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Novel bacterium *Rhizobium undicola* isolated from the upland cultivated rice *Oryza sativa* L. promoting plant growth

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Abstract
A novel endophytic nitrogen-fixing bacterium identified as *Rhizobium undicola* (Ouran110) on the basis of 16S rRNA gene sequence analysis was isolated from surface-sterilized roots of upland rice *Oryza sativa* L. var. Narendra Dhan 118. The diazotrophic nature of the bacterium was established by its close homology with typical diazotrophic bacteria and further confirmed by the amplification of *nif*H gene. Amplification of *nodC* confirmed that the strain could be a legume symbiont and this was further ascertained by the nodulation of the strain with *Phaseolus vulgaris* and *Glycine max.* Endophytic colonization ability was ascertained by molecular tagging with g*fp/Gusa* fused to a constitutive promoter followed by inoculation onto rice seedlings in axenic condition. Microscopic observation confirmed colonization of *gfp/gus*-tagged Ouran110 in the intercellular spaces of vascular zones of rice roots. Inoculation of *R. undicola* (Ouran110) with rice plants resulted in significant increase in plant growth promoting traits suggesting implications of this endophytic bacterium as bio-fertilizer in sustainable rice production agriculture.

Keywords: *R. Undicola*, Endophyte, rice, colonization, plant growth promotion

Introduction
Several species of *Rhizobium*, traditionally known as legume endosymbionts have generally been isolated from nodules of leguminous plants (De Lajudie *et al.* 1998; Van Berkum *et al.* 1998; Squartini *et al.* 2002) [6, 36, 31] with the exception of *Rhizobium selenitireducens* from bioreactors (Hunter *et al*. 2007) [12] and *R. cellulosilyticum* from sawdust of *Populus alba* (Garcia-Fraile *et al.* 2007) [8]. Large population of *Rhizobia* are found both in the bulk soil and rhizosphere soils of legumes and other plants (Sullivan *et al*. 1996) [32] and are reported to live as viable cells in water where they infect and nodulate aquatic legumes like species of *Neptunia* (Zurdo-Pineiro *et al*. 2004) [42], *Aeschynomene* and *Sesbania* (Chaintreuil *et al*. 2000) [3]. It has been reported that these legume symbionts also occur as endophytes in the roots of cereals such as rice (*Oryza breviligulata* and *Oryza sativa*) (Yanni *et al*. 1997; Chaintreuil *et al*. 2000; Elbeltagy *et al*. 2001) [40, 3, 7] and wheat and maize (Schloter *et al*. 1997; Rosenblueth and Martinez-Romero, 2004). The findings showing rhizobial colonization with non-legume plants especially cereals stimulated research on growth promotion in rice by rhizospheric and endophytic rhizobia (Yanni *et al*. 1997; James *et al*. 2000; Tan *et al.* 2001) [40, 14, 35] and led to the isolation and characterization of endophytic plant growth-promoting rhizobia capable of enhancing rice yield under greenhouse and field conditions. Upland cultivated rice is the most important food crops in developing countries and the dependency of this crop on chemical fertilizers for nitrogen is well known. This is why microbe-mediated biological options for nitrogen fixation in rice need to be searched out at a greater pace.
This study presents the isolation and characterization of an endophytic nitrogen-fixing bacteria *R. undicola* from a popular upland rice variety in Northern Indo-gangetic plains of India, Narendra Dhan 118. This *Rhizobium* isolate showed close homology with typical diazotrophic bacteria and nodulated *Phaseolus vulgaris* and *Glycine max*. Potentiality of nitrogen fixation of this isolate, its correlation with the rice plants for growth promotion and possible implications is discussed at length.

Materials and Methods
Samples of rice plants were collected at the heading stage of the crop from 50 locations in five districts (Varanasi, Balia, Chandouli, Faizabad and Sonabhadra) of Uttar Pradesh, India. Common bean was never rotated with rice in all 50 locations selected in this study but, legumes like *Cicer arietinum* and *Trifolium alexandrinum* rotated along with rice in all locations.

Standard protocols following all the precautions for sterilization were used for isolation and identification of putative rhizobial endophytes from rice roots (Singh *et al.* 2011) [30]. Plants of *Phaseolus vulgaris* and *Glycine max* were inoculated with the test strain along with the reference strains of *R. undicola* ORS992, *R. trifolii* strain ANU 843 a*nd R. leguminosarum by phaseoli* USDA 2695 to confirm symbiotic characteristics of this isolate (Chen *et al.* 1991) [4]. Uninoculated plants were used as control.

Nitrogenase activity was measured by acetylene reduction assay (Stewart *et al*. 1967) [32]. Inoculum (10^6 cfu) from each isolate was added in 3.0 ml semisolid (0.15% w/v) $JNFb^-$ medium (Singh 2008) [19] in a 7.0 ml vacutainer tube (Becton-Dickinson, Rutherford, NJ, USA) and grown for 96 h at 30 ºC. Acetylene was injected into each tube by a hypodermic syringe to attain 10% final concentration and incubated at 30 ºC without shaking. At different time intervals, 0.2 ml gas was removed and the ethylene formed was analysed in a 5700 Nucon Gas Chromatograph (Nucon Engineers Ltd. New Delhi) equipped with Porapak R column and flame ionization detector. Each assay was replicated thrice and nitrogenase activity (acetylene reduction) was expressed in terms of µM C_2H_4 formed per h per mg of protein.

The production of indole acetic acid (IAA) was tested as reported by Gordon and Weber (1951) [11]. Screening of phosphate solubilization was made as per the method of Goldstein (1986) [10] and quantitative estimation of solubilized P was done according to Marinetti (1962) [18].

