CHAPTER -3
MATERIALS AND METHODOLOGY
3. MATERIALS AND METHODOLOGY

The details of materials used and the methods adopted during the present investigation are given under appropriate heads. Altogether, two types of experiments viz., laboratory bioassay and field experiments were conducted.

3.1 MATERIALS: Only the surface soil was collected from the selected fields of different sites and was used as basic material for present study. The glasswares used in the present study were Borosil make. Washing of glasswares were done by using potassium dichromate, sulphuric acid mixture followed by washing in running tap water. After complete removal of acid from glasswares, then cleaned by detergent and washed repeatedly till the removal of detergent. After washing glasswares were dried in a hot air oven. Cultures were maintained either in Petri plates or in 250 ml conical flasks. All organic and inorganic chemicals used were of Merck Specialities Pvt. Ltd., Mumbai made.

3.2 METHODOLOGY FOR ISOLATION, IDENTIFICATION AND CULTURING OF CYANOBACTERIA:

3.2.1 Isolation of Cyanobacteria from Paddy Field Soil of Nearby Areas:

Following sites of Dayalbagh area of Agra District were chosen for the study purpose-

1. DEI Dairy farm,
2. Dairybagh,
3. Lalgarhi- village road.
4. Dayalbagh Panjabi farms
5. Nagala Talfi
Samplings were done in the month March-September, year 2008. Randomized sampling was done for collecting the soil samples. After mixing the sub-samples very well, a composite sample was collected in poly bags for laboratory studies. This experiment was conducted in following steps:

(i) Collection of Soil Sample:

Composite samples were collected from different paddy fields of nearby areas. The composite samples were composed of at least 10 core sub-samples, including the top 0.5 cm of soil. After mixing the core samples, the composite samples of paddy soil were kept in bags for further experimentations.

(ii) Preparation of Soil Dilutions:

After mixing the core samples, composite samples were used to prepare the suspension dilutions of soil. Soil dilution (10^{-1}) of different samples were prepared by suspending 10 gm soil in 90 ml distilled water gave rise to 10^{-1} suspension dilution of soil.

3.2.2 Isolation and Culturing:

For isolation of Cyanobacteria from soil samples, method of Kaushik, (1987) was followed. From the prepared soil dilutions 1 ml of each were transferred in to the flasks containing culture media with or without addition N_{2} source under aseptic conditions by using laminar air cabinet. The flask were shaken well and incubated undisturbed in a growth room for 20-25 days at 25 ^{\circ}C and 2000-3000 lux light.
Pure cultures of selected BGA genera were procured from National Facility for Utilization and Conservation of Blue Green Algae, IARI, New Delhi. The procured selected strains were again maintained in liquid BG-11 medium by following the described method to conduct further *In-vivo* and *in-vitro* studies and identification of allelochemicals from their extracts. Procured cultures were incubated in a BOD growth chamber maintained at 25 °C and 2000-3000 lux light.

BG-11 liquid culture media (Rippka et al., 1979) with and without combined N₂ was used for isolation of Cyanobacterial genera. After mixing the BG-11 media ingredients (listed in Table 3.1) in distilled water, the pH was adjusted to 8.5 using either 0.1N NaOH or 0.1N HCl. The media with or without combined N₂ were dispensed in separate sterilized conical flasks. Conical flasks, containing 250 ml medium were plugged with cotton plugs. The medium was sterilized in an autoclave at 121 °C and 15 psi for 15 min.

### 3.2.3 Identification of Blue Green Algae:

With the help of dissecting microscope slides (50 of each sample) were made from each sample for microscopic examination and identification of Cyanobacterial strains. Algal threads were picked up from culture, under a dissecting microscope, algal thread were separated from clumps by using platinum needle, slides were prepared observed under binocular microscope. Identification of Cyanobacterial genera were carried out using the taxonomic publications of Desikacharya, (1959).
3.2.4 **Succession period:** A chronological sequence of appearance of valuable Cyanobacterial genera in all studied sites was also recorded in addition of isolation and culturing of Cyanobacteria during July 2010 – March 2011 to understand their succession period under local environmental conditions. For this experiment method was followed as described for isolation and identification of Cyanobacteria.

3.2.5 **Relative abundance** (Dey *et al.*, 2010): The number of organisms of a particular kind as a percentage of the total number of organisms of a given area or community; the number of birds of a particular species as a percentage of the total bird population of a given area.

