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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

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Abstract: The potential enhancement of root colonization and nitrogenase activity of wheat cultivars (Baccross and Mahdavi) was studied with application of two *Azospirillum brasilense* strains (native and Sp7) co-inoculated with two *Rhizobium meliloti* strains (native and DSMZ 30135). The results indicated that the colonization was different due to the strains and cultivars of wheat were used. Native *A. brasilense* colonized wheat root better than Sp7 strain. However, Baccross cv. reacted better with native *Azospirillum* compared to Mahdavi cv. which reacted better with Sp7. When plants inoculated with dual inoculants (SP7 with standard *Rhizobium*), the colonization of *Azospirillum* were increased significantly (from 1.67×10^5 to 22×10^5 cfu g^{-1} FW for Baccras cv. and 3.67×10^5 to 26×10^5 cfu g^{-1} FW for Mahdavi cultivar). When the standard *Rhizobium* as co-inoculants changed to the native *Rhizobium*, the colonization of *Azospirillum* was higher when compared to the single inoculants but was almost the same when compared to the standard *Rhizobium*. When the standard or native strains of *Rhizobium* used as single inoculation of wheat roots, the number of *Rhizobium* in the wheat roots were not changed significantly. However, when plants co-inoculated with *Rhizobium* and *Azospirillum*, the colonization of *Rhizobium* was increased. Co-inoculation of standard strain of *R. melilot* with *A. brasilense* Sp7 showed that the colonization of *Rhizobium* were increased from 0.67×10^5 to 21×10^5 cfu g^{-1} FW for Baccross cv. and 0.33×10^5 to 18×10^5 cfu g^{-1} FW for Mahdavi cv. This behavior was the same when inoculation of *Rhizobium* was happened with the native one. In dual inoculation, the highest nitrogenase activity was measured in combination of the local strains (native *A. brasilense* with the native *R. meliloti*) and the lower one belongs to the combination of standard strains (Sp7 with standard *R. meliloti*). The difference in nirtogenase activity for different cultivars of wheat with Sp7 and standard *Rhizobium* is not significant but the difference for Sp7 strain plus native *Rhizobium* is significant ($p>0.05$). However, the differences were not significant ($p<0.05$) for nitrogenase activity in bacterial tubes, the difference for nitrogenase activity of co-inoculated plants with combination of Sp7 and *Rhizobium* either standard or native were significantly different

Key words: *Azospirillum brasilense*, colonization, co-inoculation, endophytic nitrogen fixation associative, nitrogenase activity, *Rhizobium meliloti*

INTRODUCTION

Biological Nitrogen Fixation (BNF) in agriculture is one of the major nitrogen sources for satisfying the demand and nutritional requirement of crops. BNF is the microbiological process which converts atmospheric N_2 into a plant-usable form. An important requirement for efficient BNF is to have diazotrophic bacteria growing endophytically within plants, as in the legume-rhizobia (Van Rhijin and Vanderleyden, 1995). One of the approaches taken to attempt to achieve BNF in non-legume crops is to determine whether certain naturally occurring rhizobial strains, which have the ability to enter legume plants, are able to internally colonize the roots of non-legumes (Cocking *et al*., 1993; Spencer *et al*., 1994). This idea leads the researchers to use chemical or biological components as an amendment for entry and also the contribution of a successful partnership (Elanchezhian and Panwar, 1997; Mostajeran *et al*., 2007). Various Auxins (2,4-D) can develop an endophytic diazotrophic symbiosis and induced cancerous root meristems (Elanchezhian and Panwar, 1997; Zeman *et al*., 1992). Introduced diazotrophs potentially inhabit paranodules as a major colonization niche that within the paranodules bacterial nitrogenase activity is less sensitive to increased oxygen tension in the roots (Chirstiansen-Weniger, 1997, 1998). Nitrogenase activity in tumor structures inhabited by bacteria significantly increased, compared to untreated plants.

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Mixed culture of microorganisms are suitable systems for studying the interactions between organisms and their impact on the environment. Combination of microorganisms with different metabolic capacities (N_2-fixation, P-mobilization, production of phytohormones and antibiotics) can partly surpass the effect of single inoculations, or can produce a positive effect where single inoculation are ineffective (Hoflich *et al*., 1994; Molla *et al*., 2001; Rueda-Puente *et al*., 2004). Co-inoculation also allows plants to achieve a more balanced nutrition and/or absorption of nutrients and consequently improved its growth (Biro *et al*., 2000). Combination of microorganisms with different metabolic capacities (N_2-fixation, P-mobilization, production of phytohormones and antibiotics) can partly surpass the effect of single inoculations, or can produce a positive effect where single inoculation are ineffective (Hoflich *et al*., 1994). Growth stimulation by inoculation requires microorganisms with phytoeffective metabolic characteristics and the ability to survive in the rhizosphere during the growth period (Hoflich *et al*., 1994) Co-inoculation allows plants to achieve a more balanced nutrition and/or absorption of nutrients (Biro *et al*., 2000), enhanced quality characteristics of the yield, higher net return and better cost-benefit ratio.