IAA was extracted from the supernatant of the cultures as reported by Manulis *et al*. (1994) [17]. High-performance liquid chromatography (HPLC) of the extract was performed in a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with two LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne (Model 7725) injector with a loop size of 20 µl. IAA present in the samples was identified by comparing retention time (Rt) with an individual reference standard and further by injection. Each analysis was replicated three times.

Chlorophyll content (SPAD reading) of the upper two fully expanded leaves was measured by SPAD-502 chlorophyll meter (Minolta, K. Arano and Co. Ltd. Tokyo, Japan). Plants were separated from medium (agar/sand) and cut from stem base. Both shoots and roots were washed thrice with double distilled water and oven dried at 60 °C for 10 days to record dry weight. N and P content were estimated according to Singh (2008) [19].

PCR amplifications were performed using the GoTaq Kit (Promega) with the extraction of genomic DNA as per the protocol of Moulin *et al.* (2004) [20]. Nearly full length 16S rRNA gene was amplified and sequenced with primers AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCC (Weisburg *et al.* 1991) [39]. 930-bp *nodC* and 783-bp *nifH* gene fragments were amplified with primers AYGTHGTYGAYGACGGTTC and CGYGACAGC CANTCKCTATTG and TACGGNAARGGSGGNATCGGC AA and AGCATGTCYTCSAGYTCNTCCA, respectively (Laguerre *et al*. 2001) [16]. PCR amplification was done by 30 cycles of 30 s at 95 °C, annealing for 30 s at 58 °C and extension at 72 °C for 45 s, with a final 5 min extension at 72 °C. The phylogenetic trees based on 16S rRNA (HQ895844), *nifH* (JF738072) and *nodC* (JF738073) partial gene sequences were constructed by neighbor-joining method using MEGA 4.0 (Saitou And Nei 1987; Tamura *et al*. 2007) [25, 34]. All bootstrap values (1,000 replications) indicating more than 80% support were placed at the nodes in the phylogenetic tree.

To study colonization with rice roots, isolate Ouran110 was tagged with constitutively expressed *gfp*/*gusA* genes according to Singh *et al*. (2009) [29]. Plasmid pHRGFPGUS containing *gfp* and *gus*A genes expressed under the control of a gentamycin promoter was introduced by biparental mating using *E. coli* S17-1. Plasmid pHRGFPGUS (kindly provided by HJO Ramos UFPR, Curbita, Brazil) is a derivative of plasmid pBBR1 and is a small (2.6 kb), broad-host range plasmid stably maintained in a number of Gram-positive and Gram-negative bacteria (Ramos *et al*. 2002) [23]. After transfer of the plasmid, derivatives showing green fluorescence on a UV transilluminator and forming blue colonies on TY medium containing X-GlucA substrate were selected for colonization.

Rice variety (Narendra Dhan 118) was surface-sterilized using 2.5% sodium hypochlorite for 3 min and washed 4-5 times with sterilized distilled water. Seeds were germinated for 24 h on 1% agar. Ouran 110 tagged with *gfp*/*gus*A was grown in TY broth with kanamycin for 48 h. One ml (about 10^6 cells) was centrifuged at 8000 g for 1 min and washed with sterile distilled water three times. The pellet was resuspended in 1 ml sterile water. Ten germinated seeds were soaked in 1 ml culture and transferred to 100 ml tubes (25×150 mm) containing 25 ml YEMA salts with 1% agar, but without mannitol and yeast extract VINCENT (1970). After 10 days of growth in an environmental chamber (22 ^{0}C, 10 h daylight), plants were removed from the tubes and sections were cut. Small root segments (0.5 cm) were fixed in 5% agarose and cut in ultra-thin sections (50 to 80 µm) on a vibratome Leica VT 1000S (Leica Microsystems Wetzlar GmbH, Germany). Sections were observed and documented on a confocal laser microscope.

For the assessment of plant growth promoting potential of rhizobial isolates, a plant infection test was performed under laboratory, greenhouse and field conditions according to Singh *et al.* (2011) [30] and Peng *et al.* (2002) [21]. Rice seeds were surface sterilized and sown in pots filled with sterilized field soil (clay loam). Exponentially growing cells of Ouran110, USDA 2695 (Dr. Peter Van Berkum, USDA, USA), ORS 992 (Prof. Bernard dreyfus, LSTM, INRA, France) and ANU 843 (Prof. Barry G. Rolfe, ANU, Australia) were used to inoculate the rice seedlings grown in pots, and the effects of inoculation were assessed by measuring various plant growth-promoting parameters at maturity. Field experiment was carried out with rice variety Narendra Dhan 118. Before sowing 5 ml of bacterial culture was suspended with 10 ml of binder solution, taken into deep tray, mixed thoroughly and mixed properly with 500g of rice seeds placed in same tray for surface coating of the bacterium on seeds. The treated seeds were sown in nursery and separate trays were used for each treatment. After attaining height up to 12 cm after two weeks of sowing, the seedlings were uprooted for transplanting (Singh 2004) [27]. Experiments were performed in CRD (field condition) and RBD (laboratory and green house conditions) with six replications. Uninoculated plants served as control.

Data related to plant growth parameters were subjected to analysis of variance with Excel (Microsoft, version 5.0) software package. Treatment means were compared at 95% and 99% probability levels (P=0.05 and 0.01), and the same set of data was further analyzed to calculate least significant difference at P=0.05 and 0.01, respectively.

Results and Discussion

Varieties of rice grown in the fields getting minimal application of synthetic chemical N fertilizer were chosen for the isolation of endophytic bacteria. Out of four varieties, the macerate of rice variety Narendra Dhan 118 showed the presence of endophytic diazotrophic bacteria when tested for growth on $JNFb^{-}$ solid medium. Based on distinct morphotypes of colonies on $JNFb^{-}$ agar medium, four root isolates RREM 3, RREM 39, RREM 62 and Ouran 110 and four culm isolates RREM 36, RREM 28, RREM 23 and RREM 17 were picked up and grown in nitrogen-free medium. The root macerates routinely showed higher number (3×10^5) of diazotrophic bacterial isolates than those of culm (3×10^4 cfu/g fresh wt). However, bacterial population was much higher (2×10^7 cfu/g fresh wt) if the root/culm macerate was plated on NA medium. No growth of any bacteria occurred on plates spread with the last wash and sections of surface sterilized roots or culms suggesting the recovery of the isolates from the interior parts of the plants only.

