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Biodegradation of PCP by the Rhizobacteria Isolated from Pentachlorophenol-tolerant Crop Species

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***Abstract*—**Pentachlorophenol (PCP) is a polychlorinated aromatic compound that is widespread in industrial effluents and is considered to be a serious pollutant. Among the variety of industrial effluents encountered, effluents from tanning industry are very important and have a serious pollution potential. PCP is also formed unintentionally in effluents of paper and pulp industries. It is highly persistent in soils and is lethal to a wide variety of beneficial microorganisms and insects, human beings and animals. 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. Microbes and plants are among the most important biological agents that remove and degrade waste materials to enable their recycling in the environment. The present investigation was carried out with the aim of developing a microbial system for bioremediation of PCP polluted soils. A number of plant species were evaluated for their ability to tolerate different concentrations of pentachlorophenol (PCP) in the soil. The experiment was conducted for 30 days under pot culture conditions. The toxic effect of PCP on plants was studied by monitoring seed germination, plant growth and biomass. As the concentration of PCP was increased to 50 ppm, the inhibition of seed germination, plant growth and biomass was also increased. Although PCP had a negative effect on all plant species tested, maize and groundnut showed the maximum tolerance to PCP. Other tolerating crops included wheat, safflower, sunflower, and soybean. From the rhizosphere soil of the tolerant seedlings, as many as twenty seven PCP tolerant bacteria were isolated. From soybean, 8; sunflower, 3; safflower 8; maize 2; groundnut and wheat, 3 each isolates were made. They were screened for their PCP degradation potentials. HPLC analyses of PCP degradation revealed that the isolate MAZ-2 degraded PCP completely. The isolate MAZ-1 was the next best isolate with 90 per cent PCP degradation. These strains hold promise to be used in the bioremediation of PCP polluted soils.

***Keywords*—**Biodegradation, pentachlorophenol, rhizobacteria.

I. Introduction

PENTACHLOROPHENOL (PCP) is a widely used biocide. It is applied to crops as a herbicide. It is also used as an insecticide, fungicide, an algicide and a disinfectant. PCP is particularly widely used 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 pesticides, appreciable quantities of pesticides and their degraded products may accumulate in the soil ecosystem. Microbes and plants are among the most important biological agents that remove and degrade waste materials to enable their recycling in the environment. Aerobic degradation of polychlorinated phenols have been studied extensively in the past (1). Several strains of bacteria that are able to completely mineralize polychlorinated phenols have been described and applied to bioremediate many PCP contaminated sites (2). The present investigation was carried out with the aim of developing a microbial system for bioremediation of PCP polluted soils.

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II. Materials And Methods

A. Screening of Plant Species to Tolerate PCP

Ten different plant species viz., Green gram, Black gram, Soybean, Sunflower, Safflower, Bengal gram, Maize, Ground nut, Wheat and Horse gram were tested for their tolerance to different concentrations of Pentachlorophenol (PCP) ie., 0 ppm, 25 ppm, 50 ppm. About 200gm sieved black soil was filled into plastic pots (7 cm dia X 10 cm height).Pots were spiked with PCP @25 ppm and 50 ppm as per the treatment schedule. The spiked soil was kept ventilated for 24 hours to let the methanol vaporize. Later, the seeds were sown @ 4 seeds per pot. The pots were maintained in a green house and their growth monitored.

B. Plant Growth Parameters

At 30 days after sowing, effect of PCP on seed germination, root and shoot length and biomass were measured and tabulated.

C. Soil Sampling and Isolation of Predominant Bacteria

Soil samples were collected from the rhizosphere of plants grown in the PCP polluted soils as per the treatments and was stored at 4^0 C till further use. They were appropriately diluted and plated out on nutrient agar plates. After 48 h of incubation at 37^0 C, the predominant bacteria were picked up and purified.

