Bull. Environ. Contam. Toxicol. (1988) 41:127–134

Bulletin of Environmental Contamination and Toxicology

Microbial Uptake and Accumulation of (^{14}C Carbofuran) 1,3-Dihydro-2,2-Dimethyl-7 Benzofuranylmethyl Carbamate in Twenty Fungal Strains Isolated by Miniecosystem Studies

Kantha Deivi Arunachalam[1] and M. Lakshmanan[2]

[1]235 Blackmarsh Road, Apartment 213, St. John's, Newfoundland, A1E 4T2, Canada, and Head of the Department, [2]Department of Microbiology, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamilnadu, India 625 021

Studies have amply demonstrated that members of the microbial world vary widely in their response to pesticides and that several factors may influence the toxicity of pesticides (Durham, 1969). Similarly, the microbial tolerance of pesticides may be affected by growth conditions, physiological conditions of cells and various stress factors which might exist in natural population.

The pesticides are incorporated into microorganisms by an active or passive accumulation mechanism. Most observations of pesticide accumulations within the cells were recorded with chlorinated hydrocarbons like DDT, dieldrin, aldrin and heptachlor. It was found that not only live bacterial cells, but autoclaved cells also, show a similar uptake of pesticides. Johnson and Kennedy (1973) found that in Aerobacter aerogenes, the uptake of methoxychlor, after autoclaving the cells was double the amount absorbed by living cells. They suggested that the molecular polarity and lipid solubility could influence the retention of the organochlorine insecticides by bacterial cells.

Adsorption and concentration of the insecticide aldrin, was determined for floc-forming bacteria and it was suggested that the adsorption capacity or flocculent bacteria might even be evaluated for the removal of pesticides in an aqueous environment (Leshniowsky et al., 1970). Since aquatic microorganisms and plankton in freshwater and marine environments are an important nutrient source for a broad spectrum of aquatic filter-feeding organisms, their accumulation of pesticides can constitute a hazardous link in the food chain to fish and higher vertebrates. Therefore, the findings of extensive biomagnification of these cultures of blue-green agae Anacystisnidulans, the green algae Scenedesmus obliques, the flagellate Euglenagracilis and two ciliates Parameciumbursaria and P.multimicronucleatum accumulated DDT and Parathion after exposure for 7 days at a rate of 100 to 964 and 50 to 116 times respectively (Gregory et al, 1969).

[1] Send reprint requests to K.D. Arunachalam
[2] Where the work was done.

MATERIALS AND METHODS

Analytical grades of carbofuran (purity 99.5%), 3-OH carbofuran (93%), carbofuran phenol (99.2%), 3-OH phenol (99.4%), 3-keto phenol (99.4%), and ^{14}C labelled carbofuran (98%) with a specific activity of 26.73mCi/mmol were gifts from Dr. Robert A. Robinson, FMC Corporation, NY, USA.

20 species of soil fungi isolated during model ecosystem studies (Arunachalam, 1980) were used as test organisms. The species were tested for their mode of uptake and mechanism of accumulation of carbofuran and its metabolites under in vitro conditions. This study is the follow-up of our earlier works (Arunachalam and Lakshmanan, 1988a). The Czapeck-Dox liquid medium containing 1.0g KH_2PO_4, 2.0g $NaNO_3$, 0.5g $MgSO_4.7H_2O$, 10mg $FeSO_4.7H_2O$, 0.5g KCl and 30.0g sucrose in 1000ml distilled water was prepared. The medium was distributed as 100ml/250ml flasks and autoclaved. Filter sterilized ^{14}C ring labelled carbofuran was mixed with cold carbofuran to obtain final concentration of 100 µg/ml medium. The flasks were inocculated with spore suspension to give 10^7 spores/ml and incubated at 37°C for 30 days. After 30 days of incubation the mycelia were harvested and the culture medium was filtered and centrifuged to remove any suspended mycelial fragments and spores. The filtrate was extracted with three repeated additions of equal amounts of benzene and concentrated. The aqueous fractions were concentrated by dialysis and dissolved in a low volume of acetone. An aliquot of 0.1ml of final extract was mixed with liquid scintillation fluid (NE 220 or NE 213) and its radioactivity was counted in automatic liquid scintillation counter (Beckman model LSS100) having 90 percent efficiency. Necessary quenching corrections were also made. The extract was also estimated spectrophotometrically. The mycelia were harvested, ground and analysed for its carbofuran content. The recovery percentage varied from 78 to 89% under the above extraction conditions. The mycelial extracts and culture filtrate extracts were analysed qualitatively for the accumulation of carbofuran and its metabolites by Think Layer Chromatography (TLC).

