American-Eurasian J. Agric. & Environ. Sci., 5 (3): 296-307, 2009
ISSN 1818-6769
© IDOSI Publications, 2009

Influence of the Co-inoculation *Azospirillum brasilense* and *Rhizobium meliloti* plus 2,4-D on Grain Yield and N, P, K Content of *Triticum aestivum* (Cv. Baccros and Mahdavi)

[1]*Mehray Askary,* [2]*Akbar Mostajeran,* [3]*Rehaeh Amooaghaei and* [4]*Maryam Mostajeran*

[1]Biology Dept., University of Arak, Arak, Iran
[2]Plant Science Division, Biology Dept., University of Isfahan, Isfahan, Iran
[3]Biology Dept., University of Sharakord, Skarakord, Iran
[4]Microbiology Division, Biology Dept., University of Isfahan, Isfahan, Iran

Abstract: Co-inoculation with symbiotic microorganisms to create a successful system of biological nitrogen fixation in a non-legumes crop can lead to many profits for plant. The aim of this work was to evaluate the single and co-inoculation effects of *Azospirillum basilense* (native or Sp7) and *Rhizobium meliloti* (native or DSMZ 30135) plus 2,4-D on grain yield and the total N, P and K content of the grain. The results indicated that co-inoculation of wheat seeds with *A. brasilense* and *R. meliloti* had positive and significant effects on the grain yield and N, P and K content of the wheat grains in compared to either single inoculation or control plants. In overall, the average grain yield was increased to 53.8% of co-inoculation plants with a mixture of *A. brasilense* and *R. meliloti* compared to the control plants. This was due to increasing number of grains (26%) and grain weight per plant (22%) in co-inoculated plants. The results showed that single inoculation with *Azospirillum* produced significantly higher grain yield by 29% and the grains contain more N (22.8%), P (59.5%) and K (34%) compared to the control plants. Simultaneously, co-inoculation increased the mentioned parameters by 53.8, 52, 114 and 82%, respectively compared to the control Therefore, the effect of either the single or dual inoculation of wheat with *A. brasilense* and *R. meliloti* could result higher grain yield with better quality. However the effect of single and/or co-inoculation on plant growth parameters varies depending on the strain of *A. brasilense* (Sp7 and native), wheat cultivars and selected of co-inoculants (type of mixed strains). The highest grain yield and also N, P and K in grain were observed in combination of the local strains (native *A. brasilense* and native *R. meliloti*) when concentration of 10^7 cfu ml^{-1} and 10^3 cfu ml^{-1} were used respectively for the strains plus 2 ppm of 2,4-D.

Key words: *Azospirillum brasilense* · *Rhizobium meliloti* · *Triticum aestivum* · Co-inoculation · Nitrogen · Phosphor · Potassium

INTRODUCTION

Inoculation with plant growth promoting bacteria (PGPB) can result a significant change in various plants growth parameters, which may affect crop yield [1, 2]. Increase in different plant parameters such as height, tiller number, dry matter yield and N uptake of rice plants that inoculated with *A. lipoferum* [3, 4], dry matter of shoot and seed of *Medicago sativa* co-inoculated with *Azospirillum* and *Rhizobium* [5] and also in dry matter of leaf and shoot and mineral elements in grain and grains yield and stover in maize plants inoculated with *A. brasilense* plus 2,4-D [6] could be mentioned as few examples among too many.

Bashan [7] reported that inoculation of wheat with *A. brasilense* significantly increased plant dry weight, number of tillers per plant, spikelt fertility and grain yield. Also, co-inoculated wheat with *Azospirillum* and *Azorhizobium caulinodans* showed significant increases in dry weight and nitrogen content as compared with no inoculated plants [8]. Even in stress conditions, *Azospirillum* inoculation increased the grain yield and mineral nutrition in plants under stress [2]. For example, in experiments of Creus [2] the grain yield loss to drought was 26.5% in no inoculated plants and in *Azospirillym*-inoculated plants was 14.1%. The grains harvested from *Azospirillum*-inoculated plants had significantly higher Mg, K and Ca than no inoculated

Corresponding Author: Akbar Mostajeran, Department of Biology, University of Isfahan, Isfahan Iran

plants. Therefore, the inoculation can increase grain yield and also the accumulation of total nitrogen of the plant and its grain yield [9-13]. The beneficial effects caused by the inoculation with PGPB are not only due to BNF [14, 15], but mainly, the best efficiency in the absorption of water and nutrients, which happens due to a more developed root system, caused by the capacity of the bacterial-plant produced growth promoters substances [16-20, 1, 13].

Co-inoculation can benefit plant growth by different mechanisms [21-24]. However one of the most commonly reported plant growth promotion mechanism by bacteria is the changing of morphological and physiological changes in root system [17, 25, 26]. An increase in the number of lateral roots and root hairs cause addition of root surface available for nutrients and water uptake. Higher water and nutrient uptake by inoculated roots caused an improved water status of plant, which in turn could be the main factor enhancing plant growth [18, 11, 27-29]. Increasing nutrient uptake such as NO_3^-, NH_4^+, PO_4^{-2}, K^+, Rb and Fe^{+2} in the various inoculated plants have reported [30, 31, 4, 12]. For example, single and double inoculation with *Azetobacter*, *Azospirillum* and *Streptomyces* increased P, Mg and N content in wheat grains [33]. Co-inoculation of *Sorghum* with *Azospirillum* and *Glomus* significantly increased P, N, Zn, Cu and Fe content. Thus, co-inoculation may substitute partially as P and N fertilizer [34].

Using co-inoculation to have more advantages for plant growth and development in one hand and different results obtain from different mixture of strains for co-inoculation in another hand lead us to use a mixture of *Azospirillum brasilense* and *Rhizobium meliloti* to evaluate the effects of single and co-inoculated strains plus 2,4-D on grain yield and also N, P and K content of wheat grain harvested. This mixture of strains was used because a rotation of wheat and alfalfa in our agriculture sector is usual and mostly the farmers use harvested grain as wheat seeds for new production.

MATERIALS AND METHODS

Bacterial Strains: Two bacterial types were used: (1) two *Azospirillum brasilense* strains, a local strain isolated from wheat roots of Arak region in central of Iran, identified by biochemical and morphological tests and confirmed by 16S rDNA amplification called native strain and the reference strain Sp7 (DSMZ 1960) as standard one; (2) two *Rhizobium meliloti* strains, a local strain isolated from *Medicago sativa* nodules of Arak region in central of Iran as native strain and the reference strain *R. meliloti* DSMZ 30135 were obtained from the Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ, Germany). Physiological and biochemical characters of the local isolated bacteria were examined according to methods described in Bergey's Manual of Systematic Bacteriology [35] and also 16S rDNA amplification

Preparation of Inoculants and Seeds: *A. brasilense* strains (Sp7 and native) were cultured in a NFB liquid medium supplemented with NH_4Cl (0.5 g L^{-1}) at 30°C [36, 37] and two *R. meliloti* strains (native and standard) were grown in YMA medium at 25°C [38, 30] in Erlenmeyer flasks for the 24 h in a rotary shaker at 200 rpm (logarithmic phase). Before inoculation, the growth was harvested by centrifuging (1000g, 10 min), washed with sterile saline phosphate buffer and then were re suspended in phosphate buffer at concentration of 10^7 and 10^3 cfu ml^{-1} for *A. brasilense* strains and *R. meliloti* strains, respectively. Two concentrations of 2,4-D (0 and 2 ppm), dissolved in water and was added to inoculants according to statistical layout.

