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TO THE STUDY OF PHOSPHATE-SOLUBILIZING MICROORGANISM FOR ENHANCEMENT OF *MURRAYA KOENIGII* GROWTH AND YIELD

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Abstract

Nineteen isolates of rhizobacteria associated with *murraya koenigii*, collected from various locations in Dehradun, were screened for phosphate solubiliza- tion and indole-3-acetic acid (IAA) production. Two potential phosphate-solubilizing bacterial isolates with substantial IAA biosynthesis capacity, Ps-5 and Ss-2, were selected for further study. Based on 16S rRNA gene sequence analysis, isolate Ps- 5 was identified as *Bacillus* sp. and Ss-2 as *Alcaligenes faecalis*. Both strains were found to be metabolically diverse in terms of the number and amount of different carbon sub- strates they utilized in the BIOLOG GN2/GP2 microplate assay. High-performance liquid chromatography analysis of the culture supernatant confirmed that *Bacillus* sp. Ps-5 pro- duced considerable amounts of both lactic and tartaric acids, while *A. faecalis* Ss-2 secreted only lactic acid. There was a strong positive correlation between phosphate solubilization and organic acid production by both strains. Following inoc- ulation, strain Ps-5 and Ss-2 were found to be good root colonizers and significantly ($P \leq 0.05$) increased *murraya koenigii* growth and phosphorus (P) uptake. However, inoculation had a non-significant ($P \leq 0.05$) effect on *murraya koenigii* yield parameters, including oil contents. Based on these results, we conclude that Ps-5 and Ss-2 are potent plant growth- promoting rhizobacteria strains with the ability to supplement the P requirements of *murraya koenigii* crops. Further field inoculation studies are needed before these strains can be recommended as bio-inoculants. To the best of our knowledge, this is first report on the association and phytobeneficial potential of *A. faecalis* with *murraya koenigii*.

Keywords :- Phosphate-solubilizing bacteria, IAA production, Oil content, curry leaf, Transmission electron microscopy

Introduction

A microbial community that is closely affiliated with plant roots and densely populates the immediate environment of these plant roots is known as the rhizosphere microbiome or rhizo-microbiome (Chaparro et al. 2013). A proportion of bacteria within the dynamic environment of the rhizosphere. These microorganisms are an important biological asset for sustainable agriculture in that they promote plant growth directly by nitrogen fixation, macro- and/or-micro nutrient solubilization/mobilization, phytohormone production and/or indirectly by controlling phytopathogens through the produc- tion of siderophores, antibiotics, lytic enzymes, among others (Compant et al. 2005). PGPR are the most widely studied group of plant growth-promoting bacteria (PGPB) that are capable of establishing a dense population in rhizosphere, the soil adhering closely to root surfaces (Kloepper et al. 1989). The PGPB also found directly attached to roots are known as rhizoplanic bacteria, while those likely to penetrate into the root epidermal and cortical tissues are referred to as endo- phytes (Sylvia et al. 2005). PGPR are important biological tools for the effective release of insoluble phosphorus (P) from organic and inorganic pools through mineralization and solu- bilization processes, respectively, thereby helping plants obtain their nutrition from the soil (Hilda and Fraga 1999).

Although, P is the second most important nutrient for plant growth, only 1–5 % of total P in soil is in a form available to plants (Arcand and Schneider 2006; Zaidi et al. 2009). This has led to the excessive use of synthetic phosphate fertilizers becoming a normal routine for the farmers around the world. However, these mineral fertilizers form complexes with cat- ions of calcium (Ca), aluminum (Al) and iron (Fe) in the soil and become insoluble (Sharan et al. 2008). In addition, synthetic mineral fertilizers have serious environmental and human health impacts and place a heavy economic burden on farmers. An alternative and environmentally friendly approach to achieve sustainable agriculture in a cost-effective manner would be to select and utilize those microorganisms and their biosystems with the mineral phosphate-solubilizing trait (Hameeda et al. 2006; Fankem et al. 2008; Park et al. 2010). Bacterial strains of genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* and fungal strains of genera *Penicillium* and *Aspergillus* are the most widely accepted P solubilizers (Whitelaw 1999; Shahid et al. 2012). The main phytobeneficial mechanism of mineral phosphate-solubilizing bacteria (PSB) is lowering of the pH of the surroundings through the production of oganic acids.

These organic acids carry hydroxyl and carboxyl groups which chelate cations (Al, Fe, Ca) bound to soil phosphates, thereby making them soluble by detaching these cations (Mullen 2005; Trivedi and Sa 2008).
Organic acids synthesized by PSB, indole-3-acitic acid (IAA) is considered to be the most important. IAA is capable of playing a crucial role in cell growth and division and has the potential to be a major factor in root proliferation/ elongation in plants (Seo and Park 2009). To date, three main IAA biosynthetic pathways have been reported in bacteria, namely, the indole-3-pyruvic acid, indole-3-acetamide and indole-3-acetonitrile pathways, respectively (Duca et al. 2014).

