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Biodegradation of Pentachlorophenol by Endophytic Bacteria Isolated from PCP-Tolerant Plant Species

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Pentachlorophenol (PCP) is widely used as a pesticide and a wood preservative. It is highly persistent in soils and is lethal to wide variety of beneficial microorganisms, insects and animals. The present investigation was carried out with the aim of developing an endophytic microbial system for bioremediation of Pentachlorophenol (PCP) polluted soils. A number of plant species were evaluated for their ability to tolerate pentachlorophenol in the soil spiked with different concentrations under pot culture conditions. The toxic effect of pentachlorophenol on plants was studied by monitoring seed germination, plant growth and biomass. In general, pentachlorophenol significantly inhibited the seed germination and growth of all plants at 25 μg ml^{-1}. As the concentration was increased to 50 μg ml^{-1}, there was further inhibitory influence on seed germination and plant growth. Although PCP had a negative effect on all the plant species tested, however, maize and groundnut showed maximum tolerance to PCP. Other tolerating plants included wheat, safflower, sunflower, and soybean. From the PCP tolerating plant species, bacterial endophytes from roots and leaves were isolated and screened for their PCP degradation potentials. It was interesting to note that most of the isolates degraded PCP. The bacterial isolate, GRN (root) 4 was found to be the most efficient PCP degrader, which degraded 94.12 % of PCP as analyzed through HPLC, and holds promise in bioremediation of PCP polluted soils.

Key words: Pentachlorophenol, endophytes, degradation, bioremediation, HPLC.

Pentachlorophenol (PCP) is applied to crops as a herbicide, and also used as an insecticide, fungicide, algicide and a disinfectant. It also finds use as a wood preservative, to inhibit molds and wood boring insects. It is present in tannery effluents and also formed unintentionally in effluents of paper and pulp industries. It is highly persistent in soils and is lethal to wide variety of beneficial microorganisms and insects, human beings and animals. Due to continuous use of PCP as a pesticide, its appreciable quantities may accumulate in the soil ecosystem. The natural processes that breakdown toxic chemicals in the environment have become the focus of much attention to develop safe and environment-friendly deactivation technologies. Microorganisms and plants are among the most important biological agents that remove and degrade waste materials to enable their recycling in the environment. There has been a significant amount of work done concerning bacteria in the rhizosphere, but little is known about endophytic bacteria residing within roots.

Endophytes are defined as microorganisms living within tissues of plants

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without causing substantive harm. Little is known about the contribution of endophytes in the degradation of contaminants. Much of what is known about microbial communities in the rhizosphere may perhaps be applicable to endophytic communities as well. The root surface and rhizosphere soil support microbial communities, presumed to be under strong selective pressure that originates from the plant (Hallmann *et al*., 1997). The microbial community structure in the rhizosphere varies with plant species and environmental stresses, such as organic contamination (Joner *et al*., 2001; Priha *et al*., 2001). If endophytic bacteria are under a pressure similar to those in the rhizosphere, they may also vary with plant species and respond to external stresses. Like rhizosphere microorganisms, endophytes may also be capable of degrading organic contaminants. The study examines the impact of PCP on the germination and growth of different crops at different concentrations of PCP and isolation of PCP degrading endophytic bacteria from the roots and leaves. The ability of the isolated endophytes to degrade PCP was also assessed.

MATERIALS AND METHODS

Screening of Plant Species to Tolerate PCP

Different plant species *viz*., Green gram, Black gram, Soybean, Sunflower, Safflower, Bengal gram, Maize, Ground nut, Wheat and Horse gram were tested for tolerance to different concentrations of PCP *ie*., 25 µg ml^{-1} and 50 µg ml^{-1}. Sieved black soil was filled into plastic pots (7 cm dia X 10 cm height) and spiked with PCP @25 µg ml^{-1} and 50 µg ml^{-1} as per the treatment schedule. The spiked soil was kept ventilated for 24 hours to let the methanol to vaporize and the seeds were sown in these pots. The pots were maintained in a green house and monitored regularly. At 30 days after sowing, effect of PCP on seed germination, root and shoot length and biomass were measured and tabulated.