D. Growth of the Bacterial Isolates on PCP

All the 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) containing PCP @ 25 ppm and incubated at 37^0 C for 4-6 days and checked for their growth. BTB was added to the medium (0.5% in

ethanol) as the pH indicator. And, the isolates were initially tested for their growth on PCP at 25 ppm and later on 50 ppm.

E. Assessing Biodegradation Potentials of the Isolates

The isolates were inoculated in to mineral salts medium containing PCP @ 25 ppm and incubated on a shaker at 30º C. At regular intervals, the samples were drawn and measurements of PCP remaining in the medium were carried out using HPLC [3] and the per cent PCP degraded computed.

F. 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 C18 column (0.5 µm, 4.5 x250mm). 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.

III. Results And Discussion

A. Effect of PCP on Seed Germination and Growth Parameters

As a preliminary experiment, soil was spiked with different concentrations of PCP to assess the adverse effects of pentachlorophenol (PCP) on seed germination and shoot, root and total length and root and shoot biomass as well. Table I shows the effect of PCP on seed germination and relative lengths of shoot and root at 30 DAS. In general, PCP significantly affected the seed germination and growth of all plants even at 25 ppm. As the concentration was increased to 50 ppm, there was further inhibitory influence on seed germination and plant growth (Fig. 1).

Although PCP had negative effect on all plant species tested, maize and groundnut showed maximum tolerance to PCP at 25 ppm compared to 50 ppm. There was no significant reduction in seed germination of maize due to PCP at 25 ppm. The total plant height of maize, groundnut, black gram and sunflower plants were unaffected by PCP at 25 ppm (Fig. 2), indicating that most of PCP was degraded, in the rhizosphere, rapidly enough to protect the plants from its phytotoxicity. Although seed germination was significantly affected at 50 ppm, in terms of height, these plants showed tolerance to PCP. Other tolerating species included wheat, safflower and soybean. Similarly, it was experimented on hycrest crested wheatgrass (Agropyron desertorum) and it tolerated PCP up to 100 ppm [4].

The effects of different PCP concentrations on plant biomass are furnished in Table 2. It was observed that PCP posed inhibitory effects on shoot and root biomass on most of the plant species tested. A significant (P> 0.01) shoot and root growth inhibition was seen at 50 ppm PCP in all the plant species tested. However, it was revealed that maize and groundnut were significantly different (P> 0.01) as compared to other seedlings. In Black gram, Soybean, Sunflower, Safflower, Bengal gram and Horse gram, fresh root biomass showed no significant (P> 0.01) differences at 0, 25 and 50 ppm of PCP. As PCP level was increased, total biomass production in all species dropped significantly (Fig. 3). The earlier work [5] illustrated the influence of PCP and its co-contaminants such as Cr, As, Cu and B on plant growth. It was showed that as PCP levels were increased, total biomass production in both poplar and willow dropped significantly.