The association of PSB with *murraya koenigii* roots and screening of the potential of these PSB for enhancing the growth and yield of *murraya koenigii* plants has not as yet been completely studied. Recently, Ambrosini et al. (2012) reported the associ- ation between bacterial species/strains belonging to the genera of *Enterobacter*, *Klebsiella*, *Grimontella*, *Novosphingobium*, *Microbacterium*, *Acinetobacter*, *Pantoea*, *Variovorax*, *Asticcacaulis*, *Chryseobacterium*, *Herbaspirillum*, *Mitsuaria*, *Moraxella* , *Serratia* , *Shinella* , *Sphingobium* and *Xanthomonas* and the *murraya koenigii* rhizosphere. Among these bacterial genera, members of the *Enterobacter* and *Burkholderia* were found to be the most abundant. In Pakistan there is a huge gap between edible oil production and market demand, resulting in *murraya koenigii* oil being imported into Pakistan on a large scale. In the context of decreasing this gap, any improvement in the yield of *murraya koenigii* crops is of keynote significance (Khan et al. 2000; Shah et al. 2013). *Murraya koenigii* yield and yield components, including the oil yield, can be increased by the application of PSB (Ekin 2010). However, compared to studies on the rhizospheres of other plants, the isolation, characterization, identification and manip- ulation of potential PSB from the *murraya koenigii* rhizosphere have been less explored worldwide, and to our knowledge no valid reports from Pakistan are available in this area of scientific research. Thus, the objective of this study was to characterize and identify promising PGPR strains which can supplement the P requirements of *murraya koenigii* in a cost-effective manner without compromising crop yield and oil contents.

Materials and methods

Isolation and morphological studies

Murraya koenigii (curry leaf plant) plants at the flowering stage were uprooted with intact roots and adhering soil from various locations in Dehradun including Haridwar area (30° 44' 27.10 N, 72° 38'18.39 E, 154 m a.s.l.). The shoots were excised using a sterilized knife, following which root portions were transferred to sterilized plastic bags (25×30 cm) and transported to the laboratory. Roots were shaken gently in sterile distilled water to remove the loosely adhering soil. One gram of the soil that remained tightly adhered to the roots was then re- moved from each sample and added in 9 mL of 0.85 % (w/v) NaCl solution; this solution was serially diluted as described by Somasegaran and Hoben (1994). A 100-μL aliquots from dilutions 10^{-4}, 10^{-5} and 10^{-6} of each sample was spread on Luria-Bertani (LB) agar plates using a sterilized glass spreader and incubated at 28±2 °C for 48 h. Bacterial isolates from each plate were selected based on prolific growth and colony appearance and streaked many times to achieve the maximum purity level. Twenty isolates purified in this manner were maintained on LB agar plates at 28±2 °C for further studies, and five copies of each isolate were stored in 20 % (v/v) glycerol at −80 °C. Colony morphology, cell shape, motility and the Gram reaction were studied under the light micro- scope as described by Vincent (1970).

Phosphate solubilization and detection of organic acids

A single colony of each purified isolate was spotted on Pikovskaya's agar (Pikovskaya 1948) plates, incubated at 28±2 °C and observed for up to 240 h for halo-zone formation. For quantification of solubilized phosphate and detection of organic acids in the culture medium, we performed triplicate experiments in which 100-mL of Pikovskaya's broth containing a single colony of each isolate was inoculated into a 500-mL Erlenmeyer flask and incubated at 28±2 °C for 240 h in an orbital shaker at 150 rpm. A 20-mL sample of bacterial culture from each flask was harvested after 168 h (for all isolates) and after 120, 168 and 240 h (for two isolates) and centrifuged at 13,000g for 10 min to collect the supernatant. The quantitative measurement of phosphate solubilization was performed ac- cording to phosphomolybdate blue colour method (Murphy and Riley 1962) using a spectrophotometer (λ=882 nm). To determine the identity and concentration of organic acids, the supernatant of two selected isolates was first filtered through 0.2-μm nylon filters (Millipore, USA). Then, 20-μL samples of each supernatant were analysed using a high-performance liquid chromatography (HPLC) system equipped with Turbochrom software (Perkin Elmer, USA) and a C18 column (length 150 mm, diameter 4 mm, pore size 120 Å). The mobile phase consisted of methanol:acetic acid (30:70, v/v), and a flow rate of 0.6 mL min^{-1} was set. Organic acids to be used as standards were purchased from Sigma (USA). The supernatant was analysed for the presence of gluconic acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid and tartaric acid by spectrometry at a 210 nm wavelength.