Azospirillum are free-living, Plant-Growth-Promoting Rhizobacteria (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. It is assumed that the bacteria affect plant growth mainly by nitrogen fixation (Elanchezhian and Panwar, 1997; Holguin and Bashan, 1996; Kucey, 1988), the production of various phytohormones (Bashan *et al*., 2004; Dobbelaere *et al*., 2001; Thuler *et al*., 2003) and also proton efflux in roots (Amooaghaie *et al*., 2002; Bashan and Levanony, 1989) which lead to an improvement in root growth, adsorption of water and minerals that eventually yield larger and in many cases, more productive plants (Bashan *et al*., 2004; Dobbelaere *et al*., 2001). *Azospirillum* is a general root colonizer and is not a plant specific bacterium (Bashan and Holguin, 1997; Fages and Arsac, 1991; Favilli *et al*., 1993; Zaady *et al*., 1993).

Rhizobia form root nodules that fix N_2 in symbiotic legumes (Matiru and Dakora, 2004). Instead of fixing N_2, rhizobia could use carbon received from the plant to synthesize energy-rich storage molecules like polyhydroxybutyrate (PHB), which could enhance *Rhizobium* survival and reproduction after they return to the soil (Denison, 2000). An individual *Rhizobium* can enhance host photosynthesis, presumably increasing the *Rhizobium*'s own photosynthate (Denison and Kiers, 2004). The rhizobia-legume interaction falls into cross inoculation groups, whereby certain rhizobial strains nodulate only certain legumes (Hirsch *et al*., 2001; Humphry *et al*., 2007).

Efforts at extending N_2-fixing ability to important non-leguminous crops such as cereals have long been a major goal of workers in the field of biological nitrogen fixation (Matiru and Dakora, 2004). Rhizobia have been isolated as natural endophytes from roots of non-legumes such as cotton, sweet corn (McLnroy and Kloepper, 1995), rice (Yanni *et al*., 1997) that either grown in rotation with legumes or in a mixed cropping system involving symbiotic legumes. Also, several experiments were showed nitrogenase activity and internal colonization of the root system of non-legumes by rhizobia (Al-Mallah *et al*., 1990; Antoun *et al*., 1998; Gough *et al*., 1997; Spencer *et al*., 1994). Inoculation of wheat and rice with *Azorhizobium caulinodanse* showed internal plant colonization and active nitrogen fixation (Cocking *et al*., 1994; Webster *et al*., 1998). *R. leguminosarum* bv. *trifolii* strain R39 stimulated the growth of maize, spring wheat, spring barley and oil radish. After seed inoculation with peat inoculant, *Rhizobium* strain R39 colonized the roots of wheat, maize, rape and sugar beet (Hoflich, 1999).

Exploitation of synergistic interactions between co-inoculated *Azospirillum* and other plant-growth-promoting microorganisms to increase further *Azospirillum*'s beneficial effect on plant growth was first discussed by Bashan and Holguin (1997). Dual inoculation studies of *Azospirillum* usually showed that this has some advantages over single inoculation in grain and plant dry matter production and N and P uptake depending on the co-inoculants and plant used (Rodelas *et al*., 1999; Bashan and Holguin, 1997; Tripathi and Mishra, 1996; Itzigsohn *et al*., 1993).

On the bases of co-inoculation information and chemical interaction on colonization of bacteria, this work was conducted to evaluate the effect of co-inoculation of *Azospirillum brasilense* and *Rhizobium meliloti* combined with 2,4-D on colonization and nitrogenase activity of co-inoculated wheat.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used in this study were: (1) two *Azospirillum brasilense* strains, a local strain isolated from wheat roots of Arak region in central of Iran and identified by biochemical and morphological tests and confirmed by 16S rDNA gene PCR amplification as native strain and the reference strain Sp7(DSMZ 1960); (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. Reference strains were obtained from the Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ, Germany). Physiological and biochemical characters of the local bacterial isolates were examined according to methods described in Bergey's Manual of systematic Bacteriology (Holt *et al*., 1994) and also 16s rDNA determination.

