World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:5, No:3, 2011

An Evaluation of Pesticide Stress Induced Proteins in three Cyanobacterial Species- *Anabaena Fertilissima*, *Aulosira Fertilissima* and *Westiellopsis Prolifica* using SDS-PAGE

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***Abstract*—**The whole-cell protein-profiling technique was evaluated for studying differences in banding pattern of three different species of Cyanobacteria i.e. *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica* under the influence of four different pesticides-2,4-D (Ethyl Ester of 2,4-Dichloro Phenoxy Acetic Acid), Pencycuron (N-[(4-chlorophenyl)methyl]-N-cyclopentyl-N'–phenylurea), Endosulfan (6,7,8,9,10,10hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide) and Tebuconazole (1-(4-Chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol). Whole-cell extracts were obtained by sonication treatment (Sonifier cell disruptor -Branson Digital Sonifier S-450D, USA) and were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analyses of the total protein profile of *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica* showed a linear decrease in the protein content with increasing pesticide stress when administered to different concentrations of 2, 4-D, Pencycuron, Endosulfan and Tebuconazole. The results indicate that different stressors exert specific effects on cyanobacterial protein synthesis.

***Keywords*—**Cyanobacteria, pesticide, SDS-PAGE

I. Introduction

LIVING organisms, especially micro-organisms, are exposed to various types of natural stresses, such as nutrient limitation, pesticides, pollution, drought, salinity, temperature, pH, light intensity and quality, etc. Cyanobacteria, a group of prokaryotic, oxygen-evolving, photosynthetic Gram-negative bacteria, survive in a wide variety of extreme environmental conditions [1]. Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no or low cost [2]. These cyanobacteria are exposed to pesticides which are indispensable to the modern agricultural practice. However, the use of these pesticides over the years has resulted in problems caused by their interactions with the biological systems in the environment and has deleterious effects on cyanobacteria [3]. To survive in the stressful conditions all organisms including bacteria will have to adopt different strategies at ecological, physiological, biochemical and molecular level. A molecular technique that has proved to be useful in typing bacterial strains is sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell bacterial proteins, wherein differences seen in protein bands in different circumstances have been successfully used to group tolerant bacteria [4].

A Protein in the cyanobacterial thylakoid membranes was identified as a sensitive protein to environmental stress conditions: under various unfavorable conditions like drought, nutrition deficiency, heat, chemical stress, ozone fumigation as well as UV-B and visible light stresses can influence the turnover of protein [5].

Many species are capable of not only surviving, but thriving in conditions previously thought to be inhabitable, tolerating desiccation, high temperatures, extreme pH, high salinity and pesticides illustrating their capacity to acclimate to extreme environments [6]. Rath and Adhikary, [7] demonstrated that the exposure of estuarine cyanobacterium *Lyngbya aestuarii* to UV-B radiation resulted in differential expression of cellular proteins. In addition, toxicity tests of Tebuconazole on *W.prolifica* confirmed inhibition in the growth and metabolic activities [8]. Bhargava et al. [9] expressed copper-induced changes in protein profiling of *Anabaena doliolum* subjected to short- and long-term treatments. SDS PAGE analyses of the total protein profile of *Anabaena* sp. showed a linear decrease in the protein content with increasing UV exposure time [10]. Changes in protein profiling newly formed proteins might be helping Cyanobacterium to tolerate adverse conditions [11]. Moreover, Nirmal Kumar et al. [12] studied the differential effects of 2, 4-D on pigments, metabolites and enzyme activities of three species of cyanobacteria.

The aim of the work was to study protein profile changes and differentially expressed proteins in three cyanobacterial species such as *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica*, exposed to 2,4-D, Endosulfan, Pencycuron and Tebuconazole as the most frequent soil contaminants in paddy fields.