Indole-3-acetic acid production

For the detection of IAA, a single colony of each purified isolate was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of LB broth supplemented with tryptophan (100 mg L^{-1}) and incubated at 28±2 °C for 48 h in an orbital shaker at 150 rpm. The supernatant was collected in separate tubes after centrifugation at 13,000g and acidified (pH 2.8) with hydrochloric acid. An equal volume of ethyl acetate was mixed with the supernatant in a separating funnel to extract the IAA (Tien et al. 1979). The upper layer of ethyl acetate containing the IAAwas collected in a separate sterilized tubes, and excessive ethyl acetate was removed under vacuum at 45 °C, using a rotary evaporator. The recovered extract was dissolved in methanol (1 mL) and filtered through a 0.2-μm nylon filter (Millipore) prior to analysis on a HPLC (λ= 260 nm) system equipped with Turbochrom software (Perkin Elmer) and a C18 column at a flow rate of 0.5 mL min^{-1} using 30:70 (v/v).

16S rRNA gene sequencing

Total genomic DNA of isolate Ps-5 and Ss-2 was isolated by the alkaline lysis method (Maniatis et al. 1982) and quantified on the Ultrospec™ 3100 pro UV/visible spectrophotometer (GE Healthcare Life Sciences, USA) at $OD_{260,\ 260/280}$. The quantified DNA was then used to amplify the 16S rRNA gene with primers fD1 and rD1 as described by Weisburg et al. (1991), with the following modifications: 50 µL reaction volume in purified water, 5 µL of Taq buffer (Fermentas, Lithuania), 3 µL of 25 mM $MgCl_2$ (Fermentas), 5 µL of 2 mM dNTPs (Fermentas), 0.5 µL of 100 % DMSO, 1.5 µL each of forward and reverse primer, 0.75 µL of 5 U μL^{-1} Taq DNA polymerase (Fermentas) and 40 ng of template DNA. Amplification by PCR was carried out in a thermal cycler (advanced Primus 96; PeQLab Biotechnologie, Germany) also under modified conditions of 30 cycles of 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. The initial denaturation and final extension steps were performed at 95 °C for 5 min and 72 °C for 10 min, respectively. Amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, USA) and sequenced directly on both sides by a commercial laboratory (Eurofins, Germany). The gene sequence was analysed using the Sequence Scanner software package; both ends were joined using Caps 3 assem- bly software and compared with others in the GenBank data- base using the NCBI BLASTn tool. The final sequences were deposited in Genbank and accession numbers were obtained.

Phenotypic microarray analysis

For the phenotypic microarray analysis, 50-mL samples of culture media containing isolates Ps-5 and Ss-2 were grown in LB broth for 20 h in 250-mL Erlenmeyer flasks to obtain a population density of 10^8 CFU mL^{-1}. A 30-mL sample of culture was then collected from each flask and centrifuged at 8,000g for 10 min; the pellet was removed and dissolved in the same volume of phosphate buffer (pH 6.5). The target $OD_{620\ nm}$ (0.34) which indicated a population density of 10^6 CFU mL^{-1} was obtained by diluting the suspension with the appropriate amount of phosphate buffer. The suspension was then incubated at room temperature for 3 h to starve the cells and deplete most of the energy reserve needed for bacterial cell growth. Each well of the Biolog GN2/GP2 micro-titer plate (Biolog, USA) was filled with 100-µL of suspension and the plates were incubated at 28±2 °C for 72 h; the reaction was analyzed qualitatively for color development and quantitatively on the VERSA max micro-plate reader (Molecular Devices, USA) with SoftMax Pro software. The $OD_{595\ nm}$ was determined after 24, 48 and 72 h of incubation.

Root colonization studies

In the pot experiment, both bacterial strains were recovered from the rhizosphere after 7, 15 and 30 days of transplanting on LB plates. To recover bacterial isolates, *murraya koenigii* plants with intact roots were carefully uprooted and shaken gently in sterilized distilled water to remove the loosely adhering soil.

Bacteria were then recovered by the dilution plating technique using 1 g of the soil which remained strictly adhering to the roots in 9-mL sterile water (Seldin et al. 1998). The bacterial colonies recovered were also examined under the stereoscope to confirm their morphology.