The method developed by Gupta and Dewan (1976) was followed for this study. Suitable aliquots of the standard compounds in acetone containing 0.1 to 1 µg were evaporated to near dryness. To this, 3ml of acetone and 7ml of coagulating solution (1gm of NH_4Cl dissolved in 400ml of distiled water plus 2ml of H_2O_4) were added. The contents were filtered after 10 min. An aliquot of 5ml was pipetted into stoppered test tubes which were placed in an ice bath. When the contents of the test tubes reached below 4°C, 2ml of ice-cold 1.5 N methanolic KOH (40% KOH) was added and mixed well. After 5 min. 0.5ml of ice-cold chromogenic reagent (saturated solution of p-nitro benzene diazonium fluoborate in ethanol with one ml of acetic acid) was added and the contents were mixed thoroughly. The mixture was allowed to stand for 2 min. and the absorbance of the pink coloured solution was measured at 550nm against a blank in spectronic 70 spectrophotometer. From the

The pink coloured spots of the authentic compounds were marked and the R_f values were calculated. Following the above procedure, standards of 0.1 - 100 µg per spot for all the six analytical compounds were chromotographed. These were eluted and their spectra were recorded on Spectronic 70 spectrophotomter.

Since this TLC method was more sensitive and needed less clean up procedures, it was followed throughout the investigation. A file on documentation of TLC was maintained. By comparing the R_f value obtained with the sample to the standard, the compounds were identified (Fig. 1). When labelled carbofuran was used for quantitative studies, the metabolites and carbofuran were quantified by scraping the silica gel areas corresponding to authentic compounds into scintillation vials mixed with 10ml of liquid scintillator (NE 220 or NE 213) and the radioactivity was measured in an automatic liquid scintillation counter (Beckman model LSS 100) having 90 per cent efficiency. Necessary quenching corrections were also made.

Table 1 Quantitative analysis of carbofuran residue in culture filtrate and mycelium of fungal culture [1] after 30 days.

Organisms	[2]Percentage of carbofuran residue	
	Culture filtrate	Mycelium
1. Trichoderma viride	13.1	12.0
2. Alternaria sp.	17.2	8.5
3. Botryosporium longibrachiatum	36.8	16.5
4. Cephalosporium sp.	23.4	21.9
5. Curvularia lunata	28.5	16.1
6. Fusarium oxysporum	39.9	14.8
7. Gliomastrix murorum	33.7	4.6
8. Gliocladium roseum	14.5	8.9
9. Lacellionopsis sacchari	-	-
10. Halminthosporium gossipi	-	-
11. Microsporum sp.	17.3	2.4
12. Myrothecium sp.	0.6	1.0
13. Mucor racemous	-	-
14. Cladosporium chlorecephalum	-	-
15. Aspergillus sp.	8.3	13.7
16. Penicillium crysogenum	23.9	3.8
17. Periconia byssoides	19.4	4.7
18. Rhizoctonia sp.	21.3	7.1
19. Rhizophus sp.	-	6.5
20. Trichocladium canadense	-	-

[1] Fungal cultures were grown in medium containing ^{14}C carbofuran (100 µg/ml) at 37°C for 30 days.

[2] Carbofuran residue levels after 30 days were calculated spectrophotometrically.

