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SELECTIVE ISOLATION AND CHARACTERIZATION OF AGRICULTURALLY BENEFICIAL ENDOPHYTIC BACTERIA FROM WILD HEMP USING CANOLA

IMRAN AFZAL[1], ZABTA K. SHINWARI[2*] AND IRUM IQRAR[1]

[1]*Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan*
[2]*Pakistan Academy of Sciences, Islamabad, Pakistan*
[*]*shinwari2008@gmail.com*

Abstract

Endophytic bacteria can provide a useful alternative to synthetic fertilizers to improve plant growth. Wild plants are little investigated as a source of growth promoting endophytic bacteria for commercial application to crops. In present study, endophytic bacteria were isolated from *Cannabis sativa* L. (hemp) using two different methods to examine their ability to promote canola growth. Besides direct isolation from the roots, endophytic bacteria were also selectively isolated from the rhizosphere of *C. sativa* using canola. Under gnotobiotic conditions, six bacteria from the selective isolation significantly improved canola root growth, as compared to the two bacteria isolated from direct method. Overall, three isolates performed distinctly well, namely, *Pantoea vagans* MOSEL-t13, *Pseudomonas geniculata* MOSEL-tnc1, and *Serratia marcescens* MOSEL-w2. These bacteria tolerated high salt concentrations and promoted canola growth under salt stress. Further, the isolated bacteria possessed plant growth promoting traits like IAA production, phosphate solubilization, and siderophore production. Most isolates produced plant cell-wall degrading enzymes, cellulase and pectinase. Some isolates were also effective in hindering the growth of two phytopathogenic fungi in dual culture assay, and displayed chitinase and protease activity. *Paenibacillus* sp. MOSEL-w13 displayed the greatest antifungal activity among all the isolates. Present findings conclude that wild plants can be a good source for isolating beneficial microbes, and validates the employed selective isolation for improved isolation of plant-beneficial endophytic bacteria.

Key words: Selective isolation, Plant-beneficial endophytic bacteria, *Cannabis sativa* L., *Brassica napus* L., Antifungal, Rhizosphere.

Introduction

Endophytic bacteria are a group of plant associated bacteria known to provide growth benefits to their host. The bacteria achieve this by producing plant hormones, increasing nutrient availability and mitigating biotic and abiotic stress (Glick, 2012). Unlike rhizobacteria, the well-known bacteria establishing symbiotic relationships with many plants, endophytes thrive within the internal tissues of plants and use specialized mechanisms to enter the host (Compant *et al.,* 2010). Primarily, they enter the host from the rhizosphere soil surrounding the roots (Conn & Franco, 2004). This entry is also regulated by the plant host itself (Dong *et al.,* 2003). As a result, plants are known to harbor specific microflora as their mutualistic symbionts. On the other hand, endophytic bacteria can have multiple hosts, being either host specific or general endophytes (Hardoim *et al.,* 2008).

Most of the work on endophytic bacteria has been focused on agricultural crops. However, a great number of plants still remain unstudied for their endophytic diversity, particularly the wild and perennial plants. Wild and perennial plants provide an interesting niche to screen for the potential plant growth promoting bacteria. Unlike crop plants, wild plants are constantly challenged by harsh and adverse conditions, including water and nutrient scarcity, extreme weather and attacks by pests and pathogens. They ensure their vitality using a range of mechanisms. This includes choosing the right endophytic partners that help them withstand such difficulties (Rout *et al.,* 2013). Therefore, identifying potentially useful endophytic bacteria of wild plants, that can also improve growth of crop plants, can be extremely beneficial for the agricultural sector.

Wild hemp (*Cannabis sativa* L.) is a good candidate for the evaluation of useful endophytic bacteria. It is a common herbaceous plant in many parts of the world, known for its medicinal use as well as a source of recreational drug. In Pakistan, it is a native plant that grows wildly and perennially in most areas of Pakistan, occurring more abundantly in northern Punjab (Ashraf *et al.,* 2012). Although some work has been done on endophytic bacteria of wild plants including *C. sativa* (Hung *et al.,* 2007; Kusari *et al.,* 2014; Zinniel *et al.,* 2002), there are only limited studies where these bacteria were tested for their usefulness on commercial crops (Ma *et al.,* 2011; Zhang *et al.,* 2011).

Researchers have used different properties of bacteria to select plant growth promoting bacteria. The most widely used method is to select bacteria with ACC deaminase, an enzyme that breaks down the precursor of plant stress hormone thereby improving plant growth (Sun *et al.,* 2009; Zheng *et al.*, 2014). Using this approach, endophytic bacteria have been isolated, both from wild plants and agricultural soils, promoting growth of a non-host canola plant (Rashid *et al.,* 2012; Zhang *et al.,* 2011). However, the growth promoting ability of ACC deaminase producing bacteria can be limited to the original host (Long *et al.,* 2008). Moreover, some non-ACC deaminase bacteria can also promote plant growth to similar extent compared to ACC deaminase producing bacteria (Ma *et al.,* 2011; Sheng *et al.,* 2008). The aim of present study was to isolate and characterize useful endophytic bacteria of *C. sativa* with the ability to promote canola growth. In addition to direct isolation from surface sterilized host tissues (Beneduzi *et al.,* 2013), endophytic bacteria were also selectively isolated using a new approach. The isolated bacteria were tested

for different traits consistent with plant growth promotion. Some growth promoting bacteria were also tested for their ability to reduce the inhibitory effects of salt stress on plant growth.

Materials and Methods

Isolation of endophytic bacteria: Endophytic bacteria were isolated from healthy *Cannabis sativa* plants growing in the wild. Two different approaches were used for the isolation. The first approach was direct isolation of endophytic bacteria from roots of *C. sativa*. Plants were collected from three sites located within the campus area of Quaid-i-Azam University, Islamabad. The roots were washed thoroughly with tap water and chopped into 2-3 cm lengths. The cuttings were surface sterilized by washing with 70% ethanol (2 minutes) followed by a wash with commercial bleach (5 minutes), and then washed 10 times with sterile distilled water. Water from the last wash was plated on tryptic soy agar (TSA) to ensure no epiphytes were selected. About 5-6 cuttings were macerated in 5 ml of sterile 0.03 M $MgSO_4$ using an autoclaved mortar and pestle, and kept in a laminar flow cabinet for 30 minutes at room temperature. The macerate was serially diluted up to 10^{-3} and plated on half strength TSA, R2A agar and nutrient agar medium and incubated for 3-5 days at 30°C. Dilutions were plated in replicates on each growth medium (Rashid *et al.,* 2012).

The second approach was novel and involved selective isolation of endophytic bacteria from rhizosphere of *C. sativa* using canola. About 1% rhizosphere soil was added to 50 ml half strength tryptic soy broth (TSB). The inoculated broth was incubated at 30°C for 48 hours with 150 rpm orbital shaking. About 1 ml of the resulting mixed culture was added to fresh 50 ml half strength TSB with 5% rhizospheric soil extracts and incubated under conditions indicated earlier. The resulting mixed culture was washed twice with 0.03 M $MgSO_4$ and adjusted to an absorbance of 0.2 at 600 nm. Agricultural soil was autoclaved twice for one hour at 120°C and 15 lbs and added to plastic pots. The resulting soil was also plated on TSA to ensure it contained no indigenous microbes. Canola seeds were surface sterilized and sown in the autoclaved soil. The soil was then thoroughly drenched with bacterial culture suspension prepared earlier. The pots were placed on a lab bench top. Plants emerging from the seeds were grown for 4 weeks to give them ample time to take up endophytic bacteria from the enriched rhizobacterial pool. The plants were then uprooted, washed thoroughly with tap water, reduced to 2-3 cm cuttings, and endophytic bacteria were isolated as described earlier.

