). Practicum of Microbiology. Faculty of Agriculture, Novi Sad.

Karayilanli, E. and Ayhan, V. (2016). Investigation of feed value of alfalfa (*Medicago sativa* L.) harvested at different maturity stages. Legume Research. 39(2): 237-247.

Koleško, O.J. (1981). Ekoligija mikrooraganizmov poèvi. Višaja škola, Minks.

Kumar, H., Dubey, R.C. and Maheshwari, D.K. (2011). Effect of plant growth promoting rhizobia on seed germination, growth promotion and suppression of *Fusarium* wilt of fenugreek (*Trigonella foenum-graecum* L.). Crop Protection 30 (11): 1396-1403.

Liu, L., Li Z., Shi, W., Li, H., Huang, F., Li, J. and Wang M. (2016). Study on the taxonomy status of alfalfa with cream flowers. Legume Research. 39 (6): 860-867.

Majumder, S.P., Chaitanya, A.K., Datta, A., Padhan, D., Badole, S. and Mandai, B. (2018) Dynamics of carbon and nitrogen in agricultural soils: role of organic and inorganic sources. In: Soil Management and Climate Change. [María Ángeles Muñoz and Raúl Zornoza (eds)]. Elsevier Academic Press: 151-169.

Meena, S.S., Mehta, R.S, Bairwa, M. and Meena, R.D. (2014). productivity and profitability of fenugreek (*Trigonella foenum-graecum* l.) As influenced by bio-fertilizers and plant growth regulators. Legume Research. 37 (6): 646-650.

Mrkovaèki, N., Bjelić, D., Maksimović, L., Ćurèić, Ž., Ćirić, M., Živanov, M. (2016). The effect of inoculation with *Azotobacter chroococcum* on microorganisms in rhizosphere and sugar beet yield in organic farming. Matica Srpska Journal for Natural Sciences. 130: 45-52.

Nath, D.J., Gogoi, D., Gayan, A. and Chelleng, A. (2015). Influence of chemical and biological properties of field grown pea and lentil rhizospheres on nodulation status and resident rhizobial population in acidic soils of Assam, India. Legume Research. 38(6): 844-850.

Poshon, J. and Tardieux, P.(1962): Tehniques d'analise en microbiologie du sol, Paris.

Reinhold-Hurek, B. and Hurek, T. (2011). Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14: 435-443.

Sahrawat, K.L. (2008). Factors affecting nitrification in soils. Communications in Soil Science and Plant Analysis. 39 (9-10): 1436-1446.

Skiba, W.M., George, T.S., Baggs, E.M. and Daniell, T.J. (2011). Plant influence on nitrification. Biochem. Soc. Trans. 39(1): 275-278.

Stamenov, D., Djurić. S., Hajnal-Jafari. T. and Andjelković, S. (2017). Influence of *Pseudomonas* and *Bacillus* strains isolated from *Lolium perenne* rhizospheric soil in Vojvodina (Serbia) on planth growth and soil microbial communities. Polish Journal of Microbiology. 66(2): 269-272.

Strbanović, R., Simic, A., Postic, D., Zivanovic, T., Vuckovic, S., Pfaf-Dolovac, E. and Stanisavljevic, R. (2015). Yield and morphological traits in alfalfa varieties of different origin. Legume Research. 38(4): 434-441.

Stres, B., Danevèević, T., Pal, L., Mrkonjic-Fuka, M., Resman, L., Leskovec, S., Hacin, J., Stopar, D., Mahne, I. and Mandic-Mulec, I. (2008). Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. FEMS Microbiology Ecology. 66: 110-122.

Subbarao, G.V., Tomohiro, B., Masahiro, K., Osamu, I., Samejima, H., Wang, H.Y., Pearse, S.J., Gopalakrishnan, S., Nakahara, K., Zakir Hossain, A.K M., Tsujimoto, H. and Berry, W.L.

(2007). Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (*Triticeae*) combat nitrification in wheat farming? Plant Soil. 299**:** 55-64.

Vasić, T., Anđelković, S., Radović, J., Lugić, Z., Hajnal-Jafari, T., Djurć, S. and Živković, S. (2014). Alfalfa inoculation: the effect on root growth and number of rhizospheric microorganisms. Romanian Biotechnological Letters. 19(4): 9457-9464.

Vasić, T., Radović, J., Lugić, Z., Marković, J., Jevtić, G. and Gajić, S. (2009). Occurrence of *C. trifolii* (Bain et Essary), the inducer of alfalfa anthracnose in Serbia. In: Sustainable use of genetic diversity in forage and turf breeding, [I C Huyghe (ed)]. Springer: 369-374.

Yadav, B.K. (2015).Variation in soil biological properties with legume crops in arid region of Rajasthan, India. Legume Research. 38 (6): 772-780.