African Journal of Biotechnology Vol. 11(84), pp. 15012-15019, 18 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3438
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Non-rhizobial bacteria for improved nodulation and grain yield of mung bean [*Vigna radiata* (L.) Wilczek]

Mohsin Tariq[1,2], Sohail Hameed[1]*, Tahira Yasmeen[1,3] and Amanat Ali[1]

[1]National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Road, Faisalabad, Pakistan.
[2]Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Allama Iqbal Road, Faisalabad, Pakistan.
[3]Department of Environmental Sciences, Government College University Faisalabad, Allama Iqbal Road, Faisalabad, Pakistan.

Accepted 28 March, 2012

Nine fast-growing bacteria were isolated from mung bean nodules and characterized with plant growth promoting properties. All the isolated bacteria were able to colonize mung bean root at varying level of 3×10^3 to 3×10^7 cfu (0.1 mg)$^{-1}$ root, whereas, bacterial isolates M2, M4, M5 and M6 showed high biofilm formation ability on abiotic surface. None of them was able to nodulate mung bean plant when reinoculated. Bacterial isolate M2 was found to be an efficient indole acetic acid producer (28.3 µg mL^{-1}), whereas M6 was an excellent phosphate solubilizer (21.8 µg mL^{-1}). Two isolates, M1 and M3, were able to fix nitrogen. 16S rRNA gene sequence analysis of potential isolates revealed that bacterial isolates M2 and M6 showed maximum similarity with *Bacillus subtilis*, M4 with *Bacillus simplex* and M5 with *Agrobacterium tumefaciens*. Further, the impact of co-inoculation of these non-rhizobial bacteria with *Bradyrhizobium* sp. MN-S on nodulation, plant growth and grain yield of mung bean was also assessed. Generally, co-inoculation significantly improved nodulation and grain yield compared with *Bradyrhizobium* sp. MN-S alone inoculation. The enhancement due to co-inoculation in nodule number and nodule dry weight was 78 and 127%, respectively when compared with the *Bradyrhizobium* sp. MN-S alone. Co-inoculation combination of *Bradyrhizobium* sp. MN-S with *B. subtilis* M6 performed best by increasing 22% grain yield, while the rest combinations also benefited plants non-significantly. The results show that non-rhizobial plant growth promoting bacteria improve nodulation and grain yield of the legumes upon co-inoculation with crop specific rhizobia.

Key words: Mung bean, nodulation, non-rhizobial, endophytes, co-inoculation.

INTRODUCTION

Mung bean [*Vigna radiata* (L.) Wilczek], is one of the important and well-known economic crops extensively cultivated in Asia during warm season. It has gained key importance in intensive crop production because of its short growing period and better storage ability. Mung bean not only has great dietary value due to its high protein contents but also improve soil fertility by fixing atmospheric nitrogen into available form by establishing a cooperative interaction with soil bacteria. Soil bacteria generally associated with legumes include *Rhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Azorhizobium*, collectively called rhizobia, more specifically α-rhizobia. Rhizobia infect plants, leading to the root nodule formation. Rhizobia belong to the genus *Bradyrhizobium* symbiotically associated with mung bean, which results in the nodule formation. Generally, *Bradyrhizobium* nodulates soybean, siratro, *Vigna* sp., *Lespedeza* sp. etc. (Appunu et al., 2009; Melchiorre et al., 2010).

Root nodules also accommodate various non-rhizobial bacteria having definite influence on the survival, nodulation and grain yield of the crop (Remans et al., 2008). This influence may be passive, but most often

*Corresponding author. E-mail: shameed@hotmail.com. Tel: +92 41 2651471.

non-rhizobial bacteria synergistically act with rhizobia and enhance nodulation and grain yield possibly by indole acetic acid (IAA) production, phosphate solubilization, fixing nitrogen, siderophore production, etc. (Mishra et al., 2009; Rajendran et al., 2008). Rhizosphere competence is one of the most important character of plant growth promoting rhizobacteria (PGPR), which enable bacteria to resist the stressed environment and exclude pathogens from rhizosphere (Compant et al., 2005; Chauhan and Nautiyal, 2010).

Symbiotic effectiveness of rhizobial inoculants for a wide variety of legumes can be improved by co-inoculation with suitable non-rhizobial plant growth promoting bacteria (PGPB) (Lazdunski et al., 2004). Co-inoculation of PGPB with crop specific rhizobia improves root infection which results in better nodulation and grain yield e.g., *Agrobacterium* sp. helps *Bradyrhizobium* sp. in infecting root and ultimately developing nodule (Hameed et al., 2004). Similarly, co-inoculation of *Pseudomonas* with rhizobia enhance nodulation, nitrogen fixation, plant biomass and grain yield in various leguminous crops such as alfalfa, pea, soybean, green gram and chickpea (Mishra et al., 2009). Co-inoculation of rhizobia with *Bacillus*, specifically *Bacillus thuringifensis*, *Bacillus megatrium* and *Bacillus cereus* significantly promotes nodulation, plant growth and grain yield (Halverson and Handelsman, 1991; Mishra et al., 2009). Moreover, bacteria belonging to *Burkholderia*, *Azotobacter*, *Azospirillum*, *Enterobacter* and *Kurthia* have also been evaluated for their co-inoculation efficacy with rhizobia and were found to improve plant growth (Pandey and Maheshwari, 2007). Recently, co-inoculation of arbuscular mycorrhizae with *Bradyrhizobium* sp. proved to be very helpful in improving mung bean growth (Yasmeen et al., 2012a, b).

Keeping in view the above facts, the objective of this study was to isolate and characterize non-rhizobial nodule endophytic bacteria from mung bean and to find out their effectiveness in nodulation and grain yield improvement upon co-inoculation with mung bean specific *Bradyrhizobia* as well as study their utility as biofertilizers for crop improvement.

