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Solubilization of tricalcium phosphate by temperature and salt tolerant *Serratia marcescens* NBRI1213 isolated from alkaline soils

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A number of stress tolerant plant growth promoting phosphate solubilizing bacteria were isolated from soils collected from different geo-climatic zones of India. Maximum numbers of these bacteria were found in Aurawan soil, followed by National Botanical Garden, Mukhateshwar and Nainital. Among these bacteria, strain NBRI1213 showed maximum phosphate solubilization activity (984 µg/mL) and was identified as *Serratia marcescens* by 16S rRNA full gene sequencing. It showed tolerance to wide range of temperature (30 to 50°C), salt (0 - 5% NaCl) and pH (7 - 10). Salicylic acid was found to be present in addition to oxalic, citric acid and acetic acid in culture filtrate. In a micro plot study, inoculation of maize seeds with phosphate solubilizing *S. marcescens* NBRI1213 showed better response than control. It did not only improve the quality but increased the soluble phosphorus content and enhanced the phosphate-solubilizing microbial populations of the soil. *S. marcescens* NBRI1213 isolated from alkaline soil was found to be an efficient phosphate solublizer and can be used as potential plant growth promoting rhizobacteria bio-inoculant in agricultural systems.

Key words: Phosphate solubilization, *Serratia marcescens,* stress tolerance, rhizosphere competence, maize.

INTRODUCTION

Microbes play an important role in agriculture as they promote the circulation of plant nutrients and reduce the need for chemical fertilizers. Phosphorus an essential nutrient for plant growth and development remains in insoluble form or in the form of insoluble metal chelates in soil (Vassilev et al., 2006a). Therefore, many soils throughout the world are deficient in phosphates (P). Phosphate is added to agricultural soil in the form of soluble chemical P fertilizers. However, only a small fraction (0.1%) of the added P is made available to plants (Scheffer and Schtschabel, 1989) since most of it is rapidly converted into insoluble forms (Reddy et al., 2002). This problem can be overcome to some extent by P-solubilizing microbes which can transform the insoluble phosphorus to accessible form for plants. The role of P-solubilizing microbes in soil has been attributed mainly to their abilities to reduce the pH of the surroundings by the production of organic acids (Sperber, 1958; Kin et al., 1998; Chen et al., 2006) and alkaline phosphatases (Rodriguez and Fraga, 1999) and to H^+ protonation (Illmer and Schinner, 1995). These organic acids can either dissolve phosphates as a result of anion exchange or can chelate Ca, Fe or Al ions associated with the phosphates (Chang and Yang, 2009; Gyaneshwar et al., 2002). Unfortunately, the effectiveness of plant growth promoting bacteria (PGPB) often lacks consistency under field conditions (Bashan and Levanony, 1990; Burr et al., 1978) due to their variability in root colonization which

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Table 1. Characteristics of sampling sites.

Sites/locations	Latitude/ longitude	Soil texture	Altitude (m)	Mean rainfall (mm)	Temperature (°C)		pH	Heterotrophic bacterial (CFU/g) soils
					Min.	Max.		
Jim Corbett National Park, Nainital	24040'N/ 72045'E	Sandy	240	326.3	8.0	32.0	6.5	10^6
(Mukhteshwar)	23019'N/ 69049'E	Sandy clay	1722	526.0	12.0	31.0	5.8	10^6
National Botanical Garden (Lucknow)	26014'N/ 78010'E	Loamy coarse	123	100	8.0	43.0	7.0	10^7
Aurawan Research Station (Aurawan)	22015'N/ 89010'E	Loamy fine	87	94.0	10.0	48.0	9.0	10^6

is affected by many different biotic and abiotic factors (Mahalakshmi and Reta, 2009). Therefore it is important that PGPB should also be tolerant to the respective stress conditions with root colonization ability.

Among various abiotic stresses, temperature and salinity are known to be one of the most important limiting factors for productivity of plants and bacteria. The detrimental effect of salinity on plants is a consequence of both water deficits resulting in osmotic stress and excess sodium ions on critical biochemical processes (Rehman and Nautiyal, 2002).

Root colonization by introduced plant growth promoting rhizobacteria (PGPR) is a complex process and has been the subject of intense research during the past two decades because variable colonization remains one of the major impediments to the widespread use of rhizobacteria in commercial agriculture. In order to be effective the introduced bacteria must attain the threshold population size in the rhizosphere (Mavrodi et al., 2006). Intensive studies have revealed that many bacterial traits are involved in the colonization process with the relative importance of individual traits varying according to the bacterial strain, host plant species and age, soil type, environmental conditions and type of assay used. Some of the trait are (i) attachment to roots, (ii) motility and chemotaxis, (iii) polysaccharide production, (iv) catabolism of nutritional sources, (v) a two component signaling system, (vi) a site-specific recombinase and (vii) NADH dehydrogenase I (Mavrodi et al., 2006; Nautiyal et al., 2010). It has been shown that rhizosphere competence confers to be effective in a site to directly promote the plants growth (Benizri et al., 2001). Though a number of PGPB have been reported however very little is known about the abiotic stress tolerant rhizosphere competence (Podile and Kishore, 2006).

Serratia marcescens a Gram negative bacterium (family Enteriobacteriaceae) widely known as an insect pathogen (Bahar and Demirbag, 2007; Fly and Xanthopoulos,1983) has been reported to promote plant growth by inducing resistance against plant pathogens (Kloepper et al., 1993) and production of antagonistic substances (de Queiroz and de Melo, 2006).

Mechanism of stress tolerant plant growth promoting phosphate solubilizing bacteria has not been explored much. To understand the physiology of phosphate solubilizing bacteria (PSB) similar stressed conditions is required. PSB, with the genetic potential for increased tolerance to high salt, high pH and high temperature, could enhance crop production. Therefore, the objective of this study was to isolate and characterize PSB *Serratia marcescens* NBRI1213 from alkaline soils which could solubilize phosphates at high salt, high pH and high temperature.

