J. Cotton Res. Dev. **28** (2) 311-315 (July, 2014)

Exploring the potential of phosphate solubilising diazotrophic *Pseudomonas* sp Db76 as plant growth promoter for *Bt* cotton

RAJESH GERA*, VARUN KUMAR, MONIKA KAYASTH, MEENU WALIA, SURJEET SINGH AND SNEH GOYAL

Department of Microbiology, CCS Haryana Agricultural University, Hisar 125 004
**E mail: rajeshgera1967@gmail.com*

ABSTRACT : A cultivable bacteria associated with rice rhizosphere was isolated by using N free media from Dadupur, Karnal. *nif* H gene amplification in strain Db76 confirmed it as nitrogen fixer. The strain exhibited the plant growth promoting attributes of IAA production, ammonia excretion and siderophore production. It was able to produce IAA (231.16 µg/ml) and ammonia (156.70µg/ml) at 30°C. A significant increase in the growth of *Bt* cotton plant was recorded on inoculations under controlled conditions. On the basis of partial 16S rRNA sequencing, Db76 was identified as *Pseudomonas* sp. These results demonstrate that *Pseudomonas* sp has the promising PGP attributes to be developed as a biofertilizer to enhance soil fertility and promote the plant growth of *Bt* cotton.

Key words *Bt* cotton, IAA, phosphate solubilisation, *Pseudomonas* sp, 16S rRNA

Soil bacteria which beneficially influence the growth of the plants have been used in crop production for decades. Free living soil bacteria beneficial to plant growth are usually referred to as plant growth promoting rhizobacteria (PGPR), capable of promoting plant growth by colonizing the plant root. PGPR have been reported to directly enhance plant growth by a variety of mechanisms. After N_2 fixation, phosphate (P) solubilization is the most important plant growth promoting activity. A large proportion of soluble inorganic phosphate added to the soil is fixed as insoluble forms soon after the application and become unavailable to the plants. Several soil bacteria particularly belonging to genera *Bacillus* and *Pseudomonas,* possess the ability to change insoluble forms into soluble form (Kumar *et al.*, 2012). The application of plant growth promoting rhizobacteria (PGPR) as crop inoculants for biofertilization would be an attractive alternative to decrease the use of chemical fertilizers which cause environmental pollution. Free living nitrogen fixing bacteria or associative nitrogen fixers, for example, bacteria belonging to the species *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas*, have been shown to attach to the root and efficiently colonize root surfaces. However, among them *Pseudomonas* sp is widespread bacteria in agricultural soils and has many traits that make them well matched as PGPR. The most effective strains of *Pseudomonas* are gram negative, rod shaped bacteria and have various phytobeneficial traits. Mantelin and Touraine, 2004. These attributes make PGPR prime interest for agricultural microbiologists in enhancing the crop yield.

Cotton (*Gossypium* sp), the "White Gold", is one of the most important commercial fibre crops. Like in non *Bt* cottons, biotic factors such as bacteria are becoming important in the *Bt* cotton cultivation (Johri *et al.*, 2003). In the past years, various researches were carried out worldwide on the use of bacterial isolates in enhancing the growth of cotton plants (Saharan and Nehra, 2011) but the effect of bacterial isolates on the growth of *Bt*-cotton has not been reported yet. By keeping this in view, the present study was designed to isolate and identify a potential strain of *Pseudomonas* sp from soil with effective plant growth promoting activity and evaluating its effect on the growth of *Bt* cotton.

MATERIALS AND METHODS

Isolation of bacteria : The soil used for bacterial isolation was collected from Dadupur, Karnal. The processed soil sample was serially diluted, spread plated on Dobereiner agar (DB) plates, which contained (in g/L); malic acid (5.0), K_2HPO_4 (0.6), KH_2PO_4 (0.4), $MnSO_4$ (0.01), $MgSO_4.7H_2O$ (0.05), NaCl (0.02), $Na_2MO_4.2H_2O$ (0.002), KOH (4.0) and bromothymol blue (2mL of

0.5% alcoholic solution); pH was adjusted to 6.6-7.0 and incubated at 28°C for 48 h. Different morphotypes were selected and purified on DB agar slants.

Genomic DNA isolation and *nif*H gene amplification : All the DNA preparations were treated with RNase A and the DNA concentrations were estimated by visual examination of ethidium bromide stained agarose gels as well as by spectrophotometric examination. The amplification of *nif*H gene was carried out by using bacterial DNA as template with primers *nif*H forward 5'- TAY GGN AAR GGN GGHATY GGY ATC- 3' and *nif*H reverse 5'-ATR TTR TTN GCN GCR TAV ABB GCC ATC AT-3' where Y represents C/T, N represents A/C/G/T, R represents A/G, H represents A/C/T, B represents G/C/T and V represents A/G/C (Sarita *et al.*, 2007).