MATERIALS AND METHODS

Bradyrhizobium strain MN-S, previously isolated from mung bean, was obtained from the BIRCEN culture collection, Plant Microbiology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. Mung bean (*V. radiata* L.) cultivar MN-92 seeds were obtained from Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Isolation of nodule endophytic bacteria

Mung bean plants grown in the field of NIBGE, Pakistan were sampled for bacterial isolation at nodulation stage (35 days after germination). Roots were washed thoroughly with tap water to remove soil. About 10 nodules were detached from the roots. Surface sterilization was carried out by immersing intact and undamaged nodules in ethanol for 15 s and then transferred to a 3% solution of calcium hypochlorite for five minutes and rinsed in five changes of sterile water. Surface sterilization of nodules was tested by incubating nodules on yeast extract mannitol (YEM) medium plates up to seven days at 28 ± 2°C. Surface sterilized nodules were crushed to prepare a suspension aseptically with a few drops of sterile water in a Petri dish. Nodule suspension was plated on Congo red YEM (CR-YEM) agar plates and incubated at 28 ± 2°C. After 24 h, the isolated bacterial colonies showing different morphotypes were selected and sub-cultured until the purity of cultures was confirmed (Marsudi et al., 1999). Pure cultures were stored in 20% glycerol at -80°C. Gram reaction was performed according to Vincent (1970).

Nodulation assay

Nodulation assays were performed according to Ma et al. (2003) with some modifications. All the nodule endophytic bacteria were grown individually in Lauria Bertani (LB) broth, and maintained at 10^6 cfu ml^{-1} in sterile water. Seeds of mung bean *cv*. NM-92 were surface sterilized with 0.1% mercuric chloride for 5 min and washed extensively with sterile water. Surface sterilized seeds were allowed to germinate in dark room at 25 ± 2°C on moist filter paper kept in sterile Petri plates containing 15 seeds. Germinated seedlings (1 cm) were inoculated by dipping in each type of endophytic bacteria, separately. Inoculated seedlings were transplanted in the sterilized assemblies of magenta boxes containing vermiculite-perlite (ratio 1:1 v/v) and incubated at 30 ± 2°C (day) and 20 ± 2°C (night) for long day photoperiod (16 h of light per day). Plants were watered with quarter strength nitrogen free Hoagland solution. After five weeks, plants were observed for nodulation.

Indole acetic acid production

Detection and quantification of IAA production by the non-rhizobial bacterial isolates was carried out by growing cultures in LB broth supplemented with tryptophan (100 mg L^{-1}) as the precursor of IAA. After one week of growth, qualitative estimation of IAA was assessed by Fe-$HClO_4$ and Fe-H_2SO_4 reagents producing pink color. IAA was quantified by ethyl acetate oxidation method (Zakharova et al., 1999) using high performance liquid chromatography (HPLC) using Turbochom software (Perkin Elmer USA).

Phosphate solubilization

For phosphate solubilization study, a single colony of each bacterial culture grown from LB plate was streaked on to Pikovskaya's plate containing tricalcium phosphate (Pikovskaya 1948) and incubated at 25 ± 2°C for seven to 10 days. The plates were observed for clear phosphate solubilization zone around colonies. Phosphate solubilization was quantified by Phospho-molybdate blue color method using spectrophotometer (Nair et al., 2007).

Nitrogen fixation

Nitrogen fixation ability of the non-rhizobial bacteria was assessed by inoculating single colony in 5 ml semisolid nitrogen free media (NFM) in 15 ml vials and incubated at 28 ± 2°C for 48 h. Acetylene (10% v/v) was injected into the vials. After incubation for 16 h at room temperature, gas samples (100 µl) were analyzed on a gas chromatograph (Thermoquest, Trace G.C, Model K, Rodono Milan, Italy) using a Porapak Q column and a H_2 -flame ionization detector (FID). Nitrogenase activity was measured as described by Hameed et al. (2004).

Root colonization

Bacterial isolates were also studied for the potential of colonization on the mung bean roots. 10 days old plants grown under aseptic conditions were harvested and the roots were cut into 1.5 cm segments. Pieces of uniform shape and size were placed into the 96-wells of a microtiter plate. 200 µl of bacterial culture maintained at OD_{600} = 0.2 were added to the wells and the plates were incubated at 28°C for 48 h. After the incubation period, the root pieces were removed from the cultures, washed with sterile water, and then added to 1 ml sterile water. Bacterial biofilms were removed from the root surface and dispersed in sterile water by vigorous shaking. An aliquot (100 µl) of the dispersed preparation was plated on LB agar and the colony forming units (cfu) were counted after five days as cfu 0.1 mg^{-1} root.

Biofilm formation

Biofilm formation was studied on abiotic surface by a microtiter plate assay according to Fujishige et al. (2006) with some modifications. The bacterial cultures were grown up to an optical density at λ600 nm (OD_{600}) = 2.0 in LB broth, pelleted by centrifugation at 8,000 rpm for 2 min, and washed with sterile distilled water. The cells were resuspended in the same medium and maintained at OD_{600} = 0.2. An aliquot (150 µl) of bacterial cell suspension was added to individual wells in a 96-well polyvinyl chloride (PVC) plate (Fisher, USA). LB alone was used as the control. The plates were covered with plastic lids and incubated at 28°C for 48 h. After the incubation period, the medium was removed and the wells were washed with sterile water. The plates were allowed to dry and the wells were treated with 150 µl of 0.001% crystal violet for 15 min. The excess dye was removed and the wells were washed with sterile water. The retained stain was solubilized with 150 µl of 95% ethanol and the amount of dye was quantified by measuring the absorbance at 570 nm on micro-plate reader.