Morphologically distinct colonies (on basis of color, shape, texture, size and gram reaction) were selected for further studies and also backed up on half strength TSA agar slants (stored at 4°C) and glycerol stocks (stored at -80°C).

Canola gnotobiotic root elongation assay: Canola seeds were surface sterilized by washing with 70% ethanol for 1 minute and 20% commercial bleach (1% NaOCl) for 10 minutes. Residual chemicals were removed by washing 10 times with sterile distilled water. Bacteria were grown in half strength TSB for 48 hours, cells were harvested by centrifugation at 5000 rpm at 4°C, washed twice with 0.03 M $MgSO_4$, and resuspended to an absorbance of about 0.1 ±0.02 at 600nm. The suspension was used to treat the surface sterilized seeds for 1 hour at room temperature. The treated seeds were placed in tubes containing 0.5% water agar (1 seed per tube and 18 tubes per treatment). Surface sterilized seeds treated with sterile 0.03 M $MgSO_4$ were used as a negative control. The tubes were placed in a plant growth chamber with 12 hour light/dark cycles, and a constant temperature (25°C) and relative humidity (60%). Root lengths of the resulting plantlets were measured on the fifth day.

For testing growth promotion of canola by selected bacteria under salt stress, water agar was supplemented with 125 mM NaCl in the gnotobiotic assay.

Confirmation of endophytic growth: The isolated plant growth promoting bacteria were tested for their endophytic presence. For this, surface sterilized seeds were treated with test bacteria as described before. The seeds were grown in flasks containing Murashige and Skoog Basal Salt (Sigma-Aldrich M5524) medium supplemented with 3% sucrose and 0.8% agar. Flasks were placed in the plant growth chamber. Plantlets were harvested after 2 weeks, and surface sterilized by washing with 70% ethanol (30 seconds) and 1% commercial bleach (2 minutes), followed by 10 washes with sterile distilled water. To confirm the sterilization process, water from the last wash was plated on TSA medium. The surface sterilized plant (root and stem) were macerated and plated on TSA medium to confirm presence of test bacteria.

Salt stress tolerance: Selected plant growth promoting bacteria were test for their ability to tolerate different salt concentration. For this, bacteria were grown in TSB in the presence of 0%, 1%, 2%, 3%, 5%, 7% and 10% NaCl concentration for 24 hours at 30°C. Bacterial growth was measured by taking absorbance at 600nm using uninoculated broth as blank.

Production of IAA: Bacteria were grown in TSB for 24 hours at 30°C. About 50 µL of the culture was used to inoculate fresh TSB broth amended with 200 µg/ml tryptophan and incubated for 24 hours at 30°C. Cells were harvested by centrifugation. One part of the culture supernatant was mixed with 2 parts of Salkowski's reagent (Gordon & Weber, 1951), and incubated for 25 minutes at room temperature. The mixture was then analyzed using spectrophotometer at 535 nm. The concentration of indole-3-acetic acid (IAA) in each sample was determined using a standard curve of IAA.

Phosphate solubilization: The tri-calcium phosphate containing minimal medium was used to qualitatively measure of bacterial phosphate solubilization (Verma *et al.,* 2001). Zone of clearing around the colonies was used to identify phosphate solubilizing bacteria.

Siderophore production: Ability of the bacteria to produce siderophore was determined qualitatively using the Chrome-Azurol S (CAS) based agar medium (Schwyn & Neilands, 1987). Bacteria were spot inoculated on the test medium and allowed to grow for 48-72 hours at 30°C. The colonies surrounded by orange to yellow halo were identified as siderophore producers.

Plant cell-wall degrading activities: Ability of the endophytic isolates to invade into plant tissues was determined using qualitative cellulolytic and pectinolytic assays. The endoglucanase activity was investigated using the carboxymethylcellulose (CMC) and Congo red based growth medium (Hendricks *et al.,* 1995). Pectin containing minimal media was used to identify pectinase producing bacteria (Hankin *et al.,* 1971). The zones of hydrolysis of pectin around bacterial colonies were visualized by flooding culture plates with iodine solution (Ouattara *et al.,* 2008).

Fungal cell-wall degrading activity: Chitinase and protease activity was tested using the modified method of Bibi *et al.* (2012). Bacterial colonies producing clear halos on half strength TSA containing 0.5% colloidal chitin were identified as chitinase producers. Protease producing isolates were identified by their ability to produce of clear halos on half strength TSA supplemented with 1% skimmed milk.

Antifungal activity: Antifungal potential of the isolated bacteria was determined against *Aspergillus niger* and *Fusarium oxysporum* obtained from the Fungal Culture Bank, University of the Punjab, Lahore. Dual culture method was used for this purpose by inoculating bacteria and the test fungi on agar medium containing 1:1 ratio of potato dextrose agar (PDA) and TSA (Kumar *et al.,* 2012). Culture plates were incubated for 5-7 days at 30°C and bacterial colonies antagonizing fungal growth were identified by presence of zone of inhibition at the point of interaction.

Identification of endophytic bacteria: Bacterial isolates were identified based on their 16S rRNA gene sequence. For this purpose, bacteria were grown on TSA for 24 hours at 30°C. A small quantity of a pure colony was mixed in 50 µL of colony lysis buffer (1% Triton X-100; 20 mM Tris HCl, pH 8.0; 2 mM EDTA, pH 8.0). Contents were heated for 10 minutes at 95°C to lyse the bacterial cells and release genomic DNA. Lysate was spun at 14000 rpm for 10 minutes to pellet cell debris. Resulting supernatant (containing genomic DNA) was used as a template for PCR reaction. Bacterial 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), as proposed by Weisburg *et al.* (1991). PCR reaction mixture (25 µL) was prepared containing 0.3 µM of forward and reverse primers, about 2 µL template DNA and 1XGoTaq® Green Master Mix (Promega, USA) in PCR grade water. PCR amplification was performed with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C for 30s), annealing (55°C for 30s) and extension (72°C for 1 minute) with a final extension at 72°C for 10 min. The resulting PCR product (approximately 1400 bp) was visualized on 1% agarose gel containing ethidium bromide and compared to a 1 kb ladder for size confirmation. The product was purified from its PCR mixture using the PureLink™ Quick PCR Purification Kit (Invitrogen, USA). Sequencing of the purified product was done commercially (Macrogen, South Korea). Sequences were analyzed with the BLASTn program (www.ncbi.nlm.nih.gov/BLAST) using the GenBank database to identify closely matching bacteria. Closely matching type strain identification was performed using the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim *et al.,* 2012).

Results

Isolation and identification of the endophytic bacteria: Out of 34 morphologically distinct bacteria isolated from *C. sativa,* sixteen were directly isolated from the surface sterilized roots, and eighteen were selectively isolated from the enriched rhizosphere bacteria using canola plants. Morphologically similar bacteria isolated from the two sources were considered among the isolates from *C. sativa* roots.