TABLE I
EFFECT OF PCP ON SEED GERMINATION, SHOOT LENGTH, ROOT LENGTH AND TOTAL LENGTH OF DIFFERENT PLANT SPECIES

Crops	Treatments	% Germination	Shoot length (cm/plant)	Root length (cm/plant)	Total length (cm/plant)
	0 ppm	44.98 d-h	4.12 g-k	26.00 a-e	31.68 c-g
Black gram	25 ppm	34.99 f-i	4.76 g-k	8.50 g-l	29.10 c-g
	50 ppm	10.00 ij	3.6 h-k	4.93 j-l	8.53 g-i
	0 ppm	74.97 a-c	12.18 c-e	39.22 a	60.83 b
Soybean	25ppm	29.99 g-i	8.00 e-i	12.17 e-l	31.33 c-g
	50 ppm	0.00 j	0.00 k	0.00 l	0.00 i
	0 ppm	69.97 a-d	9.67 d-g	7.00 i-l	23.86 d-h
Sunflower	25 ppm	54.98 b-g	8.00 e-i	6.39 j-l	20.72 d-i
	50 ppm	39.98 e-h	7.63 e-i	4.89 h-l	15.00 e-i
	0 ppm	64.97 a-e	8.56 e-h	11.12 f-l	26.71 c-h
Safflower	25 ppm	34.99 f-i	5.63 f-j	8.10 g-l	13.73 e-i
	50 ppm	29.99 g-i	2.67 i-k	2.13 kl	5.10 hi
	0 ppm	39.98 e-h	14.08 cd	13.33 e-l	30.83 c-g
Bengal gram	25 ppm	24.99 h-j	8.00 e-i	6.50 i-l	14.50 e-i
	50 ppm	10.00 ij	4.67 g-k	4.67 k-l	9.33 f-i
	0 ppm	89.96 a	17 abc	39.00 a	98.33 a
Maize	25 ppm	79.97 ab	16.94 a-c	34.72 ab	86.67 a
	50 ppm	49.98 c-h	16.22 bc	31.19 ac	47.42 bc
	0 ppm	89.96 a	7.08 e-j	20.25 c-i	36.75 c-e
Groundnut	25ppm	59.98 b-f	7.06 e-j	18.92 c-j	41.48 b-d
	50 ppm	49.98 c-h	7.00 e-j	21.78 b-g	28.78 c-g
	0 ppm	69.97 a-d	21.83 a	24.08 b-f	82.17 a
Wheat	25 ppm	39.98 e-h	19.33 ab	29.00 a-d	61.17 b
	50 ppm	24.99 h-j	10.5 d-f	20.92 c-h	25.61 c-h
	0 ppm	69.97 a-d	5.58 f-j	15.42 d-k	32.33 c-f
Horse gram	25 ppm	10.00 ij	1.73 jk	2.67 kl	4.40 hi
	50 ppm	0.00 j	0.00 k	0.00 l	0.00 i

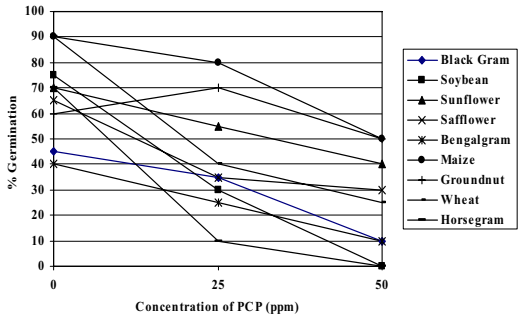

Fig. 1 Effect of different concentrations of PCP on Seed germination

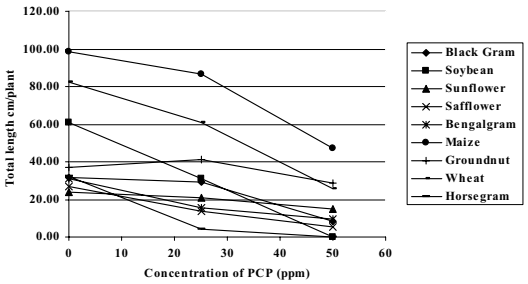

Fig. 2. Effect of different concentrations of PCP on total plant height

PCP had a significantly adverse effect on the growth of winter wheat when measured by the plant weight [6]. And, measuring plant weight was a physiological indicator of plant growth. PCP is a phyotoxic compound and known to uncouple oxidative phosphorylation by making cell membranes permeable to protons, resulting in dissipation of transmembrane pH gradients and electrical potentials [7].

B. PCP Tolerant BacterialIsolates

Since in the present investigation, many plants showed tolerance, it is likely that the microorganisms harboring the rhizosphere may be degrading PCP and reducing the phytotoxicity of PCP.

Hence, a number of predominant bacteria were isolated from rhizosphere soils of PCP tolerant plant species. They were streaked on mineral salts medium containing PCP (25ppm) as the sole carbon source. In all, 27 isolates showed growth on PCP (Table 3). From soybean, 8; sunflower, 3; safflower 8; maize 2; groundnut and wheat, 3 each. A bacterial strain, KC- 3, from industrial waste-water capable of mineralizing PCP has been isolated [8]. Two isolates with the ability to mineralize PCP were isolated [9], of which one was from the sludge of an aerated lagoon treating pulp and paper effluents, while the other was from the soil collected from a sawmill timber yard. Similarly, 10 indigenous PCP degrading bacteria from enriched soil samples have been characterized by plating out on a mineral salts medium containing 40 ppm PCP [3].

