

Plant Science

REGULAR ARTICLE

Innoculant technology in *Populus deltoides* rhizosphere for effective bioremediation of Polyaromatic hydrocarbons (PAHs) in contaminated soil, Northern India

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Abstract

Four bacterial strains viz. *Kurthia* sp. SBA4, *Micrococcus varians* SBA8, *Deinococcus radiodurans* SBA6 and *Bacillus circulans* SBA12 identified as Polyaromatic hydrocarbons (PAH) degrader, isolated from the rhizospheric soil of *Populus deltoides* growing in non-contaminated sites of Uttarakhand Himalyan region, India. Out of these four isolates, *M. varians* SBA8 degraded appreciable amount of PAH along with some plant growth promoting properties viz. indole acetic acid (IAA) 19 $\mu\text{g ml}^{-1}$, phosphate solubilization 1.8 $\mu\text{g ml}^{-1}$, produced siderophore and possessed ACC deaminase activity. Along with these traits, *M. varians* SBA8 also exhibited biocontrol activities against phytopathogenic fungi *Rhizoctonia solanii*, *Macrophomina phaseolina* and *Fusarium oxysporum* with 50, 43.3 and 34.6 percent growth inhibition (PGI). Moreover, mycelial deformities were also observed in the test fungal spp. The *M. varians* SBA8 proved to be competent rhizobacteria in rhizosphere niche in treatments T₁ (Sterile soil + plant cuttings + bacterization) and T₂ (sterile soil + anthracene + plant cuttings + bacterization). Rhizoremediation potential of *M. varians* SBA8 was also determined in PAH amended soil model system. Significant enhancement in shoot, root length, root and shoot biomass including stem girth of *P. deltoides* with respect to control was recorded and concurrently the bacterium degraded 43.6 % of PAH as determined by HPLC analysis in soil model system after 120 days of inoculation. Multipurpose inoculant was also formulated using *M. varians* SBA8 strain immobilized in different lignocellulosic waste material used as carriers. Rock phosphate, cocoa peat and wheat bran based bioinoculant were found suitable for rhizoremediation of PAH.

Key words: PAH, Plant-microbe-interactions, Bioinoculant, Rhizoremediation

Introduction

Over the last century, industrialized global economy has led to dramatically increase in production of toxic anthropogenic pollutants into the environment, causing widespread contamination of soil and water. Such anthropogenic activities which release huge amounts of petroleum

hydrocarbons (PHC), polyaromatic hydrocarbons (PAH), halogenated hydrocarbons, pesticides, solvents, metals, and salt, have caused deleterious damage on human and ecosystems (Meagher, 2000). Out of these contaminants, polyaromatic hydrocarbons (PAHs) are major hazardous environmental pollutants that possess carcinogenic and mutagenic properties (Menzie and Potokib, 1992; Wilson and Jones, 1993). Basically, PAH in the ecosystem spread through various ways viz. petrogenesis, fossil fuel combustion, waste incineration and by products of industrial processes that include coal gasification, production of metals, petroleum refining, component of wood preservatives, smokehouses, vehicles and wood stoves (Shuttleworth and Cerniglia, 1995; Wingfors et al., 2001).

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Remedial options using physico-chemical treatments are expensive and environmentally invasive, being inadequate for the treatment of large contaminated sites (Cunningham and Ow, 1996). However, bioremediation is the perfect tool for hazardous compounds with less input of chemicals, energy and time, using suitable microbes (Yuan et al., 2002). Recently, the synergistic use of plants and microbes to clean up the PAH-contaminated soil generated encouraging results (Glick, 2003; Huang et al., 2004), as the rhizosphere of plant provides better environment for bacteria to survive and proliferate (Yee et al., 1998). Very few studies have been reported regarding the directed introduction of a microbial strain for PAH degrading activities on plant seed to colonize the root and proliferate on the root system (Kuiper et al., 2001; Spirang et al., 2002). Rhizoremediation treatment can be successfully exploited by using plant growth-promoting and bioprotecting rhizobacteria. Along with bioremediation plant growth promoting bacteria are known to participate in many other important ecosystem interventions, such as the biological control of plant pathogens, nutrient cycling and/or seedling growth (Zahir et al., 2004). Rhizobacteria that produce indole acetic acid (IAA), siderophores, hydrogen cyanic acid (HCN), 1-Aminocyclopropane-1-carboxylate (ACC) deaminase and solubilize phosphate activity which are capable of stimulating plant growth (Glick et al., 1998; Rajkumar et al., 2006) are suitable biological agents for plant growth as well as bioremediation.

To take advantage of the plant-bacterium relationship for degrading PAH compounds in rhizosphere *Populus deltoides* (deciduous plant) was used as bio-injector of pollutant degrading bacteria. *P. deltoides* has a number of advantages such as a highly branched and deep root system; therefore it can be used as a vector/ bio-injector for root-colonizing bacteria, for the penetration of impermeable soil layer and transport of bacteria. Moreover, its roots can exude up to 35 % of their photosynthate as exudate carbon (Gregory and Atwell, 1991) release oxygen which provides better redox conditions and has been widely used for phytoremediation purposes. However, little work has been done on PGPR activities of forest plants in PAH contaminated soil by using selected rhizosphere-competent PAH degrading strains (Bisht et al., 2014). In the light of above facts, the present study was envisaged to utilize a beneficial plant-microbe pair for effective rhizoremediation of PAH in soil model system using *P. deltoides* and previously studied selected soil bacterial strains.

Finally a carrier based multipurpose bioinoculant was formulated for proper dissemination in agriculture system especially for PAH contaminated sites.

Materials and Methods

In vitro plant growth promoting activities of PAH degrading soil isolates

Four PAH degrading strains were isolated from the rhizosphere of *P. deltoides* growing in non-contaminated sites of Garhwal Himalayan regions (Bisht et al., 2010). These bacteria were further checked for plant growth promoting activities viz., indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore, HCN (Hydrogen cyanic acid) and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase production. Along with biocontrol activity against selected soil borne phytopathogenic fungal strains were also checked. These plant growth promoting activities were studied to screen among these four PAH degrading strains to act as potential rhizospheric competitor and beneficial plant growth promoter so that plant can be efficiently act as remediator agent for the pollutant used in our study..

IAA production was determined by Salkowski's method (Rodriguez et al., 2008). The potential to solubilize relatively insoluble tricalcium phosphate was checked by the method of Kumar and Narula (1999). HCN production was observed by following Miller and Higgins (1970) method. Siderophore production was detected qualitatively as described by Schwyn and Neilands (1987) and for ACC deaminase activity determination, Honma and Shimomura (1978) method was used.

