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Isolation, characterization, and effect of fluorescent pseudomonads on micropropagated sugarcane

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Abstract: In this study, we report on the isolation, identification, and characterization of seven fluorescent pseudomonads isolated from the roots, shoots, and rhizosphere soil of sugarcane and their impacts on the growth of sugarcane plantlets. 16S rRNA gene sequence of five isolates showed close homology with *Pseudomonas putida*, one with *Pseudomonas graminis*, and one with *Pseudomonas fluorescens*. Physiological and biochemical characterizations were determined using API50CH and QTS24 identification kits. The isolates were also subjected to tests for various known growth promoting properties including production of indole acetic acid, the ability to fix nitrogen via the presence of the *nifH* gene, and ability to solubilize phosphate. Biological control potential was determined from agar diffusion assays of HCN production and production of antifungal compounds against local isolates of *Colletotrichum falcatum* (that induces red-rot disease of sugarcane). Direct plant growth promoting effects were tested on sugarcane plantlets in tissue culture under gnotobiotic conditions. All seven isolates provided significant increases in fresh and dry masses but only five strains increased shoot height.

Key words: Pseudomonas putida, *Pseudomonas fluorescens*, 16S rRNA, indole acetic acid production, *nifH* gene, sugarcane.

Résumé : Dans cette étude, nous rapportons l'isolement, l'identification et la caractérisation de sept pseudomonades fluorescentes isolées des racines, des pousses et du sol de la rhizosphère de la canne à sucre, ainsi que leurs impacts sur la croissance des plantules de canne à sucre. La séquence du gène de l'ARNr 16S de cinq isolats a montré une forte homologie avec *Pseudomonas putida*, un avec *Pseudomonas graminis* et un avec *Pseudomonas fluorescens*. Les caractérisations physiologiques et biochimiques ont été déterminées à l'aide des trousses d'identification API50 et QTS24. Les isolats ont été aussi soumis à des tests visant à détecter des propriétés connues de promotion de la croissance, notamment la production d'acide indole acétique, la capacité de fixation d'azote via la présence du gène *nifH* et la capacité de solubilisation du phosphate. Le potentiel de contrôle biologique a été déterminé par des essais de diffusion sur gélose de production de HCN et de production de composés antifongiques dirigés contre des isolats locaux de *Colletotrichum falcatum* (qui induit la maladie de la pourriture rouge de la canne à sucre). Les effets directs de promotion de la croissance ont été testés sur les plantules de canne à sucre en culture de tissus sous des conditions gnotobiotiques. Les sept isolats ont généré des augmentations significatives du poids à l'état frais et du poids sec, mais seules cinq souches ont permis d'augmenter la longueur des pousses.

Mots-clés : Pseudomonas putida, *Pseudomonas fluorescens*, ARNr 16S, production d'acide indole acétique, gène *nifH*, canne à sucre.

[Traduit par la Rédaction]

The production of sugarcane, an important cash crop of Pakistan, has increased over time but there has been only a marginal increase in plant biomass productivity per unit area. This can be attributed to the low soil fertility in most production areas, as most growers cannot afford the cost of chemical fertilizers. Many growers report significant crop

Received 23 December 2008. Revision received 13 March 2009. Accepted 27 March 2009. Published on the NRC Research Press Web site at cjm.nrc.ca on 15 August 2009.

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Table 1. Biochemical and physiological differences among different strains of fluorescent pseudomonads isolated from sugarcane.

