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Isolation and screening of pesticide resistant cyanobacteria from pesticide contaminated agricultural soil

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Abstract

Pesticide contaminated soil samples (Grapes and Vegetables) were collected and analyzed from different locations of Baramati-taluka (Pune District). Cyanobacterial isolates were obtained by enrichment and isolation methods by using BG-11, Gerloff's medium. Isolated cultures were preserved in the laboratory in both liquids as well as on solid media containing Monocrotophos or endosulfan 100 mg/lit. In all twenty-two isolates obtained from ten soil samples were screened for its MCP and Endosulfan tolerance capacity in liquid media. Ten cyanobacterial cultures were found to be tolerating MCP. Among these *Synechocystis* isolated from the Grapes field (Khandaj Baramati) was found to be highest MCP tolerant (900mg/L). However among twelve endosulfan tolerating cultures isolated, *Anabaena* from vegetable field was the highest endosulfan tolerant (500 mg/L). Effect of pH and temperature on growth of cultures in presence of pesticides showed optimum growth at pH 7 and pH 6.4 for *Synechocystis* and *Oscillatoria* respectively. However, both the cultures show optimum growth at 30^{o} Celsius. .Both these cultures restore pesticide tolerance after subculturing but others did not.

Key words: Cyanobacteria, Pesticide, Screening.

Introduction

Cyanobacteria are excellent organisms to serve as models for the investigation of a wide variety of biological problems such as acting as environmental pollution indicators. In the last decade cyanobacteria were studied with numerous aspects especially with respect to their utility in bioremediation, human nutrition, as biofertilizer (Venkataraman, 1975), source of precious chemicals and some novel applications. It has been observed that the population of soil microflora responsible for degradation of pesticides in soil does not show a significant rise in number upon repeated application of the pesticide to soil. Using a most probable number technique the that larger population of EPTC (S- ethyl, N,N – depropyl carbamothioate) degrading microorganisms did not exist in soils exhibiting accelerated degradation of EPTC and that increased rates of metabolism were responsible for accelerated degradation rather than increased number of organisms. In India, consumption of pesticides is increasing @ 2-5 % pa. To date pesticide consumption ranges between 480 – 520 g /ha and this accounts for about 3% of the total pesticides used in the world. Cyanobacteria possess the ability to survive in extreme environments and are also reported to degrade xenobiotics has screening of different strains of cyanobacteria for their growth and tolerance to pesticides like monocrotophos and Malathion.

Materials and methods

Collection of soil samples

A survey of use of pesticides [endosulfan and monocrotophos (MCP)] in different agricultural fields from farmers in the Baramati region of Pune district, (MS) India was first carried out. Based on preliminary survey the research work was concentrated on two pesticides viz. monocrotophos (MCP) and endosulfan and 10 different sites of pesticide contaminated soil. Soil samples were collected as per standard protocol before one week of pesticide application. All soil samples were collected in sterile plastic bags and carried to the laboratory for further studies (Nayak, 2007).

Physicochemical analysis of soil

Soil analysis was done at Department of Microbiology, Tuljaram Chaturchand College, Baramati. Soil pH was measured in a 1:2.5 soil-water suspension using a glass electrode (Systronics, Digital pH meter, India). Soil electrical conductivity (EC) was determined by measuring the electrical conductance of soil-saturation extract with a conductivity meter (Equiptronics, India) (Pawar, 2008). Colony forming unit was (CFU) calculated by using standard plate count method.

Detection of Pesticide from soil samples

Soil samples were extracted with equal volumes of ethyl acetate for both types of pesticides endosulfan and monocrotophos (MCP). The organic phase was passed through $MgSO_4$ for endosulfan extraction, the both type of organic phases gently evaporated by vacuum evaporator and redissolved in acetone and ethyl acetate respectively. TLC plates were developed in either petroleum ether and acetone (8.5:1.5) or chloroform and ethyl acetate (3:1). For endosulfan, spots were visualized by spraying silver nitrate and exposure at UV light for 10 min (Bhalerao and Puranik 2007). In case of monocrotophos redissolved organic phase in ethyl acetate was developed in n-hexane and acetone (7.5:3) and visualized

under UV (Sharma 2005). Stock solutions (1000 mg/l) were prepared from technical grade pesticides in acetone. Working standard solution (50 mg/l) was used for standard reference compounds.