TABLE II
EFFECT OF PCP ON SHOOT AND ROOT BIOMASS OF DIFFERENT PLANT SPECIES

Crops	Treatments	Fresh Biomass (g/plant) Shoot biomass	Root biomass	Total biomass
Black gram	0 ppm	0.47 kl	0.37 f	0.83 fg
	25 ppm	0.40 kl	0.17 f	0.57 fg
	50 ppm	0.20 kl	0.13 f	0.33 fg
Soybean	0 ppm	2.97 c-f	0.63 ef	4.60 cd
	25ppm	1.30 h-k	0.17 f	1.47 fg
	50 ppm	0.00 l	0.00 f	0.00 g
Sunflower	0 ppm	2.67 d-g	1.03 ef	3.70 de
	25 ppm	1.73 g-j	0.43 f	2.17 ef
	50 ppm	0.93 j-l	0.27 f	1.20 fg
Safflower	0 ppm	1.03 i-l	0.30 f	1.33 fg
	25 ppm	0.67 j-l	0.20 f	0.87 fg
	50 ppm	0.20 kl	0.10 f	0.30 fg
Bengal gram	0 ppm	2.07 f-i	0.30 f	2.37 ef
	25 ppm	0.93 j-l	0.40 f	1.33 fg
	50 ppm	0.33 kl	0.13 f	0.47 fg
Maize	0 ppm	3.57 cd	5.83 a	9.40 a
	25 ppm	3.13 c-e	3.97 b	7.10 b
	50 ppm	2.33 e-h	2.33 cd	4.67 cd
Groundnut	0 ppm	7.23 a	3.00 bc	10.23 a
	25ppm	5.90 b	3.33 bc	9.23 a
	50 ppm	3.77 c	2.33 cd	6.10 bc
Wheat	0 ppm	1.70 g-j	4.00 b	5.70 bc
	25 ppm	0.63 j-l	1.67 de	2.30 ef
	50 ppm	0.27 kl	1.00 ef	1.27 fg
Horse gram	0 ppm	1.67 g-j	3.33 bc	5.00 cd
	25 ppm	0.07 l	0.33 f	0.40 fg
	50 ppm	0.00 l	0.00 f	0.00 g

Values are the means of three replications ±SE. Variants possessing the same letters (a-j) are not significantly different at $P < 0.01$.

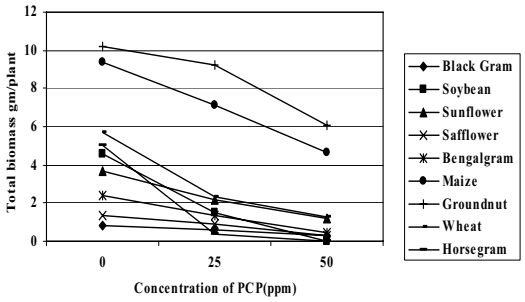

Fig. 3 Effect of different concentrations of PCP on total fresh biomass

The isolates produced yellow colored colonies on a mineral salt medium containing PCP and Bromothymol blue. As many as 85 strains, isolated from PCP contaminated soils from three geographic areas of Minnesota, were tested for their ability to degrade PCP in liquid cultures containing PCP and bromothymol blue [10]. Similar technique was used to screen and enrich PCP degrading bacteria in a chemostat by continuous enrichment [11]. Cells utilized PCP and the chloride produced by the isolate decreased the pH. The decrease in the pH was indicated by the change in the color of the indicator dye from blue to yellow.

