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Original Article

In Vitro Screening of Salt Tolerant *Bacillus* from Rhizosphere of Tomato (*Lycopersicon esculentum*) Showing Plant Growth Promoting Traits

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Abstract

PGPR enhance plant growth either by direct or indirect mechanisms. The direct growth promoting mechanisms involve nitrogen fixation, solubilization of minerals, production of phytohormones and the indirect approach occurs when PGPR lessen or prevent the deleterious effects of plant pathogens. In the present study, rhizospheric soil samples were collected from different tomato (*Lycopersicon esculentum*) cultivated fields from Raebareli district, Uttar Pradesh, India. A total of twenty three (23) bacterial strains were isolated from soil samples and identified on the basis of their morphological and biochemical characteristics and classified as genus *Bacillus.* Out of these twenty three isolates, seven bacterial isolates showed salt tolerance and three isolates (RBL-1, RBL-5 and RBL-6) showed tolerance against 10% (w/V) NaCl. All salt tolerant rhizobacterial isolates showed potential plant growth promoting (PGP)traits (production of IAA, HCN, NH_3, siderophores and phosphate solubilisation) and the isolate RBL-1 showed significantly high IAA production (78.20 µg/ml).

Keywords: *Bacillus*; PGPR; Tomato; Saline.

Introduction

PGPR (plant growth promoting rhizobacteria) are defined by three intrinsic characteristics: (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth. Plant growth promoting rhizobacteria are free-living, soil-borne bacteria which when applied to seeds/soils or crops, enhance the growth of the plant directly by providing nutrients and growth promoting substances to plants or indirectly by reducing the damage from soil-borne plant pathogens (Kloepper *et al*., 1980).The application of plant growth promoting rhizobacteria (PGPR) as crop inoculants for biofertilization, phytostimulation and biocontrol offers an attractive and eco-friendly alternative to decrease the use of chemical fertilizers which decrease soil fertility and also have adverse effectson the environment (Ali *et al.,* 2010). *Pantoea*, *Bacillus*, *Pseudomonas* etc. are widespread species in agricultural soils and have many traits that make them well-matched as PGPR. PGPR can stimulate plant growth directly as they can improve the supply of nutrients, such as nitrogen (Dobbelaere *et al*., 2003) and phosphorous (Rashid *et al*., 2004) or by production of phytohormones (Choong *et al.,* 2003; Stepanova *et al*., 2008) and ACC-deaminase synthesis (Arshad *et al*., 2007). Indirectly PGPR can also promote plant development by the suppression of pathogens mediated by different mechanisms such as antibiosis (Milner *et al*., 1996), iron sequestration by siderophores (Singh *et al.,* 2014), HCN (Keremer and Souissi, 2001), and cell wall degrading enzymes like chitinase (Ajit *et al*., 2006). Thus, plant growth is promoted through reducing or neutralizing pathogen activity. Nearly 40% of world's surface has salinity problems due to poor and unscientific water management practices and most of the saline areas are confined to the Tropics and Mediterranean region. This has made the necessity to explore and create salt tolerance organisms and requires attention on priority for the future of agriculture (Corodovilla *et al*., 1994; Gisbert *et al*., 2000). The combination of IAA production ability (Goldstein, 1995), phosphorous solubilization (Gyaneshwar *et al*., 1998) and siderophore production (Dulfy, 1994) by bacteria aid the plant

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rhizosphere in enhancing the nutrient availability / absorption potential under sodic environment for enabling economic production of commercial horticultural crops (Damodaran *et al*., 2013). The tomato belongs to the family Solanaceae (also known as the nightshade family), sub-family Solanoideae, tribe Solanaceae and genus *Lycopersicon* (Taylor, 1986). Tomato is the second most cultivated vegetable in the world, after potato and India is the fourth largest tomato producer worldwide. The tomato fruit is consumed in diverse ways, including raw, as an ingredient in many dishes and sauces, and in drinks. The fruit is rich in lycopene, which may have beneficial health effects. The most important tomato producingIndian states are Bihar, Karnataka, Uttar Pradesh, Orissa, Andhra Pradesh, Maharashtra, Madhya Pradesh and Assam. The expansion of tomato production in India was to a great extent attributed to the private seed sector, which developed high yielding cultivars (Nagaraju *et al.,* 2002).