For the ultrastructure studies, *murraya koenigii* (cv. FH-331) seeds were surface sterilized by dipping them in sodium hypochlo- rite (5 %, w/v) for 10 min, followed by five to six washings with sterilized water. The seeds were inoculated by immersion in the inoculum (1×10^8 CFU mL^{-1}) of selected isolates for 30 min and allowed to germinate on 1.5 % (w/v) water agar plates. Root hairs of 10-day-old seedlings were cut into pieces (approx. 1–3 cm) and embedded in water agar again, followed by cutting out approximately 2- to 3-mm small agar cubes. The cubes were put in 1.5-mL tubes in the presence of 5 % gluteraldehyde as fixative (in 0.2 M PIPES buffer, pH 8.0). After 16–18 h, the fixative was replaced with 0.2 M PIPES buffer {0.58 g NaCl, 3 g PIPES [piperazine-*N*,*N*' bis(2- ethanesulfonic acid)], 1 M NaOH, 0.2 g $MgCl_2{\cdot}6H_2O$, pH 6.8} (Salema and Brandao 1973), and the samples were washed 2×1 h in fresh buffer. The washed samples were treated with 0.2 % osmium tetraoxide made in PIPES buffer (0.2 M, pH 6.8) for 16–18 h and then washed again 2×30 min with sterilized distilled water. After being treated with 5 % aqueous uranyl acetate for 16–18 h, the samples were washed with sterilized distilled water for 2×30 min. The dehydration steps consisted of immersion in absolute alcohol for 2× 30 min, followed by immersion in propylene oxide (100 %) for 1×30 min. Infiltration of samples was carried out with propylene oxide in a ratio of 1:1 for 24–48 h and then with spurr resin for a further 24–72 h. The accelerator benzyl di- methyl amine was used in all infiltration steps. The samples were transferred to flat embedding moulds and polymerized for 72 h at 65–70 °C, following which the polymerized resin blocks were removed from the oven and left at room temper- ature for at least 24 h before being cut into ultrathin sections (150–200 nm) on an ultra-microtome (RMC-7000; Boeckeler Instruments, USA). The sections were carefully placed on copper grids. All of the sections were double stained with uranyl acetate (30 min) and lead citrate (10 min). The grids were then washed with deionized water and observed under a transmission electron Microscope (TEM; JEOL 1010).

Pot experiment

A pot experiment was conducted in growth room by employing Completely Randomized Design (CRD). Surface-sterilized *murraya koenigii* seeds (cv. FH-331) were germinated on 1.5 % water agar plates. For inoculum preparation, bacterial cultures were grown to a density of 1×10^9 CFU mL^{-1}, centrifuged at 8,000*g* and washed twice with saline solution (0.85 %, w/v). The cells were re-suspended in equal volumes of saline and diluted to 10^8 CFU mL^{-1}. The growth room was fumigated before the start of the experiment to achieve maximum sterility, and growth conditions were set at 25 °C and 16/8 light/dark periods until harvesting of the plants. The pots (diameter 9 cm) were filled with 400 g of steam-sterilized sand. Tricalcium phosphate (TCP) was mixed in sand (200 mg kg^{-1} sand) as an insoluble inorganic P source, and 4-day-old agar-grown plants were inoculated by dipping the roots in inoculums for 30 min. In addition, 7 mL 100 g^{-1} inoculum was mixed in sand at the time of transplanting. Each pot was transplanted with one inoculated seedling, with one treatment left uninoculated. Pots were watered with Hoagland's solution (Arnon and Hoagland 1940) without a P source (10 mL pot^{-1} daily). The data on various growth parameters, including P uptake by plants, was determined 30 days after transplanting. Root and shoot P content were determined by the vanadium phospho- molybdate yellow colour method (Yoshida et al. 1971).

Field experiment

The field experiment was conducted at the experimental field area of Motherhood University, Roorkee, India, using two inoculated treatments of *Bacillus* sp. Ps-5 and *Alcaligenes faecalis* Ss-2 with a half dose of the recommended fertilizer. Three uninoculated treatments with a full and half dose of fertilizer and no fertilizer were also included for comprehen- sive comparison. The experiment was conducted in a randomized complete block design with three replications.The seed bed was prepared by ploughing the soil two to three times with a tractor-mounted cultivator followed by planking after each cultivation. Plot size was maintained at 3×2 m^2. Sowing was done manually by the dibbling method, with three to four seeds per hill and a between-row and between-plant distance of 75 and 25 cm, respectively. Inoculum was prepared in the same way as described in the pot experiment, while seed inoculation was carried out by dipping *murraya koenigii* seeds in inoculum for 30 min. Thinning was done when the crop attained the height of 10 cm, and an 8 kg ha^{-1} seed rate was used. Nitrogen (50 kg ha^{-1}) was applied in the form of urea in two splits (at sowing and at first irrigation), and P (90 kg ha^{-1}) in the form of triple superphosphate was incorporated in the soil at the time of sowing by means of single row drill.

Plant protection measures were adopted to keep the crop free from weeds, insects, pests and diseases. All other agronomic practices were kept normal and uniform for all the treatments. After harvesting, the crop was left for 5 days to dry under the sun and then threshed by physical beating. Achene P contents were determined by the vanadium phospho-molybdate yellow colour method (Yoshida et al. 1971). Oil content analysis of *murraya koenigii* achenes was carried out com- mercially by Oil Seed Brassica Lab, Plant Breeding & Genetics Division, Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan, by gas chromatography as described by Erickson et al. (1980).

Measurements and data analysis

Data for the Biolog GN2/GP2 microplate assay were analyzed by principal component analysis (PCA) using SPSS 17 soft- ware (SPSS Inc., USA). Data for the pot and field experiments were analyzed statistically by analysis of variance(Steel et al. 1997) using Statistix (ver. 8.1) software. The least significant difference test (Fisher's LSD) at 5 % probability was used to compare the differences among treatment means.