Phylogenetic identification

Total genomic DNA of bacterial strains M2, M4, M5 and M6 was isolated by the alkaline lysis method (Maniatis et al., 1982) with slight modifications. The primers used for amplification of full length 16S rRNA gene were universal primer P1 (forward primer, 5` - CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACGCT- 3`) and P6 (reverse primer, 5` - CGGGATCCTACGGCTACC-TTGTTACGACTTCACCCC- 3), which correspond to *Escherichia coli* positions 8 to 37 and 1479 to 1506, respectively, and amplifies 1500 bp fragment (Tan et al., 1997). Each 25 µl of reaction mixture contained 1 U of *Taq* Polymerase (Promega), 2.5 µl 10x PCR buffer, 2 µl $MgCl_2$, and 1 µl dNTPs (2.5 mM), 1 µl of each primer (100 ng μl^{-1}) and 1 µl template DNA (12.5 ng μl^{-1}). Reaction mixture (25 µl), prepared for 16S rRNA gene amplification was initially denatured at 94°C for 2 min followed by 25 cycles consisting of denaturation at 94°C for 60 s, primer annealing at 52°C for 60 s and primer extension at 72°C for 3 min and finally, extension at 72°C for 20 min in a thermal cycler. The amplified 16S rRNA gene was ligated in TA cloning vector pTZ57R/T (Fermentas), which has 2886 bp length. In the case of pTZ57R/T vector, 30 µl ligation reaction was prepared in sterile water with 1.5 µl T4 DNA ligase, 3 µl ligation buffer, 3 µl pTZ57R/T vector (Fermentas), 3 µl of PEG 4000 and 4 µl amplified DNA in 1.2 ml tube. Ligation was performed overnight in water bath at 16°C. Plasmid extraction of recombinant transformants was carried out using GeneJET Plasmid Miniprep Kit (Fermentas) and clones were confirmed by restriction analysis using *EcoRI* and *PstI* (Fermentas). PCR products and clones were resolved on 1% agarose gel and GeneRuler™ 1kb ladder #SM0313 was used as DNA size marker.

Cloned PCR products were sequenced on ABI Prism 3100 Genetic Analyzer (Hitachi, Japan) using Big Dye Terminator v 1.1 Cycle Sequencing Kit. 16S rRNA gene sequences of all the four bacterial strains were compared with database sequences using Basic Local Alignment Search Tool (BLAST) at http://www.ncbi.n1m.nih.gov/blast/Blast.cgi.

***In vitro* microbial compatibility assay**

The overlay plate technique was used to access the compatibility of microbial inoculants by growing non-rhizobial bacteria in the presence of *Bradyrhizobium* sp. MN-S according to Mrabet et al. (2006) with some modifications. 3 ml of log phase grown culture of *Bradyrhizobium* sp. MN-S maintained at 10^4 cfu ml^{-1} in sterile water was mixed in 22 ml molten hand cool LB agar and poured in Petri plate. Plate was incubated at 28 ± 2°C for 24 h and then 5 µl of saturated culture (10^9 cfu mL^{-1}) of each non-rhizobial bacterial strain was inoculated in the center of *Bradyrhizobium* sp. MN-S plate, separately. Zone of inhibition was measured after five days of incubation at 28 ± 2°C. Each non-rhizobial strain was tested in triplicate for *in vitro* compatibility.

Control condition experiment

Culture of all the tested strains were grown individually in LB broth and maintained at 10^6 cfu mL^{-1} in sterile water. Each of the four non-rhizobial culture was mixed with the *Bradyrhizobium* sp. MN-S at equal concentration (ratio 1:1 v/v) in sterile tubes, so that cell concentration of 10^6 cfu mL^{-1} in sterile water remains maintained. Mild inoculum strength, 10^6 cfu ml^{-1} was selected, as higher cell densities may have an inhibitory effect on nodulation and plant growth (Mishra et al., 2009). On the other side, mung bean seeds cv. NM-92 were surface sterilized with 0.1% mercuric chloride for 10 min and washed extensively with sterile water. Surface sterilized seed were allowed to germinate in dark room at 25 ± 2°C on moist filter paper kept in sterile Petri plates containing 15 seed. Germinated seedlings were subjected to all the four co-inoculation combinations: M2 + *Bradyrhizobium* sp. MN-S, M4 + *Bradyrhizobium* sp. MN-S, M5 + *Bradyrhizobium* sp. MN-S and M6 + *Bradyrhizobium* sp. MN-S, *Bradyrhizobium* sp. MN-S alone (positive control) and sterile water (without inoculation, negative control). The coated seedlings were planted in sterile growth pouches containing 5 ml quarter strength nitrogen Hoagland solution. Growth pouches were incubated in growth chamber at 30 ± 2°C day and 20 ± 2°C night for long day photoperiod. The experiment was performed in triplicate and the plants were harvested after six weeks. Nodulation parameters: nodule number per plant, nodule dry weight per plant and total plant dry weight were determined and subjected to statistical analysis.

Field experiment

Mung bean field experiment was also conducted in spring 2008. A randomized complete block design (RCBD) based experiment was performed with the six above mentioned treatments and three replicates. 10 mL inoculum of each treatment maintained at 10^6 cfu mL^{-1} in sterile water was applied on 100 g surface sterilized seed. The seeds were sown by hand drill in a plot of 2 × 6 m in triplicate and the field was watered when required. Nodulation was recorded after six weeks. Mature plants were harvested after 90 days and grain yield was determined.

Statistical analysis

Data was analyzed statistically using CoStat window version

Table 1. Characterization of nodule endophytic bacteria for plant growth promoting attributes.