Diazotrophic nature of all the isolates was determined. All the isolates exhibited nitrogenase activity but the level of activity varied and ranged between 0.46 and 1.94 µM C_2H_4 per h per mg of protein (Table 1). Out of nine isolates, the maximum activity was noted in Ouran110, the rate being 1.94 µM C_2H_4 per h per mg of protein (Table 1).

Initial screening revealed IAA production in six isolates, namely, Ouran110, ORS992, RREM3, RREM39, RREM62 and RREM 36 (Table 1). The maximum IAA production was shown by Ouran110 (20.58 µg mg^{-1} dry wt.) followed by ORS992 (18.57 µg mg^{-1} dry wt) after growth in the medium supplemented with tryptophan. Production of IAA was also confirmed by the analysis of samples by HPLC. Peaks identical to standard IAA were recorded in all the six isolates. In addition to nitrogen-fixing ability and IAA production, six isolates, Ouran 110, ORS 992, RREM 3, RREM 39, RREM 62, and RREM 36 were found positive for P solubilization. Screening for P solubilization activity was based on the appearance of a clearing zone around the bacterial colonies on solid agar medium supplemented with insoluble phosphate. Further analysis showed that the isolate Ouran110 had the maximum level (32.54 µg mg^{-1} dry wt.) of P solubilization followed by ORS992 (29.55 µg mg^{-1} dry wt.) under normal growth conditions (Table 1). The chlorophyll content, roots and shoot dry weight also recorded maximum in plants inoculated with Ouran110 as compare to reference *Rhizobium* strains used in this study (Tables 2, 3, & 4).

Test of growth rate and plant growth promoting traits *viz.*, nitrogen fixation, IAA production and P solubilization of the trans conjugants revealed no change and were almost identical to that of parent isolate Ouran110. When *gfp/gusA*-tagged isolate was inoculated on germinated seedlings of rice variety, Narendra Dhan 118 active colonization occurred and was confirmed by reisolation of tagged strain from the inoculated rice plants. As expected, un-inoculated control plants grown under identical conditions did not show the presence of any bacteria in the roots or culms. Moreover, no disease symptoms appeared in control or inoculated plants throughout the study. At 40X resolution, *gfp/gusA*-tagged cells were apparently localized in intercellular spaces of cortical as well as vascular zones, indicating the entry of *gfp/gusA*-tagged isolate Ouran110 (Fig. 2). Rice roots of uninoculated control plants did not show any fluorescence 14 days after inoculation (DAI).

Among all the isolates tested, isolate Ouran110 appeared to be the best performing isolate in terms of nitrogenase activity, P solubilization, and IAA production. In order to identify and characterize this isolate, 16S rDNA (~1.5 kb) of Ouran110 was amplified by PCR, cloned and sequenced. On the basis of 16S rDNA sequence, the isolate was identified as *R. undicola*. Furthermore, based on percent divergence (Fig. 1) *R. undicola* shared significant similarity (<99.1%) with other strains of *Rhizobium* and related genera. Henceforth, this isolate is designated as *R. undicola* Ouran110. Diazotrophy of *R. undicola* Ouran110 was investigated by *nodC* and *nifH* sequence analysis. About 930 bp and 780 bp of *nodC* and *nifH* segment, respectively were obtained by PCR amplification using *nodC* and *nifH*, specific primers. Analysis of *nodC* and *nifH* sequence showed homology with typical diazotrophic Gram-negative bacteria (Fig 1).

Results indicated that *R. undicola* Ouran110, after inoculation resulted in significant plant growth promotion after 45/85 DAI as assessed on different growth parameters *viz.* plant height, dry shoot and root weight, chlorophyll and nitrogen content etc. (Tables 2, 3 and 4). Maximum number of bacterial count most probable number (MPN) was recorded in Ouran110 and Narendra Dhan 118 interaction (Table 5). Colonization of rice variety with Ouran110 also enhanced nitrogenase activity in gnotobiotic, greenhouse and field conditions with values of 21.20, 23.18 and 25.04 µM C_2H_4 mg^{-1} dry weight of root, respectively (Table 5).

Rhizobium-rice association followed by the bacterial partnership within the below-ground and above-ground host system, associated plant growth-promoting responses and probable underlying mechanisms, nutritional impact of the association on rice, growth physiology, the strain/variety specificity of the beneficial rhizobia-cereal association and preliminary biofertilizer inoculation trials under small-scale experimental conditions are reported (Yanni And Dazzo 2010) [41]. In the last two decades, it was realized that Rhizobia, in addition to its role in symbiotic nitrogen fixation, also play a role of plant growth promoting rhizobacteria (PGPR) either around the rhizospheric zone or as endophyte of cereals and other plants. Endophytic colonization by the bacteria in cereal crops was reported in wheat, maize, barley, rice etc. Under such association, these organisms prove themselves beneficiary in term of plant growth promotion and increase in grain yield (Mishra *et al.* 2008) [19].

We report the presence of endophytic *R. undicola* in the roots and culms of healthy rice variety Narendra Dhan 118 which mostly grows in the rainfed areas and requires low input of chemical N-fertilizer. These conditions probably favor association with endophytic diazotrophic bacteria such as *R. undicola*. Apart from diazotrophy, other beneficial activities such as P solubilization and IAA production make this isolate more efficient for enhancing plant growth.