Isolation of Endophytic Bacteria

Root samples of PCP tolerant plant species were collected and the predominant endorhizosphere bacteria were isolated and purified as per the method of Watanabe and Barraquio (1979). Similarly, the endophytic bacteria were isolated from the leaves of PCP tolerant plant species.

Growth of the Endophytic Bacterial Isolates on PCP

All the endophytic bacterial isolates were point inoculated on mineral salts medium (media composition in grams per liter: $NaNo_3$, 0.5: K_2HPO_4, 0.7: KH_2PO_4, 0.2: $MgSO_4$, 0.5: Agar, 20) containing PCP @ 25 µg ml^{-1} and BTB was added to the medium (0.5% in ethanol) as the pH indicator. The plates were incubated at 37^0 C for 6 days and checked for their growth. And, the isolates were initially tested for their growth on PCP at 25 µg ml^{-1} and later on at 50 µg ml^{-1}.

Assessing Biodegradation Potentials of the Endophytic Isolates

The endophytic isolates were inoculated in to mineral salts broth medium containing PCP @ 25 µg ml^{-1} and incubated on a shaker at 30º C. At regular intervals, the samples were drawn and measurements of PCP remaining in the broth medium were carried out using a HPLC (Yan He *et al*., 2005) and the per cent PCP degraded computed.

HPLC Analyses of PCP Degradation

For HPLC analyses of the aliquots, the culture samples were centrifuged at 6,000 rpm for 15 min. One ml of the suspension was filtered through cellulose nitrate membrane (0.45 µm) using a syringe filter. The HPLC analysis was done using the Waters (2487) HPLC System, fitted with a Symmetry C_{18} column (0.5 µm, 4.5 x250mm). The mobile phase used was methanol: 1% acetic acid (90:10 v/v); flow rate, 1.00 ml /min; and detector, UV at 250 nm. The readings were integrated by the Empower Software System. The PCP concentrations were calculated on the basis of peak area measurements by comparison with an external standard of known concentration of PCP prepared in methanol. All analyses were carried out using two replications.

RESULTS AND DISCUSSIONS

Effect of PCP on Seed Germination and Growth Parameters

The effect of PCP on different plants was assessed. In general, PCP significantly affected the seed germination and growth of all plants even at 25 µg ml^{-1}. As the concentration was increased to 50 µg ml^{-1}, there was further inhibitory influence on seed germination and plant growth indicating its phytotoxic effects (Figs. 1-3). Similarly, Pivetz

et al. (1997) observed PCP (at 400 mg kg^{-1}) strongly inhibiting seed germination and growth of eight species of grasses. Dams *et al*. (2007) showed that PCP had a significantly adverse effect on the growth of winter wheat when measured by the plant weight. Although PCP had negative effect on all the plant species tested, maize and groundnut showed maximum tolerance to PCP both at 25 and 50 µg ml^{-1}. Other tolerating species included wheat, safflower, sunflower, and soybean.

Endophytic Bacterial Isolates from PCP Tolerant Plants

Since in the present investigation, many plants showed tolerance, it is likely that the microorganisms harboring in the interior of plant species may be degrading PCP and reducing the phytotoxicity of PCP. Hence, predominant PCP degrading endophytic bacteria were isolated from roots and leaves of PCP tolerant plant species. They were streaked on mineral salts medium

