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Phosphate solubilising diazotrophic bacteria associated with rhizosphere of weedy grasses

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ABSTRACT

The present investigation hypothesizes that the weedy grass species grown in different physiographic regions do harbour potential microbes and shows lot of scope for the identification of novel functional microbes. In the present study, diazotrophic bacteria were isolated from rhizosphere of ten selected grass species and identified using 16S rRNA gene sequencing. The isolates were belonging to the members of alpha Proteobacteria and Firmicutes. Phosphorus solubilizing traits of all the selected diazotrophic isolates were analysed and results revealed that all the diazotrophs were found to solubilise phosphorous in qualitative assay. Influence of phosphorus solubilizing organisms on the pH, titrable acidity, available phosphorus and phosphatase enzyme production were studied. Maximum amount of available phosphorus and phosphatase activity was observed in *Klebsiella* sp. (OR7) (0.96 ± 0.09 µg/ml) and *Staphylococcus saprophyticus* (OR5) (12.9 ±0.10 µg of PNP released/ml/day) respectively. The present compilation of diverse diazotrophs along with phosphorous solubilisation potential suggests that these particular organisms can promote plant growth by more than one mechanism and that these traits could be better exploited as bio-inoculants.

Key words: Bacteria, Diazotrophs, Phosphate solubilising microbes, Weedy grasses

The search for diverse plant growth-promoting (PGP) diazotrophic bacteria is gaining momentum as efforts are made to exploit them as bio-inoculants for various economically important crops. There is a considerable number of rhizospheric microbial species that may have beneficial effects on plant growth and yield. These groups of species, which are known as plant growth-promoting bacteria (PGPB) (Bowen and Rovira 1999) play an important role in soils by transforming some nutrients, which are normally present in less available forms, into bio-available forms. One of the action mechanisms of PGPB to promote plant growth is the solubilization of insoluble phosphates (Rodriguezet *et al*. 2006). Phosphorus (P) is an essential element for all living beings as part of proteins, nucleic acids, membranes, and energy molecules, such as ATP, GTP, and NADPH. Depending on some environmental and biological factors, it can be the main growth-limiting nutrient (Azziz *et al*. 2012). Bacteria of the genera *Pseudomonas, Enterobacter, Bacillus, Proteus, Citrobacter, Klebsiella*, and *Serratia* and some soil filamentous fungi, such as *Aspergillus* and *Penicillium* (Rodríguez and Fraga 1999), solubilize phosphates through mechanisms that involve the production of organic and inorganic acids and the excretion of protons to the media during the assimilation of the NH_4^+. Together, these mechanisms transform insoluble forms of phosphorus into monobasic and dibasic phosphate ($HPO_4^{"2}$, $H_2PO_4^{"}$) available to plants (Whitelaw 1999). In addition, PGPB can promote plant growth by supplying the plant with other nutrients, such as nitrogen; by bio-controlling host plant diseases; byproducing phytostimulators; or by promoting the growth of cellulolytic microorganisms, which are important for nutrient cycling in the soil (Lugtenberg and Kamilova 2009). Despite the agronomic benefits that may be provided by phosphate solubilizing microorganisms (PSMs), their abundance in soil is not always sufficient to compete with other microorganisms established in the rhizosphere. This situation requires the inoculation of plants with PSMs to increase the density found in the soil and to take advantage of their properties in order to increase the productivity of the agricultural ecosystems.

There are numerous studies on PSMs (Chen *et al.* 2006, Azziz *et al.* 2012), related to their abundance, diversity, and phosphate solubilization potential of microorganisms associated with economically important crop plants but studies are lacking on weed. Weedy grass species normally thrive in adverse conditions and act as potential habitats for the diverse groups of elite bacteria with multiple beneficial characters. A more complete understanding of the diver-

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sity and functioning of rhizobacterial microorganisms, especially those that have symbiotic relationships with grass species is of great value for agricultural research and application. The aim of this study is to screen native population from the selected grass species rhizosphere for the isolation of nitrogen fixing and mineral solubilizing bacteria.