All the isolates from the roots or culms are indeed endophytic and present in the interior tissues of rice plants. Occurrence of higher population of endophytes in roots than in aerial parts has been reported (Chi *et al.* 2005) [5] and our results are in accordance with the existing reports. Earlier, we have shown that the roots are a preferred niche for growth and nitrogen fixation by endophytic rhizobia (Singh *et al.* 2009) [29] and this report again strengthens this assumption as we are reporting eight endophytic diazotrophic isolates from roots and culms from rice variety, Narendra Dhan 118. There were significant differences in nitrogenase activity and IAA production and P solubilization activity among the eight isolates and isolate Ouran 110 exhibited highest record for all the growth-promoting characters. This isolate was identified as *R. undicola* by 16S rRNA gene sequence analysis and it is

placed in the class Alpha-proteobacteria with other legume-associated nitrogen fixing species of the genera *Rhizobium, Mesorhizobium, Ensifer* (formally known as *Sinorhizobium*), *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium* and *Methylobacterium*, (Van Berkum *et al*. 2003) [37]. Phylogenetic analysis suggested significant similarity of this isolate with the strains of rhizobia which are known to perform different role as plant growth-promoting rhizobacteria (Singh 2008) [19]. ARA, which is an indirect method to test the diazotrophic nature indicated diazotrophy in Ouran110 and this was confirmed by amplification of *nifH* gene using different PCR primers (Laguerre *et al*. 2001) [16]. In this study, fidelity of the amplified segment of *nodC* and *nifH* were assured from the sequence information, which displayed similarity with *nodC* and *nifH* sequence available in database of Gen Bank (Barret *et al*. 2011) [1]. In N-limited environment, N-fixing microbes can play a crucial role in the ecosystems by counterbalancing the CO_2-induced limitation of N availability in the rhizosphere. In this context, flavonoids were assumed to have a key role. Indeed, increased root exudation of chemo-attractants such as phenolic acids and specific flavonoids with the capacity to activate *nod* genes (*nod* -gene inducing flavonoids) involved in establishing the *Rhizobium* symbiosis, have been found in plants growing under elevated CO_2 concentrations and nutritional limitations (Cesco *et al*. 2012) [2].

Based on the above physiological and molecular evidences, it may be concluded that the isolate Ouran110 is indeed a diazotrophic and active N-fixing bacterium. One prominent feature of *gfp/gusA*-marked *R. undicola* Ouran 110 noticed in this study is the active colonization of rice plants that was confirmed by reisolation of this strain from surface-sterilized roots and culms of the inoculated seedlings. Intense *gfp* activity was noted on intercellular spaces of vascular zones, which suggested that this region may be the possible site for colonization. This finding agrees with other reports on diazotrophic endophytic *Rhizobium* sp. in rice (Singh *et al*. 2009) [29]. As such, the apoplastic localization in intercellular spaces is considered to be the preferred site for a few endophytic diazotrophs (James And Olivares 1998) [13]. Some strains of rhizobia have the ability to infect rice root tissues via root hairs located at the emerging lateral roots and spread extensively throughout the rice root. Thus, the presence of non-inhibitory rhizobia in rice roots provides an opportunity to build an inoculum for the cereals delivering a biological N-fixation system and other genetically modified products (Perrine-Walker *et al*. 2007) [22]. On the other hand *in situ gus* staining has a major drawback, the presence of blue color does not unequivocally confirm the location or even the presence of the *gus*-marked bacteria because the color can diffuse into bacterium-free plant material (Jefferson *et al*. 1987) [15]. That isolate Ouran110 may be used, as a potent plant growth bio-agent because its inoculation on the Narendra Dhan 118 increased length of root and stem and enhanced formation of lateral and adventitious roots. It is well known that IAA secreted by bacterium may promote root growth due to stimulatory effect on plant cell elongation or cell division (Glick 2005) [9]. This may increase plant dry weight, chlorophyll and N content. Significant increase in the level of nitrogenase activity in rice plants 45 DAI may be due to the supply of fixed N by the colonized bacteria.

In conclusion, our results showed that the roots and culms of the rice variety Narendra Dhan 118 harbor a variety of endophytic diazotrophic bacteria including *R. undicola* Ouran 110. This strain showed high level of nitrogenase activity, solubilized P, and produced IAA. The findings of this research will give a detailed insight about novel plant growth promoting association between rhizobia and rice, the most important food crop of the world. It may be helpful in the development of eco-friendly and cost effective technology and will contribute towards sustainable agriculture.

Table 1: Nitrogenase activity, IAA production, Phosphate solubilization of various rhizobial species isolated from rice roots

Bacterial isolates	Nitrogenase activity (µM C_2H_4 mg^{-1} protein h^{-1})*	IAA production (µg mg^{-1} dry wt) *	Phosphate Solubilization (µg mg^{-1} dry wt) *	Bacterial isolates isolated from root/clum**
Ouran110	1.94±0.51	20.58±3.3	32.54±3.7	Root
ORS992	1.76±0.43	18.57±3.1	29.55±3.4	Legume nodule
RREM3	1.52±0.47	16.69±2.5	20.55±3.6	Root
RREM39	1.35±0.28	15.13±1.9	20.27±3.4	Root
RREM62	1.31±0.27	14.52±2.7	11.28±1.9	Root
RREM 36	1.29±0.23	13.21±2.2	ND	Culm
RREM 28	0.46±0.16	ND	ND	Clum
RREM 23	ND	ND	ND	Culm
RREM 17	1.23±0.28	ND	ND	Culm

*Means ±SD of three experiments conducted separately under identical conditions.
ND-not detected
** Bacterial isolates isolated from root/culm.