Characterization of *nif*H positive isolate for PGP traits

Determination of phosphate solubilising ability : The ability of the bacterial isolate to solubilize insoluble inorganic phosphate was tested by spotting 10 µl of overnight culture on Pikovskaya's agar plate and incubating at 28-30°C for 2-3 days. The isolates showed clear zone of solubilization of tricalcium phosphate (TCP) around the colony were noted as phosphate solubilizer. The dia of the zone of TCP solubilization was measured and solubilization Index (SI) was calculated.

Estimation of indole-3-acetic acid (IAA) production : For the estimation of Indole-3-acetic acid (IAA), diazotrophic bacterial culture was grown in duplicate in 25 ml TY broth to have the log phase of the cells. 10 µl of the culture was spotted on Dobereiner media plates supplemented with DL-tryptophan at a concentration of 0.05g/500ml media. The plate was incubated at 28+2°C for 4 days. After incubation, when there was a good growth on the spot, the hole was made in the centre of the spot and 300 µl of Salkowski reagent was added to the hole. The plate was incubated at 28±2°C till the development of pink colour. Salkowski reagent was taken from the hole in a microtiter plate and absorbance was taken at 500 nm on Enzyme Linked Immunosorbant Assay (ELISA) reader against the blank.

Production of ammonia : *nif*H positive bacterial isolate was tested for the production of ammonia in peptone water. Freshly grown culture was inoculated in 5 ml sterilized peptone water containing tube and incubated at 28+2°C for 4 days. After 4 days, 1 ml of Nessler's reagent was added to peptone water and shaked thoroughly. Culture broth (1.5 ml) from this tube was put in eppendorf tube and centrifuged at 12000 rpm for 15 min. Supernatant was taken in a cuvette and absorbance was measured at 450 nm in a spectrophotometer.

Siderophore production : Production of siderophore by *nif*H positive culture was assessed by plate assay. Chrome Azurol S blue agar medium (CAS) was used to detect siderophore production by the isolate using universal method. The overnight grown bacterial culture was spotted on CAS plate and incubated at 30^0C for 24 h. The culture showed yellow color zone around the colony and was taken as positive for siderophore production.

Pot house experiment : A pot house experiment was conducted to evaluate the effect of Db76 strain on the yield of *Bt* cotton. (RCH 134) Seeds were surface sterilized with 95 per cent ethanol followed by immersing in 0.2 per cent mercuric chloride solution for 3 min and rinsed 10 times with sterile distilled water prior to soaking in 3–4 day old culture (10^7–10^8 cells/ml) of Db76 strain for 10 min. The seeds soaked in un-inoculated culture media served as control. The soaked seeds (seeds/pot) were sown, filled with 5kg soil obtained from CCSHAU fields and the recommended rate of fertilizers (RDF) was given. The experiment was laid out in completely randomized block design with three treatments and three replications. Treatments were as follows- T_1- RDF (100%) (Control), T_2- RDF (100%) + *Azotobacter chroococcum* (HT54), T_3- RDF (100%) + Db 76. Plants were irrigated regularly with distilled water.Plant height and dry weight were noted 90 days after sowing (DAS). The physico chemical properties of soil were observed before the start of experiment (Table 1).

16S rRNA gene amplification and phylogenetic analysis : Polymerase chain reaction (PCR) ampliûcation of bacterial 16S rRNA gene of diazotrophic bacterial isolate was performed using the primers BAC 27 F 5'- AGA GTT TGA TCC TGG CTC AG - 3' (Lukow *et al.*, 2000) and BAC 1378 R 5'- CGG TGT GTA CAA GGC CCG GGA ACG - 3'. PCR was set up in 50.0 µl volume/reaction, containing 39 µl sterilized distilled water; 5 µl of PCR buffer (10X); 1.0 µl of dNTP mixture (10 mM); 2.0 µl of each forward and reverse primer (10 µM); 1.0 µl of taq DNA polymerase (3U/µl) and 2.0 µl of template DNA (50 ng/µl approx.). Ampliûcation conditions were as follows: 3 min of initial denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with the last cycle followed by a 10 min extension step at 72 °C. PCR product was separated on an agarose gel and photographed under UV illumination with Gel Doc (DNR Bio-Imaging Systems). The amplified PCR product was sent for sequencing and the partial sequence of 16S rRNA gene of the bacterial isolate obtained was compared with those available in Gen Bank databases using NCBI BLASTN program for identification of the isolate. The phylogenetic analysis was performed by constructing a phylogenetic tree through neighbour joining method using MEGA4 software (Tamura *et al.*, 2007).