II. Materials And Methods

A. Cyanobacteria Strains, Growth Conditions And Pesticide Treatment

Axenic cultures of *Anabaena fertilissima* Rao, *Aulosira fertilissima* Ghose and *Westiellopsis prolifica* Janet were obtained from National Facility for Blue-Green Algae, Indian Agricultural Research Institute, New Delhi and were grown photoautotrophically in nitrogen free BG_{11} medium [13] within controlled temperatures (25±2°C) under 3000 lux light with a photoperiod of 14: 10 h. Endocel (35% EC, Endosulfan

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manufactured by Excel Crop Care Ltd, Gujarat, India), Folicur (25.9 % EC, Tebuconazole manufactured by Bayer Crop Science, Mumbai), 2,4-D (38% EC, 2,4-D ethylester manufactured by Northern pesticides limited, Haryana) and Monceren (22.9% SC, Pencycuron manufactured by Bayer Crop Science, Mumbai) were used for the present study (Table: 1). Exponentially grown cyanobacterial cells were used throughout the experiment and organisms were subjected to various selected concentrations of the pesticides based upon a set of experiments for determination of LC_{50}. LC_{50} values of the organisms were determined in terms of quantitative estimation of chlorophyll-a of the pesticide treated cyanobacterial species and accordingly, various concentrations of the pesticides were used in all further experiments (Table: 2). Treated samples were analyzed for protein profiling by SDS-PAGE at intervals of four days and sixteen days respectively.

B. *Protein Extraction In Cyanobacteria*

The cyanobacteria cultures were centrifuged at 10 600 g for 5 min, the medium was poured out and cultures were resuspended in 80% acetone solution and sonicated using a sonifier cell disruptor (Branson Digital Sonifier S-450D, USA) for 20 s each in an ice bath, with 40 s cooling breaks up to one minute at 70% intensity. The sonicated samples were left overnight at 4°C. The samples were then centrifuged at 10,600 g for 5 min and the pellets obtained were suspended in the solubilization buffer containing 7.5 ml of ultra-pure water, 2.5 ml of 1MTris–HCl pH 6.8, 16 ml of 10% SDS, and 1 ml of 80% glycerol (v/v) [14]. Finally, Laemmli [15] sample buffer containing deionized water, β-mercaptoethanol, Sodium Dodecyl sulphate (SDS), 1M Tris-Hcl (pH 6.8), glycerol and bromophenol blue at a ratio of 2:1 was added to the samples followed by 3 min boiling.

C. Sodium Dodecyl Poly Acrylamide Gel Electrophoresis (Sds-Page) Assay

The extracted whole cell proteins from the isolates together with higher and lower range of protein molecular weight marker (obtained from Bangalore Genei) were mixed with SDS PAGE sample buffer in a 2: 1 ratio and the mixtures were heated in a heater block for 3 min at 100°C. After cooling the samples at room temperature, the insoluble materials were removed by centrifugation. The supernatants thus obtained were submitted to SDS-PAGE [15] followed by electrophoresis at 70 V until the bromophenol blue dye front reaches the bottom of the gel. Following electrophoresis, the gel was stained overnight with Coomassie Blue R-250 and then destained in the same solution. Finally, the whole cell protein profiles of the samples were visualized under Trans white light and captured using (Alpha Imager, EP). All experiments were performed in three independent replicates and only those spots present in at least two gels of the independent set were taken for analysis.

III. Results

Comparing the treatment for each strain with its corresponding control, the appearance of several differentially expressed significant protein bands in all the three cyanobacterial species was visualized after exposure to different concentrations of 2,4-D, Pencycuron, Endosulfan and Tebuconazole. In addition to several up regulated and down regulated proteins, newer protein bands appeared in treated cultures when compared to the corresponding control.

A. Changes In Polypeptide Pattern Of Anabaena Fertilissima Under Pesticide Stress

Synthesis of several proteins declined with increasing exposure duration to 2, 4-D and Pencycuron. At the same time, synthesis of a new set of proteins was induced. *Anabaena fertilissima* treated with 15 ppm had a protein pattern identical to that of the untreated cultures after 4-days and 16-days of exposure to 2, 4-D. In contrast, after 4 days of incubation 30 ppm and 60 ppm 2,4-D treated cultures showed new band of around 46 kDa but protein bands of 74, 50, 28, 24, 23 and 21kDa were disappeared after 16 days of treatment (Fig.1). Loss of all the protein bands was registered in the culture treated with 30 ppm and 60 ppm 2, 4-D.