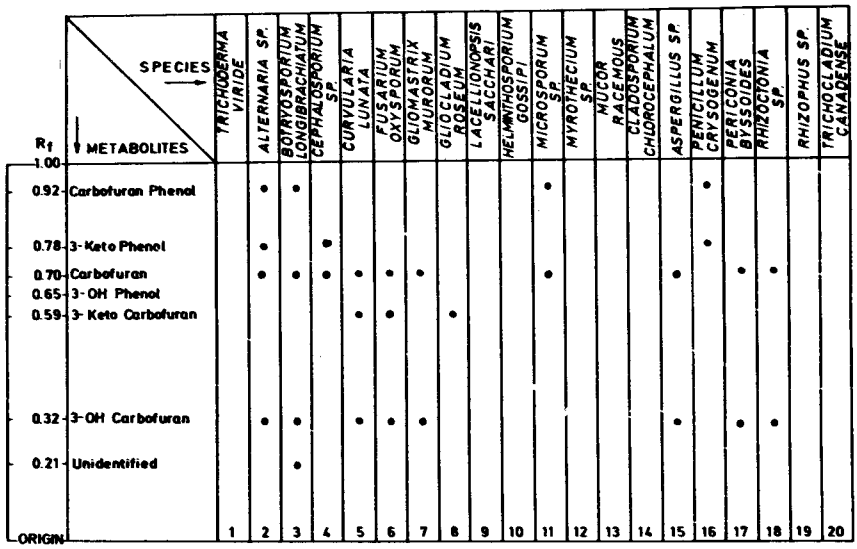

Figure 1 TLC schematic representation of ^{14}C carbofuran and its metabolites in the culture filtrates of soil fungi [Fungi grown in medium containing ^{14}C carbofuran for 30 days at room temperature and the culture filtrates were analysed qualitatively for the accumulated products by TLC]

standard curve, the concentration of carbofuran present in mycelia and culture filtrate was calculated. This method was found to be sensitive up to 0.5 µg of the analytical grade carbofuran.

The TLC estimation of residues of carbofuran and its' metabolites was carried out following a modification of the procedure given by Gupta and Dewan (1976) and the procedure given by FMC Corporation. 30g of silica gel G (E. Merck, Darmstadt, West Germany) was mixed with 60ml of distilled water for 2 min. and the slurry was spread on glass plates of 20 x 20cm size by means of an applicator to provide 250mm thickness of the gel. The plates were allowed to set for an hour at room temperature and then activated at 110°C for 12h in a hot air oven.

Standard compound concentrations of 10 µg/ml dissolved in acetone were spotted on silica gel 3cm apart using disposable microliter pipettes. Each spot contained 100 µg of the test compound. Along with this, mixtures of the standard compounds were also run on the same plate. The plates were developed to a distance of 18.5cm by employing ether: hexane 5:1 as the solvent system, 3-keto carbofuran and 3-keto carbofuran phenol were detected by the light blue fluorescence when exposed to uv light. Other authentic compounds were detected by spraying 1.5N methanolic KOH followed by a spray of p-nitrobenzene diazonium fluoborate (0.1g of the reagent was added to 50ml of 1:1 diethyl ether: methanol and shaken for one minute. The solution was filtered and used within 4 hours of preparation. It was stored in cold at 4°C until it was used).

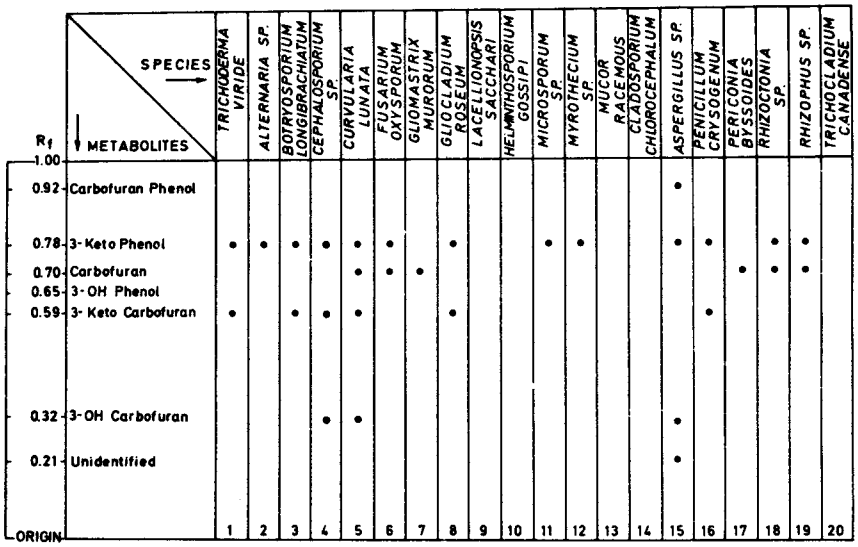

Figure 2 TLC schematic representation of ^{14}C carbofuran and its metabolites in the mycelial extracts [Fungi grown in medium containing ^{14}C carbofuran for 30 days at room temperature and the mycelia were analysed qualitatively for the metabolites as per TLC].

