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www.bti.org.in
ISSN 0974-1453
Research Article

EFFECT OF REPEATED APPLICATION OF ENDOSULFAN ON FUNGAL POPULATION OF PINE FOREST SOIL

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ABSTRACT: Fungi from the natural source can be exploited as an effective tool for biodegradation of toxic xenobiotics. The present investigation has been taken up to study the effect of endosulfan on fungal population and isolate most pesticide tolerant fungal species from pine forest soil in two different culture media (NCM and NSM). Maximum CFU count was recorded in NSM in which endosulfan was given as a sole source of sulfur as compared to NCM. When endosulfan applied in soil at lower doses (1-25 ppm), the fungal CFU count was increased between 17.67±1.15 to 23.00±2.00×10^3 CFU g soil^{-1}. Lower concentrations of endosulfan caused a stimulatory effect on fungal population as they probably utilized it as energy or other nutrient source. However, higher concentrations (50-500 ppm) of endosulfan caused inhibitory effect on the total fungal population. Thirty two fungal species belonging to twelve genera were isolated from pine forest soil in which *Cladosporium cladosporioides, Paecilomyces lilacinus, Penicillium decumbens, P. frequentans, Phoma fimeti, Trichoderma aureoviride, T. atroviride, T. harzianum* and *T. longibrachiatum* have ability to tolerate higher concentrations of endosulfan and can be utilized for its degradation studies.

Key words: Endosulfan, Fungal population, Pine forest, Soil.

INTRODUCTION

Endosulfan (6,7,8,9,10,10-hexa chloro-1,5,5ª,6,9,9ª-hexa hydro-6,9-methano-2,4,3-benzo di oxathiepin -3 - oxide), a broad spectrum chlorinated pesticide of the cyclodiene group has been used extensively through out the world. India regarded as being the world's largest producer and user of endosulfan (Li and Macdonald, 2005). It was widely used in India especially after the call for restricted use of DDT (Naina, 2000) with consumption values of 3599 metric tonnes per annum (Usha and Harikrishnan, 2005). It was classified by United States Environmental Protection Agency (US EPA) as category: "highly toxic", based on an LD_{50} of 30mg/kg body weight for rats (US EPA, 2002). It can bind to soil particles and persist for long period with half life of 60-800 days depending on the type of soil (Siddique *et al.,* 2003a). Soil is a dynamic living system which consists of a variety of micro and macro-organisms viz., bacteria, actinomycetes, fungi, arthropods, crustaceans, earthworms, etc. They have a primary catabolic role in degradation of plant and animal residues which contributes to the cycling of nutrients in soil (Doetsch and Cook, 1973). Several works have reported that native microorganisms (bacteria, actinomycetes and fungi) from soil and sediment are capable of degrading pesticides. Repeated and long term application of pesticides in the same field developed a resistant microbial population in soil with the ability to degrade determined compounds (Hernández *et al.,* 2008).

The filamentous fungi have major advantage over bacteria as they have high surface-to-cell ratio characteristic of filaments to maximize both mechanical and enzymatic contact with the environment and the extracellular nature of the degradative enzymes enables fungi to tolerate higher concentration of toxic chemicals. That is why fungi have been investigated extensively since the mid-1980s for their bioremediation capacities (Cerniglia *et al.,* 1992; Paszczynski and Crawford, 2000; Bennet *et al.,* 2001). Fungi generally biotransform pesticides and other xenobiotic compounds by introducing minor structural changes to the molecule, rendering it nontoxic; the biotransformed pesticide is released into the soil, where it is susceptible to further degradation by bacteria (Gianfreda and Rao, 2004). However, the species investigated have been primarily those studied extensively under laboratory conditions, which may not necessarily represent the ideal organisms for bioremediation. Fungi in little-explored forests of the world, for example tropical forests, may yet prove to have even better bioremediation capabilities than the temperate organisms currently studied, exhibiting more tolerance to temperature and specialist environments. The biodiversity of decomposer fungi is much higher in tropical ecosystems, especially tropical forest (Evans and Hedger, 2001).

During the present decade there is an increased awareness among the people regarding pesticide contamination and remediation created by scientific investigations. Fungi from the natural source can be exploited as an effective tool for biodegradation of toxic xenobiotics. With this idea the present investigation has been set to isolate most endosulfan tolerant fungal species from pine forest soil.

MATERIALS AND METHODS

Soil sampling

Soil (0-15 cm depth) was collected randomly from 10 different places of forest area and mixed thoroughly to prepare one composite sample. Soil used in this investigation was collected from Pine Forest, Champion Block, Forest Research Institute, Dehradun, Uttarakhand,

India (30º20□38.48□□N; 78º00□38.17□□E) with no previous history of pesticides treatment.

