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The effects of co-inoculation of PGPR bacteria and *Sinorhizobium meliloti* on nutrient contents, plant growth and yield of alfalfa

Alireza Tavasolee*[1], Kazem Khavazi[2], Ahmad Asgharzadeh[3], Hassan Monirifar[4], Saeid Ghassemi[5]

[1]*Soil and Water Research Department, East Azerbaijan Agricultural and Natural Resources Research and Education Center, AREEO, Tabriz, Iran*

[2&3]*Soil and Water Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran*

[4]*Seed and Plant Improvement Research Department, East Azerbaijan Agricultural and Natural Resources Research and Education Center, AREEO, Tabriz, Iran*

[5]*Department of Plant Eco-physiology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*

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Abstract

Two field experiments with factorial arrangement based on a randomized complete block design with three replications were conducted in 2013 and 2014 to evaluate the effects of co-inoculation of PGPR bacteria (control and mixed of azotobacter, azospirillum and pseudomonas inoculations) and *Sinorhizobium meliloti* (control, strain 1, strain 2, strain 3 and mixed of these strains) on nutrient contents, plant growth and yield of alfalfa (*Medicago sativa*). Results showed that application of PGPR bacteria enhanced nitrogen (N), phosphorus (P), manganese (Mn), zinc (Zn) and copper (Cu) contents of alfalfa in compared with control. Plant height, fresh and dry weight of forage in 2013 were significantly higher than 2014. PGPR treatment significantly increased plant height, number of branches, fresh and dry weight of forage of alfalfa compared with control.

***Corresponding Author:** Alireza Tavasolee ✉ ar.tavasolee@yahoo.ca

Introduction

One the most common forage species that cultivated in the world is Alfalfa (*Medicago sativa* L.) and about 30 Mha of this plant is grown on worldwide. This plant has good quantity and quality of production and can be to improve the characteristics of the land where it is cultivated (Jiang *et al*., 2006). Alfalfa has high protein content, good digestibility and palatability. It is widely planted throughout the world for hay, pasture, silage making and livestock feed (Hu and Cash, 2009).

Chemical fertilizers are costly and create environmental problems. Therefore, in present agricultural system use the organic, sustainable and environmentally friendly methods (Esitken *et al*., 2005). Bio-fertilizers can be used to improve plant growth and help to sustain environmental health and soil productivity of containing instead of synthetic chemicals (O'Connell, 1992). Plant growth-promoting rhizobacteria (PGPR) have beneficial effects on biocontrol function, disease-resistance mechanisms, reduction of the plant ethylene level, the production of phytohormones and promote growth, crop yield and crop quality (Babalola, 2010). These bacteria actively colonize plant roots and improved plant development. The PGPR (1) effected fixing nitrogen, producing hormones or solubilizing phosphates and promote the plant growth or increasing the enzymatic activity of the plant, enhancing root development, affected the plant metabolism by increasing the uptake of minerals and water or increased microorganisms' activities to enhance the plants yield; (2) or suppressing plant pathogens and promote the plant growth. These abilities of PGPR bacteria can reduce the negative impact of chemical fertilizers and improve the soil fertility and plant yield (Pérez-Montano *et al*., 2014).

One of the most important model systems of symbiotic nitrogen fixation is the partnership between the legumes of the genus Medicago (alfalfa and relatives) and Rhizobium *Sinorhizobium meliloti*. Rhizobia are terminally differentiated into bacteroids inside root nodules (indeterminate nodules) in this partnership and lose the ability to reproduce (Sprent, 2001; Gibson *et al*., 2008). Rhizobial invasion in the symbiotic model *Medicago sativa*/*Sinorhizobium meliloti* occurs via root hairs. The perception of bacterial nodulation factors by the host plant leads to cell division in the root per cycle and in the root cortex, where the nodule primordial forms. Simultaneously, root hairs deform and curl. The bacteria are entrapped in this curl, the local cell wall is hydrolyzed, and a plasma membrane invagination occurs, leading to the formation of an infection thread (Brewin, 2004). The aim of the study reported here was to investigate the influence of co-inoculation of PGPR bacteria and *Sinorhizobium meliloti* on nutrient contents, plant growth and yield of alfalfa.