It is evident from the results recorded after 4-days that there was no difference in the number of the protein bands in *Anabaena fertilissima* treated with 15 ppm, 30 ppm and 60 ppm Pencycuron but band intensity was less in comparison with control cultures. When culture was grown on 15ppm Pencycuron after 16-days 61, 28, 24, 23 and 21 kDa proteins were not detected. Synthesis of a new set of proteins of approximately 43 and 41 kDa was observed after 16 days of incubation at 30 ppm Pencycuron. However, only three protein bands were visible at 60 ppm after 16-days of exposure to Pencycuron from which intensity of protein band of molecular weight 50 kDa was decreased as a result of Pencycuron treatment (Fig.1).After 4 days few higher molecular weight bands of 59kDa and 53kDa were produced under Endosulfan stress conditions in *Anabaena fertilisisma*. After 16 days, remarked absence of 68 kDa and 48 kDa proteins were observed in 6 ppm and 12 ppm treatments. On the contrary, newer polypeptides of 70 kDa, 60 kDa and 50 kDa in 6ppm and 52 k Da was observed in 12 ppm of Endosulfan exposure (Fig.1). Protein profiling by SDS-PAGE revealed sharp distinct bands for *Anabaena fertilisisma*, indicating the presence of several high molecular weight proteins during the initial treatment periods of Tebuconazole (7.5 ppm). Many bands of 28 kDa, 24 kDa, 23 kDa and 21 kDa were absent in all the treatments of Tebuconazole after 4 days. However, several newer proteins were observed in the treated samples after16 days. Lower molecular weight proteins of 45, 35, 29, 25 and 24 kDa were identified in 7.5 ppm. Tebuconazole induced stress confirmed elimination of many proteins such as 68, 58, 52 and 30 kDa and expression of newer higher molecular weight proteins like 94 kDa and 48 kDa in 15 ppm and 93 kDa, 76 kDa, 61 kDa, 50 kDa and 28 kDa in 30 ppm (Fig.1).

