Materials and Methods

2.1 Experimental plant

Two species of *Azolla*, viz. *Azolla pinnata* var. imbricata and *Azolla microphylla* have been selected for present study (Photoplate 1 and 2). *Azolla pinnata* was procured from the pond of the Botany Department of university of Allahabad, and the *Azolla microphylla* was collected from National Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi and cultured in Roxburg garden, Department of Botany, University of Allahabad. The genus name, *Azolla*, is a conjugation of two Greek words, *Azo* (to dry) and *oilyo* (to kill), suggesting the fern is killed by drought. Some of the fern's vernacular names are: water velvet, mosquito fern (English); Algenfarn (German); Helechito del Agua (Spanish); Lu P'ing, Ho P’ing, Man Chiung hung shu (Chinese).

*Azolla* belongs to the salviniales which is closely related to the hymenophyllaceae. Lamarck established the genus *Azolla* in 1783 after examining specimens brought from Chile (Griffith 1845). The genus was originally included in the salviniaeaceae a family of heterosporous free-floating ferns. But some taxonomists have assigned *Azolla* to a monotypic family, Azollaceae separate from the genus *Salvinia* (Reed 1954). *Azolla* occurs in ponds, ditches and paddy fields of warm-temperate and tropical regions throughout the world.

**Morphology** - *Azolla* plants are triangular or polygonal in shape, and float on the water surface individually or in mats. They give the appearance of a dark green to reddish carpet. The main rhizome bears several alternating branches with attached lateral branches. At the point of attachment each branch has an
abscission layer which is important in vegetative reproduction. The papillose dorsal lobes are chlorophyllous except in the colorless margin and contain the symbiont (cyanobacteria) within an ovoid cavity connected to the atmosphere by a pore. Stomata are present in vertical rows on both surfaces of the dorsal lobe and on the superior surface of the ventral lobe (Inamdar et al. 1971).

2.2 Nutrient Medium

*Azolla* fronds were kept in Espianase and watanabe (E and W) medium (1976). Composition of Espianase and watanabe medium is given below

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>Concentration (µg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>87.8</td>
</tr>
<tr>
<td>MgSO(_4)(_7)H(_2)O</td>
<td>76.5</td>
</tr>
<tr>
<td>CaCl(_2)(_2)H(_2)O</td>
<td>147.7</td>
</tr>
<tr>
<td>KCl</td>
<td>40.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>Concentration (µg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_3)BO(_3)</td>
<td>1.4</td>
</tr>
<tr>
<td>MnSO(_4)(_7)H(_2)O</td>
<td>2.52</td>
</tr>
<tr>
<td>ZnSO(_4)(_7)H(_2)O</td>
<td>0.044</td>
</tr>
<tr>
<td>Na(_2)MoO(_4)(_2)H(_2)O</td>
<td>0.378</td>
</tr>
<tr>
<td>CuSO(_4)(_5)H(_2)O</td>
<td>0.039</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.087</td>
</tr>
</tbody>
</table>

2.3 Glassware, plastic pots and chemicals

Glassware (Petri plates, pipette, test tubes, flasks etc.), used in the present study, were made of “Borosil”. For experimental use, plastic pots of 220 ml capacity containing 200 ml of medium were used. The chemicals were purchased from standard companies like Sigma, Merk, Himedia and Loba Chemie, etc.

2.4 Sterilization of glassware and nutrient medium
All the glassware and nutrient medium were sterilized in an autoclave at 15 lb inch⁻² and at 121 °C for 15 min, while the plastic trays and plastic pots used in present study were rinsed and surface sterilized with absolute alcohol.

2.5 Growth Conditions

The plants were surface sterilized quickly with a solution of mercuric chloride (0.1% for 30 s) followed by dipping the plants into a large volume of sterile distilled water. Washing of the Azolla with sterile distilled water was repeated several times. Fronds were then transferred into plastic trays (32×25×6 cm) containing combined-N free Espianase and watanabe medium (1976). The pH of the medium was adjusted to 7.2. Plastic trays were placed in the Roxburg garden, Department of Botany, University of Allahabad, during the experimental period, average minimum and maximum temperature ranged from 16.7 to 36.8 °C, and relative humidity 55 to 71%. Photosynthetic active radiation (PAR) ranged between 800 -1000 µmol photon m⁻² s⁻¹.

Mode of stress

In the present study two abiotic stresses pesticide (rice field herbicide pretilachlor) and enhanced Ultraviolet-B radiation (above ambient level) were chosen for detailed study.

Pesticide used

Pesticide, pretilachlor [2-chloro-2', 6'-diethyl-N-(2-propoxyethyl) acetanilide] 50% EC [manufactured by Krishi Rasayan Exports Pvt. Ltd. 1st parallel Road, Industrial Growth Centre, Samba, Jammu (J & K)] was selected for the treatments. This is widely used as herbicide to control grasses, sedges, broad leaved weeds like Echinochloa, Cyperus iria, Cyperus difformis, Fimbristylis milliaceae, Ludwigia parviflora, Pannicum repens etc. in rice fields particularly in Asian and South American countries. The details of the herbicide are given below:
Chapter 2: Materials and Methods

**Status:** ISO 1750 (published)

**IUPAC:** 2-chloro-2',6'-diethyl-N-(2-propoxyethyl) acetalnilide

**CAS:** 2-chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetamide

**Reg. No.:** 51218-49-6

**Formula:** $C_{17}H_{26}ClNO_2$

**Activity:** herbicides (chloroacetanilide herbicides)

**Structure:**

![Chemical Structure](image)

**Pesticide concentration and selection of doses**

For the pesticide concentration stock 1 (500 µg ml$^{-1}$) from original sample (Pretilachlor 50% EC) was prepared in Espianase and Watanabe medium. From this stock 1, stock 2 (100 µg ml$^{-1}$) was prepared. From this stock various doses (2, 5, 10, 15, 20, 25, 30, 40 and 50 µg ml$^{-1}$) of pretilachlor in nutrient medium were prepared for screening experiments. On the basis of the screening experiments 5, 10 and 20 µg ml$^{-1}$ were selected for detailed study. The concentrations used in the present study are environmentally relevant and comparable to those present under natural conditions.