C. Assessing Biodegradation Potentials of the Isolates

The biodegradation efficiency of these isolates was evaluated. At periodical intervals of 10, 20 and 30 days,

measurements of PCP concentrations remaining in the medium were carried out using HPLC analyses. Most of the

TABLE III
ISOLATION OF PCP TOLERATING BACTERIA FROM THE RHIZOSPHERE SOIL OF PCP TOLERATING PLANTS

Plant	No. of PCP tolerant bacteria isolated	Code No of the Isolate	Growth on PCP (25ppm)	Code of the Isolate	Growth on PCP (50ppm)
Soybean (SOY)	8	ASOY1	++	BSOY1	+
		ASOY2	++	BSOY2	+
		ASOY3	+++	BSOY3	++
		ASOY4	++	BSOY4	+
		ASOY5	++	BSOY5	+
		ASOY6	++	BSOY6	+
		ASOY7	++	BSOY7	+++
		ASOY8	++	BSOY8	+
Sunflower (SUN)	3	ASUN1	+++	BSUN1	+
		ASUN2	+++	BSUN2	++
		ASUN3	+++	BSUN3	++
Safflower (SAFF)	8	ASAFF1	+++	BSAFF1	++
		ASAFF2	+++	BSAFF2	+
		ASAFF3	++	BSAFF3	+
		ASAFF4	+++	BSAFF4	++
		ASAFF5	++	BSAFF5	+
		ASAFF6	+++	BSAFF6	+
		ASAFF7	++	BSAFF7	+
		ASAFF8	+++	BSAFF8	++
Groundnut (GRN)	3	AGRN1	+	BGRN1	+
		AGRN2	++	BGRN2	+
		AGRN3	+	BGRN3	+
Maize (MAZ)	2	AMAZ1	++	BMAZ1	+
		AMAZ2	+	BMAZ2	++
Wheat (WHT)	3	AWHT1	++	BWHT1	+
		AWHT2	++	BWHT2	--
		AWHT3	+	BWHT3	--

Note: A-25ppm PCP B-50ppm PCP

TABLE IV
PCP BIODEGRADATION POTENTIALS OF NATIVE RHIZOSPHERE BACTERIAL ISOLATES FROM PCP TOLERANT PLANT SPECIES

Source	Code No. of the Isolate	Degradation of PCP (%)		
		10 DAI	20 DAI	30 DAI
Rhizosphere soil of Soybean	SOY1	52.4	87.2	91.4
	SOY2	45.3	63.0	72.0
	SOY3	48.0	62.3	89.0
	SOY4	32.4	58.0	88.0
	SOY5	34.0	52.0	80.4
	SOY6	42.5	68.2	92.4
	SOY7	45.2	76.0	91.2
	SOY8	52.4	72.0	90.7
Rhizosphere soil of Sunflower	SUN1	38.0	57.2	92.0
	SUN2	60.2	76.8	88.8
	SUN3	69.0	88.0	90.0
Rhizosphere soil of Safflower	SAFF1	61.0	78.0	80.8
	SAFF2	58.6	71.3	83.3
	SAFF3	63.5	70.8	87.1
	SAFF4	56.3	81.2	87.6
	SAFF5	62.6	79.1	88.8
	SAFF6	59.2	75.6	83.2
	SAFF7	54.9	80.8	85.2
	SAFF8	62.1	72.8	88.0
Rhizosphere soil of Groundnut	GRN1	41.8	62.8	88.8
	GRN2	28.0	81.2	88.0
	GRN3	66.0	79.2	87.6
Rhizosphere soil of Maize	MAZ1	57.8	86.0	97.2
	MAZ2	80.0	85.6	100.0
Rhizosphere soil of Wheat	WHT1	67.0	85.0	97.6
	WHT2	40.0	62.1	76.4
	WHT3	59.0	68.6	74.2