MATERIALS AND METHODS

Rhizosphere sampling and isolation of diazotrophs

Ten different grass species along with rhizosphere soil were collected from different physiographic regions (*Brachiaria reptans, Cenchrus glaucus, Saccharum spontaneum, Panicum repens, Cyperus rotundus, Dactyloctenium aegyptium, Chloris barbata, Oryza rufipogon, Cyanodon dactylon* and *Setaria verticillata*) (Table 1). Plants were uprooted carefully and the soil adhering to the root was separated in a sterile Petridish and mixed thoroughly so as to make a composite sample for microbiological analysis. Plant samples and soil samples collected were transported to laboratory in ice box for further analysis. The samples were used immediately for preliminary analyses and stored at 4 °C in a refrigerator for further studies (Pramer and Schmidt 1966). Diazotrophic microorganisms isolated using serial dilution technique (10^{-6} dilution) on selective N-free malate medium (NFM) (Piao *et al.* 2005). After required incubation period, colonies growing on N-free media were counted and grouped according to their morphological characteristics. Single colonies from rhizosphere soil samples picked from NFM plates and sub-cultured several times in same medium to obtain pure cultures and stored as glycerol stocks at -20 ºC.

Identification of diazotrophs by 16S rRNA gene sequencing

Nearly full-length of 16S rRNA gene was amplified from elite isolates as described earlier using universal eubacterial primers, FD1 and RP2 (Weisburg *et al.* 1991) and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using pTZ57R/T vector supplied with TA cloning kit (Fermentas, USA) prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST).

In vitro determination of phosphate solubilising activity

The bacterial cultures were inoculated in to hydroxyapetite medium (*Sperber* 1958). The test organisms were inoculated on these media and incubated (Lab Companion, Korea) at 30°C for 48 h. The diameter of the clearing zones around the colonies were measured. The solubilizing efficiency was calculated as indicated below (Srivastav *et al.* 2004).

$$\text{Solubilization efficiency (\%)} = \frac{\text{Diameter of solubilization zone} - \text{colony diameter}}{\text{Colony diameter}} \times 100$$

One ml of the culture containing 10^9 cell/ml was inoculated into the flasks of Pikovaskaya's broth containing 100 mg of tricalcium phosphate. An uninoculated control was maintained. After 7 days incubation, the contents were centrifuged at 7000 rpm for 10 min and clear supernatant was used for soluble P estimation following method described by Olsen *et al.* (1954). One ml of the culture filtrate was pipette into a 25 ml volumetric flask and diluted to 20 ml with water. Four ml of reagent (1.056 g of ascorbic acid in 200 ml of reagent A) was added and the volume was made up to 25 ml with distilled water. The intensity of blue colour was read in spectrophotometer (Cary 50 Bio, Varian) at 660 nm. The standard curve was prepared with orthophosphate (KH_2PO_4) and amount of P solubilized was calculated by referring to standard graph. The phosphorus content was expressed in terms of mg of phosphorus/ml. Influence of phosphorus solubilizing organisms on the pH and titrable acidity of growth medium was also analyzed.

Phosphatase activity

The phosphatase activity was determined based on the liberation of p-nitrophenol from p-nitrophenol phosphate by colorimetric method (Morton 1952). The phosphatase activity was estimated by adding 1 ml of substrate solution (100 mg of p-nitrophenol phosphate in 100 ml distilled water), 10 ml of acetate buffer and 2 ml of enzyme source. The contents were thoroughly mixed and incubated at room temperature. After 24 h of incubation, 10 ml of assay mixture was withdrawn, centrifuged at 5000 rpm for 15 min. 1 ml of supernatant was mixed with 1 ml of fresh Folin's reagent (prepared by mixing one part of Folin-ciocalteau reagent and one part of distilled water) and 2 ml of 20 per cent sodium carbonate were added and boiled exactly for 1 min. It was immediately removed and the volume was made up to 10 ml and the colour was read in spectrophotometer (Cary 50 Bio, Varian) at 600 nm. The phosphatase activity was calculated and expressed as µg of p-nitrophenol released per ml of culture filtrate.