DNA extraction and 16S rDNA gene PCR amplification: Genomic DNA was obtained from bacterial cultures grown in NFB (for *A. brasilense*) and YMA (for *R. meliloti*) for 24 h, in log phase growth. DNA extraction was carried out using the high yield DNA purification kit, DNTP™ KIT (DN 8115, Cinnagen Inc., Iran). 1.5 mL of the bacterial cultures (freshly grown bacterial strains) including of *A. brasilense* Sp7 (positive control), *R. meliloti* DSMZ 30135, native strains of *A. brasilense* and *R. meliloti* were added into a micro-centrifuge tube. Bacterial culture were centrifuged for 10 min at 7500 g, 20 μL of bacterial was resuspended in 100 μL of protease buffer and then 5 μL of protease was added, vortexed and was incubated at 37°C for overnight. Then 100 μL of bacterial samples were mixed with 400 μL of lysis solution and vortexed 20 sec, 300 μL of precipitation solution were added and mixed by vortexing 5 sec and placed in -20°C for 20 min. Then the tubes were centrifuged 12000 g for 10 min. The supernatant were added to 1 mL washed buffer, vortexing and centrifuge at 12000 g for 5 min, then decanted and were poured of the washed buffer completely and pellet was dried at 65°C for 5 min. The plette were suspended in 50 μL of solvent buffer by gentle shaking and placing at 65°C for 5 min, the solution contains purified DNA.

The bacterial DNA were prepared for PCR amplification of the 16S rDNA using forward primer (5'-AGA GGG GCC CGC GTC CGA TTA GGT AGT T-3', location 37-64 in *Azospirillum*) and reverse primer (5'-CCC GAC AGT ATC AAA TGC AGT TCC CAG GTT-3', location 436-407 in *Azospirillum*), PCR product length was 400 bp. Each 25 μL of PCR reaction solution contained 1 μL of each primers, 5 μL of template DNA, 0.5 μL for dNTPs 10 mM (dATP, dCTP, dGTP and dTTP), 0.25 μL (1 unit) of Taq DNA polymerase (TA7506C, Cinnagen, Iran), 2.5 μL of 10X PCR buffer, 0.75 μL of 50 mM $MgCl_2$ and 14 μL sterile distilled water, also 25 μL mineral oil. PCR amplification was performed in a automated thermal cycler (MWG Primus 25 PCR-system, Germany). The program includes an initial denaturation at 94°C for 2 min, Thermal cycling then proceeded with 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min and a final extension at 72°C for 10 min. Five microliter of each PCR reaction solution was analyzed by gel electrophoresis.

Preparation of inocula 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 (Sadovnikova *et al*., 2003; Zimmer and Bothe, 1988) and two *R. meliloti* strains (native and standard) were grown in YMA medium at 25°C (Vincent, 1970) in Erlenmeyer flasks for the 24 h in a rotary shaker at 200 rpm (logarithmic phase). Before inoculation, the growth was harvested by centrifuging (1000 g, 10 min), washed with sterile saline phosphate buffer and then were resuspended in phosphate buffer at concentration of 10^7 and 10^3 cfu mL^{-1} for *A. brasilense* strains and *R. meliloti* strains, respectively. 2,4-D (Merck) dissolved in water to 2 ppm concentration and that was added to inoculants.

The seeds of *Triticum aestivum* cv. Baccross and cv. Mahdavi were prepared from the Institute of Agricultural and Research of Isfahan in Iran. The seeds were surface sterilized according to methods described by Ogut *et al*. (2005). The experiment was conducted in University of Arak, Iran according to the experimental lay out.

Estimation of bacterial population in roots of treated plants: The results of our pervious experiment (unpublished results) indicated that the combination of inoculants and co-inoculants of *Azospirillum* and *Rhizobium* would have the best effect when the population of 10^7 and 10^3 cfu were used, respectively plus 2 ppm of 2,4-D. Therefore, the sterilized seeds of wheat cultivars were imbibed in bacterial suspended according to selected treatments for 2 h at ambient temperature under vacuum (Bashan and Levanony, 1985). Control wheat seeds were imbibed with sterile phosphate buffer under the same conditions. The co-inoculated seeds were germinated on filter paper in Petri dishes for 3 days in dark at 25°C. Three days seedlings were transferred, aseptically, to glass culture tubes (20×318 mm; 100 cm^3 capacity) containing 20 cm^3 of NFB medium (Fahraeus, 1957), one seedling per tube. The germinated seeds in glass tube incubated in chamber growth (Conviron TC30) at 25/18°C day/night cycle and 12 h light/12 h dark photoperiod for 7 days. In control tubes the bacterial were added without wheat seeds.