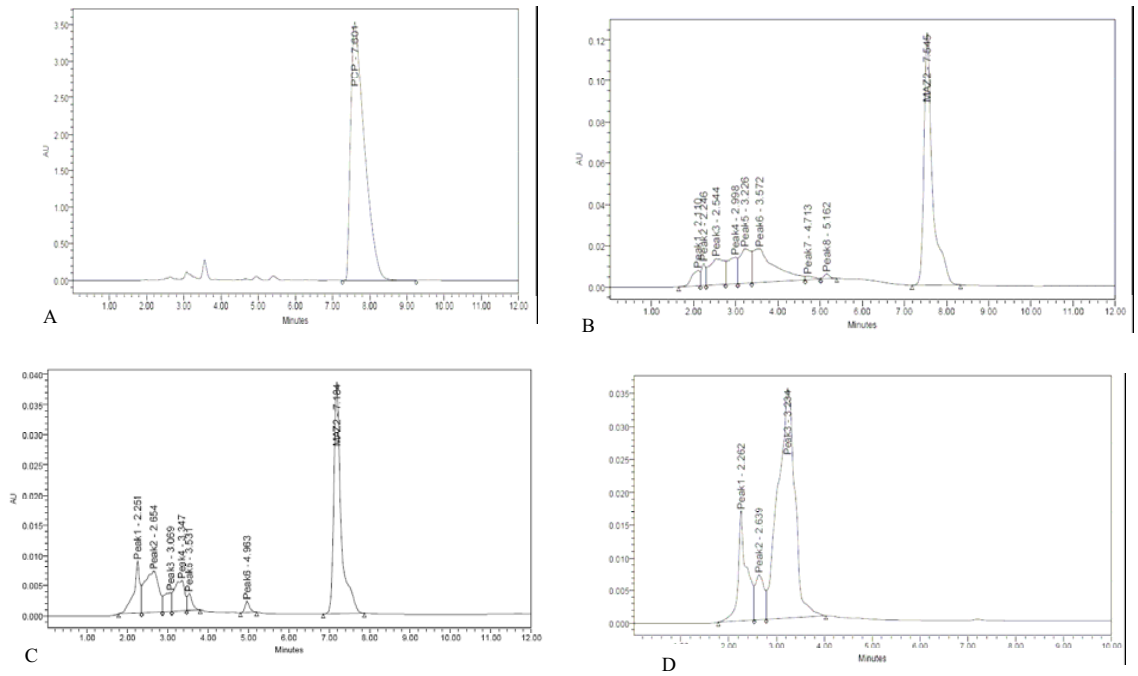

Fig. 4. HPLC profile of PCP degradation
A,B,C & D - Standard PCP and degradation of PCP by the isolate MAZ 2 at 10 DAI, 20 DAI and 30 DAI respectively.

isolates degraded PCP by 70 to 100 % after 30 DAI (Table 4). The HPLC profile of the study indicated maximum degradation of PCP by MAZ 2 isolate and cent percent PCP was degraded at 30 days after inoculation (Fig 4). The next promising isolates were MAZ 1 and WHT 1 which degraded PCP by about 97.2 % and 97.6 % respectively (Table 4). These three potent isolates hold promise in bioremediation of PCP polluted soils. Similarly, eight strains, isolated from PCP contaminated soils, degraded up to 90 % of chlorophenols within 14 days [12].

Thus, the experiment has revealed that most of the PCP (25 ppm) was degraded in 30 days indicating that plants protect themselves from the phytotoxicity effects. The bacteria present in the rhizosphere of these tolerant plants have degraded PCP effectively and protected them against the PCP toxicity, suggesting that both plants and associated microbial communities play a significant role in attenuating the PCP toxicity.

IV. Conclusion

Out of 10 plant species screened for tolerance to PCP, Maize and Groundnut tolerated the most, Wheat, Safflower, Sunflower and Soybean showed moderate tolerance. From all these species, rhizobacteria capable of PCP degradation were isolated. These bacteria degraded PCP, neglected the phytotoxic effect of PCP, thus resulting in tolerance of these plant species to PCP. Out of 27 isolates, MAZ 2 was the most efficient PCP degrading bacterium which degraded PCP completely in 30 days.

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