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Impact of rhizobial inoculation and nitrogen utilization in plant growth promotion of maize (*Zea mays* L.)

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Abstract. *Singh RK, Malik N, Singh S. 2013. Impact of rhizobial inoculation and nitrogen utilization in plant growth promotion of maize (*Zea mays *L.). Nusantara Bioscience 5: 8-14.* During the course of growing population demands, there has been an increasing interest in exploring the possibility of extending the beneficial interaction between cereals and plant growth promoting rhizobacteria (PGPR). Endophytes are a group of microorganism that resides mostly in the intercellular space of various parts of plants including cereals. Assessment of plant growth promoting properties of the five-rhizobial strains belonging to α subclass, i.e. *Rhizobium leguminosarum* bv. *phaseoli* RRE6 and *R. undicola* RRE36 and those belonging to β subclass, i.e. *Burkholderia cepacia* (RRE3, RRE5, RRE25) was done by growing maize plants inoculated with these strains. Inoculated maize plants showed a significant increase in plant height, root length, shoot and root dry weight over uninoculated control. *R. leguminosarum* bv. *phaseoli* RRE6 and *B. cepacia* RRE5 among the α and β-subclass representatives respectively, gave the best inoculation response. Effect of nitrate supplementation upon maize-RRE6 and RRE5 association was also studied and a significant increase in all the growth parameters and colonization ability was recorded when nitrate was present as a supplement over uninoculated control and maize-RRE6 and RRE5 in absence of external nitrate.

Key words: *Rhizobium leguminosarum* bv. *phaseoli, R. undicola, B. cepacia, Zea mays*, nitrate utilization

Abstrak. *Singh RK, Malik N, Singh S. 2013. Pengaruh inokulasi rhizobia dan perlakuan nitrogen terhadap pertumbuhan tanaman jagung (*Zea mays *L.). Nusantara Bioscience 5: 8-14.* Dalam penelitian pertumbuhan populasi telah terjadi peningkatan minat dalam mengeksplorasi kemungkinan mendapatkan keuntungan dari interaksi antara sereal dan pertumbuhan tanaman yang dibantu rhizobakteri (PGPR). Endofit adalah kelompok mikroorganisme yang umumnya berada di ruang antar sel berbagai bagian tanaman termasuk sereal. Penilaian sifat-sifat pertumbuhan tanaman yang dipromosikan oleh lima strain rhizobia sub kelas α, yaitu *Rhizobium leguminosarum* bv. *phaseoli* RRE6 dan *R. undicola* RRE36 serta sub kelas β, yaitu *Burkholderia cepacia* (RRE3, RRE5, RRE25) dilakukan dengan menumbuhkan tanaman jagung yang diinokulasi dengan strain-strain tersebut. Tanaman jagung yang diinokulasi menunjukkan peningkatan yang signifikan dalam tinggi tanaman, panjang akar, tunas dan bobot kering akar dibandingkan kontrol tanpa inokulasi. *R. leguminosarum* bv. *phaseoli* RRE6 dan *B. cepacia* RRE5 yang masing-masing secara berturut-turut mewakili sub kelas α dan β, memberikan respon inokulasi terbaik. Pengaruh suplementasi nitrat pada jagung yang berasosiasi dengan RRE6 dan RRE5 juga dipelajari dan peningkatan yang signifikan dalam semua parameter pertumbuhan dan kemampuan kolonisasi dicatat dimana nitrat hadir sebagai suplemen dibandingan kontrol tanpa inokulasi dan jagung-RRE6 dan RRE5 tanpa tambahan nitrat dari luar.