B. Changes In Polypeptide Pattern Of Aulosira Fertilissima Under Pesticide Stress

Exposure to 2, 4-D for 4-days resulted in a decline in protein bands of molecular weight of 18 and 16 kDa at 20 ppm and 80 ppm. Further at 40 ppm there was overcome in the intensity of two protein bands of 65 and 60 kDa, whereas at 80 ppm these bands were dense as compare to control and other treatments after 4-days of incubation. After 16-days of exposure to 20 ppm 65, 60 and 55 kDa proteins were not detected, while at 40 ppm weak bands of the same kDa were recovered. However, the intensity of 48 kDa protein was sharply decreased at 80ppm, while 65 and 60 kDa proteins were less affected than 29, 18 and 16 kDa which were completely eliminated after 16-days of incubation (Fig.2).During Pencycuron stress after 4-days revelation the 55 kDa protein could not be detected in the cells treated with 15 ppm, 30 ppm, and 60 ppm. After 4-days Polypeptides with molecular masses of 48, 44, and 29 kDa were detected in cells grown in 30 ppm and 60 ppm Pencycuron amended medium while another polypeptide of 65 and 60 kDa was found only in cells grown in medium amended with 60 ppm Pencycuron. Two polypeptides of 18 kDa and 16 kDa were inhibited in cells treated with 15 ppm, 30 ppm and 60 ppm Pencycuron. One very prominent band appeared at around 29 kDa which was comparatively little affected even after 16-days of Pencycuron exposure in all three concentrations. After 16-days in 30 ppm and 60 ppm Pencycuron, the protein bands of molecular weight 18 and 16 kDa almost disappeared while the intensity of 48 kDa was decreased. In contrast these polypeptides which disappeared due to Pencycuron treatment appeared at 15 ppm. Maximum bands were disappeared at 60 ppm of Pencycuron treatment. Thus the degree of reappearance of this polypeptide is concentration dependent (Fig.2).Protein profiles of Endosulfan and Tebuconazole treated *Aulosira fertilissima* expressed as many as approximately 38 bands (Fig.2). A marked degradation of all proteins was noticed in 30 ppm and 60 ppm treatments of Endosulfan after 4 days. Moreover, 15 ppm of Endosulfan eliminated certain proteins of 63 kDa, 60 kDa, 55 kDa and 48kDa as compared to the control which demonstrated as many as seven protein bands. However after 16 days, newer protein bands having molecular weights ranging of 16 kDa, 28 kDa, 48 kDa and 59 kDa in 15 ppm and 63 kDa, 57 kDa and 29 kDa in 60 ppm were recorded ,but no bands were observed in 30 ppm. Initially, increase in proteins was noted with rising treatments of Tebuconazole in *Aulosira fertilissima*. After 4 days, proteins were degraded in 15 ppm of Tebuconazole, where as a single protein band of 47 kDa was recorded in 30 ppm treatment. Two newer protein bands having 46 and 31 kDa molecular weights were identified in 60 ppm. Moreover, an increase in the proteins was recorded in all the treated samples after 16 days. Polypeptides having 29, 48 and 61 kDa in 15ppm, 31 kDa and 48 kDa in 30 ppm and 51 and 33 kDa in 60 ppm were noted (Fig.2).

C. Changes In Polypeptide Pattern Of Westiellopsis Prolifica Under Pesticide Stress

Different concentrations of the pesticides challenge did not seem to have any effect on the protein synthesis (7.5, 9.0, and 30.4 kDa) of the *Westiellopsis prolifica* after 4-days of incubation. At the end of 16-days only two protein bands of 16 and 14 kDa were observed at 60 and 120 ppm 2,4-D and the rest of the bands which otherwise appeared in the untreated control and 30 ppm were completely eliminated (Fig.3). Furthermore, protein pattern were not severely affected but intensity of bands were decreased within 16-days of 100 and 200 ppm Pencycuron exposure. These observations suggest that the decrease in the amount of protein band is days and concentration dependent along with the tolerance of the tested organism (Fig.3).Significant changes were observed in either Endosulfan or Tebuconazole treated *Westiellopsis prolifica* after 4 and 16 days (Fig 3). Proteins ranging from 15 kDa, 20 kDa and 22 kDa in 10ppm, 16 kDa, 20 kDa and 22 kDa in 20ppm and 21 kDa and23 kDa in 40 ppm of Endosulfan after 4 and 16 days were observed. On the other hand, a decline in the number of protein bands were observed in Tebuconazole treated samples as compared to the control. No proteins were observed when treated with the highest concentration (60 ppm) of Tebuconazole, whereas proteins ranging of 17 kDa, 52 kDa and 94 kDa in 15 ppm and 17 kDa and 94 kDa in 30 ppm of Tebuconazole were registered.

IV. Discussion

Exposure to the pesticides resulted in a qualitative and quantitative regulation of individual proteins in the cultures. Synthesis of a wide spectrum of proteins is either curtailed or enhanced, and in addition, synthesis of a specific set of proteins is coordinately induced de novo. This was manifested through appearance of protein bands in all the species under study. Changes in protein profiling and newly formed proteins might be helping Cyanobacteria to tolerate adverse stress conditions [16].The present study found that *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica* had 4 basic differences in terms of protein content under the stress conditions used: 1. Production of some new proteins not present in the untreated cultures; 2. Inhibition of some proteins that are produced by the untreated cultures; 3. Increase in the level of expression of some proteins; 4. Decrease in the level of expression of some proteins that are present in the untreated cultures. All four of the differences are directly associated with the response of different blue-green algal species to 2, 4-D, Pencycuron, Endosulfan and Tebuconazole stress conditions. Our results tend to agree with Rajendran et al. [17] who reported that the production of novel proteins or the increased production of already existing proteins, which are only produced under stress conditions due to stress response.A successive decrease in the protein profiling pattern was observed in the present investigation which could be the result of high concentrations of pesticide and time dependent stress. SDS protein profile of the UV irradiated *Lyngbya* cells showed repression of 20 and 22 kDa proteins [7]. However,