**Ultraviolet–B irradiation**

Ultraviolet–B irradiation was provided artificially by UV-B lamps (Philips, TL 40W/12, The Netherlands with emission peak at 312 nm). The
radiation was filtered through 0.127 mm cellulose acetate (Johnston Industrial Plastics, Toronto, Canada) to remove all incidents UV-C (< 280 nm). The intensity of UV-B irradiation was measured with the help of Power Meter (Spectra Physics, A-2, model 407, USA).

**UV-B treatment and selection of doses**

Enhanced UV-B radiation (above ambient level) was provided artificially by UV-B lamps hanging above and perpendicular to the pot rows on an adjustable frame. Enhanced UV-B was provided daily from 9:30 (3.30 h after the beginning of the photoperiod) to 15:30 h. Each sample was also receiving solar ambient level of UV-B (8.6 kJ m⁻² day⁻¹) radiation. Out of various doses of enhanced UV-B radiation (1.1, 2.2, 3.3, 4.4, 5.5 and 6.6 kJ m⁻² day⁻¹), two levels of enhanced UV-B doses were selected after screening experiment. For treatment, fronds were exposed to two levels of enhanced UV-B radiation, the low (UV-B₁: ambient + 2.2 kJ m⁻² day⁻¹) and high (UV-B₂: ambient + 4.4 kJ m⁻² day⁻¹) biologically effective UV-B (UV-B_{BE}), simulating 6 and 12% depletion, respectively in stratospheric ozone at Allahabad (25° 28' N latitude, 81° 54'E longitude). There were negligible differences in experimental temperature, relative humidity and PAR during two type of treatment: pretilachlor and enhanced UV-B radiation.

**Experimental Design**

Fronds of both species were harvested from culture tank and acclimatized in nutrient medium. After 24 h, they were divided into six sets with 500 mg fronds in each pot. The first set was allowed to grow without either of the stresses and was considered as control. The second and third sets were exposed to four successive exposures of enhanced UV-B radiation, UV-B₁ and UV-B₂, respectively. The fourth, fifth and sixth set were treated with various concentrations of pretilachlor (5, 10 and 20 µg ml⁻¹). The fifth and sixth sets (earlier treated with different pretilachlor concentrations) were also
exposed to four successive exposures of enhanced UV-B radiation, UV-B$_1$ and UV-B$_2$, respectively. Thus, the fifth and sixth sets were studied for analyzing the effects of different concentrations of pretilachlor and UV-B with their interactive effects. The fronds of each set were harvested and various parameters were analyzed in three replicates after 96 h of experiment set up (Experimental design 1).

2.6 Growth analysis

Biomass accumulation

Prior to initial measurements the treated and untreated fronds were washed in distilled water and gently blotted several times with pieces of absorbent paper to remove adhering liquid and epiphytic algae. The growth pattern of the *Azolla microphylla* and *Azolla pinnata* was monitored by observing the biomass accumulation in terms of fresh mass and dry mass. Fresh biomass of both *Azolla* species was harvested and weighed after 96 using digital balance (Contech- CA 223, India) and dry mass was estimated after oven drying of the plant material for 48 h at 90 °C. The growth rate was expressed as the biomass increase/decrease (mg fresh mass).

Relative growth rate

The relative growth rate (RGR) was determined using the following formula (Harper 1977).

\[
\text{Relative Growth Rate (RGR)} = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}
\]

Where $W_1$ and $W_2$ represent plant fresh mass (mg) at $T_1$ and $T_2$, respectively, and $T_1$ and $T_2$ represent the initial and final time for experiment.
2.8 Estimation of protein

Protein content of each sample was measured by the method of Lowry et al. (1951) modified by Herbert et al. (1971).

Reagents

- **NaOH**: 1.0 N
- **Reagent A**: 0.2% Na$_2$CO$_3$ in 0.1 N sodium hydroxide
- **Reagent B**: 0.5% CuSO$_4$.5H$_2$O in 1.0% sodium potassium tartarate
- **Reagent C**: 50 ml reagent C was prepared by mixing 48 ml of reagent A with 2 ml of reagent B
- **Folin-Ciocalteu’s reagent**: 1:2 (v/v in DDW)

Procedure

For the measurement of protein, 20 mg of treated and untreated fronds thoroughly washed and digested with 0.5 ml of 1N NaOH in a boiling water bath for 10 min. After sufficient cooling, 2.5 ml of reagent C was added and reaction mixture was incubated for 15 min at room temperature followed by addition of 0.5 ml of Folin-Ciocalteu’s reagent. Reaction mixture was centrifuged quickly and the intensity of the resulting blue colour was determined spectrophotometrically at 650 nm. The amount of protein was estimated using standard curve prepared with bovine serum albumin.

2.7 Extraction and estimation of pigments

2.7.1 Estimation of photosynthetic pigments chlorophylls and carotenoids

Treated and untreated fronds (12 mg) of both species were cut into small pieces and photosynthetic pigments were extracted in 80% (v/v) acetone. The extracts were centrifuged and pellets were resuspended in 80% acetone, until they became colourless. The absorbance of the resulting solutions was recorded at 663.2, 646.5 and 470 nm spectrophotometrically (Shimadzu double beam UV–Visible spectrophotometer-1700, Japan). The amount of chlorophylls (Chl $a$ and $b$), carotenoids and total chlorophylls were determined.
and Chl b) and carotenoids were calculated by using the equations of Lichtenthaler (1987).

\[
\begin{align*}
\text{Chl } a \ (\mu g/ml) & = 12.25 \ (A_{663.2}) - 2.79 \ (A_{646.5}) \\
\text{Chl } b \ (\mu g/ml) & = 21.50 \ (A_{646.5}) - 5.10 \ (A_{663.2}) \\
\text{Car} \ (\mu g/ml) & = [(1000 \ A_{470} - 1.82 \ (\text{Chl } a) - 85.02 \ (\text{Chl } b)] / 198
\end{align*}
\]

Where,

\[\begin{align*}
\text{Chl } a & = \text{Chlorophyll } a \\
\text{Chl } b & = \text{Chlorophyll } b \\
\text{Car} & = \text{carotenoids}
\end{align*}\]

2.9 Measurement of photosynthetic and respiratory activities

2.9.1 Photosynthetic oxygen yield

Photosynthetic oxygen yield of treated and untreated samples was measured as described by Kurra–Hotta et al. (1987).