Two cultivars of wheat seeds (*Triticum aestivum*) called Baccross cv. and Mahdavi cv. were prepared from the Institute of Agricultural and Research of Isfhan in Iran. The seeds were surface sterilized according to methods described by Ogut *et al*. [39]. The sterilized wheat seeds were vernalized at 4°C for the 10 day [2]. The experimental design was a factorial experiment in three replicates.

Planting and Application of Treatments: The results of our pervious experiment [40] indicated that combination of *Azospirillum* and *Rhizobium* would have the best results when the population of 10^7 and 10^3 cfu ml^{-1} were used, respectively plus 2 ppm of 2,4-D. Therefore, the vernilized and sterilized seeds of wheat cultivars were imbibed in bacterial suspended according to selected treatments for 2 h at ambient temperature under vacuum [41]. Control vernalized wheat seeds were imbibed with sterile phosphate buffer under the same conditions. Single, co-inoculated and no inoculated wheat seeds were planted in pots, 5 seeds per pot. The pots filled with agricultural sterilized soil and perlite at a ratio 1:1 (W:W dry weight). The soil used in this experiment was collected from same location that the local strain of *Azospirillum* isolated from and then sterilized by

autoclaving at 121°C for 60 min in two consecutive day. Physio-chemical analysis of the soil indicated that the texture is clay loam with pH=8 and the soil organic-C and total-N were each as much as 1% by weight; available-P and K were 12.8 and 300 ppm respectively.

The pots were incubated in growth chamber (Conviron TC30) at 25/18°C day/night cycle and 11h light/13h dark photoperiod for 30 days and then gradually photoperiod were changed to 13 h light/11h dark during the growing season. The pots were watered every week with Hoagland's solution [42] according to the layout of the experiment. The plant samples were harvested at the end of growing season for future analysis.

Analysis of Grain Yield and N, P and K in Wheat Gain: At the end of growing season, three plants were randomly selected from each pot. Then, the total number of seeds per plant, the weight of 1000-seed and the amount of grain harvested per plant were measured [2, 8].

The total nitrogen content of the grains (mg g^{-1} DW of grain) was defined by the Kjeldahal method [18, 43, 8]. The amount of Phosphor (mg g^{-1} DW of grains) and Potassium were determined by spectrophotometer [2, 44] and flame photometric method [45, 44] respectively. The data were subjected to statistical analysis using SPSS11 and Duncan's multiple rang tests.

RESULTS

Effect of Inoculation and Co-inoculation on Harvested Grain: The results of single and co-inoculation with *A. brasilense* and/or *R. meliloti* plus 2,4-D on grain yield, 1000-seed weight and the number of seeds per plant were showed in Table 1 and Fig. 1, 2. Analyze of variance and comparison within means of different treatments indicated that wheat cultivars, strains of *Azospirillum* and *Rhizobium* and 2,4-D have significant effect on the number and weight of seeds per plant. The effects of co-inoculation on wheat quality (N, P and K content) were significant compared to single or no inoculated plants.

1000-seed Weight (SW): The mean values of 1000-seed weight (SW) were measured from 34.97 g in non inoculated plants to 43.96 g for co-inoculated plant in Baccross cultivar. Single *Rhizobium* inoculation (apart from *Rhizobium* strain and wheat cultivars) did not have any significant effect on the mean of SW and there was not any significant difference between the strains of *Rhizobium* (native and standard). In contrast, inoculation of plants with *Azospirillum* had positive significant effect on SW. When the plants inoculated with *A. brasilense* Sp7, the mean values of SW were increased 8.5% and 9.2% in Baccross cv. and Mahdavi cv. respectively compared to non inoculated plants. When the Sp7 strain replaced with the native strain of *A. brasilense* in inoculation, the increasing rates were reached to 12.3% and 12.2% in Baccross cv. and Mahdavi cv. respectively. Therefore, the weight of 1000-seed was different due to both the strain and cultivars of wheat. When the inoculation of *Azospirillum* was associated with *Rhizobium* (without 2,4-D), the SW did not change significantly.

Using 2,4-D (without inoculation) increased SW by almost 3% in the two cultivars compared to the control plants (from 34.97g to 36.21 g). Adding 2,4-D to single *Rhizobium* inoculation cause almost 6% more in SW. In this case, there was significant difference between strains

Table 1: The effect of the single and co-inoculation of wheat with *A. brasilense* and *R. meliloti* plus 2,4-D on 1000-seed weight, the number and dry matter of seeds per plant (apart from the bacterial strains and wheat cultivars). Different letter(s) on the mean values indicated significant differences (p=0.05) between values of each column according to Duncan multiple rang test

		1000-seeds weight		grains number per plant		Grains yield	
Treatments	2,4-D ppm	Gram	Increase (%)	Number	Increase (%)	Gram	Increase (%)
Control (no inoculat)	0	35.01[a]	-	29.67[a]	-	1.04[a]	-
	2	36.12[b]	3.2	31.33[b]	5.6	1.19[b]	14.4
Inoculation with *Rhizobium*	0	35.08[a]	0.2	29.67[a]	0.0	1.041[a]	0.0
	2	37.08[c]	5.9	31.75[b]	7.0	1.15[b]	10.6
Inoculation with *Azospirillum*	0	38.70[d]	10.5	33.0[c]	11.2	1.28[c]	23.0
	2	40.82[e]	16.6	35.67[d]	20.2	1.46[d]	40.4
Azospirillum and *Rhizobium*	0	38.69[d]	10.5	33.33[c]	12.3	1.29[c]	24.0
	2	42.73[f]	22.0	37.38[e]	26.0	1.6[c]	53.8