Pesticide used

Endosulfan 35EC (Yash Pesticides, Mumbai) was purchased from local market.

Soil treatment with endosulfan

Soil was air dried, sieved through a 2 mm sieve and 1 kg soil was taken in an earthen pot (on oven dry basis). Endosulfan was diluted with sterilized distilled water and applied to the soil to adjust the moisture of the soil to 50% of its water holding capacity. Eight different concentrations (1, 2, 5, 25, 50, 100, 200 and 500 ppm) of endosulfan were added in the same soil in the pot on the interval of 7 days. The experiment was terminated at 56th day. Treatments and control (no pesticide) pots were kept in the glass house. Sterilized distilled water was added at two days interval to compensate for the loss of water by evaporation. After every seven days of intervals before adding a new concentration, soil samples were harvested for analysis of fungal population.

Isolation of pesticide tolerant fungi

Isolation of potential fungi from forest soil was done using serial dilution agar plate method (Aneja, 2005). One gram of soil (oven dry basis) was mixed with 9 ml sterilized distilled water and the suspension was diluted upto 10^{-3} dilution. Noncarbon nutrient culture medium (NCM; Herman and Frankenberger, 1999) and nonsulfur nutrient culture medium (NSM; Siddique *et al.,* 2003b) supplemented with 1 ml l^{-1} of Focht trace elements (Focht, 1994). Agar (1.5%; w/v) was added in media and then autoclaved for 30 min at 121ºC at 15 lbs psi pressure. Stock solution (10,000 ppm) of endosulfan was prepared in methanol and appropriate amount was added to sterilized medium using syringe filter (Whatman; 0.45 µm pore size) to bring the final concentration of pesticide range between 1-500 ppm. For control (without pesticides), NCM was used with *D*-glucose (1.0 g l^{-1}) as a carbon source to evaluate toxic effect of chlorpyrifos. Chloramphenicol was added in media to avoid bacterial contamination then media were poured in sterilized Petri plates. Soil suspension (0.1 ml) from 10^{-3} dilution was spread in plates and incubated for 3-7 days at 26±1ºC. Pure cultures of fungi were obtained by repeated sub culturing on PDA plates and maintained in PDA slants at 4ºC in a refrigerator.

Quantitative estimation of fungal population

Total fungal population (CFU count) and relative abundance (RA) of fungal species were calculated using following formulas (Aneja, 2005., Rodrigues and Jaiswal, 2008):

$$\text{CFU/g dry wt of soil} = \frac{\text{Number of colonies} \times \text{Dilution}}{\text{Amount of suspension plated (ml)}}$$

$$\text{RA (\%)} = \frac{\text{CFU count of a each fungal species} \times 100}{\text{Total CFU count of all species}}$$

Identification of fungi

Isolated fungi were examined through the stereoscopic binocular and compound research microscope, after making slides in water and cotton blue in lacto phenol as a mounting medium. The shape and size of conidiophores, conidial/spore arrangement and sporulation was studied. The fungi were identified with the help of identification keys, standard monographs (Gilman, 1957; Rifai, 1969; Ellis, 1971; Booth, 1971; Barnett and Hunter, 1972; Samson, 1974; Bissett, 1983; 1991 a, b, c, d; Boerema *et al.,* 2004) and available expertise.

Statistical analysis

Experiments were conducted in triplicate and data were analyzed using STATISTICA software. Data were analyzed using analysis of variance (ANOVA) and Tukey's test was used to compare means. P values less than 0.05 were

considered significant. The values are expressed as mean of three replicates ± SD (Standard Deviation).

RESULTS AND DISCUSSION

Microbes can utilize endosulfan as source of carbon and sulfur. The present investigation describes the effect of endosulfan treatment on fungal population of pine forest soil, along with isolation and identification of most tolerant fungi in two different media namely NCM and NSM supplemented with endosulfan as a sole source of carbon and sulfur, respectively.

Thirty two fungal species belonging to twelve genera viz., *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Gliocladium, Mucor*, *Mammaria*, *Paecilomyces*, *Penicillium*, *Phoma*, *Trichoderma* and *Verticillium* were isolated from pine forest soil. Data regarding per cent relative abundance of different fungal species isolated from pine forest soil was shown in Table 1. Species of *Aspergillus*, *Penicillium* and *Trichoderma* were most dominant fungi observed during the complete treatment period and contributed 1.03-17.02 per cent of total population count. There may be selective inhibition of some species in the presence of endosulfan but others rapidly appear to replace the sensitive species, thus maintaining the metabolic integrity of the soil. Out of thirty two fungal species, *Penicillium lilacinum, P. striatum, P. spinulosum* and *Trichoderma pseudokoningii* were observed in control soil in which no pesticide treatment was given and completely inhibited in endosulfan treated soil.

