Biodegradation of ^{14}C-chlorpyrifos by hairy root culture of *Chenopodium amaranticolor* Coste & Reynier

Chlorpyrifos (*O,O*-diethyl *O*-3,5,6-tri-chloro-2-pyridyl phosphorothioate) is a broad-spectrum insecticide used against a wide range of insect and arthropod pests. It is an anticholinesterase agent effective by contact, ingestion and vapour action. Owing to the ban on the usage of many organochlorine insecticides, organophosphorous insecticides like chlorpyrifos have attained importance. Because of its versatility, chlorpyrifos is used globally in a myriad of pest-control situations. Chlorpyrifos is registered for use in over 900 different formulations. In 1997, Dursban (a commercial formulation) was found in more than 25% of surface waters sampled for pesticide by the US Geological Survey. Elevated groundwater contamination has been found in areas where it is used as termiticide. Depending on the pesticide formulation and the form used for application (liquid, aerosol, solid or powder), chlorpyrifos residues may be detectable in water, soil and plant surface for months or even years. In contrast to several other organophosphorus pesticides like parathion, fenitrothion, dichlorovos and diazinon, persistence of chlorpyrifos is unaffected in a medium (peptone and glucose media containing 20 ppm chlorpyrifos) containing various cultures of bacteria like *Pseudomonas* spp., *Bacillus* spp. and others[1]. The degradation of ^{14}C-chlorpyrifos in model rice ecosystem was studied in our laboratory, where degradation of chlorpyrifos to 3,5,6-trichloro-2-pyridinol (TCP) was demonstrated[2], while rapid chlorpyrifos degradation in a medium was shown by a mixture of different fungi compared to the medium with single fungus[3].

Phytoremediation is the use of green plants to remove, render harmless or contain environmental contaminants[4,5]. Soils, sediments or water that show shallow contamination of organic nutrients can be treated using phytoremediation. Plant roots transformed by *Agrobacterium rhizogenes* exhibit all features of a normal plant root. They grow rapidly in aseptic conditions *in vitro* in the absence of phytohormones and have applications in phytoremediation research[6]. Due to the absence of microbial community in these axenically grown cultures, metabolism of

xenobiotics can be wholly attributed to the plant. Other organic xenobiotics like polychlorinated biphenyls[7], trinitrotoluenes[8], malathion, demeton-*S*-methyl and crufomate[9] were metabolized in a plant tissue culture system. Hairy roots cultures of *Cichorium intybus* and *Brassica juncea* that degraded dichloro-diphenyl-trichloroethane (DDT) to its different metabolites were studied in our laboratory[10]. There are also reports of degradation of nitroaromatic compounds like trinitrotoluene (TNT) by *Catharanthus rosesus* hairy root cultures[11]. Plant species belonging to Cruciferae (mustard), Chenopodiaceae (spinach) and Compositae (sunflower) possess genetic potential to extract heavy metals from soil or water and accumulate them in plant parts[12]. In order to exploit this ability of such plant species for organic xenobiotic transformation, an attempt was made to study chlorpyrifos uptake and metabolism using hairy root culture of *Chenopodium amaranticolor* Coste & Reynier.

Axillary buds grown on MS basal medium[13] were used for induction of hairy roots using *Agrobacterium rhizogenes* strain-A4 (ref. 14). The hairy roots induced were cultured on MS medium supplemented with 500 mg l^{-1} cefotaxime in a liquid medium for 4–5 passages and later maintained on an MS medium devoid of antibiotics. One clone of *C. amaranticolor* maintained for one year was used for the experiment.

After harvesting, the roots were blotted on a filter paper and the fresh weight was recorded. The roots were then dried in a hot-air oven at 100°C for 24 h for estimation of dry weight. After drying, aliquots of hairy roots were subjected to soxhlet extraction to recover the radioactivity. The results presented are average of three replicates.

