RESEARCH PAPER:

Protein profiling of nitrogen fixing cyanobacteria under pesticide stress by SDS-PAGE

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SUMMARY

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (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 different concentrations of Endosulfan and Tebuconazole. SDS-PAGE protein profile after pesticide stress revealed a decline in the synthesis of several proteins but at the same time, synthesis of a new set of proteins was induced after 4 and 16 days of incubation. However, complete elimination of the many protein bands occurred after sixteen days of exposure. The results indicate that different stressors exert specific effects on cyanobacterial protein synthesis.

Key words : *Anabaena fertilissima, Aulosira fertilissima,* protein, SDS-PAGE, *Westiellopsis prolifica*

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Cyanobacteria are among the most known widespread, morphologically distinct and abundant prokaryotes. They are oxygenic photosynthetic autotrophs, originally considered as a class of algae, the blue-green algae possessing a unique ability in fixing atmospheric nitrogen, (Holt *et al.*, 1994). With an extraordinary biosynthetic potential and a repertoire of diverse metabolic activities, they are one of the dominant genera in various ecological habitats, especially in rice fields. *Anabaena fertilissima, Aulosira fertilissima and Westiellopsis prolifica,* photoautotrophic cyanobacteria constitute an important fraction of the N-fixing microflora of the paddy. Increased production of rice for meeting the food demand of the ever-growing population requires enormous use of fertilizers and pesticides, resulting in heavy contamination of paddy fields and the cyanobacteria inhabiting therein.

Cyanobacteria are known to adapt to environmental stresses by suitably modifying their proteome (Apte and Bhagwat, 1989). Rajendran *et al.* (2007) detected the presence of newer poypeptides in *Tolypthrix scytonemoides* in response to a fungicide, insecticide and a biopesticide by SDS-PAGE. Photosynthetic, biochemical and enzymatic investigation of *Anabena fertilissima* in response to an insecticide-hexachloro-hexahydro-methano-benzodioxathiepine-oxide was also studied by Kumar *et al.* (2009). The organochlorine insecticide showed to be deleteriously affecting the activities in the cyanobacterium, *Anabena fertilissima*.

Moreover, Suroz and Palinska (2004) have studied the effect of different doses of copper on the SDS–PAGE protein profile of *Anabaena flos-aquae* and demonstrated down-regulation of the synthesis of a large number of proteins and up-regulation of only one protein of 55 kDa. In view of the above, there appears a complete lack of information on the pesticide-induced stress stimulation N-fixing cyanobacteria in particular. The current study was aimed at studying protein profile changes and differentially expressed proteins in three cyanobacterial strains, *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica*, exposed to Endosulfan (insecticide) and Tebuconazole (fungicide) as most widely used pesticides in paddy fields in Gujarat, India.

MATERIALS AND METHODS

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, IARI, New Delhi and were grown photoautotrophically in nitrogen free BG11 medium (Rippka *et al.*, 1979) within controlled temperatures (25±2°C) under 3000 lux light with a photoperiod of 14: 10 h. Among the different pesticides, Endosulfan as a broad spectrum insecticide as well as an acaricide to control aphids, beetles, foliar, etc and Tebuconazole as a systemic fungicide against sheath blight of rice need special significance and exponentially grown cyanobacterial cells were used throughout the experiment and organisms were subjected to various selected concentrations of the Organochlorine insecticide 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide and Triazole fungicide 1-(4-Chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol based upon a set of experiments for determination of LC50. Endocel (35% EC, Endosulfan manufactured by Excel Crop Care Ltd, Gujarat, India) and Folicur (25.9 % EC, Tebuconazole manufactured by Bayer Crop Science, Mumbai) were used for the present study. LC50 values of the organisms for Endosulfan and Tebuconazole were determined in terms of quantitative estimation of chlorophyll-a of the cyanobacterial species and accordingly, various concentrations of the pesticides were used in all further experiments (Table 1) and samples were analysed for protein profiling by Sodium Dodecyl Poly Acrylamide Gel Electrophoresis (SDS-PAGE) at four days and sixteen days, respectively.

Protein extraction in cyanobacteria:

The cyanobacteria cultures were centrifuged at 10 600 g for 5 min and 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 4^0C. 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) (Gentili *et al.*, 2005). Finally, Laemmli 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.