Two solid media were chosen for counts of bacteria: NFB medium and YMA medium (Vincent, 1970). The roots of 7 days treated plants were washed with water and sterilized then 0.5 g of each sterilized roots were randomly selected for the enumeration of bacterial strains. Serial dilutions in KCl of root macerates extract were performed and aliquots of each dilution were planted on PDA (potato-dextrose agar) and YMA medium, Petri dishes were incubated at 30 and 25°C, respectively. The colonies phenotypically similar to the strains of *Azospirillum* and

Rhizobium were counted as CFUs using modified Most Probable Number (MPN) method and a colony counter (Knowles, 1982). The data were subjected to statistical analysis using SPSS13 and Duncan's multiple range tests.

Nitrogenase activity or acetylene reduction assay: The reduction of acetylene to ethylene specifically was proposed as an indirect method to assay nitrogenase activity (Hardy *et al*., 1973, 1968). The co-inoculated seeds were cultured using same procedure described for bacterial count experiment. After 7 days the glass tube closures were replaced by subaseals and 10% (v/v) sample of acetylene gas was injected into each sealed tube and incubated for 24 h at 25°C. Then, 0.5 mL of gas of each sample was injected into a Gas Chromatograph (Varian 3300, chrompack capillary column, cp-Al_2O_3/KCl, 50×0.53 mm, 530 μ and flame ionization detector) and quantified for ethylene. Nitrogen gas flow was at 9 psi. Temperatures of injection port, column and detector were 120, 75 and 230°C, respectively. Nitrogen-fixation was measured as C_2H_2 reduction activity (ARA) in the treated plants. The mean nitrogenase activity was expressed as μmoles C_2H_2 formed per plant per day. The data were subjected to statistical analysis using SPSS11 and Duncan's multiple range tests.

RESULTS

Verification of bacteria using PCR: The PCR product were showed similar band 400 bp (Fig. 1) for *A. brasilense* Sp7 and *A. brasilense* native (lines #1 and 3), also the similar band patterns were observed between *R. meliloti* DSMZ 30135 and native strain of *R. meliloti* (lines # 4 and 5). The result verifies that the native bacteria were used in this experiment are *Azospirillum* or *Rhizobium* according to 16 s rDNA patterns.

Root colonization with *Azospirillum*: The wheat root cultivars were inoculated with *Azospirillum* or *Rhizobium* and co-inoculated with *Azospirillum* and *Rhizobium* squashed and then both *Azospirillum* and *Rhizobium* bacteria cells were isolated and counted. The results indicated that the single and combined strains of bacteria plus 2,4-D could differently colonize roots of wheat plants.

When the plants inoculated only with *A. brasilense* Sp7, colonization was increased in both cultivars of wheat compared to the control. In this case the bacterial populations were increased from 1.67×10^5 cfu g^{-1} FW in Baccross cv. to 3.67×10^5 in Mahdavi cv. The difference was significant ($p<0.05$) for different cultivars (Table 1).

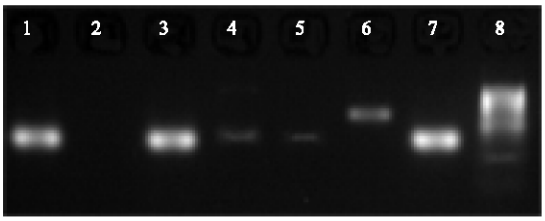

Fig. 1: Gel electrophoresis of 16 sec rDNAs prepared from *Azospirillum brasilense* and *Rhizobium meliloti*. The numbers in the figure identified as: (1) *A. brasilense* Sp7 (positive control), (2) negative control, (3) *A. brasilense* native, (4) *R. meliloti* standard DSMZ 30135, (5) *R. meliloti* native, (6) PCR check system (PR7 8011C, Cinnagen, Iran, the amplified DNA shows a band of 620 bp in an agarose gel), (7) mix of *A. brasilense* Sp7 and PCR check system (8) ladder (100 bp DNA ladder, Fermentas)

Table 1: Effect of inoculation and co-inoculation of wheat roots with *Azospirillum brasilense* 10^7 cfu (SP7 or native) and *Rhizobium meliloti* 10^3 cfu (DSMZ 30135 as standard or native) plus 2 ppm of 2,4-D on *Azospirillum* population of wheat roots