Isolate	Colony morphology	Cell morphology	Gram reaction	ARA	IAA Production (µg mL^{-1})	Phosphate solubilization (µg mL^{-1})
M1	Round, large, smooth, white	Small rods	-ve	+	9.2 ± 0.6	6.92 ± 0.63
M2	Round, small, wavy, dark creamy	Medium rods	+ve	_	28.3 ± 4.7	18.1 ± 0.8
M3	Round, small, smooth, creamy	Small rods	-ve	+	32.92 ± 0.7	11.61 ±0.46
M4	Round, large, wavy, dark creamy	Medium rods	-ve	_	3.4 ± 0.3	4.4 ± 0.5
M5	Round, small, smooth, dark creamy	Small rods	+ve	_	2.2 ± 0.3	13.8 ± 1.1
M6	Round, small, wavy, dark creamy.	Medium rods	+ve	_	26.5 ± 2.1	21.8 ± 2.7
M7	Round, medium, smooth, creamy	Small rods	-ve	_	10.94 ± 0.74	6.77 ± 0.47
M8	Round, large, wavy, white	Small rods	-ve	_	17.2 ± 0.98	6.78 ± 0.45
M9	Round, large, wavy, dark creamy	Small rods	-ve	_	1.02 ± 0.08	6.79 ± 0.48

Each value represents mean ± standard error; ARA, acetylene reduction assay; IAA, indole 3-acetic acid.

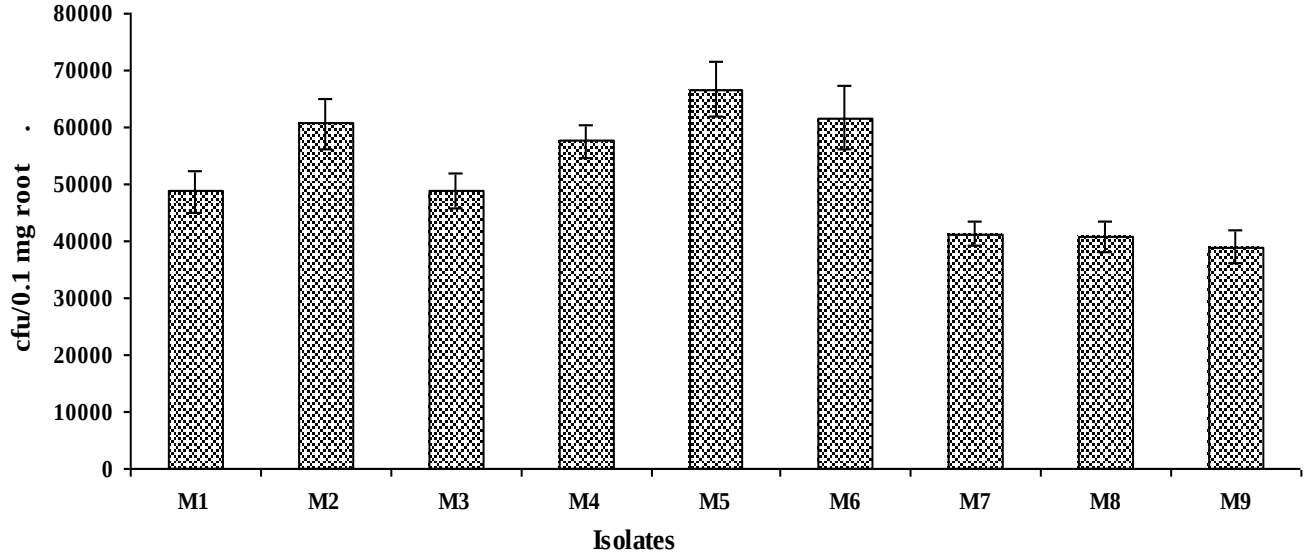

Figure 1. Colonization efficiency of nodule endophytic bacteria on plant root. Each value is plotted as the mean ± SE (n = 3).

computer program. The least significant difference (LSD) at 5% level of probability was used to test the differences among mean values (Steel and Torrie, 1980).

RESULTS

In this study, nine bacteria were isolated from the mung bean nodules on the basis of different colony morphology. The colonies appeared over night, suggesting that they are non-rhizobial in nature, as even fast growing rhizobia appear after 48 h of incubation (Marsudi et al., 1999). Selected bacterial isolates also strongly picked Congo red dye on CR-YEM agar plate, which was a second line of evidence showing that isolated bacteria are non-rhizobial (Kneen and Larue, 1983). Furthermore, bacterial isolates failed to nodulate plant in nodulation assay. All bacterial isolates showed much variation in colony and cell morphology (Table 1). Three out of nine isolates were Gram positive, while six were Gram negative.

All the non-rhizobial isolates were able to produce IAA at various levels (Table 1). Isolate M2 produced maximum amount of IAA (28.3 ± 4.7 µg mL^{-1}), whereas M5 strain produced least quantity of IAA (2.2 ± 0.3 µg mL^{-1}). All bacterial strains were also able to solubilize phosphate ranging from 4.4 to 21.8 µg mL^{-1} (Table 1). Two bacterial isolates, M1 and M3 were able to fix nitrogen in acetylene reduction assay.

Further, these bacterial isolates were checked for mung bean root surface colonization. All the isolates showed very high colonization on mung bean root surface. Similarly, equal efficient biofilm formation ability on the abiotic surface (polyvinyl chloride) was also observed. Overall, bacterial isolates, M2, M4, M5 and M6 showed high efficiency in colonization on roots and biofilm formation on abiotic surface (Figures 1 and 2).

