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Persistence And Degradation Of Carbofuran In Soil And In Microbial Cultures Without And With Added Salts

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The stability of U-phenyl-^{14}C-carbofuran in a flooded alluvial soil amended with a mixture of NaCl + $CaCl_2$ + $MgSO_4$ (3:2:1) to raise the salinity to 4, 8 and 16 dS/m was studied. The soil persistence of carbofuran was not affected by the presence of salts even at 16 dS/m. Soil enrichment cultures from both salt-amended and unamended soil retreated with carbofuran effected more rapid degradation of carbofuran in a mineral medium than did the respective soil suspension not exposed to carbofuran before. The dominant bacteria isolated included *Bacillus* sp. and *Micrococcus* sp. from the soil amended with 8 dS/m and two strains of Arthrobacter from the soil amended with 16 dS/m. *Bacillus* sp. was the most effective in degrading carbofuran. Interestingly, the *Arthrobacter* sp., isolated from the soil amended with salts at 16 dS/m effected more rapid degradation of carbofuran in the presence of NaCl + $CaCl_2$ + $MgSO_4$ than in their absence. Hydrolysis with concomitant accumulation of 7-phenol was the primary pathway in the bacterial degradation of carbofuran.

Rice is grown even under stress conditions of high salinity. Carbofuran (2,3-dihydro-2, 2-dimethyl-7-benzofuranyl *N*-methylcarbamate) is one of the widely used carbamate insecticides in agriculture. There are several reports on its fate and significance in normal agricultural soils and the role of microorganisms in its degradation[1]. But, information on the fate of this insecticide and the role of microorganisms in its degradation in saline soils is meagre. The present study is concerned with the degradation of carbofuran in a flooded soil amended with salts, in carbofuran-enrichment cultures from salt-amended soil and in pure cultures of bacteria isolated from salt-amended soil.

MATERIALS AND METHODS

U-phenyl-^{14}C-carbofuran (specific activity 39.4 mCi/mol 96% purity) was a gift from FMC Corporation, Middleport, New York, U.S.A. The labelled insecticide was dissolved in 100 ml of acetone. An aliquot of this stock solution was evaporated to dryness at room temperature and redissolved in acetone for incorporation into the soil or mineral medium.

A mixture of NaCl, $CaCl_2$ and $MgSO_4$, the major salts in coastal saline soils, was prepared in sterile distilled water to provide 3:2:1 milliequivalent ratio of Na^+, Ca^{2+} and Mg^{2+}, respectively. This mixture was added to 20g portions of an alluvial soil (Haplaquept, pH 6.2, organic matter 1.61%, total nitrogen 0.088%) at levels required to raise its salinity, in terms of electrical conductivity of saturated soil extracts, to 4, 8 and 16 dS/m. The alluvial soil, unamended and amended with salts, after 10 days of flooding with 25 ml water in test tubes (2.5 x 20 cm) was treated with U-phenyl-^{14}C-carbofuran at 2.0 X 10^5 dpm/20 g soil in 0.1 ml of acetone. ^{14}C-Residues in duplicate soil samples were solvent-extracted for radioactivity (when ^{14}C-Carbofuran was used) after separation by thin-layer chromatography (TLC).

Soil enrichment cultures were prepared by repeated additions of carbofuran to the flooded alluvial soil with and without added salts as follows: Soil samples (20 g portions) contained in pre-sterilized test tubes (2.5 x 20 cm) were treated with a mixture of NaCl, $CaCl_2$ and $MgSO_4$ (Na^+, Ca^{2+} and Mg^{2+} at 3:2:1 milliequivalent ratio) at levels to raise its salinity to 4,8, and 16 dS/m. The alluvial soil, unamended and salt-amended after

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10 days of flooding with 25 ml of water, was treated with 1 mg of technical formulation of carbofuran at 10-day intervals. The soil samples were incubated at room temperature (28 ± 2°C). Four days after the fourth addition, the contents in each tube were stirred and the resulting soil suspension was used as the soil enrichment culture. Soil suspensions were prepared from another set of the same soil similarly incubated but not amended with carbofuran.