The repeated application of endosulfan exerted different effects on the fungal species. At lower concentrations of endosulfan (1-50 ppm) variability in fungal species was observed which was decreased with progressive increase in concentration range between 100-500 ppm. The occurrence of fungal species viz., *Alternaria alternate*, *A. humicola, Aspergillus candidus, A. flavus, A. fumigatus, Fusarium lateritium, Gliocladium deliquescens, G. fimbriatum, Mucor hiemalis, M. varians, Paecilomyces inflatus, Penicillium notatum* and *Verticillium tenuissimum* was decreased as the concentration of endosulfan increased in range between 1-50 ppm. In contrast, they were inhibited at higher concentrations (100-500 ppm). Some fungi namely, *Aspergillus niger, A. terreus, Fusarium solani, Penicillium restrictum* and *P. tardum* were initially shown more percent relative abundance at lower concentrations of endosulfan (1-50 ppm) as compared to control, while they were inhibited at higher concentrations (100-500 ppm). *Cladosporium cladosporioides, Mammaria echinobotryoides, Paecilomyces lilacinus, Penicillium decumbens, P. frequentans, Phoma fimeti, Trichoderma atroviride, T. aureoviride, T. harzianum* and *T. longibrachiatum* were observed at lower as well as higher concentrations of endosulfan and showed maximum contribution for total fungal population count.

Maximum relative abundance was recorded with *Trichoderma aureoviride* (17.02 %), followed by *T. harzianum* (15.70 %), *T. atroviride* (14.56 %), *Penicillium frequentans* (12.45 %), *P. decumbens* (10.06 %), *Cladosporium cladosporioides* (8.25 %), *Paecilomyces lilacinus* (7.05 %), *Mammaria echinobotryoides* (7.02 %), *Trichoderma atroviride* (6.78 %), *Phoma fimeti* (6.57 %) and *T. longibrachiatum* (1.37 %) at a concentration of 500 ppm (Table 1). It can be concluded that these fungi have ability to tolerate higher concentrations of endosulfan and can be utilized for degradation studies of endosulfan.

The data regarding total fungal population (CFU) count in different culture media is shown in Table 2. It was observed that maximum CFU count was recorded on control (15.96×10^3 CFU g $soil^{-1}$) in which no endosulfan treatment was given, followed by NSM (15.16×10^3 CFU g $soil^{-1}$)

in which endosulfan given as a sole source of sulfur and NCM (13.62×10^3 CFU g $soil^{-1}$) in which endosulfan was give as a sole source of carbon. The different doses of endosulfan were applied in soil and it was observed that, the total CFU count increased between 15.00-19.22×10^3 CFU g $soil^{-1}$at lower doses (1-25 ppm) as compared with control. Low concentrations of endosulfan caused a stimulatory effect on microbial population supporting the microbial growth probably utilizing them as energy or other nutrient source. However, higher concentrations (50-500 ppm) inhibited the fungal population. Minimum CFU count (8.44×10^3 CFU g $soil^{-1}$) was recorded when endosulfan was applied in the soil at a concentration of 500 ppm.

In control soil microbial population was almost constant during the complete incubation period (1-56^{th} days). Highest CFU count was recorded in soil treated with endosulfan at 2 ppm ($23.00\pm2.00\times10^3$ CFU g $soil^{-1}$) which was at par with 5 ppm ($22.00\pm1.73\times10^3$ CFU g $soil^{-1}$) in NSM medium, while the lowest CFU count was recorded in soil treated at 500 ppm on NCM ($5.33\pm1.53\times10^3$ CFU g $soil^{-1}$) which was at par with NSM ($6.00\pm1.00\times10^3$ CFU g $soil^{-1}$) at a same concentration. In all applied endosulfan doses, NSM in which endosulfan was given as sole sulfur source was supported more fungal growth in term of CFU count than NCM in which endosulfan was given as carbon source. In all the treatments, when lower concentrations of endosulfan (1-25 ppm) were applied in the soil, the fungal CFU count was increased between 17.67 ± 1.15 to $23.00\pm2.00\times10^3$ CFU g $soil^{-1}$ as compared to control which was range between 13.33 ± 1.15 to $15.33\pm0.58\times10^3$ CFU g $soil^{-1}$. In contrast the increased endosulfan doses (50-500 ppm) inhibited the fungal population in all tested media (Table 2).