**Reagents**

\[
\begin{align*}
\text{CaSO}_4 \cdot 1/2 \text{H}_2\text{O} & : 0.5 \text{ mM} \\
\text{NaHCO}_3 & : 20.0 \text{ mM} \\
\text{HEPES-NaOH Buffer (pH 7.6)} & : 50.0 \text{ mM}
\end{align*}
\]

**Procedure**

Photosynthetic oxygen yield was measured in terms of oxygen evolution using Clark type oxygen electrode (Digital Oxygen System, Model-10, Rank Brothers, UK). Fronds were sliced into small pieces by keeping them in a Petri dish containing 0.5 mM CaSO\(_4\) solution. The sliced pieces were transferred in air tight vessel of oxygen electrode containing 20 mM NaHCO\(_3\) in 5 ml of 50 mM HEPES–NaOH buffer (pH 7.6), and the oxygen evolution (photosynthesis) in light (400 \mu\text{mol} \text{ photon m}^{-2} \text{ s}^{-1}, \text{PAR}) and consumption (respiration) in darkness were recorded for 5 min. The temperature around the vessel was maintained at 25 °C by water jacket. Photosynthetic and
respiratory rates were expressed as µmol oxygen evolved/consumed (g FM)\(^{-1}\) h\(^{-1}\).

### 2.9.2 Photosynthetic electron transport activity in isolated chloroplasts

Photosynthetic electron transport activities (photosystem I, photosystem II and whole chain) were determined in isolated chloroplasts of *Azolla* fronds of treated and untreated samples.

#### Isolation of chloroplasts

Chloroplasts were isolated following the method of Mishra and Singhal (1992).

#### Reagents

**Incubation buffer**

- Sucrose : 400 mM
- HEPES–NaOH Buffer (pH 7.6) : 20 mM
- NaCl : 10 mM
- MgCl\(_2\).7H\(_2\)O : 5 mM

#### Procedure

Fresh *Azolla* fronds from treated and untreated samples were cut into pieces and homogenized in a chilled isolation buffer containing 400 mM sucrose, 20 mM HEPES–NaOH buffer (pH 7.6), 15 mM NaCl and 5 mM MgCl\(_2\).7H\(_2\)O at 4 °C. The slurry was filtered through eight layers of muslin cloth. The filtrate was centrifuged at 1,000 g for 1 min to remove the cell debris. The supernatant was again centrifuged at 20,000 g at 4 °C for 15 min by using CPR 30, Remi, India. The chloroplasts containing pellets were washed and resuspended in the same buffer and kept in dark at 4 °C before use.

**(i) Photosystem I (PSI) activity**

For measurement of PSI activity, reaction mixture was (6 ml) consisted of 20 mM HEPES–NaOH buffer, 0.4 M sucrose, 5 mM MgCl\(_2\).7H\(_2\)O, 10 mM...
Materials and Methods

NaCl, 1.0 mM sodium ascorbate (ASC), 0.05 mM DCPIP (2, 6–dichlorophenol indophenol), 10 μM DCMU (3–(3, 4–dichloro diphenyl) – 1, 1–dimethyl urea), 0.05 mM sodium azide (NaN₃), 0.05 mM MV (methyl viologen) and chloroplasts equivalent to 20 μg chlorophyll. Uncoupled electron transport mediated by PSI was determined polarographically as oxygen consumption in the presence of DCPIP / ASC as electron donor system (DCPIPH₂) and MV as terminal auto–oxidizable electron acceptor, using an oxygen electrode (Rank Brothers, UK). DCMU was used to block the electron flow from PSII, while NaN₃ was used to inhibit catalase activity.

\[ \text{DCPIP/ASC} \rightarrow \text{MV} \]

(ii) Photosystem II (PSII) activity

The electron transport activity through PSII was monitored spectrophotometrically as well as polarographically. PSII dependent DCPIP photoreduction was measured spectrophotometrically (Vis-167) by observing the decrease in the absorbance at 600 nm. The reaction mixture (3 ml) consisted of 20 mM HEPES–NaOH (pH 7.6), 400 mM sucrose, 10 mM NaCl, 5 mM MgCl₂.7H₂O, 50 μM DCPIP and the chloroplasts equivalent to 3 μg chlorophyll.

\[ \text{H₂O} \rightarrow \text{DCPIP} \]

Polarographic assay of PSII activity was measured in terms of O₂ evolution in the presence of p–benzoquinone (p-BQ; 1 mM). The chloroplasts equivalent to 20 μg Chl were suspended in reaction mixture containing 400 mM sucrose, 20 mM HEPES–NaOH buffer, 5 mM MgCl₂ .7H₂O, 10 mM NaCl and 1 mM p-BQ.

\[ \text{H₂O} \rightarrow p\text{-BQ} \quad \text{(as O₂ evolution)} \]

To pin point the actual site of action of given stresses on PSII, DCPIP photoreduction was also measured by addition of various artificial electron
donors i.e. DPC (Diphenyl carbazide), 5 mM; NH₂OH (Hydroxylamine), 5 mM; MnCl₂ (Manganese chloride) 3 mM, in the reaction mixture separately.

(iii) **Whole chain electron transport activity**

The reaction mixture (6 ml) for whole chain electron transport activity contained reaction buffer, 0.05 mM NaN₃, 0.05 mM MV and chloroplasts equivalent to 20 μg Chl and rate was measured in terms of O₂ consumption.

\[ \text{H₂O} \rightarrow \text{MV (as O₂ consumption)} \]

In each assay, the photosynthetic photon flux density PPFD equivalent to 400 μmol photons m⁻² s⁻¹ at the surface of the electrode cell was used. In each assay, the temperature around the electrode vessel was maintained 25 °C.

**Source of illumination and maintenance of temperature**

- Projector lamp (halogen lamp) : with 400 μmol photons m⁻² s⁻¹
- Temperature : 25 °C was maintained in auto-regulated water bath

2.10 **Nitrogen metabolism**

2.10.1 **Nitrate reductase activity**

Nitrate reductase (NR; EC 1.6.6.1) activity, was measured according to the modified method described by Debouba et al. (2006). Measurement of the activity was based on the total nitrite formed.