The relative abundance of a particular Cyanobacteria type was calculated by employing the following formula:

\[
\text{Relative abundance} = \frac{Y}{X} \times 100
\]

Where,

\[X = \text{total number of slides were observed.}\]

\[Y = \text{number of time particular Cyanobacteria type was observed.}\]

3.2.6 **Observations:** Microscopic observations were done when green matting of Blue Green Algae was appeared in conical flasks containing liquid culture media with or without additional N₂ source. Microscopic photographs were taken by using a Nikon digital camera.

The observations were supported by their photographs.
3.2.7 Presentation of Data: Microscopic examination was done when complete growth of BGA in the form of green matting was appeared. To make easy maintenance of cultures of different samples, abbreviations were used as given below:

<table>
<thead>
<tr>
<th>Sites</th>
<th>Field no.</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DEI Dairy Farm)</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>Site 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Dairy bagh)</td>
<td>1</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>Site 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Lalgarhi village road)</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.3</td>
</tr>
<tr>
<td>Site 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Dayalbagh Panjabi farms)</td>
<td>1</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.3</td>
</tr>
<tr>
<td>Site 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nagala Talfi</td>
<td>1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.3</td>
</tr>
</tbody>
</table>
3.3 METHODOLOGY TO ANALYZE THE PHYSICAL AND CHEMICAL PARAMETERS OF SOIL SAMPLES.

The soil samples taken from each plot/field were air dried, crushed and sieved. The analysis of soil was done in two steps. In first step all 5 studied sites were examined for soil parameters viz., texture, moisture, water holding capacity (WHC), pH, EC, organic carbon, total nitrogen and C/N ratio. In second step the soil samples of experimental plots were analyzed for chemical parameters viz., pH, EC, organic carbon, total nitrogen and C/N ratio before and after application of BGA to understand their allelopathic interactions with the soil health. The experiment was conducted in following steps-

3.3.1 Texture Determination: (Pandeya et al., 1968)

Texture of soil was determined by passing 100 gm. soil samples through a set of standard sieve (BSS), which contains four containers i.e. 60, 150, 350, 500 BSS and one reservoir. The complete set was kept undisturbed on the table for 48 hours after adding 25 ml of water in 60 BSS of container. The presence of different types of soil was observed after 48 hrs, by weighing soil particles present in different containers of sieve set. The estimation of texture of soil was done according to these standards:

- 60 BSS------ Stones and Pebbles
- 150 BSS------ gravel
- 350 BSS------ Coarse and sand
- 500 BSS------ Fine sand
- Reservoir------ Silt and Clay
3.3.2 Soil moisture: (Pandeya et al., 1968)

For calculating soil moisture, 100 gm was taken and placed in the oven at 80 ºC for 24 hours, dry weight of soil samples. The decrease in the weight was considered as moisture content of the soil. The percentage of soil moisture was calculated in terms of oven dried soil.

\[
\text{Wight of the dried soil} = X
\]
\[
\text{Wight of the fresh soil} = Y
\]
\[
\text{Moisture content (\%) } = \frac{(Y-X)}{Y} \times 100
\]

3.3.3 Water holding Capacity: (Pandeya et al., 1968)

Water holding capacity of soil was measured with the help of Buchner funnel. A circular Whatman filter paper no. 1 was placed over the perforated disc of Buchner funnel. The funnel was half-filled with 100 gm soil. Water was added slowly on the soil till it becomes fully saturated. It was fitted to a filtration flask connected to the water tap to give small suction force. More water was poured in the soil 2-3 times to ensure that the soil has thoroughly wet. When water stopped to trickle out for 1-2 hours, the soil was taken out from the funnel. The soil obtained weight and then dried in an oven at 105ºC. The dried soil was weighted again. The loss of weighted in soil was expressed in percentage.

The percentage water holding capacity was calculated by following expressed,

\[
\text{Weight of the wet soil}=X
\]
\[
\text{Weight of the dried soil}= Y
\]
\[
\text{Decrease in weight of soil}= X-Y
\]
\[
\text{Percentage of water holding capacity (\%) } = \frac{(X-Y)}{X} \times 100
\]
3.3.4 Soil pH: (Pandeya et al., 1968)

The pH of soil was measured by digital pH meter. Soil sample was dissolved in distilled water in ratio of 1:5 (soil:water) to make suspension. Soil suspension was shaken interminably for one hour and kept undisturbed. pH meter was calibrated before use by means of buffer solution of pH 4. The temperature of pH meter was adjusted according to room temperature. Supernatant liquid was poured into a beaker after standardizing of the digital pH meter was dipped in it. The value, indicated on the screen was noted as pH value. The experiment was repeated thrice for more accuracy.