Table 2: Effect of rhizobial inoculation on growth and N content in rice (var. Narendra Dhan 118) under field condition after 85 days of inoculation

Treatment	Plant height (cm) [a]	No. of panicles ($hill^{-1}$) [a]	Panicle Length (cm) [a]	Chlorophyll Content (SPAD reading) [a]	Root dry weight (g $hill^{-1}$)[a]	Shoot dry weight (g $hill^{-1}$)[a]	1000 grain weight (g) [a]	Grain yield (g $hill^{-1}$) [a]	Plant N content (mg $hill^{-1}$) [a]	Plant P content (mg $hill^{-1}$) [a]
Narendra Dhan 118 + Sterile H_2O (T_1)	90.90	6.20	19.18	23.17	2.18	23.02	18.58	14.17	696.52	76.23
Narendra Dhan 118 + Ouran110 (T_2)	98.20*	10.17 *	22.27*	28.08 *	3.23 *	29.37 *	22.33*	17.38 *	2011.73 *	207.93 *
Narendra Dhan 118 + ANU843 (T_3)	94.42*	8.30*	21.20 *	25.10 *	2.38 *	27.40 *	20.32 *	15.45 *	1102.95*	152.68*
Narendra Dhan 118 + USDA2695 (T_4)	94.22*	7.42 *	20.17*	25.27 *	2.35*	28.40*	20.00 *	15.50 *	1134.40*	176.02*
Narendra Dhan 118 + ORS992 (T_5)	93.22*	8.22 *	20.20 *	26.12 *	2.88 *	26.52*	20.80 *	16.35*	1640.88 *	174.10 *
LSD 5%	0.506	0.405	0.432	0.475	0.358	0.460	0.439	0.488	122.60	9.71

[a]Values are means of six replicates.
*Significantly different from the control at 5%

Table 3: Effect of rhizobial inoculation on growth and N content in rice (var. Narendra Dhan 118) under greenhouse condition after 45 days of inoculation

Treatment	Plant height (cm) [a]	Root dry weight (g hill^{-1}) [a]	Shoot dry weight (g hill^{-1}) [a]	Chlorophyll Content (SPAD reading) [a]	Plant N content (mg plant^{-1}) [a]
Narendra Dhan 118 + Sterile H_2O (T_1)	36.38	31.17	29.35	28.57	18.47
Narendra Dhan 118 +Ouran110 (T_2)	42.38*	39.40 *	37.57 *	32.15 *	23.63 *
Narendra Dhan 118 +ANU843 (T_3)	38.33 *	37.88 *	36.48 *	31.15 *	22.13*
Narendra Dhan 118+USDA2695 (T_4)	41.07*	36.58*	35.90*	30.07*	20.23*
Narendra Dhan 118 +ORS992 (T_5)	40.13*	37.00 *	35.07 *	29.97 *	20.58 *
LSD 5%	0.495	0.453	0.484	0.300	0.452

[a] Values are means of six replicates.
*Significantly different from the control at 5%

Table 4: Effect of rhizobial inoculation on growth and N content in rice (var. Narendra Dhan 118) under gnotobiotic condition after 45 days of inoculation

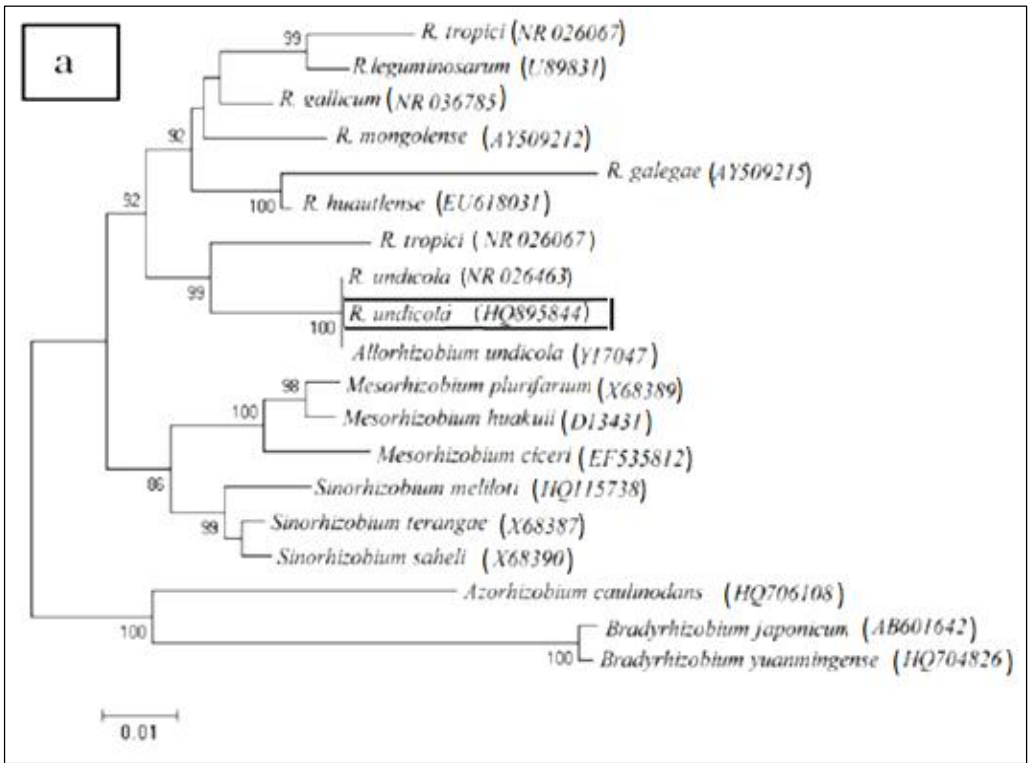
Treatment	Plant height (cm) [a]	Root dry weight (g hill^{-1}) [a]	Shoot dry weight (g hill^{-1})[a]	Chlorophyll content (SPAD reading)[a]	Plant N content (mg plant^{-1})[a]
Narendra Dhan 118 + Sterile H_2O (T_1)	16.52	10.10	5.42	21.25	0.43
Narendra Dhan 118 + Ouran110 (T_2)	19.52 *	14.37*	9.38 *	24.67 *	0.79 *
Narendra Dhan 118 + ANU843 (T_3)	18.52*	13.12*	8.12 *	23.80 *	0.67*
Narendra Dhan 118 + USDA2695 (T_4)	17.73*	12.38*	7.92*	23.08*	0.69 *
Narendra Dhan 118 + ORS992 (T_5)	18.17 *	11.78*	7.05*	22.13*	0.63*
CD at 5%	0.497	0.319	0.455	0.463	0.089