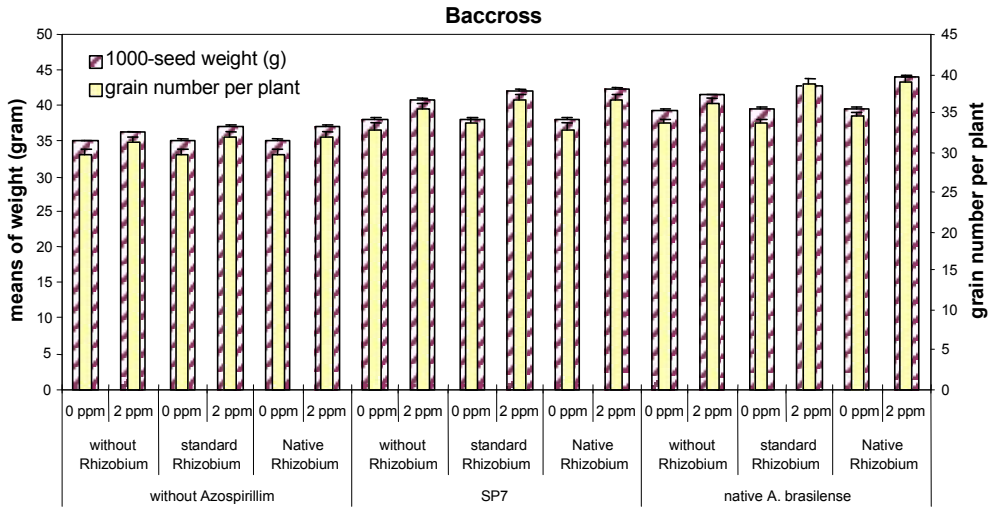

Fig. 1: Effect of the single and co-inoculation of wheat with *A. brasilense* 10^7 cfu mL^{-1} (native and and Sp7) and *R. meliloti* 10^3 cfu mL^{-1} (native and standard) plus 2,4-D (0 and 2 pp) on 1000-seed weight, the number of grain per plant

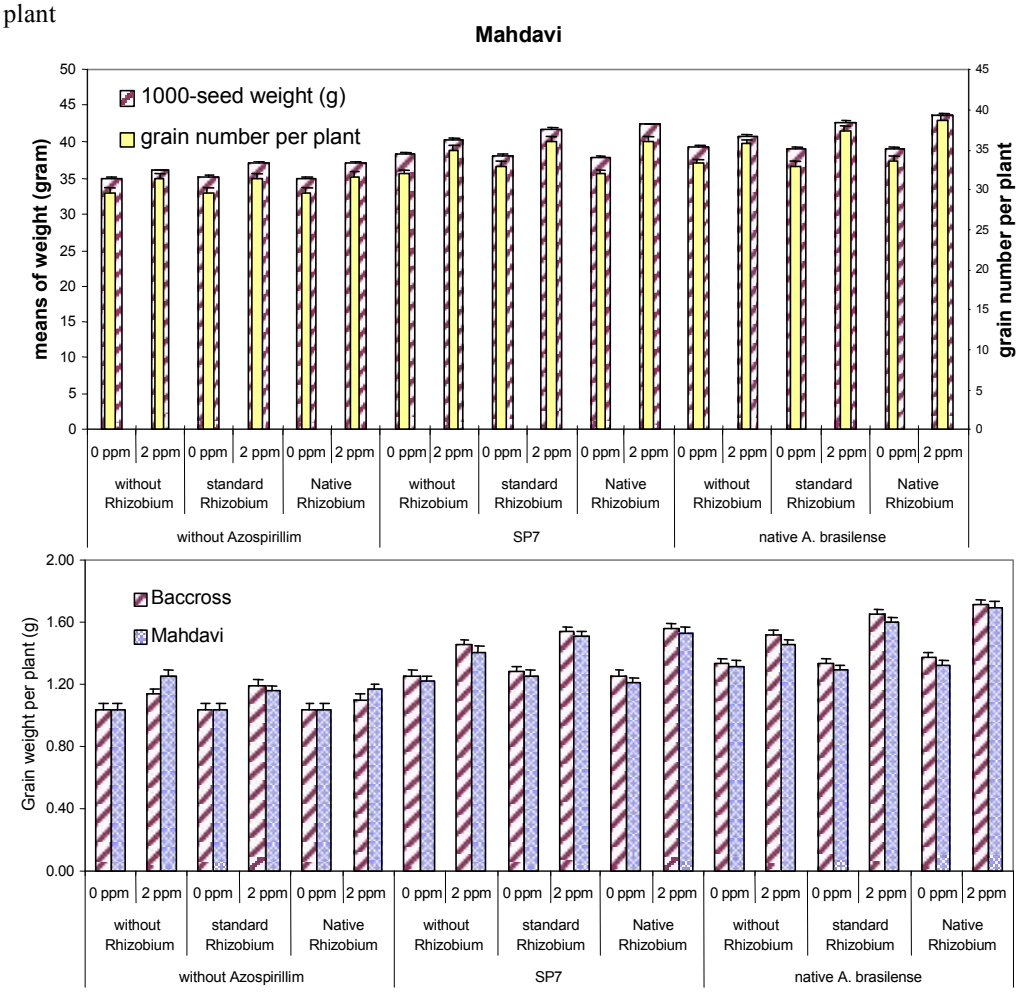

Fig. 2: The effect of the single and co-inoculation of wheat with *A. brasilense* 10^7 cfu mL^{-1} (native and and Sp7) and *R. meliloti* 10^3 cfu mL^{-1} (native and standard) plus 2,4-D (0 and 2 ppm) grain weight (g) per plant

Table 2: Effect of the single and co-inoculation of wheat with *A. brasilense* and *R. meliloti* plus 2,4-D (0 and 2 ppm) on the amounts of N, P and K of harvested grain in mg g^{-1} Dw (apart from the bacterial strains and wheat cultivars). Different letter(s) on the mean values indicated significant differences (p=0.05) between values of each column according to Duncan multiple rang test

		N content		P content		K content	
Treatments	2,4-D ppm	mg g^{-1} Dw	Increase (%)	mg g^{-1} Dw	Increase (%)	mg g^{-1} Dw	Increase (%)
Control (no inoculat)	0	15.85[a]	-	2.37[a]	-	2.53[a]	-
	2	16.88[b]	6.5	2.8o[b]	18.1	2.95[b]	16.7
Inoculation with *Rhizobium*	0	15.78[a]	0.0	2.41[a]	1.7	2.51[a]	0.0
	2	17.65[c]	11.4	3.33[c]	40.5	3.18[c]	25.7
Inoculation with *Azospirillum*	0	19.46[d]	22.8	3.78[d]	59.5	3.39[d]	34.0
	2	21.43[e]	35.2	4.33[e]	82.7	3.88[e]	53.3
Azospirillum and *Rhizobium*	0	19.56[d]	23.4	3.86[d]	62.9	3.4[d]	34.4
	2	24.10[f]	52.1	5.09[f]	114.8	4.62[f]	82.6

of *Rhizobium* (native and standard) and also the cultivars of wheat (Baccross and Mahdavi culivars). Similarity, application of 2,4-D in addition to inoculation with *Azospirillum* further enhanced SW. However, co-inoculation of *Azospirillum* and *Rhizobium* plus 2,4-D had more stimulatory effect on SW. In this case, the mixture of native bacterial strains of *Azospirillum* and *Rhizobium* performed better than the other combinations. The amounts of SW were increased in plant co-inoculated with Sp7 and standard *Rhizobium* to 42.35 and 42.43 g for Baccross and Mahdavi cultivars respectively. The highest values for SW were recorded in combination of the local strains (native *A. brasilense* and native *R. meliloti*) when the population of 10^7 and 10^3 cfu ml^{-1} were used for each strain respectively, plus 2 ppm of 2,4-D. In this case the mean values were reached to 43.96 g and 43.67g in Baccross and Mahdavi cultivars, respectively.