3.3.5 Soil Electrical Conductivity (EC): (Jackson, 1979)

10 gm of soil extract or soil suspension (1:2) was taken in 100 ml beaker and 20 ml of distilled water was added. The soil water suspension was measured by electrical conductivity meter. After this EC meter was adjusted at room temperature for reading with the KCl. The EC (dsm\(^{-1}\)) was calculated as follows.

EC (dsm\(^{-1}\)) = Meter reading × C.C × 5.4 + 0.7

Where C.C = 12.88/ Kcl reading.

3.3.6 Carbon Estimation:

Carbon estimation of soil samples was analyzed by method of Jackson, (1979).

Requirements: (i) Sulphuric acid (H\(_2\)SO\(_4\))

(ii) N/10 Iodine solution
(iii) 4g of Potassium Sulphate (K₂SO₄)

(iv) 0.5g of Copper Sulphate (CuSO₄)

(v) Sodium Thiocyanate (NaSCN)

Procedure: 5 grams of oven dried soil of each soil sample was taken in around bottom flask (Kjeldhal’s flask). Take each sample, 30ml of conc. H₂SO₄ was added and mouth of the flask closed with the help of rubber stopper. Through rubber stopper thistle funnel was inserted, touching its one end with H₂SO₄ and soil mixture. The side tube of the kjeldhal flask was connected to two washing bottles arranged in series contacting 75ml of N/10 iodine solution. In turn the washing bottles were connected to suction pump. Before inserting the thistle funnel, 4g of K₂SO₄ and 0.5 g of CuSO₄ were added to the mixture of H₂SO₄ in soil. As the whole apparatus get ready, the heating of flask with the help of burner allowed till the blue colour obtained. The iodine present in wash bottles was used for analysis. The following equation has been used to calculate the amount of organic carbon:

\[ 1 \text{ ml of N/10 iodine used} = 0.003 \text{ gm of organic carbon} \]

3.3.7 Nitrogen Estimation: (Jackson, 1979)

Estimation of nitrogen in soil sample was carried out by Kjeldhal method.

Requirements:

(i) Sodium hydroxide (NaOH)

(ii) 0.1N H₂SO₄

(iii) Phenolphthalein indicator
Procedure: The soil sample left after digestion in carbon estimation experiment was used in the process in which 80ml of hot distilled water was added. Then in the suspension, saturated solution of sodium hydroxide was added till the appearance of brown precipitate. Now the Kjeldhal flask was connected to vapour condensing tube from one end. On the other end of condensing tube, beaker containing 0.1N sulphuric acid was connected.

In the obtained assembly, round bottom flask was heated to half an hour. The solution of the beaker was used for the purpose of nitrogen estimation. Nitrogen was estimated by titrating the solution of 0.1N sulphuric acid against 0.1 sodium hydroxide using phenolphthalein as indicator.

The percentage nitrogen content present in the following was calculated using following formula:

\[
\text{Nitrogen (\%)} = (B-T) \times 0.14 \times N \times 100
\]

Where, B= blank titration reading
T=Actual titration reading
N=Normality of the alkali used.
3.4 METHODOLOGY TO FIND OUT THE EFFECT OF ALGAL LEACHATES ON SEED GERMINATION AND SEEDLING GROWTH OF PADDY (IN VITRO).

This study was based on laboratory bioassays which consisted five treatments i.e. leachates of *Nostoc* sp. (Treatment A), *Anabaena variabilis* (Treatment B), *Aulosira fertilissima* (Treatment C), Combination of these three strains (Treatment D) and Water Control (WC). These treatments were applied on seeds of *Oryza sativa* L. (var. PR 114) to test their allelopathic effect on seed germination and seedling growth of paddy. In nature, allelochemicals are released in to the environment in water soluble form, i.e., leachate, root exudates, volatiles and decomposition products from plant residues (Narwal and Tauro, 1996). Therefore, distilled water was used as solvent for preparation of algal leachate. The experiment consist of following steps-