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

Table 1 : LC_{50} values and pesticide treatments of the test organisms for Endosulfan and Tebuconazole

Sr. No.	Xenobiotic compound	Organisms selected for study	LC_{50} values determined (ppm)	Treatments decided based upon LC_{50} (ppm)
1.	Endosulfan	*Anabaena fertilissima*	6	3
				6
				12
		Aulosira fertilissima	30	15
				30
				60
		Westiellopsis prolifica	20	10
				20
				40
2.	Tebuconazole	*Anabaena fertilissima*	15	7.5
				15
				30
		Aulosira fertilissima	30	15
				30
				60
		Westiellopsis prolifica	30	15
				30
				60

mixed with SDS PAGE sample buffer in a 2: 1 ratio and the mixtures were heated in a heater block for 3 min at 100^0C. After cooling the samples at room temperature, the insoluble materials were removed by centrifugation. The supernatants thus obtained were submitted to SDS-PAGE (Laemmli, 1970) 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 innotech).

All the 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.

RESULTS AND DISCUSSION

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 observed after exposure to different concentrations of 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.

The protein profiles of Endosulfan stressed samples of *A.fertilissima* expressed significant changes when compared to the control after 4 and 16 days (Fig. 1A and 1B). The molecular weights of polypeptides ranged from 17-57 kDa in control, 16-60 kDa (3 ppm), 51 kDa (6ppm) and 18-51 kDa (12 ppm). Few higher molecular weight bands were produced under stress conditions in *Anabaena* after 4 days. However, after 16 days, proteins ranging from 15-68 kDa (control), 15-69 kDa (3ppm), 16-70 kDa (6ppm) and 15-52 k Da (12ppm) were noted, confirming absence of any noteworthy changes in the treated protein profiles.

Protein profiling by SDS-PAGE revealed sharp distinct bands for *A.fertilisisma*, indicating the presence of several high molecular weight polypeptides during the initial treatment periods of Tebuconazole (7.5 ppm). Proteins ranged from 21-74 kDa in control, 18-74 kDa in 7.5 ppm in addition to the higher molecular weight proteins, 18-71 kDa in 15 ppm and 20-67 kDa in 30 ppm treatments of Tebuconazole after 4 days (Fig. 1C). No major changes in the polypeptide patterns of 15 and 30 ppm treatments were observed after 4 days. However, several newer proteins were observed in the treated samples after16 days. Lower molecular weight proteins were identified in 7.5 ppm, while 15 ppm and 30 ppm Tebuconazole confirmed elimination of many proteins and expression of new higher molecular weight proteins. Proteins ranging from 30-68 kDa in control, 24-70 kDa in 7.5 ppm, 48-94 k Da in 15 ppm and 28-93 k Da in 30 ppm were expressed after 16 days (Fig. 1D).

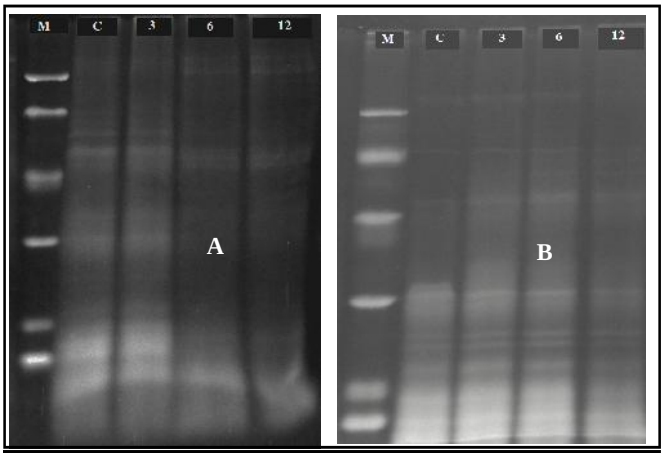

Fig. 1 : Gel images of the total protein of *A. fertilissima* exposed to 0, 3, 6 and 12 ppm of Endosulfan for 4 days (Fig. 1A) and 16 days (Fig. 1B)

