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Nitrogen Fixation and Indole Acetic Acid Production Potential of Bacteria Isolated from Rhizosphere of Sugarcane (*Saccharum officinarum* L.)

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Abstract: Twelve bacterial strains were isolated from root and rhizosphere samples collected from different sugarcane growing areas. Of these strains, ten strains were identified as *Pseudomonas* and two as *Azotobacter* on the basis of colony- and cell-morphology. Among isolates the presence of nif*H* gene was detected only in two *Azotobacter* strains (Azoto1 and Azoto2). Acetylene reduction activity of the strains Azoto1 and Azoto2 determined in N-free medium was 1966 and 4210 nmole C_2H_4/vial/24h, respectively. All isolates showed IAA production in growth medium containing tryptophane as a precursor. Maximum IAA production (4.49mg/L) was detected in isolate A17 where as IAA production in strains A4 and A11 was also significant. Values for IAA production by nitrogen fixing isolates Azoto1 and Azoto2 were comparatively low (0.2 and 0.1mg/L respectively). For rapid screening of bacterial isolates from sugarcane, heterologous plant host (sorghum) was used as a test plant. Most of the strains showed beneficial effects on root length, root area and plant dry weight which were comparable to those observed in treatments where confirmed PGPR were used as positive control. Beneficial effects of inoculation on sugarcane grown in pots were also observed.

Key words: Indole acetic acid · *Azotobacte* · Rhizobacteria · Nitrogen fixation · Pseudomonas

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are living, soil borne bacteria isolated from rhizosphere, which when applied to the seeds and crops enhance the growth of the plant or reduce the damage through the soil borne plant pathogens [1]. PGPR are important for the agriculture because PGPR can only be the best alternative to decrease the production cost of the crops. PGPR colonize the rhizosphere of the plants and impose beneficial effects on the plants either symbiotically or non-symbiotically. During the late 19th and early 20th centuries inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers. Due to the growth in human population, fertilizers were used to increase the crop production to meet the rising demands for food. Increase in the production cost and the hazardous nature of chemical fertilizers for the environment has led to the resurgence of interest in the use of biofertilizers for the enhanced environmental stability, crop production and good crop yield. Biofertilizers are products containing living cells of different types of microorganisms, which have the ability to convert the nutritionally important elements from unavailable to available form through biological processes [2, 3].

Plant growth promoting rhizobacteria may increase the plant growth directly or indirectly [4, 5]. Direct mechanisms of actions of PGPR include; nitrogen fixation, production of phytohormones such as indole acetic acid, lowering of ethylene concentration and solublization of phosphorous. Whereas; antibiotic production, depletion of iron from rhizosphere, synthesis of antifungal metabolites, synthesis of antifungal cell wall lysis enzymes, competition for the sites on the roots and induced systemic resistance, are included in the indirect mechanisms of plant growth promotion by PGPR.

The most intensively studied application for free living PGPR is agriculture. Several tests have been conducted to study the effects of PGPR on different crops. Plant growth benefits due to PGPR include increase in germination rates, root growth, yield, leaf area,

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chlorophyll contents, magnesium content, nitrogen content, hydraulic activity tolerance to drought, shoot and root dry weights and delayed leaf senescence. Another major benefit of PGPR use is disease resistance conferred to the plant, known as biocontrol [6].

It is a well established fact that inoculation of crops with PGPR generally causes changes in root growth and morphology [7-9]. More importantly, the increase in the root length and root surface area is reported [10, 11]. Fallik *et al.* [12] found that the inoculation of maize with *Azospirillum brasilence* resulted in the proliferation of root hair which could have dramatic effects on increasing root surface area. PGPR can also increase the plant growth, development and yield in non-legume crops such as potato, sorghum and sugarcane [13, 14].

Keeping in view the important role of plant growth promoting rhizobacteria in agriculture, this study was conducted to isolate PGPR from roots and rhizosphere of sugarcane, identify and characterize bacterial isolates on the basis of Indole-3-acetic acid production (IAA) production and nitrogen fixing ability in the culture and finally the inoculation of original plant (sugarcane) and other heterologous plant host (sorghum) to study the effects on plants.