PCR amplification of the 16S rRNA gene of isolates, using 27F and 1492R universal primers, yielded a product close to 1500 bp. A partial sequence of the product was used to identify the endophytic isolates. Identification revealed the isolates were in fact different and belonged to diverse genera. The most prominent isolated genera included *Acinetobacter*, *Chryseobacterium*, *Enterobacter*, *Microbacterium* and *Pseudomonas*. Most of the isolates shared more than 99% homology to the closest type strain based on the analysis of EzTaxon server. However, four isolates (MOSEL-w4, MOSEL-p22, MOSEL-w2.5 and MOSEL-r15) shared about 98% similarity, while MOSEL-w13 and MOSEL-n5 shared about 97% similarity to the closest type strain. Further, all isolates shared 99-100% similarity to a GenBank submission, analyzed using BLASTn program. Details about the identification of bacteria isolated from selective and direct isolation, and their GenBank accessions are given in Tables 1 and 2, respectively.

Plant cell-wall degrading enzymes: All bacteria isolated from canola plants possessed cellulase activity, and with the exception of MOSEL-tnc3, all isolates also showed a positive pectinase activity. Most isolates (88%) from *C. sativa* roots were also cellulase positive, with the exception of MOSEL-t7 and MOSEL-r7, which tested negative. While, only nine isolates (56%) were found to possess pectinase activity. Overall, some isolates showed a more pronounced cellulase (MOSEL-w4, MOSEL-w12, MOSEL-w13, MOSEL-tnc1, MOSEL-tnc3 and MOSEL-n5) and pectinase (MOSEL-w12, MOSEL-w2.5, MOSEL-w13, MOSEL-w1, MOSEL-r13 and MOSEL-n11) activity then others based on the size of zone of hydrolysis produced on respective media (Table 3). Isolates with pronounced activity were more abundant in the bacteria isolated from canola. MOSEL-t7 and MOSEL-r7, isolated from the *C. sativa* roots, were the only two isolates lacking both cellulase and pectinase activities.

Table 1. Identification of endophytic bacteria resulting from selective isolation method based on the partial 16S rRNA gene sequence. GenBank match similarity was ≥99% for all isolates while Eztaxon match similarity is given in the table.

Strain ID (MOSEL)	GenBank closest match	EzTaxon closest match (type strain)	Similarity (%)	Genbank accession
w4	*Chryseobacterium* sp. YU-SS-B-43	*Chryseobacterium indologenes* LMG 8337	98.57	KF307668
p22	Bacterium 1A5	*Chryseobacterium tructae* 1084-08	98.49	KF307670
w15	*Curtobacterium* sp. EP_L_2	*Curtobacterium flaccumfaciens* LMG 3645	100	KF307671
p6	*Enterobacter hormaechei* strain SR3	*Enterobacter cancerogenus* LMG 2693	99.62	KF307674
w7	*Enterobacter cloacae* strain RM20	*Enterobacter cloacae* subsp. dissolvens LMG 2683	99.81	KF307675
w12	*Exiguobacterium acetylicum* strain ZYJ-7	*Exiguobacterium indicum* HHS31	99.62	KF307677
w2.16	*Microbacterium* sp. ZL2	*Microbacterium ginsengiterrae* DCY37	99.14	KF307678
w2.5	*Microbacterium* sp. Am3	*Microbacterium natoriense* TNJL143-2	98.86	KF307679
w2.1	*Microbacterium* sp. THG S3-10	*Microbacterium phyllosphaerae* DSM 13468	99.52	KF307680
w13	*Paenibacillus* sp. 512(2014)	*Paenibacillus hunanensis* FeL05	97	KF307683
w1	*Paenibacillus* sp. BL14	*Paenibacillus tundrae* A10b	99.14	KF307684
w6	*Pantoea anthophila* strain L8-457	*Pantoea anthophila* LMG 2558	99.52	KF307685
w16	*Paracoccus marcusii* strain BF13-3	*Paracoccus marcusii* DSM 11574	99.62	KF307687
tnc1	*Pseudomonas geniculata* strain R6-798	*Pseudomonas geniculata* ATCC 19374	99.71	KF307689
tnc2	*Pseudomonas* sp. DT1	*Pseudomonas koreensis* Ps 9-14	99.9	KF307691
p18	*Pseudomonas plecoglossicida* strain S20411	*Pseudomonas plecoglossicida* FPC951	100	KF307693
w2	*Serratia marcescens* strain MUGA199	*Serratia marcescens* subsp. sakuensis KRED	99.9	KF307696
tnc3	*Stenotrophomonas rhizophila* strain HR89	*Stenotrophomonas rhizophila* e-p10	99.62	KF307697

Table 2. Identification of endophytic bacteria resulting from direct isolation method based on the partial 16S rRNA gene sequence. GenBank match similarity is ≥99% for all isolates while Eztaxon match similarity is given in the table.

Strain ID (MOSEL)	GenBank closest match	EzTaxon closest match	Similarity (%)	Genbank accession
r2	*Acinetobacter* sp. SZ-1	*Acinetobacter gyllenbergii* 1271	99.44	KF307663
n6	*Acinetobacter* sp. R4-413	*Acinetobacter nosocomialis* LMG 10619	99.62	KF307664
t7	*Acinetobacter gyllenbergii* A207	*Acinetobacter parvus* DSM 16617	99	KF307665
r8	*Acinetobacter oleivorans* strain Z-A18	*Acinetobacter pittii* LMG 1035	99.33	KF307666
r4	*Bacillus anthracis* strain UM-5	*Bacillus anthracis* ATCC 14578	100	KF307667
n5	*Chryseobacterium kwangjuense* strain KJ1R5	*Chryseobacterium vrystaatense* LMG 22846	97.28	KF307669
t15	Bacterium OX_LEAF4	*Enterobacter asburiae* JCM 6051	99.43	KF307672
r7	*Enterococcus casseliflavus* strain ALK061	*Enterococcus casseliflavus* CECT969	99.52	KF307676
r13	*Nocardioides albus*	*Nocardioides albus* KCTC 9186	99.52	KF307681
r15	Bacterium 405	*Nocardioides kongjuensis* A2-4	98.67	KF307682
t13	*Pantoea agglomerans* strain PGHL1	*Pantoea vagans* LMG 24199	99.81	KF307686
n9	*Planomicrobium chinense* strain L10-2	*Planomicrobium chinense* DX3-12	99.71	KF307688
t14	*Pseudomonas putida* strain CSM10	*Pseudomonas taiwanensis* BCRC 17751	99.78	KF307694
n12	*Agrobacterium tumefaciens* strain R6-409	*Rhizobium radiobacter* ATCC 19358	99.43	KF307695
n11	*Streptomyces werraensis* strain 1165	*Streptomyces eurocidicus* NRRL B-1676	99.52	KF307698
r5	*Xanthomonas arboricola*pv. pruni strain BCRC80481	*Xanthomonas gardneri* ATCC 19865	100	KF307699

Nutrient availability: In the qualitative assay of phosphate solubilization, eight isolates (44%) from canola solubilized tri-calcium phosphate. Such isolates were more abundant (56%) in bacteria isolated from roots of *C. sativa*, and displayed more noticeable activity apparent from larger halos formed around their colony. Overall, MOSEL-p6, MOSEL-t13 and MOSEL-t15 possessed the highest mineral phosphate solubilization ability. Moreover, thirteen isolates (72%) from canola produced siderophore, apparent from the production of orange to yellow halos around the colonies after incubation, while only nine isolates (56%) from *C. sativa* roots were siderophore producers (Table 3).