In vitro antifungal activity of selected soil isolates

The test fungal strains *Fusarium oxysporum* and *Fusarium solanii* were procured from Microbial Type Culture Collection center (MTCC), Chandigarh, India (accession numbers-MTCC 284 and 350 respectively) while *Macrophomina phaseolina*, *Rhizoctinia solanii* and *Sclerotinia sclerotium* were provided by Forest Research Institute, Dehradun, India. *In vitro* fungal mycelium growth inhibition was observed by dual culture technique (Verma et al., 2001). The percentage growth inhibition (PGI) was calculated using the following formula:

$$PGI = [(R - r)/R \times 100]$$

Where, r is the radius of the fungal growth opposite the bacterial colony and R is the maximum radius of the fungus growth away from the bacterial colony. Furthermore, fungal mycelia growing towards the zone of inhibition were processed for

morphological studies. The mycelia were picked up from zone of inhibition and control plates, thereafter, transferred to a drop of lacto-phenol on a clean glass slide. Deformities, if any occurred due to antagonism were observed under the fluorescent microscope.

Survival of potential PAH degrading isolates under different osmotic stress regime

The efficient strains which had the ability to metabolize PAH, and further narrowed down for the plant growth promoting attributes were chosen for stresses of different salt concentrations i.e. 1, 1.5, 1.7, 1.9 and 2.1 percent using the method of Elsheikh and Wood (1990).

Determination of antibiotic susceptibility and cell surface hydrophobicity of PAH degrading isolates

Antibiotic resistance of selected PAH degrading isolates was determined by disc diffusion assay on Mueller Hinton agar using Himedia octodiscs. Results were recorded by observing and monitoring the zone of inhibition (if any), around the antibiotic discs. Cell surface hydrophobicity of isolates was determined by their adherence to hydrocarbons which is based on the partitioning of cells in a two phase system with a slight modification from Zhang and Miller (1994) method. For, this isolates were grown in nutrient broth and cells of exponential phase were collected by centrifugation at 5000xg for 15 min at 4 °C, washed twice with sterile distilled water (SDW) to remove any interfering solutes and then resuspended in a 0.2 M phosphate buffer. The bacterial suspension (4.0 ml) with n-Heptanol (2 ml) was vortexed vigorously for 3 min in a test tube and left at room temperature for 30min. The aqueous phase was recovered with the help of pipette. The optical density was measured at 600nm and hydrophobicity was calculated by the formula:

$$\text{Percentage of adherence to hydrocarbon} = 100 \times 1 - \frac{\text{OD of aqueous phase}}{\text{OD of initial cell}}$$

Field trial application of efficient PAH degrading isolates

***P. deltooides* cutting bacterization**

The *P. deltooides* cuttings with uniform shape and size were selected and soaked in water overnight for pre-sowing treatment. Cuttings were surface-sterilized with 70% alcohol for 30 sec followed by treating with 0.1% HgCl₂ for one minute and then washings with sterile distilled water 5-6 times. Sterilized dried cuttings were

bacterized with a slight modification as described by Shim et al. (2000).

Evaluation of rhizoremediation potential in soil model system

Terracota pots (24cm × 12cm × 12cm) were filled with sterilized field soil (approx. 3 kg / pot). Soil characteristics were: sand (63.3 %), silt (16.8 %), clay (20.0 %), organic carbon (2.33 %), nitrogen (0.222 %), pH (7.83) and electrical conductivity (0.96 dsm⁻¹). Anthracene was used as PAH. The experiment was conducted with three sets of following treatments:

T₀ (Control) - Sterile soil + *P. deltooides* cuttings.

T₁ - Sterile soil + *P. deltooides* cuttings + bacterization.

T₂ - Sterile soil + *P. deltooides* cuttings + bacterization + anthracene.

T₃ - Sterile soil + *P. deltooides* cuttings + anthracene.

All the pots were watered as and when required routinely and were kept outdoors and partially covered to protect the plants from rainfall. During cultivation, minimal and maximal temperature ranged from 24 to 38°C respectively and the experiment was conducted from 5 May 2010 to 5 September 2010, as described previously by Bisht et al. (2014). Observations on various growth parameters of *P. deltooides* were recorded such as shoot and root length, fresh and dried shoot and root biomass, stem girth and number of leaves after every 30 days. The data was analyzed using ANOVA, where the means of studied treatments were compared using LSD at *P* = 0.05 significant level. MSTAT-C software was used for computing the data.

Root colonization studies

Root colonization study by selected soil isolate SBA8 was determined by its intrinsic antibiotic resistant pattern. Bacterial strain was inoculated onto cutting of *P. deltooides* by bacterization method with 10⁸ CFU/ml of target strain. To enumerate the viable cells, plants were carefully uprooted from their respective pots after 30, 60, 90 and 120 d of inoculation, and all root segments 5 mm below stem remnants were excised. This was done to ensure that only the bacteria that colonized the roots and not the bacteria that remained on the stem were assayed (Nautiyal, 1997). Roots were washed thoroughly with 0.85% sterile saline water, to remove the soil particles. The associated rhizosphere soil was studied to determine the population density of target strain with its

antibiotic-resistant marker system along with other normal indigenous bacteria (NIB) in rhizosphere soil using serial dilution plate technique on nutrient Agar (NA) medium containing resistant antibiotic (100 µg/ml). A rhizosphere or root segment was considered to be colonized when the target microbe was detected on NA plates after 24 - 48 h of incubation at 27°C.

Quantitative removal of hydrocarbon in soil model system

For determination of residual anthracene content in soil, *P. deltooides* were uprooted and anthracene amended soils attached to roots were carefully collected from respective pots. The soil samples were air dried in the dark and the anthracene was extracted from rhizospheric soil as described by Gao and Zhu (2004) and anthracene biodegradation in soil was determined by HPLC (Shimadzu) analysis (Filonov et al., 2006).

Bioinoculant formulation

Saw dust, wheat bran, cocoa peat, sugar bagasse, rock phosphate and dried leaves of *P. deltooides* were used as solid lignocellulosic waste carrier material, although in present investigation the dried leaves of *P. deltooides* were also used as solid carrier material. For bioinoculant formulation, the test culture was grown in nutrient broth for 24 h at 150 rpm, the cells $\approx 10^6$ CFU/ml was used for inoculants preparation (Somasegaran and Hoben, 1994). For preparation of carrier materials autoclavable, grinded and air dried material i.e. ≈ 40 g of materials were packed in polythene bags made up of 50-75 mm thick, low density and flexible sheets. The polythene bags were sealed leaving about 2/3 vacant space to give proper aeration to the inoculant. 1 ml of bacterial suspension was introduced aseptically with a hollow needle directly through the wall of the bag and the small hole was immediately covered with the help of self-adhesive tape. Immediately, after sealing, the inoculum and carriers were hand mixed by shaking or kneading between the fingers. The materials inside the sealed polythene bags were spread and kept at 27°C for 2 days for curing so as to attain maximum number of cells in the formulations. After curing, the polythene bags having inoculants were stored at room temperature in dark to prevent from U.V radiation and heat. Three replicates of each treatment were done. Physiochemical properties of

carrier material such as inherent moisture content, water holding capacity (WHC), pH, total phosphorous, C/N ratio and total potassium were also measured in accordance to standard procedure (Page et al., 1982; Jimenez and Ladha, 1993).