3.4.1 Preparation of algal leachate (Hamdi et al., 2001):

To prepare the leachate of selected strains of BGA, cultures were filtered from liquid medium through Millipore filters (0.45 µ). This filtrate was allowed to dry at room temperature then crushed to make its powder carefully. Then 10 gm powder of each tested BGA (individual as well as in combination form) was suspended in 100 ml distilled water and placed it on electronic shaker for 72 hr and then placed undisturbed for 2 hr. Supernatant was collected carefully in a separate beaker. This supernatant was considered as 100% concentration of algal leachate. Other concentrations (25%, 50%, 75%) were prepared by diluting 100% leachate with distilled water.
The abbreviations used for denoting the leachates of selected BGA strains were as follows:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Leachate of BGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Nostoc</em> sp.</td>
</tr>
<tr>
<td>B</td>
<td><em>Anabaena variabilis</em></td>
</tr>
<tr>
<td>C</td>
<td><em>Aulosira fertilissima</em></td>
</tr>
<tr>
<td>D</td>
<td>Combination (A+B+C)</td>
</tr>
<tr>
<td>WC</td>
<td>Water control</td>
</tr>
</tbody>
</table>

3.4.2 Sterilization of seeds Healthy seeds of Paddy (100 gm) were surface sterilized in 0.1% HgCl₂ solution for 10 minutes then repeatedly washed with six changes of sterilized distilled water in a laminar air flow chamber to remove all traces of mercuric chloride.

3.4.3 Procedure: 50 sterilized seeds were separately soaked in the different concentrations (25 - 100%) of leachates of tested treatments separately for 12 hrs. Seeds soaked in distilled water were considered as water control (WC). Paddy seeds were then collected separately. Total 12 per treatment (three per concentration) 150x15 mm pre-sterilized covered glass Petri dishes were taken, whose base were covered with Watman No. 1 filter paper. Ten Paddy seeds were arranged on the surface of filter
paper moisten with leachates of four treatments (A, B, C, D) and water control (WC) accordingly. Petri dishes were then placed under a florescent light for 4 days. Used concentrations of leachates of different treatments were poured with the help of separate pipette to the related Petri dishes at regular intervals. Distilled water was used in the control set of experiment. This test was repeated thrice following the same procedure.

3.4.4: Parameter for observations: Total three parameters were taken for recording the observations-

(i) Germination (%): Germination of treated seeds along with control was recorded at the end of 6th Days after sowing (DAS). Total germination percentage was analyzed by observing number of germinated seeds out of total number of sowing seeds.

(ii) Root length (cm): Root length of seedlings of treated seeds in triplicate set of experiment at regular intervals (6th, 12th, and 18th DAS) along with control was recorded. For this three well germinated seeds of each replicate (total 9 seeds) ware randomly selected and measured for root length in centimeter.

(iii) Shoot length (cm): Shoot length was also measured along with root length at same intervals of time and procedure as described for root length measurement.
3.5 To study the effect of BGA on growth and yield attributes of Paddy crops (Under field conditions).

The field experiment was designed at the agricultural farms of Dayalbagh dairy, where less animal disturbance found. Although around the experimental field wires were polled to protect the crop with animals.

3.5.1 Layout of the experimental paddy field: Detailed of the treatments are given below. Total experimental area including irrigation canal was 30.375 m².

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Randomized Block Design with Dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plot size</td>
<td>1 x 1 m = 1 m²</td>
</tr>
<tr>
<td>Total number of treatments</td>
<td>5</td>
</tr>
<tr>
<td>Replication</td>
<td>3</td>
</tr>
<tr>
<td>Total number of plots</td>
<td>3 x 5 = 15</td>
</tr>
<tr>
<td>Detail of treatments</td>
<td>(A) Nostoc sp.</td>
</tr>
<tr>
<td></td>
<td>(B) Anabaena variabilis</td>
</tr>
<tr>
<td></td>
<td>(C) Aulosira fertilissima</td>
</tr>
<tr>
<td></td>
<td>(D) Combination (A+B+C)</td>
</tr>
<tr>
<td></td>
<td>(WC) Water control</td>
</tr>
<tr>
<td>Net Experimental plot area</td>
<td>15 m²</td>
</tr>
<tr>
<td>Gross area</td>
<td>6.75 x 4.5 = 30.375 m²</td>
</tr>
</tbody>
</table>
3.5.2 Experimental field design: The experiment was conducted in randomized block design with three replicates (R1, R2, and R3). The experimental variable consisted of 5 treatments with the crop of paddy (*Oryza sativa*; var. PR 114). Design of experimental field was as follows-
3.5.3 Preparation of soil based cyanobacterial bio-fertilizers (Venkataraman, 1981).