Results and Discussion

Morphological and physiological characterization

Most of the bacterial isolates were motile short rods, but some long rods and few cocci were also present. One isolate, Ps-3, was found to be oval shaped. Of the 19 isolates collected, 14 were Gram-negative and five, i.e. one each from Multan (Ms- 4), Peshawar (Ps-5) and Tandojam (Ss-1) and two from Faisalabad (Fs-1 and Fs-4), were Gram-positive. Six isolates (Fs-6, Ms-3, Ps-2, Ps-3, Ps-5 and Ss-2) produced considerable halo-zones on TCP-supplemented agar medium. Analysis of the culture supernatants revealed that isolates Ps-5 and Ss-2 had the maximum P-solubilizing and IAA biosynthesis poten- tials, solubilizing 28.02±1.85 and 28.91±2.56 μg mL^{-1} TCP, respectively, and synthesizing 10.65 ± 2.08 and 2.27 ± 0.10 μg mL^{-1} IAA, respectively (Table 1).

Relationship between phosphate solubilization, pH change and organic acid synthesis

In the time-course studies, a trend towards increasing in vitro phosphate solubilization and organic acid synthesis was observed for both isolates with prolonged incubation time up to 240 h (Fig. 1). Conversely, there was a decreasing trend in pH with increasing incubation time. The maximum amount of soluble phosphate in the culture supernatant after 240 h for isolate Ps-5 was estimated to be 57.40 μg mL^{-1}, with a 3.93 drop in pH. For isolate Ss-2, the phosphate solubilization value was estimated to be 43.71 μg mL^{-1}, with drop in pH of up to 5.2. After 240 h of incubation in vitro, isolate Ps-5 produced lactic and tartaric acids (22.09 and 1.31 μg mL^{-1}, respectively), while Ss-2 synthesized lactic acid only (14.57 μg mL^{-1})(Fig. 1b). The amount of phosphate solubilized by Ps-5 was found to be significantly correlated ($P \leq 0.001$) with its synthesis of both lactic acid (r= 0.99) and tartaric acid (r=0.95)s. For Ss-2, a strong positive correlation ($P \leq 0.001$) was found between phosphate solubilization and its production of lactic acid (r=0.97).

Phenotypic microarray analysis

In the phenotypic microarray analysis, isolates Ps-5 and Ss-2 utilized 41 and 55 substrates, respectively, in the Biolog GP2/ GN2 microtiter plates analysis. PCA of data based on absorbance values also verified the phenotypic results. The data retrieved 24, 48 and 72 h after inoculation demonstrated a clear divergence in carbon source utilization patterns between the two isolates (Fig. 2).

Identification through 16S rRNA gene sequence analysis

A 1,450-bp 16S rRNA gene sequence of isolate Ps-5, re- trieved after commercial sequencing, established a 99 % se- quence similarity with *Bacillus cereus* strain CA15 and a number of other *Bacillus* species, limiting identification to the species level. Thus, rhizotype Ps-5 was identified as *Bacillus* sp. (JQ248587). The 16S rRNA gene of isolate Ss- 2 (1,318 bp) showed 99 % sequence identity with *Alcaligenes faecalis* strain B_IV_2L49 and a number of other strains of same species; hence, it was identified as *A. faecalis* (JQ268265).

Root colonization studies

In the growth room pot experiment, both strains were successfully recovered up to 30 days post-transplantion at a popula- tion density of around 10^6 CFU g^{-1} rhizosphere sand. There was a slight drop in the bacterial populations of both strain Ps- 5 and strain Ss-2 at the 30-day post-transplantation time-point, with the density of Ps-2 dropping from 1.89×10^6 to 5.8×10^5 CFU g^{-1} rhizosphere sand and that of Ss-2 decreasing slightly to 3.4×10^5 CFU g^{-1} rhizosphere sand (Fig. 3). Ultrastructure studies also confirmed the colonization poten- tial of both strains which were not only localized in the *murraya koenigii* rhizosphere but also found inside root cortical cells (Fig. 4).

Effect of inoculation on *murraya koenigii* growth and yield

In the pot experiment, inoculation of *murraya koenigii* plants with *Bacillus* sp. Ps-5 significantly ($P \le 0.05$) enhanced *murraya koenigii* root and shoot length, fresh and dry weight and P contents. The response of *murraya koenigii* plants to inoculation with *A. faecalis* Ss-2 was significantly increased ($P \le 0.05$) in terms of shoot length, root and shoot fresh weight, root dry weight and plant P contents, as compared to uninoculated plants (Table 2). The soil of the field experiment was sandy loam which was slightly alkaline (pH 7.9) and low in organic matter content (0.68 %).