Fungal cell-wall degrading enzyme: Three isolates from canola (16%) showed positive chitinase activity (MOSEL-w2, MOSEL-13, and MOSEL-tnc3), forming clear halos around them on chitin containing medium, while only MOSEL-r4 from *C. sativa* root showed positive activity. Regarding protease activity, ten isolates (55%) from canola and seven (43%) from *C. sativa* root were able to hydrolyze milk casein to produce zone of clearing on the test medium. Overall, four isolates, MOSEL-w2, MOSEL-w13, MOSEL-tnc3 and MOSEL-r4, possessed both chitinase and protease activity (Table 3).

Table 3. Plant growth promotion, host invasion and antifungal characteristics of endophytic bacteria isolated from *C. sativa* using selective isolation by canola and direct isolation from *C. sativa* roots.

	Isolate	GR	CEL	PEC	PRO	CHI	SID	AN	FO	PHO	IAA (µg/ml)	IAA-T (µg/ml)
Selective isolation	*Chryseobacterium* sp. MOSEL- w4	-	+++	++	++	-	+	-	-	-	0.51	1.29
	Chryseobacterium sp. MOSEL-p22	-	++	++	++	-	-	-	-	-	0.4	1.4
	Curtobacterium flaccumfaciens MOSEL-w15	+	+	+	+	-	+	-	-	-	1.8	5.1
	***Enterobacter cancerogenus* MOSEL-p6**	-	+	+	-	-	-	-	-	+++	2.92	6.94
	***Enterobacter* cloacae MOSEL-w7**	-	+	+	-	-	-	+	+	+	2.38	4.56
	Exiguobacterium indicum MOSEL-w12	+	+++	+++	++	-	+	-	-	+	2.22	4
	Microbacterium ginsengiterrae MOSEL-w2.16	+	++	++	-	-	-	-	-	-	0.62	2.69
	Microbacterium sp. MOSEL-w2.5	+	++	+++	++	-	+	-	-	-	0.13	1.6
	Microbacterium phyllosphaerae MOSEL-w2.1	+	+	++	-	-	-	-	-	-	0.15	1.48
	Paenibacillus sp. MOSEL-w13	+	+++	+++	+++	++	+	++	++	+	0.2	0.82
	Paenibacillus tundrae MOSEL-w1	+	+	+++	-	-	+	-	-	-	1.4	3.01
	Pantoea anthophila MOSEL-w6	-	+	+	-	-	+	+	-	+	1.6	3.5
	Paracoccus marcusii MOSEL-w16	-	+	+	-	-	+	-	-	-	3.9	5.36
	***Pseudomonas geniculata* MOSEL-tnc1**	-	+++	+	++	-	+	-	-	-	1.2	5.42
	***Pseudomonas koreensis* MOSEL-tnc2**	-	++	+	+	-	+	++	-	++	1.78	6.5
	Pseudomonas plecoglossicida MOSEL-p18	-	+	++	-	-	+	-	-	+	1.84	2.92
	***Serratia marcescens* MOSEL-w2**	-	++	+	++	+++	+	+	+	++	3.02	5.64
	***Stenotrophomonas rhizophila* MOSEL-tnc3**	-	+++	-	++	+	+	+	+	-	1.92	4.02
Direct isolation	*Acinetobacter gyllenbergii* MOSEL-r2	-	++	-	+	-	-	-	-	++	4.56	7.2
	Acinetobacter nosocomialis MOSEL-n6	-	+	+	-	-	+	-	-	++	2.54	4.02
	Acinetobacter parvus MOSEL-t7	-	-	-	-	-	-	-	-	-	2.02	3.14
	Acinetobacter pittii MOSEL-r8	-	+	-	-	-	-	-	-	++	5.14	10.68
	Bacillus anthracis MOSEL-r4	+	++	++	+++	+	+	++	++	+	1.68	3.04
	Chryseobacterium sp. MOSEL-n5	-	+++	++	++	-	+	-	-	-	2.12	3.26
	***Enterobacter asburiae* MOSEL-t15**	-	+	+	-	-	-	+	-	+++	1.94	4.28
	Enterococcus casseliflavus MOSEL-r7	+	-	-	+	-	-	-	-	+	1.84	4
	Nocardioides albus r13	+	++	+++	++	-	+	-	-	-	1.34	4.56
	Nocardioides kongjuensis MOSEL-r15	+	+	+	-	-	+	-	-	-	0.24	1
	***Pantoea vagans* MOSEL-t13**	-	+	-	-	-	+	-	+	+++	2.92	7.7
	Planomicrobium chinense MOSEL-n9	+	++	-	-	-	-	-	-	-	0.92	3.52
	Pseudomonas taiwanensis MOSEL-t14	-	+	+	+	-	+	+	+	++	3.64	4.82
	*Agrobacterium tumefaciens*MOSEL-n12	-	+	-	-	-	-	-	-	+	3.8	13.34
	Streptomyces eurocidicus MOSEL-n11	+	++	+++	-	-	+	-	-	+	0.98	1.8
	Xanthomonas gardneri MOSEL-r5	-	+	++	+	-	+	+	+	-	1.4	5.1

GR, Gram reaction (positive or negative); CEL, cellulase; PEC, pectinase; PRO, protease; CHI, chitinase; SID, siderophore; AN, *Aspergillus niger*; FO, *Fusarium oxysporum*; PHO, tri- calcium phosphate solubilization; IAA, indole acetic acid; T, tryptophan (200 µg/ml); +, positive activity; -, negative activity; +, smaller halos around colonies (<0.5 cm); ++, medium halos around colonies (0.5-1 cm); +++, large halos around colonies (>1.0 cm). Values represent average of three replicates. Isolates in bold letters significantly promoted canola root growth

Antifungal ability: In the dual culture assay to identify isolates with antagonistic activity against phytopathogenic fungi, four isolates from selective isolation (MOSEL-w2, MOSEL-w7, MOSEL-w13 and MOSEL-tnc3) were effective against both *A. niger* and *F. oxysporum,* while MOSEL-tnc2 was effective against *A. niger* only. Among the isolates from *C. sativa* roots, MOSEL-r4, MOSEL-t14 and MOSEL-r5 were effective against both the test fungi, whereas MOSEL-t13 and MOSEL-t15 were effective against *F. oxysporum* and *A. niger* respectively. Overall, MOSEL-w13 possessed the prominent antifungal activity against the two test fungi (Table 3; Figs. 3 and 4).

Production of IAA: All isolates were able to produce IAA like molecule when tested using the colorimetric assay. The amount of IAA produced ranged from 0.2-5.1 µg/ml in the absence of tryptophan, the precursor of IAA. MOSEL-r8 (5.1 µg/ml) produced the highest amount of IAA among all the isolates followed by MOSEL-w16 (3.9 µg/ml) and MOSEL-w2 (3.02 µg/ml). Ability of the isolates to produce IAA was increased by 1.5 to 4 folds in the presence of tryptophan (200 µg/ml). After supplementation with tryptophan, maximum IAA production was observed for MOSEL-n2 (13.2 µg/ml) followed by MOSEL-r8 (10 µg/ml) and MOSEL-t13 (7.7 µg/ml) (Table 3).