Statistical analyses

All the data were subjected to statistical analysis with softwares, SPSS (Kirkpatrick and Feenay 2005) and Microsoft Excel for Windows 2007 add-ins with XLSTAT Version 2010.5.05 (XLSTAT 2010). Data was subjected to ANOVA and statistically significant differences between the treatments were analyzed using Duncan's Multiple Range Test (DMRT) at 5 % level of significance.

RESULTS AND DISCUSSION

Soil microorganisms play an important role in soil processes that determine plant productivity. Rhizosphere microbial communities are influenced by the plant exudates, roots as mechanical support and competition for nutrients. Equally, plants are affected by rhizosphere microbial communities through their participation in fast soil nutrients cycle, water dependence and growth promoting metabolites (Buscot and Varma 2005). One of the various mechanisms by which rhizobacteria promote plant growth is by solubilization of insoluble minerals.

Altogether sixty diazotrophic isolates were obtained by using four N-free media after 5 days of incubation. Out of 60 isolates, there were about 16 unique bacterial colonies from different grass species were further reconfirmed as putative diazotrophs by polymerase chain reaction. Based on such data, 16 isolates were selected for further study.

Identification of rhizosphere isolates by 16S rRNA gene sequence homology

The total genomic DNA of all the 16 isolates from rhizosphere were and the 16S rRNA gene of all the isolates was amplified using universal primers FD1 and RP2. All amplified products produced a single band with approximately 1500 bp length and the differences among them were not visible in 1 per cent agarose gel. Comparative BLAST analyses which include the closest species and per cent homology of full length 16S rRNA revealed the presence of diversity of Gamma proteobacteria and Firmicutes (Table 2).

Nearly 19% of diazotrophic isolates showed similarity to *Klebsiella* sp. and *K. pneumonia* respectively. Chelius and Triplett (2000) reported that *K. pneumoniae* as an endophyte in maize. In wheat, Iniquez *et al*. (2004) demonstrated and confirmed the nitrogen fixing activity of *K. pneumoniae*. The nitrogen fixing activity of *K. pneumoniae* isolates were again confirmed by our work. Among the diazotrophic isolates *Serratia* sp. accounted for 12% of which, 6% of isolates belonged to *S. marcescens*. Diverse species of *Serratia* have been isolated from cotton and sweet corn, rice rhizosphere rice seed.

In the present work, firmicutes were mainly dominated by different groups of *Bacillus*, which have been isolated from selected grass species is in accordance with the findings of Chowdhury *et al*. (2009). In the present investigation, *Enterobacter* sp. accounts for 12% of the total diazotrophs members of enterobateriales are known N_2-fixers and one of the most universal of endophytic genera. *Enterobacter* has been identified as endophytes of several plants such as *Citrus sinensis*, soybean, sweet potato and maize Kuklinsky-Sobral *et al*. 2004). Among the diazotrophs, one isolate from *Saccharum spontaneum* (SS4) identified as *Stenotrophomonas* sp. is ubiqui-

Table 1. Grass species from different physiographical regions of India used for the present study