Number of Seeds per Plant (NSPP): The results indicated that co-inoculation were significantly increased the number of seeds per plant (NSPP). The mean values for NSPP was 29.67 in non inoculated plants and in co-inoculated plants were increased to 39.0 and 38.67 in Baccross cv. and Mahdavi cv. respectively. Single inoculation with *Rhizobium* did not have any significant effect on NSPP. In contrast, 2,4-D was increased NSPP by almost 5.6% compared to no inoculated plants. Adding 2,4-D to inoculated plants with *Rhizobium*, the NSPP was increased 7.0% compared to the control plants. However, there was not significant difference between strains of native and standard *Rhizobium*.

Inoculation with *Azospirillum* had positive and remarkable effect on NSPP. Using Sp7 as inoculants increased NSPP to 33 and 32 in Baccross and Mahdavi cultivars, respectively. When Sp7 replaced with the native *A. brasilense*, the NSPP were increased to 33.7 (13.5% more) and 33.3 (12.3% more) in Baccross and Mahdavi respectively compared to the control plants. The mean values of NSPP was not significantly changed when mixture of *Azospirillum* and *Rhizobium* were used without 2,4-D. However, application of 2,4-D in addition to co-inoculants of *Azospirillum* with *Rhizobium* further enhanced the number of seed per plant. The results were remarkable, when plants co-inoculated with the combination of the native strains of *Azospirillum* and *Rhizobium* plus 2 ppm of 2,4-D. In this case, the NSPP was increased 30% compared to the control plants. The heist NSPP (39) were counted in the co-inoculated wheat with the combination of the native strains of *A. brasilense* and *R. meliloti* plus 2ppm of 2,4-D in Baccross cultivar (almost 31% more compare to the control plant).

Harvested Yield: The lowest grain yield (1.04 g seed per plant) was obtained in no inoculated plants and the highest (1.71 g) was observed when plants co-inoculated with the combination of the local strains (*A. brasilense* and *R. meliloti*) plus 2ppm of 2,4-D in Baccross cv. This is almost 64.8% more with respect to the control plants. Inoculation with *Rhizobium* alone (native or standard) had no significant effect on the grain yield. However, the 2,4-D alone were increased significantly grain yield (Fig. 2).

Plants inoculated with *Azospirillum* produced more yield. However the amount of yield depends on the wheat cultivar and the strain of bacteria. When Sp7 was used the mean values of grain yield in Baccross and Mahdavi were increased to 20.2% and 17.3% respectively in compare to the control plants. Substitutions of Sp7 with the native *A. brasilense* in the mentioned cultivars would increase

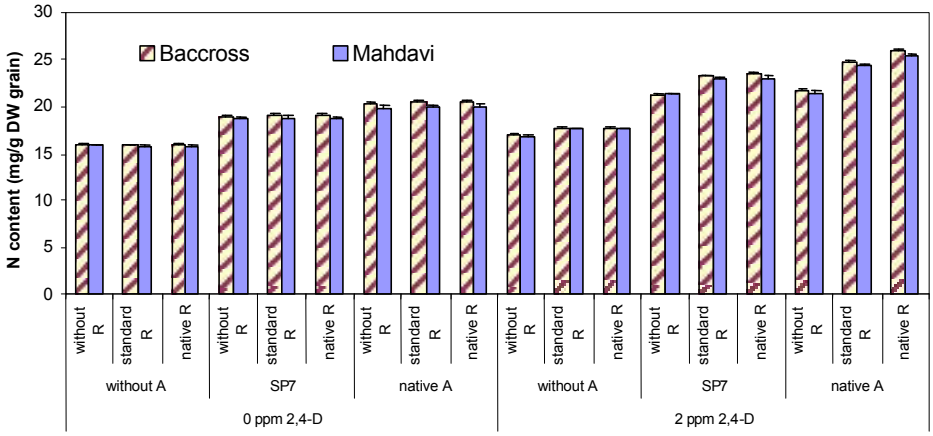

Fig. 3: The effect of the single and co-inoculation of wheat with *A. brasilense* 10^7 cfu mL^{-1} (native and and Sp7) and *R. meliloti* 10^3 cfu mL^{-1} (native and standard) plus 2,4-D on the nitrogen content of grain. A for *Azospirillum brasilense* and R for *Rhizobium meliloti*

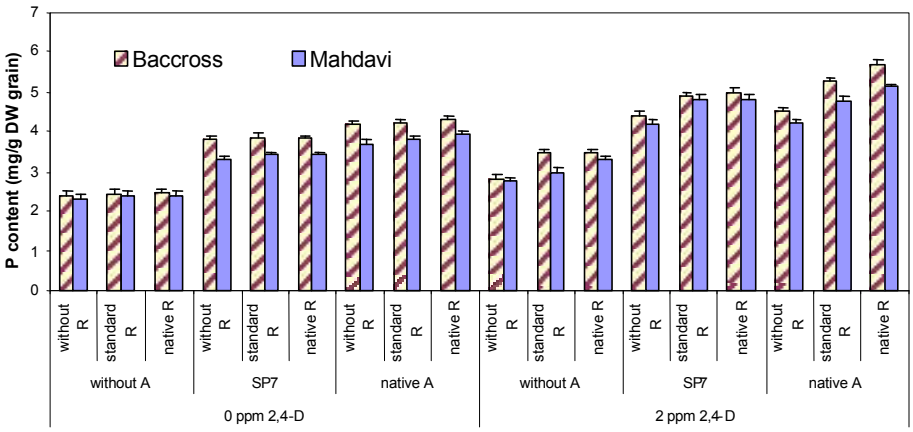

Fig. 4: The effect of the single and co-inoculation of wheat with *A. brasilense* 10^7 cfu mL^{-1} (native and and Sp7) and *R. meliloti* 10^3 cfu mL^{-1} (native and standard) plus 2,4-D on the phosphorus content of grain. A for *Azospirillum brasilense* and R for *Rhizobium meliloti*

the grain yield up to 26% and 27.9% respectively. Addition of 2,4-D to a mixture of *A. brasilense* and *R. meliloti* further enhanced grain yield. In dual inoculation (Sp7 with standard *Rhizobium*) the grain yield were increased significantly from 1.04 to 1.54g for Baccross and to 1.50 g for Mahdavi cultivars. However, when plants co-inoculated with the local strains of *A. brasilense* and *R. meliloti*, the grain yield were enhanced to 1.71 and 1.69 g for Baccross and Mahdavi cultivars, respectively.

