INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–1054/2015/17–6–1110–1116
DOI: 10.17957/IJAB/15.0033
http://www.fspublishers.org

Full Length Article

First Report of *Providencia vermicola* Strains Characterized for Enhanced Rapeseed Growth Attributing Parameters

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Abstract

Ever-increasing prices of synthetic fertilizers in developing countries put a huge burden on farmer community in terms of per unit cost of production. Supplementing chemical fertilizers with bacterial inoculants offers an alternative strategy to sustain yield by reducing cost of produce. Thus, in current study, two *Providencia vermicola* strains KP-21 and KR-29 were identified through 16S rRNA gene sequence analysis. The KP-21 and KR-29 strains were originally isolated from rhizosphere of rapeseed grown in Peshawar and Rawalakot, respectively. Morphologically, both strains were similar with round creamy colonies, short rod and Gram-negative cells under light microscope. *P. vermicola* KP-21 was found promising in phosphate soubilization and auxin biosynthesis. Likewise, strain *P. vermicola* KR-29 was found to be a potential phosphate solubilizer, auxin producer in addition to its considerable acetylene reduction capacity. High performance liquid chromatography (HPLC) analysis revealed that strain KP-21 produced gluconic and lactic acids, while strain KR-29 synthesized gluconic, malic and lactic acids in considerable amounts. Both strains densely colonized rapeseed rhizosphere area as depicted in transmission electron micrographs. Moreover, the strains significantly ($P \leq 0.05$) augmented rapeseed root and shoot length, dry weight, and phosphorus contents, while a significant increase in root and shoot nitrogen contents was only recorded in plants inoculated with strain KR-29 as compared to non-inoculated plants. We concluded that *P. vermicola* strains are being reported for the first time as rapeseed growth promoting agents. In addition, strain KP-21 and KR-29 were prospective PGPR rhizotypes which were recommended to be evaluated further under field conditions comprising of different agro-climatic locations before using them as commercial bio-inoculants.

Keywords: *Providencia vermicola*; 16S rRNA; Nitrogen fixation; Phosphate solubilization; Transmission electron microscopy

Introduction

Current agricultural technologies stress the use of microbes in terms of their innate potential to provide essential nutrients to plants from environment (Bashan *et al.*, 2014). Physiologically the most explored portion of this microbiome inhabits closely to plant roots, thus referred to as plant growth promoting rhizobacteria (PGPR). Mechanistically, PGPR promote plant growth by ensuring micro and macro nutrient availability, synthesizing various phytohomones, inducing stress resistance and producing siderophores, antifungal compounds and lytic enzymes (Compant *et al.*, 2005; Son *et al.*, 2014). Another important plant growth-promoting mechanism of PGPR is production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and subsequent lowering of ethylene levels in plant tissues (Glick, 1995).

Inoculating plants with PGPR and other biological approaches is becoming popular worldwide, especially in developing countries. This has led agronomists and environmentalists to focus on integrated nutrient management systems (Ma *et al.*, 2011). Increasing costs of synthetic fertilizers, in addition to their environmental impacts, has led farmers to accept the alternative strategy of using biofertilizers (Bashan *et al.*, 2014). Several PGPR inoculants have been commercialized for their use as agricultural inputs. Bacterial genera *Pseudomonas*, *Bacillus*, *Streptomyces* and *Agrobacterium* are bio-control agents, which has been commercialized and marketed. They produce antibiotics or siderophores for the induction of systemic resistance against phytopathogens (Gardener and Fravel, 2002). In addition, biofertilizers with atmospheric

To cite this paper: Hussain, K., S. Hameed, M. Shahid, A. Ali, J. Iqbal and D. Hahn, 2015. First report of *Providencia vermicola* strains characterized for enhanced rapeseed growth attributing parameters. *Int. J. Agric. Biol*., 17: 1110–1116

dinitrogen fixing and phosphate solubilizing bacteria have also been commercialized and marketed (Chabot *et al.*, 1996; Bashan and Holguin, 1997).