MATERIALS AND METHODS

Isolation and Identification of Bacteria from Sugarcane Roots and Rhizosphere: Roots of sugarcane (*Saccharum officinarum* L.) along with rhizosphere soil were collected from Shakarganj, Jhang, Shakot, Faisalabad and NIBGE fields (Pakistan). Rhizosphere samples were collected carefully by uprooting the root system and placed in the cool box for transport to the laboratory. One gram of the roots along with adhering soil was ground well with the help of a sterile pestle and mortar. Serial dilutions (10X) were made and 0.1 mL aliquots from 10^{-3} to 10^{-5} dilutions were spread on LB (Luria-Bertani) plates [15]. The plates were incubated for overnight at 30°C and morphologically different colonies appearing on the growth medium were selected for further purifications. Isolated colonies were streaked on fresh plates with LB medium to get single cell colonies. The bacterial cultures obtained were grown at 30°C for 24 h and preserved in glycerol (20%) at -80°C. Bacterial cultures were grown in LB broth and LB agar plates at 30°C for 24 h and used for studying colony and cell morphology. Single colonies appearing on the LB plates were transferred to a drop of sterilized water on a microscope glass slide and observed under the light microscope (Nikon, Japan). Bacterial growth (50 µL) from LB broth was also directly examined under the microscope to record cell morphology and motility.

PCR Amplification of Partial nif*H* Gene: In order to identify any nitrogen-fixers among the bacterial isolates, nif*H* primers were used in PCR to confirm the presence of nitrogen fixing genes or structural genes of nitrogenase enzyme. DNA from bacterial cultures was extracted by following the method of Birnboim [16]. One µL of each nif*H* gene primer, i.e., PolF and PolR (5' TGC GAY CCS AAR GCB GAC TC 3' and 5' ATS GCC ATC ATY TCR CCG GA 3', respectively) was used [42]. These primers amplify a 360 bp region between sequence positions 115 and 476 (referring to *Azotobacter* nif*H* coding sequence M20568). Each reaction mixture (50 µL) contained 0.2 µL taq DNA polymerase (5 U/µL; Fermentas), 5 µL taq buffer, 5 µL dNTPs (final concentration 20 mM each), 1 µL of each nif*H* primer (100 ng/µL), 1 µL of template DNA and the volume of the reaction mixture was made to 50 µL with double ionized water. Thirty five rounds of temperature cycling (94°C for 1 min, 55°C for 2 min and 72°C for 3 min) were followed by incubation at 72°C for 7 min in Perkin Elmer GenAmp PCR system 2400, Germany.

Acetylene Reduction Assay (ARA): Nitrogen fixing ability of the isolates was determined in semi-solid NFM by the acetylene reduction assay [17]. Acetylene reduction activity of the isolates was estimated in 17 mL screw cap vials containing 5 mL of semi-solid NFM. The vials were then inoculated with 100 µL of 24 h old bacterial cultures and incubated at 30°C. The bacterial cells used to inoculate vials were washed with saline solution prior to inoculation. The screw caps of the vials were replaced with the rubber stopper and acetylene (10% V/V) was injected into the vials after the growth was visible. Ethylene production was measured on a gas chromatograph (Fractonvap Series 2150, Italy).

Indole-3-acetic Acid Production (IAA): IAA produced by the cultures was estimated by growing the isolates at 30°C in malate (nitrogen-free medium) [18] supplemented with L-tryptophane (100 mg/L) and NH_4Cl (1 g/L). Study IAA production in the absence of the precursor, tryptophane-free media were also used. The supernatant of the culture fluid was obtained by centrifuging the stationary phase cultures at 10,000 rpm for 15 min and pH was adjusted to 2.8. The auxins from the acidified cultures

were extracted with equal volumes of ethyl acetate [19], evaporated to dryness and re-suspended in 1 mL of ethanol. The samples were analyzed by HPLC (Varian Pro Star, United Kingdom) using UV detector and C-18 column. Methanol: Acetic acid: Water (30:1:70 V/V/V) was used as a mobile phase at the rate of 0.6 mL min^{-1}. Pure indole acetic acid was used as a standard. The IAA of the samples was identified and quantified by comparing the retention time and peak area by using computer software (Varian Inc.).