Quality of Harvested Yield: Results of both single inoculation with *A. brasilense* and /or *R. meliloti* as well as their combination plus 2,4-D on the amount of nitrogen, phosphors and potassium content of harvested grains were showed in Table 2 and Fig. 3 to 5. Analyze of variance and compare means of the effect of single and co-inoculation showed that wheat cultivars, strains of *Azospirillum* and *Rhizobium* and 2,4-D have significant effect on the amount of N, P and K of grain. The effect of co-inoculation on grain quality was significant compared to single inoculation or non inoculated control plants.

Nitrogen Content: The lowest amount of N in harvested grains was observed in non inoculated plants (almost 15.7± mg g^{-1} DW for both cultivars). Inoculation with *Rhizobium* alone did not have significant effect on N content of grain. However, if 2,4-D was combined with inoculation of *Rhizobium*, the amount of nitrogen of grains was increased 12% compared to the control plants and in this case, the difference were in significant for native or standard *Rhizobium* (Fig. 3).

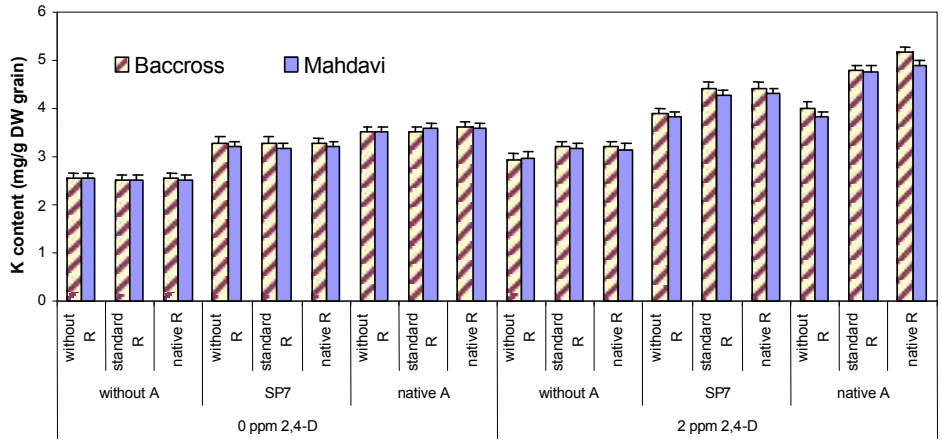

Fig. 5: Effect of the single and co-inoculation of wheat with *A. brasilense* 10^7 cfu ml^{-1} (native and and Sp7) and *R. meliloti*10^3 cfu ml^{-1} (native and standard) plus 2,4-D on the potassium content of harvested grain. A for *Azospirillum brasilense* and R for *Rhizobium meliloti*

In contrast, *Azospirillum* inoculation had positive significant effect on N content of grains. In inoculation of plants with Sp7, the amount of nitrogen of the grain in both cultivars was increased to almost 18.8 mg g^{-1} DW (19% more). If Sp7 was replaced by native *A. brasilense*, the amount of nitrogen would increase to 20.3 and 19.87 mg g^{-1} DW in Baccross and Mahdavi cv., respectively. Therefore, the amount of N in harvested grain in inoculated with native *A. brasilense* were increased up to 7.6% (Baccross cv) and 5.7% (Mahdavi cv) compared to the inoculated plants with Sp7.

Co-inoculation of *Rhizobium* with *Azospirillum*, without 2,4-D, had significant effect on the amount of N in harvested grains. However using 2,4-D in a mixture of *Azospirillum* and *Rhizobium* had marked significant effect on the amount of N in grain. Co-inoculation of wheat with standard strains (Sp7 and standard *R. meliloti)* caused an increase of 45% in both wheat cultivars compared to the control plants. Co-inoculation of wheat with the local strains of *A. brasilense* and *R. meliloti* plus 2,4-D showed that the amount of N in grain were increased to 26.03 (65.8% more) and 25.4 mg g^{-1} DW (61.8% more) in Baccross and Mahdavi cultivars, respectively. This difference was significant between Baccross cv. and Mahdavi cultivars. The highest amount of nitrogen (26.03 mg g^{-1} DW) was observed in the Baccross cv. that co-inoculated with the combination of the local strains (native *A. brasilense* 10^7 cfu ml^{-1} and native *R. meliloti* 10^3 cfu ml^{-1}) plus 2,4-D.

Phosphorus Content: The amounts of phosphor (P) in harvested grain were increased from almost 2.4 (in control plants) to 5.13 and 5.7 mg g^{-1} DW in co-inoculated cultivars of Mahdavi cv. and Baccross cv. respectively (Fig. 4)

Inoculation of plants with *Rhizobium* alone or *Rhizobium* with *Azospirillum* as co-inoculants (without 2,4-D) had not significant effect on P content of harvested grains. In all cases, addition of 2,4-D to the inoculants were significantly increased the amount of phosphorus in grains. Application of 2,4-D alone would increase the amounts of seed's phosphorus to 2.77 and 2.83 mg g^{-1} DW in Mahdavi and Baccross, respectively. Therefore, 2,4-D as an amendment in co-inoculation caused increase of 20% the amount of the grain's phosphorus compared to the control plants. In this case, there were not insignificant differences between the two cultivars. In single inoculation with *Rhizobium* plus 2,4-D, the amount of phosphorus of grain were enhanced to 3.47 (44.5% more) and 3.3 mg g^{-1} DW (42% more) for Baccross and Mahdavi cultivars, respectively. Similarity, there was insignificant differences between strains of *Rhizobium* (native and standard).

In single inoculation with Sp7, the amount of phosphorus were increased to 3.3 (Mahdavi) and 3.8 mg g^{-1} DW (Baccross) being 42% and 58% more compared to the control plants. If Sp7 changed to the native *A. brasilense* (in single inoculation), the amount of phosphorus were increased to 3.7 (Mahdavi cv.) and 4.17 mg g^{-1} DW (Baccross cv.) being 59% and 74% more than the control plants. This difference was significant between the cultivars of wheat.

Co-inoculation of the standard strains (Sp7 and standard *R. meliloti)* plus 2,4-D were increased the P

content to 4.83 (107% more) and 4.9 mg g^{-1} DW (104% more) in Mahdavi and Baccross cultivars, respectively. Replacing the standard strains of *A. brasilense* and *R. meliloti* with the native ones caused the highest P content (5.13 and 5.7 for Mahdavi and Baccross cultivars respectively) in co-inoculation of wheat. In this case, the amounts of P were increased 120% and 137% in Mahdavi cv. and Baccross cultivars respectively in compared to the control plants.

Potassium Content: The lowest amount of potassium in harvested grain (2.5 mg g^{-1} DW) was recorded in non inoculated control plants and the highest (5.17 mg g^{-1} DW) were observed in Baccross cv. that co-inoculated with a mixture of local strains of *A. brasilense* and *R. meliloti* (Fig. 5).