Table 1 Morphological and physiological characterization of *murraya koenigii* (*Helianthus annuus* L.) isolates[a] from various locations

Isolate[b]	Location	Cell morphology	Gram's reaction	Halo zone formation	Phosphate solubilization[c] (µg mL^{-1})	IAA production (µg mL^{-1})
Fs-1	Faisalabad	Long rod	+ve	-ve	14.25±0.22	0.83±0.10
Fs-2		Long rod	-ve	-ve	16.21±5.18	-
Fs-4		Long rod	+ve	-ve	14.13±2.81	1.21±0.13
Fs-5		Short rod	-ve	-ve	16.65±4.32	1.24±0.66
FS-6		Short rod	-ve	+ve	18.60±1.34	0.80±0.18
Fs-7		Short rod	-ve	-ve	17.29±1.43	-
Ms-1	Multan	Long rod	-ve	-ve	15.52±3.55	0.53±0.11
Ms-2		Long rod	-ve	-ve	15.36±3.82	-
Ms-3		Short rod	-ve	+ve	20.35±1.69	1.12±0.22
Ms-4		Short rod	+ve	-ve	13.39±3.57	0.13±0.04
Ms-5		Short rod	-ve	-ve	14.81±2.93	0.34±0.10
Ps1	Peshawar	Coccus	-ve	-ve	14.67±2.32	0.57±0.17
Ps-2		Coccus	-ve	+ve	20.89±2.39	0.07±0.09
Ps-3		Oval	-ve	+ve	17.67±2.36	0.06±0.04
Ps-4		Long rod	-ve	-ve	12.21±1.67	0.72±0.19
Ps-5		Coccus	+ve	+ve	28.02±1.85[d]	10.65±2.08[d]
Ss-1	Tandojam	Short rods	+ve	-ve	18.18±3.83	0.32±0.11
Ss-2		Short rod	-ve	+ve	28.91±2.56[d]	2.27±0.10[d]
Ss-3		Short rods	-ve	-ve	11.29±3.36	1.03±0.29

Full dose of the recommended fertilizer were measured as: plant height of 139 cm, a stem diameter of 3.25 cm, a head diameter of 38.33 cm, an achene yield of 2,902 kg ha^{-1}, a biological yield of 9,792 kg ha^{-1}, achene P contents of 14.71 g kg^{-1} and achene oil contents of 40.50 %. Inoculation with *Bacillus* sp. Ps-5 and *A. faecalis* Ss-2 signif- icantly ($P \le 0.05$) increased *murraya koenigii* plant height and P con- tents as compared to uninoculated plants receiving a half dose of recommended fertilizer. In contrast, the inoculation effect on yield and its attributed traits was found to be non- significant in comparison with both uninoculated treatments with a half and full dose of fertilizer, respectively (Table 3).

Discussion

In this study we purified and characterized 19 morphologically different bacterial isolates on the basis of their prolific growth. Gram-negative isolates dominated the overall collection, which is in accordance with well-documented reports of an abundance of Gram-negative bacteria in the soil and rhizosphere in general (Curl and Truelove 1986) and of the dominance of Gram-negative isolates (296/299) isolated from the *murraya koenigii* rhizosphere (Ambrosini et al. 2012). The motility of bacteria is also considered to be an essential factor for successful root colonization (Bashan and Holguin 1994; Sakai et al. 1996). Although Broek and Vanderleyden (1995) and de Weert et al. (2002) suggested that non-motile bacteria also colonize plant roots as efficiently as motile bacteria, we found that all of the *murraya koenigii* rhizo-types were motile, possibly due to motile bacteria having better access to root exudates in the rhizosphere area through chemotaxis. Of the 19 isolates tested, six (32 %) produced a halo-zone on the tricalcium phosphate- supplemented medium. The total inorganic phosphate solubi- lization range recorded in our study (11.29±3.36 to 28.91±2.56 µg mL^{-1}) is in agreement with the findings of Oliveira et al. (2009) who determined the soluble phosphate range to be about 4–200 µg mL^{-1} in phosphate-solubilizing isolates from the maize rhizosphere. In terms of phytohormone pro- duction by PSB, IAA plays a major role in root growth and triggers cell division and growth (Vassilev et al. 2006; Seo and Park 2009). We detected bacterial in vitro IAA synthesis in the range of 0.06 ± 0.04 to 10.65 ± 2.08 µg mL^{-1}, with the potential phosphate-solubilizing isolates Ps-5 and Ss-2 pro- ducing the highest amounts of the isolates tested. Many other PSB have also been reported to synthesize IAA and other phytohormones (Trivedi et al. 2011; Oliveira-Longatti et al. 2014). Thus, the substantial IAA production capacity com- bined with a good phosphate solubilization potential