Inoculation of Plants with Bacterial Isolates: For inoculation of plants, bacterial strains were grown in 100 mL of LB liquid medium in a water bath (25°C; 150 rpm) for overnight. The cell suspensions were pelleted in a sterile centrifuge tube at 10,000 rpm for 10 min, washed once with sterile distilled water and re-suspended in 100 mL of sterile distilled water. The selected strains were used to inoculate heterologous plant host sorghum (*Sorghum bicolor*; Var. PC-1). The seeds were obtained from Ayub Agriculture Research Institute, Faisalabad. The seeds were surface sterilized with sodium hypochloride for 5 min and then washed with sterilized water. The seeds were sown in sterilized sand in plastic beakers and kept in the growth room (2544 $\mu Em^{-2} S^{-2}$ and 25°C). One mL of inoculum was used to inoculate each seedling two days after germination. One mL of nitrogen-free Hoagland solution (1/2 strength) was added twice a week as a nutrient source. The root area and root length were measured by using root image analysis programme, created by Washington State University Research Foundation Programme, USA. In this programme, roots are scanned with the scanner and a computer image is created and analyzed. The roots and aerial parts of the seedlings were dried to a constant weight in an oven at 70°C and the dry weight was recorded.

Inoculation of Sugarcane in Pot Experiment: Selected bacterial strains were tested as inoculants for sugarcane plants grown in pots (33x29 cm). The pots were filled with 10.0 kg of soil collected from NIBGE fields. Sets with two nodes and approximately equal length (9 cm) and thickness (2.5 cm) were used in this experiment. One mL of the inoculum was diluted to 50 mL with sterile distilled water and used to inoculate one week after emergence. Plants were harvested after 60 days.

RESULTS

Isolation and identification of bacterial isolates and their place of collection is given in Table 1. PCR product of expected size (360 bp) was obtained from DNA template of only two isolates (Azoto1 and Azoto2) (Fig. 1).

Table 1: Identification of bacterial isolates of sugarcane roots and rhizosphere based on colony- and cell-morphology

Isolates	Colony characteristics	Identification	Site of collection
A1	Whitish colonies on L.B. medium, actively motile, short rods, mostly single cells	*Pseudomonas*	NIBGE, Faisalabad
A2	Whitish colonies on L.B medium; long thin rod shaped cells; joined together but single cells were also found, motile	*Pseudomonas*	NIBGE, Faisalabad
A3	Whitish or off-white colonies on L.B. long rods, mostly the cells are joined together, but single cells are also found, motile	*Pseudomonas*	NIBGE, Faisalabad
A4	White colonies on L.B., long rods slightly motile, no sheath formation observed	*Pseudomonas*	NIBGE, Faisalabad
A7	White colonies, rod shaped cells, joined together, or occurred singly, motile	*Pseudomonas*	Shakarganj Sugarcane Institute, Jhang
A8	Whitish or off white colonies; long rod shaped cells, slightly motile	*Pseudomonas*	Shakarganj Sugarcane Institute, Jhang
A11	Yellowish colonies, rod shaped cells, motile	*Pseudomonas*	Shakarganj Sugarcane Institute, Jhang
A12	White colonies, slightly curvedcells but not helical, not surrounded by sheaths; motile	*Pseudomonas*	Shahkot, Faisalabad
A14	Whitish colonies, rod shaped cells, motile	*Pseudomonas*	Shahkot, Faisalabad
A17	Whitish or off white colonies, curved cells but not helical not surrounded by sheaths, motile	*Pseudomonas*	Shahkot, Faisalabad
Azoto1	Large cells, pleomorphic; ranging from rods to coccoid cells, occurred singly, in pairs or; irregular clumps and sometimes; in chains of varying length, form cysts, motile, pigments are produced; nitrogen fixers	*Azotobacter*	Shahkot, Faisalabad
Azoto2	Identical to Azoto1	*Azotobacter*	Shahkot, Faisalabad