Table 1. Isolation of Endophytic bacteria from PCP tolerating plants

Crops	Code No. of the Isolate (Leaf)	Growth on PCP (25ppm) (Leaf)	Code No. of the Isolate (Root)	Growth on PCP (25ppm) (Root)
Soybean	SOY(leaf)1	+	SOY(root)1	+
	SOY(leaf)2	+	SOY(root)2	+
Sunflower	-		SUN(root)1	++
	-		SUN(root)2	+
Safflower	SAFF(leaf)1	++	SAFF(root)1	++
			SAFF(root)2	+++
			SAFF(root)3	+++
			SAFF(root)4	+
Groundnut	GRN(leaf)1	++	GRN(root)1	+
			GRN(root)2	++
			GRN(root)3	+
			GRN(root)4	++
Wheat	WHT(leaf)1	+	WHT(root)1	++

Table 2. PCP biodegradation potentials of native endophytic bacterial isolates from PCP tolerant plants

Sl. No	Sources	Code No. of the Isolates	Percentage of PCP degradation	
			10 DAI	20 DAI
1	Soybean root	SOY(root)1	22.09	77.56
2		SOY(root)2	32.14	86.16
3	Safflower root	SAF(root)1	25.14	82.80
4		SAF(root)2	31.16	85.78
5		SAF(root)3	30.58	86.06
6		SAF(root)4	38.42	74.94
7	Sunflower root	SUN(root)1	21.86	81.10
8		SUN(root)2	37.26	73.12
9		SUN(root)3	32.14	81.74
10	Groundnut root	GRN(root)1	32.84	77.48
11		GRN(root)2	70.62	89.28
12		GRN(root)3	61.78	94.08
13		GRN(root)4	64.58	94.12
14	Wheat root	WHT(root)1	28.38	83.52
		SEM±	4.88	2.81
		CD@1 %	20.55	11.82

Note: DAI – Days after inoculation

containing PCP (25 µg ml^{-1}) as the sole carbon source. As many as 18 endophytic bacteria were isolated from roots and leaves of different plant species (Table 1). From soybean, 2 each from leaves and roots; 2 from roots and 1 from leaves of sunflower; 4 from roots of safflower; 1 from leaves and 4 from roots of groundnut and one each from leaves and roots of wheat. The results, here, suggest that the endophytic community composition, size and response to contamination, depend on plant species. Brannock (2004) isolated a total of 345 endophytes from PAH spiked soil planted with clover (*Trifolium pratense*), milkweed (*Asclepias syriaca*) and primrose *(Oenothera biennis)*.

The isolates produced yellow colored colonies on a mineral salts medium containing PCP and Bromothymol blue. Thakur *et al*. (2000) also used similar technique to screen and enrich PCP degrading bacteria in a chemostat by continuous enrichment. Cells utilized PCP and the chloride produced by the isolates decreased the pH. The decrease in the pH was indicated by the change in the color of the indicator dye from blue to yellow.

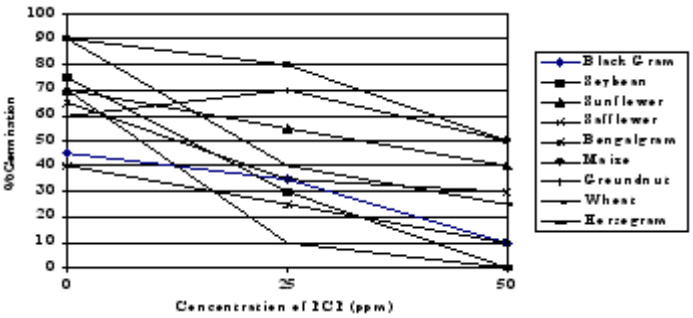

Fig 1. Effect of different concentrations of PCP on Seed germination of different plants

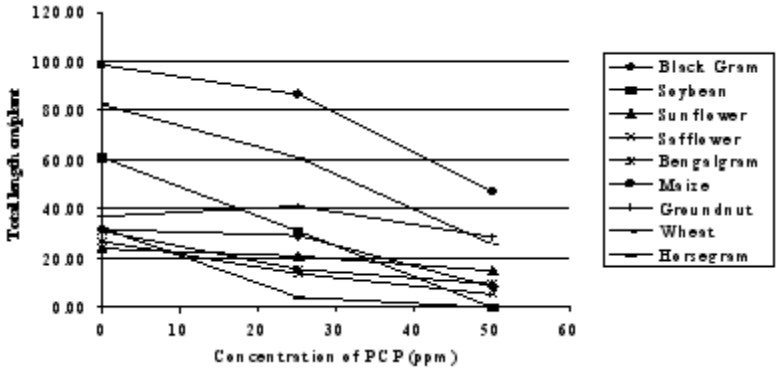

Fig 2. Effect of different concentrations of PCP on plant height of different plants