irradiation with UV-B for 6–24 h led to overproduction of 84, 73, 60, 46, 40, 37 kDa proteins, and possibly conferring protection to the organism from UV-B.Among the four tested pesticides, 2, 4-D and Tebuconazole expressed the most deleterious effect on the protein synthesis, remarkably inhibiting protein production of *Anabaena fertilissima* and *Westiellopsis prolifica* respectively. However, no species can be always identified as the most or least susceptible [18]. To overcome the stress, organisms generally might have two types of defense strategies: (i) mechanisms that prevent interaction with the stress factors and (ii) those that counteract the stress-induced damages [19].

V. Conclusion

Experiments were conducted with a view to determining the deleterious and differential effects of four pesticides-2,4-D, Pencycuron, Endosulfan and Tebuconazole on the protein profiling of *Anabaena fertilissima, Aulosira fertilissima* and *Westiellopsis prolifica*. However, the effect of pesticides on the populations of nitrogen-fixing cyanobacteria in rice fields also depends on pesticide concentrations, and the water regimes of flooding or non-flooding associated with paddy rice fields [20]. Moreover, toxicity is affected not only by the types of pesticide, but also by the taxonomic groups and species.

Acknowledgements

Authors thank UGC (United Grants Commission, NewDelhi) for financial assistance in support of the study.

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TABLE I
PROPERTIES OF DIFFERENT XENOPHOBIC COMPOUNDS SELECTED FOR STUDY

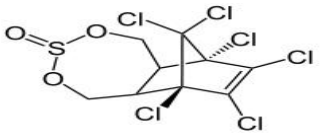
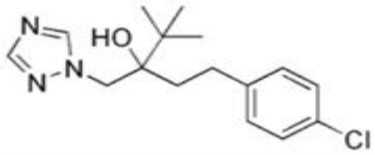
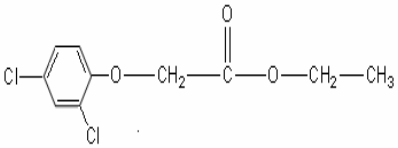
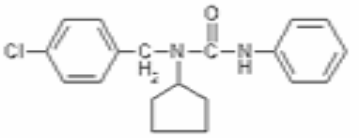
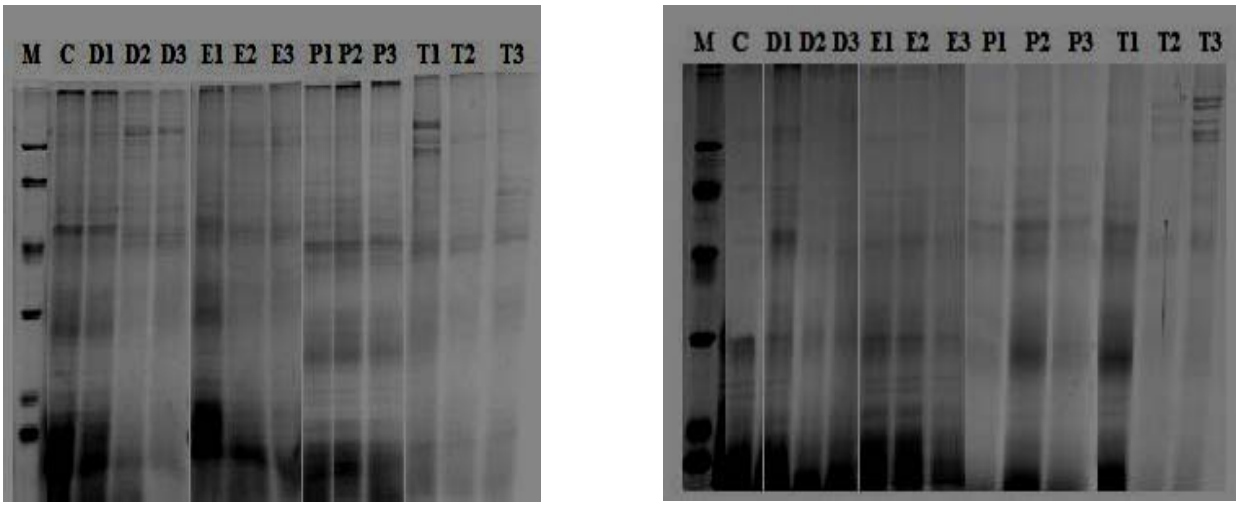
Xenobiotic compound	Class of compound	Structure	IUPAC name
Endosulfan	Organochorine Insecticide		6,7,8,9,10,10 hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide
Tebuconazole	Triazole Fungicide		1-(4-Chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol
2,4-D	Phenoxy Herbicide		Ethyl Ester of 2,4-Dichloro Phenoxy Acetic Acid
Pencycuron	Urea Fungicide		N-[(4chlorophenyl)methyl]-N-cyclopentyl-N'–phenylurea