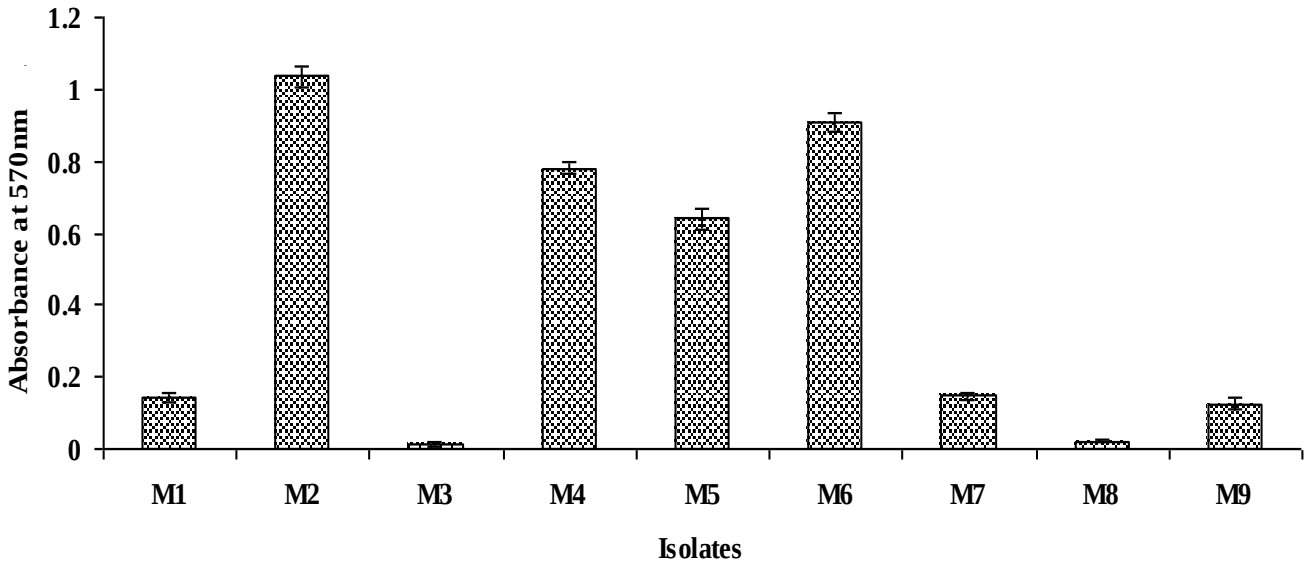

Figure 2. Biofilm formation efficiency of nodule endophytic bacteria on abiotic surface (PVC). Each value is plotted as the mean ± SE (n =16). PVC, Polyvinyl chloride.

Table 2. Effect of co-inoculation of *Bradyrhizobium* sp. MN-S and non-nodulating endophytic bacteria on nodulation and plant growth of mung bean under control conditions.

Treatment	Nodule number plant^{-1}	Nodule dry wt. plant^{-1} (mg)	Total plant dry wt. (mg)
Bradyrhizobium sp. MN-S	34 ± 3.1^{c}	3.9 ± 0.61^{c}	407.3 ± 38^{bc}
Bradyrhizobium sp. MN-S + Isolate M2	53 ± 6.1^{ab}	6.4 ± 0.67^{b}	504 ± 6.1^{ab}
Bradyrhizobium sp. MN-S + Isolate M4	45.7 ± 5.3^{bc}	4.13 ± 0.71^{c}	314 ± 62.1^{c}
Bradyrhizobium sp. MN-S + Isolate M5	54.7 ± 4.1^{ab}	6.8 ± 0.74^{b}	406 ± 11.2^{bc}
Bradyrhizobium sp. MN-S + Isolate M6	60.7 ± 6.8^{a}	8.87 ± 0.14^{a}	552.7 ± 39.1^{a}
Uninoculated control	0 d	0 d	328.7 ± 36.7^{c}
LSD (P=0.05)	12.3	1.42	94.2

LSD, Least significant difference; each value represents mean ± standard error (n = 3). Values followed by the different letters in same column indicate significant difference.

Potential bacterial isolates, M2, M4, M5 and M6, were phylogenetically identified by 16S rRNA gene sequencing. DNA fragments of ~1500 bp were amplified using primers P1 and P6 set. The DNA fragments were cloned in vector pTZ57R/T. When the clones were restricted with *EcoRI* and *PstI*, DNA fragment of ~2900 and 1500 bp appeared, which revealed that 16S rRNA fragments of tested bacteria do not have *EcoRI* and *PstI* restriction site. The 16S rRNA gene sequence analysis revealed that non-rhizobial bacterial strains M2 and M6 have 99% homology with *Bacillus subtilis*. Strain M4 showed 100% sequence similarity to *Bacillus simplex*, while strain M5 showed 99% similarity to *Agrobacterium tumefaciens*. 16S rRNA gene sequences of almost full length of bacterial strains M2, M4, M5 and M6 were deposited at NCBI GenBank under the accession numbers EF443161, EF443162, EF443163 and EF443164, respectively.

In vitro compatibility assay showed that the selected bacterial isolates were able to grow in the presence of *Bradyrhizobium* sp. MN-S as the mats of both cultures in each treatment was touching each other and there was no inhibition zone between the edges/boundaries of dually platted cultures. There was no inhibitory effect of any of the tested non-rhizobial bacteria on *Bradyrhizobium* sp. MN-S or vice versa. The results suggest the use of these non-rhizobial bacteria for co-inoculation studies with *Bradyrhizobium* sp. MN-S in mung bean crop.

In growth pouch experiment, highly significant difference in nodule numbers was observed among treatments at a confidence level of 0.001 (Table 2). Microbial consortia of *Bradyrhizobium* sp. MN-S + *B. subtilis* M6 significantly increased nodule number per plant (60.7), which is 78% higher as compared to the

Table 3. Effect of co-inoculation of *Bradyrhizobium* sp. MN-S and non-nodulating endophytic bacteria on nodulation and plant growth of mung bean under field conditions

Treatment	Nodule number plant^{-1}	Nodule dry weight plant^{-1} (mg)	Grain yield (kg ha^{-1})	Grain yield increase (%)
Bradyrhizobium sp. MN-S	39.3 ± 5.2^{b}	12.4 ± 1.3^{c}	1216 ± 112^{b}	0
Bradyrhizobium sp. MN-S + Isolate M2	49.7 ± 4.7^{ab}	16.3 ± 2.1^{b}	1461 ± 105^{ab}	20
Bradyrhizobium sp. MN-S + Isolate M4	51 ± 5.1^{ab}	11.9 ± 2.2^{c}	1219 ± 72^{b}	0.25
Bradyrhizobium sp. MN-S + Isolate M5	58.7 ± 8.8^{a}	15.9 ± 2.5^{b}	1335 ± 81^{ab}	10
Bradyrhizobium sp. MN-S + Isolate M6	61 ± 8.2^{a}	20.3 ± 3.9^{a}	1478 ± 108^{a}	22
Uninoculated control	22 ± 5^{c}	6.5 ± 1.1^{a}	947 ± 79^{c}	-22
LSD (P=0.05)	17.5	2.85	238	