To be certain that the compounds were not altered chemically during TLC procedures and to characterize the metabolites formed during the study, uv spectral analyses of the various metabolites wre carried out. After separation of the residue and the authentic compound mixtures in the TLC plates, the metabolites of the residues from the test samples and the standards were isolated without application of any chromogenic spray by scraping the silica gel at the particular R_f value. These were individually extracted in acetone and concentrated. The concentrated extracts were finally dissolved in 1ml of acetone and their absorption was followed from 260nm to 380nm keeping acetone as blank.

RESULTS AND DISCUSSION

Microbial uptake and accumulation studies of carbofuran by the 20 selected fungi showed variations in their mode of operation in the culture medium. The residue accumulation in culture filtrate and in the mycelial extracts were given in table 1. From the analysis of this study it could be seen that about 25% of the tested organisms, namely, species of Lacellionopsis, Helminthosporium, Mucor, Cladosporium and Trichocladium did not show any trace of labelled compound after 30 days of incubation. Of the remaining 75% of the organisms, species of Trichoderma, Botryosporium, Curvularia, Fusarium and Aspergillus (30% of organisms) showed accumulation of ^{14}C carbofuran above 10% level, while mycelium of Myrothecium sp. alone had shown less than 1% level of ^{14}C carbofuran accumlation. (The other 40% of the organisms, species

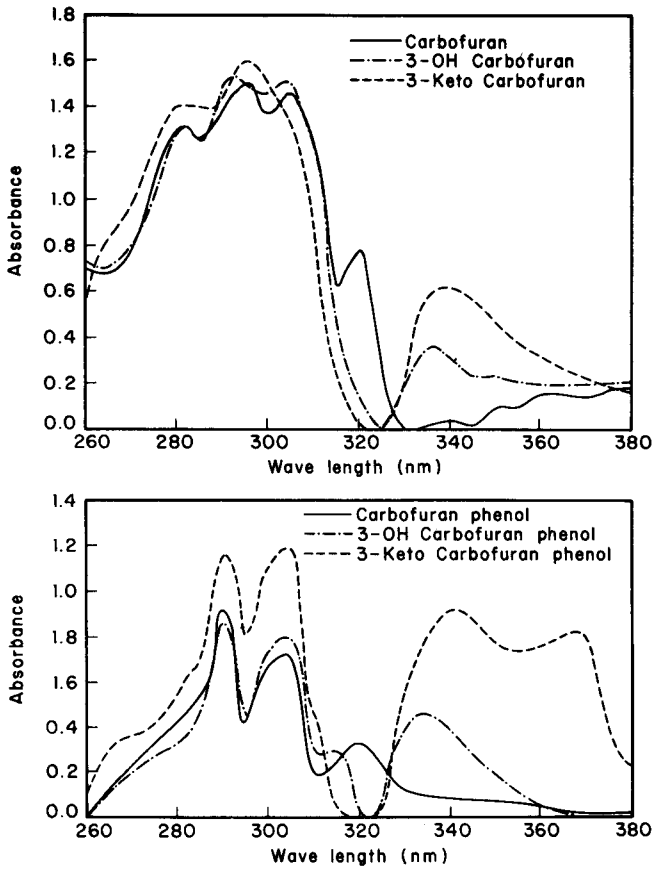

Figure 3 Ultraviolet absorption spectra of carbofuran and its degraded products (100 µg/ml in acetone).

of Alternaria, Rhizoctonia and Rhizophus showed less than 10% of residue accumulation in the mycelial extracts).

From spectrophotometry and isotopic analysis of the culture filtrates for the residue analysis, it was evident that about 30% of the organisms namely species of Lacellionopsis, Helminthosporium, Mucor, Cladosporium, Rhizophus, Trichocladium showed no traces of carbofuran. Species of Myrothecium and Aspergillus (10% of organisms) showed less than 10% of the residue. High residue levels of 36.8%, 39.9% and 33.7% of the ^{14}C carbofuran were observed respectively in the culture filtrates of Botryosporium, Fusarium, Gliomastrix after 30 days of incubation.

The results of the TLC studies for carbofuran metabolites are depicted in figures 1 and 2. The metabolites formed by the biodegradation were identified to their respective compounds by co-chromotography and also evidenced by uv absorption (Fig. 3).