Accession number : The partial 16S rRNA gene sequence of strain Db76 obtained in this study was submitted in GenBank database under accession number: JQ437543.

RESULTS AND DISCUSSION

Bacterial isolation was done by spreading serially diluted sample on nitrogen free media (Dobereiner) and a total of 5 different morphotypes were obtained which were selected on the basis of their colony color, shape and size. Genomic DNA of all the selected isolates was extracted and then amplified for *nif* H gene. Only one isolate Db76 was found *nif*H positive (Fig. 1). This isolate was screened for gram nature and other morphological characteristics and found gram negative rods, colony color was observed as pale yellow. Db76 strain was further assessed for other plant growth promoting attributes.The PGP traits of the selected bacterial isolate are shown in Table 2. The isolate was able to produce significant amount of indole-3-acetic acid (231.16 µg/ml) in the presence of L Tryptophan; the later considered as the IAA precursor in bacteria, because its addition to IAA producing bacterial cultures promote and increases IAA synthesis. Production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several PGPR belonging to the genera *Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Pantoea, Pseudomonas*, and *Serratia*. The isolate Db76 also exhibited strong production of ammonia (156.70 µg ml^{-1}) which is taken up by plants as a source of nitrogen for their growth (Ahmad *et al.*, 2008). In addition, Db76 showed ability to solubilize P on Pikovskaya medium plate which was confirmed by formation of a clear zone around the culture spot (Fig. 2).

Table 1. Evaluation of physico chemical properties

Soil characteristics	Values
pH	6.87
EC (d/Sm)	1.02
Organic C (%)	0.34
Available N (kg/ha)	133.0
Available P (kg/ha)	24.59
Available K (kg/ha)	658.0

Table 2. Characterization of strain Db76 for specific PGP traits

Morphological characters and PGP traits	Db 76
Gram nature	-
Colony shape	Rod
Colony colour	Pale yellow
Ammonia excretion (µg/ml)	156.70
IAA production (µg/ml)	231.16
Phosphate solubilization	4.2
Siderophore production	+

Table 3. The effect of reference strain (HT54) and isolate (Db76) on the plant height and dry weight of *Bt* cotton plants

Treatment	Height (cm)	Dry weight (g/plant)
T_1	42.58±1.409	5.79±0.040
T_2	49.66±1.045*	6.02±0.167¥
T_3	55.49±2.073**	8.13±0.109***

Data represent the means ± SEM in each group. ***P<0.001; **P<0.01; *P<0.05 and ¥non significant

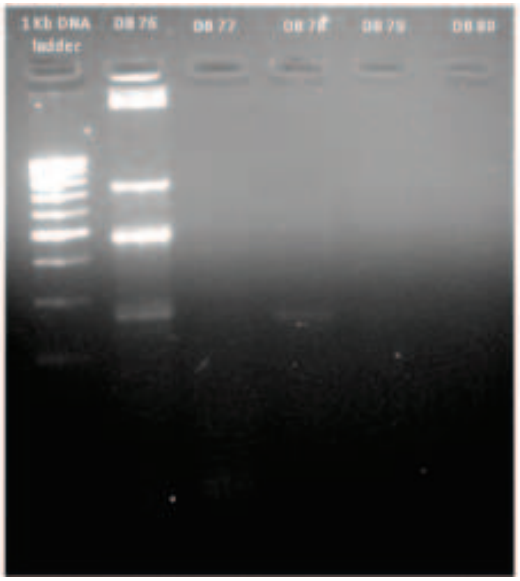

Fig 1. Amplification of *nifH* gene
Lane 1: 100 bp DNA ladder; lane 2: Db76; lane 3: Db77; lane 4: Db78; lane 5: Db79 and lane 6: Db80.

Fig 2: Phosphate solubilising activity of strain Db76.

This is supported by a study which showed that free-living P solubilizing bacteria solubilize precipitated phosphates and enhance phosphate availability to plant that represent a possible mechanism of plant growth promotion under field conditions. Db76 also exhibited positive siderophore production which was indicated by change in color of CAS agar plate from blue to yellow around the culture spot. The siderophore producing microorganisms suppress some soil borne fungal pathogens in the rhizosphere by chelating iron. The plant growth promoting potential of strain Db76 was tested under pot house conditions by inoculating the bacterial culture on *Bt* cotton seeds. The strain caused statistically significant increases in plant height (30.3%) and dry weight (40.4%) compared to the uninoculated control plants (Table 3). Positive effects across *Bt* cotton plants were also observed with reference strain HT54 but in contrast to the strain DB76, inoculation of HT54 resulted in less increase in plant height (16.6%) and dry weight (3.9%) over uninoculated controls. This is probably due to the possession of multiple plant growth promoting traits by the strain DB76 and thus, indicates that this strain has the potential to be used as bioinoculant for *Bt*-cotton. Molecular analysis based on 16S rRNA homology of 1351-bp partial sequence confirmed that strain Db76 belongs to *Pseudomonas* genus. The phylogenetic analysis performed by the construction of phylogenetic tree also showed that the strain DB76 fell within the cluster comprising *Pseudomonas* sp (Fig. 3). The bacterial identification by analyses of partial 16S rRNA gene has been proved to be a powerful tool and is supported by many researchers (Naz and Bano, 2010). Apart from diazotrophy, the strain Db76 (*Pseudomonas* sp.) may be better exploited as bioinoculant since it possess other plant growth promoting characteristics, in particular IAA, ammonia excretion, phosphate solubilisation and siderophore production. Further elucidation of its role in plant growth under pot culture will clarify the potential of this associative diazotroph as potential bioinoculant for *Bt*-cotton.