	No. of *Azospirillum* *10^5	
Treatments*	*T. aestivum* cv. Baccross	*T. aestivum* cv. Mahdavi
Not treated root	0^a	0^a
$R._{standard}$ (control)	0^a	0^a
$R._{native}$ (control)	0^a	0^a
$A._{Sp7}$	1.67^a	3.67^b
$A._{Sp7} + R._{standard}$	22^f	26^g
$A._{Sp7} + R._{native}$	19^e	27^g
$A._{native}$	12.67^d	8.67^c
$A._{native} + R._{standard}$	34^k	32^i
$A._{native} + R._{native}$	35^k	30^h

Different letter(s) on the mean values indicated significant differences ($p = 0.05$) between values according to Duncan multiple rage test, *R. stand for *Rhizobium* and A. for *Azospirillum*

When the Sp7 strain changed to a native strain for single inoculation, the number of *Azospirillum* in the wheat roots increased in comparison to *A. brasilense* Sp7. In this case, the number of *Azospirillum* was increased from 8.67×10^5 cfu g^{-1} FW in Mahdavi cv. to 12.67×10^5 in Baccross cv. This is almost 2.36 to 7.59-fold higher than Sp7 strain for Mahdavi and Baccross cultivars, respectively. Therefore, the colonization was different due to the strain and cultivars of wheat were used. Native *A. brasilense* colonized wheat root better than Sp7 strain. However, Baccross cv. reacted better with native *Azospirillum* compared to Mahdavi cv. which reacted better with Sp7.

When plants inoculated with dual inoculants (*Azospirillum* with *Rhizobium*), the colonization of *Azospirillum* was increased dramatically. A comparison between colonization for single Sp7 strain and Sp7 with

standard *Rhizobium* as co-inoculant indicated that the colonization were ranged from 1.67×10^5 to 22×10^5 cfu g^{-1} FW for Baccras cv. and 3.67×10^5 to 26×10^5 cfu g^{-1} FW for Mahdavi cultivar. The addition of colonization due to use of co-inoculant (in this case standard *Rhizobium*) is so high and different for different cultivar. When the standard *Rhizobium* as co-inoculant changed to a native strain of *Rhizobium* and compared with single inoculant of *Azospirillum*, the colonization was higher when compared to the single inoculate but was almost the same when compared to the standard *Rhizobium*. Although colonization of *Azospirillum* was higher in dual inoculation using native *Azospirillum* with native *Rhizobium*, the difference in *Rhizobium* strains was almost the same. From statistical point of view, the type of strain, wheat cultivars and co-inoculant affected the colonization differently and the differences and their interactions were significant (Table 3). In conclusion, the wheat cultivars reacted better with co-inoculants compared to the single strain of *Azospirillum* and also this reaction is much positive with the native strains compared to the standard ones. Although the wheat cultivars react differently with different inoculants, Mahdavi cv. reacts better with standard strains compared to the Baccras cv. (Table 1).

Root colonization with *Rhizobium*: Colonization of wheat cultivars with *Rhizobium* shows that when plants inoculated only with *R. meliloti* DSMZ 30135 (as standard strain), colonization were increased in both wheat cultivars compared to the non treated roots. In this case the *Rhizobium* populations were ranged from 0.33×10^5 cfu g^{-1} FW in Mahdavi cv. to 0.67×10^5 in Baccross cv. (Table 2). From statistical point of view the difference between different cultivars and the non inoculated roots was not significant ($p<0.05$).

When the standard *Rhizobium* changed to a native strain for single inoculation, the number of native *Rhizobium* in the wheat roots were not changed. Therefore, bacterial population and colonization of wheat roots were not depended to *Rhizobium* type. The difference was not significant ($p<0.05$) for this case.

When plants co-inoculated with *Rhizobium* and *Azospirillum*, the colonization for *Rhizobium* increased dramatically. A comparison between colonization of single standard *R. melilot* with *R. melilot* co-inoculated with *A. brasilense* Sp7 indicated that the colonization of *Rhizobium* were increased from 0.67×10^5 to 21×10^5 cfu g^{-1} FW for Baccross cv., Respectively. These values were 0.33×10^5 to 18×10^5 cfu g^{-1} FW for Mahdavi cv., respectively. When the Sp7 strain changed to a native *A. brasilense* as co-inoculant for *Rhizobium* inoculation, the colonization of *Rhizobium* was higher compared to the single inoculant and also higher compare to Sp7. This behavior was almost the same for the native *Rhizobium*. In conclusion, the *Rhizobium* population was higher in dual inoculation using native *Rhizobium* with native *Azospirillum*. The wheat cultivars reacted better with co-inoculants compared to the single strain of *Rhizobium* and also this reaction is much positive with the native strain compared to the standard ones. In contrast to inoculation of roots with single *Rhizobium*, the colonization of co-inoculated plants was depended to *Rhizobium* strain, wheat cultivars and also co-inoculants. The most number of *Azospirillum* and *Rhizobium* in the wheat varieties (Baccross and Mahdavi cultivars) were found when the local strains of bacteria were used. From statistical point of view, the differences and their interactions were significant (Table 3).