Use of bioinoculants for agricultural benefits is expanding in Pakistan. After attaining satisfactory results in cereals, the focus of inoculating plants with beneficial bacteria is now on oilseed crops (Shahid *et al.*, 2012). Pakistan is importing a huge amount of edible oil from other countries and there is a huge gap between edible oil production and its consumption (Khan *et al.*, 2000). To minimize this gap and reduce the large export bill on the import of edible oil, the constraints in terms of local edible oil production and marketing need to be addressed. High costs and adverse environmental consequences of synthetic fertilizers are main limiting factors of low cultivation of oilseeds in addition to their marketing issues. Thus, the current study aims at characterization and identification of promising PGPR strains and their subsequent inoculation to rapeseed plants, in order to use these strains as bioinoculants for enhanced growth and yield attributing traits in oilseed rapeseed (*Brassica napus* L.) crop.

Materials and Methods

Isolation and Morphological Studies

Isolates KP-21 and KR-29 were isolated from rhizosphere of rapeseed cultivated at two agro-ecologically different zones of Pakistan, Peshawar (34º 00′ 56″N, 71º 42′ 46″E) and Rawalakot (33°5′ 13″N, 73° 45′ 34″E) by serial dilution plate method (Somasegaran and Hoben, 1994). Intact rapeseed roots with adhering soil, at maturity stage, were transferred to sterile plastic bags (20 × 25 cm) after excising aerial portions with sterilized knife. The samples were transported to Microbial Physiology Laboratory, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad Pakistan. The roots were then shaken gently in the sterile distilled water to detach the loosely adhering soil. One gram of strictly bound soil was added in 9 mL 0.85% (w/v) NaCl solution and serially diluted to isolate bacteria on Luria-Bertani (LB) agar media by spreading 100 µL from each of the dilutions 10^{-3}, 10^{-5} and 10^{-7}. Selection of the two isolates from huge number of rhizotypes obtained was based on their prolific phyto-beneficial traits. Pure bacterial colonies after repeated streaking on LB-agar were stored in 20% (v/v) glycerol at -80ºC. Colony and cell shapes, motility, and Gram reaction were studied under light microscope (Olympus). Physico-chemical analyses of soils were commercially carried out from Soil Fertility Laboratory, Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Soil electrical conductivity and pH were measured on EC and pH meters (ATAGO®, Japan). Soil texture was determined by using USDA soil texture calculator, while organic matter was estimated by standard ignition and weight loss method. Total nitrogen of soil was calculated through micro-Kjeldahl procedure (He *et al*., 1990) and available phosphorus was measured through Olsen method (Olsen *et al*., 1954).

Nitrogen Fixation

Acetylene reduction assay (ARA) was carried out to determine nitrogen fixing ability of bacterial isolates as described by Hardy *et al.* (1968). Both isolates individually inoculated in 30 mL semisolid nitrogen-free malate medium (NFM) in 125 mL Erlenmeyer flasks and grown in an orbital shaker at 150 rpm and 28 ± 2ºC for 72 h. Glass vials of 12 mL capacity, containing 6 mL NFM semisolid media were then inoculated with 5 mL well-grown culture and vials were incubated at 28±2ºC for 24 h. Afterwards, the steel caps of vials were replaced with rubber seals. Similarly, air (10%, v/v) was replaced with an equal volume of acetylene (C_2H_2) using a sterilized syringe followed by incubation at 28±2ºC for 24 h. Conversion of C_2H_2 to ethylene (C_2H_4) was recorded through a gas chromatograph (Thermoquest, Trace GC, Model K, Rodono Milan, Italy) equipped with a Porapak N column (2 mm × 2 m) and a flame ionization detector (FID). The operating specifications were: oven temperature, 80ºC, right inlet 100ºC, right detector 180ºC, nitrogen flow rate 42 mL min^{-1}, hydrogen flow rate 30 mL min^{-1}, and air flow rate was maintained at 300 mL min^{-1}. A 200 µL of standard ethylene (Spancan Calibration Gas, Spantach Products, England) was run before injecting the same volume of test samples and peak area was recorded. Protein concentration in vial mixture was calorimetrically estimated according to an already described method (Bradford, 1976).