Population dynamics of PAH degrading isolates in different substrate carrier based formulation

For inoculant preparation, selected isolates were grown in Nutrient broth at 27°C and when cell concentration exceeded 10^7 colony forming unit (CFU)/ml, it was inoculated into the carrier material (Somasegaran and Hoben, 1994). Samples were collected from bio-formulation after different time intervals under aseptic conditions and viable cells were determined by inoculating suitable dilutions on NA medium having antibiotic (100 µg/ml) and colony forming unit (CFU) were enumerated (Pandey and Maheshwari, 2007a,b). The CFU/g of inoculants was monitored after every 30 days for a period of 180 days.

Results

Four bacterial strains isolated from the rhizosphere of *P. deltooides* have already been identified as *Kurthia* sp. SBA4, *Micrococcus varians* SBA8, *Deinococcus radiodurans* SBA6 and *Bacillus circulans* SBA12 which have the ability to metabolize PAH and are chemotactically active against PAH (Bisht et al., 2010), were subjected for further rhizoremediation studies. Among these four isolates, only one soil isolate *M. varians* SBA8 exhibited higher PGP and biocontrol activity, therefore, anthracene biodegradation studies was conducted with this bacterium only in PAH contaminated soil-model system.

Plant growth promoting attributes

On the basis of appreciable biodegradation abilities of PAH in solution, *M. varians* SBA8 was selected for further studies. IAA production and phosphate solubilization were quantified for this selected isolate. *M. varians* SBA8 produced maximum amount of IAA after 96h of incubation i.e. $19 \mu\text{g.ml}^{-1}$ (Figure 1). The IAA production profile of all the strains had a declining trend after 96h and kept falling subsequently. Apparently highest amount of phosphorous liberated by this efficient isolate was $1.8 \mu\text{g.ml}^{-1}$ after 120h (Figure 2). The pH of the broth also dipped down from 7.2 \pm 0.5 to 4.4 ± 0.5 after 192 h respectively.

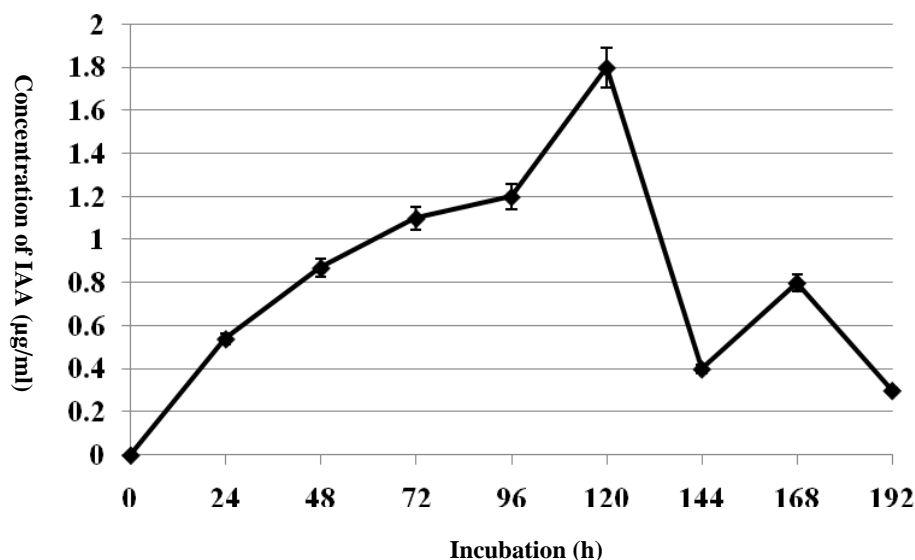


Figure 1. Quantitative estimation of IAA by *M. varians* SBA8.

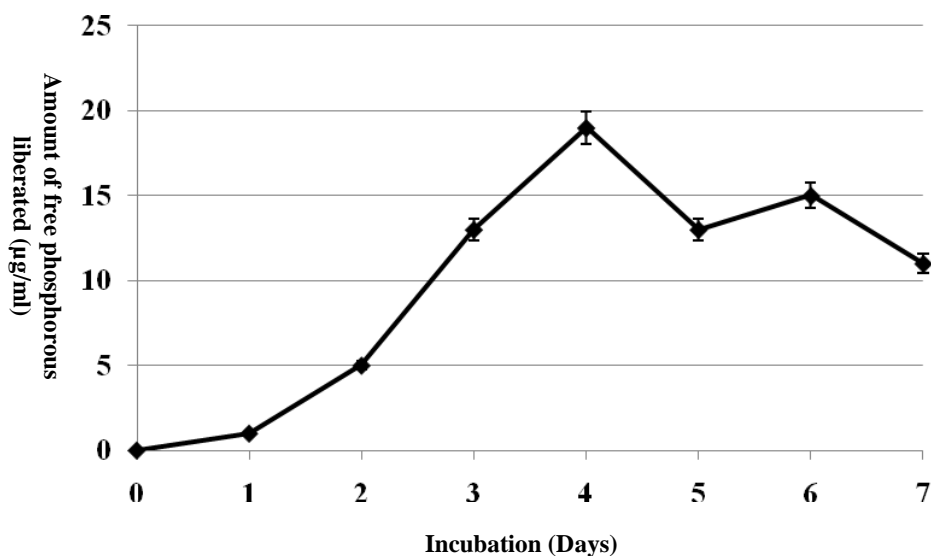


Figure 2. Amount of phosphate solubilized by *M. varians* SBA8.

In vitro antagonism test and cell surface hydrophobicity

The *M. varians* SBA8 inhibited the growth of *M. phaseolina*, *R. solani* and *F. oxysporum*, by 43.3%, 50% and 34.6%, respectively (Table 1). The mycelia picked from the interaction zone showed morphological deformities like lysis of hyphal cell, halo cell formation, loss of cytoplasm and granulation in hyphae (Figure 3). However, relative

cell surface hydrophobicity of *M. varians* SBA8 isolate was observed in n-Heptanol and it was calculated as 20% of the percentage of the cells adsorbed to Heptanol phase.

Field study for rhizoremediation

Increase of 6.6% and 23.5% and 4.7% and 33.5% in shoot and root length was observed in T₁ and T₂ treatments after 60 days whereas, it remained 1.8% and 8.4% and 2.2% and 4.7% in the

same treatments after 120 days. An increase of 6.6%, and 33.1% and 1.6% and 30.5% in fresh and dry shoot biomass was recorded in T₁ and T₂ treatments while 10.7% and 48.2% and 8.7% and 51.6% increase was obtained in fresh and dry root biomass in T₁ and T₂, respectively. Moreover, an

increase of 2.8% and 10.5% in stem girth was observed in T₁ and T₂ treatments, respectively, after 120 days (Table 2). However, in treatment T₃ all the plants growth parameters were relatively less than control and other treatments due to application of PAH.

Table 1. Plant growth promoting attributes.