[a]Values are means of six replicates.
*Significantly different from the control at 5%

Table 5: In planta nitrogenase activity and bacterial count most probable number (MPN) in rice variety Narendra Dhan 118 after 45 days of inoculation

Bacterial Strains	Gnotobiotic condition		Greenhouse condition		Field condition	
	Nitrogenase activity (µM C_2H_4 mg^{-1} dry weight of root)[a]	Bacterial count in rice root (log10 cfu g^{-1} root dry weight)[a]	Nitrogenase activity (µM C_2H_4 mg^{-1} dry weight of root)[a]	Bacterial count in rice root (log10 cfu g^{-1} root dry weight)[a]	Nitrogenase activity (µM C_2H_4 mg^{-1} dry weight of root)[a]	Bacterial count in rice root (log10 cfu g^{-1} root dry weight)[a]
Control (Uninoculated)	ND	0	ND	0	ND	0
Ouran110	21.20 *	8.26 *	23.18*	9.31*	25.04 *	10.29 *
ANU843	20.17 *	7.42 *	22.14*	8.18 *	24.15*	9.20 *
USDA2695	19.31*	7.02 *	21.19 *	7.20 *	23.05 *	7.87 *
ORS992	18.22 *	6.31 *	20.12*	7. 20*	22.16*	7. 77 *
LSD 5%	0.244	0.221	0.128	0.252	0.315	0.268

[a]Values are mean of six replications.
* Significant at 5%



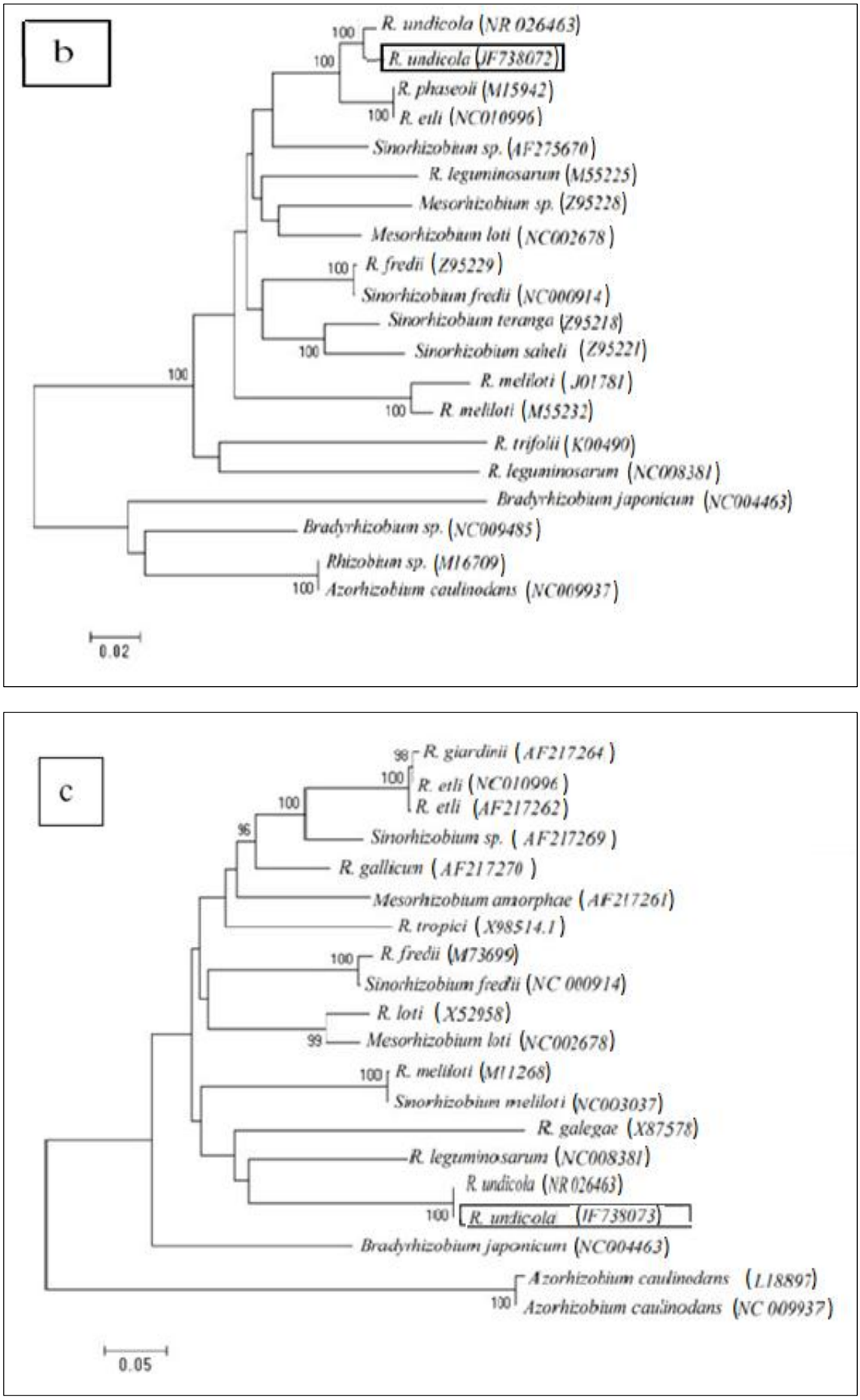


Fig 1: The phylogeny of *Rhizobium undicola* (Ouran110) with different *Rhizobium* species. (a) Phylogenetic tree based on 16S rRNA gene sequences. (b) Phylogenetic tree based on partial *NifH* DNA sequences. (c) Phylogenetic tree based on partial *nodC* DNA sequences. The strain *Rhizobium undicola* (Ouran110) used in this study was indicated in Box. Phylogenetic trees were generated by neighbor-joining method using MEGA 4.0. The percentage of bootstrap values (1,000 resampling) that supported more than 80% were indicated at the branches