Test	BN-St	LH-S1	LH-R1	OK-St	PB-St1	QR2	Q-Stw3
API50CH							
D-arabinose	−	−	−	−	+	−	−
D-arabitol	−	−	−	−	+	−	−
D-maltose	−	−	−	−	+	−	+
D-mannose	+	+	+	−	+	+	+
D-mannitol	−	−	−	−	+	−	+
D-trehalose	+	−	−	−	+	−	+
D-lyxose	−	−	+	−	+	+	+
D-saccharose	−	−	−	−	−	−	+
L-rhamnose	−	−	−	−	−	+	−
L-xylose	−	−	−	−	+	−	−
Dulcitol	+	−	−	−	−	−	−
N-acetyl glucosamine	−	−	−	−	+	−	+
Esculin ferric citrate	+	−	+	+	−	−	+
Methyl-β-D-xylopyranoside	−	−	−	+	−	−	−
Potassium gluconate	−	+	+	−	+	+	+
Potassium 2-ketogluconate	−	+	+	−	+	−	+
Potassium 5-ketogluconate	−	−	+	+	+	+	+
QTS24							
Acid from D-maltose	−	−	−	−	+	−	−
Acid from L-arabinose	+	+	−	−	+	+	−
Acid from D-glucose	−	−	−	−	+	+	+
Sodium malonate	−	+	+	−	−	+	−
Gelatinase	−	−	−	−	+	−	−
Nitrogen gas	−	+	−	+	+	+	−
Growth at 4 °C	−	−	−	−	+	+	+
Growth at 41 °C	−	+	+	−	−	−	+
Growth at pH 11	−	+	+	−	+	−	+
Growth at pH 12	−	+	+	−	−	−	+
Growth with 5% NaCl	+	+	+	−	+	+	+
Antibiotics resistance							
Gentamycin (25 $\mu g \cdot mL^{-1}$)	−	+	+	−	−	+	+
Streptomycin (100 $\mu g \cdot mL^{-1}$)	+	+	+	+	−	−	+
Tetracyclin (25 $\mu g \cdot mL^{-1}$)	+	+	+	+	−	−	−

Note: All isolates were positive for growth at 25–37 °C, pH 5–10, tolerance to 3% NaCl, utilization of glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-melibiose, D-fucose, gentiobiose, presence of arginine dihydrolase, catalase, cytochrome oxidase, trisodium citrate, tolerance to 100 $\mu g \cdot mL^{-1}$ ampicillin, chloramphenicol, and spectinomycin and negative for utilization of erythritol, D-adonitol, L-sorbose, inositol, D-sorbitol, methylα-D-manno pyranoside, methylα-D-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-lactose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, D-turanose, D-tagatose, L-fucose, L-arabitol, acid production from D-adonitol, inositol, D-mannitol, D-melibiose, D-raffinose, L-rhamnose, D-sucrose, D-sorbitol, tolerance to 25 $\mu g \cdot mL^{-1}$ kanamycin, rifampicin, presence of lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, indole production, nitrate reduction, acetoin, 2-nitrophenyl-β-D-galactopyranoside, and sodium thiosulfate.

losses owing to soilborne diseases caused by fungal pathogen. There are no inexpensive chemical means of controlling such diseases but biofertilizers and biocides offer a promising and affordable means of reducing production cost and crop losses. Fluorescent pseudomonads are a likely candidate for biofertilizers and biocides through a multiple number of mechanisms by which they can exert beneficial effects on plant growth including production of phytohormones, siderophores, and antibiotic compounds that reduce disease severity caused by pathogenic soilborne microorganisms (Benizri et al. 1997). A limited number of researchers have reported being able to isolate fluorescent pseudomonads from sugarcane (Herrera et al. 1994; de Lima et al. 1999; Kumar et al. 2002; Viswanathan and Samiyappan 2002) but their effects on plant growth have not been fully explored.

Indian researchers recently isolated fluorescent pseudomonads from indigenous sugarcane varieties and reported that they were an effective means to increase plant growth and bring about control of red-rot disease (Viswanathan and Samiyappan 2002). Since Indian production is similar to that found in Pakistan, we set out to examine the microbial ecol-

Fig. 1. Neighbor-joining tree of 16S rRNA gene sequences of bacterial isolates from sugarcane. Sequences of type strains were obtained from databases and accession numbers are given in parentheses. The isolates from sugarcane are in bold type. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