Radiolabelled chlorpyrifos (supplied by IAEA, Vienna) and analytical-grade chlorpyrifos (Dow Elanco) were used for the experiment. All other chemicals were of analytical grade. To obtain the same stage culture approximately equal weights of hairy root tissues were cultured in 250 ml Erlenmeyer flasks containing 50 ml MS medium. Cultures were maintained in a shaker at 100 rpm at 24°C. After sufficient growth of cultures

(5 days), ^{14}C-labelled chlorpyrifos (uniformly labelled, specific activity 1.106 MBq mmol^{-1}, radiochemical purity 95.2% as checked using thin layer chromatography (TLC)) was added along with analytical-grade chlorpyrifos to give a final concentration of 1 µg l^{-1}. Flasks without hairy roots served as control. All the flasks were taken in triplicate.

Hairy root samples were taken at 3rd hour and 3rd, 7th, 17th and 24th day. The medium was decanted and hairy root culture was washed thrice with sterile distilled water. Each washing was for half an hour, with shaking at 100 rpm on a shaker. The medium was extracted with hexane and ethyl acetate alternatively by vigorous shaking to get adsorbed activity on the root surface. Hairy roots were frozen, lyophilized and then extracted with hexane in a Soxhlet extractor for 8 h. ^{14}C residues in solvent or medium were quantified in liquid scintillation counter (LSC; Packard Tricarb 2100 TR). One-millilitre sample in 10 ml of scintillation cocktail (PPO 4 g, naphthalene 100 g and dioxane 900 ml) was used for counting. The efficiency of LSC for ^{14}C was 92%. Quench corrections were applied whenever necessary.

The fraction of ^{14}C-chlorpyrifos residues bound with plant roots was determined by combusting plant samples in a biological material oxidizer (OX-500, R. J. Harvey Instrument, USA) according to the manufacturer's recommended procedure. Residues remaining in extracted roots (0.5 g) were combusted in duplicate and ^{14}CO$_2$ collected to estimate the proportion of radiolabel that was unextractable. The ^{14}CO$_2$ was trapped in the cocktail and quantified in the scintillation counter. The cocktail for trapping ^{14}CO$_2$ was prepared using B-phenyl ethylamine (270 ml), methanol (270 ml), toluene (460 ml), PPO (5 g) and POPOP (100 mg). Hexane extract of root was counted on LSC and further analysed by TLC after purification with fluorosil. The solvent system used for developing the plate was toluene : methanol : hexane (18 : 1 : 1 V/V/V). R_f values for chlorpyrifos and TCP were 0.73 and 0.3 respectively. Analytical-grade chlorpyrifos was used in co-chromatographic procedure. One set of TLC plates was sub-

Table 1. Per cent ^{14}C-activity in various fractions of root culture (applied ^{14}C-activity is 100%)

Sample time	Hexane extract from medium		Ethyl acetate extract from medium		Remaining residues in medium		Root wash with water		Root extract with hexane		Polyurethane foam trap		Bound activity to roots		Total mass balance	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
Third hour	54.55 ±0.10	7.89 ±0.30	9.41 ±1.2	7.93 ±0.41	8.96 ±0.10	16.01 ±0.46	nd	17.15 ±1.74	nd	29.43 ±4.98	27.06 ±0.15	7.7 ±0.05	nd	10.20 ±1.62	98.98 ±6.3	96.3 ±5.1
Third day	50.14 ±0.21	2.66 ±0.26	7.21 ±0.1	6.60 ±0.04	7.87 ±0.02	19.07 ±1.11	nd	12.14 ±0.8	nd	21.76 ±0.55	25.78 ±0.20	6.17 ±2.48	nd	19.51 ±10.42	92.00 ±4.21	85.91 ±6.3
Seventh day	41.95 ±1.02	1.76 ±0.54	7.36 ±0.3	4.03 ±0.42	8.67 ±1.10	17.97 ±1.40	nd	14.73 ±0.5	nd	18.54 ±3.74	30.08 ±1.10	3.16 ±1.5	nd	18.93 ±0.66	91.06 ±3.10	79.12 ±1.15
Seventeenth day	25.32 ±0.20	1.28 ±0.65	9.83 ±0.2	3.10 ±1.46	9.69 ±0.30	14.95 ±2.53	nd	15.48 ±1.2	nd	8.68 ±1.62	45.28 ±0.54	3.74 ±1.25	nd	20.15 ±6.82	90.08 ±4.81	62.36 ±0.52
Twentieth day	8.32 ±0.04	0.31 ±0.75	1.81 ±1.3	0.00	9.63 ±0.11	17.48 ±1.05	nd	13.00 ±2.05	nd	3.78 ±0.06	66.59 ±0.62	3.95 ±0.08	nd	25.75 ±3.05	88.41 ±5.10	57.27 ±2.07