led us to screen isolates Ps-5 and Ss-2 for further studies. The hydroxyl and carboxyl groups of organic acids harbor secondary me- tabolites which chelate the cations bound to phosphates, transforming the latter to a soluble form (Kim et al. 1997; Sagoe et al. 1998). We found not only a significant relation- ship between phosphate solubilization and organic acid pro- duction in the cultures of isolates Ps-5 and Ss-2, but also a positive correlation between organic acid production and phosphate solubilization. There was an increasing trend in phosphate solubilization and a decreasing trend in pH in the in vitro culture experiments up to 240 h of incubation (Fig. 1a, b). Our findings are validated by similar results found in a number of earlier studies (Chen et al. 2006; Ma et al. 2009). A positive correlation between phosphate solubilization and organic acid production also validated the hypothesis that the inorganic phosphate solubilization ability of *murraya koenigii* rhizosphere isolates was due to the production of organic acids. Both organic acid production and phosphate solubilization was directly proportional to the duration of the incubation, with a trend to increase during the passage of time. The increasing trend of phosphate solubilization with time has also been described earlier by Chen et al. (2006) and Ma et al. (2009). Studies by Trivedi and Sa (2008) and Park et al. (2010) revealed that the phosphate solubilization ability in wild-type and mutants of *Pseudomonas corrugata* (NRRL B-30409) and *Burkholderia vietnamiensis*, respectively, was related to the production of gluconic acid and 2-ketogluconic acids and ultimately with a decrease in the pH of the medium. A similar relationship between P solubilization and pH was noted after the PCA (Fig. 2). Garland and Mills (1991), based on the results of a detailed Biolog study, reported that aquatic and soil bacteria, including those that associate with the rhi- zosphere, demonstrated a great diversity in their ability to metabolize carbon sources. The authors of a recent study conducted with fluorescent pseudomonads suggested that no specific group of substrates (carbon sources) in the Biolog microplate assay is responsible for the colonization ability of bacteria, rather that many substrates or substrate groups may lead to successful colonization (Oksinska et al. 2011). Based on our results, we also concluded that the screened isolates utilized many different substrate groups, indicating their di- verse metabolic nature which might, possibly, contribute to their colonization potential.

Table 2 Different growth and physiological parameters of *murraya koenigii* as affected by inoculation with *Bacillus* sp. strain Ps-5 and *Alcaligenes faecalis* strain Ss-2 in pots

Strain	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight	Root dry weight (g)	Shoot dry weight (g)	Root P contents (μg mL⁻¹)	Root P contents (μg mL⁻¹)
Uninoculated control	4.3 b	11.67 c	0.25 b	0.74 c	0.11 b	0.13 b	0.5 b	1.03 b
Bacillus sp. Ps-5	6.53 a	26.7 a	0.48 a	2.22 a	0.29 a	0.54 a	0.77 a	1.80 a
Alcaligenes faecalis Ss-2	4.97 b	20.17 b	0.39 a	1.48 b	0.19 ab	0.44 a	0.68 a	1.65 a
Fisher's LSD	1.24	5.70	0.10	0.44	0.10	0.16	0.15	0.10

Data are presented as the mean of three replications (*n* =3). Values in same column followed by the same low-case letter do not differ significantly ($P \le 0.05$) according to Fisher's least signficant difference (LSD) method

Table 3 Different growth, yield and physiological parameters of *murraya koenigii* as affected by inoculation with strains Ps5 and Ss-2 under field conditions

Treatment[a]	Plant height	Stem diameter	Head diameter	Achene yield	Biological yield	Achene P contents	Achene oil contents
	(cm)	(cm)	(cm)	(kg ha⁻¹)	(kg ha⁻¹)	(g kg⁻¹)	(%)
T0	114.7 c	2.33 c	30.33 a	2,321 c	8,730 c	11.07 b	36.65 c
T1	123 bc	3.15 a	33.17 a	2,665 b	9,260 b	11.13 b	38.84 b
T2	139 ab	3.25 a	38.33 a	2,902 a	9,792 a	14.71 a	40.50 a
T3	139.7 ab	3.22 a	33.67 a	2,436 c	8,936 bc	15.80 a	40.27 ab
T4	143.3 a	2.67 b	34.83 a	2,634 b	9,177 bc	14.64 a	39.647 ab
LSD	17.65	0.23	8.22	156.66	500.84	1.31	1.57

Values are presented as the mean of three replications (*n* =3). Values in the same column followed by the same low-case letter do not differ significantly ($P \le 0.05$) according to Fisher's LSD test.