Table 2: Effect of inoculation and co-inoculation of wheat roots with *Rhizobium meliloti* 10^3 cfu (DSMZ 30135 as standard or native) and *Azospirillum brasilense* 10^7 cfu (SP7 or native) plus 2 ppm of 2,4-D on *Rhizobium* population of wheat roots

	No. of *Rhizobium* $*10^5$	
Treatments*	*T. aestivum* cv. Baccross	*T. aestivum* cv. Mahdavi
Not treated root	0^a	0^a
$A._{native}$ (control)	0^a	0^a
$A._{Sp7}$ (control)	0^a	0^a
$R._{standard}$	0.67^a	0.33^a
$R._{standard}+A._{native}$	23^e	$20^{c,d}$
$R._{standard}+A._{Sp7}$	21^d	18^b
$R._{native}$	0.67^a	0.33^a
$R._{native}+A._{native}$	27^f	29^g
$R._{native}+A._{Sp7}$	17^b	$19^{b,c}$

Different letter(s) on the mean values indicated significant differences ($p = 0.05$) between values according to Duncan multiple range test. *R. stand for *Rhizobium* and A. for *Azospirillum*

Table 3: The F-values for the effect of *A. brasilense* and *R. meliloti* types (native and standard), co-inoculated of *A. brasilense* and *R. meliloti* and their interaction with wheat cultivars on the number of *Azospirillum* and/or *Rhizobium* in treated wheat roots

		F-value+	
Source of variation	df	*Azospirillum* No.	*Rhizobium* No.
Plant variety	1	6.92^{**}	1.11^{ns}
Azo-type	1	641.72^{**}	270^{**}
Rhizo-type	1	4.22^{*}	46.88^{**}
Variety*Azo-type	1	169.22^{**}	0.0^{ns}
Variety*Rhizo-type	1	0.47^{ns}	46.88^{**}
Azo-type*Rhizo-type	1	0.47^{ns}	120^{**}
Variety*Azo-type*Rhizo-type	1	22.97^{**}	0.0^{ns}
Error	20		
Total	30		

+Symbols in the upright corner of the values indicated as: **Highly significant (1%), *Significant (5%), ^{ns}Not significant

Nitrogenase activity using dual inoculation: The nitrogenase activity of wheat seedlings (*Triticum aestivum* Baccross cv. and Mahdavi cv.) were compared using different co-inoculants plus 2 ppm 2,4-D as

Table 4: Nitrogenase activity (acetylene reduction assay, ARA, μmol C_2H_4 produced plant^{-1} day^{-1}) of wheat roots co-inoculated with mixture of *A. brasilense* 10^7 cfu (SP7 or native) and *R. meliloti* 10^3 cfu (DSMZ 30135 as standard or native) plus 2 ppm of 2,4-D

Treatment*	Bacterial without plant	*T. aestivum* cv. Baccross	*T. aestivum* cv. Mahdavi
Plants without-bacterial		0.0[a]	0.0[a]
$A._{Sp7} + R._{native}$	0.0112[a]	0.664[d]	0.403[c]
$A._{Sp7} + R._{standard}$	0.0182[a]	0.169[b]	0.169[b]
$A._{native} + R._{native}$	0.0089[a]	3.126[h]	2.243[g]
$A._{native} + R._{standard}$	0.0107[a]	1.719[f]	1.133[e]

*Different letter(s) indicated significant differences (p = 0.05) between mean values according to Duncan multiple test range, *R.* stand for *Rhizobium* and A. for *Azospirillum*

combined treatments. Nitrogen-fixation was measured as C_2H_2 reduction activity in co-inoculated plants. The Acetylene Reduction Assay, (ARA) shows that both wheat cultivars (Baccross and Mahdavi cultivars) created symbiotic system for nitrogen fixing activity following the co-inoculation with combined treatments (Table 4).

In tubes (without plants) contains different bacterial combinations, the values of nitrogenase activity were ranged from 0.0089 to 0.0182 μmol C_2H_4 tube^{-1} for 24 h. Although the lower nitrogenase activity was measured in combination of the local strains (native *A. brasilense* with native *R. meliloti*) and the higher one belongs to the combination of standard strains (Sp7 with standard *R. meliloti*) the differences were not significant ($p<0.05$) for nitrogenase activity in the tubes. No ethylene was also detected in non-inoculated plants as control treatment.