There was not significant difference between single *Rhizobium* inoculated and uninoculated control plants. The results indicated that the treated plants with 2,4-D would increase the amount of K to 2.93 (17% more) and 2.97 mg g^{-1} DW (18.8% more) in harvested grain of the Baccross and Mahdavi respectively compared to the control plants. These differences between wheat cultivars using 2,4-D was not significant. Addition of 2,4-D to single *Rhizobium* inoculation were enhanced the amount of potassium in grain from 2.53 mg g^{-1} DW (in control) to 3.2 (28% more for Baccross cv) and 3.17 (27% more for Mahdavi cv). There were insignificant difference between strains of *Rhizobium*. However, single *Azospirillum* inoculation had positive marked significant effect on the K content. When plants inoculated with *A. brasilense* Sp7 alone, the amount of potassium in grain were increased to 3.3 (30% more) and 3.2 mg g^{-1} DW grain (26% more) in Baccross cv. and Mahdavi cultivar, respectively. If *A. brasilense* Sp7 replaced with native *A. brasilense*, the amount of K was increased to 3.53 mg g^{-1} DW (39.5% more). Addition of 2,4-D to the native *A. brasilense* inoculation would increase the amount of K from 2.53 mg g^{-1} DW (in control) to 3.8 (in Mahdavi cv) and 4 mg g^{-1} DW (in Baccross cv.). However co-inoculation with *Rhizobium* and *Azospirillum*, without 2,4-D, had not significant effect on the amount of potassium. When wheat co-inoculated with the combination of the standard strains of *A. brasilense* and *R. meliloti* plus 2,4-D, the amount of K was increased to almost 4.4 (70-74% more) in two cultivars. The results indicated that co-inoculation with the native *A. brasilense* and *R. meliloti* were increased the amount of K to 4.87 (92.4% more) and 5.17 mg g^{-1} DW (104.3% more) in Baccross and Mahdavi cultivars, respectively.

Therefore, co-inoculation with *Azospirillum* and *Rhizobium* (apart from the bacterial strains and wheat cultivars) had positive significant effects on the N, P and K content of the wheat grains in comparison with single inoculation or non inoculated control plants (Table 2).

DISCUSSION

Co-inoculation of wheat seeds with *A. brasilense* and *R. meliloti* had positive significant effects on the grain yield and the amount of N, P and K in harvested grains in comparison with single inoculation or no inoculated control plants. Apart from the bacterial strains and wheat cultivars, the average grain yield resulting from co-inoculation was 53.8% higher compared to non inoculated plants. The recorded increase in wheat grain yield by co-inoculation of plants with *A.brasilense* and *R. meliloti* could be due to the increase in the number of grains per plant or/and due to the increased weight of grains per plant. Some researchers found that the increase in grain yield of inoculated wheat were due to the increased weight of wheat grains [46], in contrast, some other researchers reported that the increases in grain yield in wheat, sorghum and maize results of increased number of grains [47, 6, 12]. Our results indicated that increase of wheat grain yield (53.8%) is a result of the increase in the number of grains (26% more) and the weight of grains per plants (22% more) when mixture of inoculants were used. However increases in grain yield (23%) were observed in single *Azospirillum* inoculation. Our results in single inoculation were almost similar to Dobbelaere *et al*. [10]. They reported that the grain yield of the inoculated wheat with *Azospirillum* was increased to 30%.

In single and co-inoculated wheat, all measured parameters (grain yield, the number of grains per plant, 1000-seeds weight, N, P and K content of grains) were higher in cv. Baccross than cv. Mahdavi. Also, in single inoculation, the native *A. brasilense* increased all parameters better than Sp7. Mostajeran *et al*. [48] reported homolog effect between local strains of *A. brasilense* and local wheat cultivars on plant growth and harvested yield. The combination of the local strains clearly increased the grain quantity compared to the combination of the standard strains. Similar result was proposed by many researchers [49, 44, 43, 8]. The results of this experiment showed that, the effect of the single and co-inoculation on plant growth parameters varies depending to the strain of *A. brasilense* (Sp7 and native), wheat cultivars, co-inoculants (type of mixed strains). This difference may be due to their superior adaptation to the environment and compatibility to local plant varieties. Therefore, it is needed a wide isolation and research, to select the best combination between genotype of the plant and bacteria

strain. Dalla Santa *et al*. [18] also indicated that the intensive use of inoculants with associative bacteria, to select the best combination between genotype of the plant and bacteria strain needs a wide isolation of strains.

Numerous reports has been shown from the positive effects of the single inoculation [4, 8, 29, 46, 50-52] and co-inoculation [4, 53, 54] on grain yield accompanied with N, P and K uptake by inoculated plant root. However, our results showed that single inoculation with *Azospirillum* produced significantly higher grain yield by 29% which the produced grains contain more N (22.8%), P (59.5%) and K(34%) compared to the non inoculated plants. Simultaneously, co-inoculation increased the mentioned indexes by 53.8, 52, 114 and 82%, respectively compared to no inoculated plants. Hoflich [55] reported that in inoculated wheat with *Rhizobium leguminosarum*, the amounts of N, P and K increased about 16-30%. Also, the amounts of phosphor in co-inoculated wheat with *A. lipoferum* and *Bacillus megaterium* increased about 37 to 53% in compared to the single *Azospirillum* inoculated and control wheat, respectively. Therefore, inoculation of plant with *Azospirillum* could be very important, not only to have higher grain yield, but also to have better seed quality. Similar results indicated significantly higher Mg, K and Ca for inoculated plants than no inoculated plants [2]. They indicated that grains with a higher Mg, K and Ca content could become seedlings with better capability in drought stress.

In all measurements, the single *Rhizobium* inoculation did not have any effects on all measured parameters. Because, rhizobial strains are specific to certain legumes and *Rhizobium meliloti* can create a successful symbiosis system with species of *Medicago, Melilotus* and *Trigonella* [56, 57]. In our case, *R. meliloti* can not enter into the wheat roots [40]. While with presence of 2,4-D, *Rhizobium* can penetrate and colonized in root of wheat and therefore helped the growth of wheat plant. Using 2,4-D as an amendment and addition to inoculants of *Rhizobium* or/and *Azospirillium* would have positive effects on all growth parameters directly (by facilitation of *Rhizobium* penetration and stimulus of root system growth) and indirectly (increase of root surface).

The results of this study have demonstrated a clear synergistic effect of *A. brasilense* and *R. meliloti* plus 2,4-D and additive effects in plants co-inoculated with *Azospirillum* and *Rhizobium*. Thus, *Azospirillum* and *Rhizobium* could be used as co-inoculants for better crop productivity. Similar result were obtained by Sindhu *et al*. [58] for co-inoculation of chickpea with *Pseudomonas* spp and *Mesorhizobium* spp, which enhanced grain yield and N content of grain.

N_2 fixation by PGPB in association with plants was the first major mechanism of successful symbiosis system suggested for the enhancement of plant growth by the *Azospirillum* [21, 17]. Therefore, N_2 fixation can explain a portion of the addition of nitrogen content of harvested grain. But, large accumulation of N, P and K in grain and the difference between the amount of N, P and K accumulated in grain (selection phenomenon) in single and co-inoculated wheat showed that nitrogen fixation alone can not be a sufficient explanation for accumulation of different elements in grain. Increases in mineral uptake by plants might be the major contribution of bacterial inoculation. The mineral uptakes are due to a general increase in the volume of the root system [21, 17, 31, 4] and also proton efflux in roots [59, 60].