±, Standard deviation; nd, Non-detectable (detection limit 10 Bq); C, Control; E, Experimental.
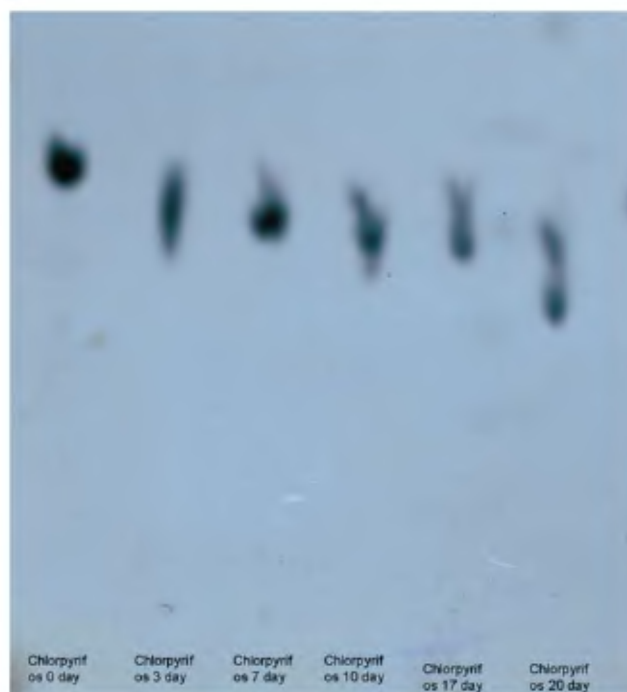
Figure 1. Thin layer chromatograph of ^{14}C-chlorpyrifos activity in the root extract after different days of treatment.

jected to autoradiography using Indu X-ray films (Hindusthan Photo Films, Ootacamund, India). The other set was used for scraping at various spot levels and counting ^{14}C activity to estimate the quantity of ^{14}C residues in each fraction. Part of the sample (1 µl) was subjected to gas liquid chromatography (Shimadzu model GC-17A) with electron-capture detector. Operating conditions were injector port, 230°C; detector 280°C; column temperature, 250°C and carrier gas, nitrogen (1 ml min^{-1}).

Chlorpyrifos did not cause any growth inhibition in hairy root cultures of *C. amaranticolor* at the added concentration (1 µg l^{-1}). The roots showed growth and differentiation patterns similar to those of untreated control cultures. Maximum biomass (dry wt) of 19.5 g was recorded for *C. amaranticolor* cultures after 24 days from an initial inoculum.

Details of the results of the experiment are presented in Table 1. It was observed that chlorpyrifos was adsorbed to the hairy roots immediately after incubation. A small fraction of activity (7.89%) was left in the medium immediately after the start of the experiment and it decreased up to 0.3% on the last day. Ethyl acetate

did not improve extraction from the medium. As this extractable activity was low (5–7%), it could indicate a more polar nature of the activity. Decrease in the hexane extractable activity in the control flask may be due to abiotic degradation or volatilization. This was again confirmed by trapping of radioactivity in polyurethane foam. As a direct consequence of the non-polar nature of this insecticide, chlorpyrifos has a strong tendency to favour the adsorbed state over the dissolved state. An adsorptive equilibrium is usually reached after a few hours. Since chlorpyrifos is more soluble in water compared to 0.01 M CaCl$_2$, solubility ratio being 1.12 : 0.78 mg l^{-1} at 24°C, it was preferred to wash the roots with water. Chlorpyrifos residues up to 15–17% were observed in the root washes. This activity recovered from washings remained constant throughout the experimental period, thus confirming the concept of equilibrium. Due to adsorption in the roots, chlorpyrifos volatilization was controlled in the experimental sets. However, in case of the control, chlorpyrifos volatilized and later got trapped in polyurethane foam probably due to continuous shaking. The decrease in extractable activity from the third hour to the twenty fourth day was met with a proportionate increase in the bound activity.