**Reagents**-

- Potassium phosphate buffer (pH 7.5) : 0.1 M
- Cysteine : 5 mM
- EDTA : 2 mM
- PVP : 0.5%
- KNO₃ : 7 mM
- NADH : 0.14 mM
- Zinc acetate : 0.5 M
EDTA : 5 mM  
Sulfanilamide : 1% (w/v, 1:4 HCl: Water)  
NEDD : 0.01% (w/v, in water)  

Procedure  
For estimation of NR activity, fresh fronds (100 mg) were homogenized (1:5, w/v) in an ice-cold mortar using 0.1 M potassium phosphate buffer pH 7.5 containing 5 mM cysteine, 2 mM EDTA, and 0.5% PVP. After centrifugation (20,000 g for 20 min), the supernatant was used for the determination of NR activity. For estimation of NR activity, the reaction mixture consisted of 0.1 M potassium phosphate buffer pH 7.5 containing 5 mM EDTA, 7 mM KNO₃, 0.14 mM NADH, and enzyme extract. The reaction was started by addition of NADH. After 30 min of incubation at 27 °C, the reaction was stopped by addition of 0.5 M zinc acetate and then incubate was centrifuged (3000 g for 10 min). The nitrite formed was determined colorimetrically after diazotation with 1% sulfanilamide and 0.01% naphthylenediamine dihydrochloride (NEDD) according to the method of Snell and Snell (1949). After 20 min of incubation at room temperature, the absorbance was measured at 540 nm and the amount of nitrite formed was calculated using a standard calibration curve prepared for NaNO₂. Activity of nitrate reductase is expressed in terms of μmol nitrite formed (g FM)⁻¹ h⁻¹.

2.12.2 Nitrite reductase activity  
Nitrite reductase (NiR; EC 1.7.7.1) activity was measured as the reduction in the amount of nitrite in the reaction mixture according to the modified method of Debouba et al. (2006).

Reagents-  
Potassium phosphate buffer (pH 7.5) : 0.1 M
### Materials and Methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>5 mM</td>
</tr>
<tr>
<td>Potassium phosphate buffer (pH 6.8)</td>
<td>0.1 M</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>2.3 mM</td>
</tr>
<tr>
<td>Sodium dithionite</td>
<td>4.3 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>100 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
</tr>
<tr>
<td>PVP</td>
<td>0.5%</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>1% (w/v, 1:4 HCl: Water)</td>
</tr>
<tr>
<td>NEDD</td>
<td>0.01% (w/v, in water)</td>
</tr>
</tbody>
</table>

### Procedure

For estimation of NiR activity, fresh fronds (50 mg) were homogenized (1:5, w/v) in an ice-cold mortar using 0.1 M potassium phosphate buffer pH 7.5 containing 5 mM cysteine, 2 mM EDTA, and 0.5% PVP. After centrifugation (20,000 g for 20 min), the supernatant was used for the determination of NiR activity. The reaction mixture consisted of 0.1 M potassium phosphate buffer pH 6.8, 0.4 mM NaNO₂, 2.3 mM methyl viologen, enzyme extract, and 4.3 mM sodium dithionite in 100 mM NaHCO₃, which started the reaction. After 30 min of incubation at 27 °C, the reaction was stopped by vortexing and boiling for 1 min and nitrite that remained in the reaction mixture were determined at 540 nm after reaction with Sulfanilamide and NEDD according to the method of Snell and Snell (1949). Activity of nitrite reductase is expressed in terms of μmol nitrite consumed (g FM)⁻¹ h⁻¹.

#### 3.5.2. Enzymes of ammonium assimilation

#### 3.5.2.3. Glutamine synthetase activity
Glutamine synthetase (GS; EC 6.3.1.2) activity was measured by the method of Lillo (1984).

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 7.8)</td>
<td>50 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15% (v/v)</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>14 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>500 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>100 mM</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>300 mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1% (v/v)</td>
</tr>
<tr>
<td>NH$_2$OH.HCl (neutralized by NaOH)</td>
<td>200 mM</td>
</tr>
<tr>
<td>tris-HCl</td>
<td>200 mM</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>2.5% (w/v)</td>
</tr>
<tr>
<td>TCA</td>
<td>5% (w/v)</td>
</tr>
<tr>
<td>HCl</td>
<td>1.5 M</td>
</tr>
</tbody>
</table>

**Procedure**

Fresh *Azolla* fronds (100 mg) were homogenized in extraction medium which contained 50 mM Tris-HCl (pH 7.8) contained 15% (v/v) glycerol, 14 mM 2-mercaptoethanol, 1 mM EDTA and 0.1% (v/v) Triton X-100 in cool condition. The homogenate was centrifuged for 15 min at 6000 g at 4 °C. The supernatant was used as enzyme extract. The reaction mixture (2 ml) contained 500 mM L-glutamate, 100 mM ATP (adenosine triphosphate), 300 mM MgSO$_4$, 200 mM NH$_2$OH.HCl (neutralized by NaOH) and 200 mM tris-HCl. The final pH of reaction was adjusted to 8.0. The reaction was started by the addition of 0.2 ml enzyme extract and then the reaction mixture was incubated for 30 min at 25 °C. The reaction was terminated by the addition of
4 ml of 2.5% (w/v) FeCl₃ and 0.5 ml of 5% (w/v) TCA (tri chloro acetic acid) in 1.5 M HCl. The absorbance was read spectrophotometrically at 540 nm and the enzyme activity was calculated by using standard curve prepared with γ-glutamyl hydroxamic acid. One unit of enzyme activity is defined as 1 nmol γ-glutamylhydroxamate produced (mg fresh mass)⁻¹ min⁻¹.

3.5.2.2. Glutamine 2-oxoglutarate aminotransferase activity

Glutamine 2-oxoglutarate aminotransferase also called as glutamate synthase (NADH-GOGAT; EC 1.4.1.14) activity was determined by the method of Singh and Srivastava (1986).