The pure cultures of tested strains procured from Center for Cultivation and Utilization of BGA, IARI, New Delhi was maintained in BG-11 liquid medium under standard conditions for their mass cultivation. The developed green scum of pure BGA cultures were filtered from liquid medium through Millipore filters (0.45 µ). This filtrate was mixed in the ratio of 1:10 with the culture base of pure clay soil (Multaani Mitti) carefully. This mixture then allowed to dry at room temperature then crushed to make its powder carefully and stored in separate poly bags for field analysis (Plate 5).

3.5.4 Details of the Experiment:

3.5.4.1 Preparation of field: Field was ploughed once by disk harrow, shallow harrowed by spring tyne cultivator and leveled in the first week of July. The plots were separated into three rows by earthen bends and layout of field experiment is included in this study were-

i. Control plots (Neither chemical fertilizer nor Cyanobacterial).
ii. Three plots with individual Cyanobacterial inoculants only.
iii. Plot with the combination of all three Cyanobacterial inoculants.

Three replicates of described experimental plots were designed randomly in experimental field.

3.5.4.2 Raising of Paddy nursery and transplantation in experimental field: 500 gm paddy seeds (var. PR 114) were first dipped in water for 10 min. The grains floating on water surface were removed and discarded. Healthy grains were selected and then surface sterilized in 0.3% HgCl₂ solution for 10 minutes then repeatedly washed with six changes of sterilized distilled water to remove all traces of mercuric chloride. Sterilized
healthy seeds were taken and sown in pre-prepared seed beds for raising the Paddy in
to nursery. Plants from nursery were raised during first week of June. The seedlings
were uprooted from the nursery after four to six weeks and one or two healthy seedlings
were transplanted in experimental plots at the distance of 30 cm. From line to line 20
cm, from plant to plant in the first week of July. Before transplanting the seedlings, all
the plots were flooded with water up to 4-5 cm height.

3.5.5 Detail of observations recorded: The observations were taken on the bases of
following parameters –

i. Plant height (cm): Height of the paddy crop was measured at different stages of
growth i.e., 35 DAS (Days after sowing), 70 DAS and 110 DAS or transplantation of
seedlings. Three hills were selected randomly from each plot and then three plants
tagged out randomly from selected hills. Length between the base of stem touching
the ground and tip of the top of the most leaf and / or panicle was considered as
height of plant and panicle.

ii. Leaf length: The length of the leaf of the tagged plants was measured in centimeter
(cm) at regular interval of time i.e. 35 DAS, 70 DAS and 110 DAS.

iii. Number of tillers per hill: Number of tillers per hill was counted finally after 110 days
of the transplantation. Three hills were tagged for the purpose. Only healthy and
panicle bearing tillers were counted. Average value of tillers per hill was calculated.
iv. Root length: The root length of paddy crop was finally measured 110 DAS or transplantation of seedlings. From each plots three hills were carefully digged out (40 cm deep) and washed well with water and measured the length of the main root in centimeter.

v. Number of seeds per ear: Number of seeds/ear was counted after maturity of the crop.

vi. Number of seeds per hill: Three hills were selected to count total number of seeds after harvesting of the crop.

vi. Seeds weight: Random samples were collected from the cleaned grain counted and weight of 100 healthy seeds was recorded.

vii. Grain yield: After threshing, the grain yield was estimated by weighing the seeds from each plot (1/m²) separately and was converted into the (q ha⁻¹).

3.5.6 Post transplantation operation:

3.5.6.1 Water management: The crop was grown under well irrigated condition for the proper growth of Cyanobacterial inoculants as well as paddy.

3.5.6.2 Weed management: To avoid the interaction of weedicide with crop and Cyanobacterial inoculants weeding was carried out manually at different times depending upon needs.
3.5.6.3 **Harvesting:** The crop for each experimental plot was harvested separately in the last week of month October, on its complete maturity. The produce was kept for drying in sun for a week and was threshed.