Kata kunci: *Rhizobium leguminosarum* bv. *phaseoli, R. undicola, B. cepacia, Zea mays*, pemupukan nitrat

INTRODUCTION

PGPR is plant growth promoting rhizobacteria that directly or indirectly induce beneficial effects on plant growth and development. They can occur naturally as rhizospheric, endophytic or symbiotic component of bacteria-plant association. PGPR can affect plant growth and development either indirectly or directly. Direct mechanisms include production of phytohormones, synthesis of 1 aminocyclopropane-1-carboxylate (ACC) deaminase, phosphorous solubilization, nitrogen fixation, and siderophore production. Some indirect mechanisms are that they act as biocontrol agents and induce systemic resistance in plants (Ashraf et al. 2013). Occurrence of endophytic association has been reported in many non-leguminous crops such as maize, wheat, millets, kallar grass, sugarcane, etc. (Webster et al. 1997; Chaintreuil et al. 2000; Gutierrez-Zamora and Martinez-Romero 2001; Matiru and Dakora 2004). Plant-microbe interactions may occur at phyllosphere, endosphere, and rhizosphere (Bhattacharyya and Jha 2012).

Maize (*Zea mays*) is widely cultivated throughout the world and it is one of the most important staple grain crops in the world. Nitrogen and phosphorus are two of the essential nutrients for maize plant growth and development. Large quantities of chemical fertilizers are used to replenish soil N, and P. Use of high levels of nitrogenous fertilizers in crop production has its drawbacks. Only one-third of the nitrogen applied as a chemical fertilizer is used up by the crop. The non-assimilated nitrogen results in nitrate (NO_3^-) contamination of groundwater supplies (Mytton 1993; Shrestha and Ladha 1998), a potential health

hazard; soil acidification (Kennedy and Tchan 1992) and increased denitrification. Soil acidification and denitrification results in high emission of nitrous oxide (N_2O), a potent greenhouse gas, which enhances global warming (Bronson et al. 1997). Thus we are in dire need of exploring alternate or supplementary non-polluting sources of N for agriculture (Ladha et al. 1997). This problem could be solved if maize and other cereals were able to establish more intimate associations with plant growth promoting microorganisms. It, therefore, would be a noteworthy achievement if maize could profit from biological nitrogen fixation thereby decreasing its requirement and dependence on chemical nitrogenous fertilizers (Chelius and Triplett 2000; 2001). We have therefore endeavored to see if inoculation of some endophytic bacteria can improve growth performance of maize.

MATERIALS AND METHODS

Details of bacterial strains used in the study are listed in Table 1. Spontaneous mutants of *Burkholderia* strains RRE3, RRE5, RRE25, and *Rhizobium* strains RRE6 and RRE36 were isolated which were resistant to various antibiotics to be used as genetic marker during the study.

Table 1. Bacterial strains used in the present study

Strains	Accession number
Burkholderia cepacia RRE3	AY 946010
B. cepacia RRE25	EU 246850
B. cepacia RRE5	AY 946011
Rhizobium leguminosarum bv. *phaseoli* RRE6	AY 946012
R. undicola RRE36	EU 512923

Characterization of the strains

For growth studies and utilization of different nitrogen sources, all the bacterial strains were inoculated in Yeast Extract Mannitol (YEM, Vincent 1970) and grown up to 10^9 cells mL^{-1} and Rhizobial Minimal Medium (RMM) for specific experiments (Diebold and Noel 1989). Sodium glutamate of minimal medium was replaced by nitrogen sources like sodium nitrite, sodium nitrate and ammonium sulfate (10mM each) to study nitrogen utilization. Indole acetic acid (IAA) quantification and siderophore production were assayed as described by Patten and Glick 2002; Penrose and Glick 2003. Phosphatase enzyme activity was studied using Pikovskaya's broth medium. Pectinase assay was done according to the method of Mandels (1985). To estimate nitrogenase activity the acetylene reduction assay of Stewart et al. (1967) was used.

Plant growth experiment (greenhouse conditions)

To study the effect of inoculation with different antibiotic marked endophytic strains on growth of maize plant, seeds of the maize cultivar Malaviya 1 were selected and surface sterilized following standard protocol according to Singh et al. (2006). Three days old maize seedling, with root length ranging from 2.0 cm to 3.0 cm, were inoculated separately with one mL each of exponentially grown bacterial culture having cell population of 10^9 CFU (colony forming units) mL^{-1}. Uninoculated seedlings served as control. The germinated maize seedlings were transferred aseptically to plastic pots containing sterile sand. Sand was washed three times with tap water and dried in hot air oven. Washed and dried sand was then autoclaved twice for 20 minutes at 120 °C with an interval of 24 hours. All treatments were arranged in 25 pots i.e. 5 replicates with 5 pots per replication. The plants were incubated in plant growth chamber under a combination of fluorescent and incandescent light with a light intensity of 16000 lux, with a cycle of 16 hrs light and 8 hrs dark cycle temperatures of 28°/23°C and relative humidity of 55/75%. Plants were regularly watered with Nitrogen free Fahraeus medium (NFM, Fahraeus 1957) and were harvested at 35 days after inoculation.

