Transformation of pWWO in *Rhizobium leguminosarum* DPT to Engineer Toluene Degrading Ability for Rhizoremediation

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Abstract Rhizoremediation of organic xenobiotics is based on interactions between plants and their associated micro-organisms. The present work was designed to engineer a bacterial system having toluene degradation ability along with plant growth promoting characteristics for effective rhizoremediation. pWWO harboring the genes responsible for toluene breakdown was isolated from *Pseudomonas putida* MTCC 979 and successfully transformed in *Rhizobium* DPT. This resulted in a bacterial strain (DPT^T) which had the ability to degrade toluene as well as enhance growth of host plant. The frequency of transformation was recorded 5.7×10^{-6} . DPT produced IAA, siderophore, chitinase, HCN, ACC deaminase, solubilized inorganic phosphate, fixed atmospheric nitrogen and inhibited the growth of *Fusarium oxysporum* and *Macrophomina phaseolina* in vitro. During pot assay, 50 ppm toluene in soil was found to inhibit the germination of *Cajanus cajan* seeds. However when the seeds bacterized with toluene degrading

P. putida or *R. leguminosarum* DPT were sown in pots, again no germination was observed. Non-bacterized as well as bacterized seeds germinated successfully in toluene free soil as control. The results forced for an alternative mode of application of bacteria for rhizoremediation purpose. Hence bacterial suspension was mixed with soil having 50 ppm of toluene. Germination index in DPT treated soil was 100% while in *P. putida* it was 50%. Untreated soil with toluene restricted the seeds to germinate.

Keywords Petroleum hydrocarbons ·
Pseudomonas putida · *Rhizobium leguminosarum* ·
Rhizoremediation · Toluene

Introduction

Petroleum hydrocarbons are common pollutants as a result of leaking underground storage tanks and spills, release of petroleum hydrocarbons in the environment from leaks of storage tanks pollutes the soil, and many petroleum hydrocarbons are soluble in water and bind strongly to soil. A major risk of petroleum hydrocarbon release is contamination of drinking water sources by the mobile petroleum hydrocarbons, which can migrate through the soil matrix [1–3]. Contamination with these compounds is difficult to remediate because these compounds are relatively soluble in water and can diffuse rapidly once introduced into an aquifer. Techniques for in situ bioremediation of these compounds are used to eliminate or reduce contamination levels in an aquifer. In the United States, toluene is ranked 27th among the top 50 chemical products by production volume, with 931 million gallons (3.5×10^9 l) of toluene manufactured in 1992 [4]. Industry uses toluene in refining gasoline, chemical manufacturing, manufacture of paints,

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lacquers and adhesives, and some printing and leather-tanning processes. Toluene is usually disposed as a used solvent. In a 1988 study, toluene was detected in ground-water, surface water, or soil at 29% of the hazardous waste sites surveyed, the average amounts detected were 0.2 μM in ground water, 0.1 μM in surface water and 77 $\mu\text{g kg}^{-1}$ in soil (US Public Health Service, 1989). Since toluene is ubiquitous in the environment, it is not surprising that micro-organisms capable of degrading toluene have been isolated from a variety of environments [5].

In many studies describing rhizoremediation, the degrading rhizosphere communities emerge and develop during plant growth in the studied microcosm or field. Very few studies report the directed introduction of a microbial strain for xenobiotics degrading activities on plant seed, which subsequently is able to efficiently colonize the root and sustain on the root system [6–8]. The present work was designed in which characteristics responsible for toluene degradation were transferred from a known toluene degrader into an established plant growth promoting rhizobacteria. The basic idea behind the work was to construct a microbial system, which not only have a toluene degrading ability but also flourish and spread in the soil using root injection system of the host plant and also enhance its growth.