**Reagents**

- Sodium phosphate buffer (pH 7.5) : 0.2 M
- Sodium phosphate buffer (pH 7.3) : 25 mM
- L-Glutamine : 20 mM
- EDTA : 2 mM
- 2-Oxoglutarate : 5 mM
- NADH : 1 mM
- Triton X-100 : 0.5% (v/v)
- KCl : 50 mM
- Mercaptoethanol : 0.1% (v/v)

**Procedure**

Fresh *Azolla* fronds (100 mg) were homogenized in extraction medium which contained 0.2 M sodium phosphate buffer (pH 7.5) with 2 mM EDTA, 50 mM KCl, 0.1% (v/v) mercaptoethanol and 0.5% (v/v) Triton X-100 under cool condition. The homogenate was centrifuged for 15 min at 6000 g 4 °C. The supernatant was used as enzyme extract. The reaction mixture (3 ml) contained 25 mM sodium phosphate buffer (pH 7.3) containing 1 mM EDTA, 20 mM L-glutamine, 5 mM 2-oxoglutarate, 100 mM KCl, 1 mM NADH and 0.3
ml enzyme extract. Reaction was initiated by the addition of L-glutamine and decrease in absorbance was read spectrophotometrically at 340 nm for 5 min. The enzyme activity was calculated from the standard curve prepared with NADH. Activity of Glutamate dehydrogenase is expressed in terms of nmol NADH oxidized (mg fresh mass)$^{-1}$ min$^{-1}$.

3.5.2.1. Glutamate dehydrogenase activity

Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity (aminating) was assayed by the method of Singh and Srivastava (1983).

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate buffer (pH 7.4)</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Sodium phosphate buffer (pH 8.1)</td>
<td>0.5 M</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.2 M</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
</tr>
<tr>
<td>α Ammonium sulphate</td>
<td>1.5 M</td>
</tr>
<tr>
<td>NADH</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.4 M</td>
</tr>
</tbody>
</table>

**Procedure**

Fresh *Azolla fronds* (250 mg) were homogenized in extraction medium which contained 0.5 M sodium phosphate buffer (pH 7.4), 0.4 M sucrose and 2 mM EDTA in cool condition. The homogenate was centrifuged for 15 min at 15000 g at 4 °C. The supernatant was used as enzyme extract. The assay mixture (6 ml) consisted of 0.1 M sodium phosphate buffer (pH 8.1), 0.2 M 2-oxoglutarate, 1.5 M ammonium sulphate, 1 mM NADH and 0.2 ml of enzyme extract. Each component of assay mixture was prepared in 0.1 M sodium phosphate buffer (pH 8.1). The reaction was initiated by adding the substrate ammonium sulphate and decrease in absorbance was recorded for 5 min at
340 nm. The enzyme activity was calculated from the standard curve prepared with NADH. Activity of Glutamate dehydrogenase is expressed in terms of nmol NADH oxidized (mg fresh mass) \(^{-1}\) min\(^{-1}\).

2.11 Estimation of active oxygen species (AOS)

2.11.1 Superoxide radical

The amount of superoxide radical (SOR; O\(_2^•\)¯) was measured by the method of Elstner and Heupel (1976) with some modifications as described by Jiang and Zhang (2001) by monitoring the nitrite formation from hydroxylamine in the presence of O\(_2^•\)¯ in supernatant obtained from homogenates of each sample.

**Reagents**

- Potassium phosphate buffer (pH 7.8): 65 mM
- Hydroxylamine hydrochloride (NH\(_2\)OH.HCl): 10 mM
- Sulfanilamide: 17 mM
- \(\alpha\)-N (N-1)-Naphthylethylene diamine dihydrochloride (NEDD): 7 mM
- Diethyl ether: Commercial supply

**Procedure**

Treated and untreated *Azolla* fronds (1 g) from each set were homogenized in 3 ml of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 g for 10 min. The reaction was initiated by incubating the mixture containing 0.9 ml of 65 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine and 1 ml of supernatant. After incubation at 25 °C for 20 min, 17 mM sulfanilamide was added followed by 7 mM \(\alpha\)-naphthylene diamine dihydrochloride and mixed well. The reaction further proceeded up to 20 min. After completion of the reaction equal volume of diethyl ether was mixed well to each sample and left for the separation of the diazo compound
in aqueous phase. The absorbance of the pink color was read at 530 nm. The amount of NO$_2^-$ was determined with the help of standard curve prepared with NO$_2^-$.

2.11.2 Hydrogen peroxide

Hydrogen peroxide (H$_2$O$_2$) content in treated and untreated *Azolla* fronds was estimated by following the method Velikova et al. (2000).

**Reagents**

- Trichloroacetic acid (TCA): 0.1% (w/v)
- Potassium phosphate buffer (pH 7.0): 10 mM
- Potassium iodide: 1 M

**Procedure**

Treated and untreated fronds (40 mg) were harvested and homogenized in 3 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 8,000 g for 15 min; the supernatant obtained was used for H$_2$O$_2$ estimation. The reaction mixture contained supernatant, 10 mM potassium phosphate buffer (pH 7.0) and 1 mM potassium iodide. The absorbance of the solution was read at 390 nm against the blank and the concentration of H$_2$O$_2$ in each sample was calculated from standard curve prepared with H$_2$O$_2$.

2.12 Analysis of the indices of oxidative damage

2.12.1 Determination of lipid peroxidation

Lipid peroxidation in terms of malondialdehyde (MDA) was determined in *Azolla* fronds following pretilachlor and enhanced UV–B exposure. MDA is a product of polyunsaturated fatty acid peroxidation formed after reaction with thiobarbituric acid (TBA). MDA concentration was estimated by the method of Heath and Packer (1968).

**Reagents**

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Phosphate buffer (pH 7.0) : 50 mM
2-Thiobarbituric acid (TBA) : 0.5% (w/v) in 0.2 N HCl
Trichloroacetic acid (TCA) : 20% (w/v) in TBA solution
Trichloroacetic acid (TCA) : 5% (w/v) in DDW

Procedure

Treated and untreated fronds (250 mg) were homogenized with 2 ml 5% (w/v) TCA. The resulting homogenate was centrifuged at 10,000 g for 10 min. To an aliquot of 0.5 ml of the supernatant, 2 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 90 °C for 20 min and then quickly cooled in ice-bath followed by centrifugation. Absorbance of the supernatant was read at 532 nm and 600 nm. The value for non-specific absorption of each sample at 600 nm was subtracted from absorption recorded at 532 nm. MDA concentration was calculated using the extinction coefficient 155 mM$^{-1}$ cm$^{-1}$.