MATERIALS AND METHODS

Description of sites

Four different geoclimatic zones of India namely Jim Corbett National Park, Nainital (240 m); Mukhateshwar (1722 meter); National Botanical Garden, Lucknow, (123 meter) and Aurawan Research Station, Uttar Pradesh (87 meter), India (Table 1) were selected in order to study the role of phosphate solublizer in India. Soil samples were aseptically collected between April and June 2003 from 0-30 cm depth after removing 3 cm of the soil and put into Whirl bags. The samples were immediately taken to the laboratory and stored at 4°C till further analysis.

Isolation of P- solubilizing microorganism

Phosphate solubilizing bacteria were isolated from these soil samples using dilution plating and enrichment techniques. Root samples containing the adhering soils were shaken vigorously using vortexes and homogenized to remove loosely adhering soil and macerated in 0.85% distilled sterile saline water. Serial dilutions of the homogenate (10 fold log dilution series down to 10^{-6}) were prepared from each representative samples and 0.025 mL aliquots of each dilution were spread over the surface of Nutrient Agar (NA) plates with a sterile glass spreader (Mehta and Nautiyal, 2001). Phosphate solublizer microbes were isolated on Pikovaskaya agar (PKV) (Reddy et al., 2002) plates having tri calcium phosphate (TCP). Distinct colonies which showed halo zones were selected for further qualitative testing in National

Botanical Research Institute's Phosphate medium-Bromo phenol blue (NBRIP-BPB) (Mehta and Nautiyal, 2001). Quantitative determinations of 400 positive isolates (showed decolorization in NBRIP-BPB) were carried out further in National Botanical Research Institute's Phosphate medium (NBRIP). Quantitative estimation of phosphate solubilization in broth was carried out using Erlenmeyer flasks (150 ml) containing 50 ml of medium inoculated in triplicate with the bacterial strain. Autoclaved uninoculated medium served as control. The cultures were harvested by centrifugation at 10 000 rpm for 10 min, using Sorvall RC 5C centrifuge, Dupont, USA. Phosphate in culture supernatant was estimated using the Fiske and Subbarow method and expressed as equivalent phosphorus (µg/mL). For each sample, phosphate solublizer having the ability to solubilize TCP in an excess of 200 µg/mL were considered to be phosphate solubilizers. Out of 400, 40 isolates showed P solubilization in the range of 500 to 1000 µg/mL (data not shown). One dominant and efficient strain, NBRI1213 was selected which showed maximum p solubilization activity (984 µg/mL).

Characterization and identification of NBRI1213

The strain NBRI1213 was studied for substrate utilization by Biolog GN2 microplates (Biolog, Inc., Hayward, CA, USA. The strain was grown on Biolog® BUG® agar for identification by the Biolog® system (Biolog Inc., Hayward, CA, USA). In preparation for analysis colony was picked from pure culture of bacteria and was plated as a lawn of bacteria on to a BUG® agar plate was analyzed. The Biolog Microlog® Bacterial Identification System consists of databases combined with specialized 96-well plates (test panels). A panel of 95 different substrates gives a very distinctive and repeatable pattern of purple wells for "Metabolic Fingerprint'. The Gram-positive and Gram-negative panels and databases were used in this study (Nautiyal et al., 2010). Absorbance value for the wells with glucose as carbon sources was blanked against the control well. Average color development (AWCD) for plates was calculated as the mean of the blanked absorbance values for all wells per reading time. Data was recorded on the 3rd day of incubation. NBRI1213 was identified by 16S rRNA full length gene sequencing. Genomic DNA of NBRI1213 was isolated as described by Agrawal et al. (2010). The 16S rRNA full gene was amplified by PCR using MicroSeq full Gene Module (Applied Biosystems, UK) as directed in the manufacturer's protocol with an automatic DNA sequencer (Model 310; Genetic Analyzer, Applied Biosystems, USA). The 16S rRNA sequences was checked with Microseq software and Chromaspro version 1.41 (http: //www.technelysium.com. au/ChromasPro.html). Tree topology was evaluated by performing bootstrap analysis of 1000 data sets with the MEGA 4 package (Tamura et al., 2007). The 16S rRNA gene sequence of NBRI1213 has been submitted to the NCBI Genbank database with the accession number JF713819.

Biolog analysis

The Biolog MT plates (Biolog, Inc., Hayward, CA, USA) were used to screen the ability of isolated bacteria to solubilize other phosphorus sources. This technique is more commonly used for identification and community analysis where specific carbon source are provided and active metabolism results in the production of color product. Fifteen different phosphate sources namely ammonium di-hydrogen orthophosphate (ADOP), ammonium sodium hydrogen orthophosphate (ASHP), calcium hydrogen phosphate (CHP), di-potassium hydrogen phosphate (DPHP), di-sodium hydrogen phosphate (DHP), glucose-6-phosphate (GP), hydroxyapatite (HA), orthophosphoric acid (OPA), potassium di-hydrogen phosphate (PHP), sodium di-hydrogen phosphate (SDP), sodium hexametaphosphate (SHP), sodium phytate (SP), sodium tripolyphosphate (STP), tributylphosphate (TBP) and tricalcium phosphate (TCP) were selected. The rate of utilization is indicated by the reduction of tetrazolium, a redox indicator dye, which changes from colorless to purple. Soil (10 g) was shaken in 100 ml of sterile 0.85% saline for 60 min and then made up to a final dilution of 10^{-3}. A 150 µl aliquot was inoculated into each microplate well. The plates were incubated at 30°C. The absorbance of wells was measured at 590 nm using automated microplate reader. Microbial activity in each microplate was determined by average well color development (AWCD) as described (Garland, 1996).