3.6 **METHODOLOGY FOR ESTIMATION OF PROTEIN, CARBOHYDRATE, AMINO ACIDS:**

The aim of experiment was to understand the impact of Cyanobacterial application on the nutritional value of germinated paddy grains (de-husked) and compared with the grains grown without Cyanobacterial application; taken as control. Experiment was conducted in three steps (i) by estimation of protein (ii) estimation of carbohydrate (iii) and estimation of amino acids of paddy grains grown with and without Cyanobacterial application. GABA (γ- amino butyric acid) was also estimated as additional work under this experiment because of its biochemical importance.

3.6.1 **Germination of Paddy seeds:** One gram of seeds per sample (in three replicates) was soaked in distilled water for their germination. Prior to development of germinated Paddy grains, all grain samples were surface-sterilized with 0.1% HgCl₂ for 1 min followed immediately by rinsing five times in distilled water. The seeds were then soaked in distilled water for 12 hrs.

The Paddy seeds were then wrapped with moistened cheesecloth and left in an open vessel for 12 hrs. The hull, roots and shoot were removed carefully followed by surface sterilization as described above and all Paddy grain samples were collected separately for the biochemical analysis (Protein, Amino acids and Carbohydrate).
3.6.2 ESTIMATION OF PROTEIN: Protein estimation was done with the help of Lowry’s method (1951).

3.6.2.1 Reagents:

1. Phosphate buffer (0.1 M, pH 7.6)*,

2. Alkaline Na$_2$CO$_3$ solution (20g/lit Na$_2$CO$_3$ in 0.1 mol/lit NaOH),

3. Copper sulphate reagent,

4. Alkaline solution** prepared on a day of use,

5. Folin’s reagent,

6. 20% (w/v) TCA,

7. Acetone, 0.1 N NaOH,

8. Standard protein (Bovine serum albumin (BSA) 0.2 mg/ml).

Potassium phosphate buffer*

Solution A: 0.2 M monobasic sodium phosphate (31.20 gm of sodium dihydrogen orthophosphate dissolve per liter of distilled water).

Solution B: 0.2 M dibasic disodium orthophosphate dissolve per liter of distilled water.
Working solution was made by adding 16 ml of solution A with 84 ml of solution B. The working solution was diluted with distilled water to make the total volume 200 ml. The pH was maintained at 6.8.

**Alkaline solution**

Solution A: 400 mg of NaOH + 2 gm Na2CO3 was dissolved in 200 ml distilled water.

Solution B: 50 mg of CuSO4.5H2O + 1% sodium potassium tartarate dissolved in 200 ml of distilled water.

Working solution was prepared by dissolving, 50 ml of solution A and 1 ml of solution B.

3.6.2.2 Details of the experiment: Paddy grains (germinated and de-hulled samples) grown with and without Cyanobacterial application were separately grinded in pestle mortar and air dried to powder. Then 1 gm of grain powder separately macerated in 5ml of phosphate buffer and transferred to centrifuge tubes. Keep it for one hour then samples were centrifuged at 8000 rpm for 20 min. at room temperature, collect the supernatant and repeated the extraction 4-5 times. Supernatants were combining and made the volume to 50 ml with phosphate buffer. In 1 ml of extract added 1 ml of 20% TCA. It was kept for half an hour and centrifuge at 8000 rpm for 20 min. The pellet was washed with acetone twice. Supernatant was discarded after again centrifuge it. The pallet was then dissolved in 5 ml of 0.1 N NaOH and mix well till it gets dissolved.

Take suitable aliquot (1 ml) of above solution and add to it 5 ml of freshly prepared alkaline copper sulphate reagent. Mix properly and after 10 min add 0.5 ml of Follin’s reagent. The contents were mixed instantaneously. Allow the colour to develop (blue)
for 30 min. The absorbance was recorded at 660 nm after setting the instrument with reagent blank which contains 1 ml of 0.1 N NaOH instead of the sample aliquot. In another set of tubes suitable aliquot of BSA solution was taken to make the total volume of 1 ml with 0.1 N NaOH and allowed the color to develop for 30 min. A standard curve of absorbance at 660 nm versus µg of BSA was drawn. From this standard curve the amount of protein was calculated as per gm of the sample.