Grass species	Sampling site	Latitude	Longitude	Physiographic region
Brachiaria reptans (water grass)	Barrackpur, Kolkata,West Bengal	88° 34´ 5.1" E	22° 19´ 49.6" N	Indo Gangetic alluvial plain
Cenchrus glaucus (buffel grass)	Chadrapur Ganjam, Orissa	88° 24´ 22.8" E	19° 24´ 21.09" N	Eastern Ghats
Saccharum spontaneum (Wild sugarcane)	Madan Mahal, Jabalpur, Madhya Pradesh	79° 40´ 50.33" E	22° 51´ 17.03" N	Central highlands
Panicum repens (torpedo grass)	Maruteru, West Godaveri, Andrapradesh	80° 59´ 38.86" E	16° 30´ 39.7" N	Deccan Plateau
Cyperus rotundus (nut grass)	Chickarasinikere, Mandya, Karnataka	77° 3´ 35.9" E	12° 17´ 34.78" N	Reverain land form
Dactyloctenium aegyptium (crowfoot grass)	Kasargod, Kerala	75° 7´ 59.81" E	12° 24´ 31.4" N	Kerala plains
*Chloris barbata (*finger grass*)*	Thavalakuppam, Pudhucherry	76° 46´ 54.7" E	11° 23´ 12.6" N	Coastal plains
Oryza rufipogon (wild rice)	Gudalur, Ooty, Tamil Nadu	79° 51´ 33.1" E	11° 54´ 32.52" N	Western Ghats
Cyanodon dactylon (bermuda grass)	Navalurkutapattu, Trichy, Tamil Nadu	79° 46´ 34.9" E	10° 33´ 21.32" N	Reverain land form
Setaria verticillata (bristly foxtail)	Thirupoondi, Nagapattinam, Tamil Nadu	79° 53´ 37.6" E	10° 46´ 25.67" N	Coastal plains

Table 2. Authentication of diazotrophic isolates from grass species of different physiographic regions by 16S rRNA gene sequence homology

Isolate	Grass species	Species homology[a]	Percent homology[b]	Gen bank accession no.
BR1	*B. repens*	*Enterobacter* sp.	99	KF906826
CG1	*C. glaucus*	*Klebsiella* sp.	94	KF906827
CG3	*C. glaucus*	*Enterobacter* sp.	98	KF906828
CG5	*C. glaucus*	*Bacillus* sp.	99	KF906830
SS4	*S. spontaneum*	*Stenotrophomonas* sp.	98	NS
CR2	*C. rotundus*	*Klebsiella pneumoniae*	98	NS
CR3	*C. rotundus*	*Klebsiella pneumoniae*	99	KF906829
CB2	*C. barbata*	*Serratia* sp.	99	KF906831
CB3	*C. barbata*	*Bacillus subtilis*	98	NS
CB4	*C. barbata*	*Klebsiella* sp.	98	NS
OR3	*O. rufipogon*	*Serratia* sp.	96	KF906832
OR5	*O. rufipogon*	*Staphylococcus saprophyticus*	99	KF906833
OR7	*O. rufipogon*	*Klebsiella* sp.	98	KF906834
CD1	*C. dactylon*	*Serratia marcescens*	97	KF906835
CD2	*C. dactylon*	*Bacillus* sp.	99	NS
SV1	*S.verticillata*	*Klebsiella pneumoniae*	99	KF906836

[a]Species identified based on the 16S rRNAgene sequence similarity by BLAST ; [b] Per cent similarity of the isolate's sequence in BLAST result; NS-Sequence not submitted

Table 3. Mineral solubilizing potential of diazotrophs isolated from selected grass species collected from different physiographic regions

Isolate	Colony diameter (mm)	Solubilization zone (mm)	Solubilization efficiency (%)
Enterobacter sp.(BR1)	5.0	12	140 (± 11.64)^{ef}
Klebsiella sp.(CG1)	9.0	12	33 (± 0.98)^{j}
Enterobacter sp.(CG3)	4.0	6.0	50 (± 2.17)^{ij}
Bacillus sp. (CG5)	5.0	8.0	60 (± 1.14)^{ij}
Stenotrophomonas sp. (SS4)	4.0	13	225 (± 12.16)^{ab}
K. pneumonia (CR2)	3.0	9.0	200 (± 2.64)^{bc}
K. pneumonia (CR3)	4.0	10	150 (± 11.19)^{def}
Serratia sp. (CB2)	4.0	14	250 (± 13.53)^{a}
B. subtilis (CB3)	4.0	9.0	125 (± 11.29)^{fg}
Klebsiella sp.(CB4)	4.0	10	150 (± 12.10)^{def}
Serratia sp.(OR3)	3.0	10	233 (±1 3.10)^{a}
S. saprophyticus (OR5)	3.0	8.0	167 (± 11.54)^{de}
Klebsiella sp.(OR7)	3.0	9.0	200 (± 12.64)^{bc}
S. marcescens (CD1)	8.0	14	75(±1.14)^{hi}
Bacillus sp.(CD2)	5.0	10	100 (± 13.19)^{gh}
K. pneumonia (SV1)	3.0	8.0	167 (± 12.63)^{de}
P. fluorescens (Pf1)*	5.0	15	200 (± 12.69)^{bc}