P- solubilization under abiotic stress conditions

P- Solubilization activity was studied in 150 mL Erlenmeyer flasks containing 50 mL of NBRIP medium initially inoculated with 2 x 10^8 CFU/ mL under different stress conditions such as 0 - 5 % NaCl, pH 7.0 - 10 and temperature 30 and 50C. Cell count was monitored by dilution plating at 30°C with the intervals of 0, 3, 5, 7, 10 days (Nautiyal 1999). Morphology of NBRI1213 under stress conditions was studied by scanning electron microscopy. The 1.5 mL of 10 days old culture was centrifuged at 8000 rpm for 5 min at 4°C. The cell pellet was washed four times with sterile MQW at 4000 rpm and cells were fixed in 2.5% glutaraldehyde for 2 h at 4°C, followed by another two washes in MQW. The cells were dehydrated by successive 30 min washing with 30, 50, 70, 90, 95 and 100% ethyl alcohol. At absolute ethanol stage cells were dried in a CO_2 atmosphere (BAL-TEC CPD 030 Critical Point Dryer, Germany) and mounted on aluminum stubs and gold coated for 5 min in a BAL-TEC SCD 005 sputter coater and examined under a Philips XL-20 (Holland) scanning electron microscope. The cell dimensions were measured directly from the microscope to calculate the size as expressed in µm. Values represent the means of 7 replicates.

HPLC analysis for organic acid determination

NBRI1213 was grown in NBRIP medium for 10 days at 30°C. Extracted organic acids from culture filtrate were analyzed by HPLC (Agilent 1100 HPLC, Palo Atlo, CA, USA) with G1312A binary pump, using Aminex Column (HPX 87H ion exchange column, 300 × 7.8 mm) and UV detector fixed at 210 nm. Column temperature was maintained at 50°C and 0.01N H_2SO_4 was used as mobile phase with a flow rate of 0.6 mL/min. Standards samples of salicylic acid, gluconic acid, succinic acid, citric acid, oxalic acid, lactic acid, acetic acid and formic acid were used for comparison.

Tracking of the strain NBRI1213 in the rhizosphere of maize

In order to monitor the presence of NBRI1213 on plant roots grown in un-sterilized soils, a spontaneous rifampicin-resistant (Rif^r) mutant of NBRI1213 was developed on 250 µg rifampicin. A spontaneous rifampicin-resistant mutant of NBRI1213 was isolated on nutrient agar (NA) plates containing 250 µg rifampicin (from Sigma Chemical Co., St Louis, MO, USA) by plating 100 µl of overnight grown culture per plate. Resistance to rifampicin was used as it is mediated by a mutation in the β subunit of RNA polymerase, unusual among soil bacteria (Nautiyal, 1997). The heterotrophic rhizosphere bacterial population was recovered by serial dilution plating on NA and for NBRI1213R 250 µg rifampicin/mL was used. Average rhizosphere colonization of NBRI1213R was determined at the time of harvesting. No naturally occurring Rif^r bacteria were observed when root homogenates of uninoculated controls were plated from non-sterilized soils.

Plant growth promotion assay

Micro plot trial was carried out in NBRI farmhouse using a randomized block design with three replicates. Unsterile field soil was used to evaluate the plant growth promotion potential of NBRI1213 using maize (*Zea mays* cv. Arkil), host plant (Nautiyal et al., 2010). Each treatment was raised in eight rows, each of 8 m length 6 m width, with an inter and intra row spacing of 10 and 60 cm. Inoculum of NBRI1213 was prepared by growing the culture up to 48 h at 30°C till 8 $\log_{10}$ CFU/mL was obtained. Surface-sterilized maize seeds were then soaked with this bacterial suspension for 4 h at 30°C on a reciprocal shaker at 100 rpm. Control seeds were soaked in 0.85% saline. Harvesting was carried out after 105 days of sowing and data was recorded at the same time. Each analysis was carried out using four plants per row. Values for shoot lengths (cm), shoot dry weights (g) and number of fruits/plant are the mean of 20 plants with three replicates. Whereas number of seeds per fruit was calculated from 12 fruits each chosen at random interval. Values for the dry weights (g) are the mean of 100 seeds. The yield per plot was recorded and calculated as described earlier (Nautiyal et al., 2010). The harvested 12 plants were taken at random interval per treatment, rinsed with MQW and oven-dried at 75°C for 72 h. The dried shoot tissues were ground and then digested using concentrated HNO_3 (Page et al., 1982) for the determination of K using an atomic absorption spectrometer. Total N and P were extracted by digesting shoot tissue with 3 mL concentrated H_2SO_4 and 1 mL H_2O_2 at 360 °C, and determined by the Berthelot reaction and molybdenum blue method, respectively (Page et al., 1982). The amount of $NaHCO_3$-extractable P (available inorganic P) from the soil was determined by extracting samples with 0.5 M $NaHCO_3$ (pH 8.5) at a solution/solid ratio of 20:1 for 30 min (Olsen and Sommers, 1982).

Data analysis

Levels of significance (p<0.05) of main treatments and their interactions were calculated by analysis of variance (ANOVA). Critical difference was calculated (p<0.01) level in plant growth promotion by NBRI1213R under micro plot conditions.

RESULTS AND DISCUSSION

Little is known about how abiotic factors influence plant growth promotion. Understanding the factors influencing the abundance and activity of bacteria with plant growth potential is of practical importance because it might enable us in the near future to manage the indigenous microbial communities towards optimal plant growth. Application of biofertilizers has been reported to result in improved plant growth. Though direct correlation has been established between *in vitro* solubilization of P and organic acid production, the results of this study make these striking as phosphate solublizer.

