Advancements in agri-biotechnology and genetic engineering have opened up new avenues of potential for increasing food production without the use of agro-chemicals. The soil of rice fields contains high density of cyanobacteria especially heterocystous filamentous forms (over 50%). Cyanobacteria are observed to be more resistant to pesticides than eukaryotic algae, leading to selective consequences by some algicides and insecticides, which promote cyanobacterial growth. Other possible beneficial effects of cyanobacteria on rice include competition with weeds, increased soil organic matter content, excretion of organic acids that increase P availability to rice, inhibition of sulfide injury in sulfate-reduction-prone soils by $O_2$ production, increased water temperature and possible production of plant growth regulators (Roger, 1996).

Cyanobacteria are known to survive in a wide range of environmental stress through changes in growth rate as well as qualitative and quantitative changes in their cell constituents that may be of biotechnological significance. The success of cyanobacteria in many past and modern environments can be attributed to a large extent to their metabolic versatility, flexibility and reactivity. Cyanobacteria have a choice of different metabolic pathways and physiologies. The cyanobacteria are very reactive and respond instantaneously to the changing conditions. Among pesticides other than algicides, herbicides are the most detrimental to cyanobacteria.

During the present investigation, an attempt has been made to evaluate the effect of some of the common rice field pesticides (herbicide and fungicide) on three representative genera of nitrogen-fixing bluegreen algae in the laboratory conditions. The blue green algae, *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* have been employed as the experimental materials. These have been preferred over others because of their common occurrence in the paddy fields, ability to form homogeneous suspension in the liquid culture medium, differentiation of some cells into heterocysts at specific position in the filaments and an important role in increasing the fertility of paddy fields.

The pesticides put on test comprised 2,4-D ethyl ester (herbicide) and pencycuron (fungicide). Among many classes of fungicides, pencycuron (Monceren 250 SC) is introduced in the Indian subcontinent by Bayer Crop Science, India. It controls the growth of *Rhizoctonia*...
solani at different minimum inhibitory concentration depending on the strains. It is a non-systemic protective fungicide and used widely in agricultural production, particularly in Asia (Sylvanie & Comis, 1989). However, information as regards to the toxic effects of pencycuron on cyanobacteria is lacking. While literature is available as regards to 2,4-D acid but it is seldom used in commercial forms as it is less soluble. Commercially available 2,4-D consists of the more soluble forms such as alkali salts, amine salts, or esters. Furthermore, ester formulations were observed to be more toxic than the free acids or salts but there is scarcity of literature for toxicity of ester forms of 2,4-D.

In the current study several morphological changes introduced in three selected cyanobacterial species were recorded after the 4th and 16th day of pesticide treatments. Several biochemical parameters such as pigment content (chlorophyll \(a\), carotenoids, phycocyanin, phycoerythrin and allophycocyanin), metabolite content (total carbohydrate, total amino acids, total protein and total phenol content) and enzyme activity (nitrate reductase, glutamine synthetase and succinate dehydrogenase) were estimated after every 4-day interval up to sixteen days. For further verification of the results molecular approaches like functional group variation (FT-IR), biotransformation (GC-MS), protein profiling (SDS-PAGE), DNA profiling (RAPD-PCR) and 16S rDNA sequencing were carried out.

There were significant differences in morphological, biochemical and molecular pattern of these test algae. In regard to the above aspects following significant observations were recorded during the present study.