ND- Solubilization not detected. Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT (p=0.05); *Standard strain

tous and often associated with plants has reportedly been isolated from rhizosphere of grass, wheat, oat, cucumber, maize, oilseed rape, potato and lettuce. It is not yet defined as nitrogen fixing bacteria despite their nitrogen fixing activities have been investigated and confirmed (Liu *et al*. 2007). Cibichakravarthy *et al*. (2011) isolated the diazotroph *Stenotrophomonas maltophila* from the rhizosphere of *Prosopis*. Roots of *Spartina alterniflora*, a common smooth cordgrass growing in salt marsh of North America harboured several gamma proteobacterial diazotrophs (Bagwell and Lovell 2000).

In vitro phosphorus solubilizing potential of diazotrophic isolates

A survey of Indian soils revealed that 98 per cent of these need phosphorus fertilization either in the form of chemical or biological fertilizer. Application of

Table 4. *In vitro* P-solubilizing potential of diazotrophic isolates from the rhizosphere of grass species collected from different physiographic regions

Isolate	pH	TA (%)	Available P (µg/ml)	Phosphatase (µg of PNP released/ml/day)
Enterobacter sp.(BR1)	4.0 (± 0.04)^{a}	2.4 (± 0.01)$^{c-g}$	0.47 (± 0.04)^{ghi}	5.8 (± 0.16)^{def}
Klebsiella sp.(CG1)	4.0 (± 0.03)^{a}	3.0 (± 0.03)^{abc}	0.69 (± 0.03)^{cde}	5.3 (± 0.10)^{def}
Enterobacter sp.(CG3)	4.0 (± 0.04)^{a}	2.1 (± 0.05)^{fgh}	0.81 (± 0.01)^{ab}	5.3 (± 0.11)^{def}
Bacillus sp. (CG5)	4.4 (± 0.04)^{a}	1.8 (± 0.01)^{gh}	0.36 (± 0.03)^{hij}	5.3 (± 0.15)^{def}
Stenotrophomonas sp. (SS4)	4.0 (± 0.01)^{a}	1.9 (± 0.01)^{gh}	0.68 (± 0.02)$^{c-f}$	2.4 (± 0.14)^{g}
K. pneumoniae (CR2)	4.0 (± 0.01)^{a}	1.8 (± 0.04)^{gh}	0.56 (± 0.06)^{efg}	4.5 (± 0.11)^{f}
K. pneumoniae (CR3)	4.0 (± 0.00)^{a}	2.9 (± 0.04)$^{a-d}$	0.86 (± 0.01)^{ab}	6.5 (± 0.08)^{cd}
Serratia sp. (CB2)	4.2(± .001)^{a}	2.3 (± 0.03)$^{d-g}$	0.68 (± 0.04)$^{c-f}$	5.4 (± 0.34)^{def}
B. subtilis (CB3)	4.3 (± 0.01)^{a}	3.1 (± 0.14)^{ab}	0.54 (± 0.01)^{efg}	4.4 (± 0.21)^{f}
Klebsiella sp.(CB4)	4.0 (± 0.04)^{a}	2.2 (±0.05)$^{e-h}$	0.25 (± 0.01)^{j}	5.6 (± 0.26)^{def}
Serratia sp.(OR3)	4.0 (± 0.04)^{a}	3.4 (± 0.01)^{a}	0.26 (± 0.01)^{j}	5.3 (± 0.25)^{def}
S. saprophyticus (OR5)	4.0 (± 0.06)^{a}	2.9 (± 0.04) $^{a-d}$	0.35 (± 0.03)^{ij}	4.5 (± 0.04)^{f}
Klebsiella sp. (OR7)	4.0 (± 0.14)^{a}	3.2 (± 0.02)^{ab}	0.36 (± 0.02)^{hij}	4.4 (±0.16)^{f}
S. marcescens (CD1)	4.0 (± 0.01)^{a}	2.3 (± 0.05)$^{d-g}$	0.85 (± 0.04)^{ab}	12.9 (± 0.10)^{a}
Bacillus sp.(CD2)	4.0 (± 0.05)^{a}	3.1 (± 0.06)^{ab}	0.96 (± 0.09)^{a}	10.9 (± 0.29)^{b}
K. pneumoniae (SV1)	4.0 (± 0.07)^{a}	2.8 (± 0.08)$^{a-e}$	0.48 (± 0.03)^{ghi}	7.5 (± 0.24)^{c}
P. fluorescens (Pf1)*	4.0 (± 0.03)^{a}	2.3 (± 0.02)$^{d-g}$	0.75 (± 0.01)^{bcd}	5.6 (± 0.08)^{def}
Control	6.6 (± 0.01)^{b}	0.2 (± 0.06)^{i}	0.02 (± 0.04)^{k}	0.05 (± 0.02)^{h}