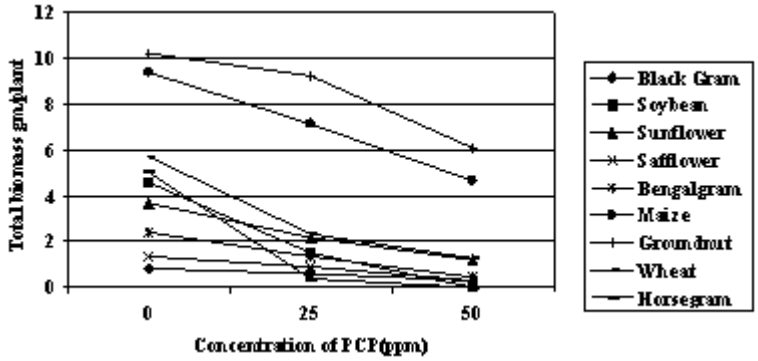

Fig 3. Effect of different concentrations of PCP on total fresh biomass of different plants

Scanty literature is available on the role of endophytes in environmental bioremediation. Much of what is known about microbial communities in the rhizosphere may perhaps be applicable to endophytic communities as well. The microbial community structure in the rhizosphere varies with plant species and the environmental stresses, such as organic contamination (Germida *et al*., 1998). If endophytic bacteria are also under a pressure similar to those in the rhizosphere, they may also vary with plant species and respond to external stresses. It seems possible that endophytes may "protect" plants from contaminants in the soil. Harboring endophytes may be expensive for the plant, but in contaminated systems, the benefits may outweigh these costs if they enhance plant growth (which would have otherwise been inhibited by soil contamination) or detoxify organic contaminants within plant tissues.

Assessing Biodegradation Potentials of the Isolates

The biodegradation efficiency of the endophytic isolates was evaluated. At periodical intervals, measurements of PCP concentration remaining in the mineral salts broth medium was carried out using HPLC. Most of the isolates degraded PCP by 70 to 90 per cent after 20 DAI (Table 2). The HPLC profile of the study indicated maximum degradation of PCP by the isolates, GRN (root)4 and GRN (root)3 *ie*., 94.12 and 94.08 per cent degradation respectively (Fig. 4). The next promising isolates were, GRN (root)2 and SOY(root)2 which degraded PCP by 89.28 and 86.16 per cent respectively (Table 2). These endophytic potent isolates hold promise in bioremediation of PCP polluted soils. Barac *et al.* (2004) evaluated lupine seeds inoculated with the engineered toluene degrading endophytic bacteria and observed a marked decrease in its phytotoxicity, and a 50"70 per cent reduction of its evapotranspiration through the leaves. Siciliano *et al*. (2001) reported that bacteria degrading recalcitrant compounds were more abundant among endophytic populations than in the rhizosphere of plants in contaminated sites, which could mean that endophytes have a role in metabolizing these substances. Endophytes may directly detoxify contaminants or protect plants from toxicity.

Thus, the results have clearly indicated that maize, soybean and groundnut plants exhibited maximum tolerance to Pentachlorophenol up to 50 µg ml^{-1}. Most of the endophytic bacterial isolates from the tolerant plant species degraded PCP by 70 to 90 per cent in 20 days. The isolate, GRN (root) 4 was found to be the most potent PCP degrader with 94.12 per cent degradation, and holds promise in bioremediation of PCP polluted soils.

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