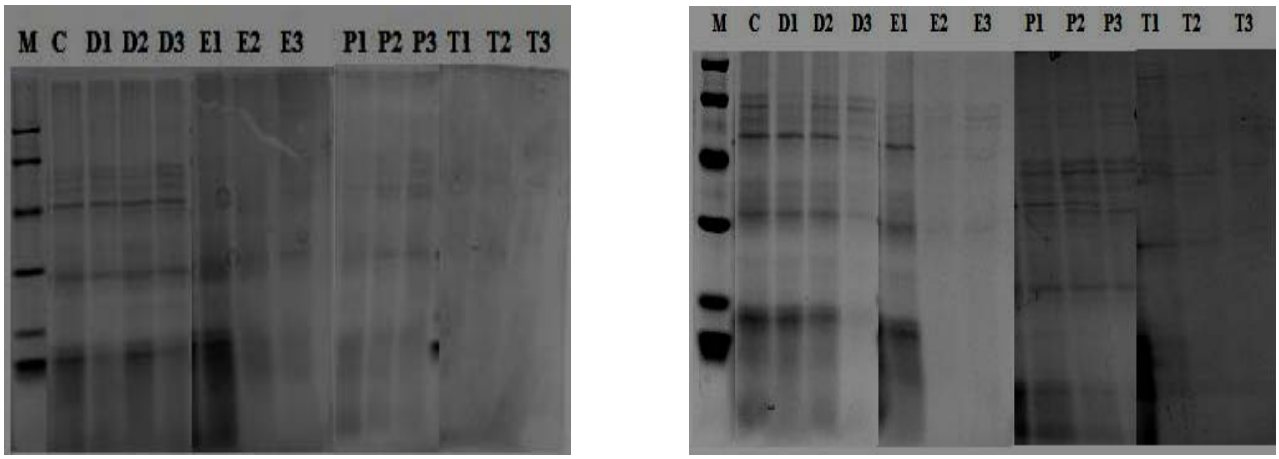
TABLE II
LC_{50} VALUES AND TREATMENTS CHOSEN FOR SELECTED TEST ORGANISMS IN RESPONSE TO DIFFERENT PESTICIDES

Xenobiotic compound	Organisms selected for study	LC_{50} values determined (ppm)	Treatments decided based upon LC_{50} (ppm)
Endosulfan	*Anabaena fertilissima*	6	3 6 12
	Aulosira fertilissima	30	15 30 60
	Westiellopsis prolifica	20	10 20 40
Tebuconazole	*Anabaena fertilissima*	15	7.5 15 30
	Aulosira fertilissima	30	15 30 60
	Westiellopsis prolifica	30	15 30 60
2,4-D	*Anabaena fertilissima*	30	15 30 60
	Aulosira fertilissima	40	20 40 80
	Westiellopsis prolifica	60	30 60 120
Pencycuron	*Anabaena fertilissima*	30	15 30 60
	Aulosira fertilissima	30	15 30 60
	Westiellopsis prolifica	100	50 100 200