Canola gnotobiotic root elongation and endophytic presence: Ability of the isolates to confer growth benefit on canola was assessed using gnotobiotic assay in water agar. The bacteria isolated from two approaches performed differently in the assay. Isolates from canola were much better in the growth promoting ability, where six isolates significantly enhanced root lengths of the test plant (Least significant difference; $p<0.05$). However, the performance of the six isolates did not differ significantly from each other. On an average, MOSEL-w7 (29.2% increase), MOSEL-tnc1 (31.41%) and MOSEL-w2 (32.15%) produced longer root lengths than MOSEL-p6 (17.39%), MOSEL-tnc2 (19.6%) and MOSEL tnc3 (16.28%) (Fig. 1A).On the other hand, only two isolates from *C. sativa* roots significantly enhanced root length of test plant, where MOSEL-t13 (38.63%) performed better than MOSEL-t15 (16.97%). However, their performance also did not vary significantly compared to each other, or compared to the six isolates from canola (Fig. 1B).

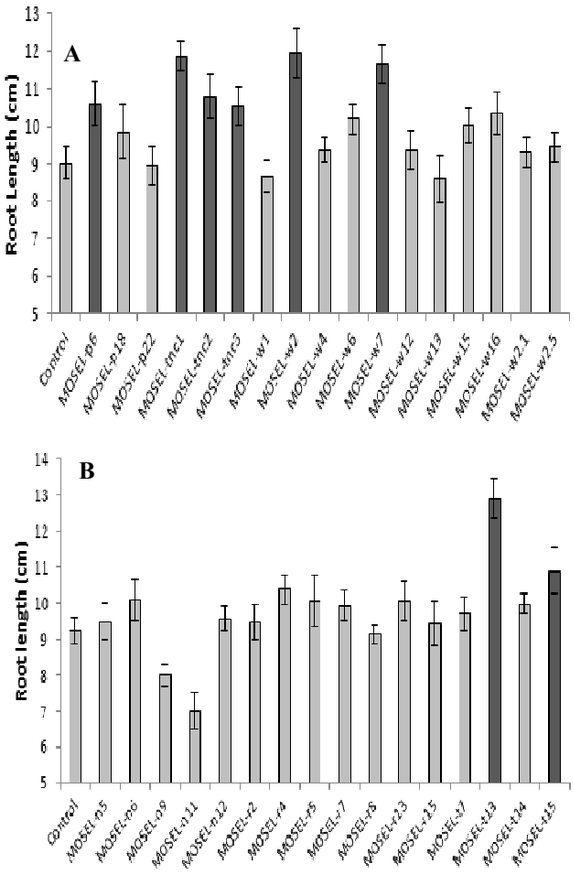

Fig. 1. Canola gnotobiotic root elongation assay of endophytic bacteria isolated from *C. sativa* (A) Selective isolation using canola (B) Direct isolation from *C. sativa* roots. Each bar represents mean root length (± SE, n=12) of five day old plantlets treated with sterile 0.03M $MgSO_4$ (Control) or bacterial suspension in 0.03M $MgSO_4$ (0.1 ± 0.02 OD at 600nm). Dark bars belong to isolates significantly increasing root length compared to control (Least significant difference, $p<0.05$).

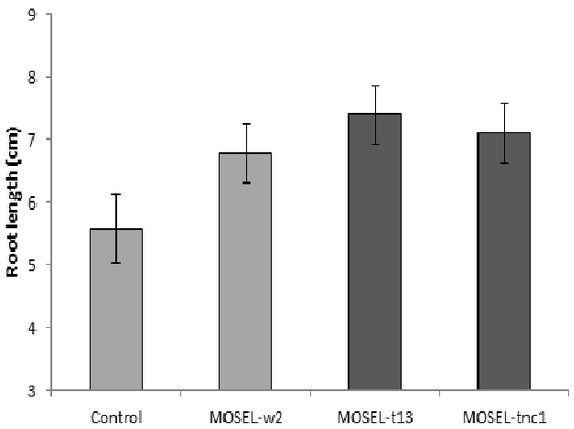

Fig. 2. Canola gnotobiotic root elongation assay of three growth promoting bacteria in the presence of 125mM NaCl stress. Each bar represents mean root length (± SE, n=12) of five day old plantlets treated with sterile 0.03M $MgSO_4$ (Control) or bacterial suspension in 0.03M $MgSO_4$ (0.1 ± 0.02 OD at 600nm). Dark bars belong to isolates significantly increasing root length under stress compared to control (Least significant difference, $p<0.05$).

Three prominent plant growth promoting bacteria, MOSEL-tnc1, MOSEL-w2 and MOSEL-t13 were also tested for their ability to ameliorate the inhibitory effects NaCl on canola. Under 125 mM NaCl stress, plants showed stunted growth compared to non-stressed plants, and under stress condition, plants treated with MOSEL-t13 and MOSEL-tnc1 produced significantly longer roots (LSD; $p<0.05$) then non-bacterized plants. MOSEL-w2 treated plants also produced longer roots then control plants although the difference was insignificant. Overall, bacterized plants showed better growth compared to non-bacterized plants (Fig. 2; Figs. 5 and 6).

The eight growth promoting bacteria were recovered from the surface sterilized canola plants inoculated with these bacteria, indicating their endophytic presence. The endophytic bacterial counts were between 10^3 to 10^4 colony forming units (CFU) per gram fresh weight of plant.

Growth under salt stress: The three bacteria tested for salt tolerance were able to grow under most salt concentrations tested. MOSEL-w2 (Turbidity 0.265±0.01) appeared to be most salt tolerant at 7% NaCl followed by MOSEL-t13 (0.185 ± 0.01) and MOSEL-tnc1 (0.08 ± 0.01). At 10% salt concentration, only MOSEL-w2 showed measurable but little growth.

Discussion

In the present study, endophytic bacteria were isolated from *C. sativa* and investigated for their potential as bio-inoculants for canola, a commercial crop. Selective isolation procedure yielded six bacteria that significantly promoted the root growth of test plant under gnotobiotic conditions, as compared to two isolates from direct isolation. Recovery of these bacteria from the surface sterilized canola plants, originating from bacterized seeds, confirmed their endophytic presence (Rashid *et al.,* 2012). Although statistically insignificant, remaining isolates from selective isolation also performed better in promoting canola growth as compared to isolates from direct isolation (Fig. 1A). The difference in performance could be due to the host plant type. Long *et al.* (2008) noticed this for plant growth promoting bacteria of *Solanum nigrum* that were unable to produce growth enhancement in *Nicotiana attenuate*, a non-host plant. Plant genotype can also influence the ability of bacteria to promote plant growth. This was reported by Kim *et al.* (2012) in their work on growth promotion of switch grass cultivars by *Bukholderia phytofirmans* PsJN. Hence, selecting endophytic bacteria using canola produced more isolates that positively affected the growth of the same host plant. On the other hand, endophytic bacteria selected by *C. sativa* performed less efficiently when tested on canola, an unrelated plant host (Fig. 1).