Phosphate Solubilization and Organic Acid Production

In vitro phosphate solubilization capacity of both isolates was estimated calorimetrically by inoculating single colony of each purified isolate into 500 mL Erlenmeyer flask before subsequent incubation at 28±2°C for 240 h in an orbital shaker at 150 rpm. A sample containing 20 mL from each flask was then harvested and spun at 13,000 *g* for 10 min to collect supernatant. Measurement of soluble phosphate in culture supernatant was carried out according to phosphomolybdate blue color method (Murphy and Riley, 1962) using M350 double beam scanning spectrophotometer (Camspec M350, UK) at 882 nm.

The same supernatant was filtered through 0.2 µm nylon filters (Millipore, USA) to measure the nature and amount of organic acids. For this purpose, a sample of 20 µL was analyzed by using a high-performance liquid chromatography (HPLC) system equipped with Turbochrom software (Perkin Elmer, USA) and a C18 column (length 150 mm, diameter 4mm, pore size 120 Å). The composition of mobile phase was methanol acetic acid (30 70, v/v) and a flow rate of 0.6 mL min^{-1} was set. The organic acids to be analyzed were commercially purchased (Sigma, USA). The test samples

were analyzed through spectrometry at 210 nm for the presence or absence of gluconic, malic, lactic, citric, succinic and tartaric acids.

Indole-3-acetic Acid Production

Both the isolates KP-21 and KR-29 were inoculated into 100 mL LB-broth supplemented with tryptophan (100 mg L^{-1}) in 500 mL Erlenmeyer flasks and incubated at 28±2°C for 48 h in an orbital shaker at 150 rpm. A sample of 40 mL from these well-grown cultures was collected in falcon tubes and spun at 13,000 *g* and harvested supernatant was acidified (pH 2.8) with hydrochloric acid. For indole-3-acetic acid (IAA) extraction, an equal amount of ethyl acetate was mixed in separating funnel (Tien *et al.*, 1979). The ethyl acetate upper layer containing IAA was collected in separate tubes which were then placed in a rotary evaporator under vacuum at 45°C to remove the ethyl acetate. The remaining extract was dissolved in 1 mL methanol followed by filtration through 0.2 µm nylon filter (Millipore). The filtrates were analyzed on HPLC system equipped with Turbochrom software (Perkin Elmer) and C18 column at 260 nm. The composition of mobile phase and flow rate were; methanol: water (30: 70, v/v) and 0.5 mL min^{-1}, respectively.

Amplification and Sequencing of 16S rRNA Gene

To extract total genomic DNA of isolates KP-21 and KR-29, alkaline lysis method was employed as described by Maniatis *et al.* (1982). Quantification of isolated DNA was accomplished using ultraspec™ 3100 ($OD_{260,\ 260/280}$) which then served as a template to amplify 16S rRNA gene. The primers used for amplification were FD1 and rD1 with slight modifications into an already described method (Weisburg *et al.*, 1991) as: for 50 µL reaction in purified water, 5 µL of Taq buffer (Fermentas, USA), 3 µL of 25 mM $MgCl_2$ (Fermentas, USA), 5 µL of 2 mM dNTPs (Fermentas, USA), 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, USA) and 40 ng of template DNA. The reaction was carried out in thermal cycler ((peQlab, advanced Primus 96, UK) with modified temperature conditions; 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. Purification of amplicons was made with QIAquick PCR purification kit (Qiagen, USA) and subsequently commercial sequencing was carried out directly on both sides (Eurofins, Germany). The retrieved sequences were analyzed, trimmed and assembled using Sequencher (ver. 5.2.4) software. Assembled sequences were compared with others in GenBank database using NCBI BLASTn tool and deposited in data bank for accession numbers.