The effect of a pesticide on soil microorganisms is governed not only by the chemical and the physical properties of the pesticide itself, but also by the soil type, soil properties, and prevailing environmental conditions (Malkomes and Wohler, 1983). It has been known that pesticides may stimulate, inhibit, or have no effect on microbial numbers (Grossbard, 1976). Some workers have stressed the fact that insecticides do not in general have much effect, except at concentrations greatly exceeding normal recommended field rates on soil microbial numbers and activities (Greaves, 1987; Kale and Raghu, 1989). No effects of insecticides on fungi have been reported by Sivasithamparam (1969; 1970). Fungitoxic effects of many insecticides were demonstrated by Richardson and Miller (1960), Adjust (1970) and Singh and Alma (1998) whereas stimulatory effects have been confirmed also (Neumann, 1970). In the present study, Endosulfan at low concentrations (1-25 ppm) caused a stimulatory effect on microbial population supporting the microbial growth probably utilizing them as energy or other nutrient source. However higher concentrations (50-500 ppm) inhibited the fungal population. Ismail and Enema (2005) also reported the same finding. Jayashree and Vasudevan (2007) reported that the CFU count increased in endosulfan treated soil in the presence of Tween 80 which emulsified the endosulfan, thereby increasing the amount of insecticide in contact with the soil microbes.

Microbial breakdown of pesticides is considered one of the most important activities in soil. Biological transformation of pesticides in soil, fresh water, or estuarine ecosystem, appears to be caused primarily by bacteria, actinomycetes and fungi (Bollag, 1974). In soil, it occurs when microorganisms use pesticides as a source of carbon and energy, or consume the pesticides along with other sources of food or energy (Singh and Ward, 2004; Diaz, 2004). In the present investigation, when soil treated with endosulfan,

NSM medium in which endosulfan itself given as a sole sulfur source supported the maximum CFU count at all the concentrations followed by NCM medium in which endosulfan given as carbon source. It may be concluded that endosulfan might be utilized as sulfur source more efficiently than carbon. Guerin (1999) and Sutherland *et al.* (2000) considered endosulfan as a poor biological energy source and attributed previous unsuccessful enrichment attempts to poor utilization of endosulfan as a sole source of carbon. Hussain *et al.* (2007) also reported that fungi utilized endosulfan in NSM broth as a sole source of sulfur. This is most likely attributed to the fact that carbon is required by microbial cells in the greatest amount followed by nitrogen, phosphorus, and sulfur for the construction of cell components as well as for obtaining energy. In the presence of easily available carbon source, microbes obtain maximum growth of microbes and utilize other resistant compounds as other nutrients viz., S, P, N, etc. (Alexander, 1998). In contrast to this, some microbes utilized endosulfan as a sole carbon source (Kullman and Matsumura, 1996; Awasthi *et al.*, 1997). Siddique *et al.* (2003b) also reported that the amount of endosulfan degraded by bacteria as a sulfur source was lower than the amount removed as a sole carbon source.

Table 1. Relative abundance (%) of different fungal species isolated from endosulfan treated pine forest soil.

Fungi	Endosulfan applied in soil (ppm)								
	Control	**1**	**2**	**5**	**25**	**50**	**100**	**200**	**500**
Alternaria alternate	4.98	3.21	1.84	1.15	0.68	0.45	0.12	-	-
A. humicola	2.34	1.54	1.32	1.05	0.55	0.32	0.09	-	-
Aspergillus candidus	2.03	1.85	1.55	1.20	0.95	0.51	0.25	-	-
A. flavus	1.78	1.25	1.05	0.82	0.52	0.25	-	-	-
A. fumigatus	1.23	1.15	1.01	0.75	0.48	0.12	-	-	-
A. niger	3.09	4.98	5.02	5.15	5.25	5.32	4.02	2.01	-
A. terreus	3.09	3.45	3.82	3.95	4.01	4.05	2.05	-	-
Cladosporium cladosporioides	5.09	6.75	6.95	7.02	7.12	7.15	7.55	7.85	8.25
Fusarium lateritium	1.23	1.15	1.02	0.85	0.35	0.15	-	-	-
F. solani	4.09	4.95	5.03	5.14	5.21	5.25	4.12	2.11	-
Gliocladium deliquescens	1.98	1.08	0.72	0.52	0.25	-	-	-	-
G. fimbriatum	2.56	1.08	0.85	0.56	0.28	0.12	-	-	-
Mammaria echinobotryoides	3.09	4.48	4.85	4.90	4.95	5.02	5.68	5.75	7.02
Mucor hiemalis	5.09	3.05	2.22	1.54	1.55	1.01	-	-	-
M. varians	2.09	1.56	1.15	0.95	0.75	0.5	-	-	-
Paecilomyces inflatus	2.87	2.38	2.02	1.55	0.85	0.35	-	-	-
P. lilacinus	5.89	6.35	6.62	6.7	6.75	6.8	6.84	6.92	7.05
Penicillium decumbens	5.78	6.24	6.45	6.5	6.68	7.37	7.42	7.82	10.06
P. frequentans	9.01	9.75	9.82	9.85	9.89	9.95	10.02	11.05	12.45