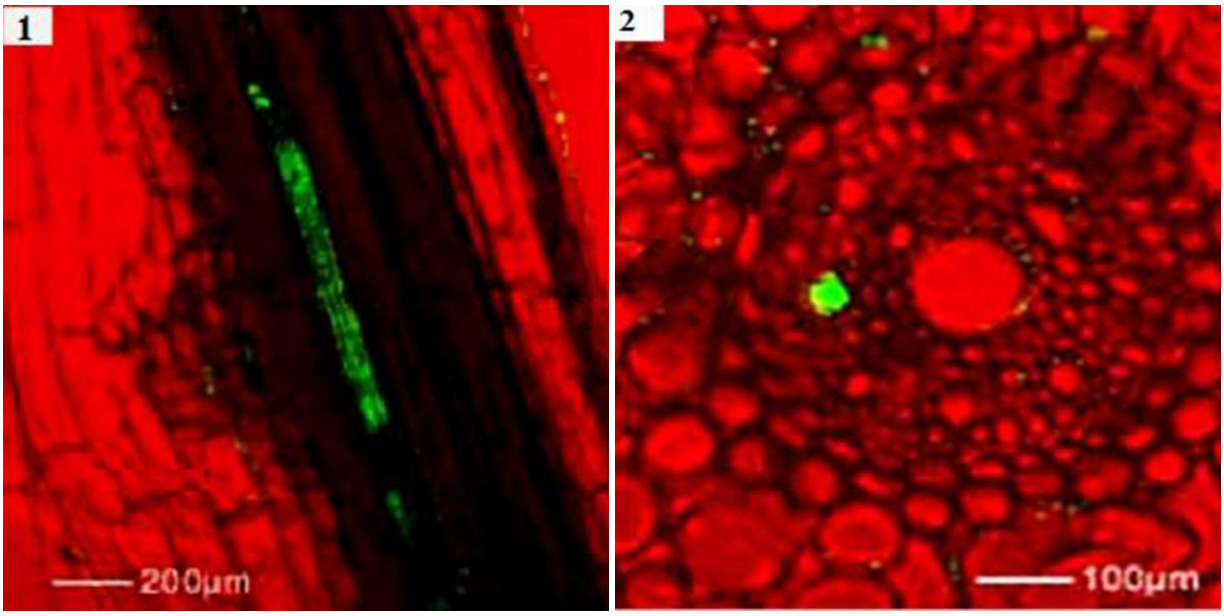


Fig 2: Confocal laser scanning micrograph of *gfp/gusA* tagged *Rhizobium undicola* (Ouran110) cells. Rice root inoculated with Ouran110 and grown for 14 days in the growth chamber showed fluorescence (green colour) after colonizing vascular tissues (1). The infection is spreading to the intercellular spaces and vascular tissues (2)

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References

1. Barret M, Morrissey JP, O'gara F. Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biol. Fertil. Soils. 2011; 47:729-743.
2. Cesco S, Mimmo T, Tonon G, Tomasi N, Pinton R, Terzano R *et al*. Plant-borne flavonoids released into the rhizosphere: impact on soil bio-activities related to plant nutrition. Biol. Fertil. Soils. 2012; 48:123-149.
3. Chaintreuil C, Giraud E, Prin Y, Lorquin J, ba A, Gillis M, *et al*. Photosynthetic *Bradyrhizobia* are natural endophytes of the African wild rice *Oryza breviligulata*. Appl. Environ. Microbiol. 2000; 66:5437-5447.
4. Chen WX, Li GS, Qi YL, Wang ET, Yuan HL, Li JL. *Rhizobium huakuii* sp. nov, isolated from the root nodules of *Astragalus sinicus*. Int. J Syst. Bacteriol. 1991; 41:275-280.
5. Chi F, Shen SH, Cheng HP, Jing YX, Yanni YG, Dazzo FB. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl. Environ. Microbiol. 2005; 71:7271-7278.
6. De Lajudie P, Laurent-Fulele E, Willems A, Torck U, Coopman R, Collins MD *et al*. *Allorhizobium undicola* gen. nov, sp. nov, nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J Syst. Bacteriol. 1998; 48:1277-129.
7. Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T *et al*. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 2001; 67:5285-5293.
8. Garcia-Fraile P, Rivas R, Willems A, Peix A, Martens M, Martinez-Molina E, *et al*. *Rhizobium cellulosilyticum* sp. nov, isolated from sawdust of *Populus alba*. Int. J Syst. Evol. Microbiol. 2007; 57:844-848.
9. Glick BR. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS* Microbiol. Lett. 2005; 251:1-7.
10. Goldstein AH. Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am. J Alter. Agric. 1986; 1:51-57.
11. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. Plant Physiol. 1951; 26:192-195.
12. Hunter WJ, Kuykendall LD, Manter DK. *Rhizobium selenireducens* sp. nov.: a selenite-reducing alfa-*Proteobacteria* isolated from a bioreactor. Curr. Microbiol. 2007; 55:455-460.
13. James EK, Olivares FL. Infection and colonization of sugarcane and other gramineous plants by endophytic diazotrophs. Crit. Rev. Plant. Sci. 1998; 17:77-119.
14. James EK, Gyaneshwar P, Barraquio WL, Mathan N, Ladha JK. Endophytic diazotrophs associated with rice. In: Ladha JK, Reddy PM, editors; Ladha JK, Reddy PM, editors. The quest for nitrogen fixation in rice. Manila, Philippines: IRRI. 2000; 119-140.
15. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: b- glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J, 1987; 6:3901-3907.
16. Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P, Amarger N. Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiol. 2001; 147:981-993.
17. Manulis S, Shafrir H, Epstein E, Lichter A, Barash I. Biosynthesis of indole-3-acetic acid via the indole-3-acetamide pathway in *Streptomyces* spp. *Microbiol*. SGM. 1994; 140:1045-1050.
18. Marinetti GV. Chromatographic separation, identification and analysis of phosphatides. J Lipid. Res. 1962; 3:1-20.
19. Mishra RPN, Singh RK, Jaiswal HK, Singh MK, Yanni YG, Dazzo FB. Rice-Rhizobia Association: Evolution of an Alternate Niche in Beneficial Plant-Bacterial Association. In: Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth 1st ed. (Ahmad,