Community profiling for substrate was done by Biolog MT plates. Additional 15 different phosphorus (P) sources were tested with each of the soil samples. Significant difference (at p>0.01) was found in the utilization of CHP, DPHP, DHP, GP, OPA, STP among 15 phosphate substrates by Aurawan and Nainital soils. TCP, DPHP, GP was utilized more by Aurawan soil when compared with other soils. However, Nainital soil showed maximum utilization of ADOP, ASHP, TBP, SP, PHP, SHP, SDP as compared to National Botanical Garden, Mukhateshwar and Aurawan soils (Figure 1). Son et al. (2005) has also reported TCP solubilization significantly greater than hydroxyapatite by *Pantoea agglomerans*. Xiao et al. (2013a) demonstrated the solubilization of rock phosphate and its uptake in wheat plant by a filamentous fungi *Aspergillus niger*.

To elucidate the role of phosphate solubilizing bacteria under stress condition, the samples were collected from 4 sites consisting of different stages of abiotic stresses. The highest heterotrophic bacterial population was found in the National Botanical Garden site (10^7) (Table 1). On the basis of potential discoloration ability of isolates in NBRIP-BPB medium, 100 isolates from each site were selected for further quantification. Quantitative test showed solubilized P in the range of 500-1000 µg/mL (data not shown). On the basis of quantitative estimations maximum number of phosphate solublizer (TCP) were found to be present in Aurawan soil (96%) followed by National Botanical Garden soil (75%) and Mukhateshwar soil (55%). However in Nainital soils, 33% of phosphate solubilizers were obtained (Figure 2) and an isolate 1213 from Aurawan soil showed solubilization of TCP 984 µg/mL. Therefore, strain 1213 from Aurawan soil was selected as potential solublizer and named as NBRI1213 for further characterization. Soil is a complex environment where bacterial growth and development can be influenced by several environmental stresses. Among them, salinity and temperature are very important and prevalent in India. The quantity and activity of microorganism represent sensitive indicators related to soil development processes (Perez et al., 2007). Recently, Xiao et al. (2013) reported rock phosphate solubilization with yeast strains.

Substrate utilization of strain NBRI1213 was tested by Biolog GN2 plates which contained 95 carbon sources which were categorized into six groups: (1) amine/amides, (2) amino acids, (3) carbohydrates, (4) carboxylic acids, (5) miscellaneous and (6) polymers (Figure 3). Significant difference was observed in substrate utilization rate in all groups except for amino acids. In general, utilization of amine/amides, carboxylic acids, carbohydrates and polymers group were utilized.

Strain NBRI1213 was identified by 16S rRNA full gene sequencing. The closest match of NBRI1213 was *S. marcescens* DSM 30121 (98%) as presented in Figure 4. Phylogenetic dendogram were mapped with closely related members. The topology shown is an unrooted tree obtained with a neighbor joining algorithm (Jukes-cantor corrections) with bootstrap value expressed as percentage of 1000 replications. *S. marcescens* strains have been frequently reported from the food industry (Nitschke and Costa, 2007), but this is a report where we studied presence of stress tolerant phosphate solubilizing (PGPB) *S. marcescens* from the alkaline soils.

Effect of abiotic stresses on phosphate solubilization

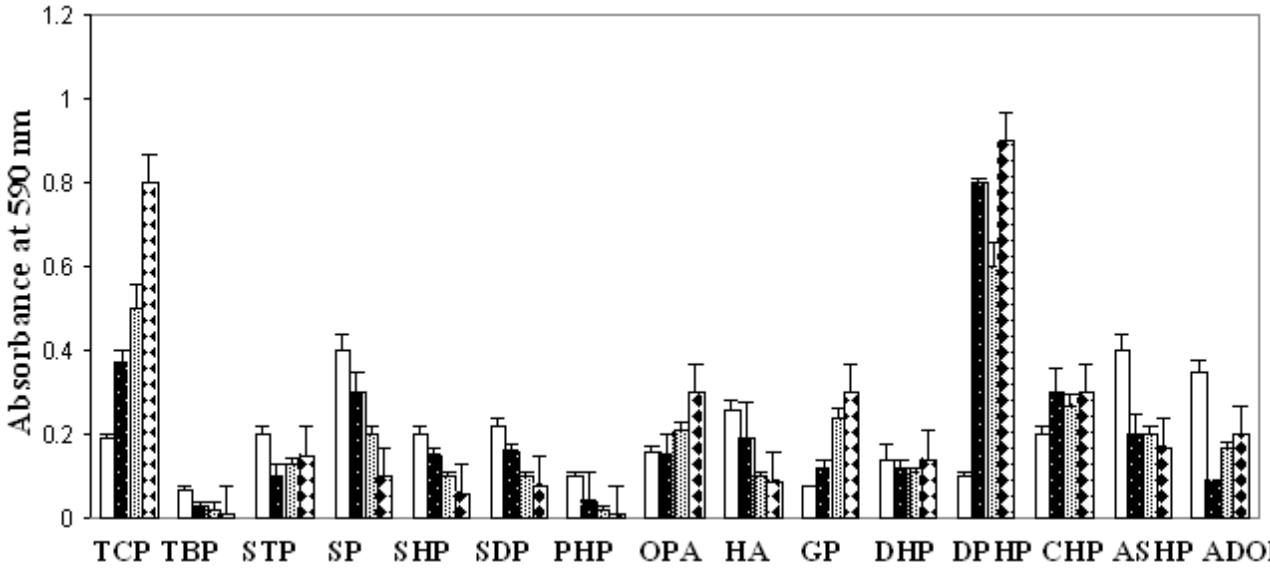

Figure 1. Specific substrate utilization pattern using Biolog MT plates containing 15 different phosphate sources in soil samples collected from Jim Corbate National Park, Nainital (□) Mukhteshwar (■), National Botanical Garden, Lucknow (▨) and Aurawan Research, Aurawan (▩). Tricalcium phosphate (TCP), Tributyl phosphate (TBP), Sodium tripolyphosphate (STP), Sodium phytate (SP), Sodium hexametaphosphate (SHP), Sodium dihydrogen phosphate (SDP), Potassium dihydrogen phosphate (PHP), Orthophosphoric acid (OPA), Hydroxapatite (HA), Glucose-6-PO_4 (GP), di Sodium hydrogen phosphate (DHP), di Potassium hydrogen phosphate (DPHP), Calcium hydrogen phosphate (CHP), Ammonium sodium hydrogen phosphate (ASHP), Ammonium dihydrogen ortho phosphate (ADOP).