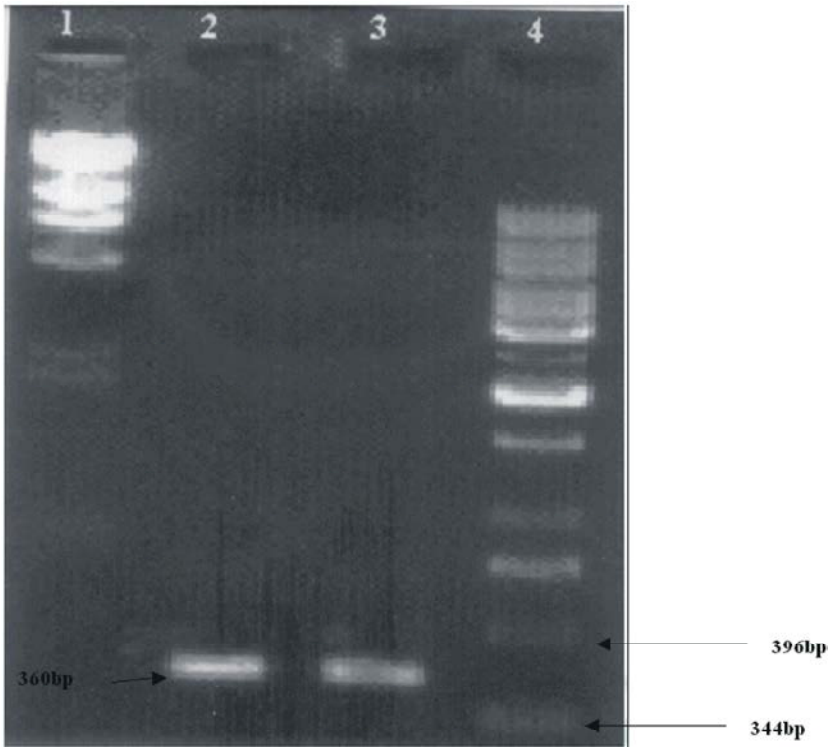

Fig. 1: PCR amplification of partial nif*H* from *Azotobacter* strain
Lane 1: λ/HindIII marker;
Lane 2: *Azotobacter* strain 1
Lane 3: *Azotobacter* strain 2; Lane 4: 1Kb size marker

Table 2: Indole acetic acid (IAA) production and Acetylene reducation assay of bacterial isolates from sugarcane roots

Isolates	IAA (mg/L)	ARA (nmol. C_2H_4/vial/24h)
A1	0.91cde	Not detected
A2	1.35bcde	Not detected
A3	2.01bcd	Not detected
A4	2.82b	Not detected
A7	1.32bcde	Not detected
A8	1.03bcde	Not detected
A11	2.11bc	Not detected
A12	0.92cde	Not detected
A14	0.74cde	Not detected
A17	4.49a	Not detected
Azoto1	0.20de	1966.4b
Azoto2	0.11e	4210a

Means containing same letter within the column are non-significant at 5% level (*n*=3)

Only two strains (Azoto1 and Azoto2) reduced acetylene to ethylene (Table 2). Acetylene reduction activity of the strain Azoto2 was higher than Azoto1 (4210 and 1966 nmol C_2H_4/24h, respectively).

All the isolates produced IAA in the medium containing tryptophane while IAA production was not detected by any of the isolates in the tryptophane-free medium (Table 2). Maximum IAA production (4.49 mg/L) was detected in isolate A17 while IAA production by the nitrogen-fixing isolates Azoto1 and Azoto2 were comparatively low (0.2 and 0.1 mg/L, respectively).

In growth room experiment, 12 bacterial isolates were tested as inoculants for sorghum grown in plastic beakers filled with sterilized sand (Table 4). These bacterial strains included 12 isolates from sugarcane and two PGPR strains *Pseudomonas* strain K1 and *Azospirillum* strain N4 from kallar grass and rice respectively. The strains K1 and N4 were used as positive controls. Among the inoculants tested in this experiment, maximum root area (37.40 mm^2) and root length (288.47 mm) was recorded in plants inoculated with the isolate A17 from sugarcane while the maximum increase in the shoot fresh and dry weights was recorded in plants inoculated with Azoto1. Taken overall, plants inoculated with K1, N4, A17, Azoto 1 and Azoto 2 showed significantly higher root fresh and dry weights as compared to the plants inoculated with other stains (Table 3).