Figure 1 shows the qualitative analysis of metabolite accumulation

in the culture filtrate. 55% of the tested organisms showed carbofuran, the parental compound, along with other degraded products, whereas 3-OH carbofuran phenol was not observed in any of the tested organisms. But the other four products, that is, 3-OH carbofuran, 3-keto carbofuran, 3-keto carbofuran phenol,carbofuran phenol were identified. The culture filtrate analysis of Botryosporium longibrachiatum showed an unidentified product of R_f 0.21 which was absent in others.

Figure 2 shows the TLC schematic representation of carbofuran and its metabolites in the mycelial extracts of 20 tested soil fungi. Here again 25% of the tested organisms did not show any trace of the compound. Among the 75%, 3-keto phenol was observed in 35% of the organisms. The parental compound carbofuran was observed in 35% of the organisms. Just like in the culture filtrates, no accumulation of 3-OH phenol, 3-keto carbofuran, 3-OH carbofuran were observed in 30% and 15% of the total tested organisms. Carbofuran phenol was observed as one of the end products along with another unidentified product of R_f value 0.21 in the mycelial extracts of Aspergillus sp.

Analysis of microbial accumulation of ^{14}C carbofuran in fungal mycelium after 30 days showed that the percentage of labelled compound varied with the organisms and so the process is species specific. The mechanism of accumulation may be due to absorption or genuine uptake (Grossbard, 1970). Uptake of ^{14}C herbicides by fungi has been observed both in pure culture and in soil (Kaufman et al., 1965 and Grossbard, 1970). However, the role of cellular lipid content with the uptake of pesticide have been reported by Enebo et al., (1946) in Rhodotorula gracilis with DDT. Variations observed in the accumulated metabolites both in the culture filtrates and in mycelial extracts depends on the chemical structure of carbofuran as well as the nature of the tested organisms and their biological activities. Factors like cellular permeation of the chemical and its steric and electronic characteristics influence the biological activity of the tested compounds. Whether the real mode of operation or the mechanism of uptake and accumulation depends on the physical, chemical or biological or involves all the three processes opens a wide field of research. But for our studies in vitro experiments were conducted with the tested soil fungi to verify further the role of the exocellular and microsomal enzymes in the uptake and accumulation processes (Arunachalam, 1980). Reports have also been made in predicting the metabolic pathways operating in these 20 tested soil fungi by Arunachalam and Lakshmanan, (1988b).

REFERENCES

Arunachalam KD (1980) Studies on pesticide-soil-microbes interactions with reference to carbofuran and crop plants, Ph.D. thesis, Madurai Kamaraj University, Madurai, Tamilnadu, India, pp: 116-145

Arunachalam KD and Lakshmanan M (1988a) Decomposition of ^{14}C labelled carbofuran in a black tropical soil under laboratory conditions, Soil Biol and Biochem (accepted for publication)

Arunachalam KD and Lakshmanan M (1988b) Metabolic pathways of 2,3-Dihydro-2, 2 Dimethyl-7-Benzofuranyl Methyl Carbomate in twenty soil fungi, Pesticide Biochem and Physiol (Communicated)

Durham WF (1969) The influence of other factors on the toxicity of pesticides, In: Chemical Fall Out, (M W Miller and G G Berg, eds) C.C. Thomas, Springfield Illinois, pp: 433-467

Enebo L, Anderson LG and Lundin H (1946) Microbiological fat synthesis by means of Rhodotorula yeast, Arch Biochem , 11, 383-395

Gregory WW, Reed JK and Priester LE (1969) Accumulation of parathion and DDT by some algae and protozoa, J Protozool , 16: 69-71

Grossbard E (1970) Distribution of ^{14}C labelled Simazine and Atrazine before and after incubation detected by autoradiography of soil particles, Meded Fac Landbouw Gent , 35: 531-542

Gupta RC and Dewan RS (1976) Estimation of combined residues of carbofuran (Furadan) and its toxic metabolites by an enzymatic method, Pesticides, 10: 29-31

Johnson BT and Kennedy JO (1973) Biomagnification of P, P - DDT and methoxychlor by bacteria, Appl Microbiol , 26: 66-71

Kaufman DD, Kearney PC and Sheets TJ (1965) Microbial degradation of simazine, J Agric Food Chem 13: 238-242

Leshniowsky WO, Dugan PR, Pfister RM, Frea JI and Randles CI (1970) Aldrin: Removal from lake water by floculent bacteria, Science, 169: 993-995

Received August 18, 1987; accepted March 1, 1988.