The nitrogenase activity of wheat cultivars was different in different co-inoculation mixture. The nitrogenase activity was lowest and also the same (0.169 μmol C_2H_4 produced plant^{-1} day^{-1}) in two wheat cultivars when the standard strains of bacterial were used as co-inoculant. Substitution of standard *Rhizobium* with native one as co-inoculant with standard *A. brasilense* causes the variation of nitrogenase activity in different wheat cultivars. The nitrogenase activities were 0.664 and 0.403 μmol C_2H_4 produced plant^{-1} day^{-1} for Bacross and Mahdeh wheat cultivars, respectively. From statistical point of view, the difference in nirtogenase activity for different cultivars is significant. Using native *Azospirillum* with either standard or native *Rhizobium* as co-inoculant would increase the nitrogenase activity much higher compared to standard ones in both cultivars. However, the activities of nitrogenase were higher in all cases, the reaction were different in different cultivars and also mixture of bacterial were used. Native *Azospirillum* co-inoculated with standard *Rhizobium* has less effect on nitrogenase activity compared to the native *Rhizobium* especially in Mahdvi cv. The highest amount of nitrogenase activity was measured in the combination of native *A. brasilense* and native *R. meliloti* in Baccross cv. of wheat. In conclusion, the acetylene reduction assay

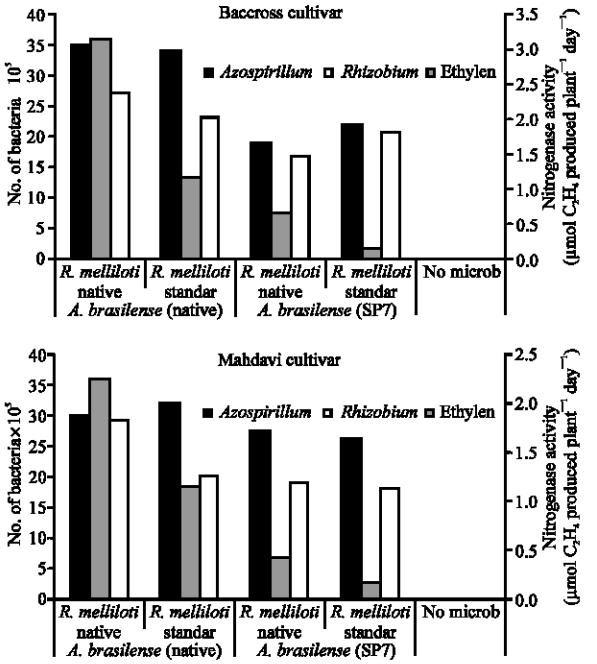

Fig. 2: Comparison between number of bacteria and nitrogenase activity in wheat roots for different cultivars of wheat

Table 5: The F-values for the effect of *A. brasilense* and *R. meliloti* type of strains in mixture of inoculants, wheat cultivars and their interaction on nitrogenase activity in treated wheat roots

Source of variation	df	F-value+
Wheat cultivars	2	1263.83**
Azospirillum type	1	3976.26**
Rhizobium type	1	898.37**
Variety * *Azospirillum*	2	1096.49**
Variety * *Rhizobium*	2	248.36**
Azospirillum * *Rhizobium*	1	276.39**
Variety * *Azospirillum* * *Rhizobium*	2	67.97**
Error	30	
Total	45	
Total	45	

+The symbol in the upright corner of the values indicated as: **-Highly significant (1%)

showed that combination of stains affected significantly on N_2-fixation and also different wheat cultivars behaves differently according to the strains combinations (Table 5, Fig. 2).

DISCUSSION

Exploitation of synergistic interactions between co-inoculated *Azospirillum* and other plant-growth-promoting microorganisms to increase further *Azospirillum*'s beneficial effect on plant growth was first discussed by Bashan and Holguin (1997). Efficient colonization and adaptation to plant varieties are the option for beneficial association in wheat root. Survival

strategies depend on the physiological adaptation in the introduced cells, such as adaptation to specific interactions with plants (Baladani and Baldani, 2005; Bashan and de-Bashan, 2005). Efficient root colonization is a major factor when assessing the effect of beneficial plant-associated bacteria. One of the most commonly reported mechanism for non-legume growth promotion by bacteria is intracellular colonization of root by viable batteries with nitrogenase activity, means symbiotic biological nitrogen fixation into the non-legume crops (Bashan and de-Bashan, 2005; Cocking *et al*., 1994).