TLC of root extract showed accumulation of chlorpyrifos inside the roots and also simultaneous degradation over a period of time (Figure 1). It is also clear that a different metabolite was formed over the incubation period. Gas chromatographic (GC) analysis also showed similar results where cholorpyrifos con-

centration decreased with the time and completely mineralized after the 17th day. Retention time of parent compound was observed at 6.4 min.

Advantage of hairy roots over suspension culture has been shown for biodegradation of DDT, where the effectiveness of hairy roots for degradation of xenobiotics has been emphasized[10]. Although our basic knowledge of degradation of organic pollutants by plants lags behind that of bacteria and animals, plants can transform a wide variety of complex organics too[15]. Plant enzymes oxidize, reduce or hydrolyse a xenobiotic compound, thus introducing a reactive group for subsequent conjugation to moieties such as glutathione or glucuronate, after which these conjugated xenobiotics are stored in the cell either in the vacuole (soluble conjugates) or the cell wall (insoluble conjugates)[16].

Biodegradation of chlorpyrifos has been reported in different systems, including soil, bacteria and fungi, where it showed similar pathways through formation of TCP. However, the present study shows that hairy roots have accelerated the process of biodegradation and metabolized chlorpyrifos to polar products. Whether these polar products are formed in the medium and are then taken inside or are formed within the roots is not clear. But these products were found to be present in the plant roots as is evident from the increase in the bound activity in the roots. These findings were also supported by analysis using TLC as well as GC data. The accumulation of chlorpyrifos inside the roots and its consequent degradation over a period was evident during the experiment. Further studies are essential with a view to find the metabolites to which chlorpyrifos is converted.

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Spatial redistribution of *Soliva anthemifolia* (Juss) R. Br. – possible manifestation of changing climate

Soliva anthemifolia (Juss) R. Br., a diffuse, creeping, stoloniferous herb that bears characteristic sessile heads (Figure 1) belongs to the family Asteraceae and commonly occurs in South America and Australia. In India, the plant was reported for the first time in 1963 from Uttar Pradesh[1]. In 1966, its extended distribution northward into the Himalayan states was reported from Dehradun (now in Uttarakhand), where it was growing at an altitude of 640 m asl (ref. 2). In 1973, it was reported as new addition to the Flora of Delhi[3]. Later, its extension to Jammu, where the plant was collected from an altitude of 400–700 m asl, was reported[4]. The plant was also reported from Rajasthan and Haryana[5,6].

Recently *S. anthemifolia* was observed growing in the mesic areas of Ranser Island between 500 and 600 m asl and has been reported as a new generic addition to the flora of Himachal Pradesh (HP)[7]. Soon after, the plant was observed growing along water channels at an altitude of more than 1000 m asl in the Patti locality of HP. It should be noted that the higher limit of its distribution in the Himalayas has been mentioned to be 1000 m (ref. 8). Subsequently, the plant has now been observed in Palampur at an altitude of 1300 m (Figure 2).

The plant appears to be a new entrant to the area as earlier floristic surveys and inventories dedicated to Palampur[9,10], did not report its occurrence. This area is frequently visited by us and we had also not encountered this plant in our earlier surveys, until the recent one. This points to the fact that the plant has not been overlooked during the previous surveys, but rather is a new colonizer (Table 1).

Range shift and spatial redistribution of species are amongst the best indicators of climate change in the mountainous regions of the world, such as the Himalayas[11,12]. Changing distribution patterns, especially at upper altitudinal limits of species distribution, have been hypothesized as a strategy to mitigate temperature increase[13]. So, is the movement pattern of *S. anthemifolia* any indication of changing environmental conditions? As reported, Palampur has shown an increase of 0.6°C in temperature over the last three decades[14], i.e. 1978–2008 (ref. 14). Also, Palampur is a relatively wet place that receives substantial rainfall. Both these factors are favourable for *S. anthemifolia* – a mesic habitat-loving