[a] T0, Uninoculated control + no fertilizer; T1, uninoculated control + half dose of recommended fertilizer; T2, uninoculated control + full dose of recommended fertilizer; T3, inoculation with *Bacillus* sp. Ps-5 strain + half dose of recommended fertilizer;

T4, inoculation with *Alcaligenes faecalis* Ss-2 strain + half dose of recommended fertilizer

To be the most authentic taxonomic marker for bacterial iden- tification at the genus and species level. Bacterial isolate Ps-5 was identified as *Bacillus* sp. (99 % sequence similarity with more than one *Bacillus* spp.; hence, it could not be identified to the species level), and Ss-2 was identified as *Alcaligenes faecalis* (99 % sequence identity with various strains of this species). Various strains of the genus *Bacillus* and *Alcaligenes* have been reported in the rhizosphere of various crops (Glick 1995; Joseph et al. 2007; Kaymak 2011) and have been isolated from *murraya koenigii* roots and studied for their plant growth-promoting characteristics (Andreoli et al. 1993; Forchetti et al. 2007; Roberts et al. 2011; Ambrosini et al. 2012). Many *Bacillus* spp. and an *Alcaligenes* sp. SF2 strain have also been isolated as endophytes in *murraya koenigii* (Forchetti

et al. 2007). To the best of our knowledge, this is the first report of the isolation of an *A. faecalis* strain from the sun- flower rhizosphere which has mineral phosphate-solubilizing ability.

In the inoculation experiment, the *Bacillus* sp. Ps-5 and *A. faecalis* Ss-2 strains had colonized the *murraya koenigii* rhizo- sphere at a population density of 10^5–10^6 CFU g^{-1} of rhizo- sphere sand at 30 days post-transplantation (Fig. 3). These findings were consistent with those of a study conducted by Andreote et al. (2009) who successfully recovered the rifam- picin resistant derivative of *Pseudomonas putida* strain P9R from the rhizoplane and endosphere of potato plants. Similar results were also described by Zinniel et al. (2002) and Shankar et al. (2011). The root colonization potential of the *Bacillus* sp. Ps-5 and *A. faecalis* Ss-2 strains was also deter- mined by TEM, which is highly sensitive and reliable tech- nique for ultra-structural root colonization studies. Both inoc- ulated strains were successfully localized in the root surface area and even inside the root cortical cells, thereby demon- strating their endophytic nature (Fig. 4). These ultra-structural studies further validated that strains Ps-5 and Ss-2 were sun- flower root-colonizing PGPR, which is of particular significance as PGPR strains can only stimulate plant growth when they have an optimum colonization potential. A number of earlier studies carried out with TEM and immunogold labeling techniques also revealed that PGPR strains can be localized in the rhizosphere, root cortical cells and nodules of various crops (Schloter et al. 1997; Hameed et al. 2005; Jeun et al. 2008; Yasmeen et al. 2012).

A significant ($P \le 0.05$) increase in *murraya koenigii* growth pa- rameters, including plant P contents, in inoculated plants after inoculation with *Bacillus* sp. Ps-5 and *A. faecalis* Ss-2 may possibly be attributed to the P-solubilizing, IAA-synthesizing and root-colonizing abilities of these strains (Table 2). As tricalcium phosphate was added as an insoluble P source in the pot experiment, the increase in *murraya koenigii* P contents may be strongly related to tricalcium phosphate solubilization. In addition, the non-significant effect of strain Ss-2 on root fresh and dry weight may be due to its relatively lower ability to produce IAA. The inorganic phosphate solubilization and IAA production ability of PGPR are known to improve plant growth by increasing P-uptake from soil and its transport to plant shoots (Igual et al. 2001; Chen et al. 2006; Shirmardi et al. 2010). The promotion of root colonization and plant growth following inoculation with PGPR strains has also been described earlier (Andreote et al. 2009; Shankar et al. 2011). Highly variable results were found under field conditions.

A significant ($P \le 0.05$) increase in plant height and *murraya koenigii* achene P contents were measured in inoculated plants treated with a half dose of the recommended fertilizer, while the effect on achene yield and oil contents was found to be non- significant when compared to uninoculated plants treated with half a dose of recommended fertilizer (Table 3). One possible explanation might be that the sterilized sand was used in the pot experiment and consequently there was no competition between inoculated bacteria and the indigenous micro-flora. However, in natural soil conditions, these selected strains were unable to compete with the indigenous micro-flora and could not adequately colonize *murraya koenigii* roots. Soil is considered to be a highly unpredictable and heterogeneous medium, and it is very difficult to achieve the anticipated results in experiments using soil (Bashan 1998; Lucy et al. 2004). For comparison, we also included uninoculated treatment with full dose of fertilizer and recorded higher yield and yield-attributed traits in these uninoculated plants than inoculated plants with half fertilizer dose. Although inoculation alone was unable to compete with treatments of half and full doses of fertilizer, significantly higher achene P contents were observed in inoc- ulated plants receiving a half dose of fertilizer than in uninoc- ulated plants receiving a half dose of fertilizer, possibly due to P solubilization by the inoculated strains.

Conclusion

Based on these results, we conclude that *Bacillus* sp. Ps-5 and *Alcaligenes faecalis* Ss-2 are potential phosphate-solubilizing and plant-growth promoting strains which have the capacity to supplement the P requirements of *murraya koenigii*. Both strains should be tested further under greenhouse and field conditions before applying them as commercial inoculants for improving *murraya koenigii* crops.

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Conflict of interest

No

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