The ability of soil enrichment culture to degrade carbofuran was tested in a mineral medium as follows. The mineral medium [$MgSO_4$. $7H_2O$, 0.2 g; K_2HPO_4, 0.1 g; $CaSO_4$, 0.04 g; $FeSO_4$. $7H_2O$, 0.001 g; distilled water 1 litre; pH 6.2] supplemented with carbofuran as the sole source of both carbon and nitrogen was used in the study. ^{14}C-carbofuran (2.0×10^5 dpm) in 0.1 ml of acetone was added aseptically to pre-sterilized 100-ml Erlenmeyer flasks. After 24 h for evaporation of acetone at room temperature, 20 ml portions of mineral medium were dispensed into the flasks and equilibrated for 24 h. The medium was then inoculated with o.1 ml of the carbofuran-enrichment culture from salt-amended and unamended soil. In another set, the medium was inoculated with the soil suspension from salt-amended and unamended soil which was not treated with carbofuran. Unino culated medium served as control. After incubation at room temperature, residues in duplicate samples were solvent-extracted and analyzed by liquid scintillation after separation by TLC.

For isolation of bacteria, the mineral medium supplemented with carbofuran was inoculated with carbofuran-enrichment culture from salt-amended (8 and 16 dS/m) and unamended soil. After 10 days of incubation, serial dilutions of the inoculated medium were plated on nutrient agar medium (peptone, 5 g; beef extract, 3 g; agar 16g; distilled water, 1 litre; pH 7.0). Dominant bacteria appearing on the plate were characterized. Several bacterial colonies appearing on the agar plate were tested for their ability to degrade carbofuran in the mineral medium (as described for enrichment culture) and active isolates were identified based on their morphological, physicochemical and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology[2].

Degradation of carbofuran by pure cultures of bacteria in mineral medium at two salinity levels (8 and 16 dS/m) was studied. The mineral medium amended with a mixture of NaCl + $CaCl_2$ + $MgSO_4$ (3:2:1) milliequivalent of Na^+ + Ca^{2+} + Mg^{2+}) at levels required to raise its salinity in terms of electrical conductivity of the medium to 8 and 16 dS/m. The mineral medium unamended and amended with NaCl + $CaCl_2$+$MgSO_4$ solution was supplemented with U-phenyl-^{14}C-carbofuran (2.0×10^5 dpm) as the sole source of carbon and nitrogen. The salt-amended (8 and 16 dS/m) and unamended medium were inoculated with bacterial cultures (*Bacillus* sp. and *Arthrobacter* sp.) isolated from carbofuran enriched soil amended with salts. Uninoculated medium served as control. The amount of carbofuran in duplicate samples at periodical intervals was estimated by liquid scintillation after separation by TLC.

For extraction of residues of carbofuran from the soil, the contents of each of the duplicate tubes were quantitatively transferred to 250-ml Erlenmeyer flask by rinsing with 50 ml of chloroform-diethyl ether (1:1) and then stirred in a wrist-action shaker for 1 h. After centrifugation at 7000 rpm for 10 min, the supernatant was transferred to a separating funnel and the lower fraction was collected in a beaker. This process was repeated twice with 30 ml portions of chloroform-diethyl ether. The pooled solvent fractions from the three successive extractions were evaporated to dryness at room temperature and the residues redissolved in 2 ml of methanol. In studies with enrichment of pure cultures, the residues were extracted from the medium three times with 30 ml portion of chloroform-diethyl either (1:1). The solvent fractions were pooled and after evaporation of the solvent to dryness, the residues were redissolved in 2 ml methanol. Methanol dissolved residues were then separated on 300μm thick silica gel-G plates as described earlier[3].