Re-isolation and characterization of putative endophytes

Root portion of uprooted maize plants was cut into small pieces of 5 mm length. Surface sterilization was done twice once with 95% ethanol and then with 0.2% acidified $HgCl_2$. Sterilized root pieces were macerated in 10 mL sterilized distilled and were decimally diluted (10^{-5}) and spread on YEM agar plates supplemented with appropriate antibiotics.

Growth of maize seedling as influenced by nitrate availability and PGPR inoculation

Two antibiotic resistant derivatives of endophytic rhizobia, i.e., *Rhizobium leguminosarum* bv. *phaseoli* RRE6^{strR} (resistant to streptomycin 100 µg/mL) and *Burkholderia cepacia* RRE5^{strR} (resistant to streptomycin 500µg/mL) were used for this study. Surface sterilized seeds of maize (Malaviya 1) were germinated in Petri plates containing moistened filter paper under aseptic conditions. Three-day-old seedlings, with root length ranging from 2 to 4 cm, were selected and soaked in 25 mL of bacterial suspension of RRE-5^{strR} and RRE-6^{strR} for 90 min. Uninoculated seedlings served as control. Soaked seedlings were then removed and transferred to agar-water plates supplemented without and with 10mM NO_3^-. Four seedlings were planted on each Petri plate and five replicates were considered for each treatment. These seedlings were allowed to grow for 48 h and then removed from agar water plates and washed in distilled water in order to remove the agar adhering to the roots. Shoot length, root length, and fresh weight were recorded. Two grams of root were taken from each treatment separately and macerated. Macerate was decimally diluted; 100 µl of each of these solutions were spread on streptomycin-containing YEM plates. Subsequently, the plates were incubated at 28°C and CFU was counted. For analysis of data, the completely randomized design was used.

Statistical analysis

Data related to plant growth parameters were subjected to analysis of variance using SPSS software. Treatment

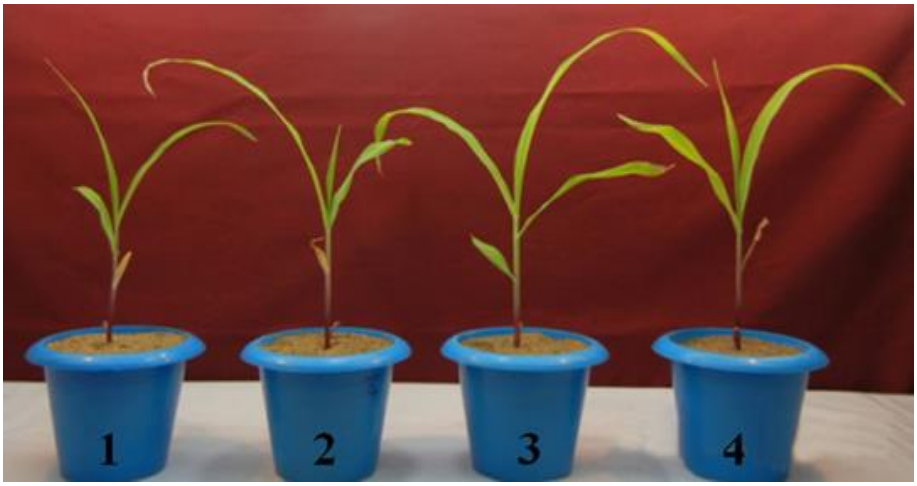

Figure 1. Maize plants inoculated with endophytic bacterial isolates. Note: 1. Control (uninoculated), 2. *R. undicola* RRE36, 3. *B. cepacia* RRE3, 4. *R. leguminosarum* RRE6

means were compared at 95% and 99% probability level (P = 0.05 and 0.01), and the same set of data was further analyzed to calculate the least significant difference at P = 0.05 and 0.01, respectively.