**Differential effects of 2,4-D ethyl ester (herbicide) on three cyanobacterial species**

- Morphological examination showed that the *Anabaena fertilissima* filaments disintegrated and the cells lysed gradually after 4-days of 60 ppm 2,4-D ethyl ester exposure till the end of the experiment. While, the highest concentration of 2,4-D ethyl ester markedly affected the morphology of *Aulosira fertilissima* and *Westiellopsis prolifica* after 16-days. Furthermore, reduction in the heterocyst frequency ranged from 28% to 53% in *Anabaena fertilissima* reflecting severe inhibitory action of 2,4-D ethyl ester which was followed by *Aulosira fertilissima* (16% to 41%) and *Westiellopsis prolifica* (12% to 38%).
• A concentration of 80 ppm (22 to 79% reduction) and 120 ppm (15 to 67% reduction) after 16-days was recorded to be most lethal (P<0.05, ANOVA) for pigment content of *Aulosira fertilissima* and *Westiellopsis prolifica* respectively, whereas only 60 ppm (32 to 95% reduction) of 2,4-D ethyl ester diminished the pigments of *Anabaena fertilissima*. However, among pigments, phycoerythrin concentration showed highest sensitivity towards 2,4-D ethyl ester concentrations in all the three cyanobacterial species. In *Anabaena fertilissima* carotenoid content was less affected, whereas in *Aulosira fertilissima* and *Westiellopsis prolifica* phycocyanin (64%) and chlorophyll *a* (54%) showed sparsely reduced when compared with other pigments.

• The different concentrations of 2,4-D ethyl ester significantly reduced total carbohydrates which was followed by total amino acids, whereas total proteins showed an initial stimulation period which was succeeded by a period of inhibition. However, phenol content was stimulated throughout the experiment with maximum percentage increased at highest concentration from 10% to 39% in *Anabaena fertilissima*, 13% to 27% in *Aulosira fertilissima* and 10% to 24% in *Westiellopsis prolifica*. Carbohydrate content under highest concentration of 2,4-D ethyl ester was reduced (P<0.05, ANOVA) by 81%, 70% and 65% in *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica*, respectively.

• Highest concentration of 2,4-D ethyl ester was most toxic (P<0.05, ANOVA) to glutamine synthetase activity in *Anabaena fertilissima* (97% reduction), whereas in *Aulosira fertilissima* and *Westiellopsis prolifica* the succinate dehydrogenase was highly suppressed by 85% and 73% respectively, after 16-days of exposure. On other hand, nitrate reductase was less affected with respect to other enzyme activities by 78%, 73% and 62% reduction was registered in *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* respectively.

• *Anabaena fertilissima* when treated with three different concentrations of 2,4-D ethyl ester generated new peaks of 3511 cm\(^{-1}\), 1348 cm\(^{-1}\), 869 cm\(^{-1}\) and 1599 cm\(^{-1}\) frequency attributed to O-H of phenols & alcohols, N-O symmetric stretch of nitro compounds, N-H wag of 1°, 2° amines and C-H of aromatics respectively. In 80 ppm 2,4-D ethyl ester treated *Aulosira fertilissima*, new peaks of 1347 cm\(^{-1}\) (nitro
compounds; N-O symmetric stretch), 869 cm⁻¹ (1°, 2° amines; N-H wag), 1285 cm⁻¹ (aromatic amines; C-N stretch), 1425 cm⁻¹ (aromatics; C-C stretch (in-ring)) and 1630 cm⁻¹ (1° amines; N-H bend) were observed. While in Westiellopsis prolifica occurrence of functional groups like aromatic amines (C-N stretch) with a wavelength of 1288 cm⁻¹, aliphatic amines (C-N stretch) with a wavelength range of 1237-1247 cm⁻¹, nitro compounds (N-O symmetric stretch) with a wavelength range of 1479-1339 cm⁻¹ and aromatics (C-H “oop”) with a wavelength of 867 cm⁻¹ were encountered.

- GC-MS study revealed different by-products of 2,4-D ethyl ester when highest concentrations of herbicide were exposed to three cyanobacterial species. All three species of cyanobacteria were proficient enough to metabolize the mother compound (2,4-D ethyl ester) and release various byproducts during the interaction period. Isobutyrlic acid allyl ester, 3-bromobutyric acid and 2,4-D butyl ester were the products of biotransformation of 2,4-D ethyl ester by Anabaena fertilissima; Hydroxyurea, trifluroacetic acid, 2-methyl propyl ester and acetic acid 2-propenyl ester were resulted by Aulosira fertilissima, while acetic acid (2,3-dichlorophenoxy) and 2,4-D methyl ester were produced by Westiellopsis prolifica.