Uttar Pradesh region represent unique combination of plant and soil type which changes drastically with altitude. However, limited efforts have been done so far to explore the available bacterial diversity. In the present study, rhizospheric soil samples from tomato fields were collected from Raebareli district for assessment of PGPR. The objectives of this study have been to isolate and characterize rhizobacteria from the rhizosphere of tomato, and to screen them for salt tolerance and the possession of direct and indirect plant growth promoting rhizobacteria attributes.

Materials and Methods

Collection of Sample

The rhizosphere soil samples were collected from the roots of tomato (*Lycopersicon esculentum*) plants growing at different sites in Raebareli district, Uttar Pradesh, India. Intact root system was dug out at a depth of 5 to 10 inch and the rhizospheric soil samples were carefully taken in plastic bags with proper labelling and stored at 4°C. Total of five soil samples were collected for the isolation of rhizosphere bacterial isolates.

Preparation of Soil Samples

The soil samples were first ground with sterile mortar and pestle to liberate the adhering microorganism before their suspension was prepared. One gram of the soil sample was weighed out and dissolved in 9 ml of distilled water in a beaker and homogenized properly.

Isolation and Characterization of Rhizobacteria

One (1) gram of soil sample was serially diluted in sterile distilled water and 0.1 ml of soil suspension from 10^{-1} to 10^{-5} dilutions was spread on the nutrient medium agar (NA) plate and subsequently incubated at 37ºC for 48 hrs. Fine isolated and distinct colonies were picked up and streaked freshly on NA plates and incubated at 37ºC. After the recovery isolates in pure form, the rhizobacteria were identified on the basis of the standard protocols. Selected twenty-three rhizobacterial isolates were characterized morphologically and biochemically. The biochemical tests (Table 1) were carried out separately for gram reaction, pigmentation, oxidase test, indole test, utilization of citrate, etc. as per the standard methods (Cappuccino and Sherman, 1992).

Salt tolerance

Tolerance of bacterial strains to NaCl was evaluated on Nutrient agar medium supplemented with increasing NaCl concentrations (1.0, 2.5, 5.0, 8.0 and 10.0% w / V).The nutrient agar media were poured in Petri plates and bacterial strains were streaked on each plate and incubated at 37°C for 24 hrs (Dubey and Maheshwari, 2012). The influence of NaCl concentrations on degree of inhibition of bacterial growth was recorded.

Production of Indole acetic acid

Qualitative (Brick *et al*., 1991) and quantitative (Patten and Glick, 1996) analyses of indole acetic acid (IAA) were carried out on bacterial isolates. Development of pink colouration indicates positive result. Absorbance of supernatant mixture was measured at 535 nm and quantified using tryptophan as standard.

Production of HCN

Production of HCN was detected according to the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g / L glycineand bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2°C for 4 days. Development of orange to red colour indicated HCN production.

Phosphate Solubilization

Phosphate solubilization of isolates was evaluated from the ability to solubilize inorganic phosphate. Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate was used in assay. A loopful of bacterial culture were streaked on the plates and kept for incubation at 28°C for 4-5 days. The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

Production of Ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Results

Isolation and Identification of Bacteria

On the basis of cultural, morphological and biochemical characteristics (Table 1), total 23 isolates were identified as *Bacillus* from the rhizosphere of tomato (*Lycopersicon esculentum*) plants growing at different sites at Raebareli district, Uttar Pradesh, according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Among the 23 isolates, 7 bacterial isolates (RBL-1,RBL-2, RBL-3, RBL-4, RBL-5, RBL-6 and RBL-7) were selected for further studies based on the salt tolerance profile and efficiency for multiple plant growth promoting activities (Table 2 and 3).

Table 1: Morphological, Cultural and Biochemical characteristics of bacterial isolates

Biochemical and cultural characterization	*Bacillus spp.*
Number of isolates	Seven
Grams reaction	G +ve
Shape	Rods
Pigments	-
Dextrose	+
Sucrose	+
Mannitol	+
Oxidase	-
OF test	-
H_2S production	-
Indole	-
Methyl red	-
Vogues Proskauer	+
Citrate utilization	+
Starch hydrolysis	+
Gelatin hydrolysis	+

+ve = Positive; -ve = Negative

Salt tolerance profile of bacterial isolates

In the present study all the 23 bacterial isolates were tested for growth at different NaCl concentrations, and seven isolates (RBL-1, RBL-2,