Strains	IAA	P solubilization	Siderophore	HCN	Antagonistic Activity*			ACC Deaminase
					FO	RS	MP	
SBA4	–	–	–	–	–	–	–	–
SBA8	++	++	++	+	34.6	50	43.3	+
SBA6	–	+	–	–	–	–	–	–
SBA12	+	–	–	–	–	–	–	–

– No production; + Slight production; ++ Good production; +++ Excellent production; *percent inhibition; FO – *Fusarium oxysporum*; RS – *Rhizoctonia solani*; MP – *Macrophomina phaseolina*

Figure 3: Post interaction events by *M. varians* SBA8 (A) Lysis of hyphal cell in *R. solanii* (B) Halo cell formation in *M. phaseolina* (C) Deformity in hyphae of *F. oxysporum*. (D) Normal hyphae of *R. solanii* from control plate (E) Normal hyphae of *M. phaseolina* from control plate (F) Normal hyphae of *F. oxysporum* from control plate.

Table 2. Effect of inoculation of *M. varians* SBA8 on the growth of *P. deltoides* in pot trial at different durations.

Treatments	Length (cm)		Shoot mass (g)		Root mass (g)		Stem girth (mm)	Total no. of leaves
	Shoot*	Root*	Fresh*	Dry*	Fresh*	Dry*		
After 30 days of sowing								
T ₀	14.3	8.7	9.2	8.0	6.8	4.8	0.17	2
T ₁	15.7*	8.8*	9.3*	8.2*	6.0*	5.2*	0.16*	2
T ₂	17.3*	9.1*	10.6*	9.0*	7.3*	6.1*	0.17*	2
T ₃	13.0	7.4	8.3	7.1	5.7	3.9	0.16	2
After 60 days of sowing								
T ₀	28.4	14.6	12.8	10.6	7.8	5.4	0.23	2
T ₁	30.3*	15.3*	11.1*	10.7*	7.8*	6.0*	0.23*	2
T ₂	35.1*	19.5 ^{ns}	14.3*	13.0*	8.2*	6.8*	0.24*	3*
T ₃	25.1	11.9	10.5	8.8	6.1	4.2	0.22	2
After 90 days of sowing								
T ₀	42.5	22.6	14.5	12.1	9.8	6.9	0.33	5*
T ₁	46.5*	22.8*	14.2*	13.1 ^{ns}	9.9*	7.8*	0.34*	5*
T ₂	48.9*	24.6*	18.0**	17.3*	12.2*	10.4*	0.36*	6*
T ₃	36.7	17.5	11.9	9.5	7.2	5.1	0.28	4*
After 120 days of sowing								
T ₀	98.7	44.6	18.1	17.7	11.2	9.1	0.38	8
T ₁	100.5**	45.6*	19.3*	18.0*	12.4*	9.9*	0.39*	8*
T ₂	107**	46.7*	24.1*	23.1*	16.6*	13.8*	0.42*	9*
T ₃	76.4	29.5	14.0	13.8	8.7	8.0	0.32	6*

– no leaves were found during this duration; Mean values were calculated from three replicates of each treatment: *not significant; **significant at $P = 0.05$ LSD.

Root colonization assay

For root colonization antibiotic marker strategies were performed for evaluation of CFU/ml count. Based on its intrinsic antibiotic resistance profile, *M. varians* SBA8 isolate was found to be resistant against a large number of antibiotics (Table 3) therefore; it was further screened to find out the tolerance limit to the maximum concentration of individual antibiotic. It was established that *M. varians* SBA8 showed highest resistance to erythromycin (100 µg/ml), hence

abbreviated as *M. varians* SBA8^{Erth⁺}. The result of root colonization assay of *M. varians* SBA8 suggested it as a good colonizer, which is a desirable property in a suitable PGPR. It was observed that increase in population count was maintained after 60 days in T₁ treatment then started decline whereas, continuous decrease in cell population was observed in T₂ treatment. The normal indigenous bacterial population (NIB) was comparatively higher till 90 days study (Table 4).

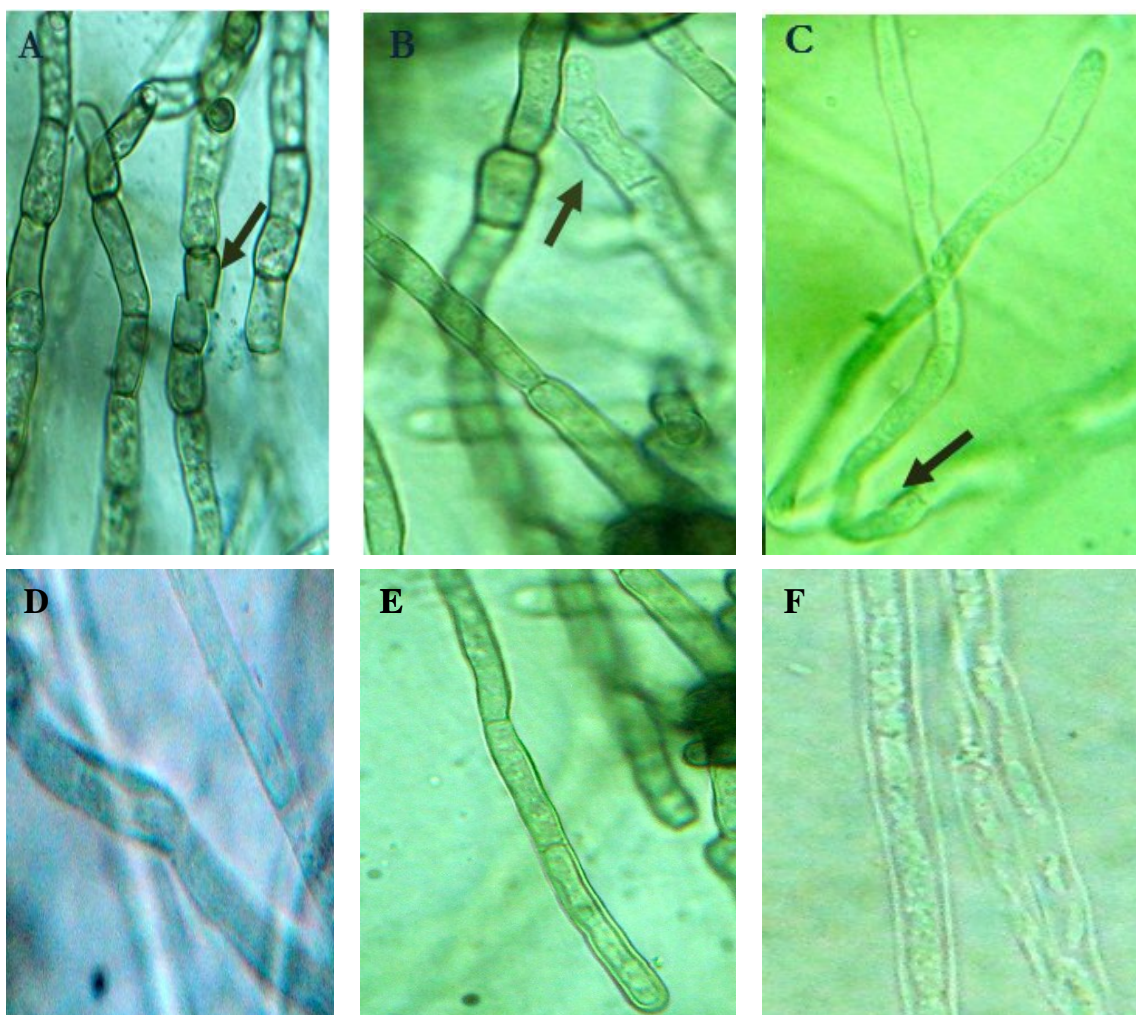


Figure 3. Post interaction events by *M. varians* SBA8 (A) Lysis of hyphal cell in *R. solanii* (B) Halo cell formation in *M. phaseolina* (C) Deformity in hyphae of *F. oxysporum*. (D) Normal hyphae of *R. solanii* from control plate (E) Normal hyphae of *M. phaseolina* from control plate (F) Normal hyphae of *F. oxysporum* from control plate.