Materials and Methods

Microorganisms

The bacterial strains used in the present study were *Pseudomonas putida* MTCC 979 and *Rhizobium leguminosarum* DPT. *Pseudomonas putida* MTCC 979 was obtained from MTCC, IMTECH, Chandigarh, while the DPT was isolated from *Cajanus cajan* root nodules by standard method [9]. *Pseudomonas putida* MTCC 979 was maintained on nutrient agar (peptone—0.5%, beef extract—0.3%, NaCl—0.5%, agar—1.5%, pH—7.0) slants and *R. leguminosarum* DPT was maintained on YEMA (yeast extract—0.1%, mannitol—1.0%, NaCl—0.1%, K_2HPO_4 —0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.02%, agar—2.0%, pH—6.8) slants. The bacterial cultures were stored at 4°C and revived after every 15 days. *Rhizobium leguminosarum* DPT was identified on the basis of 16S rDNA sequence analysis. 16S rDNA was amplified as described by Weisberg [10]. Searches in the EMBL/Gen bank/DDBJ/PDB data libraries were performed using BLAST. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 software.

Plant Growth Promoting Characteristics of DPT

The plant growth promoting activity of DPT was determined by accessing the factors, which directly or indirectly

benefits the plants [11]. ACC deaminase production [12], nitrogen fixation [13], phosphate solubilization [14], IAA production [15], siderophore production [16] and HCN production [17] were determined as per standard procedures.

Isolation of pWVO and Its Transformation in DPT

Plasmid DNA (pWVO) was isolated from *P. putida* MTCC 979 as described [18]. Plasmid DNA isolated was transformed in CaCl_2 treated, competent *R. leguminosarum* DPT cells by heat shock method. Transformation was done according to standard protocol [18]. Transformed strain of DPT, growing on minimal medium (ammonium nitrate—0.7%; K_2HPO_4 —0.1%; KH_2PO_4 —0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.05%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ —0.013%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —0.0013%; pH—6.8) and able to utilize toluene was picked (termed DPT^T) and stored for further studies. The morphological and biochemical characteristics of DPT and DPT^T were compared to check the possibility of contamination.

Screening for Biodegradation of Toluene

Toluene biodegradation was determined by transformed DPT and *P. putida* MTCC 979 [19, 20]. Toluene was used as sole source of carbon and energy, and provided in vapour phase to the organisms growing on minimal medium. The minimal medium was inoculated with respective bacterial strains in sterile conditions, and incubated at room temperature (27–30°C) in glass desiccator, which contained a beaker of toluene at the bottom. The toluene concentration in the gas or in vapour phase was regulated by toluene solution in the beaker, as the concentration of toluene dissolved in an aqueous solution in the beaker, was dependent on vapour phase of toluene. The preparations were incubated in desiccators. Opening of desiccators with equilibrated atmosphere was kept to a minimum. The toluene in beaker was replaced in order to maintain a constant vapour pressure of toluene. Uninoculated minimal medium was experimented in parallel as control.

Effect of DPT^T on seed germination of *Cajanus cajan* in presence of toluene

Cajanus cajan seeds were surface sterilized with 95% alcohol for 10–15 s followed by 0.1% HgCl_2 for 1–2 min. The seeds were washed with sterile distill water (DW) 5–6 times. The seeds were then dried overnight under sterile air stream. The *C. cajan* seeds were bacterized as described [21]. The organism was cultivated on YEM broth at $28 \pm 1^\circ\text{C}$ for 24 h and finally centrifuged at 8,000 rpm for 15 min 4°C. Supernatant was discarded and pellets were washed with DW and resuspended in DW to obtain a

population density of 1×10^7 cfu ml⁻¹. Bacterized, as well as non-bacterized seeds were placed on sterile 1% agar separately, and incubated at 28°C. Seed germination was noted after 48 h. For determination of emergence index, bacterized seeds were sown in small pots with sterile soil (160°C for 2 h twice in hot air oven), supplemented with 50 ppm of toluene. The pots were incubated at room temperature and irrigated routinely with sterile DW. Non-bacterized seeds sown in toluene treated served as negative control, while seeds sown in untreated soil served as positive control. Later, bacterial suspension (10^9 cfu ml⁻¹) was mixed with soil having 50 ppm of toluene and preincubated for overnight before sowing the non-bacterized seeds. Emergence index was measured after 2 weeks. Seedling weight of different treatments was also recorded. Three replications of each treatment were included in the study. The data were subjected to analysis of variance, and means compared using Duncan's new multiple range test.