ACKNOWLEDGEMENT

The authors wish to thank the office of Graduate Studies of the University of Isfahan for their support and also the University of Arak for facilities and laboratory spaces. We thanks Dr. Zarkesh and Dr. Vaez for providing standard strains.

REFERENCES

1. Ravi, G., C. Mukesh and S.A. Tongmin, 2002. Influence of diazotrophicus *Azospirillum* on growth, flower yield and N uptake of *Chrysanthemum*. 17th WCSS.14-21 August 2002. Thailand. 645(1-7)
2. Creus, C.M., R.J. Sueldo and C.A. Barassi, 2004. Water relations and yield in *Azospirillum* inoculated wheat exposed to drought in the field. Can. J. Bot., 82: 273-281.
3. Nayak, D.N., J.K. Ladha and I. Watanabe, 1986. The fate of marker *Azospirillum lipoferum* inoculated into rice and its effect on growth, yield and N_2 fixation of plants studied by acetylene reduction, ^{15}N feeding and ^{15}N dilution techniques. Biology and Fertility of Soils. 2: 7-14.
4. Murty, M.G. and J.K. Ladha, 1988. Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions. Plant Soil., 108: 281-285.
5. Itzigsohn, R., Y. Kapulnik Y. Okon and A. Dovrat, 1993. Physiological and morphological aspects of interactions between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) in association with *Azospirillum brasilense*.Can. J. Microbiol., 39: 610-615.

6. Saikia, S.P., V. Jain, S. Khetarpal and S. Aravind, 2007. Dinitrogen fixation activity of *Azospirillum brasilense* in maize (*Zea mays*). Current Science, 93(9,10): 1296-1300.
7. Bashan, Y., 1986. Enhancement of wheat root colonization and plant development by *Azospirillum brasilense* Cd. Following temporary depression of rhizosphere microflora. Applied and Environmental Microbiology, 51: 1067-1071.
8. Sabry, S.R.S., S.A. Saleh, C.A. Batchelor, J. Jones, J. Jotham, G. Webster, S.L. Kothari, M.R. Davey and E.C. Cocking, 1997. Endophytic establishment of *Azorhizobium caulinodans* in Wheat. Proc. Royal Society Lond., 264: 341-346.
9. Boddey, R.M. and J. Döbereiner, 1988. Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. Plant Soil, 108: 53-65.
10. Dobbelaere, S., A. Croonenborghs, A. Thys, D. Ptacek, J. Vanderleyden, P. Dutto, C. Labandera-Gonzalez, J. Caballero-Mellado, J.F. Aguirre, Y. Kapulnik, S. Brener, S. Burdman, D. Kadouri, S. Sarig and Y. Okon, 2001. Responses of agronomically important crops to inoculation with *Azospirillum*. Aust. J. Plant Physiol., 28: 871-879.
11. Fallik, E. and Y. Okon, 1996. Inoculants of *Azospirillum bras*ilense: biomass production, survival and growth promotion of *Setaria italica* and *Zea mays*. Soil Biol. Biochem., 28: 123-126.
12. Sarig, S., A. Blumand and Y. Okon, 1988. Improvement of the water status and yield of field-grown grain Sorghum (*Sorghum bicolor*) by inoculation with *Azospirillum brasilense*. J. Agri. Sci., 110: 271-277.
13. Steenhoudt, O. and J. Vanderleyden, 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev., 24: 487-506.
14. Yuming, B., Z. Xiaomin and L.S. Donald, 2003. Enhanced Soybean Plant Growth Resulting from Coinoculation of *Bacillus* Strains with *Bradyrhizobium japonicum,* Crop Sci., 43: 1774-1781
15. Creus, C.M., R.J. Sueldo and C.A. Barassi, 1997. Shoot growth and water status in *Azospirillum*-inoculated wheat seedlings grown under osmotic and salt stresses. Plant Physiol. Biochem. (Paris), 35: 939-944.
16. Barbieri, P., T. Zanelli, E. Galli and G. Zanetti, 1986. Wheat inoculation with *Azospirillum brasilense* Sp6 and some mutants altered in nitrogen fixation and indole-3-acetic acid production. FEMS Microbiol. Lett., 36: 87-90.
17. Bashan, Y. and H. Levanony, 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbial., 36: 591-608.
18. Dalla Santa, O.R., R.F. Hernández, G.L.M. Alvarez, P.R. Junior and C.R. Soccol, 2004. *Azospirillum* sp. Inoculation in wheat, barley and oats seeds greenhouse experiments. Brazilian Archives of Biology and Technology. 47(6): 843-850.
19. Umali-Garcia, M., D.H. Hubbel, M.H. Gaskins and F.B. Dazzo, 1980. Association of *Azospirillum* with grass roots. Appl. Environ. Microbiol., 39: 212-226.
20. Vande Broek, A., M. Lambrecht, K. Eggermont and J. Vanderleyden, 1999. Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. J. Bacteriol., 181: 1338-1342.
21. Bashan,Y., G. Holguin and L.E. de-Bashan, 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural and environmental advances (1997-2003). Can. J. Microbiol., 50: 521-577.
22. Cattelan, A.J., P.G. Hartel and J.J. Fuhrmann, 1999. Screening for plant growth-promoting rhizobacteria to promote early soybeen growth. Soil Science Society of America J., 63: 1670-1680.
23. Okon, K. and R. Itzigsohn, 1995. The development of *Azospirillum* as a commercial inoculant for improving crop yields. Biotechnology Advances, 13: 415-424.
24. Rodriguez, H., R. Fraga, T. Gonzalez and Y. Bashan, 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and Soil, 287: 15-21.
25. Pacovsky, R.S., 1990. Development and growth effects in the *Sorghum-Azospirillum* association. J. Appl. Bacteriol., 68: 555-563.
26. Sarig, S., Y. Okon and A. Blum, 1992. Effect of *Azospirillum brasilense* inoculation on growth dynamics and hydraulic conductivity of *Sorghum bicolor* roots. J. Plant Nutr., 15: 805-819.
27. Boddey, R.M., V.L.D. Baldani, J.I. Baldani and J. Döbereiner, 1986. Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field grown wheat. Plant and Soil, 95: 109-121.