Table 3. Intrinsic resistant antibiotic profile of *M. varians* SBA8.

Antimicrobial agent	Symbols	Disc content (μ g)	Zone of diameter (mm)	Inference
Chloramphenicol	C	25	3.8	Sensitive
Erythromycin	E	5	-	Resistant
Fusidic acid	Fc	10	-	Resistant
Methicillin	M	10	-	Resistant
Novobiocin	Nv	5	-	Sensitive
Penicillin G	P	1*	-	Resistant
Streptomycin	S	10	14	Sensitive
Tetracycline	T	25	19	Sensitive

*= content of Penicillin G in octodisc is 1 unit.

Table 4. Survival of *M. varians* SBA8^{Erth+} in various treatments of *P. deltoides* at 30, 60 and 90 days in non-sterilized soil.

Treatments	Population of <i>M. varians</i> SBA8 ^{1etr+} in the <i>Populus</i> rhizosphere (10^6 CFU/g soil) ^a		
	30days	60days	90days
T ₁	6.4 \pm 2	20.2 \pm 1	16 \pm 2.5
T ₂	5.7 \pm 1	3 \pm 1	2 \pm 1
NIB	11 \pm 4.5	38 \pm 1	72.5 \pm 6.5

^aValues are mean of three replicates $\square \pm SD$, NIB = normal indigenous bacteria

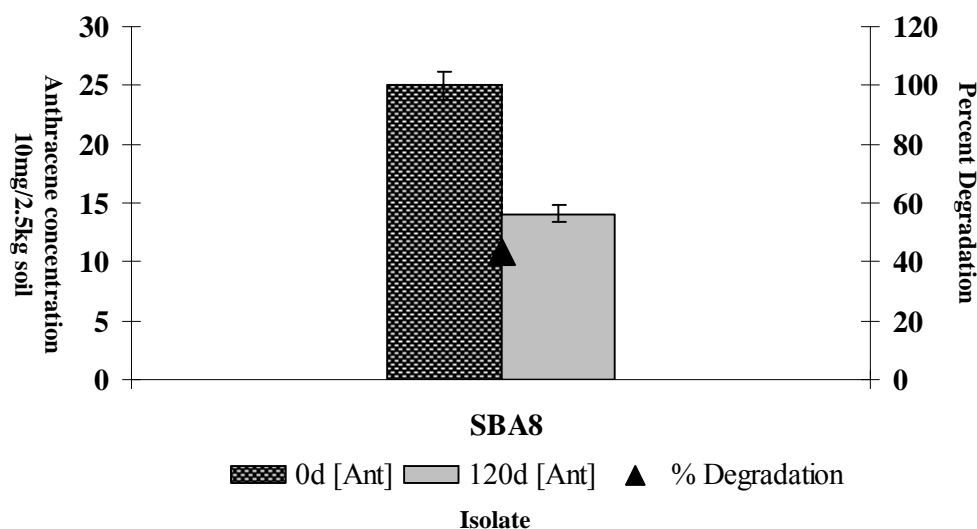


Figure 4. Anthracene removal in *P. deltoides* planted soil inoculated with *M. varians* SBA8 amended with 10 mg.kg⁻¹ of anthracene (Ant).

Viability of *M. varians* SBA8 in different solid lignocellulosic waste as a carrier

Biodegradation of anthracene in soil

Rhizoremediation of anthracene with *M. varians* SBA8 was analyzed by HPLC analysis. It

was noticed that the presence of several intermediate compounds were eluted at different retention times ranging from 1.2 to 28 min. The residual concentration of anthracene was determined by calculating the peak area relative to

standard with pure anthracene. *M. varians* SBA8 resulted in 43.6% degradation of anthracene after 120 days in treatment T₂ (Figure 4).

Under *in vivo* conditions, *M. varians* SBA8 showed an appreciable biodegradation activity and also possessed PGP attributes including antagonistic activity against soil borne phytopathogens. Therefore, suitable system was provided for proper reproduction and dispensing of this isolate for agriculture and other industrial uses. According to Indian standard: specification for rhizobial inoculants (1997), the survival of these potential isolates in different carriers were checked for 6 months, to find out the possibilities of effective carrier material for these multidimensional isolates for formulation. Analysis of bagasse for viable count showed that initial population was started with 10⁸ CFU/g, then there was continuous ≈ 10 times decreases in population count in the first 60 days, after that it remain constant for next month and then a $\approx 10^4$ times decline in population count were observed after 180 days. Similar trend was followed with wheat bran as it showed approx. constant cell density as the initial count i.e. $\approx 10^8$ for 60 days after that it reduced to $\approx 10^3$ times after 180 days respectively. Cocoa peat and rock phosphate proved out to be better carrier material as they gives ≈ 10 times increase in cell density for 60 days and then declined to $\approx 10^3$ and $\approx 10^2$ times respectively after 180 days. Saw dust was considered a moderate carrier material comparatively to saw dust and bagasse as it continued to ≈ 10 times decline after 4 months and then reduced to $\approx 10^3$ times after 180 days. Dried leaves of *P. deltoides* showed an evidence of poor carrier material as there was a continuous reduction in CFU/g and after 120 days it showed no CFU/g (Table 5).

Discussion

Present investigation assessed the potential of a selected soil isolate to be used for rhizoremediation of PAH contaminated soil model system. However, this study tried to establish a perfect 'plant-microbe pair' for bioremediation of PAH in soil. Bisht et al. (2010) found that soil isolates *Kurthia* sp. SBA4, *M. varians* SBA8, *D. radiodurans* SBA6 and *B. circulans* SBA12 have promising characteristics for rhizoremediation of PAH using *P. deltoides* rhizosphere system in soil. To explore this, the potential of the best soil isolate i.e. *M. varians* SBA8 was evaluated as it possessed all the PGP traits and rhizospheric bioremediation activity. Various research studies from rhizobacterial isolates were focused on the plant growth promoting agents, because these improve plant

growth by synthesizing phytohormones precursors (Ahmad et al., 2008), solubilize inorganic P and nutrient cycling (Khan et al., 2002). In remediation context, rhizobacterial system has been proved to be more effective in minimizing the bioavailability and biotoxicity of pollutants (Khan 2005a,b; Wani et al., 2008). Previously, *Bacillus* sp, *Micrococcus* sp. and *Pseudomonas* sp. have been reported to be efficient candidates for phosphate solubilization and IAA application in agriculture systems (Lucas Garcia et al., 2004; Antoun et al., 2004; Gupta et al., 2005; Rajkumar et al., 2006). Therefore, the rhizobacterium used in this study was a promising plant growth stimulator and a biocontrol agent for *P. deltoides* root rot disease. Soil isolate *M. varians* SBA8 used in current study caused significant inhibition of mycelial growth of *M. phaseolina*, *R. solanii* and *F. oxysporum*. Several workers have reported the inhibition of soil borne phytopathogen by rhizobacteria (Barnard, 1994) but the reports for *Micrococcus* sp. are scanty.