3.6.3 ESTIMATION OF AMINO ACIDS: In this experiment free amino acids content of paddy seeds (germinated and de-hulled) grown with and without Cyanobacterial application, were quantitatively estimated for individual free amino acids by following the standard methods as followed-

3.6.3.1 Estimation of Glycine (Gly):

Quantitative estimation of Glycine was carried out by Ninhydrin method described by Sawhney and Shing, (1999).

Reagents

1. 0.2 M Citrate buffer (pH 5): 10.5 gm citric acid dissolved in 100 ml of 1N NaOH. Make up to 250 ml with distilled water.
2. Ninhydrin Reagent: 2 gm of ninhydrin dissolved in 25 ml ethylene glycol monoethyle. To it add 25 ml of 0.2 M acetate buffer*.
3. 0.2 M Acetate buffer (pH 5.5)* -
   (A) 0.115 ml acetic acid make up to 10 ml with distilled water.
   (B) 0.82 gm sodium acetate in 50 ml distilled water.
   4.4 ml of A + 20.6 ml of B = Make up to 50 ml with distilled water.
**Details of the experiments:** For extraction of free concentration of Glycine 1.0 gm of de-hulled germinated paddy seeds were grinded in 10 ml of 70% ethanol in a pestle and mortar. Homogenate was centrifuged at 5000 rpm for 10 min, decant the supernatant. Combine the supernatant and made the volume to 50 ml with 70% (v/v) ethanol. 10 ml of this ethanolic extract of germinating paddy seeds taken and evaporate it to dryness on a boiling bath and dissolved the residue in 5 ml of 0.2 m citrate buffer. Pipette 2 ml of the prepared sample in a test tube. 0.2 M citrate buffer was used as a reagent blank.

In another set take 11 test tubes and add Glycine solution of concentration 100 µg/µl in order starting from 0-1 ml (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml) respectively. Now to it add 0.2 M citrate buffer (pH 5.0) in order starting from 2 ml to 1.0 ml (for make total volume 2 ml) respectively. Now, 1 ml of KCN- acetone ninhydrin reagent was added in each tube and mixed thoroughly. Kept the test tubes in boiling water bath for 20 min, cool under running tap water and made the volume to 10 ml with distilled water. The absorbance was taken at 570 nm after setting the instrument with reagent blank. A standard curve was prepared by plotting $A_{570}$ against amount of Glycine. Free concentration of Glycine in the seeds of paddy was expressed in terms of mg of Glycine equivalents to per gm of seeds with the help of standard curve.
3.6.3.2 Estimation of Proline:

For rapid quantitative analyses of Proline from de-hulled germinated Paddy seeds, Bates et al., (1973) method was followed.

**Materials**

1. Sulphosalicylic acid
2. Glacial acetic acid
3. Ninhydrin solution
4. Toulene or benzene

**Procedure**

Free concentration of Proline was extracted by grinding 1.0 gm of de-hulled germinated Paddy seeds in 5 ml of 3% sulphosalicylic acid in a pestle and mortar. Homogenate was centrifuged at 5000 rpm for 10 min, decant the supernatant. In supernatant 5 ml glacial acetic acid and 5 ml freshly prepared ninhydrin solution was added. The mixture was boiled for 1 hour in water bath and then cooled at room temperature. 10 ml toulene or benzene was added to mixture. Mixed well and allowed to stand for sometime till it become clear. Separate the benzene layer carefully and absorbance of transmittance was taken at 520 nm. A standard curve was prepared by plotting $A_{520}$ against amount of Proline. Free concentration of Proline in the seeds of Paddy was expressed in terms of mg of Proline equivalents to per gm of seeds with the help of standard curve.
3.6.4 Estimation of GABA: (Kitaoka and Nakano, 1969)

Each of the de-hulled germinated ground Rice samples (3 mg) was dissolved with 80% ethanol in a test tube (18 x120 mm), shaken thoroughly, and then filtered with filter paper (no. 1). The filtered solution was boiled in a water bath (80 °C) to evaporate the ethanol. This was followed by addition of 0.5 ml distilled water, and then centrifugation at 10 000 rpm for 10 min. The floating portion on top was aspirated, and 0.2 ml of 0.2 M borate buffer and 1.0 ml of 6% phenol were added. For the standard GABA solution (0.1–0.3 ml) was added to test tubes (18 x120 mm) together with 0.2 ml of borate buffer and 1.0 ml of phenol reagent. The solutions were mixed thoroughly and cooled in a cooling bath for 5 min. Next, 0.4 ml of 10– 15% NaOCl was added, and the solution was shaken vigorously for 1 min, and again cooled in a cooling bath for 5 min. Finally, the solution was boiled in a water bath (100 °C) for 10 min, and allowed to cool. Optical density was recorded with the help of spectrophotometer at a wavelength of 630 nm, with ethanol 2.0 ml as a blank. GABA content was quantified by comparing the optical density reading with the standard GABA content curve.