Values are mean (± SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as detected by DMRT (p=0.05); *Standard strain

chemical phosphatic fertilizers is practised though a majority of the soil P reaction products are only sparingly soluble. Under such conditions, microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. P solubilization by plant-associated bacteria has been well documented in a number of studies (Stoltzfus and de Bruijn 2000).

In the qualitative assay, all the sixteen rhizosphere diazotrophs were found to be positive where, *Serratia* sp. (CB2) and *S. marcescens* (CD1) exhibited maximum solubilization efficiency of 250 and 233 per cent respectively were observed in the present study (Table 3). However, the most efficient phosphate solubilizing bacteria were reported from genera *Bacillus* and *Pseudomonas* from the rhizosphere of legumes, cereals (rice and maize), arecanut palm, oat, jute and chilli (Kole *et al.* 1998).

Influence of phosphorus solubilizing organisms on the pH titrable acidity, available phosphorus and phosphatase enzyme production was studied and the results are given in Table 4. In general, pH of the medium was decreased with growth of all phosphorus solubilizing isolates. Among the sixteen rhizosphere isolates, not much variation pH reduction was observed but for the titrable acidity increased due to the growth of phosphorus solubilizing organisms. Maximum titrable acidity of 3.4 ±0.01 per cent was found with *S. marcescens* (CD1). The amount of available phosphorus was significantly higher in *Klebsiella* sp. (OR7) (0.96 ± 0.09 µg/ml) compared to the other isolates. The phosphatase activity was higher in *Staphylococcus saprophyticus* (OR5) (12.9 ±0.10 µg of PNP released/ml/day) followed by *Klebsiella* sp. (OR7) (10.90 ± 0.29 µg of PNP released/ml/day) (Table 4). These results confirm the known relation of phosphate solubilization with pH and the release of organic acids as one of the mechanism for the solubilization of $Ca_3(PO4)_2$, as was also reported by Vazquez *et al.* (2000). Solubilization can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion or production of chelating agents (Nahas 1996). The production of gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Several other mechanisms such as production of other inorganic acids such as sulphuric acid, nitric acid and carbonic acid have also been reported (Seshadre *et al.* 2002). In the present investigation, nitrogen fixing and mineral solubilizing activities were found in most of the diazotrophic strains. It is therefore probable that they can be used as bacterial inoculate to support growth and development of crop plants *in vitro* and in field experiment. These novel efficient isolates with plant growth promoting activity obtained from the weedy grass rhizosphere may be employed in nutrient deficient and problematic soils for stress mitigation and sustainable crop cultivation with fewer chemical inputs.

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