The bacteria isolated from *C. sativa* roots represented endophytes selected by that plant from the rhizosphere bacterial pool. These bacteria were found to be different from the isolates selected by canola from the same pool. Hence, *C. sativa* and canola can take up their respective endophytic bacteria differently. Germida *et al.* (1998) reported similar observation for their work on canola and wheat plants grown in the same field. Nevertheless, the enriched rhizospheric community of *C. sativa* did yield a unique set of microbes when selected using canola plant

(Table 1). Most of these bacteria were not found to be part of the endophytic community of canola reported by earlier researchers (Germida *et al.,* 1998; Granér *et al.,* 2003; Misko *&* Germida, 2002; Siciliano *&* Germida, 1999). Further, Kusari *et al.* (2014) recently reported their work on the endophytic bacteria of *C. sativa* sampled from Bedrocan BV, Netherlands. They recovered only two bacterial genera with *Bacillus* being more predominant than *Mycobacterium*. The isolates collected from *C. sativa* roots in the present study appear to be different from these findings, and more bacterial genera were recorded (Table 2). Collectively, these observations support the idea that endophytic community of a plant is defined by the nature of its surrounding soil (McInroy *&* Kloepper, 1995; Rashid *et al.,* 2012).

In the present study, most of the bacterial isolates were found to possess cellulase and pectinase activity (Table 3). This is not surprising, since plant cell-wall degrading enzymes are known to play an important role in the invasion and systematic dissemination of endophytic bacteria in the host tissues (Reinhold-Hurek *&* Hurek, 2011). Endophytic bacteria are also known to increase the accessibility of nutrients like mineral-bound Phosphorus for the host plant (Young *et al.,* 2013). A number of isolates also solubilized inorganic phosphate, and the activity was more pronounced in *Acinetobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas*and *Serratia* (Table 3). These genera are well documented for their phosphate solubilizing abilities (Sharma *et al.,* 2013).

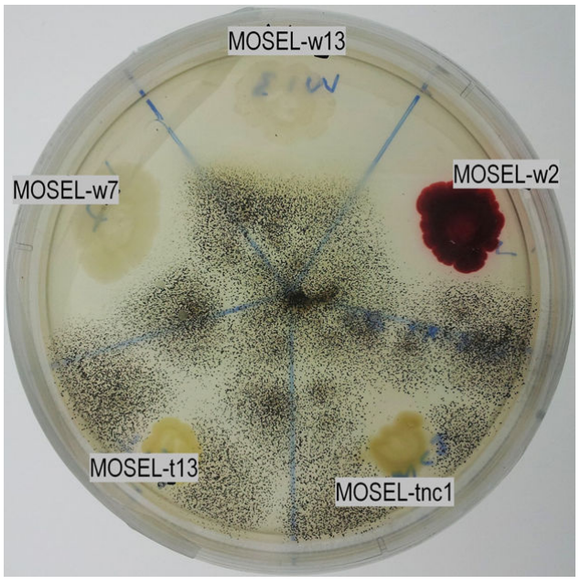

Fig. 3. Dual culture antifungal assay of five selected isolates against *Aspergillus niger*.

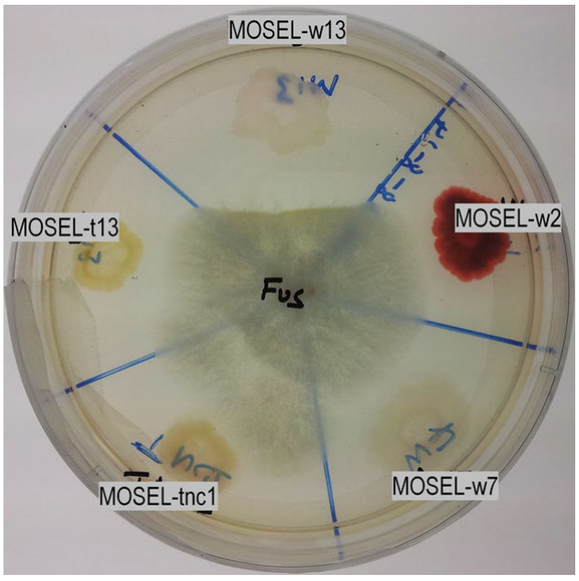

Fig. 4. Dual culture antifungal assay of five selected isolates against *Fusarium oxysporum.*

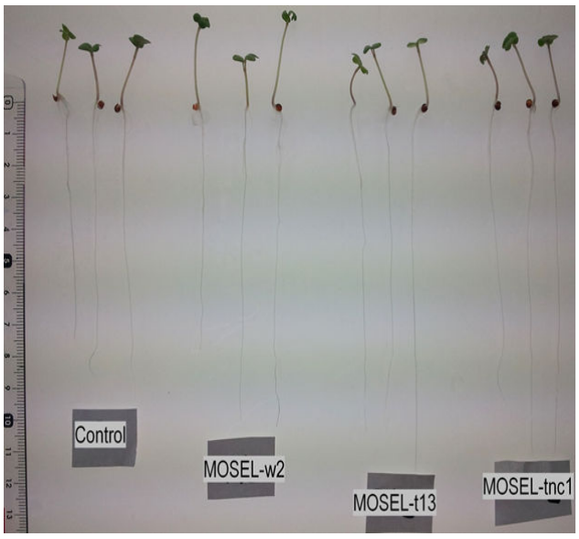

Fig. 5. Root elongation of canola by three selected growth promoting bacteria under gnotobiotic condition. Scale indicates values in centimeters.

Fig. 6. Root elongation of canola by three selected growth promoting bacteria under gnotobiotic condition and 125mM NaCl stress. Scale indicates values in centimeters.

All isolates were found to produce IAA like molecule albeit a moderate to low level. While high IAA production is known to be a characteristic of plant pathogens and can cause stunting of root growth (Malik & Sindhu, 2011; Kunkel & Chen, 2006), lower amounts of IAA production by bacteria can produce positive effect on plant growth (Marques *et al.,* 2010). In the present study, many isolates did promote root length in the inoculated plants compared to non-bacterized plants while only few adversely affected root growth. Interestingly, two highest IAA producing bacteria, *Agrobacterium tumefaciens* MOSEL-n12 and *Acinetobacter pittii* MOSEL-r8, produced comparably lesser root lengths in canola than the bacteria that improved host root growth significantly. In fact, bacteria producing IAA in the range of 4-8 µg/ml produced a more pronounced root elongation of the host plant (Table 3; Fig. 1; Fig. S3). Long *et al.* (2008) also reported similar effects by growth promoting bacteria on *Solanum nigrum*. Hence, plant growth promoting ability of bacteria appears to be dependent on the amount of IAA produced by them, where lower amounts tend to favor plant growth.

Endophytic bacteria can also benefit their host plant by discouraging the growth of phytopathogens. They can accomplish this by using strategies like limiting nutrient availability and colonization sites, by producing antagonistic substances, and by forming biofilms (Compant *et al.,* 2010). In the present work, dual culture assay identified useful endophytic bacteria that retarded the growth of two phytopathogenic fungi, *A. niger* and *F. oxysporum*. Interestingly, all chitinase producing bacteria were antagonistic towards the two test fungi, and most of them also produced protease. The antifungal ability of bacteria can be due to their chitinolytic and proteolytic activity (Kim & Chung, 2004). Among the antifungal bacteria, *Paenibacillus* sp.MOSEL-w13 exhibited the most prominent antifungal activity against the two fungi. The isolate was also positive for various enzyme activities tested (Table 3; Fig. 3; Fig. 4). *Paenibacillus* are well known for their antifungal potential (Aktuganov *et al.,* 2008; Beatty & Jensen, 2002). However, the antifungal potential of *P. hunanensis*, the bacteria most similar to MOSEL-w13, has not been reported before. Other interesting antifungal isolates were *Enterobacter cloacae* MOSEL-w7, *Enterobacter asburiae* MOSEL-t15, *Pantoeavagans* MOSEL-t13, *Pseudomonas koreensis* MOSEL-tnc2, *Serratia marcescens* MOSEL-w2 and *Stenotrophomonas rhizophila* MOSEL-tnc3, and they also significantly promoted root growth of canola (Fig. 3; Fig. 4).