3.6.5 Estimation of carbohydrate (Dubois et al., 1956):

Dried powder of de-hulled germinated Paddy grains (50 mg) was extracted with 80% ethanol at room temperature for one hour and centrifuged at 1000 g at 25 °C for 60 minutes. The pallet was re-extracted with 80% ethanol for 10 minutes and centrifuged at 10,000 g at 25 °C. The two supernatant were poured and the volume was reduced in vacuo to 25 ml, which was used for the analysis of the total soluble sugar.
**Materials and Reagents:** Phenol reagent was freshly prepared by mixing 10 ml of distilled water with 90 ml of phenol solution (90% w/v). Standard Glucose solution was prepared by dissolve 100 mg glucose in 100 ml of distilled water.

**Procedure:**

The extract 0.1 ml was made up to 2 ml with distilled water. To this, 0.5 ml phenol reagent was added. The colour was developed by rapidly adding 5 ml conc. H₂SO₄ and the solution was allowed to stand at room temperature (25 °C) for 30 minutes. The absorbance was recorded at 485 nm.

The sugar concentration in gm per 100 gm of Paddy grains was calculated using a standard curve prepared by different concentration (20-200 µg of D- Glucose).

3.7 METHODOLOGY FOR EXTRACTION AND CHARACTERIZATION OF ALLELOCHEMICALS.

3.7.1 EXTRACTION: For extraction of allelochemicals from algal extracts, following attempts were made-

(i) **Selection of a Solvent:** (Hughes, 2002)

Successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in extraction of secondary metabolites or allelochemicals include:

- Low toxicity,
- Ease of evaporation at low heat,
- Promotion of rapid physiological absorption of the extract,
- Preservative action and inability to cause the extract to complex or dissociate.

(ii) **Method:** (Green, 2004)

Variations in extraction method are usually depend on the

- Length of the extraction period,
- Solvent used,
- pH of the solvent,
- Temperature,
- Solvent-to-sample ratio.

The basic principle is to grind the sample (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal. In the present study methanol was selected as solvent.

(iii) **Centrifugation for extraction:** (Starr et al., 1962)

The air dried cultures of selected strains were crushed to power with the help of pastel mortar and put in to centrifuge tubes in the ratio of 10:1 solvent to sample and applied for centrifugation at 5000 rpm for 20 min. After centrifugation the supernatant was collected carefully. This process was repeated three times and combined the supernatant separately. This crude algal methanolic extract was used for characterization of allelochemicals.
3.7.2 CHARACTERIZATION:

In this part of experiment, qualitative and quantitative analyses of methanol extracts (crude) of *Nostoc* sp., *Anabaena variabilis*, *Aulosira fertilissima*, were carried out with a Shimadzu GC-2010 gas chromatography (GC) coupled with a Shimadzu GCMS QP-2010 series system (AIRF, JNU, New Delhi) equipped with a AB inno-wax column (60 m x 0.25 mm id, film thickness 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas a constant flow rate of 1.2 ml min$^{-1}$. Injector and mass transfer line temperature were set at 270 °C and 280 °C, respectively. The oven temperature was programmed from 50 to 180 °C at 3 °C min$^{-1}$ with hold time of min$^{-1}$ and from 180 to 250 °C at 5 °C min$^{-1}$ with hold time 20 min respectively.

Diluted samples (prepared in methanol) of 0.2 µl were manually injected in the split less mode. Identification of compounds of the samples was based on GC retention time on AB inno-wax column, computer matching of mass spectra with standards (Mainlab, Rapid and Tutorial data of GC/MS systems). Total GC running time was 30 min. The relative percentage amount of each compound was calculated by comparing its average peak area to the total areas.
3.8 **Statistical analysis:** During course of present study all observations were supported by statistical analysis i.e., Mean, Standard deviation (SD), Analysis of Variance (ANOVA) by using Microsoft Excel- 2007 data analysis tool and SPSS software respectively.