In the dual culture assay, *M. varians* SBA8 not only inhibited the mycelial growth but also caused deformities of hyphae cell as clearly depicted in Figure 3. In some of these processes bacterial cells attached to the mycelia and penetrated into the hyphal wall resulting in lysis in both *F. oxysporum* and *R. solani* (Chung et al., 1998). On the other hand, *M. varians* SBA8 used in present study did not show mycelial or conidial attachment but showed the involvement of diffusible secondary metabolites responsible for inhibition of fungal pathogens. Earlier also, Lee and Kobayashi (1989) showed the deformities in the mycelial morphology of *Rhizoctonia solani* caused by the action of antifungal metabolites of *B. cepacia*.

However, in present study we have evaluated the *M. varians* SBA8 not only for plant growth promoting and biocontrol activities but also for bioremediation potential. Previously, Glick et al. (1998) has reported the production of ACC deaminase from rhizobacteria that results in improved root growth by lowering the concentration of plant ethylene. The effect on different plant-root systems under the influence of ACC deaminase from rhizobacteria is well explained (Belimov et al., 2005). Glick (2003) reported the development of plant seedlings in presence of PGPR having ACC deaminase activity under stress conditions like salt stress. *M. varians* SBA8 used in this study has the ability to produce ACC deaminase (Table 1).

Additionally, *M. varians* SBA8 also produced IAA and solubilized P with reduction in broth pH

which may be due to organic acids production (Kumar and Narula, 1999) after 96 h and 120 h in culture condition (Figure 1 and 2). Similarly, Pandey and Maheshwari (2007b) observed that a rhizobacterial strain *S. meliloti* PP3 produces maximum 80 µg/ml IAA after 7 days of incubation. Leinhos and Bergmann (1995) reported that the addition of IAA to soil enhanced the uptake of iron and other elements (e.g. zinc, calcium etc.) by plant roots which lead to an increase in plant growth. Very few reports are available for phosphate solubilizing microorganisms from rhizosphere of *Populus* sp. growing in hilly regions. The present study showed the potential of rhizobacteria as plant growth promoter as well as P solubilizer, suggesting it can be used as a suitable biofertilizers for agricultural improvement (Kumar et al., 2001; 2011). The PGP attributes provided by efficient soil isolate like *M. varians* SBA8 enhanced the growth of host plant in rhizosphere and also leads to PAH degradation in rhizospheric soil due to perfect plant-microbe interaction. Appreciable surface hydrophobicity was observed with *M. varian* SBA8 i.e. 20% against the hydrocarbon. This is a desirable property responsible for the increase in the aqueous PAH solubility by facilitating transport of anthracene in soil and efficient substrate-to-cell contact mechanisms. Earlier, Glick (2003) suggested that PAH increasingly bound in soil particles as the time passes. So, it is necessary to increase the movement of tightly bound hydrophobic PAH to the microbes where they can be effectively metabolized.

A key concern for the safety, efficacy, and commercial potential for any environmental application of an isolate is the ability of the microbe to survive in target habitats. Henceforth, the efficient isolate *M. varians* SBA8 with ability to biodegrade PAH along with PGP traits was subjected for its ability to compete for an ecological niche in the rhizosphere of *P. deltoides* for 90 days in hydrocarbon contaminated soil as well as in non-contaminated soil. Though, resistance against antibiotic was also utilized for colonization studies (Table 4). Similarly, Nautiyal (1997) established a procedure for selecting a rhizosphere-competent biocontrol bacterium *Pseudomonas fluorescens* NBRI1303 with its antibiotic marker system and checked its effect on plant growth and its ability to compete with other indigenous population of rhizosphere in chick pea. Earlier, Pandey et al. (2005) also monitored the survival of an antibiotic-resistant marker strain *Pseudomonas* GRC₁ which exhibited efficient root colonization after different time intervals of observation (i.e., 30, 60, and 90

days) and enhanced plant growth and growth yield. In current study the population density of *M. varians* SBA8 increased up to 60 days of inoculation, which became almost stable thereafter. It has been suggested, that bacteria that attain colony-forming units of about 10³ per gram or higher on root mass can be considered a good colonizers (Meyer and Abdallah, 1978) and in this study we have obtained the population density higher than 10³ per gram of soil. Successful application of plant-microbe systems for rhizoremediation relies on *in situ* establishment of a high level of competence of introduced bacteria (Liu et al., 2007).

Rhizoremediation study demonstrated that the PAH-degrading and growth-promoting *M. varians* SBA8 exhibited a significant enhancement in shoot and root length, root and shoot biomass including stem girth of *Populus* plant in hydrocarbon contaminated as well as non-contaminated soil with respect to control. Previous studies indicated that inoculation of barley seeds with phenanthrene degrading bacteria improved the growth of plants in the peat mixture containing (5 mg/g) phenanthrene (Anokhina et al., 2004), while in our work *Populus* sp. were planted with 10 mg/kg anthracene contaminated soil. Additionally, with respect to other treatment i.e. T₀ and T₃, maximum plant growth was achieved in treatment T₂ (Table 2). Therefore, it clearly indicate that PAH amended in soil act as carbon source and it might accelerate the growth rate of our target strain i.e. *M. varians* SBA8 so that plant growth promoting substance can be secreted at higher amount which conferred the remarkable change in plant growth parameters. Present results demonstrated that *M. varians* SBA8 substantially degraded 43.6% anthracene respectively after 120 days in soil model system as determined by HPLC analysis (Figure 4). Earlier, Wischmann and Steinhart (1997) reported substantial removal of benzo(a)anthracene, chrysene, and benzo(a)pyrene, with 11%, 19%, and 54% respectively after 15 weeks whereas in our study remediation with suitable plant microbe system i.e. *P. deltoides* and its rhizobacterial system were used for efficient removal of anthracene within 120 days only. Reports on *Micrococcus* sp. as good root colonizers are scanty.