2.12.2 Protein oxidation

Determination of reactive carbonyl groups in protein was carried out according to method of Levine et al. (1994).

Reagents

Potassium phosphate buffer (pH 7.4) : 50 mM
Hydrochloric acid (HCl) : 2 N
2, 4-dinitrophenyl hydrazine (DNPH) : 10 mM
Potassium phosphate buffers (pH 2.3) : 2 mM
(pH adjusted by Trifluoro acetic acid)
Trichloroacetic acid (TCA) : 20% (w/v)
Trichloroacetic acid (TCA) : 40% (w/v)
Ethanol ethyl acetate : 100 ml (v/v)
Guanidine : 6 M
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Procedure

Fresh Azolla fronds (500 mg) from each set were homogenized in 3 ml 50 mM phosphate buffer (pH 7.4) at 4 °C and centrifuged at 8,000 g for 15 min; the supernatant obtained was used for carbonyl group estimation (protein oxidation). The supernatant obtained was added with 20% trichloroacetic acid, so it gave final strength of 10% TCA (w/v) and then samples were incubated at 4 °C. The homogenate was centrifuged at 8,000 g for 10 min, protein pellets obtained were mixed with 0.5 ml of 10 mM 2, 4 dinitrophenyl hydrazine (DNPH) prepared in 2 N HCl and vortexed for 1h. After vortexing, samples were again precipitated with 0.5 ml of 40% TCA for 10 min. Then samples were centrifuged at 8000 g for 5 min. Protein pellets were washed and centrifuged several times with ethanol and ethyl acetate mixture (1:1, v/v) until supernatant becomes colourless. During washing supernatant was discarded at each time and protein pellets were collected. Then pellets were added with 1 ml of 6 M guanidine prepared in 2 mM potassium phosphate buffer (pH 2.3, adjusted by adding few drops of trifluoro acetic acid) and incubated at 37 °C for 15 min for dissolution of protein pellets. If dissolution of protein pellets does not occur, sonication was carried out in ice cold water bath with the help of Vibracell Ultrasonicator, USA and samples were again incubated at 70 °C for 5 min. Samples were centrifuged at 8000 g for 3 min and absorbance of supernatant was read at 375 nm with the help of Schimadzu UV-VIS spectro-photometer series UV-1700. The amount of carbonyl group was calculated by using the extinction coefficient ($\varepsilon = 22000 \text{ M}^{-1} \text{ cm}^{-1}$).

2.12.3 Membrane permeability measurement

Membrane stability index (MSI)
The intactness of plasma membrane in plants of each set was measured in terms of leakage of electrolytes as described by Premchandra et al. (1990) as modified by Sairam (1994).

**Procedure**

Fresh *Azolla* fronds (200 mg) from each set of sample were cut into pieces and placed in test tubes containing 10 ml deionized water at 40 °C for 30 min. The samples were centrifuged and the initial electrolyte conductivity of the supernatant (C₁) was measured by Digital Conductivity Meter (Systronics Conductivity meter, 304). After this, all the samples were boiled at 100 °C for 15 min to release all electrolytes, cooled and centrifuged, and final electrical conductivity (C₂) of the supernatant was measured. MSI was calculated using the formula given below:

\[ \text{MSI} = \left[ 1 - \frac{C_1}{C_2} \right] \times 100 \]

2.13 Estimation of antioxidative enzyme activities and amount of non-enzymatic antioxidants

2.13.1 Enzymatic antioxidants

(i) Superoxide dismutase activity

Superoxide dismutase activity (SOD; EC 1.15.1.1) was estimated by measuring the inhibition in the reduction of nitroblue tetrazolium chloride (NBT) by the method as described by Giannopolitis and Ries (1977).

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>1.3 μM</td>
</tr>
<tr>
<td>L–Methionine</td>
<td>13 μM</td>
</tr>
<tr>
<td>Nitroblue tetrazolium chloride (NBT)</td>
<td>63 μM</td>
</tr>
<tr>
<td>Sodium carbonate (Na₂CO₃) (pH 10.2)</td>
<td>0.05 M</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid (EDTA)</td>
<td>100 mM</td>
</tr>
<tr>
<td>Di-potassium hydrogen phosphate</td>
<td>100 mM</td>
</tr>
</tbody>
</table>
Procedure

For extraction and assay of SOD, fresh *Azolla* fronds (500 mg) were homogenized under ice–cold condition with 100 mM EDTA–phosphate buffer (pH 7.8). The homogenate was centrifuged at 10,000 g for 20 min and the supernatant was used for enzyme assay. The reaction mixture 3 ml contained 1.3 µM riboflavin, 13 mM L- methionine, 0.05 M Na₂CO₃, (pH 10.2), 63 µM p-nitroblue tetrazolium chloride (NBT) and 0.1 ml of crude extract. Reaction was carried out for 20 min in similar test tubes at 25 °C under an illumination of 100 µmol photons m⁻² s⁻¹ obtained from fluorescent lamp. The initial rate of reaction as measured by the difference in increase in absorbance at 560 nm in the presence and absence of extract was proportional to the amount of enzyme. The one unit of SOD activity was defined as the amount of enzyme which is required to cause 50% inhibition in reduction of the NBT.

(ii) Catalase activity

Catalase (CAT; EC 1.11.1.6) activity was determined by the method of Aebi (1984).

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>40 mM (v/v)</td>
</tr>
<tr>
<td>Potassium phosphate Buffer (pH 7.0)</td>
<td>50 mM (w/v)</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM (w/v)</td>
</tr>
</tbody>
</table>

Procedure

Frozen *Azolla* fronds (100 mg) of each set were homogenised with 3 ml 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) and centrifuged at 6,000 g for 15 min. The supernatant obtained was used as enzyme. The reaction mixture contained 0.5 ml 40 mM H₂O₂, 1.3 ml 50 mM...
potassium phosphate buffer (pH 7.0) having 1 mM EDTA, and 0.2 ml crude extract. The decrease in absorbance of the solution was recorded at 240 nm using UV-VIS spectrophotometer (Shimadzu, Japan) for 1 min at 25 °C. The consumption of H₂O₂ was calculated using the extinction coefficient (ε = 39.4 mM⁻¹ cm⁻¹). One unit of CAT activity is the amount of enzyme dissociating 1 nmol H₂O₂.