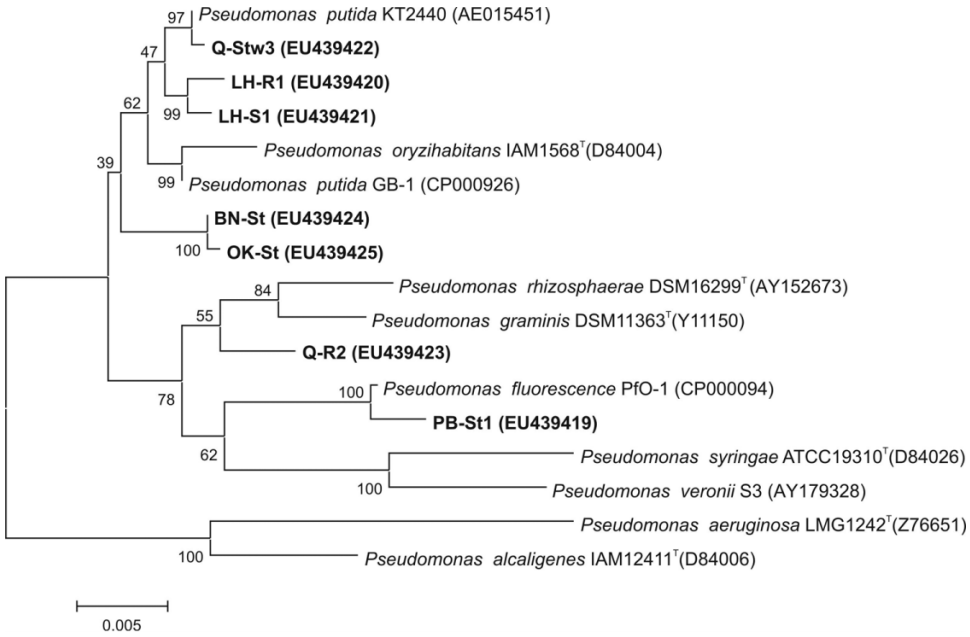

Table 2. Effects of fluorescent pseudomonads on in vitro growth of sugarcane plantlets.

Treatment	Shoot height (cm)	Fresh mass (mg·plant^{-1})	Dry mass (mg·plant^{-1})
Uninoculated	17.1±0.71e	288±12e	30.1±1.0d
BN-St	20.4±0.73cd	800±76d	96.2±8.0bc
LH-S1	16.9±0.82e	682±70d	71.3±6.9c
LH-R1	20.0±0.96d	895±92cd	89.7±8.8bc
OK-St	29.5±1.63a	1569±155a	161.1±16.2a
PB-St1	23.5±1.00b	1060±49bc	106.7±6.6b
Q-R2	23.0±0.75bc	1210±73b	137.8±7.4a
Q-Stw3	19.1±0.61de	670±48d	75.0±5.8c

Note: Means followed by the same letter are not statistically different at the 5% level according to Duncan's multiple range test. Results are the average ± SE of three experiments, each with 10 replicates.

ogy of Pakistani sugarcane varieties, with specific focus on the fluorescent pseudomonads that reside on indigenous sugarcane varieties. The bacteria that we isolated were identified and tested for their growth-promoting and biocontrol activity.

Plants of local sugarcane varieties were collected from Punjab. Bacteria were isolated from washed and surface-sterilized tissues and rhizosphere soil of sugarcane. In total, 50 phenotypes of colonies were obtained and seven of them showed fluorescence under UV light. Fluorescent cultures were maintained on King's B medium (King et al. 1954). Identification of these isolates was carried out using colony morphology and ability to grow at various pH conditions (5–12), temperatures (4 and 25–41 °C), and NaCl concentrations (0.5%–7%) on LB medium. Biochemical characterization was done using API50CH (BioMerieux, Marcy l'Etoyle, France) and QTS24 bacterial identification kits (Desto Laboratories, Karachi, Pakistan). The ability to grow in the presence of the antibiotics was also examined. Colony morphology of these strains varied in color (off-white, beige, yellow) and (or) shape (convex, flat, watery) when grown on King's B medium. The results of physiological and biochemical tests including tolerance to different antibiotics are presented in Table 1. 16S rRNA gene sequence analysis was carried out using amplicons generated from primers FGPS4-281 and FGPS1509-153 and PCR conditions as described in Mehnaz et al. (2001). All sequences were deposited in GenBank (Accession Nos. EU439419 to EU439425). A phylogenetic tree based on 1442 nucleotides is shown in Fig. 1. Q-Stw3, LH-R1, and LH-S1 were found to be clustered together and grouped with *Pseudomonas putida* KT2440. In this group, Q-Stw3 showed the highest similarity to *P. putida* KT2440 as compared with the other two strains. Strains LH-S1 and LH-R1 showed the highest similarity to each other. Strain BN-St was grouped with OK-St, as both showed the closest homology with *P. putida* GB-1 and strain Q-R2 was grouped with *Pseudomonas graminis* and PB-St1 with *Pseudomonas fluorescens* PfO-1.