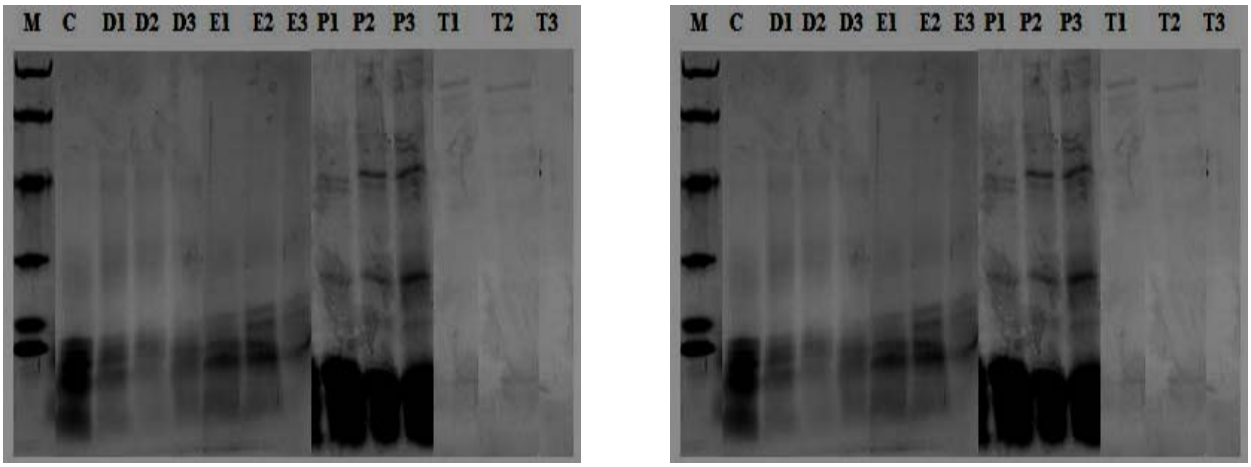
A **B**

Fig. 1 Effects of four pesticides on polypeptide patterns of total proteins from *Anabaena fertilissima* as analyzed on SDS-PAGE. Lanes C, D, E, P and T (1-3) from left to right represent proteins extracted from control and respective chosen concentrations of 2,4-D (D), Endosulfan(E), Pencycuron (P) and Tebuconazole (T) treated *Anabaena fertilissima* after 4 days (**A**) and 16 days (**B**) of treatment, respectively, and lane M represents the molecular weight marker. Equal amounts of proteins were loaded into each well.



A **B**

Fig. 2 Effects of four pesticides on polypeptide patterns of total proteins from *Aulosira fertilissima* as analyzed on SDS-PAGE. Lanes C, D, E, P and T (1-3) from left to right represent proteins extracted from control and respective chosen concentrations of 2,4-D (D), Endosulfan(E), Pencycuron (P) and Tebuconazole (T) treated *Aulosira fertilissima* after 4 days (**A**) and 16 days (**B**) of treatment, respectively, and lane M represents the molecular weight marker. Equal amounts of proteins were loaded into each well.



A **B**

Fig. 3 Effects of four pesticides on polypeptide patterns of total proteins from *Westiellopsis prolifica* as analyzed on SDS-PAGE. Lanes C, D, E, P and T (1-3) from left to right represent proteins extracted from control and respective chosen concentrations of 2,4-D (D), Endosulfan(E), Pencycuron (P) and Tebuconazole (T) treated *Westiellopsis prolifica* after 4 days (**A**) and 16 days (**B**) of treatment, respectively, and lane M represents the molecular weight marker. Equal amounts of proteins were loaded into each well.