- The Anabaena fertilissima was the most sensitive to 2,4-D ethyl ester at higher concentrations because of disappearance of all the protein bands of 74, 46, 50, 28, 24, 23 and 21 kDa. While in Aulosira fertilissima and Westiellopsis prolifica there was slight modification in bands after 4-days when compared with control. The intensity of three protein bands of 65, 60 and 48 kDa was sharply decreased in 2,4-D ethyl ester treated Aulosira fertilissima culture while protein bands of 65, 60, 55, 29, 18 and 16 kDa were completely eliminated. After 4-days of incubation, the 2,4-D ethyl ester treated Westiellopsis prolifica was unaffected as all the bands of 62, 59, 30.4 and 14 kDa were present but after 16-days only two bands of 16 and 14 kDa were encountered.

- DNA profiling of all three species were different depending upon their susceptibility towards the herbicide. Anabaena fertilissima was found to be the most sensitive among other two test organisms due to disappearance of bands i.e. 1500, 1300, 700,
500 and 200 bp while in *Westiellopsis prolifica* some new bands 2500, 1400, 1300, 950, 900, 600 and 500 bp were generated at 60 and 120ppm in 2,4-D ethyl ester treatments which were absent in control.

- The obtained 16S rDNA sequence of 2,4-D ethyl ester treated cyanobacteria was aligned with its untreated control using BLAST. The results confirmed that 16S rDNA region of *Anabaena fertilissima* was more affected by 2,4-D ethyl ester as there was no homology in the region of 39 basepairs also several mismatches and gaps were encountered with 94% identity in the nucleotides 1-203 while only 83% sequence similarity was recorded from nucleotide 242-1159 of control. However, less difference in 16S rDNA was observed in case of *Aulosira fertilissima* (83% identities with 5% gaps) and *Westiellopsis prolifica* (98% identities with 2 gaps). Thus, *Westiellopsis prolifica* was found to be reluctant to the changes caused due to 2,4-D ethyl ester stress at the DNA level.

**Differential effect of Pencycuron (fungicide) on three cyanobacterial species**

- The morphological study revealed that out of the three selected cyanobacteria one species i.e. *Westiellopsis prolifica* not much effected / remained unchanged throughout the experimental period. On the other hand *Anabaena fertilissima* and *Aulosira fertilissima* when treated with higher concentration of pencycuron resulted into bleaching of cells and distortion in filamentous structure thereby generating trichomes of lesser length. Heterocyst frequency recorded in response to different concentrations of pencycuron displayed significant reductions in *Anabaena fertilissima* (15% to 35% inhibition) followed by *Aulosira fertilissima* (6% to 30% inhibition) and *Westiellopsis prolifica* (0% to 24% inhibition).

- The water soluble pigment (phycoerythrin) was highest responsive to the pencycuron among pigments in all the experimental organisms and was proficiently declined with increasing concentrations of pencycuron. This rate of inhibition was highest in *Anabaena fertilissima* (86%) followed by *Aulosira fertilissima* (67%) and *Westiellopsis prolifica* (40%) in the presence of higher concentrations of the fungicide at the end of the experiment period. Hence it is clear that *Westiellopsis*
prolifica is the most tolerant species as the reduction was less than 50%. In Anabaena fertilissima, there was a gradual and highest decrease (P<0.05, ANOVA) in optical density of pigments, however carotenoid content was less affected by 65% when compared with other study pigments.

- Pencycuron treatment adversely affected (P<0.05, ANOVA) total amino acids with respect to other two metabolites in Anabaena fertilissima and Westiellopsis prolifica, while in Aulosira fertilissima total carbohydrates (57% reduction) was exceedingly affected. However, protein content was little influenced by pencycuron treatment in all the three cyanobacteria as the highest reduction observed was only 47% (Anabaena fertilissima). Like 2,4-D ethyl ester, pencycuron treatment showed enhancement (5% to 50%) in total phenol content which confirmed its stimulation at each stage of investigation. Maximum phenol content (50% stimulation) was recorded in most sensitive cyanobacteria i.e. Anabaena fertilissima at 60 ppm of pencycuron.