The viability of the inoculum in an appropriate formulation for a certain length of time is of utmost importance as contribution to provide adequate nutrition. Effectively in agriculture plant nutrients may be limiting factors, particularly as regards nitrogen and phosphorus which are major elements required for plant growth and higher seeds

production (Pandey et al., 2007a). Materials having high organic content, high water holding capacity and good aeration has been considered as good carrier for bioinoculants (Brockwell and Bottomley, 1995). In this study also, all the physical parameters measured were found optimum for bagasse, cocoa peat, saw dust, wheat bran and rock phosphate were determined (data not shown). All the carrier materials used in this study were also a good source of P and K and inorganic content. Our target strain *M. varians* SBA8 was studied to check the viability in six different solid carrier materials (Table 5). Based on its viability study, rock phosphate, cocoa peat and wheat bran were most suitable materials as its final population was found in the range of $\approx 10^5$ CFU/g after 6 months of incubation. Earlier also peat is supposed to be an excellent carrier for bioinoculant and has been accepted worldwide for this purpose (Smith, 1995). However, unavailability of peat in tropical countries including India raised a concern for a suitable replacement. In view of this, we studied cocoa peat along with other carrier for the bioinoculant formulation. Similarly, wheat bran was successfully used as a carrier for microbial strains (Jackson et al., 1991). Sawdust was found to be a moderate carrier material as the viability of isolate in sawdust was not comparable with that in other lignocellulosic carrier materials. The addition of rock phosphate with bacterial cells has been suggested to serve as a good replacement for a chemical mixture of fertilizers (Bashan, 1986).

Conclusion

Conclusively, potential soil isolate *M. varians* SBA8 observed with rhizosphere competence, PGP and biocontrol activities qualifies to be an appropriate candidate for rhizoremediation of PAH. The strategy for using plant-microbe interactions for PAH degradation instead of direct amendment of contaminated soil with bacteria was adapted because in the latter strategy inoculation with bacteria is readily removed by indigenous microbial population. Also its formulation in wheat bran, cocoa peat rock phosphate based carrier materials presents immense possibilities of environmental restoration especially PAH contaminated sites. However it offers a multidimensional benefit for application on PAH contaminated sites. The potential of these formulations applied through *Populus* rhizosphere should be exploited for environmental and commercial purpose.

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References

- Ahmad, F., I. Ahmad and M. S. Khan. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* 163:173-181.
- Anokhina, T. O., V. V. Kochetkov, N. Zelenkova, V. V. Balakshina and V. M. Boronin. 2004. Biodegradation of Phenanthrene by *Pseudomonas* bacteria bearing rhizospheric plasmids in model plant-microbial associations. *App. Biochem. Microbiol.* 40(6):568-572.
- Antoun, H., C. J. Beauchamp, N. Goussard, R. Chabot and R. Lalonde. 2004. Potential of *Rhizobium* and *Bradyrhizobium* species as growth promoting bacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). *Plant Soil.* 204:57-67.
- Barnard, E. L. 1994. The nursery to field carryover and post outplanting impact of *M. phaseolina* on loblolly pine on a cutover forest site in North Central Florida. *Tree Planter's Not.* 45:68-71.
- Bashan, Y. 1986. Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. *Appl. Environ. Microbiol.* 51:1089-1098.
- Belimov, A. A., N. Hontzeas, V. I. Safronova, S. V. Demchinskaya, G. Piluzza, S. Bullitta and B. R. Glick. 2005. Cadmium-tolerant plant growth promoting rhizobacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biol. Biochem.* 37:241-250.
- Bisht, S., P. Pandey, G. Kaur, H. Aggarwal, A. Sood, V. Kumar, S. Sharma and N. S. Bisht. 2014. Utilization of endophytic strain *Bacillus* sp. SBER3 for biodegradation of polyaromatic hydrocarbons (PAH) in soil model system. *Eur. J. Soil Biol.* 60:67-76.

- Bisht, S., P. Pandey, A. Sood, S. Sharma and N. S. Bisht. 2010. Biodegradation of anthracene and naphthalene by chemotactically active rhizobacteria of *Populus deltoids*. Braz. J. Microbiol. 41:922-930.
- Brockwell, J and P. J. Bottomley. 1995. Recent advances in inoculant technology and prospects for the future. Soil Biol. Biochem. 27:683-697.
- Chung, H. S., E. S. Chung and Y. H. Lee. 1998. Biological control of post harvest roots rots of *Ginseng*. Kor. J. Plant Pathol. 14:268-277.
- Cunningham, S. D. and D. W. Ow. 1996. Promises and prospects of phytoremediation. Plant Physiol. 110:715-719.
- Elsheikh, E. A. E and M. Wood. 1990. *Rhizobia* and *Bradyrhizobia* under salt stress: possible role of trehalose in osmoregulation. Lett. Appl. Microbiol. 10:127-129.
- Filonov, A. E., I. F. Puntus, A. V. Karpov, I. A. Kosheleva, L. I. Akhmetov, D. R. Yonge, J. N. Petersen and A. M. Boronin. 2006. Assessment of naphthalene biodegradation efficiency of *Pseudomonas* and *Burkholderia* strains tested in soil model systems. J. Chem. Technol. Biotechnol. 81:216-224.
- Gao, Y. Z and L. H. Zhu. 2004. Plant uptake, accumulation and translocation of phenanthrene and pyrene in soils. Chemosphere 55:1169-1178.
- Glick, B. R., D. M. Penrose and J. Li. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. 190:63-68.
- Glick, B. R. 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol. Adv. 21:383-393.
- Gregory, P. J and B. J. Atwell. 1991. The fate of carbon in pulse labeled crops of barley and wheat. Plant Soil. 136:205-213.
- Gupta, A., V. Rai, N. Bagdwal and R. Goel. 2005. *In situ* characterization of mercury resistant growth promoting fluorescent *pseudomonads*. Microbiol. Res. 160:385-388.
- Honma, M. and T. Shimomura. 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agri. Biol. Chem. 42:1825-1831.
- Huang, X. D., Y. S. El-Alawi, D. Penrose, B. R. Glick and B. M. Greenberg. 2004. A multiprocess phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ. Poll. 130:465-476.
- Jackson, A. M., J. M. Whipps and J. M. Lynch. 1991. Production, delivery systems, and survival in soil of four fungi with disease biocontrol potential enzymes. Microb. Technol. 13:636-642.
- Jimenez, R. R. and J. K. Ladha. 1993. Automated elemental analysis: a rapid and reliable but expensive measurement of total carbon and nitrogen in plant and soil samples. Commun. Soil Sci. Plant Anal. 24:1897-1924.
- Khan, A. G. 2005a. Mycorrhizas and phytoremediation. In: N. Willey (Ed.), Method in biotechnology-phytoremediation: methods and reviews, Humana Press, Totowa.
- Khan, A. G. 2005b. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J. Trace Elem. Med. Biol. 18:355-364.
- Khan, M. S., A. Zaidi and M. Aamil. 2002. Biocontrol of fungal pathogens by the use of plant growth promoting rhizobacteria and nitrogen fixing microorganisms. Ind J. Bot. Soc. 81:255-263.
- Kuiper, I., G. V. Bloembergen and B. J. J. Lugtenberg. 2001. Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. Mol. Plant Microb. Interact. 14:1197-1205.
- Kumar, V., R. K. Behl and N. Narula. 2001. Establishment of P- solubilizing analogue resistant strains of *Azotobacter chroococcum* in rhizosphere and their effect on wheat under greenhouse conditions. Microbiol. Res. 156(1):87-94.
- Kumar, V. and N. Narula. 1999. Solubilization of inorganic phosphates by *Azotobacter chroococcum* mutants and their effect on seed emergence of wheat. Biol. Fertil. Soil 28:301-305.
- Kumar, V., A. S. Singh and S. Sharma. 2011. AM fungi *A. chroococcum*, yield, nutrient uptake and economics of *Plantago ovata* in Indian arid region. Thailand J. Agric. Sci. 44(1):53-60.