Table 2: Screening of isolates for tolerance to salinity

*Bacillus*isolates	1% salt (NaCl)	2.5% salt (NaCl)	5% salt (NaCl)	8% salt (NaCl)	10% salt (NaCl)
RBL-1	+++	+++	+++	++	+
RBL-2	+++	+++	++	+	-
RBL-3	+++	++	+	-	-
RBL-4	+++	++	+	-	-
RBL-5	+++	+++	++	+	+
RBL-6	+++	++	+	+	+
RBL-7	+++	+++	+	+	-

- = No growth; + = Less growth; ++ = Good growth; +++ = Excellent growth

Table 3: Plant Growth Promoting (PGP) activities of the test isolates *in vitro*

Bacillus isolates	IAA µg/ml	Siderophore	NH_3	HCN	PO^{-}_4 Solubilization
RBL-1	78.20	+	+++	+	+
RBL-2	65.90	+	+++	+	+
RBL-3	76.00	-	-	+	-
RBL-4	70.60	+	++	-	-
RBL-5	42.00	-	+	-	-
RBL-6	19.80	-	+	-	-
RBL-7	65.00	-	+	+	-

- = No growth; + = Poor growth; ++ = Good growth; +++ = Excellent growth

RBL-3, RBL-4, RBL-5, RBL-6, and RBL-7) growing luxuriantly in 2-10% NaCl were selected for further evaluations (Table 2). Among these seven, only three bacterial strains (RBL-1, RBL-5 and RBL-6) showed tolerance at 10% NaCl concentration.

Plant Growth Promoting (PGP) activities of thetest Isolates

The test bacterial isolates were screened for multiple plant growth promoting activities which are showed in the table 3. IAA production was shown in all the isolates of *Bacillus*. Siderophore production was detected in (43% isolates), whileas, Ammonia production was shown in most of the isolates of *Bacillus* (except RBL-3). Among the seven isolates, four isolates (RLB-1, RLB-2, RLB-3 and RLB-7) responded positively for hydrogen cyanide production and isolate RBL-1 and RBL-2 showed positively phosphate Solubilization, isolate RBL-1 showed highest production of IAA.

Discussion

A number of different bacteria may be considered to be PGPR including *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Enterobacter* and *Bacillus.* In the present study to isolate and identify salt tolerant plant growth promoting rhizobacteria were isolated, from tomato (*Lycopersicon esculentum*) rhizosphere.Test isolates were screened for their plant growth promoting activities *viz*., indole acetic acid (IAA) production, ammonia production, phosphate solubilization, Siderophore, and HCN production, at high salt concentrations. Salinity of the soil plays a prominent role in the microbial selection process as environmental stress has been shown to reduce bacterial diversity (Borneman *et al.*, 1996), In our study, we have isolated 23 isolates from tomato (*Lycopersicon esculentum*) rhizosphere grown in sodic soils at Raebareli district, Uttar Pradesh, and screened them into seven isolates, *Bacillus spp.* Rhizobacterial IAA production plays a significant role in the host plant's growth. Indole acetic acid production in microbial has been investigated by several researchers (Singh *et al.,* 2013; Ghosh *et al.*, 2008; Gulati *et al.*, 2009), Production of IAA is a general characteristic of our test isolates, RBL-1 are highly efficient IAA producer. Besides IAA production, microorganisms also enhance plant growth by scavenging available iron (Fe^{3+}), which involves secretion of high affinity, low molecular weight iron chelating ligands called siderophores (Anitha and Kumudini, 2014; Singh *et al.,* 2014). This study has demonstrated that the 3 isolates (RBL-1, RBL-2 and RBL-4) produced siderophores.whileas two (RBL-1, RBL-2) rhizobacterial isolates showed *in vitro* phosphate solubilizing efficiency and has been tested in plant growth. Phosphate solubilization by *Bacillus sp.* isolated from salt stressed environment had been observed by earlier researchers (Son *et al.*, 2006). HCN known to be both beneficial and harmful property for plants (Cattelan *et al.*, 1999), The production of HCN in excess may play a critical role in the control of fungal diseases (Flaishman, 1996), Hydrogen cyanide (HCN) synthesized by some rhizobacteria inhibits diseases in plant and thereby increasing the biocontrol mechanisms (Schippers, 1990). In present study HCN detected in four test isolates. Most of the Gram-positive, endospore forming rods with halotolerant properties have been assigned to the genus Bacillus (Yoon *et al,* 2003). From this study, out of the 23 bacteria isolated from tomato (*Lycopersicon esculentum*) plants growing at different sites at Raebareli district, Uttar Pradesh, seven showed tolerance to high salt concentration (1-10 % NaCl) and among them isolate RBL-1, RBL-5 and RBL-6 in vitro efficiency to grow in 10% NaCl concentration was found in similar range as reported by(Upadhyay *et al.*, 2009, Damodaran *et al*., 2013).