(iii) Guaiacol peroxidase activity

Guaiacol peroxidase activity (POD; EC 1.11.1.7) in treated and untreated fronds of each set was determined according to Zhang (1992).

Reagents

Potassium phosphate buffer (pH 6.1) : 50 mM
Guaiacol : 1% (v/v)
H₂O₂ : 0.4% (v/v in phosphate buffer)

Procedure

Fresh Azolla fronds (100 mg) were homogenized in 2 ml 50 mM phosphate buffer (pH 6.1). The homogenate was centrifuged at 10,000 g and the supernatant was used as the crude enzyme extract. Guaiacol peroxidase activity was measured with guaiacol as the substrate in a total volume of 3 ml. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H₂O₂ and enzyme extract. Increase in the absorbance due to oxidation of guaiacol (ε = 25.5 mM⁻¹ cm⁻¹) was measured at 470 nm. Enzyme activity was calculated in terms of Unit (g FM)⁻¹. One unit of POD activity is the amount of enzyme oxidizing 1 μM guaiacol min⁻¹ at 28 °C.

(vi) Glutathione-S-transferase activity
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Glutathione-S-transferase (GST; EC 2.5.1.18) activity was measured by the method of Habig et al. (1974) using CDNB (1-chloro, 2, 4-dinitro benzene) as a substrate.

**Reagents:**

- 1-chloro, 2, 4- dinitrobenzene (CDNB): 3.0 mM (w/v)
- Reduced glutathione (GSH): 120 mM
- Potassium Phosphate buffer (pH 6.3): 100 mM

**Procedure**

Fresh *Azolla* fronds (100 mg) were homogenized in 1 ml of 100 mM potassium phosphate buffer (pH 6.3) and centrifuged at 6,000 g for 15 min. The supernatant obtained was used for glutathione-S-transferase. The reaction mixture contained 0.5 ml of 3 mM CDNB, 0.5 ml of 120 mM GSH, 1.0 ml of 100 mM potassium phosphate buffer (pH 6.3) and 0.2 ml crude extract. The increase in absorbance of the solution was monitored at 340 nm using UV-VIS spectrophotometer (Schimadzu, Japan), after 1 min at room temperature. Increase in absorbance is due to the formation of the conjugate between GSH and CDNB. The activity of glutathione-S-transferase was calculated using the extinction coefficient ($\varepsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of GST activity is expressed as 1 nmol CDNB conjugates min$^{-1}$.

(iv) Ascorbate Peroxidase activity

Ascorbate peroxidase (APX; EC 1.11.1.11) activity in *Azolla* fronds of each set was estimated by the method described by Nakano and Asada (1981).

**Reagents**

- Ascorbate: 2 mM (w/v)
- Hydrogen peroxide: 0.4 mM (v/v)
- Potassium phosphate buffer (pH 7.0): 50 mM (w/v)
EDTA : 1 mM

Procedure

Fresh *Azolla* fronds (100 mg) were homogenized in chilled 1 ml of 50 mM potassium phosphate buffer (pH 7.0) having 1 mM EDTA, and it was centrifuged at 5,500 g for 15 min. The supernatant obtained was maintained with potassium phosphate buffer and used for ascorbate peroxidase activity. The reaction mixture contained 0.5 ml of 0.4 mM $H_2O_2$, 0.5 ml of 2 mM ascorbate, 0.8 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.2 ml of crude extract. The decrease in absorbance of the solution was monitored at 290 nm by UV–VIS spectrophotometer (Schimadzu, Japan) for 1 min at 25 °C. The activity of ascorbate peroxidase was measured using the extinction coefficient ($€ = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of APX activity is the amount of enzyme oxidizing 1 nmol ascorbate.

(v) Glutathione reductase activity

Glutathione reductase (GR; EC 1.6.4.2) activity was determined by the method of Schaedle and Bassham (1977).

Reagents:

- Potassium phosphate buffer (pH 7.8) : 50 mM
- Ethylene diamine tetra acetic acid (EDTA) : 2 mM
- Glutathione oxidised (GSSG) : 2 mM
- Nicotinamide adenine dinucleotide phosphate (NADPH) : 0.6 mM

Procedure

Treated and untreated fresh *Azolla* fronds (100 mg) were homogenized in chilled 1.5 ml potassium phosphate buffer containing 2 mM EDTA (pH 7.8)
and centrifuged at 6,000 g for 15 min. The supernatant obtained was used for measurement of glutathione reductase activity. The reaction mixture contained 0.8 ml of 50 mM potassium phosphate buffer having 2.0 mM EDTA (pH 7.8), 0.5 ml of 2.0 mM GSSG, 0.2 ml of crude extract and 0.5 ml of 0.6 M of NADPH. Reaction initiates when NADPH was added. The decrease in the absorbance was monitored at 340 nm using UV-VIS spectrophotometer (Schimadzu, Japan) for 3 min. The GR activity was calculated using the extinction coefficient (€= 6.2 mM⁻¹ cm⁻¹). One unit of GR activity is the amount of enzyme oxidizing 1 nmol NADPH min⁻¹.

(vii) Dehydroascorbate reductase activity

Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) in Azolla fronds of each set was determined spectrophotometrically according to Nakano and Asada (1981).

Reagents

\[
\begin{align*}
\text{K}_2\text{HPO}_4 & : 0.3982 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 0.0576 \text{ g} \\
\text{EDTA} & : 3.72 \text{ mg} \\
\text{Glycerol} & : 8 \text{ ml} \\
\text{Mercaptoethanol} & : 7.81 \text{ ml}
\end{align*}
\]

Extraction buffer (50 mM, pH 7.8)

\[
\begin{align*}
\text{K}_2\text{HPO}_4 & : 0.9549 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 0.4734 \text{ g} \\
\text{EDTA} & : 6.6 \text{ mg} \\
\text{GSH} & : 12.5 \text{ mM} \\
\text{DHA} & : 1 \text{ mM}
\end{align*}
\]

Reaction buffer (90 mM, pH 7.0)

Procedure

Fresh Azolla fronds (100 mg) were homogenized in 1 ml of 50 mM phosphate buffer (extraction buffer, pH 7.8). The homogenate was
centrifuged at 10,000 g for 10 min and the supernatant was used as the crude enzyme extract. Dehydroascorbate reductase activity was measured in a total volume of 2.5 ml containing 1.4 ml reaction buffer (phosphate buffer 90 mM, pH 7.0), 0.5 ml of 12.5 mM GSH, 0.5 ml of 1.0 mM DHA (dehydroascorbate) and 0.1 ml of enzyme extract. The absorbance was measured at 265 nm. The activity was calculated using an extinction coefficient of ($\varepsilon = 7.0 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit is the amount of enzyme that reduces 1 µmol of DHA min$^{-1}$.