RESULTS AND DISCUSSION

Response of bacterial inoculation on the growth of maize plant under greenhouse conditions

The maize cultivar Malaviya 1 was used in plant growth experiment test to study the effect of inoculation of various endophytic rhizobial isolates on its growth. The statistical analysis showed that there was significant effect of inoculation over the control (uninoculated). Statistically significant differences were also observed among the isolates. Significant increase in plant height was observed in all rhizobial treatments (Figure 1). *R. leguminosarum* RRE6 gave the best inoculation response with maize and resulted in maximum increase in plant height, whereas *B. cepacia* RRE3 gave the minimum plant height increase over the control (Table 2). *R. undicola* RRE36 also showed a very good response to inoculation in terms of plant height. When strains were compared among themselves, *B. cepacia* RRE5, *R. undicola* RRE36 and *B. cepacia* RRE3 differed significantly from each other.

Insofar as root length is concerned, it was found that there was significant increase over the control in the cases of *R. leguminosarum* RRE6, *B. cepacia* RRE5 and *R. undicola* RRE36. But in cases of *B. cepacia* strains RRE3 and RRE25, differences were statistically non-significant (Table 2). In the case of RRE6, there was a significant increase compared to the control (60% increase) due to inoculation. RRE36 also showed a significant increase when compared with the control plant. Statistically, significant difference was observed between both *Burkholderia* strains (RRE5 and RRE25); RRE5 was found to be more effective. A significant increase in shoot dry weight was observed in maize plants inoculated with all endophytic rhizobial isolates. Maximum shoot dry weight was observed in plants inoculated with *R. leguminosarum* RRE6 followed by *R. undicola* RRE36 (Table 2). Shoot dry weight was found to be more in the case of *Rhizobium* strains when compared with *Burkholderia* strains.

A significant increase in root dry weight was exhibited by all the bacterial endophytes. The best performance for dry weight was shown by *R. leguminosarum* RRE6 followed by *R. undicola* RRE36. When *Burkholderia* strains were considered, significant increase over the control was apparent (Table 2). The cell densities from the sterilized root macerates of uprooted maize plants were calculated and found to be similar in each case (Table 3).

Table 2. Effect of inoculation of endophytic rhizobia on promotion of growth of maize plant (greenhouse conditions)

Treatment	Plant height in cm plant^{-1}	Root length in cm plant^{-1}	Shoot dry weight in g plant^{-1}	Root dry weight in g plant^{-1}
Control	35.97	23.6	0.060	0.051
RRE5	45.47* (26.41)	28.62* (21.2)	0.167* (178)	0.079* (54.9)
RRE6	56.39* (56.76)	37.72* (59.8)	0.236* (293)	0.100* (96.07)
RRE25	47.20* (31.22)	24.17* (2.41)	0.166* (176)	0.074* (45.09)
RRE36	51.59* (43.42)	32.22* (36.52)	0.208* (246)	0.088* (72.5)
RRE3	40.80* (13.42)	23.87* (1.1)	0.107* (78.3)	0.068* (33.33)
CD at 5%	4.32	3.433	0.036	0.017
SEM ±	2.06	1.624	0.017	0.008

Note: RRE6 and RRE36 = *Rhizobium* strains; RRE5, RRE3 and RRE25 = *Burkholderia* strains; CD = Critical difference; SEM = Standard error of mean (n = 4). Values in parentheses indicate % increase over control. *significant at 5%

Table 3. Cell density of endophytic bacterial strains isolated from inoculated maize plants

Bacterial strains	Cell density* (X 10^8cells mL^{-1})
RRE5	7.88±1.05
RRE3	8.93±0.09
RRE25	7.12±1.08
RRE6	8.26±0.09
RRE36	7.25±0.08
Control	0

Note: *Values are expressed as g^{-1} root fresh weight mL^{-1}

Biological activity of the rhizobial endophytes

All the 5 endophytes i.e. *R. leguminosarum* bv. *phaseoli* RRE6, *R. undicola* RRE36, *B. cepacia* (RRE3, RRE5, RRE25) grew well in the presence of different nitrogen sources (Table 4). It was found that all the strains were similar in their ability to produce auxin, at an average of 3.00 μg mL^{-1} (Table 5). The strains were also able to solubilize phosphate and pectin (Figure 2a, b). Nitrogenase activity was found to similar in all the strains. The production of siderophores by bacterial endophytes was positive as detected on chrome azurol S (CAS) plates. Growth of all the strain was found to be identical in both rich medium and minimal medium.