REFERENCES

Ahmad, F., Ahmad, I. and Khan, M.S. 2008. Screening of free living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* **163** : 173–81.

Johri, B.N., Sharma, A. and Virdi, J.S. 2003. Rhizobacterial diversity in India and its influence on soil and plant health. *Adv Biochem Engin/Biotechnol* **84** : 49-89.

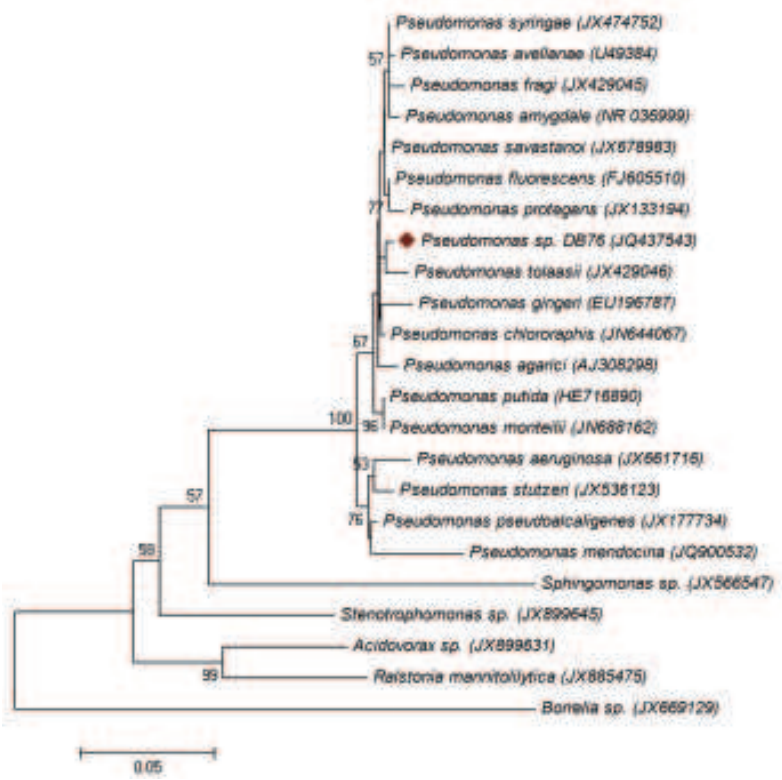

Fig 3. Neighbour joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of *Pseudomonas* sp Db76 and other related taxa. *Borrelia* sp. was used as an outgroup. Bootstrap percentages (based on 1000 replicates) more than 50 per cent are shown at branch points. Bar, 0.05 changes/ nucleotide position.

Kumar, A., Kumar, A., Devi, S., Patil, S., Payal, C. and Negi, S. 2012. Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an *in vitro* study. *Recent Res. Sci. Technol.* **4** : 01-05.

Mantelin, S. and Touraine, B. 2004. Plant growth promoting bacteria and nitrate availability impacts on root development and nitrate uptake. *J. Exp. Bot.* **55** : 27-34.

Naz, I. and Bano, A. 2010. Biochemical, molecular characterization and growth promoting effects of phosphate solubilising *Pseudomonas* sp. isolated from weeds grown in salt range of Pakistan. *Plant soil.* **334** : 199-207.

Saharan, B.S. and Nehra, V. 2011. Assessment of plant growth promoting attributes of cotton (*Gossypium hisutum*) rhizosphere isolates and their potential as bio inoculants. *J Environ Res. Dev.* **5** : 575-83.

Sarita, S., Priefer, U., Prell, J. and Sharma P.K., 2007. Diversity of *nifH* gene amplified from rhizosphere soil DNA. *Curr. Sci.* **94** : 109-114.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24** : 1596-99.

Received for publication : June 8, 2012
Accepted for publication : February 26, 2013