Bacterial isolates were screened for the presence of the *nifH* gene by using the primer set PolF/PolR and the PCR

conditions described in Poly et al. (2001). *Azospirillum brasilense* ATCC 29145 was used as positive control. The *nifH* gene was amplified by these primers and an amplicon of approximately 360 bp product was found with all strains except PB-St1. The sequence of the PCR product of the *nifH* gene of Q-R2 was deposited in GenBank (Accession No. FJ404470) and it showed 96% similarity (340 of 354 identities) with *A. brasilense* Sp7 (Accession No. X51500).

Indole acetic acid production in King's B medium containing 100 $mg \cdot L^{-1}$ L-tryptophan was carried out using the colorimetric method of Gordon and Weber (1951). All bacterial isolates produced indole acetic acid in the presence of tryptophan. Phosphate solubilizing activity was tested on NBRIP (Nautiyal 1999) and on calcium phytate (Rosado et al. 1998) agar media. All isolates were found to be positive for phosphate solubilization on both media. The ability of the bacteria to inhibit the growth of four local isolates of *Colletotrichum falcatum*, which causes red-rot disease of sugarcane, was tested on potato dextrose agar medium. HCN production was determined as described in Millar and Higgins (1970). All bacteria failed to inhibit the growth of *C. falcatum* on agar medium but Q-R2 and PB-St1 were positive for production of HCN.

The growth-promoting effects of fluorescent pseudomonads on sugarcane plantlets were studied in tissue culture. Three-month-old micropropagated plantlets of sugarcane variety 'HSF 240' were transferred into 30 mm × 200 mm glass tubes (one plantlet per tube), each containing 10 mL of MS medium (Murashige and Skoog 1962) without any growth hormone. The experiment consisted of eight treatments (a control and seven isolates) and 10 replicates of each treatment. The plants were individually inoculated with 100 µL (10^8 cells) of individual bacterial suspension prepared in phosphate-buffered saline except the control plantlets, which received 100 µL of phosphate-buffered saline. Plants were grown at 25 ± 2 °C in a growth chamber in a 16 h photoperiod (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with white fluorescent tube lights. Plants were harvested after 8 weeks of growth and data were collected for shoot height and fresh and dry plant masses. Experiments were repeated three times. The data were analyzed using one-way analysis of variance with Co-Stat statistical software (version 6.4) (CoHort Software, Berkeley, California). The significance of values differing at the 5% level was tested using Duncan's multiple range test. All isolates increased the fresh and dry masses of plants significantly compared with noninoculated plants (Table 2). Shoot height of inoculated plants was significantly increased by all strains except LH-S1 and Q-Stw3. The highest growth-promoting effects were observed with plants inoculated with the OK-St strain.

In this study, a number of fluorescent pseudomonads were isolated from Pakistani sugarcane varieties. Isolation of these strains from root and shoot and soil is an indicator that these bacteria grew well in tissues and rhizosphere of indigenous Pakistani sugarcane varieties. Similar results have been reported by Kumar et al. (2002) and Viswanathan and Samiyappan (2002) for indigenous sugarcane varieties of India. Mendes et al. (2007) isolated *P. fluorescens* from the sterilized roots of sugarcane growing in Brazil. Some of our bacterial strains were isolated from sterilized root and shoot and this increases the chances that these bacteria possess endophytic capacity, an ideal feature for a potential bioinoculant. Furthermore, the ability of some isolates to grow at 4 °C (PB-St1, Q-R2, and Q-Stw3) and 41 °C (LH-S1, LH-R1, and Q-Stw3) suggests that they would be able to withstand the summer (above 30 °C) as well as the winter (below 10 °C) temperatures found in the Punjab region where sugarcane is an annual crop. The bacteria also tolerate harsh pH ranges (pH 10–12) and can grow in 5% NaCl, which indicates adaptability to diverse soil conditions.

Further experiments examining the beneficial effects of the association of these isolates with sugarcane are in progress under greenhouse and field conditions. On the basis of reports in the literature and the presence of growth-promoting traits in these strains, it is conceivable that they will act as part of a formulation of biofertilizer for sugarcane. Further studies on this aspect will help growers to use these bacteria in fields and reduce their dependence on chemical fertilizers.

Acknowledgement

This work was partially supported by a grant from the Agriculture and Agri-Food Canada MII Initiative.

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