From the present observations it was clear that among the α and β subclass representatives, *R. leguminosarum* RRE6 and *B. cepacia* RRE5 were the most effective in increasing different growth parameters of maize. The present results clearly indicate that the α-subclass representatives were better growth enhancer as compared to β-subclass ones, though representatives of both the classes showed significant increase in the growth when compared to the control or uninoculated plants. For further study on these aspects, *B. cepacia* RRE5 and *R. leguminosarum* RRE6 were used to compare between *Burkholderia* and *Rhizobium*.

Table 4. Utilization of nitrogen sources by rhizobial strains in minimal medium (MM)

Strain	Absorbance at 420 nm (after 4 days of inoculation)		
	MM+NO_3	MM+NO_2	MM+NH_4
RRE3	1.75	1.68	0.66
RRE36	1.45	1.52	0.86
RRE25	1.91	1.85	0.84
RRE5	2.07	1.47	0.91
RRE6	2.25	1.77	0.98

Table 5. Biological activity of the rhizobial endophytes

Rhizobial strains	IAA production (μgmL^{-1})	Siderophore production	Phosphate solubilization (mm h^{-1})	Pectinase activity (mm h^{-1})	Nitrogenase activity (nmol C_2H_4 $h^{-1}mg^{-1}$ protein)
RRE5	3.11	++	0.14	0.10	22.75
RRE6	3.35	++	0.17	0.11	21.52
RRE3	2.17	++	0.09	0.10	21.27
RRE25	2.87	++	0.11	0.04	18.74
RRE36	3.01	++	0.10	0.05	19.75

Effect of nitrate availability and PGPR inoculation on the growth of maize seedlings

In this experiment, observations were recorded 48 h after inoculation. In the presence of nitrate, the growth of maize seedling was enhanced significantly upon inoculation with both the strains (Figure 3). The combined effect of nitrate enrichment and strains was much more significant than the individual effects of these two variables. Root length, shoot length and fresh weight significantly increased in all the cases when compared to the control (uninoculated) plants. Effect was more pronounced in *R. leguminosarum* RRE6 than in *B. cepacia* RRE5 when nitrate was added as supplement (Table 6).

Number of bacterial colonies recovered from root macerates of inoculated plants of maize

The number of endophytes recovered from the surface-sterilized roots in case of *R. leguminosarum* RRE6 was found to be 3.39 x 10^6, which was higher than *B. cepacia* RRE5 (2.35 x 10^6). In both the cases, nitrate addition increased the number of colonies and in case of RRE6+NO_3^- it was more than that in RRE5+NO_3^- (Table 7).