In studies using ^{14}C-carbofuran, the silica gel areas of the samples corresponding to authentic standards of carbofuran and its metabolities were scraped into 5 ml of scintillation solution (5 g PPO and 0.3 g POPOP in 1 litre of toluene) and radioactivity determined in a liquid scintillation system LSS 20. (Electronics Corporation of India Ltd., Hyderabad)

RESULTS AND DISCUSSION

The persistence of carbofuran in a flooded alluvial soil amended with a mixture of NaCl, $CaCl_2$ and $MgSO_4$ was examined using ring-^{14}C-carbofuran. These salts were selected, because of their common occurrence in coastal saline soils. In saline soils, Na^+ and Cl^- ions contribute 50% of salinity and the remaining 50% is contributed by Ca^{2+}, Mg^{2+} and SO^{2-}_4 The degradation of carbofuran was little affected by the presence of added salts. The concentration of the insecticide decreased, almost equally rapidly, in both salt-amended and unamended soil (Table 1). Thus, after 40 days, 6 to 7% of applied radioactivity was recovered as carbofuran from almost all soil samples irrespective of the salinity level (4, 8 or 16 dS/m) under flooded conditions. A simple correlation analysis between salinity level and half-life values indicated a non-significant correlation ($r = 0.931$ with df $n = 2$) between salinity and persistence of carbofuran. 7-Phenol was recovered as the major metabolite in both salt-amended and unamended soil. Its concentration reached the maximum at 20 days and then declined to low levels at 40 days. Despite reported inhibition of overall microbial activity in a flooded soil by high salinity[4], carbofuran degradation proceeded fairly rapidly in salt-amended flooded soil. Possibly, selected microorganisms involved in carbofuran degradation was not inhibited by the added salts. Alternately, chemical degradation proceeded without hindrance in the presence of salts.

According to recent reports [5-8], repeated applications of carbofuran to normal agricultural soils have led to a distinct build-up of microorganisms capable of degrading carbofuran. It is not clear whether repeated applications of carbofuran to a flooded soil under stress condition of increasing salinity can lead to a similar build-up of carbofuran-degrading microbes in the soil. This aspect was investigated in this experiment. Carbofuran was applied repeatedly to flooded alluvial soil that had been unamended or amended with $NaCl + CaCl_2 + MgSO_4$ to provide salinity levels of 8 and 16 dS/m. Soil suspensions from salt-amended or unamended soil that had been retreated with four applications of carbofuran or untreated with carbofuran were tested for their ability to degrade U-phenyl-^{14}C-carbofuran in a mineral salts medium as a sole source of carbon and nitrogen.

Carbofuran disappeared faster from the medium inoculated with carbfuran-enrichment culture from salt-amended or unamended soil than from uninoculated medium or medium inoculated with

Table 1 Persistence of U-phenyl-^{14} C-carbofuran[a] in a flooded alluvial soil with and without[b] added salts

Incubation (days)	Soil at salinity level	Per cent radioactivity recovered/20 g of soil			
		Aqueous phase	Methanol extract	Carbofuran[c]	7-Phenol[c]
0	1.2 dS/m	0.8±0[d]	80.0±0	75.0±0	0.4±0
	4 dS/m	0.9±0	79.0±0	75.0±0	0.3±0
	8 dS/m	0.7±0	81.0±0	76.0±0	0.5±0
	16 dS/m	0.9±0	80.0±0	76.0±0	0.4±0
20	1.2 dS/m	4.4±0.1	47.0±0.5	22.5±0.5	17.0±0.5
	4 dS/m	4.0±0.2	40.5±0.5	22.0±0.5	17.0±0.2
	8 dS/m	4.0±0.2	40.0±1.0	21.0±1.0	17.5±0.5
	16 dS/m	3.9±0.1	43.8±2.0	28.8±0.9	12.5±0.5
40	1.2 dS/m	4.0±0.1	25.0±0.5	5.5±0.5	12.0±1.0
	4 dS/m	4.3±0.5	28.0±1.0	7.0±0	11.0±0.8
	8 dS/m	3.3 ±0.2	28.7±1.0	6.0±0.2	13.5±0.5
	16 dS/m	3.3±0.3	21.0±1.0	6.8±0.2	6.0±0