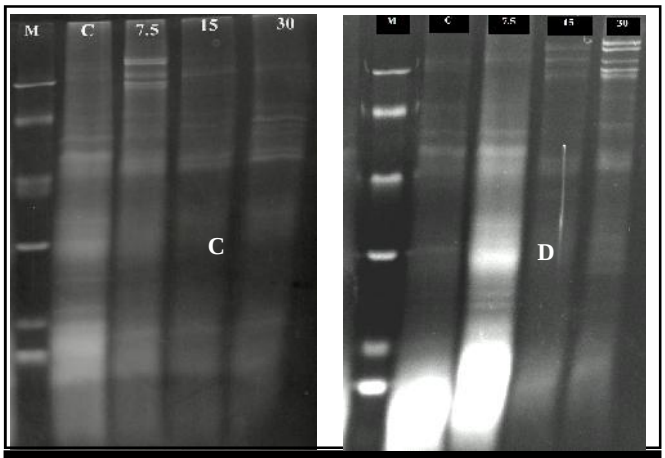

Fig. 1 : 1C and 1D demonstrate the protein profiling of *A.fertilissima* when exposed to 0, 7.5, 15 and 30 ppm of Tebuconazole after 4 and 16 days, respectively

Protein profiles of Endosulfan and Tebuconazole treated *Aul.fertilissima* expressed as many as approximately 38 bands. A marked elimination of protein bands were observed in 30 ppm and 60 ppm treatments of Endosulfan after 4 days (Fig. 2A). Moreover, 15 ppm of Endosulfan eliminated certain proteins as compared to the control which demonstrated as many as 7 protein bands. Proteins in the control ranged from 16-63 kDa,

while those in 15 ppm ranged from 18-31 kDa. However after 16 days, newer protein bands having molecular weights ranging from 16-59 kDa in 15 ppm and 29-63 kDa in 60 ppm were recorded. Although no bands were observed in 30 ppm, proteins of 18-68 kDa were determined in control (Fig. 2B).

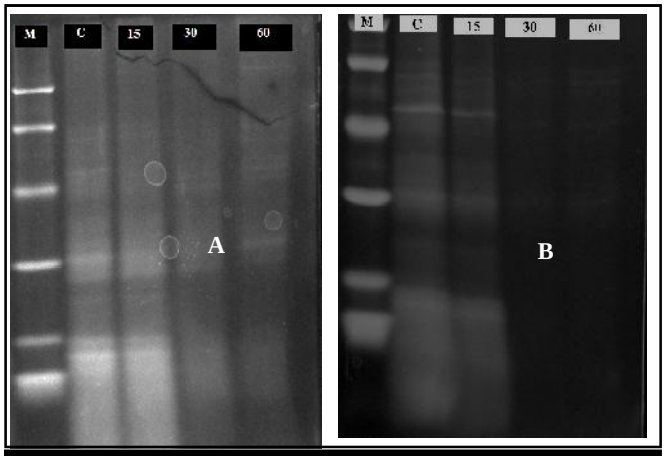

Fig. 2 : shows the gel images of the total protein of *Aul.fertilissima* exposed to 0, 15, 30 and 60 ppm of Endosulfan for 4 days (Fig. 2A) and 16 days (Fig. 2B)

Initially, increase in proteins was noted with rising treatments of Tebuconazole in *Aul.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. Protein bands ranging from 15-47 kDa were found in control (Fig 2C). After 16 days, proteins observed in control ranged from 17-63 kDa. Moreover, an increase in the proteins was also observed in all the treated samples. Proteins having molecular weights of 29-61 kDa in 15 ppm, 31-48 kDa in 30 ppm and 33-51 kDa in 60 ppm were noted (Fig 2D).