Root Colonization Studies thorough Transmission Electron Microscopy

For transmission electron microscope (TEM) studies, seeds of rapeseed (*Brassica napus* L. cv. Durre-NIFA) were immersed in 5% (w/v) sodium hypochlorite for 10 min followed by 6 washings with sterilized water for surface sterilization. The seeds were inoculated by immersing them in the bacterial inoculum (1×10^7 CFU mL^{-1}) of both strains for 30 min followed by germination water agar plates (1.5%, w/v). Roots of seven days old seedlings were chopped (approximately 1–3 cm pieces) inserted in water agar again to cut approximately 2–3 mm^3 small agar cubes which were then placed into 1.5 mL tubes in the presence of gluteraldehyde (5% v/v in 0.2 M PIPES[a] buffer, pH 8.0) as fixative. After incubation of 16–18 h, the fixative was replaced with 0.2 M PIPES buffer (0.58 g NaCI 3 g PIPES, 1 M NaOH, 0.2 g $MgCl_2.6H_2O$, pH 6.8)[1]. The samples were washed twice for 1 h in fresh buffer followed by treatment with aqueous uranyl acetate (5%, w/v) for 16–18 h. After washing twice with sterilized distilled water for 30 min, first dehydration with absolute alcohol was carried out twice for 30 min. and then with 100% (v/v) propylene oxide in ratio 1:1 for 24–48 h followed by spur resin for further 24–72 h. The accelerator Benzyl Di-Methyl Amine (BDMA) was used in all infiltration steps. Samples were shifted to flat embedding molds and polymerized for 72 h at 65–70°C. After polymerization, resin blocks were left at room temperature for 24 h before cutting ultra-thin sections of 150–200 nm on ultramicrotome (RMC-7000 UK,). The sections were carefully positioned at copper grids and were double stained with uranyl acetate and lead citrate for 30 min. and 10 min., respectivley. The grids were washed with deionized water and observed under TEM (JEOL 1010, Japan).

Pot Experiment

The pot experiment was conducted in growth room using a completely randomized design (CRD). Surface sterilized rapeseed seeds (cv. Durre-NIFA) were germinated on water agar plates (1.5%, w/v). Inoculum was prepared by growing both strains, KP-21 and KR-29, in LB-both up to 1×10^9 CFU mL^{-1}. The cultures were pelleted at 8000 *g* and washed twice with NaCl solution (0.85 %, w/v). Cell density was adjusted to 10^8 CFU mL^{-1} by dilution with a saline solution. Maximum possible sterility of growth room was achieved by fumigation before the start of experiment and a cycle of 16 h light/8 h dark at 25°C was set. Pots (9 cm diameter) were added with 400 g of steam sterilized sand. Tricalcium phosphate (TCP) was mixed in sand (200 mg kg^{-1} sand) as insoluble inorganic P source. For inoculation, roots of three days old agar-grown rapeseed seedlings were dipped in inoculum for 20 min. The remaining inoculum was

[a](N,N′-bis(2-ethanesulfonic acid)

subsequently added (7 mL 100 g^{-1} sand) and mixed in sand at the time of transplanting. Except non-inoculated control treatment, each pot was transplanted with one inoculated seedling. Pots were sprayed with half-strength Hoagland's solution (Arnon and Hoagland, 1940) without nitrogen and phosphorus sources (10 mL pot^{-1} daily). Various growth and physiological parameters, including nitrogen and phosphorus contents, were measured 30 days after transplanting. Root and shoot nitrogen contents were determined by wet digestion with H_2SO_4 by microkjeldahl method (Sparks *et al.*, 1996), while that of phosphorus contents were determined by the vanadium phospho-molybdate yellow color method (Yoshida *et al.*, 1971).