[a] U-Phenyl-^{14} C-Carbofuran was added to the soil at 2.0×10^5 dpm/20 g of soil

[b] EC of alluvial soil without added salts was 1.2 dS/m

[c] Carbofuran and 7-phenol were quantified by liquid scintillation after thin-layer chromatographic separation of the residues in methanol extract

[d] Mean of duplicate estimations ± deviation/20 g of soil

Table 2 Degradation of U-phenyl-^{14} C-carbofuran[a] in a mineral medium inoculated with suspensions from carofuran-treated and untreated alluvial soil with and without added salts

Incubation (days)	Mineral medium inoculated with suspension from	Per cent radioactivity recovered/20 ml of medium				
		Aqueous phase	Methanol extract	Carbofuran[b]	Keto-Carbofuran[b]	7-Phenol[b]
0	uninoculated[c]	0.4±0[f]	85.0±0	81.0±0	nd	0.6±0
	Soil[d]	0.4±0	85.0±0	80.0±0	nd	0.8±0
	Soil + carbofuran	0.5±0	84.0±0	79.0±0	nd	0.9±0
	Soil + salts[e]	0.4±0	84.0±0	80.0±0	nd	0.9±0
	Soil +salts + carbofuran	0.5±0	85.0±0	80.0±0	nd	0.9±0
20	Uninoculated[c]	1.8±0.1	80.0±2.0	76.0±2.0	nd	1.0±0.02
	Soil[d]	3.0±0.2	55.0±1.0	50.0±1.5	0.6±0.06	2.0±0.15
	Soil + carbofuran	3.7±0.5	41.0±1.0	30.0±1.0	1.5±0.20	7.0±0.35
	Soil + salts[e]	3.0±0.5	53.0±1.0	49.0±1.0	nd	1.0±0.10
	Soil + salts + carbofuran	5.0±0	47.0±0	40.0±0.2	1.0±0.10	2.0±0.50
40	Uninoculated[c]	6.0±0	75.0±0	70.5±0.5	nd	2.0±0
	Soil[d]	21.0±2.0	52.0±2.0	42.0±2.0	nd	4.5±0.50
	Soil + carbofuran	15.5±0.5	30.0±1.0	20.0±1.0	nd	10.0±0.50
	Soil + salts[e]	15.0±0.6	42.0±0.5	40.0±1.0	nd	1.0±0
	Soil + salts + carbofuran	14.0±1.5	35.0±0.6	29.0±0.9	nd	2.2±0.70

[a] U-Phenyl-^{14}C-carbofuran was added to the medium at 2.0x 10^5 dpm/20 ml of medium
[b] Carbofuran, ketocarbofuran and 7-phenol were quantified by liquid scintillation after thin-layer chromatographic separation of residues in methanol extract
[c] Mineral medium was not inoculated with soil suspension
[d] Mineral medium was inoculated with suspension from carbofuran untreated soil
[e] Salts ($NaCl + CaCl_2 + MgSO_4$) added to provide the salinity of 8 dS/m
[f] Mean of duplicate estimations ± deviation/20 ml of medium
nd-not detected

Table 3 Metabolism of U-phenyl-^{14} C-carbofuran[a] by *Bacillus* sp. (isolated from flooded alluvial soil amended with mixture of salts at 8 dS/m) in mineral medium without and with added salts ($NaCl + CaCl_2 + MgSO_4$)

Incubation (days)	Treatments	Per cent radioactivity recovered/20 ml of the medium				
		Aqueous phase	Methanol extract	Carbo-furan[b]	Keto-carbo-furan[b]	7-Phenol[b]
0	Uninoculated[c]	0.5	85	81	0.2	0.58
	Inoculated[c]	0.5	83	80	0.4	0.80
	Uninoculated medium[d] at 8 dS/m	0.4	83	80	0.3	0.73
	Inoculated medium[d] at 8 dS/m	0.5	82	80	0.5	0.90
40	Uninoculated[c]	6.0	80	75	1.2	2.00
	Inoculated[c]	15.0	50	10	10.0	15.50
	Uninoculated medium[d] at 8 dS/m	6.0	62	54	2.0	4.00
	Inoculated medium[d] at 8 dS/m	20.2	48	13	7.0	26.00