Enrichment, Isolation and purification of cyanobacterial cultures

The visual growth of cyanobacteria in respective field was collected and used for enrichment in liquid media (BG-11 medium) in the laboratory. The bottles were incubated at 24± 2ºC at 1800-2000 lux light intensity intermittently for 30 days. The growth of cyanobacteria was judged on the basis of visual observation after 30 days of incubation (Kaushik, 1987). For isolation of unicellular cyanobacteria enriched sample were streaked on respective agar plates and incubated at proper environmental conditions. Visibly distinct, well isolated cyanobacterial colonies were selected. In case of filamentous cultures, selective inoculation of single filaments under aseptic conditions was the methods of isolation used. Purification of the unicellular isolates was done using liquid media (serial dilution method) followed by solid media. Filamentous cultures were purified on solid medium by solid agar plate method.

Identification of Cyanobacteria

The cultures were further maintained in the broth medium at low intensity of light and at 20^0C temperature. On the basis of morphological characteristics cyanobacterial cultures were identified at the genus level. The morphology of isolated cyanobacteria was observed under high power microscope (Labomed, model-LX 300). The identification of isolated cyanobacteria was done using the key given by (Desikachary, 1959), (Ripika *et al.*, 1979).

Screening of pesticide resistant Cyanobacterial cultures

The cultures obtained from soil enrichment were screened for MCP and endosulfan tolerance capacity by following the gradient plate method. The MCP/ endosulfan concentration gradient was prepared by adding a base layer of 15 ml of BG-11 agar with MCP (1000 mg/l) / Endosulfan (500mg/l) to a 9 cm × 9 cm square Petri plate tilted at an angle of 30° (as shown in Fig: 1.) and the agar was allowed to solidify at room temperature into a wedge shape layer. Onto the set base, another 15 ml of BG-11 agar without pesticide was poured to give a MCP/ endosulfan gradient across the plate surface. Cyanobacterial cultures 0.1ml (2.6 x10^6) cells/ml for unicellular and for filamentous culture, properly homogenized (1 gm biomass/ 100ml) were poured along the pesticide gradient using a sterile cotton swab. Plates were incubated at 24 ± 2°C for 8 days. After incubation, the length of Cyanobacterial growth along the gradient was recorded.

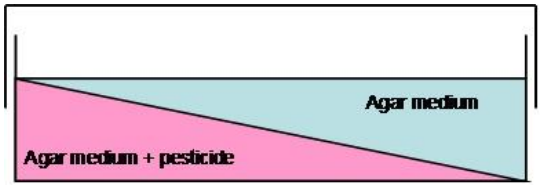

Fig: 1. Gradient plate assay

Cultures showing the highest length of growth along the gradient were selected for further experiments. In order to check the minimum inhibitory concentration (MIC) of MCP and endosulfan a series of 250 ml Erlenmeyer flasks containing 100 ml of BG-11 Medium was amended with increasing pesticide concentrations ranging from 100–1000 mg/l and 300 -500 mg/l, of MCP and endosulfan, respectively. The flasks were inoculated with 0.1ml (2.6 x10^6) cells/ml for unicellular and for filamentous culture, properly homogenized (1 gm biomass/ 100ml) incubated at 24 ± 2°C on a rotary shaker in cultivation room at 120 rpm. After 8 days of incubation the flasks were observed for Cyanobacterial growth. The MIC was noted as the concentration of MCP and endosulfan resulting in complete inhibition of Cyanobacterial growth in flasks.

Effect of pesticides on growth of cyanobacteria

To study the effect of pesticide on growth of cyanobacteria, the BG- 11 medium with Minimal inhibitory concentrations of pesticides were used. The flasks were inoculated and incubated at 24± 2ºC under 1800-2000 lux light intensity for 15 day. Effect of pesticide was observed on growth of cyanobacteria and measured in terms of its chlorophyll content (Mckinney, 1941).

Results and Discussion

Sample collection and sampling sites

From the oral information of farmers, it was concluded that up to 2005 –2006 there was a tremendous use of monocrotophos and endosulfan. According to this information monocrotophos and endosulfan were the leaders of Baramati region up to 2005 -2006. Currently, the use of monocrotophos and endosulfan has been decreased significantly. Mainly monocrotophos controls a broad spectrum of pests including sucking, chewing and boring insects and spider mites on cotton, citrus, rice, maize, sorghum, sugarcane, groundnut, potatoes, soybeans, vegetables, ornamentals and tobacco. Endosulfan controls sucking, chewing and boring insects and mites on a very wide range of crops, including fruit vines, vegetables, ornamentals, potatoes, cucurbits cotton, tea, coffee, sugarcane, tobacco, forestry, glasshouse crops, etc. The formulators and trade names of monocrotophos and endosulfan vary with diff. regions.