A good number of isolates including most antifungal bacteria also produced siderophores. Siderophore producing bacteria can discourage the growth of competing organisms by limiting iron availability in the environment (Marques *et al.,* 2010). Bacterial siderophore production can also benefit plant host by improving its iron acquisition (Masalha *et al.,* 2000; Khalid *et al.*, 2015).

Most interesting plant growth promoting isolates were identified as *Pseudomonas geniculata* MOSEL-tnc1, *S. marcescens* MOSEL-w2 and *P. vagans* MOSEL-t13 (Fig. 1; Fig. 5). *P. geniculata* has been reported as endophytic bacteria in switch grass and rice although it was not shown to be plant growth promoting (Nhu & Diep, 2014; Xia *et al.,* 2013). The isolate was able to tolerate upto 7% NaCl stress and also promoted growth of canola under salt stress (Fig. 2; Fig. 6). Interestingly, *S. marcescens* MOSEL-w2 was the only isolate that showed activity in all the assays. The isolate was able to produce plant and fungal cell-wall degrading enzymes, produced IAA and siderophore, solubilized inorganic phosphate, retarded growth of phytopathogenic fungi, and promoted canola growth under normal and stressed condition (Table 3; Fig. 1; Fig. 2). It is a well-known plant associated bacteria that was found to be endophytic and growth promoting in rice (Gyaneshwar *et al.,* 2000), and conferred cold tolerance in wheat (Selvakumar *et al.,* 2007). In the present study, highest root elongation under normal and stressed conditions was noticed for *Pantoea vagans* MOSEL-t13. The isolate also produced highest IAA (7.7 µg/ml) among all the growth promoting bacteria and antagonized growth of *F. oxysporum*. A strain of *Pantoea vagans* has been commercialized as a bacterial biocontrol agent for fire blight (Smits *et al.,* 2011). Further, all three isolates belonging to *Enterobacter* significantly promoted plant growth, and have been reported previously for the ability (Ahemad & Khan, 2010; Jha *et al.,* 2012; Saleh & Glick, 2001).

Conclusion

Wild *C. sativa* is a good source of agriculturally beneficial endophytic bacteria. The selective isolation approach used in the present study can improve the isolation of endophytic bacteria promoting growth of an unrelated non-host plant. The new approach is simple and can overcome the limitation experienced using other isolation procedures, particularly the effect of host plant type on bacterial performance. The approach can be further improved by combining with other isolation methods like selecting for ACC deaminase producing bacteria. The present study also identifies several bacteria that can be used as bio-inoculants for canola. However, their plant growth promotion and biocontrol potential needs to be further investigated to fully exploit their biotechnological potential. Moreover, the relationship between the amount of IAA produced by the bacteria and their ability to promote plant growth needs to be properly established.

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References

Ahemad, M. and M.S. Khan. 2010. Plant growth promoting activities of phosphate-solubilizing *Enterobacter asburiae* as influenced by fungicides. *Eurasia. J. Biosci.,* 4: 88-95.

Aktuganov, G., A. Melentjev, N. Galimzianova, E. Khalikova, T. Korpela and P. Susi. 2008. Wide-range antifungal antagonism of *Paenibacillus ehimensis* IB-X-b and its dependence on chitinase and beta-1,3-glucanase production. *Can. J. Microbiol.,* 54(7): 577-587.

Ashraf, M., M. Öztürk, M.S.A. Ahmad and A. Aksoy. 2012. Crop production for agricultural improvement. Springer, Netherlands.

Beatty, P.H. and S.E. Jensen. 2002. *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. *Can. J. Microbiol.,* 48: 159-169.

Beneduzi, A., F. Moreira, P.B. Costa, L.K. Vargas, B.B. Lisboa, R. Favreto, J.I. Baldani and L.M.P. Passaglia. 2013. Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil. *Appl. Soil Ecol.,* 63: 94-104.

Bibi, F., M. Yasir, G.C. Song, S.Y. Lee and Y.R. Chung. 2012. Diversity and characterization of endophytic bacteria associated with tidal flat plants and their antagonistic effects on oomycetous plant pathogens. *Plant Pathol. J.,* 28: 20-31.

Compant, S., C. Clement and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.,* 42: 669-678.

Conn, V.M. and C.M.M. Franco. 2004. Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticumaestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA clones. *Appl. Environ. Microbiol.,* 70: 1787-1794.

Dong, Y., A.L. Iniguez and E.W. Triplett. 2003. Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342. *Plant Soil,* 257: 49-59.

Germida, J.J., S.D. Siciliano, R. de Freitas and A.M. Seib. 1998. Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticumaestivum* L.). *FEMS Microbiol. Ecol.,* 26: 43-50.

Glick, B.R. 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica. Article ID 963401.

Gordon, S.A. and R.P. Weber. 1951. Colorimetric estimation of indoleacetic acid. *Plant Physiol.,* 26: 192-195.

Granér, G., P. Persson, J. Meijer and S. Alstrom. 2003. A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen, *Verticillium longisporum*. *FEMS Microbiol. Lett.,* 29: 269-276.

Gyaneshwar, P., E.K. James, N. Mathan, P.M. Reddy, B.R. Hurek and J.K. Ladha. 2000. Endophytic colonization of rice by a diazotrophic strain of *Serratiamarcescens*. *J. Bacteriol.,* 183: 2634-2645.

Hankin, L., M. Zucker and D.E. Sands. 1971. Improved solid medium for the detection and enumeration of pectolytic bacteria. *Appl. Environ. Microbiol.,* 22: 205-209.

Hardoim, P.R., L.S. van Overbeek and J.D. van Elsas. 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.,* 16: 463-471.

Hendricks, C.W., J.D. Doyle and B. Hugley. 1995. A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Appl. Environ. Microbiol.,* 61(5): 2016-2019.

Hung, P.Q., S.M. Kumar, V. Govindsamy and K. Annapurna. 2007. Isolation and characterization of endophytic bacteria from wild and cultivated soybean varieties. *Biol. Fertil. Soils,* 44: 155-162.

Jha, C.K., B. Patel and M. Saraf. 2012. Stimulation of the growth of *Jatrophacurcas* by the plant growth promoting bacterium *Enterobacter cancerogenus* MSA2. *World J. Microbiol. Biotechnol.,* 28(3): 891-899.

Khalid, S., H.N. Asghar, M.J. Akhtar, A. Aslam and Z.A. Zahir. 2015. Biofortification of iron in Chickpea by plant growth promoting rhizobacteria. *Pak. J. Bot.*, 47: 1191-1194.

Kim, P.I. and K.C. Chung. 2004. Production of antifungal protein for control *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908. *FEMS Microbiol. Lett.,* 234: 177-183.