- In Anabaena fertilissima the effect of lower concentrations (15 ppm and 30 ppm) of pencycuron, on the enzymatic activity was not favorable after 16-days of incubation (P<0.05, ANOVA). As compared with nitrate reductase (60% reduction), glutamine synthetase also resulted into remarkable suppression (66%), whereas, succinate dehydrogenase was comparatively less affected (58% reduction) in Anabaena fertilissima. However, in Aulosira fertilissima nitrate reductase activity was adversely inhibited by 56% followed by glutamine synthetase (53% reduction) and succinate dehydrogenase activity (45% reduction). Similarly, even in Westiellopsis prolifica, nitrate reductase showed highest reduction by 47% followed by succinate dehydrogenase (41% reduction) and glutamine synthetase activity (36% reduction).

- More functional group variation was observed in pencycuron treated Anabaena fertilissima whereas less variation was detected in Aulosira fertilissima and Westiellopsis prolifica. In Anabaena fertilissima three new groups with a frequency of 1337 cm⁻¹ (aromatic amines (C-N stretch)), 1284 cm⁻¹ (nitro compounds (N-O symmetric stretch)) and 2106 cm⁻¹ (alkynes (-C=C- stretch)) were observed. On other hand interaction of Aulosira fertilissima with pencycuron induced nitro compounds (N-O symmetric stretch) of 1348 cm⁻¹, alcohols, carboxylic acids, esters, ethers (C-O...
stretch) of 1313-1244 cm⁻¹, carboxylic acids (O-H bend) of 929 cm⁻¹, 1° amines (N-H bend at 1601 cm⁻¹) and aliphatic amines (C-N stretch at 1241 cm⁻¹). In Westiellopsis prolifica after 16-days new functional groups were produced with a wavelength range of 1448-1499 cm⁻¹ (aromatics (C-C stretch (in- ring)), 1311-1346 cm⁻¹ (nitro compounds (N-O symmetric stretch)), 1628 cm⁻¹ (1° amines (N-H bend)), 929 cm⁻¹ (carboxylic acid (O-H bend)) and 868 cm⁻¹ (1°, 2°amines (N-H wag)).

- In pencycuron treated Anabaena fertilissima and Aulosira fertilissima there was no change because not a single unique by-product was detected while in Westiellopsis prolifica one new by-product i.e. benzoxazole was recorded after 4-days of exposure when compared with standard (100 ppm of pencycuron). Hence, Westiellopsis prolifica was more reactive to pencycuron as compared with other two species.

- The results recorded after 4-days of incubation demonstrated the minimal effect of pencycuron while 16-days results revealed severe effect on Anabaena fertilissima when compared with the Aulosira fertilissima and Westiellopsis prolifica. In Anabaena fertilissima complete elimination of protein bands of 61, 28, 24, 23 and 21 kDa occurred after 16 days while at the same time two new proteins of 43 and 41 kDa were generated. Further in Aulosira fertilissima protein bands of 18 and 16 kDa were inhibited while the intensity of a protein band (≈29 kDa) was decreased.

- RAPD technique is used for the detection of genetic heterogeneticity revealed the higher concentrations of pencycuron were not suitable for Anabaena fertilissima since disappearance of bands (1500, 1300, 700 500 and 200 bp) were recorded. While in case of Westiellopsis prolifica, which is considered to be the most tolerant species, new bands like 2500, 1400, 1300, 950, 900, 600 and 500 bp were generated along with the bands present in the control and also recovery of two bands of 1179 and 965 bp was noticed at highest concentration of pencycuron.