The result of this study using *Azospirillum brasilense* and *Rhizobium meliloti* as a co-inoculated system in wheat cultivars indicated that the interaction of wheat roots and co-inoculant would establish a niche for better intercellular nitrogen fixation association. Co-inoculation with *A. brasilense* and *R. meliloti* significantly ($p<0.05$) increased colonization and nitrogenase activity of root wheat. The increase in the bacterial population was not associated with enhanced nitrogenase activity. However Holguin and Bashan (1996) showed that the mixed culture of *A. brasilense* Cd with *Staphylococcus* spp were increased the nitrogen fixation. Cocking (2005) also demonstrated that the cells of root meristems of maize, rice, wheat and other major non-legume crops can be colonized intracellularity by the non-rhizobial, non-nodulating, nitrogen-fixing bacterium, *Gluconacetobacter diazotrophicus*, that occurs naturally in sugarcane. *G. diazotrophicus* fixing N_2 in membrane-bounded compartments in the cytoplasm of cells of the root meristems of the target cereals and non-legume species, similar to the intracellular colonization of legume root nodule cells by rhizobia.

The combination of *Azospirillum* with other PGPB enhanced plant growth following co-inoculation is due to the synergistic effect of the both bacteria and *Azospirillum* functioning as a helper bacterium to enhance the performance of other PGPB. For instance, the involvement of polysaccharide degrading enzymes to explain the mechanism of root infection of legume by *Rhizobium* (Mateos *et al*. 1992; Reinhold-Hurek *et al*., 1993) showed the dependence of root endophytic colonization and spreading on cellulolytic enzymes of *Azoarcus* BH72 in rice. *A. brasilense* enhanced cellulose activity of wheat roots but this effect was directly depend on the strain-plant combination (Mostajeran *et al*., 2007) therefore, different colonization of *Rhizobium* in this experiment may be due to the interaction of *Azospirillum* stains and wheat cultivars. Similar results were obtained by Shaban *et al*. (1997) on maize co-inoculated with *Azospirillum* spp. and species of cellulose-decomposing fungi.

The nitrogenase activity of co-inoculated wheat's root has shown that using combination of *Azospirillum* and *Rhizobium* were differing compared to single inoculants. Similar result was reported for *Azospirillum* and wheat by Saubidet *et al*. (2002). Therefore, *A. brasilense* and *R. meliloti* would form a beneficial association in wheat root in related to nitrogenase activity. Simultaneously, the combination of local strains clearly increased nitrogenase activity in co-inoculated plants compared to the single inoculation. Plants co-inoculated with local strains have better stimulatory effect on root colonization and nitrogenase activity of wheat cultivars than co-inoculants of standard strains. Mostajeran *et al*. (2007) reported homolog effect between local strains of *A. brasilense* and local wheat cultivars on nitrogenase activity. Study results indicated that, the effect of co-inoculation on nitrogenase activity varies depending on the strain of *A. brasilense* (Sp7 and native), wheat cultivars and co-inoculant strains (*Rhizobium*; standard and native). Indigenous strains can perform more nitrogenase activity than standard strains in inoculated wheat cultivars due to their superior adaptation to the environment and compatibility to local plant varieties. Hoflich (1999) reported that the importance of plant growth promotion by factors such as phytohormones, N_2 fixation and antagonism may vary due to multiple interactions between inoculated bacteria, native microflora, crops and other ecological factors. Dalla Santa *et al*. (2004) also indicated that the intensive use of inoculants with associative bacteria, it is needed a wide isolation, to select the best combination between genotype of the plant and bacteria strain. They were also observed specify in the association of the plant and bacteria strain in the experiment on *Azospirillum* spp. Inoculation in wheat, barley and oats.

Study result indicated that the interaction effect of different cultivars and the strains of bacteria in co-inoculation of the root wheat in nitrogenase activity are significantly different. Similar result was proposed by Tsavkelova *et al*. (2006). Although the number of bacteria in wheat root is almost higher in the co-inoculation of *A. brasilense* (Sp7) with standard *Rhizobium* compared to the native *Rhizobium*, the nitrogenase activity is higher when native *Rhizobium* used as co-inoculant. This pattern is more obvious when the native *Azospirillum* co-inoculated with native *Rhizobium*. Therefore the number of bacteria in the root is not a good index for nitrogenase activity rate in compare to the type of inoculant and co-inoculant strains were used. However the co-inoculants strains effect on nitrogenase activity, the cultivar of wheat significantly effect colonization as well as nitrogenase activity.

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