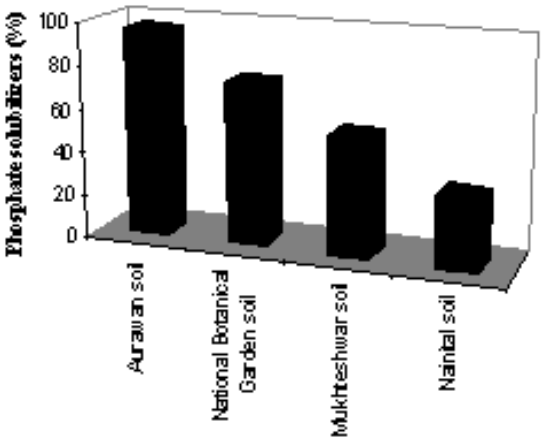

Figure 2. Percent phosphate solublizer present in stressed sites namely: soil samples collected from Jim Corbett National Park, Nainital: Mukhteshwar: National Botanical Garden, Lucknow and Aurawan Research, Aurawan.

was studied with NBRI1213. The stress environment is characterized by salt and temperature as soil is rich in soluble minerals like Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+}, K^+ but deficient in N, P and Fe (Page et al., 1982). P is normally insoluble in soil when the pH is 7.5 - 8.5 making soil deficient in available form of P. Therefore, the bacteria present in soil should possess the ability to solubilize it for uptake. Till date no detailed study has been made on stress tolerant P-solubilizing PGPB.

In the present study, strain NBRI1213 was able to solubilize TCP 984 µg/mL (Figure 5A). No significant difference in the growth of NBRI1213 was observed when it was grown under high salt (5% NaCl) as compared to control conditions (0% NaCl, pH-7 and 30°C) (Figure 5A). However, slight decrease in growth and TCP solubilization was observed at 50°C (Figure 5B). Alkaline pH hardly made any difference in terms of growth and TCP solubilization (Figure 5C). Son et al. (2005) has reported work on the bacteria which is able to solubilize insoluble phosphate under high saline environment. Rock phosphate solubilization was seen in *A. niger*, *Aspergillus japonicus*, and *Penicillium simplicissimum* isolated from wheat rhizosphere. These strains demonstrated high level rock [hosphate solubilization under stress conditions temperature (10 - 45°C), pH (4 - 11), NaCl (0 - 3.5%) and drough (0 - 35% PEG 10000) (Xiao et al., 2011).

Strain NBRI1213 showed changes in morphology under high salt and temperature conditions as compared to control (Figure 6A, B and C). The shape of cells changed to spherical and cell size reduced from 2.07 to 0.33 µm at 50°C (Figure 6A). When grown in the presence of 5% NaCl, the cells appeared to be short rods with rounded edges (Figure 6B). However when relieved from the stress conditions it regained control conditions. Kulkarni and Nautiyal (1999) reported morphological changes in rhizobial strains under abiotic stress. A study

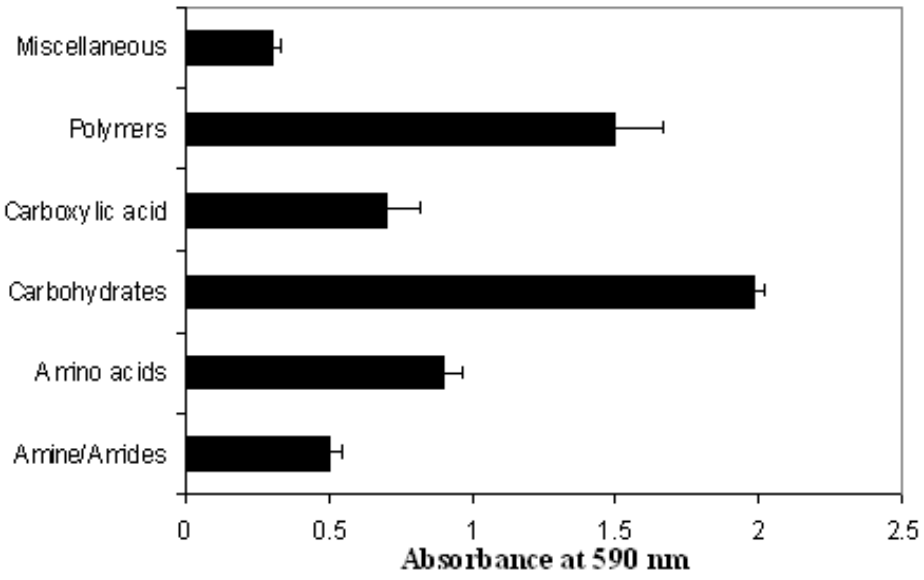

Figure 3. Categorized substrate utilization pattern of potential strain NBRI1213 by Biolog GN2 plate containing 95 carbon sources.