- Lee, W and D. Y. Kobayashi. 1989. Isolation and identification of anti-fungal *Pseudomonas* spp. from sugar beet roots and its antibiotic products. Kor. J. Plant Pathol. 4:264-269.
- Leinhos, V. and H. Bergmann. 1995. Influence of auxin producing rhizobacteria on root morphology and nutrient accumulation of crops. 2. Root-growth promotion and nutrient accumulation of maize (*Zea-mays* L.) by inoculation with indole-3-acetic acid (IAA) producing *Pseudomonas* strains and by exogenously applied IAA under different water-supply conditions. Angew Bot. 69:37-41.
- Liu, L., C. Y. Jiang, X. Y. Liu, J. F. Wu, J. G. Han and S. J. Liu. 2007. Plant microbe association for rhizoremediation of chloronitroaromatic pollutants with *Comamonas* sp. strain CNB-1. Environ. Microbiol. 9:465-473.
- Lucas Garcia, J. A., A. Probanz, B. Ramos, J. Barriuso and F. J. Gutierrez Manero. 2004. Effects of inoculation with plant growth promoting rhizobacteria (PGPRs) and *Sinorhizobium fredii* on biological nitrogen fixation, nodulation and growth of *Glycine max* cv. Osumi. Plant Soil. 267:143-153.
- Meagher, R. B. 2000. Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. 3:153-162.
- Menzie, C. A and B. Potokib. 1992. Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26:1278-1284.
- Meyer, J. M and M. A. Abdallah. 1978. The fluorescent pigment of *Pseudomonas fluorescens*- biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. 107:319-328.
- Miller, R. L. and B. B. Higgins. 1970. Association of cyanide with infection of birds foot trefoil by *Stemphylium loti*. Phytopathol. 60:104-110.
- Nautiyal, C. S. 1997. Selection of Chickpea-Rhizosphere-Competent *pseudomonas fluorescens* NBRI1303 antagonistic to *Fusarium oxysporum* f. sp. ciceri, *Rhizoctonia bataticola* and *Pythium* sp. Curr. Microbiol. 35:52-58.
- Page, A. L., R. R. H. Miller and D. R. Keeny. 1982. Methods of soil analysis, In: Chemical and microbiological properties, part 2. American Society of Agronomy, Inc. pp. 11-59. Soil Science Society of America, Inc, Madison, Wis.
- Pandey, P., S. C. Kang, C. P. Gupta and D. K. Maheshwari. 2005. Rhizosphere Competent *Pseudomonas aeruginosa* GRC1 Produces Characteristic Siderophore and Enhances Growth of Indian Mustard (*Brassica campestris*). Curr. Microbiol. 51:303-309.
- Pandey, P. and D. K. Maheshwari. 2007a. Bioformulation of *Burkholderia* sp. MSSP with a multispecies consortium for growth promotion of *Cajanus cajan*. Can. J. Microbiol. 53:213-222.
- Pandey, P. and D. K. Maheshwari. 2007b. Two-species microbial consortium for growth promotion of *Cajanus cajan*. Curr. Sci. 92(8):1137-1142.
- Rajkumar, M., R. Nagendran, K. J. Lee, W. H. Lee and S. Z. Kim. 2006. Influence of plant growth promoting bacteria and Cr (vi) on the growth of Indian mustard. Chemosphere 62:741-748.
- Rodriguez, A. H., M. Y. Perez, Y. A. Guerrero, M. G. V. Valle and A. N. N. H. Lauzardo. 2008. Antagonistic activity of Cuban native rhizobacteria against *Fusarium verticillides* (Sacc.) Nirenb. in maize (*Zea-mays* L.) Appl. Soil Ecol. 39:180-186.
- Schwyn, B. and J. B. Neilands. 1987. Universal assay for the detection and determination of siderophores. Anal Biochem. 160:47-56.
- Shim, H., S. Chauhan, D. Ryoo, K. Bowers, S. M. Thomas, K. A. Canada, J. G. Burken and T. K. Wood. 2000. Rhizosphere competitiveness of trichloroethylene-degrading, poplar-colonizing recombinant bacteria. Appl. Environ. Microbiol. 66(11):4673-4678.
- Shuttleworth, K. L. and C. E. Cerniglia. 1995. Environmental aspects of PAH biodegradation. Appl. Biochem. Biotechnol. 54:291-302.
- Smith, R. S. 1995. Inoculants formulation and application to meet changing needs in nitrogen fixation; fundamental and applications, In: I. A. Tikhanovich, N. A. Provovor, V. I. Roamniov and W. E. Newton (Eds.), pp. 653-657. Kluwer Academic publisher Dordrecht The Netherlands.
- Somasegaran, P. and H. J. Hoben. 1994. Handbook for rhizobia: methods in legume *Rhizobium*

- technology. Springer-Verlag, New York, Inc. New York.
- Spirang, R., M. Hayashi, M. Yamashita, H. Ono, K. Saeki and Y. Murooka. 2002. A novel bioremediation system for heavy metals using the symbiosis between leguminous plant and genetically engineered *rhizobia*. J. Biotechnol. 99:279-293.
- Verma, S., V. Kumar, N. Narula and W. Merbach. 2001. Studies on *in vitro* production of antimicrobial substances by *Azotobacter chroococcum* isolates/mutants. J. Plant Dis. Protec. 108(2):152-165.
- Wani, P. A., M. S. Khan and A. Zaidi. 2008. Chromium reducing and plant growth promoting Mesorhizobium improves chickpea growth in chromium amended soil. Biotechnol. Lett. 30:159-163.
- Wilson, S. C and K. C. Jones. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ. Pollut. 81:229-249.
- Wingfors, H., A. Sjodin, P. Haglund, E. Brorstrom-Lunden. 2001. Characterization and determination of profiles of polycyclic aromatic hydrocarbons in traffic tunnel in Gothenburg, Sweden. Atmos. Environ. 35:6361-6369.
- Wischmann, H. and H. Steinhart. 1997. The formation of PAH oxidation products in soils and soil/compost mixtures. Chemosphere 35(8):1681-1698.
- Yee, D. C., J. A. Maynard and T. K. Wood. 1998. Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene *ortho*-monooxygenase constitutively. Appl. Environ. Microbiol. 64:112-118.
- Yuan, S. Y., L. C. Shiung and B. V. Chang. 2002. Biodegradation of polycyclic aromatic hydrocarbons by inoculated microorganisms in soil. Bull. Environ. Contam. Toxicol. 69:66-73.
- Zahir, Z. A., M. Arshad and W. T. Frankenberger. 2004. Plant growth promoting rhizobacteria: applications and perspectives in agriculture. Adv. Agro. 81:97-168.
- Zhang, Y. and R. M. Miller. 1994. Effect of *Pseudomonas rhamnolipid* biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ. Microbiol. 60:2101-2106.