LSD, Least significant difference; each value represents mean ± standard error (n = 3). Values followed by the different letters in same column indicate significant difference.

treatment of *Bradyrhizobium* sp. MN-S alone (34). *A. tumefaciens* M5 and *B. subtilis* M2 upon co-inoculation with *Bradyrhizobium* sp. MN-S was also reasonably beneficial to increase nodule number, producing 55 and 53 nodules per plant, respectively. Nodule dry weight was also significantly different upon inoculation of various treatments at a confidence level of 0.001. A similar trend for nodule number in nodule dry weight was noticed (Table 2). *Bradyrhizobium* sp. MN-S + *B. subtilis* M6 significantly promoted nodule dry weight per plant (8.87 mg), which is 127% higher as compared to the inoculation of *Bradyrhizobium* sp. MN-S alone (3.9 mg). As far as plant yield is concerned, there was a highly significant effect of inoculation on plant dry weight (Table 2). A maximum increase of 36% in plant dry weight was recorded upon inoculation of *Bradyrhizobium* sp. MN-S + *B. subtilis* M6 as compared to the *Bradyrhizobium* sp. MN-S alone. It was noted that plant dry weight is somehow independent on nodulation as bacterial consortia of *Bradyrhizobium* sp. MN-S + *B. simplex* M4 showed reasonably good nodulation, but plant yield was lower than un-inoculated control and the *Bradyrhizobium* sp. MN-S alone treatment as well, which was poorly nodulated.

Under field conditions, nodulation trend was almost similar with that of control condition experiment. A significant difference in grain yield was observed among the inoculation treatments in field trail at a confidence level of 0.01 (Table 3). Co-inoculation combination, *Bradyrhizobium* sp. MN-S + *B. subtilis* M6 remarkably improved grain yield to 1478 kg ha^{-1}, which is 22% higher than the *Bradyrhizobium* sp. MN-S alone (1216 kg ha^{-1}) and 44% higher than the un-inoculated control (947 kg ha^{-1}). Co-inoculation combination, *Bradyrhizobium* sp. MN-S + *B. simplex* M4 was found to be least effective.

DISCUSSION

PGPB is a group of soil bacteria which promote plant growth by developing a positive relationship with roots (Tariq et al., 2010), while rhizobia is a subdivision of PGPB, which establish a symbiotic relationship with legume and results in the root nodule development. Occurrence of bacteria other than rhizobia in root nodule was first reported by Beijerinck and Van Delden (1902) in the clover plant and identified as *Agrobacterium radiobacter*. Mung bean nodule contains 90% of the analyzed strains which belonged to *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium elkanii*, while the rest belong to *Sinorhizobium* and *Rhizobium* (Zhang et al., 2008). In this study, we isolated nine fast growing bacterial morphotypes from the mung bean nodule. These bacterial isolates were found to be non-rhizobial, as they were unable to infect roots and develop nodules on mung bean. These bacterial isolates were characterized for plant growth promoting attributes including IAA production, phosphate solubilization, N-fixation, rhizosphere competence and biofilm formation. Potential bacterial strains were phylogenetically identified by 16S rRNA gene sequencing, showing maximum similarity with *B. subtilis, B. simplex* and *A. tumefaciens*. We speculated that these non-rhizobial bacteria infected nodules by the crack entry. Previously, Zakhia et al. (2006) reported that various non-rhizobial bacteria inhabit nodule and infect nodule by crack entry. *Bacillus* is known for its endophytic nature in various legume and non-legume crops (Bai et al., 2002; Zakhia et al., 2006). *Bacillus* sp. has been isolated from the nodule of common bean and soybean. Selvakumar et al. (2008) isolated *Bacillus thuringiensis* from the root nodule of leguminous vine *Kudzu* (*Pueraria thurbegiana*), which is in conformity with our results, as these *Bacillus* sp. was able to produce IAA, solubilize phosphate and thus promote plant growth. Recently, Yu et al. (2009) reported the isolation of copper resistant *Agrobacterium* from nodules of *Lespedeza cuneata*. It is believed that non-rhizobial *Agrobacterium*-like strains enter nodule during the infection of rhizobia and are able to maintain its high

density (Mhamdi et al., 2002). These evidences of occurrence of *Bacillus* and *Agrobacterium* in the nodule support our findings.