Results

Morphological Characteristics and Soil Analysis

Electrical conductivity of sample soils collected from Peshawar and Rawalakot was 0.79 and 0.71 dS m^{-1}, soil pH was 7.8 and 8.0, respectively. Both soil samples were found to be clay loam in nature with organic matter of 0.77% (Peshawar) and 1.13% (Rawalakot). Total nitrogen (N) and available phosphorus (P) of experimental soils of Peshawar and Rawalakot were 0.35 and 0.47 g kg^{-1}, and 5.2 and 6.9 mg kg^{-1}, respectively (Table 1). Both the isolates KP-21 and KR-29 were morphologically similar with round and creamy colonies and short rod-shaped cells under light microscope (Table 2). Gram staining of pure cells exhibited that both types of cells were Gram-negative.

Plant-beneficial Traits and Organic Acids Synthesis

Both isolates were found potent tricalcium-phosphte (TCP) solubilizers. The conversion of TCP to soluble phosphate by isolate KP-21 and KR-29 was estimated at 34.48 and 37.15 µg mL^{-1}, respectively (Table 2). Out of the two isolates, only KR-29 showed acetylene reduction capacity of 462.95 ηMol mg^{-1} protein h^{-1} which indirectly represented its diazotrophic nature. The amount of IAA synthesized in culture medium by the isolates KP-21 and KR-29 was recorded as 4.41 and 5.52 µg mL^{-1}, respectively. Nature and amount of organics acids bio-synthesized is presented in Table 3. Isolate KP-21 synthesized appropriate amounts of gluconic and lactic acids (15.65 and 20.35 µg mL^{-1}, respectively) in vitro. On the other hand, Rawalakot isolate KR-29 produced gluconic, malic and lactic acids in culture medium (14.37, 7.54 and 12.21 µg mL^{-1}, respectively).

Molecular Identification and Ultrastructure Studies

On molecular basis, strains KP-21 and KR-29 were identified as *Providencia vermicola* after 16S rRNA gene sequence analysis. A 16S rRNA gene sequence length of 1461 and 1525 bp for strains KP-21 and KR-29, respectively, showed 99% sequence identity with *P. vermicola* strain FFA9 in GenBank database (Table 4). *P. vermicola* strains KP21 and KR-29 were localized in the rhizosphere area of rapeseed roots (Fig. 1). Both strains were found densely populated in rhizosphere locality indicating their root colonization capacity.

Pot Experiment

Rapeseed plants inoculated with strains KP-21 and KR-29 showed significantly ($P \leq 0.05$) higher root and shoot length, and shoot dry weight as compared to non-inoculated plants. Root dry weight of *P. vermicola* KR-29 inoculated plants was found to be significantly ($P \leq 0.05$) higher than untreated plants. On the other hand, inoculation response of strain *P. vermicola* KP-21 on root dry weight was determined statistically at par with non-inoculated plants. Inoculation with strain KR-29 significantly ($P \leq 0.05$) enhanced root and shoot nitrogen and phosphorus contents, while inoculation with strain KP-21 resulted in significant increase in root and shoot phosphorus contents as compared to non-inoculated plants.

Discussion

Plant growth-promoting rhizobacteria have gained worldwide significance to supplement the synthetic fertilizers and sustain plant productivity in a cost-effective and environment friendly manner. A continuous interest of scientists to understand the mechanisms involved in phyto-beneficial multi-traits exhibited by PGPR and their adaptation to variable environmental conditions shows the importance of these biological factories for plant growth (Figueiredo *et al.*, 2011). In the current study, we isolated and screened two PGPR strains, KP-21 and KR-29, on the basis of their *in vitro* plant-beneficial traits like acetylene reduction, tricalcium phosphate solubilization and IAA synthesis. Isolate KP-21 and KR-29 were purified from rhizosphere of flowering rapeseed plants cultivated at Peshawar and Rawalakot, respectively and rhizosphere soils of both locations were clay loam, alkaline, and low in organic matter, total N and available P (Table 1). Such soils are already documented to harbor many PGPR strains (Goldstein and Krishnaraj, 2007). The dominance of Gram-negative short rod PRPRs in these soil conditions is also described (Ambrosini *et al.*, 2012; Shahid *et al.*, 2014). Although both the isolates were purified from different locations, but they are found morphologically similar in light microscopy studies.