These 12 strains were also used as inoculants for sugarcane plants grown in pots (Table 4). The increase in both root and shoot biomass was recorded in plants

Table 3: Growth attributes of 30-day old sorghum (*Sorghum bicolor*) plants inoculated with bacterial strains isolated from sugarcane rhizosphere

Strains	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Root area (mm^2)	Root length (mm)
Control	10d	0.88e	3.5b	0.65c	7.04e	64.86f
A1	13b	1.34c	5a	0.98a	10.99d	215.98b
A2	12c	1.82a	4.5a	0.95a	21.43b	182.37c
A3	14a	1.44b	5.3a	1.00a	33.29a	211.19b
A4	10d	1.95a	4a	0.98a	14.52c	128.40
A7	11c	1.22c	3.5b	0.88b	11.18d	189.84c
A8	12.5c	1.34c	3b	0.78b	9.93d	161.89d
A11	12c	1.83a	3.5b	0.68c	14.74c	106.63e
A12	13.2b	1.12d	3b	0.77b	32.08a	245.8a
A14	12c	1.32c	3.8b	0.89b	22.06b	199.27c
A17	11c	1.20c	4.5a	1.01a	37.40a	288.47a
Azoto 2	14a	1.78a	3b	0.74b	28.11b	143.32d
Azoto1	16a	1.98a	3.5b	0.77b	19.95b	129.90d
K1 (+ve control)	14a	1.55b	4.8a	0.98a	15.05c	125.15d
N4 (+ve control)	13.5b	1.71a	4.5a	0.96a	13.28c	205.10b

Means containing same letter within the column are non-significant at 5% level (n=6)

Table 4: Growth attributes of 60-day old sugarcane (*Saccharum officinarum* L.) plants inoculated with bacterial strains isolated from sugarcane rhizosphere

Strains	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Root area (mm^2)	Root length (mm)
C	7e	0.65c	2d	0.28d	4.04e	54.86e
A1	13b	1.2b	4.6b	0.55a	7.99d	115.98c
A2	10.8	0.98d	4.8b	0.56a	11.43c	142.37b
A3	9d	0.78d	5.7a	0.60a	8.29d	111.19c
A4	9.7d	0.85c	6.4a	0.7a	4.52e	78.40d
A7	11c	0.99c	3.2c	0.49b	11.18c	159.84b
A8	14.5a	1.82a	3c	0.39c	6.93d	111.89c
A11	12.4b	1.04b	4.5b	0.56a	4.74e	86.63d
A12	12b	1.11b	6.2a	0.70a	12.08c	145.8b
A14	13b	1.08b	5.7a	0.7a	12.06c	159.27b
A17	13.8a	1.44a	4.8b	0.66a	7.40d	294.47a
Azoto 2	15.6a	1.59a	6.5a	0.55a	18.11a	153.32b
Azoto1	14.8a	1.43a	6a	0.45b	6.95d	119.90c
K1 (+ve control)	15a	1.45a	5.5a	0.67a	15.05b	145.15b
N4 (+ve control)	14.5a	1.33a	4.3b	0.56a	13.28b	215.10a

Means containing same letter within the column are non-significant at 5% level (n=6)

inoculated with A17, A8, Azoto1 and Azoto2. However, maximum increase in root area was observed in plants inoculated with Azoto2 while plants inoculated with A17 and N4 also showed maximum increase in root length.