Pichtel and Hayat) WILEY-VCH Verlag Publishers, Germany, 2008, 165-190.
20. Moulin L, Bena G. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol. Phylo. Evol. 2004; 30:720-732.
21. Peng S, Biswas JC, Ladha JK, Gyaneshwar P, Chen Y. Influences of *rhizobial* inoculation on photosynthesis and grain yield of rice. Agron. J, 2002; 9:4925-929.
22. Perrine-Walker FM, Prayitno J, Rolfe BG, Weinman JJ, Hocart CH. Infection process and the interaction of rice roots with rhizobia. J Experi. Bot. 2007; 58:3343-3350.
23. Ramos HJ, Roncato-Maccari LD, Souza EM. Monitoring *Azospirillum*-wheat interactions using the *gfp* and *gusA* gene constitutively expressed from a new broad-host range vector. J Biotechnol. 2002; 97:243-252.
24. Rosenblueth M, Martinez-Romero E. *Rhizobium etli* maize populations and their competitiveness for root colonization. Arch. Microbiol. 2004; 181:337-344.
25. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 1987; 4:406-425.
26. Schloter M, Wiehe W, Assmus B, Steindl H, Becke H, Hoflich G *et al*. Root colonization of different plants by plantgrowth- promoting *Rhizobium leguminosarum* bv. *Trifolii* R39 studied with monospecific polyclonal antisera. Appl. Environ. Microbiol. 1997; 63:2038-2046.
27. Singh MK. M.Sc. thesis. Banaras Hindu University, Varanasi, India, 2004.
28. Singh MK. Ph.D. thesis. Banaras Hindu University, Varanasi, India, 2008.
29. Singh MK, Kushwaha C, Singh RK. Studies on endophytic colonization ability of two upland rice endophytes, *Rhizobium* sp. and *Burkholderia* sp, using green protein reporter. Curr. Microbiol. 2009; 59:240-243.
30. Singh MK, Singh DP, Mesapogu S, Babu BK, Bontemps C. Concomitant colonization of *nifH* positive endophytic *Burkholderia* sp. in rice (*Oryza sativa* L.) promotes plant growth. World J Microbiol. Biotechnol. 2011; 27:2023-2031.
31. Squartini A, Struffi P, Doring H, Selenska-Pobell S, Tola E, Giacomini A *et al*. *Rhizobium sullae* sp. nov. (Formerly *'Rhizobium hedysari'*), the root-nodule microsymbiont of *Hedysarum coronarium* L. Int. J Syst. Evol. Microbiol. 2002; 52:1267-1276.
32. Stewart WDP, Fitzgterald GP, Burris RH. *In situ* studies on N_2 fixation using acetylene reduction techniques. Proc. Natl. Acad. Sci. USA. 1967; 58:2071-2078.
33. Sullivan JT, Eardly BD, Van Berkum P, Ronson CW. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. 1996; 62:2818-2825.
34. Tamura K, Dudley J, Nei M. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 2007; 24:1596-1599.
35. Tan Z, Hurek T, Vinuesa P, Muller P. Ladha JK, Reinhold-Hurek B. Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S-23S ribosomal DNA intergenic spacer-targeted PCR. Appl. Environ. Microbiol. 2001; 67:3655-3664.
36. Van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD. *Rhizobium mongolense* sp. nov. Is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* L. Int. J Syst. Bacteriol. 1998; 48:13-22.
37. Van Berkum P, Terefework Z, Paulin L, Suomalainen S, Lindstrom K, Eardly BD. Discordant phylogenies within the *rrn* loci of rhizobia. J Bacteriol. 2003; 185:2988-2998.
38. Vincent JM. A manual for practical study of root nodule bacteria. Blackwell Scientific Publication Ltd. Oxford. UK, 1970.
39. Weisburg G, Barns M, Pelletier A, Lane J. 16S Ribosomal DNA Amplification for Phylogenetic Study. J Bacteriol. 1991; 173:697-703.
40. Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S *et al*. Natural endophytic association between *Rhizobium leguminosarum* bv. *Trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil. 1997; 194:99-114.
41. Yanni YG, Dazzo FB. Enhancement of rice production using endophytic strains of *Rhizobium leguminosarum* bv. *Trifolii* in extensive field inoculation trials within the Egypt Nile delta. Plant Soil. 2010; 336:129-142.
42. Zurdo-Pineiro JL, Velazquez E, Lorite MJ, Brelles-Marino G, Schroder EC, Bedmar EJ *et al*. Identification of fast-growing rhizobia nodulating tropical legumes from Puerto Rico as *Rhizobium gallicum* and *Rhizobium tropici.* Syst. Appl. Microbiol. 2004; 27:469-477.