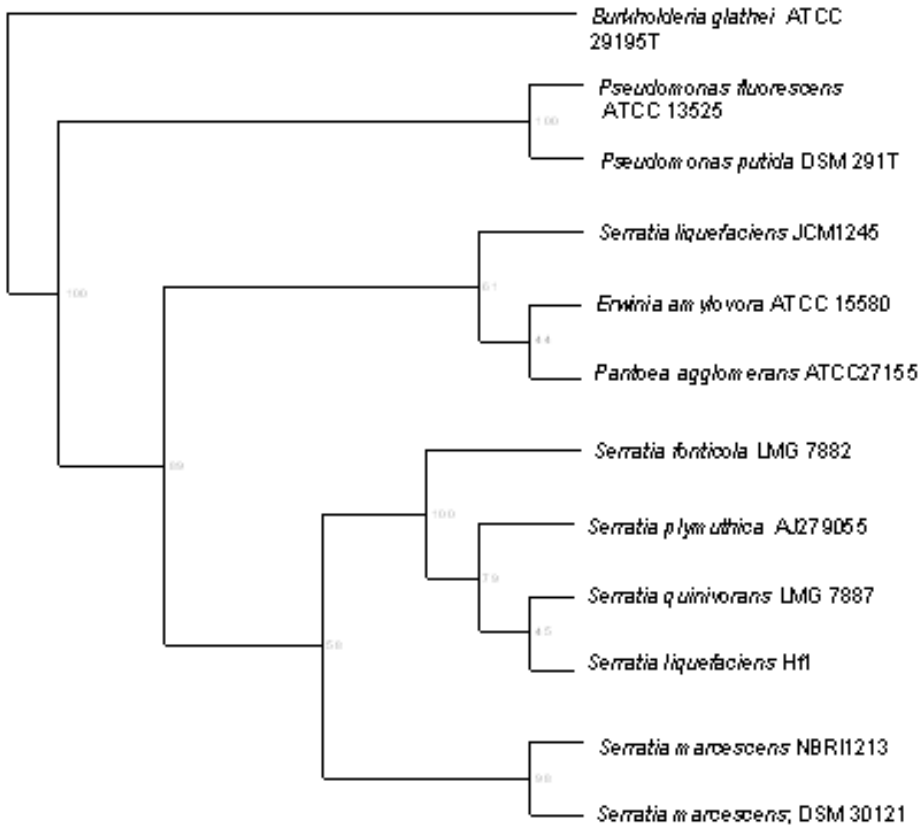

Figure 4. Phylogenetic dendrogram based on 16S rRNA full gene sequences indicating the position of strain NBRI1213 among closely related members. The topology shown is an unrooted tree obtained with a neighbor joining algorithm (Jukes-cantor corrections) with bootstrap value expressed as percentage of 1000 replication. Phylogenetic analyses were conducted in MEGA 4.0. Accession number obtained was JF713819.

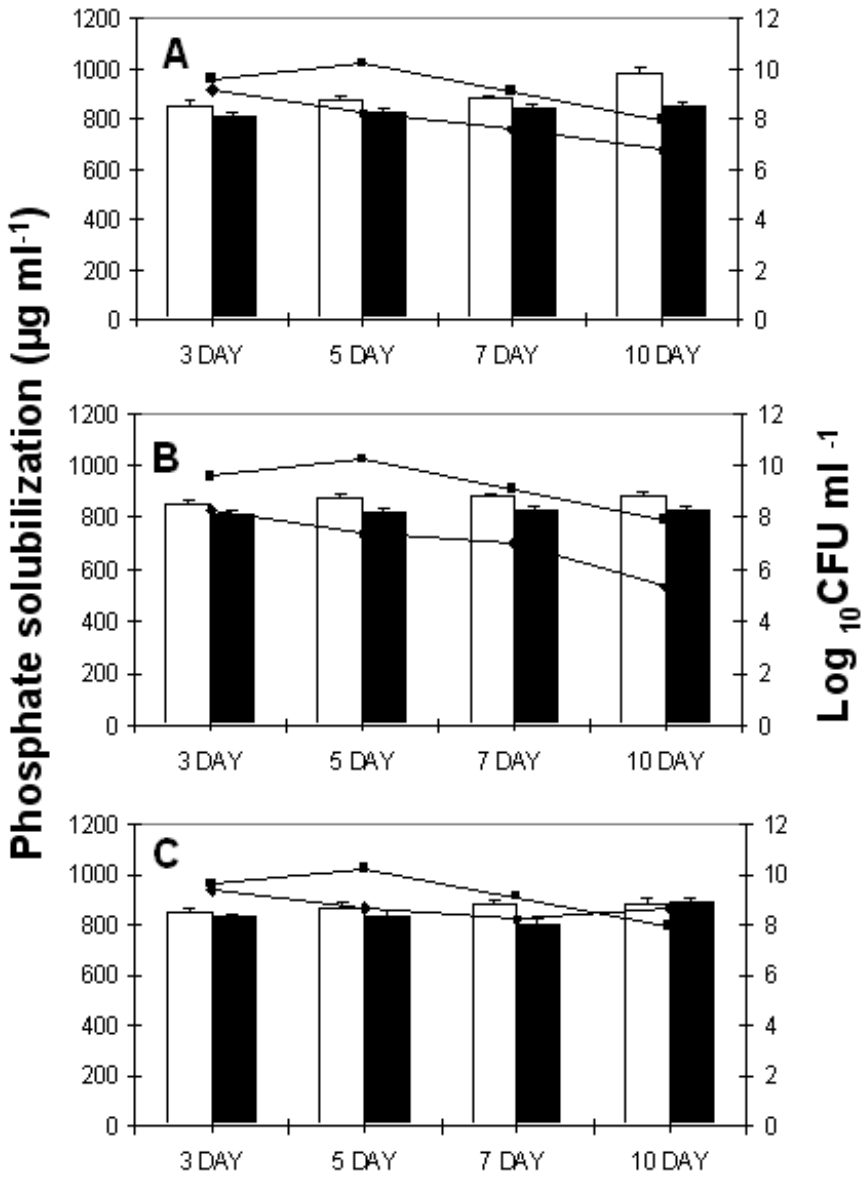

Figure 5. Effect of salt and temperature on growth and TCP-solubilization of NBRI 1213. Phosphate solubilization (Bar chart): **A.** Salt 0% (□), 5% (■); **B.** Temperature 30°C (□), 50°C (■). **C.** pH-7 (□), 10 (■). Growth (Line chart): **A**. Salt 0% (■), 5% (◆); **B**. Temperature 30°C (■), 50°C (◆); **C.** pH-7 (■), 10 (◆).

proposed bacterial suicidal response hypothesis (Atlas and Bartha, 1998) and provided a possible explanation for both the destruction and cessation of cell division under abiotic stress conditions. This hypothesis predicts that when stressful conditions are imposed on organism's morphology is abruptly impeded while metabolism is unaffected. The imbalance between anabolism and catabolism results in a burst of free-radical production that causes significant damage to intracellular components including DNA and proteins and it is the free-radical burst rather than the stress itself that leads to destruction of the cell (Bihong and Xuhua, 2003). Generally, Gram-negative bacteria tend to decrease their cell size after exposure to stress conditions (Bihong and Xuhua, 2003). These changes in morphology of NBRI1213 could be the possible strategies to overcome the stress.