Materials and methods

Two field experiments with factorial arrangement based on randomized complete block design with three replications was conducted in 2013 and 2014 in an experimental farm (latitude 38.15° N, longitude 46.45° E, altitude 1349 m) to evaluate the effects of co-inoculation of PGPR bacteria (control and mixed of azotobacter, azotobacter and pseudomonas treatments) and *Sinorhizobium meliloti* (control, strain 1, strain 2, strain 3 and mixed of these strains) on nutrient contents, plant growth and yield of an ecotype of alfalfa (Kara yonje). The averages of maximum and minimum temperatures and rainfall during the work in 2013 and 2014 were shown in Table 1. The Main properties of the experimental soil appear in Table 2. Three strains of *Sinorhizobium meliloti* were chosen according the pretests, and then ecotype of alfalfa (Kara yonje) was inoculated with these bacterias (Seeds of this ecotype were inoculated with 10^8 bacterias per ml). Before the sowing, 20 kg urea per hectare was added to the farm (Starter fertilizer for legumes) and inoculated seeds were sown.

Table 1. Averages of maximum and minimum temperatures and rainfall during the work in 2013 and 2014.

Month	Temperature (°C)		Rainfall (mm)	
	2013	2014	2013	2014
April	13.3	13.1	47.3	22
May	16.6	19.3	39.5	53.9
June	23	24.2	7.8	0.1
July	26.4	27.2	4.5	18.8
August	25.3	28.4	0	1
September	21.8	22.9	0.4	2.4

Table 2. Some physical and chemical properties of experimental soil.

Depth (cm)	EC (ds/m)	PH	Organic Carbon (%)	P (mg/kg)	K (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Sand (%)	Silt (%)	Clay (%)
0-35	3.23	8	0.9	6.64	209	0.7	2.5	4.2	1.4	78	14	8

For measurement of morphological traits and yield of alfalfa, the forage at $1m^2$ of the middle part of each plot was separately harvested (Cut off 5cm from the soil surface at the 25% of flowering stage) than fresh weight and number of branches were determined. The dry weight of each sample was determined after oven drying at 80°C for 48 h.

For measurement of nutrient contents, 1 gram of dried samples was powdered and used for determination of nitrogen (N_2) content in plant tissues with kjeldahl (Nelson and Sommers, 1973). Phosphorus (P) was measured by yellow method, in which vanadate-molybdate (Tandon *et al.*, 1968) is used as an indicator. P content was determined at 430nm using a spectrophotometer (Shimadzu UV3100, Japan). To determine the iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) content in plant tissues of alfalfa, plant samples were dry-ashed at 500°C for 7h and then, 500mg of dried samples was digested in 5ml HNO_3. Tubes were filled up to volume (50mL) with double-distilled water and analyzed for ion content (mg g^{-1} dry weight) with atomic absorption spectrophotometry (Shimadzu model: AA-7000, Kyoto, Japan).

SPSS 16 software used for the data analyzed and the means of traits were compared using LSD tests at $P \leq 0.05$. To draw fig. s, excel software was used.

Results

PGPR bacteria significantly influenced N_2, P, Mn, Zn and Cu contents of alfalfa (Table 3).

Table 3. Analysis of variance of nutrient contents of alfalfa in treatments by PGPR bacteria and *Sinorhizobium meliloti* in 2013.

Source	df	Mean Square					
		N_2	P	Fe	Mn	Zn	Cu
Repeat	2	7662.39	43.749	54082.7	14396.4	4021.5	802.76
Sinorhizobium meliloti (Sm)	4	1387.17 ns	10.043 ns	57961.4 ns	11332.5 ns	1132.3 ns	7833.4 ns
PGPR bacteria (PGPR)	1	17275.3 *	95.027 *	308743.4 ns	104200.2 *	14462.4 *	5119.5 *
PGPR × Sm	4	1570.12 ns	10.287 ns	28423.6 ns	8261.5 ns	626.1 ns	691.07 ns
Error	18	2194.64	12.442	78223.5	16134.6	1908.687	794.6
CV (%)		28.69	25.33	36.76	32.91	32.84	34.51

ns,*,**: No significant and significant at P ≤ *0.05* and P ≤ *0.01*, respectively

Application of PGPR bacteria enhanced the N_2, P, Mn, Zn and Cu contents of alfalfa compared with control plants. The Iron content in treating plants with PGPR increased by about 30.77%, but, this superiority was not statistically significant (Table 4).

Table 4. Means of of total element concentration of alfalfa in treatments by PGPR bacteria and *Sinorhizobium meliloti* in 2013.

Treatment	N (mg/kg)	P (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Cu (mg/kg)
control	139.26 b	12.15 b	659.36 a	327.02 b	111.06 b	68.63 b
PGPR bacteria	187.26 a	15.71 a	862.29 a	154.86 a	154.86 a	994.76 a

Different letters in each column indicate significant difference at $p \leq 0.05$.

Combined analyses of variance for morphological traits and yield of alfalfa showed significant effects of the year and PGPR bacteria on plant height, fresh and dry weight of forage. The effect of PGPR for branches per plant was also significant (Table 5).