Results

Plant Growth Promoting Characteristics of DPT

DPT was found to produce appreciable amount of IAA in tryptophan supplemented medium, maximum 31 µg ml⁻¹ of IAA was recorded after 48 h (Fig. 1). However, no IAA was released in medium without tryptophan. DPT formed clear zone around the colonies on Pikovskaya's agar, which confirmed its ability in solubilize inorganic phosphate. Quantitative determination of P solubilization revealed that maximum 6.0 mg ml⁻¹ of free P was released after 72 h (Fig. 1). The pattern of free P concentration in broth was random. Siderophore production was detected by observing the orange zone around the inoculated bacterial colonies after 2–3 days of incubation, on otherwise blue chrome-azuroil agar medium, indicating siderophore-mediated iron removal from the ternary complex CAS-Fe(III) HDTMA. DPT was able to grow on minimal medium

Fig. 1 Quantitative determination of IAA production (open triangle) and P solubilization (open circle) by *Rhizobium* sp. DPT at different time intervals. Error bars indicate standard error of the mean

having ACC as sole source of nitrogen. The ACC deaminase activity was estimated in intracellular fractions of DPT. 0.4 IU ml⁻¹ of enzyme was released after 48 h at incubation. Nitrogenase activity was recorded 3.14 n mol C₂H₂/min/mg protein *ex planta*. HCN and chitinase production was also positive. DPT was found to inhibit the growth of fungal phytopathogens—*Fusarium oxysporum* and *Macrophomina phaseolina*. The results are given in Table 1.

Transformation of pWVO in *Rhizobium leguminosarum* DPT

Extransformants were grown on plates with toluene as sole source of carbon. Colonies growing on the medium were presumed to carry pWVO. One large colony was selected for further studies and named as DPT^T. *Rhizobium*

Table 1 Plant growth promoting characteristics of bacteria

Strains	Phosphate solubilization	N ₂ fixation	IAA (µg ml ⁻¹)	Siderophore	HCN	Chitinase	ACC-deaminase (IU ml ⁻¹)	Antagonism against		
								MP	FO	RS
DPT	+++	+	31	++	+	++	0.4	+	+	-
<i>P. putida</i>	-	-	-	+	-	-	-	-	-	-
Method ^a	Subbarao [13]	Pagan et al. [12]	Gupta et al. [14]	Schwyn and Neilands [15]	Gupta et al. [14]	Cattelan et al. [32]	Honma and Shimomura [11]	Skidmore and Dickinson [33]		

IAA Indole-3-acetic acid, ACC I Amino-cyclopropane-1-carboxylate, MP *M. phaseolina*, FO *Fusarium oxysporum*, RS *Rhizoctonia solani*

^a References for methods adapted

leguminosarum DPT^T was similar to wild type *R. leguminosarum* DPT in its biochemical and morphological properties except that it was able to utilize toluene unlike DPT. The frequency of transformation was recorded 5.7×10^{-6} .

Seed Germination

50 ppm toluene in soil was found to inhibit the germination of *C. cajan* seeds. However when the seeds were bacterized with toluene degrading *P. putida* and *R. leguminosarum* sp. DPT were sown in pots, again no germination was observed. Non-bacterized as well as bacterized seeds germinated successfully in toluene free soil as control. These results compel us to find an alternative method for application of bacteria for rhizoremediation purpose. Hence bacterial suspension (10^9 cfu ml⁻¹) was mixed with soil having 50 ppm of toluene and preincubated for overnight before sowing the seeds. Surprisingly, germination index DPT treated soil was 100% while in *P. putida* it was 50%. The seedling weight was significantly high for the seeds inoculated with DPT^T as compared to positive control. Untreated soil with toluene restricted the seeds to germinate (Table 2).

Discussion

Emerging phytoremediation technologies have been applied at various scales to treat moderately hydrophobic pollutants, such as benzene, toluene, ethylbenzene and xylene (BTEX) compounds, chlorinated solvents, nitrotoluene, ammunition wastes and excess nutrients. Phytoremediation of organic xenobiotics is based on interactions between plants and their associated microorganisms in a process whereby plants draw pollutants, including polyaromatic hydrocarbons, into their rhizosphere via the transpiration stream, subsequently, microorganisms mediated degradation occurs in plant itself, in the rhizosphere or in both [22].