As per our survey report of pesticide contaminated agricultural soils 10 different sites were selected on the basis of the input of pesticides in respective fields (Table 1). The cyanobacteria are especially recognized among the photosynthetic prokaryotes for their ability to grow in a wide range of conditions (Mahendra, 1997)

Table 1. Sampling sites and crops under cultivation

Sr. No.	Name of sampling place	Crops under cultivation
1	Motibag 1	Grams
2	Pimpli 1	Cotton
3	Pimpli 2	Grapes
4	Pimpli 3	Pomegranate
5	Pimpli 4	Lady's finger
6	Tandulwadi 1	Spinach
7	Tandulwadi 2	Grapes
8	Tandulwadi 3	Brinjal
9	T. C. College road 1	Grapes
10	T. C. College road 2	Grams

Physicochemical analysis of soil

The mean values of various physicochemical parameters are given in Table 2. pH was found in between 7.0 to 9. Electrical conductivity of soil ranges from 0.10 to 0.95 dS/m. The highest SPC was observed at 0.11 EC and 7.8, pH.

Table 2. Physicochemical analysis of soil

Sr. No.	Name of sampling place	pH	Electrical conductivity (dS/m)	SPC (CFU×10^8/gm)
1.	Motibag 1	7.8	0.11	48
2.	Pimpli 1	8	0.85	40
3.	Pimpli 2	9	0.9	30
4.	Pimpli 3	7.2	0.6	26
5.	Pimpli 4	7.9	0.7	29
6.	Tandulwadi 1	7.0	0.5	32
7.	Tandulwadi 2	7.2	0.55	24
8.	Tandulwadi 3	7.2	0.8	36
9.	T. C. College road 1	7.5	0.2	15
10.	T. C. College road 2	7.3	0.75	42

Pesticide residues can adversely affect the soil biota and biological processes, depending on the soil and climatic conditions, and management practices. Various microbial groups such as bacteria, fungi, blue green algae, and microflora (micro arthropods) are all influenced to a different degree by different types of pesticides. Soil microbes have a primary catabolic role in the environment and contribute to the global cycling of carbon, nitrogen, sulfur, phosphorus and other elements through degradation of plants and animals residues. Among the soil microbes, bacteria are most abundant in soil varying from 10^6 to 10^{14} per g soil. They extensively participate in all the vital organic transactions to support the higher forms of life and occupy a significant position in the global cycling of nutrients. The numbers of bacteria occurring in soils are usually higher than those of the other groups, however, because of their small size in relation to the large cell size and extensive filaments of the other groups, bacteria account for less than half of the total microbial biomass in soil (Alexander, 1977).

Isolation and identification of cyanobacterial cultures

Occurrence of one heterocystous, five non-heterocystous genera were found. Out of those four unicellular genera was seen during the present investigation (Table. 3). Dominance of non -heterocystous genera over heterocystous cyanobacterial genera was in accordance with earlier reports (Tiwari 2001). However more modern approaches in the last four decades have emphasized important structural and molecular characteristics of these organisms with plantlike organisms (Stanier and Cohen- Bazire, 1977; Ripika et al. 1979). Knowledge of the full diversity and evolutionary hierarchy of cyanobacterial taxonomic entities is still unclear (Tripathi, 2006).

Table 3. Identification of cyanobacteria

Name of sampling place	Isolate identified
Motibag 1	*Anabaena spp.*
Pimpli 1	*Synechocystis spp.*
Pimpli 2	*Gloeothece spp.*
Pimpli 3	*Synechococcus spp.*
Pimpli 4	*Synechocystis spp.*
Tandulwadi 1	*Oscillatoria spp.*
Tandulwadi 2	*Chroococcus spp.*
Tandulwadi 3	*Synechocystis spp.*
T. C. College road 1	*Synechococcus spp.*
T. C. College road 2	*Anabaena spp.*

Detection of Pesticide from soil samples

All collected soil samples from Baramati region were primarily analyzed by thin layer chromatography to know that whether there is a need of this study or not. The significant number of soil samples did show presence of both pesticides even after six days after application. No detection of pesticides in few samples for both pesticides might be due the transformation of pesticides other intermediate metabolites, e.g. endosulfan gets converted to endosulfan-sulfate. In literature numerous studies have reported the environmental fate of endosulfan in different types of contaminated soils (Antonious *et al.*, 1998; Kaur *et al.*, 1998). Vig *et al.* (2001b) reported the detection of endosulfan in 0-15 cm layer of soil, which was reduced gradually to 11.22% in 30 days with a half life of 9.49 days. Dependence of endosulfan persistence on the initial concentration of residues was observed by Rao and Murty (1980). Less persistence of endosulfan residues in soil was reported by many workers (Stewart and Cairns, 1974; Rao and Murty, 1980; Agnihotri *et al.*, 1996). Persistence of monocrotophos was observed until 45 days in cotton soils and half life was ranged from 6.7 to 11.6 days (Vig *et al.*, 2001c). The fate of pesticides in soil is dependent on involvement of both biotic and abiotic factors (Gundi and Reddy, 2006).