There is a large body of literature showing that bacteria function less as individuals and more as consortia (Lazdunski et al., 2004). Various studies have shown that co-inoculation of rhizobia with *Bacillus* remarkably enhanced nodulation and growth of legume crops (Elkoca et al., 2008). In this study, we initially tested bacterial combinations for antibiosis and found that all the isolated *Bacillus* sp. and *Agrobacterium* sp. were compatible with *Bradyrhizobium* sp. MN-S. Our results are in line with the findings of Mishra et al. (2009) and Mrabet et al. (2006), who observed no inhibitory effect of *Agrobacterium* and *Bacillus* sp. on the growth of rhizobia. Co-inoculation significantly improved nodulation and plant dry weight under control condition experiment (Table 2). The results of nodulation and plant dry weight reveal an independent relationship between these parameters. Our results are in line with Dakora and Atkins (1990) who also reported such an independent relationship between nodule dry matter and plant yield in cowpea. Co-inoculation of *B. subtilis* M6 and *B. subtilis* M2 with *Bradyrhizobium* sp. MN-S significantly promoted nodulation and grain yield of mung bean as compared to *Bradyrhizobium* sp. MN-S alone, while combination of *B. simplex* M4 could not significantly improve nodulation and grain yield (Table 2). Co-inoculation of *B. subtilis* M6 + *Bradyrhizobium* sp. MN-S significantly promoted nodulation (78%) and grain yield (22%) of mung bean as compared to *Bradyrhizobium* sp. MN-S alone. Previously, Tilak et al. (2006) reported a similar enhancement in nodulation and grain yield in pigeon pea (*Cajanus cajan*). Improved nodulation and plant growth might be due to the high IAA production by these bacterial isolates which is required for the the early steps of nodulation and root elongation (van Noorden et al., 2008). Moreover, the selected bacterial isolates were also able to solubilize phosphate which is one of the limiting factors for plant growth in the soil of Pakistan (Mahmood-ul-Hassan et al., 1993), specifically *B. subtilis* M2 and M6 showed very high potential for phosphate solubilization. Better biofilm formation and root colonization efficiency might also benefit plant as these attributes ensure rhizosphere competence of microbe (Bloemberg and Lugtenberg, 2001; Lagendijk et al., 2010). Among *Bacillus* species, *B. subtilis* and *Bacillus magetrium* are found to be best studied for co-inoculation with rhizobia and were significantly beneficial to legume crops (Elkoca et al., 2008; Kumar and Chandra, 2008). Some researchers showed that combination of dual inoculation neither induced root hair proliferation nor enhanced nodulation (Sirnivasan et al., 1996). Previously, co-inoculation strategy was found to be very effective in the case of *Azospirillum* sp. + rhizobia, promoting grain yield to 54 and 29% in wheat and common bean, respectively (Askary et al., 2009). As far as co-inoculation of *Agrobacterium* sp. M5 with *Bradyrhizobium* is concerned, this study describes the beneficial impact of its co-inoculation on nodulation and grain yield. Previously, Mhamdi et al. (2005) observed that co-inoculation of *Agrobacterium* and *Rhizobium* enhanced neither nodulation nor grain yield of common bean. Recently, they noticed an adverse effect of *Agrobacterium* on the growth of *Rhizobium* and subsequently on nodulation and plant growth (Mrabet et al., 2006). Our positive results might be due to IAA production, phosphate solubilization and *in vitro* compatibility of *A. tumefaciens* M5 with *Bradyrhizobium* sp. MN-S. Previously, we reported the co-occupancy of *Agrobacterium* Ca-18 with *Bradyrhizobium* TAL-102 in cowpea nodules (Hameed et al., 2005).

Microbial consortia behave synergistically by considerably increasing the amount of solubilized nutrients, IAA production, and ultimately promoting nodulation and plant yield (Mishra et al., 2009; Pandey and Maheshwari, 2007). In this study, we found that *Bacillus* sp. endophytically colonize root nodules and upon co-inoculation with *Bradyrhizobium*, it significantly promote nodulation and grain yield of mung bean as compared to the inoculation of *Bradyrhizobium* alone. Microbial consortia of *B. subtilis* with *Bradyrhizobium* sp. MN-S was excellent, whereas the rest of the tested microbial combinations were also marginally beneficial to plant. The above findings suggest that *B. subtilis* upon co-inoculation with crop specific rhizobia significantly promoted nodulation and grain yield, and can be used as biofertilizer for a wide variety of legume.

REFERENCES

Appunu C, N'Zoue A, Moulin L, Depret G, Laguerre G (2009). *Vigna mungo, V. radiata and V. unguiculata* plants sampled in different agronomical-ecological–climatic regions of India are nodulated by *Bradyrhizobium yuanmingense*. Syst. Appl. Microbiol. 32:460-470.

Askary M, Mostajeran A, Amooaghaei R, Mostajeran M (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). American-Eurasian J. Agric. Environ. Sci. 5:296-307.

Bai Y, D'Aoust F, Smith DL (2002). Isolation of plant growth promoting *Bacillus* strains from soybean root nodules. Can. J. Microbiol. 48:230-238.

Beijerinck MW, Van Delden A (1902). Über die Assimilation des freien Stickstoffs durch Bakerien. Centralbl Bakt Abt II. 9:3-43.

Bloemberg GV, Lugtenberg BJJ (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4:343-350.

Chauhan PS, Nautiyal CS (2010). The purB gene controls rhizosphere colonization by *Pantoea agglomerans*. Lett. Appl. Microbiol. 50: 205-210.

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. 71:4951-4959.

Dakora FD, Atkins CA (1990). Effect of PO_2 on growth and nodule functioning of symbiotic cowpea (*Vigna unguiculata* L. Walp.). Plant Physiol. 93:948-955.