These ameliorative effects of PGPRs can be due to their ability to secret biologically active secondary metabolites including phytohormones. Previously, B. *cepacia*, A. *calcoaceticus* and *Promicromonospora* sp. were found to produce gibberellins and auxins during their growth. Such bioactive PGPRs can extend additional support to plant growth during abiotic stresses as shown by other studies of Asghar et al. (2002), Lugtenberg and Kamilova (2009), Noorieh et al. (2013) and Rakshapal et al. (2013). Exogenously

supplied phytohormones have already been identified to ameliorate plant growth and development during abiotic stresses (Hamayun et al. 2010; Iqbal and Ashraf 2013; Kang et al. 2014). This is in correlation with our findings as well. The present growth stimulatory effects were due to their potential to produce gibberellins (Kang et al. 2009, 2010, 2014). In conclusion, such phytohormones producing PGPRs can be applied to crops to increase their productivity, as well as their association will reduce the negative impacts of salinity and short-term drought periods. Their plant growth promotion activities ought to be determined, as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers. Study of their tolerance capacity in extremely saline conditions may bring new insight and application in environmental stress areas. Proper efficacy testing by pot / field trials is also necessary for sustainable use of these microorganisms in agricultural purpose.

References

1. Ajit NS, Verma R, Shanmugan V. Extracellular chitinase of fluorescent pseudomonas antifungal to *Fusarium oxysporum* f.sp. *dianti* causing carnation wilt. Curr Microbiol 2006; 52: 310-316.
2. Ali B, Sabri AN, Hasnain S., Rhizobacterial potential to alter auxin content and growth of *Vigna radiata* (L.). World J Microbiol Biotechnol 2010; 26: 1379-1384.
3. Anitha G, Kumudini BS. 2014. Isolation and characterization of fluorescent pseudomonads and their effect on plant growth promotion. J Environ Biol 2014; 35: 627-634.
4. Arshad M, Saleem M, Hussain S. Perspectives of bacterial ACC deaminase in phytoremediation. Trends Biotech 2007; 25: 356-361.
5. Borneman J, Skroch PW, O'Sullivan KM. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 1996; 62: 1935-1943.
6. Brick JM, Bostock RM, Silverstone SE. Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 1991; 57: 535-538.
7. Cappuccino JC, Sherman N. In: Microbiology. A Laboratory Manual, 3rd edn. Benjamin / Cummings Publ. Co., New York; 1992.
8. Cattelan J, Hartel PG, Fuhrmann J. Screening for Plant Growth–Promoting Rhizobacteria to Promote Early Soybean Growth.Soil Science Society America J 1999; 63: 1670-1680.
9. Choong MR, Mohamed AF, Chia-Hui H, Munagala SR, Hun-Yun W, Puul WP. Bacterial volatiles promote growth in *Arabidopsis*. Plant Biol 2003; 100: 4927-4932.
10. Corodovilla MP, Ligero P, Lluch C. The effect of salinity on nitrogen fixation and assimilation in *Vicia faba*. J Exp Bot 1994; 45: 1483-1488.
11. Damodaran T, Mishra VK, Sharma, DK, Jha SK, Verma CL, Rai RB, Kannan R, Nayak AK and Dhama K. Management of sub-soil sodicity for sustainable banana production in sodic soil - an approach. Int J Curr Res 2013; 5: 1930-1934.
12. Damodaran T, Sah V, Rai RB, Sharma DK, Mishra VK, Jha SK, Kannan R. 2013. Isolation of salt tolerant endophytic and rhizospheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigour in tomato under sodic environment. Afric J Microbiol Res 2013; 7: 5082-5089.
13. Dobbelaere S, Vanderleyden J, Okon Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 2003; 22: 107-149.
14. Dubey RC, Maheshwari DK. Practical Microbiology, S. Chand & Company Ltd. New Delhi, India; 2012.
15. Duffy BK, Défago G. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl Environ Microbiol 1994; 65: 2429-2438.
16. Flaishman MA, Eyal ZA, Zilberstein A, Voisard C, Hass D. Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide producing strains of *Pseudomonas putida*. Mol Plant Microbe Inter1996; 9: 642-645.
17. Ghosh S, Sengupta C, Maiti TK, Basu PS. Production of 3-indolylacetic acid in root nodules and culture by a *Rhizobium* species isolated from root nodules of the leguminous pulse *Phaseolus mungo*. Folia Microbiol 2008; 53: 351-355.
18. Gisbert C, Rus AM, Carmen Bolarin M, Isabel Arrillaga JM, Montensinos C, Caro M, Moreno R. 2000. The Yeast HAL1 gene improves salt tolerance of Tra Tomato. Plant Physiol 2000; 123: 393-402.