Screening of pesticide resistant Cyanobacterial cultures

Pesticide gradient plate assay was applied to screen the isolates for highest tolerance to monocrotophos and endosulfan independently. Growth performance was recorded as length of growth (in cm) across the pesticide gradient (Table 4). Among the isolates tolerating endosulfan, identified as *Anabaena spp.* had exhibited highest tolerance to endosulfan. From the seven monocrotophos tolerating isolates, identified as *Synechocystis spp.*, had the highest length of growth (8.8 cm) along the gradient exhibiting the highest tolerance. Cultures showing growth of >5 cm on gradient plate

were further assessed using broth assay. Results of the gradient plate assay were confirmed by growing these isolates in broth cultures with varying pesticide concentrations, 100-1000 mg/l MCP and 100-500 mg/l endosulfan. The *Synechocystis spp.* could tolerate 900 mg/l of MCP and grow well up to 500 mg/l of Endosulfan. The isolate, *Anabaena spp.* showed luxuriant growth up to 500 mg/l of endosulfan. Based on these results of *Anabaena spp.* and *Synechocystis spp.* was selected for further biodegradation studies.

Table 4. Screening of pesticide resistant Cyanobacterial cultures

Isolate No.	Identification	Growth on gradient plate (cm)		MIC* (mg/l)	
		Monocrotophos	Endosulfan	Monocrotophos	Endosulfan
1	*Oscillatoria spp.*	3.2	5.6	500	300
2	*Synechocystis spp.*	8.8	7.1	900	400
3	*Anabaena spp.*	7.5	7.9	800	500
4	*Gloeothece spp.*	6.2	4.7	700	300
5	*Chroococcus spp.*	5.9	6.9	800	200
6	*Synechococcus spp.*	7.2	5.1	600	300

MIC*: the concentration of pesticide resulting in inhibition of growth of cyanobacteria

Microorganisms play an important role in the metabolism of organochlorine insecticides. However, the persistence of a number of organochlorine insecticides in soil and water for very long periods has been reported. This may be either due to the resistance of the insecticide to microbial degradation or to the formation of a complex with some component of the environment which is largely resistant to microbial attack (Aleder, 1965). Many reviewers have indicated the effects of pesticides on soil microorganisms. There are increasing evidences to suggest that even at normal field rates, insecticides have some impact on soil microorganisms.

Effect of pesticides on growth of cyanobacteria

Prolonged persistence of pesticides in soil can have an adverse impact on the soil health and its ability to sustain productivity (Kookana *et al.*, 1998). In contrast to most aquatic ecosystems, soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts. Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant of acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence on the ability of microbial populations to degrade pesticides. Environmental fate of organic pollutants in soils is influenced significantly by the pH and texture of the soil, and also the presence of organic matter and co- pollutants (Awasthi *et al.*, 2000). Persistence of pesticides, observed in present study could be attributed to physicochemical properties of soil. The average pH of soils under investigation was around neutrality. Awasthi *et al.* (2000) reported increased degradation of endosulfan under un-inoculated conditions when pH of soil was in alkaline range.

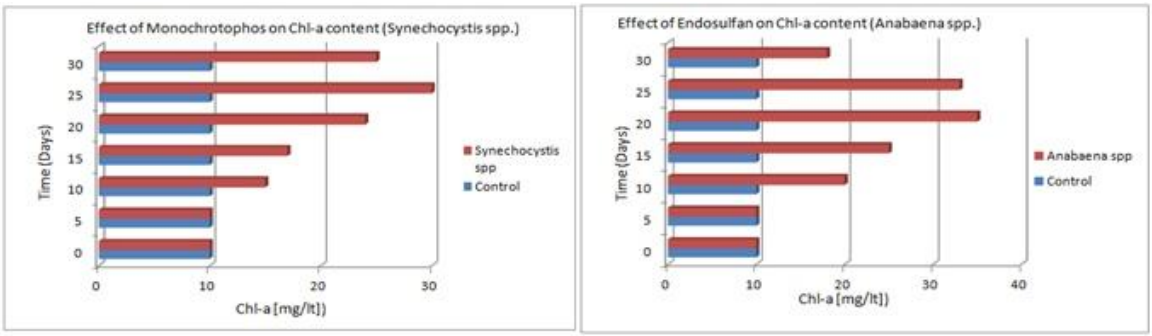

Fig. 1(a) Effect of Monocrotophos on *Synechocystis spp.*; (b) Effect of Endosulfan on *Anabaena spp.*