DISCUSSION

Plant beneficial bacterial strains belonging to the diverse genera colonize the roots and rhizosphere of plants including sugarcane [20-23]. In the present study, 12 bacterial strains were purified from the roots and rhizosphere of the field grown sugarcane. Tentative identification of the isolates on the basis of colony and cell morphology indicated that ten strains belong to genus *Pseudomonas* while two strains showed the characteristics of genus *Azotobacter*. Isolation of *Azotobacter* strains from kallar grass was also made by Bilal [24]. Isolation of nitrogen fixing as well as non-nitrogen fixing *Pseudomonas* strains has been reported from the rhizosphere of a number of plant species [25, 26]. Isolation of two nitrogen fixing *Pseudomonas* strains K1 and Ky1 from kallar grass, from Pakistan initially identified as *Azospirllum* and *Zoogloea*, has been reported [27, 28]. These strains have been re-identified recently as *Pseudomonas* strains on the basis of 16SrRNA sequence analysis [26].

Nitrogen-fixation is one of the major mechanisms utilized by PGPR for the plant growth promotion [20]. Therefore, acetylene reduction activity and the presence of nif*H* gene were also studied in the bacterial isolates from sugarcane to detect nitrogen fixing ability of the isolates. Acetylene reduction activity was detected in

only two isolates, i.e. Azoto1 and Azoto2. Partial nif*H* gene was also amplified by PCR using template DNA from bacterial isolates. PCR product of expected size (360 bp) with the particular set of primers was detected only in two *Azotobacter* strains (Azoto1 and Azoto2) showing the presence of structural genes for nitrogenase enzyme. Thus the strains Azoto1 and Azoto2 were nitrogen-fixers and good candidates for inoculum production. Among the *Pseudomonas* strains, none showed acetylene reduction activity or the presence of nif*H*. However, the isolation of nitrogen-fixing *Pseudomonas* strains from the rhizosphere of several plant species has been reported [29, 30] and at least five species of *Pseudomonas* have been included in the list of diazotrophs [26].

Phytohormone (IAA) production by the bacterial isolates in pure cultures was detected and quantified by high pressure liquid chromatography (HPLC). Among the 12 bacterial strains tested, indole acetic acid production was not detected in any of the isolates in tryptophane-free growth media. Production of only traces of IAA or no IAA production has been reported by bacterial strains in the growth media lacking tryptophane as a precursor of IAA production [31]. Maximum IAA production was recorded in *Pseudomonas* strain A17 followed by *Pseudomonas* strains A4 and A11. Phytohormone production by *Pseudomonas* strains has been reported earlier [32, 26]. Thus the strains producing high amounts of IAA have a great potential for use as PGPR for large scale production of inoculum for sugarcane and other crops. In the present study, the level of IAA production by *Azotobacter* strains Azoto1 and Azoto2 was relatively low as compared to *Pseudomonas* bacterial isolates from sugarcane. Production of auxins, gibberllins and cytokinins by *Azotobacter* strains in chemically defined media has been reported in a number of studies [33, 34].

Bacterial strains from sugarcane were used to inoculate sorghum as heterologous host. Two PGPR strains, i.e. *Azospirillum* strain N4 and *Pseudomonas* strain K1 were also included in this study as positive controls. Phyothormone production, nitrogen fixation and other beneficial effects of these strains on plants have been reported earlier [26]. The beneficial effects of the bacterial strains on the sorghum were observed as evidenced by the increase in the root area, root length and plant fresh and dry biomass of the inoculated plants as compared to the non-inoculated controls. The performance of the bacterial isolates from sugarcane was comparable to those of confirmed PGPR (strain K1 and N4). A number of research papers have been published reporting mostly growth enhancement and increased yield of sorghum due to PGPR inoculations [35-38].

The effect of these bacterial isolates from sugarcane was also studied on sugarcane grown in pots. Plants harvested after 8 weeks of growth showed increase in the plant biomass of the inoculated plants as compared to the non-inoculated control plants. A number of studies originating from Brazil showed that some sugarcane varieties can obtain large contributions of plant-associated nitrogen fixation, ranging from 60-80% of total plant N [39-41]. Additional beneficial effects to sugarcane due to phytohormone production by PGPR have also been reported earlier [23]. It can be concluded that the use of heterologous host in experiment conducted under control conditions proved helpful for rapid screening of bacterial isolates for their effects on plant growth. The bacterial strains Azoto1, Azoto 2 and A17 can be used as biofertilizers for their beneficial effects on plants.

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