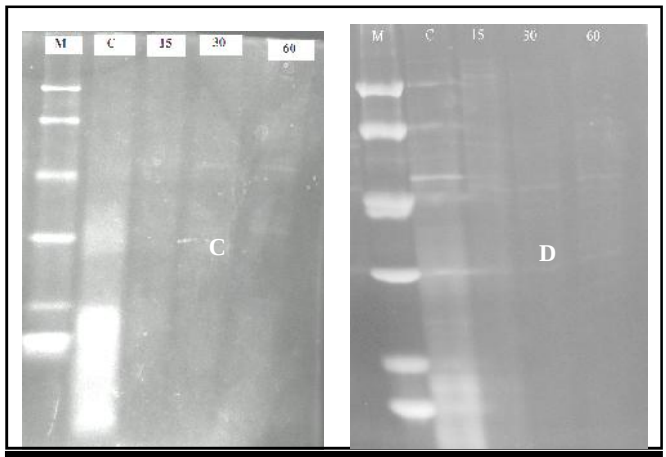

Fig. 2 : 2C and 2D demonstrate the protein profiling of *Aul.fertilissima* when exposed to 0, 15, 30 and 60 ppm of Tebuconazole after 4 and 16 days, respectively

No significant changes were observed in either Endosulfan or Tebuconazole treated *W.prolifica* after 4 and 16 days. Proteins ranging from 15-21 kDa, 16-22 kDa and 21-23 kDa were observed in 10, 20 and 40 ppm of Endosulfan after 4 and 16 days (Fig. 3A and 3B)). Moreover, control ranged from 16-52 kDa after 4 days and 15-83 kDa after 16 days, respectively. On the other hand, a decline in the number of protein bands were observed in Tebuconazole treated samples as compared to the control (Fig. 3C and 3D). No proteins were observed when treated with the highest concentration (60 ppm) of the fungicide, whereas proteins ranging from 17-94 kDa in 15 ppm and 30 ppm of Tebuconazole were noted.

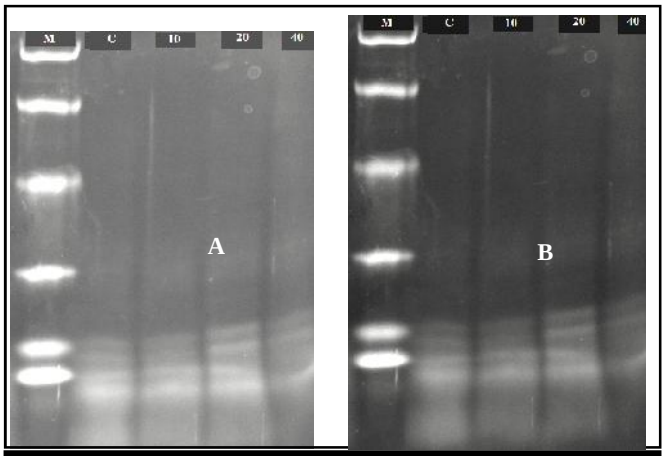

Fig. 3 : shows the gel images of the total protein of *W.prolifica* exposed to 0, 10, 20 and 40 ppm of Endosulfan for 4 days (Fig. 3A) and 16 days (Fig. 3B)

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 (Weber and Jung, 2002).

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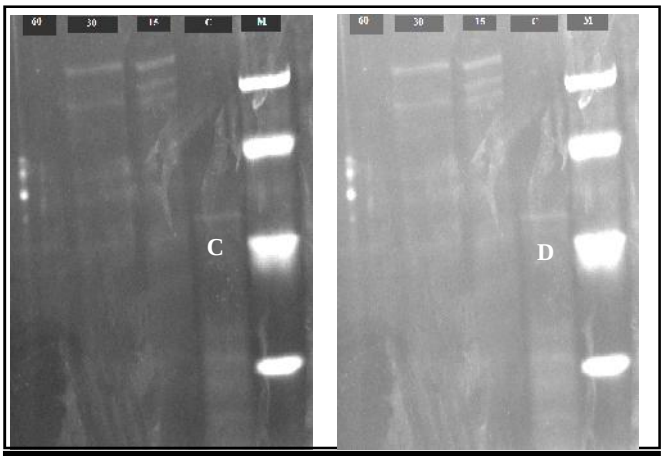

Fig. 3 : 3C and 3D demonstrate the protein profiling of *W.prolifica* when exposed to 0, 15, 30 and 60 ppm of Tebuconazole after 4 and 16 days, respectively

in all the species under study. Changes in protein profiling and newly formed proteins might be helping Cyanobacteria to tolerate adverse stress conditions (Weber *et al.*, 2002).