Table 2 Effect of different treatments on seed germination of pigeon pea

Soil treatment	Germination index (%)	Emergence index (%)	Seedling weight (g)
DPT ^{Ta}	100	100	0.75 s
<i>P. putida</i> ^a	50	25	0.41 ns
Positive control ^b	100	80	0.55
Negative control ^a	00	00	Nil

s-Value significantly different as compared to control ($P < 0.05$) using Duncan's multiple range test, ns not significant

^a With toluene

^b Without toluene

The idea behind the present work was to develop a bacterial strain having dual property of plant growth promotion and toluene bioremediation, merge together in a single organism. The strain such developed is supposed to have great use as this may serve the purpose of eco-restoration and biofertilization simultaneously. The work originated from the background of rhizoremediation as reviewed [6, 8]. *Rhizobium* sp. DPT was found to have several characteristics known to enhance the plant growth. It produced appreciable amount of IAA in broth. IAA is one of the most active physiological auxin, which can stimulate plant growth. Several workers have reported production of plant hormones by *Rhizobium* sp. [23, 24] and have considered their physiological action in altering plant growth and development. The phosphate (P) content in average soil is about 0.05% (w/w) but only 0.1% of the total is available to plants [25]. The phosphorus fertilizers applied regularly are rapidly immobilized to unavailable forms and thus account for low available phosphorus status. Thus, even if the total phosphorus is high and also if P fertilizers are applied regularly, the phosphorus is rapidly fixed to unavailable forms and account for low phosphorus use efficiency. DPT solubilized appreciable amount of inorganic P in vitro. In fact, *R. leguminosarum* with dual function of symbiotic N₂ fixation and superior phosphate solubilizing ability has been reported previously [26].

DPT also produced ACC deaminase. ACC deaminase affects the plant growth by hydrolyzing ACC, which is the immediate biosynthetic precursor of ethylene in plants and thereby lower the level of ethylene in a developing or stressed plant [27]. ACC deaminase activity has been observed earlier in *R. leguminosarum* bv. *viciae* and *Rhizobium hedysari* [28]. Also, the ACC deaminase produced by *R. leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* has been reported to promote nodulation in pea and alfa alfa plants [29, 30]. DPT was found to produce siderophore, HCN and chitinases, fixed nitrogen, and moreover, it inhibited the growth of *M. phaseolina* and *F. oxysporum*. All of these characteristics made *R. leguminosarum* DPT a suitable candidate for plant growth promotion as suggested [31, 32].

pWWO plasmid having the genes responsible for toluene degradation was successfully transferred in DPT. Earlier also, several workers have transformed toluene or other hydrocarbon degrading genes [33–36] in suitable target organisms. In an unrelated report, Fellay et al. [37] verified the properties of omega transposon to mutagenize a broad-host range plasmid which contains the entire metaclevage pathway of the toluene degradation plasmid pWWO of *P. putida*. They showed that when the plasmid containing an omega transposon was transferred by conjugal mobilization from *E. coli* to *R. leguminosarum* or in few other bacteria, the appropriate interposon drug resistance was expressed.

Unfortunately, the seeds treated with transformed variant failed to germinate in the presence of toluene, in contrast to control. It is known that when a suitable rhizosphere strain is introduced together with a suitable plant e.g. by coating bacteria on plant seed these well equipped bacteria settle on the root together with the indigenous population, thereby enhancing the bioremediation process. In addition, such efficiently root colonizing, pollutant-degrading bacteria then profit from the growing root system because this act as an injection system to spread the bacteria through the soil [6]. However, the bacteria coated seeds failed to survive. Instead when transformed bacteria were applied directly in the soil, the seeds germinated successfully. There was 36.3% increase in seedling weight, with seeds treated with DPT^T as compared to control. This was in accordance to the findings of PGP characteristics of DPT. The results clearly indicated the importance of method adapted for delivery of microbial agent in soil for getting optimum effect.

Conclusively, construction of engineered DPT^T invites great interest from environmental, industrial and ecological background and the technique holds high potential for ecorestoration and sustainable agriculture, if adopted. Further research on optimization of process, scale up and mode of application is required and recommended.

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