28. Mostajeran, A., R. Amooaghaie and G. Emtiazi, 2002. Root hair density and deformation of inoculated roots of wheat cultivars by *Azospirillum brasilense* and role of IAA in this phenomenon. Iranian Biol. J., 13: 18-28.
29. Wu, S.C., Z.H. Caob, Z.G. Lib, K.C. Cheunga and M.H. Wonga, 2005. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma, 125: 155-166.
30. Bai, Y., X. Zhou and D.L. Smith, 2003. Crop ecology, Management and quality: Enhanced Soybeen plant growth resulting from coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Science, 43: 1774-1781.
31. Morgenstern, E. and Y. Okon, 1987. The effect of *Azospirillum brasilense* and auxin on root morphology in seedlings of *Sorghum bicolour* X *Sorghum sudanense*. Arid Soil Res. Rehabil., 1: 115- 127.
32. Zarei, M., N. Saleh-Rastin, H.A. Alikhani and N. Aliasgharzadeh, 2006. Responses of lentil to co-inoculation with phosphate-solubilizing rhizobial strains and arbuscular mycorrhizal fungi, Journal of Plant Nutrition, 29: 1509-1522
33. Elshanshoury, A.R., 1995. Interactions of *Azotobacter chroococcum*, *Azospirillum brasilense* and *Streptomyces mutabilis*, in relation to their effect on wheat development. J. Agron. Crop Sci., 175: 119-127.
34. Veeraswamy, J., T. Padmavathi and K. Venkateswarlu, 1992. Interaction effects of *Glomus intraradices* and *Azospirillum lipoferum* on sorghum. Indian J. Microbiol., 32: 305-308.
35. Holt, J.G., N.R. Kreig, P.H.A. Sneath, J.T. Staley and S.T. Williams, 1994. Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, USA.
36. Sadovnikova, Yu.N., L.A. Bespalova and L.P. Antonyuk, 2003. Wheat germ agglutinin is a growth factor for bacterium *Azospirillum brasilense.* Doklady Biochemistry and Biophysics, 389: 103-105.
37. Zimmer, W. and H. Bothe, 1988. The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil, 110: 239-247.
38. Vincent, J.M. 1970. The cultivation, isolation and maintenance of rhizobia. In: A Manual for the Practical Study of Root-Nodule Bacteria, pp. 1-13. Edited by J. M. Vincent. Oxford: Blackwell Scientific, UK.
39. Ogut, M., C. Akdag, O. Duzdemir and M.A. Sakin, 2005. Single and double inoculation with *Azospirillum/Trichoderma*: the effects on dry bean and wheat. Biology and Fertility of Soils, 41: 262-272.
40. Askary, M., A. Mostajeran and G. Emtiazi, 2008. Colonization and Nitrogenase Activity of *Triticum aestivum* (cv. Baccross and Mahdavi) to the Dual Inoculation with *Azospirillum brasilense* and *Rhizobium meliloti* plus 2,4-D. Pak. J. Biol. Sci., 11(12): 1541-1550.
41. Bashan, Y. and H. Levanony, 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can. J. Microbiol. 31: 947-952.
42. Hoagland, D.R. and D.L. Arnon, 1950. The water-culture method of growing plants without soil. Calif Agric. Exp. Stn., 374: 1-32
43. Rueda-Puente, E.O., T. Castellanos, E. Troyo-Diéguez and J.L. Díaz de León-Alvarez, 2004. Effect of *Klebsiella pneumoniae* and *Azospirillum halopraeferens* on the growth and development of two *Salicornia bigelovii* genotypes. Australian J. Exp. Agri., 44: 65-74.
44. Kohler, J. and F. Caravaca, 2007. Interactions between a plant growth-promoting rhizobacterium, an AM fungus and a phosphate-solubilising fungus in the rhizosphere of *Lactuca sativa*. Applied Soil Ecology, 35(3): 480-487.
45. El-Komy, H.M.A., 2005. Coimmobilization of *Azospirillum lipoferum* and *Bacilus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. Food Technol. Biotehnol., 43(1): 19-27.
46. Kapulnik, Y., S. Sarig, I. Nur and Y. Okon, 1983. Effect of *Azospirillum* inoculation on yield of field-grown wheat. Can. J. Microbiol., 29: 895-899.
47. Bhattarai, T. and D. Hess, 1998. Growth and yield responses of a Nepalese spring wheat cultivar to the inoculation with Nepalse *Azospirillum* spp. at various levels of Nitrogen fertilization. Biol. Fertil Soils. 26: 72-77.
48. Mostajeran, A., R. Amooaghaie and G. Emtiazi, 2007. The participation of the cell wall hydrolytic enzymes in the initial colonization of *Azospirillum brasilense* on wheat roots. Plant and Soil, 291: 239-248.
49. Jain, D.K. and D.G. Patriquin, 1984. Root hair deformation, bacterial attachment and plant growth in wheat-*Azospirillum* associations. Applied and Environ. Microbiol., 48(6): 1208-1213.

50. Okon, Y. and Y. Kapulnik, 1986. Development and function of *Azospirillum*-inoculated roots, Plant and Soil, 90: 1-3
51. Saubidet, M.I., N. Fatta and A.J. Barneix, 2002. The effect of inoculation wiyh *Azospirillum brasilense* on growth and nitrogen utilization by wheat plants. Plant Soil. 245: 215-222.
52. Zavalin, A.A., T.M. Kandaurova and L.V. Vinogradova, 1998. Influence of nitrogen fixing microorganisms on the nutrition and productivity of spring wheat and on the characteristics of photosynthesis of different varieties of spring wheat. In: Elmerich, C., Kondorosi, A., Newton, W.E. Eds. Biological nitrogen fixation for the 21st century. Dordrecht, the Netherlands: Kluwar Academic Publishers, pp: 413-414.
53. Alagawadi, A.R. and A.C. Gaur, 1992. Inoculation of *Azospirillum brasilense* and phosphate-solubilizing bacteria on yield of sorghum (*Sorghum bicolor* L. Moench) in dry land. Trop. Agri., 69: 347-350.
54. Belimov, A.A., A.P. Kojemiakov and C.V. Chuvarliyeva, 1995. Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. Plant Soil, 173: 29-37.
55. Höflich, G., 1999. Colonization and growth promotion of non-legumes by *Rhizobium* bacteria. Plant-Microbe Interactions. Available: http://socrates.acadiau.ca/isme/Symposium26/hoflich.PDF
56. Hirsch, A.M., M.R. Lum and J.A. Downie, 2001. What makes the rhizobia-legume symbiosis so special? Plant Physiology, 127: 1484-1492.
57. Matiru, V.N. and F.D. Dakora, 2004. Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. African J. Biotech., 3(1): 1-7.
58. Sindhu, S.S., S. Suneja, A.K. Goel, N. Parmar and K.R. Dadarwal, 2002. Plant growth promoting effects of *Pseudomonas* sp. On coinoculation with *Mesorhizobium* sp. Cicer strain under sterile and "Witt sick" Soil conditions. Applied Soil Ecology, 19(1): 57-64.
59. Amooaghaie, R., A. Mostajeran and G. Emtiazi, 2002. The effect of compatible and incompatible *Azospirillum brasilense* strains on proton efflux of intact wheat roots. Plant and Soil, 243: 155-160.
60. Bashan, Y. and H. Levanony, 1989. Effect of root environmental on proton ef?ux in wheat roots. Plant Soil, 119: 191-197.