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 wild-type strain; 2. Inhibition production of some proteins that are produced by the wild-type strain; 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 wild-type strain. All of the above differences are directly associated with the response of cyanobacterial species to different pesticide induced stress conditions. The results were supported by Rajendran *et al*. (2007) who detected newer polypeptides of ~ 280, 152, and 25 kDa (in 250 ppm Bavistin),~58 and 28 kDa (in 0.3 and 0.2–0.4 ppm Monocrotophos, respectively) and ~31, 28, and 26 kDa (in 0.5 and 1.0 ppm Nimbicidin) in the treated cells of *Tolypthrix scytonemoides*. Similar major changes in the polypeptide synthesis and alterations in the protein content after salt stress in Synechocystis have been previously reported by Hagemann *et al*. (1990). Geeta, (2000) studied on the nitrogenase regulation and differentiation in two cyanobacteria and established that newer polypeptides of 104 and 106 kDa were present in the cyanobacteria grown in BG11 medium amended with 10 mM and 20 mM sodium nitrate, while proteins of 75 kDa was present only in 10 mM sodium nitrate.

Moreover, the production of novel proteins or the increased production of already existing proteins, which are only produced under stress conditions, is responsible for stress responses. The decrease in production or the inhibition of production of certain proteins is most probably the result of high levels of protein modification or gene regulation, caused by a decrease in metabolic activity.

Conclusion:

The impact of the pesticides on the organisms could be differentiated on the basis of whole cell protein profiling by SDS-PAGE. The profiles exhibited varied phenotypic differences among the studied species in response to different pesticides. Although newer proteins were generated in Tebuconazole treatments, Endosulfan proved to be less lethal in *Anabaena fertilissima.* Moreover, elimination of proteins increased with increasing pesticide treatments and exposure in *Aulosira fertilissima* and *Westiellopsis prolifica*. Tebuconazole proved to be comparatively more harmful to *Aulosira fertilissima* and *Westiellopsis prolifica*. Treated cyanobacterial species exhibited polypeptides ranging from 15 kDa-52 kDa.

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REFERENCES

Apte, S. K. and Bhagwat, A. A. (1989). Salinity stress induced proteins in two nitrogen fixing *Anabaena* strains differentially tolerant to salt. *J. Bacteriol*., **171**: 909–915.

Gentili, **Francesco,** Marie-Charlotte Nilsson, Olle Zackrisson, DeLuca Thomas H. and Anita Sellstedt. (2005). Physiological and molecular diversity of feather moss associative N_2-fixing cyanobacteria. *J. Exp Bot*., **56**(422): 3121–3127.

Geeta (2000). Nitrogenase regulation and differentiation in two cyanobacteira. Ph.D Thesis, Madurai University.

Hagemann, M., Wolfel, L. and Kruger, B. (1990) Alterations of protein synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803 after a salt shock. *J. Gen. Microbiol*., **136**: 1393-1399.

Holt, J.G., Krieg, N.R.., Sneath, P.H.A., Stanley, J.T. and Williams S.T. (1994). *Bergey's manual of determinative bacteriology*: group 11, oxygenic phototrophic bacteria, 377-475.

Kumar Nirmal, JI., Kumar Rita, N., Bora, Anubhuti and Amb, Manmeet Kaur (2009). Photosynthetic, biochemical and enzymatic investigation of *Anabaena fertilissima* in response to an insecticide hexachloro-hexahydromethano-benzodioxathiepine-oxide. *J.Stress Physiol. & Biochem.*, **5**(3): 4-12.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.*, **227**:680-685.

Rajendran, U.M., Elango, Kathirvel and Narayanaswamy, Anand (2007). Effects of a fungicide, an insecticide and a biopesticide on *Tolypothrix scytonemoides. Pest. Biochem & Physiol.*, **87**(2): 164-171.

Rippka, R., Deruelles, S., Waterbury, JB., Herdman, M. and Stanier, RY. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*, **111**: 1-61.

Suroz, W. and Palinska, K. A. (2004). Effects of heavy metal stress on cyanobacterium *Anabaena flos-aquae. Arch. Environ. Contam. Toxicol.*, **48** : 40–48.

Weber, A. and Jung, K. (2002). Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. *J. Bacteriol.*, **184**: 5502-5507.