TCP-solubilization by NBRI1213 is accompanied by drop in pH from 7.0 to 3.47. When it was grown at 5% NaCl and 50°C, the final pH of medium became 4.04, 4.09 and 4.40, respectively, this drop in pH is due to abiotic stresses. HPLC analysis of acids produced by NBRI1213 in NBRIP medium (at 30°C, 0% NaCl and pH-7) (Table 2) demonstrated the production of salicylic acid (65.0 mM), citric acid (23.0 mM), oxalic acid (12.7 mM) and acetic acid (0.50 mM) on the 10th day. The produc-

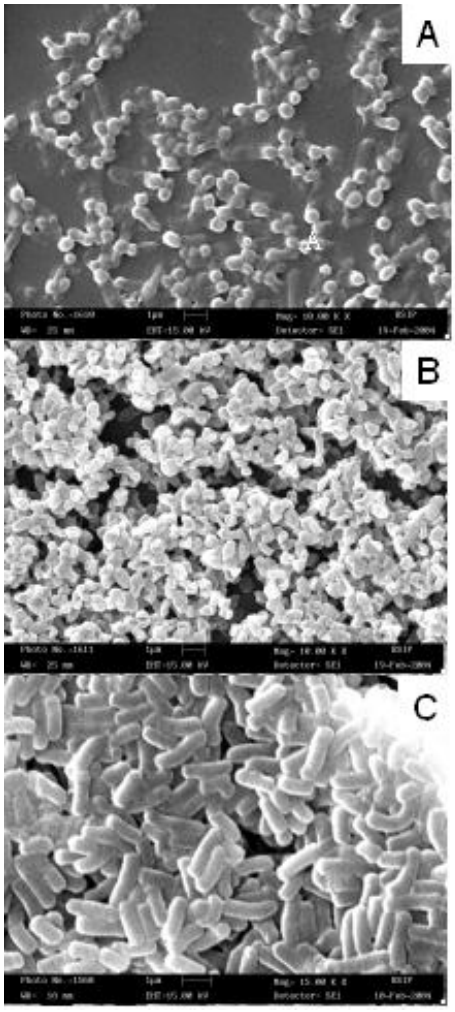

Figure 6. Scanning electron micrographs of NBRI1213 in NBRIP medium under stress conditions. A. At 50°C temperature; B. At 5% NaCl. C. Control (0% NaCl, pH-7 at 30°C temperature)

tion of acetic acid in addition to oxalic acid is likely to be responsible for P-solubilizing ability of NBRI1213 (Table 2). The mechanisms of phosphate solubilization by microorganisms are complex and are not completely known at present.

Microbial mechanisms used to solubilize phosphate include acidification, chelation and exchange reactions. Tricarboxylic acids, such as citric and oxalic and other lower molecular weight organic acids are considered to be the main contributors to phosphate solubilization and a decrease in pH (Hoberg et al., 2005). Hase et al. (2001) reported that production of acetic acid might be due to Na^+ dependent uptake of citrate wherein citrate is transported into the cell at the expense of Na^+ gradient. It is then split into acetate and oxaloacetate by citrate lyase. Oxaloacetate is further metabolized into acetate with other intermediates. This suggests that signaling between bacteria and plant root regulates expression of direct oxidation pathway. The resultant acidification of the rhizosphere played a key role in nutrient availability and/or other eco physiological parameters essential for the survival of the plant. To the best of our knowledge current study clearly demonstrate significant role of salicylic acid in solubilization of TCP by NBRI1213. Gunes et al. (2007) has investigated that salicylic acid acts as an endogenous signal molecule responsible for inducing abiotic stress tolerance in plants.

Plant growth promotion of NBRI1213R was evaluated on *Z. mays* under the micro plot. The growth and colonization by NBRI1213R in non-sterile soil in the maize rhizosphere along with heterotrophic bacterial population was monitored using a chromosomally located rifampicin-marked mutant NBRI1213R. The population of NBRI1213R increased from 2.0×10^8 CFU/g root on 5th day to 1.7×10^9 CFU/g root in non-sterilized soil. After 20th day colonization, NBRI1213R was 10^4 CFU/g root throughout the growing season of 105 days. While during the same period population of heterotrophic bacteria was in the range of 10^7 to 10^9 CFU/g roots (Figure 7).