- The 16S rDNA sequencing revealed that the pencycuron was not efficient enough to alter the conserved regions of all the three test organisms, as there was no significant change in the sequence pattern of the treated cultures after 16-days of exposure. However, pencycuron treated Anabaena fertilissima resulted in 99% identity with only 3 gaps which was followed by Westiellopsis prolifica (99.9% identity with zero gaps) and Aulosira fertilissima (100% identities with zero gaps).
Among two pesticides used in the present study, 2,4-D ethyl ester has been recorded to be the most toxic pesticide. It severely affected the cyanobacterial filaments at 60 ppm dose in *Anabaena fertilissima*, at 80 ppm dose in *Aulosira fertilissima* and at 120 ppm dose in *Westiellopsis prolifica*. *Anabaena fertilissima*, however showed little more sensitivity than *Aulosira fertilissima* and *Westiellopsis prolifica*, to both the pesticides (2,4-D ethyl ester and pencycuron). Although, the morphological characters of cyanobacteria were more affected by 2,4-D ethyl ester than pencycuron. Herbicide (2,4-D ethyl ester) also reduced the frequency of heterocyst in the test cyanobacteria. A significant change in filament shape and bleaching of cells was also observed. Both the pesticides identically reduced the chlorophyll $a$ and other accessory photosynthetic pigments. The fall in carbohydrates and amino acids content of all cyanobacterial species were fixed by the trend (concentration and time dependent) with both the pesticides. However the protein content was initially (after 4-days) stimulated by 2,4-D ethyl ester treatment at all concentrations, while all the concentrations of pencycuron consistently decreased the protein content in time-dose dependent manner. Phenol was found to be greater at relatively higher concentration for both the pesticides. Phenol content also increased with the increase in time. Among other biochemical parameters enzyme activity was generally reduced on addition of various concentrations of 2,4-D ethyl ester and pencycuron. The percentage reduction in nitrate reductase, glutamine synthetase and succinate dehydrogenase, however, differed in all three cyanobacteria at different doses of these pesticides.

In 2,4-D ethyl ester treated cultures, more new functional groups and intoxicants were recorded, when compared with pencycuron treated cultures. However, based on FTIR and GC-MS profiles, it can be inferred that *Anabaena fertilissima* is prominent among other two test organism in reacting with and transforming 2,4-D ethyl ester. Nevertheless, both the pesticides were relatively less toxic to *Westiellopsis prolifica* than *Aulosira fertilissima* and *Anabaena fertilissima*. In RAPD analysis, several bands were totally disappeared at higher concentration of 2,4-D ethyl ester in *Anabaena fertilissima*, which could be likely due to the toxic effect of herbicide at genomic level. While this was further confirmed by sequencing of 16S rDNA gene, since 2,4-D ethyl ester treated cultures showed greater percentage change than pencycuron treated cultures.
Statistical analysis:

Test of significance (Student ‘t’ test) of all the parameters estimated in response to different concentrations of 2,4-D ethyl ester and pencycuron in *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* was studied. A positive correlation was found between all the parameters except phenols since phenols showed negative correlation with all the parameters of *Anabaena fertilissima* when treated with 2,4-D ethyl ester and pencycuron. In *Aulosira fertilissima* carotenoids, phycocyanin, phycoerythrin, allophycocyanin, carbohydrates, amino acids, glutamine synthetase and succinate dehydrogenase exhibited a negative correlation with phenols and a positive correlation between each other and with other parameters after 2,4-D ethyl ester treatment. Similarly in pencycuron treated *Aulosira fertilissima* chlorophyll a, carotenoids, phycocyanin, phycoerythrin, carbohydrates and glutamine synthetase exhibited a negative correlation with phenols and a positive correlation between each other and with other parameters. However, under 2,4-D ethyl ester exposure *Westiellopsis prolifica* demonstrated positive correlation of phenols with proteins while negative correlation with all other tested parameters. The correlation matrix of *Westiellopsis prolifica* after pencycuron treatment revealed negative correlation of phenol with chlorophyll a, phycoerythrin, allophycocyanin, amino acids and glutamine synthetase.