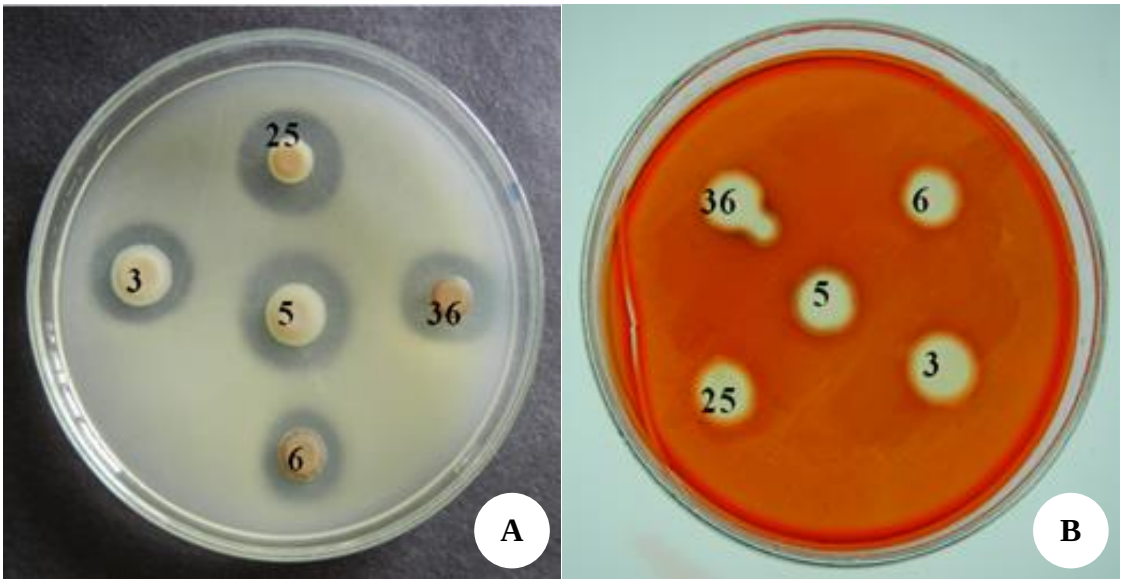

Figure 2. YEMA plate showing zone of clearance formed due to (A) phosphate solubilization and (B) pectin solubilization by *B. cepacia* (RRE5, RRE3, and RRE25), *R. leguminosarum* RRE6 and *R. undicola* RRE36

Figure 3. Effect of nitrate and *R. leguminosarum* RRE6 (A) and *B. cepacia* RRE5 (B) on seedling growth of maize. Note: 1-uninoculated control, 2-maize-RRE6 (A)/RRE5 (B), 3-maize-RRE6 (A)/RRE5 (B)+ NO_3^-

Table 6. Effect of nitrate and bacterial inoculation on the growth of seedling of maize

Treatment	Root length in cm plant^{-1}	Shoot length in cm plant^{-1}	Fresh weight in g plant^{-1}
Control	4.76	2.7	0.56
RRE5^{strR}	7.33* (53.99)	4.32* (60)	0.70* (25)
RRE5^{strR}+NO_3^-	9.95* (109)	6.0* (122)	0.84* (50)
RRE6^{strR}	12.51* (162)	7.12* (163)	0.98* (75)
RRE6^{strR}+NO_3^-	15.32* (221)	7.54* (64.1)	1.12* (100)
CD at 1%	2.275	1.055	0.110
SEM ±	1.068	0.495	0.055

Note: RRE6^{strR} = Streptomycin resistant mutant of *Rhizobium;* RRE5^{strR} = Streptomycin resistant strain of *Burkholderia;* CD = Critical difference; SEM = Standard error of mean (n = 4). Values in parentheses indicate % increase over control. *significant at 1%

Table 7. Cell density recovered from root macerates of inoculated plants of maize

Treatment	Cell density* (X 10^6cells mL^{-1})
RRE5^{strR}	2.35±0.09
RRE5^{strR} + NO_3^-	5.72±1.02
RRE6^{strR}	3.39±0.65
RRE6^{strR} + NO_3^-	6.79±1.35

Note: *Values are expressed as g^{-1} root fresh weight mL^{-1}

Discussion

The main objective of this study was to demonstrate the effect of bacterial strain on maize genotype that provides increased plant productivity compared with the uninoculated control under fully sterilized conditions. Representatives of α subclass of Proteobacteria (*R. undicola* RRE36 and *R. leguminosarum* RRE6) and β subclass of Proteobacteria *B. cepacia* (RRE5, RRE3, and RRE25) were used for the plant growth promotion experiment. Results clearly indicated a significant increase in various plant growth parameters in presence of the above strains when compared to the control or uninoculated plant. The most important parameter studied in this experiment was dry weight. When dry weight was considered both shoot and root dry weight were increased significantly when inoculated with the different endophytic strains. A similar association was described between maize and *Rhizobium etli* (Gutierrez-Zamora and Martinez-Romero 2001). The later authors reported an increase in maize plant dry matter upon *R. etli* inoculation. PGPR strains *Pseudomonas* and *Bacillus* significantly affected the height and dry weight of maize plants as was found by Jarak et al. (2012).