Kim, S., S. Lowman, G. Hou, J. Nowak, B. Flinn and C. Mei. 2012. Growth promotion and colonization of switch grass (*Panicumvirgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. *Biotechnol. Biofuels.,* 5: 37.

Kim, O.S., Y.J. Cho, K. Lee, S.H. Yoon, M. Kim, H. Na, S.C. Park, Y.S. Jeon, J.H. Lee, H. Yi, S. Won and J. Chun. 2012. Introducing EzTaxon: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.,* 62: 716-721.

Kumar, P., R.C. Dubey and D.K. Maheshwari. 2012. Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiol Res.,* 167: 493-499.

Kunkel, B.N. and Z. Chen. 2006. Virulence strategies of plant pathogenic bacteria, In: *The Prokaryotes. A Handbook on the Biology of Bacteria, Ecophysiology and Biochemistry.* (Eds.): Dworkin, M., S. Falkow, E. Rosenberg, K.H. Schleifer & E. Stackebrandt. 3rd ed. Springer., New York, pp. 421-440.

Kusari, P., S. Kusari, M. Lamshöft, S. Sezgin, M. Spiteller and O. Kayser. 2014. Quorum quenching is an antivirulence strategy employed by endophytic bacteria. *Appl. Microbiol. Biotechnol.,* 98: 7173-7183.

Long, H.H., D.D. Schmidt and I.T. Baldwin. 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One*, 3(7): e2702.

Ma, Y., M. Rajkumar, Y. Luo and H. Freitas. 2011. Inoculation of endophytic bacteria on host and non-host plants—effects on plant growth and Ni uptake. *J. Hazard. Mater.,* 195: 230-237.

Malik, D.K. and S.S. Sindhu. 2011. Production of indole acetic acid by Pseudomonas sp.: effect of co-inoculation with *Mesorhizobium sp. Cicer* on nodulation and plant growth of chickpea (*Cicerarietinum*). *Physiol. Mol. Biol. Plants.,* 17(1): 25-32.

Marques, A.P.G.C., C. Pires, H. Moreira, A.O.S.S. Rangel and P.M.L. Castro. 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biol. Biochem.,* 42: 1229-1235.

Masalha, J., H. Kosegarten, O. Elmaci and K. Mengel. 2000. The central role of microbial activity for iron acquisition in maize and sunflower. *Biol. Fertil. Soils,* 30: 433-439.

McInroy, J.A. and J.W. Kloepper. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil*, 173: 337-342.

Misko, A.L. and J.J. Germida. 2002. Taxonomic and functional diversity of pseudomonads isolated from rots of field grown canola. *FEMS Microbiol. Ecol.,* 42: 399-407.

Nhu, V.T.P. and C.N. Diep. 2014. Isolation, charcaterization and phylogenetic analysis of endophytic bacteria in rice plant cultivated on soil of Phu Yen Province, Vietnam. *Am. J. Life Sci.,* 2(3): 117-127.

Ouattara, H.G., L. Ban-Koffi, G.T. Karou, A. Sangare, S.L. Niamke and J.K. Diopoh. 2008. Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. *World J. Microbiol. Biotechnol.,* 24: 1753-1760.

Rashid, S., T.C. Charles and B.R. Glick. 2012. Isolation and characterization of new plant growth-promoting bacterial endophytes. *Appl. Soil Ecol.,* 61: 217-224.

Reinhold-Hurek, B. and T. Hurek. 2011. Living inside plants: bacterial endophytes. *Curr. Opin. Plant Biol.,* 14: 435-443.

Rout, M.E., T.H. Chrzanowski, T.K. Westlie, T.H. Deluca, R.M. Callaway and W.E. Holben. 2013. Bacterial endophytes enhance competition by invasive plants. *Am. J. Bot.,* 100: 1726-1737.

Rudrappa, T., K.J. Czymmek, P.W. Paré and H.P. Bais. 2008. Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiol.,* 148: 1547-1556.

Saleh, S.S. and B.R. Glick. 2001. Involvement of gacS and rpoS in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. *Can. J. Microbiol.,* 47: 698-705.

Schwyn, R. and J.B. Neilands. 1987. Universal chemical assay for detection and estimation of siderophores. *Anal. Biochem.,* 160: 47-56.

Selvakumar, G., M. Mohan, S. Kundu, A.D. Gupta, P. Joshi, S. Nazim and H.S. Gupta. 2007. Cold tolerance and plant growth promotion potential of *Serratia marcescens* strain SRM (MTCC 8708) isolated from flowers of summer squash (*Cucurbitapepo*). *Lett. Appl. Microbiol.,* 46: 171-175.

Sharma, S.B., R.Z. Sayyed, M.H. Trivedi and T.A. Gobi. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus.,* 2: 587.

Sheng, X.F., J.J. Xia, C.Y. Jiang, L.Y. He and M. Qian. 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environ. Pollut.,* 156: 1164-1170.

Siciliano, S.D. and J.J. Germida. 1999. Taxonomic diversity of bacteria associated with the roots of field grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. *FEMS Microbiol. Ecol.,* 29: 263-272.

Smits, T.H.M., F. Rezzonico, T. Kamber, J. Blom, A. Goesmann, C.A. Ishimaru, J.E. Frey, V.O. Stockwell and B. Duffy. 2011. Metabolic versatility and antibacterial metabolite biosynthesis are distinguishing genomic features of the fire blight antagonist *Pantoeavagans* C9-1. *PLoS One*, 6:e22247.

Sun, Y., Z. Cheng and B.R. Glick. 2009. The presence of a 1-aminocyclopropane- 1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. *FEMS Microbiol. Lett.,* 296: 131-136.

Verma, S.C., J.K. Ladha and A.K. Tripathi. 2001. Evaluation of plant growth promoting and colonization ability of endo-phyticdiazotrophs from deep water rice. *J. Biotechnol.,* 91: 127-41.

Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.,* 173: 697-703.

Xia, Y., E. Greissworth, C. Mucci, M.A. Williams and S.D. Debolt. 2013. Characterization of culturable bacterial endophytes of switchgrass (*Panicumvirgatum* L.) and their capacity to influence plant growth. *GCB Bioenergy,* 5(6): 674-682.

Young, L.S., A. Hameed, S.Y. Peng, Y.H. Shan and S.P. Wu. 2013. Endophytic establishment of the soil isolate *Burkholderia* sp. CC-Al74 enhances growth and P-utilization rate in maize (*Zea mays* L.). *Appl. Soil Ecol.,* 66: 40-47.

Zhang, Y.Z., L.Y. He, Z.J. Chen, Q.Y. Wang, M. Quian and X.F. Sheng. 2011. Characterization of ACC deaminase-producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus*. *Chemosphere,* 83: 57-62.

Zheng, P., L. Zhang, L. Tian, L. Zhang, F. Chen, B.Z. Li and Z. Cui. 2014. Isolation and characterization of novel bacteria containing ACC Deaminase from the rhizosphere resource on Dry-Farming Lands. *Pak. J. Bot.*, 46: 1905-1910.

Zinniel, D.K., P. Lambrecht, B.N. Harris, Z. Feng, D. Kuczmarski, P. Higley, C.A. Ishimaru, A. Arunakumari, R.G. Barletta and A.K. Vidaver. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.,* 68: 2198-2208.

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