Nitrogen and phosphorus are found to be the major limiting factors for crop productivity. Conversely, a huge amount of fixed P is present in acidic and alkaline soils in the form of Ca-phosphates, and Fe and Al-phosphates, respectively (Ahemad and Kibret, 2014). The in vitro acetylene reduction capacity is indirectly considered to be at par with atmospheric N_2-fixing ability of the isolates. Thus, acetylene reduction trait is only exhibited by isolate KR-29

Table 1: Different physico-chemical properties of soils of sampling locations

Location	Electrical conductivity (dSm^{-1})	pH	Type[a]	Organic matter (%)	Total nitrogen ($g\ kg^{-1}$)	Available phosphorus ($mg\ kg^{-1}$)
Peshawar	0.79	7.8	Clay loam	0.77	0.35	5.2
Rawalakot	0.71	8.0	-do-	1.13	0.47	6.9

[a]Soil type was determined by putting sand, silt and clay values in USDA soil texture calculator

Table 2: Morphological and physiological characteristics of rapeseed isolates KP21 and KR-29

Isolate	Colony Shape	Cell Shape	Gram reaction	N_2- fixation (ηMol mg^{-1} protein h^{-1})[a]	Phosphate Solubilization ($\mu g\ mL^{-1}$)[a]	IAA production ($\mu g\ mL^{-1}$)[a]
KP-21	Round, creamy	Short rods	-ve	-	34.48±2.12	4.41±0.11
KR-29	-do-	-do-	-do-	462.95±18.22	37.15±4.22	5.52±0.86

[a]Values represent mean of three replications ± standard deviation

Table 3: Measurement of organic acids synthesized by isolate KP-21 and KR-29 after 240 h of incubation

Isolate	Gluconic acid ($\mu g\ mL^{-1}$)[a]	Malic acid ($\mu g\ mL^{-1}$)[a]	Lactic acid ($\mu g\ mL^{-1}$)[a]	pH change[b]
KP-21	15.65±2.39	-	20.35±1.23	1.9±0.47
KR-29	14.37±2.37	7.54±1.42	12.21±1.24	2.6±0.65

[a]Values represent mean of three replications ± standard deviation
[b]Initial pH of medium was adjusted at 7, thus pH decrease denotes the difference between initial and pH at harvest

Table 4: Molecular identification of isolate KP-21 and KR-29 based on 16 rRNA gene sequence analysis

Isolate	Sequence length	Closest GenBank match	% identity	Strain identified[a]	Accession no.
KP-21	1461	*Providencia vermicola* strain FFA9	99	*Providencia vermicola*	JQ612517
KR-29	1525	*Providencia vermicola strain* FFA9	99	*Providencia vermicola*	JQ612520

[a]*The quality of sequence before BLASTn analysis was guaranteed by trimming and assembling the both forward and reverse sequences through Sequencher (ver. 5.2.4) software package*

Table 5: Growth and physiological parameters of rapeseed measured 30 days post inoculation

Treatment	Root length (cm)	Shoot length (cm)	Root dry wt. (g)	Shoot dry wt. (g)	Root N contents ($mg\ g^{-1}$)	Shoot N contents ($mg\ g^{-1}$)	Root P contents ($mg\ g^{-1}$)	Shoot P contents ($mg\ g^{-1}$)
KP-21	4.56b	15.97b	0.18b	0.39b	0.99b	1.61b	1.04a	2.32a
KR-29	5.84a	20.26a	0.26a	0.59a	1.51a	3.30a	0.63b	1.57b
Non-inoculated	3.12c	12.86c	0.14b	0.26c	1.09b	1.56b	0.37c	0.98c
LSD ($p \leq 0.05$)	1.12	3.09	0.5	0.11	0.13	0.66	0.19	0.49