Table 5. Combined analysis of variance of morphological traits and yield of alfalfa in treatments by PGPR bacteria and *Sinorhizobium meliloti* in 2013 and 2014.

Source	df	Mean Square			
		Plant height	Number of branches	Fresh weight of forage	Dry weight of forage
Year (Y)	1	1734.19 **	7580.2 ns	523.62 **	44.712 **
Repeat	4	18.718 ns	2439.9 ns	16.518 ns	1.4 ns
Sinorhizobium meliloti (Sm)	4	51.609 ns	3473.5 ns	9.865 ns	0.872 ns
Sm × Y	4	5.774 ns	1392.6 ns	8.892 ns	1.027 ns
PGPR bacteria (PGPR)	1	239.48 *	28967.009 *	226.01 **	26.432 **
PGPR × Y	1	25.415 ns	1230.35 ns	0.852 ns	0.109 ns
PGPR × Sm	4	68.193 ns	4941.6 ns	11.17 ns	1.138 ns
PGPR × Sm × Y	4	18.435 ns	3273.388 ns	10.659 ns	0.673 ns
Error	36	55.544	4335.88	15.43	1.231
CV (%)		12.25	21.27	30.36	26.50

ns,*,**: No significant and significant at P ≤ *0.05* and P ≤ *0.01*, respectively

Plant height, fresh and dry weight of forage in 2013 was significantly higher than 2014. PGPR treatment significantly increased plant height, number of branches, fresh and dry weight of forage of alfalfa compared with control (Table 6).

Table 6. Means of morphological traits and yield of alfalfa in treatments by PGPR bacteria and *Sinorhizobium meliloti* in 2013 and 2014.

Treatments		Plant height (cm)	Number of branches (per m²)	Fresh weight of forage (tons per hectare)	Dry weight of forage (tons per hectare)
Year	2013	66.19 a	320.86 a	15.89 a	50.51 a
	2014	55.44 b	298.38 a	9.98 b	3.325 b
PGPR bacteria	control	58.82 b	287.64 b	11 b	3.524 b
	PGPR	62.81 a	331.59 a	14.88 a	4.852 a

Different letters in each column indicate significant difference at $p \leq 0.05$

Discussion

N_2, P, Mn, Zn and Cu contents increased by plant growth-promoting rhizobacteria (PGPR) (Table 4). Increased the uptake of nutrient elements from the soil, enhanced the N_2 contents of plants (Marschner, 1995). This evidence confirms the data showing that the percentage of N_2, P, Mn, Zn and Cu was significant and relatively increased in the bacteria-treated plants, which may be explained by higher concentrations of N_2 stimulated by bacterial application. PGPR can improve the nutrient status of plants by increasing plant phytohormones production. This response has been demonstrated in many plant species and increases the nutrient contents in these plants (Cappellari *et al.*, 2017).

The high plant height, fresh and dry weight of forage of alfalfa (Table 6) in 2013 may be caused by low temperature and rainfall before harvesting, compared with 2014 (Table 1). Competition of plants for nutrients and water availability, reduced the plant height due to water stress (Ghassemi-Golezani *et al.*, 2010). Leaf and stem growth rate dependent on cell expansion so, these organs are very sensitive to water stress (Hearn, 1994). Decline in the cell enlargement and more leaf senescence reduced the plant fresh and dry weight under water stress (Shao *et al.*, 2008).

PGPR bacteria may stimulate plant growth (Table 6) by several mechanisms. That includes mechanisms such, nitrogen fixation, active the microorganisms, suppression of disease and production of plant growth regulators such as auxins and gibberellins (Holl *et al.*, 1988; Chanway, 2002). Auxin or indol-3-acetic acid (IAA), is a class of plant hormones stimulate both rapid and long term responses in plants (Cleland, 1971) and increased plant height (Table 6). *Pseudomonas* strains have increased root and shoot elongation in tomato, lettuce and canola (Glick *et al.*, 1997). They regulate growth by affecting physiological and morphological processes at very low concentrations (Arshad and Frankenberger, 1998). The IAA and nitrogenase activity was detected in two bacterial suspensions in this study and they are forming a beneficial association with the plants as evidence of the increase in plant dry weight and nutrient level uptake (Lindberg and Granhall, 1984).

Conclusions

The results of the present study showed co-inoculation of PGPR bacteria improved the nutrient contents, plant height and forage yield of *Medicago sativa, but*, *Sinorhizobium meliloti* doesn't have a significant effect on these traits. Therefore, PGPR bacteria are useful tool for alfalfa plants.

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