Elkoca E, Kantar F, Sahin F (2008). Influence of nitrogen fixing and

phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea. J. Plant Nutr. 31:157-171.
Fujishige, NA, Kapadia NN, De Hoff PL, Hirsch AM (2006). Investigations of *Rhizobium* biofilm formation. FEMS Microbiol. Ecol. 56:195-206.
Halverson LJ, Handelsman J (1991). Enhancement Halverson of soybean nodulation by *Bacillus cereus* UW85 in the field and in a growth chamber. Appl. Environ. Microbiol. 57:2767-2770.
Hameed S, Mubeen F, Malik KA, Hafeez FY (2005). Nodule co-occupancy of *Agrobacterium* and *Bradyrhizobium* with potential benefit to legume host. Biological Nitrogen Fixation, Sustainable Agriculture and the Environment, Springer, Netherlands, pp. 295-296.
Hameed S, Yasmin S, Malik KA, Zafar Y, Hafeez FY (2004). *Rhizobium, Bradyrhizobium* and *Agrobacterium* strains isolated from cultivated legumes. Biol. Fertil. Soils, 39:179-185.
Kneen BE, Larue TA (1983). Congo red absorption by *Rhizobium-Leguminosarum*. Appl. Environ. Microbiol. 45:340-342.
Kumar R, Chandra R (2008). Influence of PGPR and PSB on *Rhizobium leguminosarum* Bv. *viciae* strain competition and symbiotic performance in lentil. World J. Agric. Sci. 4:297-301.
Lagendijk EL, Validov S, Lamers GEM, de Weert S, Bloemberg GV (2010). Genetic tools for tagging Gram-negative bacteria with mCherry for visualization *in vitro* and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol. Lett. 305:81-90.
Lazdunski AM, Ventre I, Sturgis JN (2004). Regulatory circuits and communication in gram-negative bacteria. Nat. Rev. Microbiol. 2:581-592.
Ma W, Guinel FC, Glick BR (2003). *Rhizobium leguminosarum* Biovar viciae 1-Aminocyclopropane-1-Carboxylate Deaminase promotes nodulation of pea plants. Appl. Environ. Microbiol. 69:4396-4402.
Mahmood-ul-Hassan M, Rashid A, Akhtar MS (1993). Phosphorus requirement of corn and sunflower grown on calcareous soils of Pakistan. Commun. Soil Sci. Plan. 24:1529-1541.
Maniatis T, Fritsch EF, Sambrook J (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, U.S.A, p. 545.
Marsudi NDS, Glenn AR, Dilworth MJ (1999). Identification and characterization of fast and slow growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. Soil Biol. Biochem. 31:1229-1238.
Melchiorre M, de Luca MJ, Anta GG, Suarez P, Lopez C, Lascano R, Racca RW (2010). Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. Biol. Fertil. Soils 47:81-89.
Mhamdi R, Laguerre G, Aouani ME, Mars M, Amarger N (2002). Divergent species and symbiotic genotypes of Weld rhizobia can nodulate *Phaseolus vulgaris* in Tunisian soils. FEMS Microbiol. Ecol. 41:77-84.
Mhamdi R, Mrabet M, Laguerre G, Tiwari R, Aouani ME (2005). Colonization of *Phaseolus vulgaris* nodules by *Agrobacterium* like strains. Can. J. Microbiol. 51: 105-111.
Mishra PK, Mishra S, Selvakumar G, Bisht JK, Kundu S, Gupta HS (2009). Co-inoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). World J. Microbiol. Biotechnol. 25:753-761.
Mrabet M, Mnasri B, Romdhane SB, Laguerre G, Aouani ME, Mhamdi R (2006). *Agrobacterium* strains isolated from root nodules of common bean specifically reduce nodulation by *Rhizobium gallicum*. FEMS Microbiol. Ecol. 56:304-309.
Nair A, Juwarkar A, Singh S (2007). Production and characterization of siderophores and its application in arsenic removal from contaminated soil. Water Air Soil Pollut. 180:199-212.
Pandey P, Maheshwari DK (2007). Two-species microbial consortium for growth promotion of *Cajanus cajan*. Curr. Sci. 92:1137-1142.
Pikovskaya R (1948). Mobilization of P in soil in connection with vital activity by some microbial species. Microbiologica 17:362-370.
Rajendran G, Sing F, Desai AJ, Archana G (2008). Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* sp. Bioresour. Technol. 99:4544-4550.
Remans R, Ramaekers L, Schelkens S, Hernandez S, Garcia A, Reyes JL, Mendez N, Toscano V, Mulling M, Galvez L, Vanderleyden J (2008). Effect of *Rhizobium–Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. Plant Soil 312:25-37.
Selvakumar G, Kundu S, Gupta AD, Shouche YS, Gupta HS (2008). Isolation and characterization of nonrhizobial plant growth promoting bacteria from nodules of Kudzu (*Pueraria thunbergiana*) and their effect on wheat seedling growth. Curr. Microbiol. 56:134-139.
Srinivasan M, Peterse DJ, Holl FB (1996). Influence of IAA-producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. Can. J. Microbiol. 42:1006-1014.
Steel RGD, Torrie JH (1980). Principles and Procedures of Statistics. McGraw Hill, New York.
Tan Z, Xu X, Wang E, Gao J, Martinez-Romero E, Chen W (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. Int. J. Syst. Bacteriol. 47:874-879.
Tariq M, Yasmin S, Hafeez FY (2010). Biological Control of Potato Black Scurf by Rhizosphere Associated Bacteria. Braz. J. Microbiol. 41:439-451.
Tilak K, Ranganayaki N, Manoharachari C (2006). Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*). Eur. J. Soil Sci. 57:67-71.
van Noorden GE, Wasson AP, Pellerone FI, Prayitno J, Rolfe BG, Mathesius U (2008). Regulation of nodule organogenesis by auxin transport and flavonoids. Biological Nitrogen Fixation: Towards Poverty Alleviation through Sustainable Agriculture, Springer. 42:183-184.
Vincent JM (1970). A manual for the practical study of root nodule bacteria. Blackwell Sci. Publ., Oxford, UK.
Yaseem T, Hameed S, Tariq M, Iqbal J (2012a). *Vigna radiata* Root Nodule Associated Mycorrhizae and its Helping Bacteria for Improving Crop Productivity. Pak. J. Bot. 44:87-94.
Yasmeen T, Hameed S, Tariq M, Ali S (2012). Significance of arbuscular mycorrhizal and bacterial symbionts in a tripartite association with *Vigna radiata*. Acta Physiol. Plant DOI: 10.1007/s11738-012-0950-x.
Yu J, Fan L, Yang S, Tang M, Yang W, Li H, Wei G (2009b). Characterization of copper-resistant *Agrobacterium* isolated from legume nodule in mining tailings. Bull. Environ. Contamin. Toxicol. 82:354-357.
Zakharova E, Shcherbakov A, Brudnik N, Ignatov V (1999). Biosynthesis of indole-3-acetic acid in *Azospirillum brasilense*. Insights from quantum chemistry. Eur. J. Biochem. 259:572-579.
Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, De Lajudie P (2006). Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*-like gene within the genera *Microbacterium* and *Starkeya*. Microb. Ecol. 51:375-393.
Zhang YF, Wang ET, Tian CF, Wang FQ, Han LL, Chen WF, Chen WX (2008). *Bradyrhizobium elkanii, Bradyrhizobium yuanmingense* and *Bradyrhizobium japonicum* are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. FEMS Microbiol. Lett. 285:146-154.