[a] U-phenyl-^{14}C-carbofuran added at 2.0 x 10^5 dpm/20 ml of medium
[b] Carbofuran, keto-carbofuran and 7-phenol were quantified by liquid scintillation after thin-layer chromatographic separation of residues in methanol extract
[c] Medium without added $NaCl + CaCl_2 + MgSO_4$
[d] Medium with added $NaCl + CaCl_2 + MgSO_4$

suspension from soil not treated with carbofuran before (Table 2). Degradation of carbofuran was slightly faster in the medium inoculated with carbofuran enrichment culture from the soil without added salts than in the medium inoculated with carbofuran-enrichment culture from salt-amended soil. Keto-carbofuran and 7-phenol accumulated especially in the medium inoculated with the enrichment cultures from both salt-amended and unamended soil at 20 days. But, at 40 days, keto-carbofuran disappeared totally from the medium, probably because of its further metabolism. Evidence suggested that repeated application of carbofuran to salt-amended soil led to the development of an enrichment culture capable of degrading carbofuran, but this enrichment culture was slighly less active than the enrichment culture from soil without added salts.

The dominant bacteria isolated from mineral medium inoculated with suspension from carbofuran-retreated soil were: *Arthrobacter* sp. and an unidentified bacterium from soil without added salts, *Micrococcus* sp., *Arthrobacter* sp. and *Bacillus* sp. (from soil with added salts at 8dS/m) and *Arthrobacter* sp. strains 1 and 2 and *Micrococcus* sp. (from soil with added salts at 16 dS/m).

Bacillus sp., isolated from the soil with added salts at 8 dS/m was the most effective in degrading carbofuran. In 40 days, 87 to 90% of the carbofuran was lost from the medium inoculated with *Bacillus* sp. at salinity levels of 8 dS/m (Table 3). About 15 to 26% of the ^{14}C in carbofuran was accounted for as 7-phenol in the inoculated medium at both salinity levels.

In another experiment, *Arthrobacter* sp. strain that had been isolated from the flooded alluvial soil amended with salts at 16 dS/m and carbofuran were tested for its ability to degrade ^{14}C-carbofuran in a mineral medium in the presence (8 dS/m) and absence of NaCl + $CaCl_2$ + $MgSO_4$. The degradation of carbofuran in uninoculated medium was not considerable in the absence of NaCl + $CaCl_2$ + $MgSO_4$ during 40-day incubation; while some decrease in its concentration was noticed in the presence of these salts. In the medium inoculated with *Arthrobacter* sp. strain 1, the concentation of carbofuran decreased considerably in the presence and absence of NaCl + $CaCl_2$ + $MgSO_4$, but more rapidly in the presence of these salts (Table 4).

Table 4 Degradation of U-phenyl-^{14}C-carbofuran[a] by *Arthrobacter* sp. (strain 1) (isolated from flooded alluvial soil amended with a mixture of salts at 16 dS/m) in mineral medium without and with added salts at 8 dS/m