19. Goldstein AH (1995). Evidence for mutualism between a plant growing in a phosphate-limited desert environment and a mineral phosphate solubilizing rhizobacteria. FEMS Microbiol Ecol. 1999; 30: 295-300.

20. Gulati A, Vyas P, Rahi P, Kasana RC. Plant growth promoting and rhizosphere-competent *Acinetobacter* rhizospherae strain BIHB 723 from the cold deserts of the Himalayas. Curr Microbiol 2009; 58: 371-377.

21. Gyaneshwar P, Naresh KG, Parekh LJ. Role of soil microorganisms in improving P nutrition of plants. Plant Soil 1998; 245: 83-93.

22. Keremer RJ, Souissi T. Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. Curr Microbiol2001; 43: 182-186.

23. Kloepper JW, Leong J, Teintze M., Schroth MN. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature 1980; 286: 885-886.

24. Lorck H. Production of hydrocyanic acid by bacteria. Plant Physiol 1948; 1: 142 -146.

25. Milner JL, SiloSuh L, Lee JC, He H, Clardy J, Handelsman L. Production of kanosamine by *Bacillus cereus* UW85. Appl Environ Microbiol 1996; 62: 3061-3065.

26. Nagaraju N, Venkatesh HM, Warburton H, Muniyappa V, Chancellor TCB and Colvin J. Farmer's perceptions and practices for managing tomato leaf curl virus disease in Southern India. Int J Pest Manag2002; 48: 333–338.

27. Norris JR, Chapman HM. Classification of *Azotobacter.* In: Identification methods for microbiologists. In: Gibbs BM, Shapton DA, Editors. Academic press, London & New York. 1968; pp. 19-27.

28. Patten CL, Glick. Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 1996; 42: 207-220.

29. Rashid M, Khalil S, Ayub N, Alam S, Latif, F. Organic Acids productions solubilization by phosphate solubilizing microorganisms (PSM) under *in vitro* conditions. Pak J Biol Sci 2004; 7: 187-196.

30. Schippers B, Bakker AW, Van Peer R. Beneficial and deleterious pseudomonads on rhizosphere interactions. Plant Soil 1990; 129: 75-83.

31. Schwyn B,Neilands JB. Universal chemical assay for detection and determination of siderophore. Anal Biochem 1987; 160: 47-56.

32. Singh Y, Ramteke PW, Shukla PK, Khan, K. Isolation and characterization of multi heavy metal resistant *Bacillus* spp. and their plant growth promoting activities. Int J Recent Scientific Res 2013; 4: 148-150.

33. Singh Y, Ramteke PW, Shukla PK. Characterization z of *Pseudomonas* spp. isolated from sewage irrigated soils and their plant growth promoting traits *in vitro*. Int J Microbial Resour Technol 2014; 2 : 19-22.

34. Son HJ, Park GT, Cha MS, Heo MS. Solubilization of insoluble inorganic phosphates by a novel salt- and pH tolerant *Pantoea agglomerans* R-42isolated from soybean rhizosphere. Bioresour Technol 2006; 97: 204-210.

35. Stepanova A, Robertson-Hoyt J, Yun J, Benavente LM, Xie D, Dolezal K, Schlereth A, Jürgens G, Alonso JM. TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 2008; 133: 177-191.

36. Taylor IB. Biosystematics of the tomato. In: Atherton J and Rudich G, Editors. The Tomato Crop. A Scientific Basis for Improvement. Chapman & Hall, New York;1986. pp. 1-34.

37. Upadhyay SK, Singh DP, Saikia R. Genetic Diversity of Plant Growth Promoting Rhizobacteria isolated from Rhizospheric Soil of Wheat under saline condition. Curr Microbiol 2009; 59: 489-496.

38. Yoon JH, Kim IG, Kang KH, Oh TK, Park YH. *Bacillus marisflavi* sp. *nov* and *Bacillus aquimaris* sp. *nov* isolated from sea water of a tidal flat of the yellow sea in Korea. Int J Syst Evol Microbiol2003, 53: 1297–1303.