The effect of monocrotophos and endosulfan on growth of *Synechocystis spp. and Anabaena sp.* were studied respectively. The isolate *Synechocystis spp.* could tolerate 900 mg/l of monocrotophos. The figure 1 (a) showed that on 25th day incubation Chl-a was highest (30 mg/lit). The isolate, *Anabaena spp.* showed luxuriant growth up to 425 mg/l of endosulfan. The figure 1 (b) showed that on 20th day incubation Chl-a was highest (35 mg/lit). Whereas fig 2 (a) and 2(b) showed that effect of monocrotophos and endosulfan on pH of soil in the presence of *Synechocystis spp. and Anabaena sp.* respectively.

In fig. 2(a) pH of soil was decreased after 5th day of incubation. In fig. 2(b) pH of soil was decreased after 10th day of incubation. As there is decrease in pH, it indicates that cyanobacteria could degrade the pesticides present in soil.

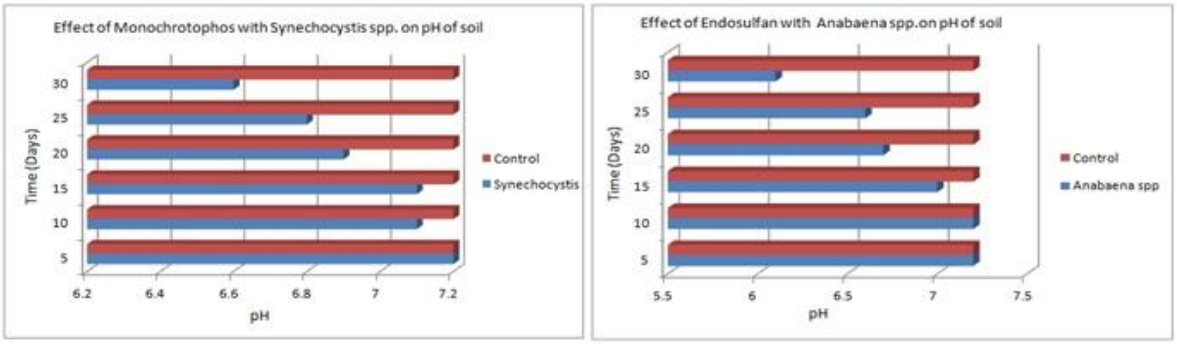

Fig. 2 (a) Effect of Monocrotophos on pH of soil (*Synechocystis spp.*); (b) Effect of Endosulfan on pH of soil (*Anabaena spp.*)

Extensive and intensive use of pesticides in agro-ecosystem has resulted in a global contamination of the environment. Besides combating insect pests, insecticides also affect the population and activity of beneficial microbial communities in soil (Singh and Parsad, 1991 and Bhuyan *et al*., 1992). In soil, it may alter the microbial activities (Bhuyan *et al.*, 1992) and have impact on microbial population (Ambrogioni *et al*., 1987). This may lead to stimulation, reduction or modification of soil biological process, which are essential for soil fertility and crop yield (Heinonen-Tanski *et al.*, 1985). Possibility that insecticide residues in soil may have deleterious effects on soil microorganisms and their activities has received considerable attention (Iqbal *et al.*, 2001). Insecticide concentrations exceeding the normal recommended field rates have effect on the soil microbial population and their activities (Tu and Miles, 1976).

Conclusion

This study based on the present survey conducted, monocrotophos and endosulfan emerged as the major pesticides used in the Baramati region. The physicochemical analysis revealed the effect of pesticides on soil properties. Although, the effect of these pesticides seems to be temporary, the results have aimed at deleterious effects. Thin layer chromatographic detection method showed the persistence of monocrotophos and endosulfan in the soil at least for a week. These observations had given direction to the study. Based on these observations further studies were restricted to pesticides, monocrotophos and endosulfan. Pesticide resistant cyanobacterial cultures were isolated from selected soil samples. Dominance of non-heterocystous genera over heterocystous cyanobacterial genera was confirmed with earlier reports which may play a unique role in bioremediation of pesticide contaminated soil.

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