Under micro plot conditions seeds treated with NBRI1213R showed significant increase in plant growth parameters as compared to un-inoculated control (Table 3). The total concentration of Ct, Nt and Pt of micro plot were 160-240, 7.6-15.3, 60-130 mg/g of soil and soluble phosphate was 0.10 mg/100 of soil. The treatment of maize with NBRI1213R resulted in significantly greater ($p < 0.05$) 12.12 15.0, 54.64, 11.52 and 9.75% increase in shoot length, number of leaves/plant, number of fruits/plant, number of seeds/fruit and weight of 100 seeds, respectively, as compared to un-inoculated control (Table 3). The plant-growth promotion ability of the NBRI1213R was also evident based on the significantly higher (at $p < 0.05$) N, P, and K content than in untreated maize (control), represented by a 65.52, 52.14 and 52.28% increase, respectively (Table 3). PGPB directly affect the metabolism of plants by providing substances that are usually in short supply. These bacteria are capable of solubilizing phosphorus, fixing atmospheric nitrogen, and iron, and producing plant hormones, such as auxins, gibberellins, cytokinins, and ethylene and nitrite and nitric oxide as reported (Bashan and Holguin, 1998; Singh and Raddy, 2011). Additionally, they improve plant tolerance towards drought, high salinity, metal toxicity and pesticide load (Bashan and Bashan, 2005; Bashan et al., 2004; Lucy et al., 2004; Rodriguez et al., 2006). One or more of these mechanisms may contribute to increase in plant growth and development that are higher than the plants grown under standard cultivation.

Conclusion

Now days there are growing demands for biologically based agricultural practices. Recent surveys of both conventional and organic growers indicated an interest in

Table 2. Release of organic acid by NBRI1213 under stress conditions.

Stress condition	Production of organic acid (mM)	Final pH
5% NaCl	Salicylic acid: 56.0 Citric acid: 28.0 Oxalic acid :15.0	4.09
50°C	Salicylic acid: 49.0 Citric acid: 20.0 Oxalic acid :18.0	4.40
pH-10	Salicylic acid: 50.0 Citric acid: 29.0 Oxalic acid :22.0	4.04
Control (at 30°C, pH-7 and 0% NaCl	Salicylic acid: 65.0 Citric acid: 23.0 Oxalic acid :12.7 Acetic acid: 0.50	3.47

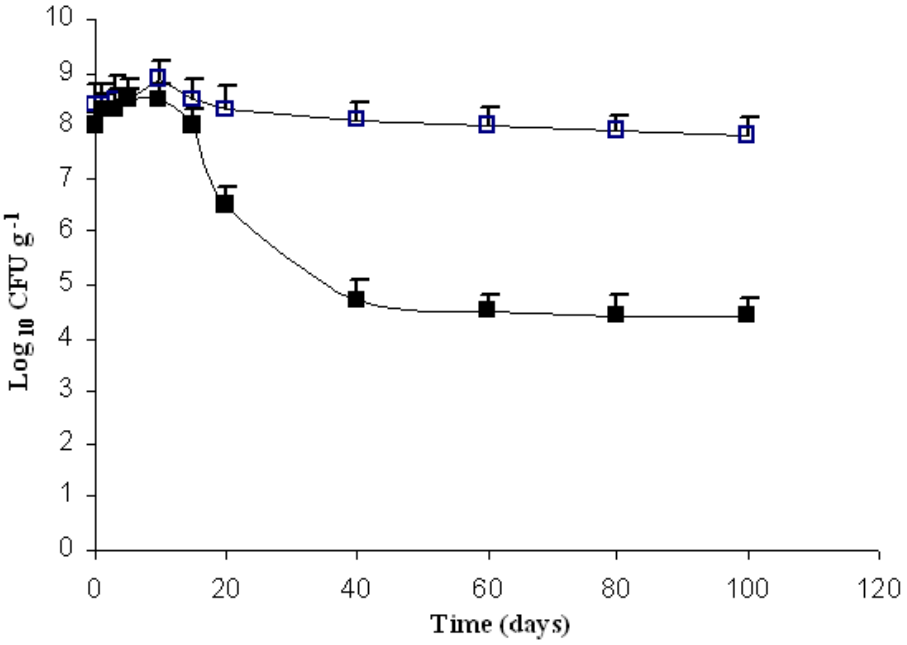

Figure 7. Growth and colonization of NBRI1213R in *Z. mays* rhizosphere. Average rhizosphere colonization of NBRI1213R (log_{10} CFU/g root) was determined at the time of harvesting from 4 plants each at different time interval as indicated. Hetrogenous population (□ ; NBRI1213R (■). Error bars indicate standard deviation.

using biofertilizer products suggesting that the market potential of biofertilizer products will increase in the coming years. *S. marcescens* NBRI1213 can therefore be used as a PGPR agent because its application resulted in better growth even under stressed environment. This strain may be helpful in minimizing the impact of salt and temperatures stresses which is currently limiting crop production under low input conditions and give rise to a more sustainable agriculture.

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Table 3. Evaluation of plant growth promotion by NBRI1213R on *Z. mays* under micro plot conditions.

Evaluation of plant growth promotion	Un-inoculated control [a]	NBRI1213R treated [a]	CD* at 1%
Plant growth parameters			
Shoot length (cm) [b]	187.00±10.11	209.67±11.23	21.87
Number of leaves/plant [b]	153.00±4.82	176.00±5.90	12.55
Number of fruits/plant [b]	1.83±0.22	2.83±0.21	0.50
Number of seeds/fruit [c]	496.02±13.87	553.17±18.17	35.74
Weight of 100 seeds (g) [d]	24.60±0.45	27.00±0.65	1.47
Nutrients uptake			
Nitrogen [N] (mg/g of tissue)	12.01±0.80	19.88±0.74	2.81
Phosphorous [P] (mg/g of tissue)	1.63±0.06	2.48±0.06	0.28
Potassium [K] (mg/g of tissue)	1.53±0.06	2.33±0.10	0.29
Microbial population			
Heterogeneous bacterial population (log_{10} CFU/g root)	8.18	7.79	
NBRI1213R (log_{10} CFU/g root)	0.00	4.02	

[a]Un-inoculated control (untreated) and treated seeds of *Z .mays* with NBRI1213R. [b]mean of shoot length, number of leaves, number of fruits, 3 replicates ± SE, based on 20 plants. [c]mean of number of seeds/fruit 3 replicates ± SE, based on 12 fruits; [d] mean of weight of 100 seeds 3 replicates ± SE, based on 12 fruits; * CD represents critical difference.

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