Incubation (days)	Treatments	Per cent radioactivity recovered/20 ml of medium				
		Aqueous extract	Methanol extract	Carbofuran[b]	Keto-carbofuran[b]	7-Phenol[b]
0	Uninoculated[c]	0.5±0[e]	88.0±0	85.0±0	nd	0.5±0
	Inoculated[c]	0.5±0	87.0±0	84.0±0	nd	0.4±0
	Uninoculated medium[d] at 8 dS/m	0.4±0	87.0±0	85.0±0	nd	0.5±0
	Inoculated medium[d] at 8 dS/m	0.6±0	86.0±0	84.0±0	nd	0.5±0
20	Uninoculated[c]	2.0±0.5	80.0±2.0	75.0±1.0	0.5±0.1	3.0±0.5
	Inoculated[c]	3.0±1.0	70.0±2.0	63.0±3.0	1.5±0.2	2.5±0.4
	Uninoculated medium[d] at 8 dS/m	3.0±0.4	71.0±1.0	64.0±1.0	0.5±0.1	6.0±0.8
	Inoculated	5.0±1.0	48.0±1.0	34.0±0.5	5.0±0.5	9.0±0.3
40	Uninoculated[c]	6.0±0.5	75.0±2.0	70.0±1.0	1.0±0.5	2.0±1.0
	Inoculated[c]	11.5±0.5	64.0±2.0	50.0±2.0	8.0±0.5	4.0±0.5
	Uninoculated medium[d] at 8 dS/m	4.0±0.4	60.0±2.0	50.0±1.0	2.0±0.5	7.0±0.5
	Inoculated medium[d] at 8 dS/m	15.5±1.0	43.0±3.0	18.0±3.0	6.5±0.5	17.0±0

[a] U- Phenyl-^{14}C-carbofuran was added at 2.0 x 10^5 dpm/20 ml of medium

[b] Carbofuran, ketocarbofuran and 7-phenol were quantified by liquid scintillation after thin-layer chromatographic separation of residues in methanol extract

[c] Medium without added NaCl + $CaCl_2$ + $MgSO_4$

[d] Medium with added NaCl + $CaCl_2$ + $MgSO_4$

[e] Mean of duplicates estimations ± deviation/20 ml of medium

This bacterial strain, isolated from salt-amended soil (16 dS/m) and hence adapted to a saline environment, effected more rapid degradation of carbofuran in the presence of NaCl + $CaCl_2$ + $MgSO_4$ than in their absence. During bacterial degradation, 7-phenol accumulated in greater amounts in salt-rich medium than in the absence of NaCl + $CaCl_2$ + $MgSO_4$. Keto-carbofuran was also detected in substantial amounts during bacterial degradation especially in the presence of salts. According to these data, microbially-mediated degradation of carbofuran can be considerable even in soil under stress conditions of high salinity especially after its repeated applications.

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REFERENCES

1. Rajagopal, B.S., Brahmaprakash, G.P., Reddy, B.R., Singh, U.D. and Sethunathan, N. 1984, Effect and persistence of selected carbamate pesticides in soils. *Residue Reviews* **93** : 1-199.
2. *Bergey's Manual of Determinative Bacteriology*. 8th edition. 1974, R.E. Buchanan and Gibbons, N.E. (Eds.). The Williams and Wilkins Co., Baltimore, U.S.A.
3. Ramanand, K., Panda, S., Sharmila, M., Adhya, T.K. and Sethunathan, N. 1988, Development and acclimatization of carbofuran degrading soil-enrichment cultures at different temperatures. *Journal of Agricultural and Food Chemistry* **36** : 200-205.
4. Reddy, B.R. and Sethunathan, N. 1985. Salinity and the persistence of parathion in flooded soils. *Soil Biology and Biochemistry* **17** : 235-239.
5. Read, D.C. 1986, Accelerated microbial degradation of carbofuran in soil from previously treated fields. *Agriculture Ecosystems Environment* **15** : 51-61.
6. Read, D.C. 1987, Greatly accelerated microbial degradation of aldicarb in retreatd field soil, in flooded soil and in water. *Journal of Economic Entomology* **80**: 156-163.
7. Chapman, R.A., Harris, C.R. and Harris, G. 1986, Observations on the effect of soil type, treatment intensity, insecticide formulation, temperature and moisture on the adaptation and subsequent activity on biological agents associated with carbofuran degradation in soil. *Journal of Environmental Science and Health* 21B : 125-141.
8. Racke, K.D. and Coats, J.R. 1988, Enhanced degradation and the comparative fate of carbamate insecticides in soils. *Journal of Agricultural and Food Chemistry* **36**: 1067-1072.