PGPR can enhance the growth and development of associated crops by improving nutrient uptake (Biswas et al. 2000). Shoot growth increased several folds in the present experiment. The exact mechanism of plant growth promotion by these isolates is not well understood in the case of maize. Phytohormone production, phosphate solubilization, nitrogen fixation and certain phenotypic changes like root proliferation could be the possible ways through which the host plants were benefited. PGPR uses one or more mechanisms to improve the growth and health of plants and can act simultaneously or independently at different stages of plant growth. Among these, phosphosolubilization, nitrogen uptake, and phytohormone production (indole-3-acetic acid), pectin solubilization was found to be present in all the strains. Large proportion of phosphorus in soil is insoluble and therefore unavailable to plants and hence phosphate solubilization is a desired property to be present in the bacteria. All tested rhizobial endophytes were able to solubilize phosphates and act as PGPR. They also act as biological agents through the production of siderophores.

Rhizobium-cereal associations are quite dynamic, enhancing the plant's root architecture as well as the overall growth physiology. This finding suggests that endophytes get intimately associated with roots of maize seedling in a very early stage of development of plant. Nitrate uptake and root architecture are affected by PGPR and nitrate availability. Nitrate transporter genes are induced by the presence of external nitrate which elicits root elongation and biomass. The effects of PGPR on

nitrate uptake are similar to those of low nitrate availability (Mantellin et al. 2004). Changes in root architecture similar to those induced by PGPR are due to the changes in nitrate availability in the medium (Wiersum 1958). In the present study, it was found that in the presence of nitrate (10mM) along with the strains, the growth of maize seedling was enhanced. It was found to be the highest in the case of *R. leguminosarum* RRE6, supplemented with nitrate. Alami et al. (2000) found that *Rhizobium* could be used in association with non-legume crops to utilize the nutrients better. The ability to use various nitrogenous compounds by rhizobial endophytes for their growth may be correlated with their evolutionary history. Due to extensive use of nitrogenous fertilizer in field, only those microorganisms could survive who had an inherent capacity to utilize the various nitrogenous compound. In the present experiment, it has been found that rhizobial inoculation to maize cultivar Malaviya 1 resulted in proliferated root architecture.

When nitrate was added as the supplement, growth was found to be more significant in all the cases (Table 6). Endophytic rhizobia may alter the morphological and physiological development of maize plant in ways that make them better miners of the existing resources of plant nutrients in soil. This has been reported in previous studies showing significantly increased production of root biomass in plants inoculated with certain rhizobial endophytes (Yanni et al. 1997, 2001; Prayitno et al. 1999; Biswas et al. 2000). It was found that maize seedling roots become intimately associated with bacterial cells in 48 h. During this initial period, it was found that there was significant increase in all the growth parameters studied and endophytes recovered from the surface-sterilized roots in case of *R. leguminosarum* RRE6+NO_3^- were found to be 6.79 x 10^6, which is higher than *B. cepacia* RRE5+NO_3^- (5.72 x 10^6). Earlier, it was always thought that rhizobia did not enter non-leguminous root tissues in any substantial manner (Sprent 1989). In the present study, it was found that bacterial population obtained from nitrate enriched root macerates of maize was very high in both the strains. So it might be possible that these endophytes are entering inside the roots of maize seedling.

CONCLUSION

Both the bacterial groups, i.e., *Burkholderia cepacia* RRE5, RRE3 and RRE25 (member of β-subclass) and *Rhizobium leguminosarum* bv. *phaseoli* RRE6 and *R. undicola* RRE36 (member of α-subclass) were capable of establishing as endophytes of maize plant. In the presence of nitrate, the plant growth promotion effect produced by endophytes (RRE6 and RRE5) was more enhanced; RRE6 showed better effect as compared to RRE5. From this study, it can be suggested that one of the sites of infection by endophytic bacteria is root of the host plant. The endophytic behavior and colonization ability of bacterial endophytes (RRE5 and RRE6) in the maize plants can be enhanced by nitrate supplementation.

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