P. lilacinum	1.9	-	-	-	-	-	-	-	-
P. notatum	3.48	3.09	2.75	1.04	0.87	0.45	-	-	-
P. restrictum	2.91	3.43	3.65	3.72	3.82	3.92	1.15	1.06	-
P. spinulosum	2.32	-	-	-	-	-	-	-	-
P. striatum	1.04	-	-	-	-	-	-	-	-
P. tardum	3.09	3.55	3.65	3.71	3.75	3.78	1.78	0.72	-
Phoma fimeti	2.67	3.05	3.45	3.51	3.87	4.05	4.25	4.82	6.57
Trichoderma atroviride	1.03	3.55	3.99	5.06	5.51	6.02	11.56	14.02	14.56
T. aureoviride	2.09	3.68	4.98	7.05	9.00	10.23	12.08	16.02	17.02
T. harzianum	5.06	5.75	5.99	7.45	7.65	8.08	12.34	13.1	15.7
T. longibrachiatum	1.67	3.82	4.05	4.1	4.15	4.46	6.67	6.75	1.32
T. pseudokoningii	2.45	-	-	-	-	-	-	-	-
Verticillium tenuissimum	2.98	3.83	4.18	4.21	4.31	4.32	2.01	-	-

Table 2. Effect of different concentrations of endosulfan on total fungal population on different media

Days after treatment	Dose (ppm)	Total fungal population ($\times 10^3$ CFU g soil^{-1})			
		Control	**NCM**	**NSM**	**Mean**
7	1	13.33±1.15 (4.12)^{defg}	14.00±1.00 (4.15)^{defgh}	17.67±1.53 (4.25)^{fghi}	15.00 (4.17)^{cd}
14	2	15.33±0.58 (4.19)^{efgh}	17.00±1.00 (4.23)^{fghi}	23.00±2.00 (4.36)^{i}	18.44 (4.26)^{ef}
21	5	16.67±1.53 (4.22)^{fghi}	19.00±1.00 (4.28)^{ghi}	22.00±1.73 (4.34)^{i}	19.22 (4.28)^{f}
28	25	18.67±0.58 (4.27)^{ghi}	17.67±1.15 (4.25)^{fghi}	20.00±1.00 (4.30)^{hi}	18.78 (4.28)^{f}
35	50	18.00±1.00 (4.25)^{fghi}	17.67±0.58 (4.25)^{fghi}	13.00±2.00 (4.11)^{def}	16.22 (4.20)^{de}
42	100	16.33±1.15 (4.21)^{efgh}	11.67±2.08 (4.06)^{cde}	10.67±0.58 (4.03)^{cd}	12.89 (4.10)^{c}
49	200	15.33±0.58 (4.19)^{efgh}	6.67±1.53 (3.82)^{ab}	9.00±1.00 (3.95)^{bc}	10.33 (3.98)^{b}
56	500	14.00±1.00 (4.15)^{defgh}	5.33±1.53 (3.72)^{a}	6.00±1.00 (3.77)^{a}	8.44 (3.88)^{a}
Mean		15.96 (4.20)^{c}	13.62 (4.09)^{a}	15.16 (4.14)^{b}	

*Values within parenthesis are $\log_{10}$ transformed and different letters in superscripts indicate significantly different ($P \leq 0.05$, Tukey's test) mean values.

CONCLUSION

Endosulfan usually enter the soil environment in lower concentration is not considered as toxic for soil health but higher concentrations inhibited the fungal population, which may have a direct or an indirect effect on their activity or ultimately the soil fertility. Some species are sensitive to pesticides present in soil at higher concentration, but *Cladosporium cladosporioides, Paecilomyces lilacinus, Penicillium decumbens, P. frequentans, Phoma fimeti, Trichoderma aureoviride, T. atroviride, T. harzianum* and *T. longibrachiatum* have ability to tolerate higher concentrations of

endosulfan and can be utilized for its degradation studies.

ACKNOWLEDGEMENTS

The authors express thanks to the Director, Forest Research Institute, Dehradun, Uttarakhand, India, for providing necessary facilities.

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