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 ($P \leq 0.05$) according to Fisher's least significant difference (LSD) method

and the amount of acetylene reduction (462.95 ηMol mg^{-1} protein h^{-1}) was found similar to that already reported in diazotrophic bacteria isolated from field-grown barley wheat and oat (Venieraki *et al.,* 2011). On the other hand, the inorganic tricalcium phosphate solubilized in vitro by isolates KP-21 and KR-29 (34.48 to 37.15 µg mL^{-1}, respectively) was in agreement with the outcomes of Oliveira *et al.* (2009) who found the soluble phosphate range of 4–200 µg mL^{-1} in phosphate solubilizing isolates purified from maize rhizosphere. In soil ecosystem, bacterial phosphate solubilization is greatly attributed to the production of organic acids carrying hydroxyl (-OH) and carboxyl (-COOH) groups, which chelate the cations (Ca, Al, Fe) attached to soil phosphates to make them plant available monobasic (HPO_4^{2-}) and dibasic ($H_2PO_4^-$) phosphates (Kim *et al.*, 1997; Sagoe *et al.,* 1998). Thus, we measured the nature and amount of organic acids produced by isolates KP-21 and KR-29. Isolate KP-21 produced gluconic acid (15.65 µg mL^{-1}) and lactic acid (20.35 µg mL^{-1}), while isolate KR-29 synthesized gluconic acid (14.37 µg mL^{-1}), malic acid (7.54 µg mL^{-1}) and lactic acid (12.21 µg mL^{-1}). Similar results of organic acid production by phosphate solubilizing bacteria, retrieved in earlier studies, validated our findings (Chen *et al.*, 2006; Ma *et al.*, 2009; Shahid *et al.*, 2012). Moreover, both the isolates produced considerable amounts of IAA in tryptophan supplemented medium (Table 2). IAA plays a key role in cell division and growth in addition to its significance root proliferation (Vassilev *et al.*, 2006; Seo and Park, 2009). Many phosphate solubilizing bacteria have also been reported to synthesize IAA and other phytohomones (Trivedi *et al.*, 2011; de Oliveira-Longatti *et al.*, 2014). The promising results on acetylene reduction to ethylene, phosphate solubilization, and organic acid synthesis, and IAA production led us to screen isolates KP-21 and KR-29 among many other isolates purified from rapeseed rhizosphere.

Isolates KP-21 and KR-29 were subjected to molecular identification and ultrastructure studies before inoculating them directly to rapeseed plants. For molecular identification, 16S rRNA gene is considered to the authentic taxonomic marker in bacterial world. Surprisingly, both rhizotypes KP-21 and KR-29 were identified as *P. vermicola*

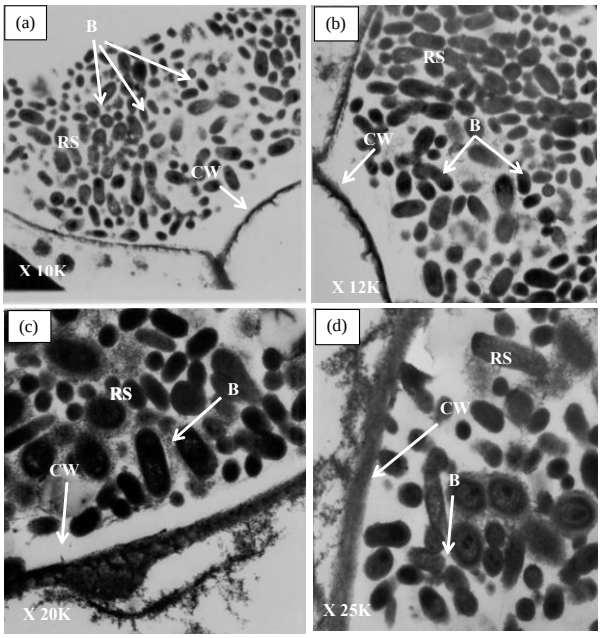

Fig. 1: Root colonization potential of Providencia vermicola strain KP-21 (a, b) and Providencia vermicola strain KR-29 (c, d) in gnotobiotically-grown rapeseed rhizosphere. *B* Bacteria, *RS* Rhizpsphere, *CW* Cell wall