### 2.13.2 Non-enzymatic antioxidants

#### (i) Proline

Proline content in *Azolla* fronds was estimated according to the method of Bates et al. (1973).

**Reagents**

- Toluene : Pure
- Sulfosalicylic acid : 3% (w/v)
- Glacial acetic acid : 3% (v/v)
- Phosphoric acid : 6 M
- Acid ninhydrin : 1.25 g ninhydrin warmed in 30 ml glacial acetic acid (100%) and 20 ml 6 M phosphoric acid

**Procedure**

Fresh *Azolla* fronds (1 g) were crushed in 3% (w/v) aqueous sulfosalicylic acid, centrifuged at 10,000 g and then reacted with 3% glacial acetic acid and acid ninhydrin. Samples were heated for 1 h in water bath at 95 °C, cooled and extracted with 4 ml toluene by vortexing for 15 s with test tube mixer. The toluene layer was then aspired and the absorbance was read at 520 nm using toluene as blank. The proline content in each sample was calculated from the standard curve.

#### (ii) Ascorbate (total, reduced and oxidised)
Total ascorbate (AsA+DHA), reduced ascorbate (AsA) and oxidized ascorbate (dehydroascorbate; DHA) were determined by the method of Gossett et al. (1994). This assay is based on reduction of Fe$^{3+}$ into Fe$^{2+}$ with ascorbic acid in acid solution followed by formation of red chelate between Fe$^{2+}$ and 2, 2’-bipyridyl.

**Reagents**

- $m$-phosphoric acid: 5% (w/v)
- Potassium phosphate buffer: 150 mM (pH 7.4)
- EDTA: 5 mM
- Dithiothreitol (DTT): 10 mM
- N-ethylmaleimide: 0.5% (w/v)
- Trichloroacetic acid: 10% (w/v)
- o-phosphoric acid: 44% (v/v)
- 2, 2’-bipyridyl: 4% (w/v)
- Ethanol: 70% (v/v)
- FeCl$_3$: 3% (w/v)

**Procedure**

Frozen *Azolla* fronds (1 g) was ground with inert sand and 10 ml of 5% (w/v) $m$-phosphoric acid using mortar and pestle. The homogenate was centrifuged at 22,000 g for 15 min. Total ascorbate was determined in a reaction mixture 200 µl of supernatant, 500 µl of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 100 µl of 10 mM dithiothreitol (DTT) to reduce dehydroascorbate to reduced ascorbate. After 10 min at room temperature, 100 µl of 0.5% (w/v) N-ethylmaleimide was added to remove excess DTT. AsA was assayed in a similar manner except that 200 µl of deionized H$_2$O was substituted for DTT. Colour was developed in both reaction mixtures with the addition of 400 µl of 10% (w/v) TCA, 400 µl of 44% (v/v) o-phosphoric acid, 400 µl of 2, 2’-bipyridyl in 70% (v/v) ethanol and 200 µl of 3% FeCl$_3$. The reaction mixtures were incubated at 40 °C for 1 h and
absorbance was recorded spectrophotometrically at 525 nm. DHA was determined by subtracting AsA from AsA+DHA. Ascorbate content was found out by using standard curve prepared with L-ascorbic acid.

(iii) Non-protein thiol

The non-protein thiol (NP-SH) content was measured following the method of Ellman (1959).

**Reagents**

- Sulphosalicylic acid : 6.67% (w/v)
- Ellman reagent : DTNB
- EDTA : 5 µM
- 5, 5′-dithio-bis 2-nitrobenzoic acid : 0.6 mM
- Potassium phosphate buffer (pH 7.5) : 120 mM

**Procedure**

For estimation of NP-SH content, treated and untreated *Azolla* fronds (100 mg) were homogenized in 6.67% sulphosalicylic acid in a chilled mortar and pestle. After centrifugation at 10,000 g at 4 °C, NP-SH content was measured in the supernatant by reaction with Ellman Reagent containing 5 µM EDTA and 0.6 mM 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 120 mM phosphate buffer (pH 7.5) and absorbance was recorded at 412 nm. The value was calculated with the help of standard calibration curve.

(iv) UV–B absorbing pigments (Flavonoids)

UV–B absorbing pigments in treated and untreated *Azolla* fronds were estimated as described by Mirecki and Teramura (1984).

**Reagents**

- Methanol : Pure
HCl : 12 N
Water : DDW

**Procedure**

UV–B absorbing pigments were extracted from *Azolla* fronds (20 mg), by keeping them in acidified methanol (methanol: water: HCl :: 78: 20: 2; v/v) for 24 h at 4 °C. The filtered extract was then used for measuring the absorbance at 315 nm, which is indicative of relative concentration of UV–B absorbing pigments. Flavonoid contents are expressed as absorbance (g FM)$^{-1}$ of tissue at 315 nm.

**2.7.2 Anthocyanin content**

Anthocyanin content was determined by the method of Wanger (1979).

**Reagents**

<table>
<thead>
<tr>
<th>Hydrochloric acid (HCl)</th>
<th>Pure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>Pure</td>
</tr>
</tbody>
</table>

Fresh *Azolla* fronds (50 mg) from each set were taken and anthocyanin was extracted in 5 ml of acidified methanol solution (methanol : HCl :: 99 : 1, v/v). Samples were kept overnight in darkness. Further supernatant was obtained after centrifugation and the absorbance was recorded at 550 nm. The amount of anthocyanin was calculated using an extinction coefficient of 33000 M$^{-1}$ cm$^{-1}$.

**2.16 Statistical Analysis**

Results were statistically analyzed by analysis of variance (ANOVA). Duncan’s multiple range test was applied for mean separation for significant differences among treatments at P<0.05 levels. The results presented are the means of three independent experiments.