strains (JQ612517 and JQ612520, respectively) after BLAST analysis of their 16S rRNA gene despite their diffent agroclimatic niches (Table 4). Both strains showed 99% sequence identity with *P. vermicola* strain FFA9, which was considered enough to describe the strains up to species level. Like morphologicaly similarity, genetic similarity of both strains might be due to the root enviroment created by similar crop plant. This may also be attributed to the similar culture conditions in laboratory. *P. vermicola* strain Ama-2 has recently been reported to augment mung bean growth throgh ACC-deaminase activity and auxin production (Akhtar and Ali, 2011). To the best of our knowledge, *P. vermicola* has seldom been documented as PGPR. In addition, this is the first report of isolation, characterization of *P. vermicola* strains from rapeseed roots and their reinoculation to rapeseed plants for enhanced growth and productivity.

Ultrastructure root colonization studies through TEM is considered to be authentic and reliable approach to check rhizosphere competance of screened strains. A dense bacterial colonization for both strains was localized in rapeseed roots as depicted in Fig. 1. Thus, both *P. vermicola* strains were found to be effective rapeseed root colonizers which can be described as key factor to execute PGPR activities after inoculation to plant roots or seeds. Localization of PGPR strains in roots and nodules through TEM and immunogold labelling techniques has already been well documented (Hameed *et al.*, 2005; Jeun *et al.*, 2008; Yasmeen *et al.*, 2012; Shahid *et al.*, 2014). When inoculated to rapeseed plants in pots, a significant ($P \leq 0.05$) increase in length, dry weight, P contents and N contents in both roots and shoots was measured in strain KR-29 inoculated plants as compared nonincoculated control plants. On the other hand, a significant ($P \leq 0.05$) increase in root and shoot length, shoot dry weight and root and shoot P contesnts was estimated in strain KP-21 treated plants, while the effect of inoculation on root dry weight, and root and shoot N contents was found non-significant ($P \leq 0.05$) compared to noninoculated control plants. The stimulatory effect on these growth parameters for both strains may be due to their nitrogen fixing, phosphate solubilizing and auxin production ability. Conversely, non-significant ($P \leq 0.05$) effect of strain KP-21 on rapeseed root and shoot N contents may be due to lack of its diazotrophic nature. The results retrieved in number of other studies conclude that inoculation of PGPR strains enhance plant growth and productivity through nutrient aquisition and transport, and auxin biosynthesis (Igual *et al.*, 2001; Chen *et al.*, 2006; Shirmardi *et al.*, 2010; Shahid *et al.*, 2012; 2014).

Conclusion

The current study, for the first time, chracterized two promising *P. vermicola* strains from rapeseed rhzosphere, which augmented the growth of rapeseed plants considerably after inculation. Considering the issues of high prices of synthetic fertilizers, their nonavailablity to farmers in thirdworld countries like Pakistan in addition to the environmental pollution, *P. vermicola* strains KP-21 and KR-29 can be used as bioinoculants to supplement chemical fertilizers. Further studies are in progress to evaluate the strains under field conditions and variable environments before using them as commercial biofertilizers.

Acknowledgments

We are highly grateful to Higher Education Commission (HEC), Pakistan for providing funds for this research. We are also thankful to Mr. Ghulam Abbas for his technical assistance to compile the data

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(Received 29 December 2014; Accepted 16 April 2015)