3.1 Plant material

Rice is a cereal grain belonging to the family Poaceae. Seeds of 13 high yielding common rice varieties (Table 1) were collected from Regional Agricultural Research Station (RARS) of Kerala Agricultural University, Pattambi, Kerala, India. Healthy and uniform sized seeds of rice were selected and pre-washed for 1 min with 0.25% Triton X-100 to remove the dirt. The seeds were surface sterilized with 0.1% HgCl$_2$ solution for 5 min and further seeds were washed thoroughly with distilled water. The seedlings were raised in plastic bottles (27×11 cm) containing absorbent cotton soaked with modified half strength Hoagland medium (Epstein, 1972). Seedling growth was carried out in a plant growth chamber (INLABCO) set at 14/10 h light-dark cycles at 300 µmolm$^{-2}$s$^{-1}$, 24±2°C temperature and 55±5% relative humidity.

**Table 1: O. sativa varieties used in this study.**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Variety</th>
<th>Developed in year</th>
<th>Parental lines/pedigree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ptb 35 - Annapoorna</td>
<td>1966</td>
<td>Ptb 10 x Taichung 1</td>
</tr>
<tr>
<td>2</td>
<td>Ptb 39 - Jyothi</td>
<td>1974</td>
<td>Ptb 10 x IR 8</td>
</tr>
<tr>
<td>3</td>
<td>Ptb 43 - Swarnaprabha</td>
<td>1985</td>
<td>Bhavani x Triveni</td>
</tr>
<tr>
<td>4</td>
<td>Ptb 45 - Mattatrvini</td>
<td>1990</td>
<td>Reselection from Triveni</td>
</tr>
<tr>
<td>5</td>
<td>Ptb 47 - Neeraja</td>
<td>1990</td>
<td>IR 20 x IR 5</td>
</tr>
<tr>
<td>6</td>
<td>Ptb 50 - Kanchana</td>
<td>1993</td>
<td>IR 36 x Pavizham</td>
</tr>
<tr>
<td>7</td>
<td>Ptb 51 - Aathira</td>
<td>1993</td>
<td>BR 51-46-1 x Cul 23332-2</td>
</tr>
<tr>
<td>8</td>
<td>Ptb 52 - Aiswarya</td>
<td>1993</td>
<td>Jyothi x BR 51</td>
</tr>
<tr>
<td>9</td>
<td>Ptb 53 - Mangalamahsuri</td>
<td>1998</td>
<td>Reselection from Mashuri</td>
</tr>
<tr>
<td>10</td>
<td>Ptb 54 - Karuna</td>
<td>1998</td>
<td>CO.25 x H4</td>
</tr>
<tr>
<td>11</td>
<td>Ptb 55 - Harsha</td>
<td>2001</td>
<td>Ptb 10 x Ptb 28</td>
</tr>
<tr>
<td>12</td>
<td>Ptb 56 - Varsha</td>
<td>2002</td>
<td>M210 x Ptb 28</td>
</tr>
<tr>
<td>13</td>
<td>Ptb 57 – Swetha</td>
<td>2002</td>
<td>IR 50 x C14-8</td>
</tr>
</tbody>
</table>
3.2 Composition and preparation of Hoagland nutrient solution

Hoagland nutrient solution was prepared according to Epstein (1972) with some modifications and used for growing rice seedlings (Table 2). A stock solution for each nutrient was prepared separately and appropriate volume of each was mixed together to make up the final volume and concentration of the nutrient solution. pH of the nutrient solution was adjusted to 6.8 using 0.1 N HCl or NaOH.

Table 2: Composition of modified Hoagland nutrient solution employed in the present study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Concentration of stock solution</th>
<th>Concentration of stock solution</th>
<th>Volume of stock solution/L of Hoagland solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g mol⁻¹</td>
<td>mM</td>
<td>g L⁻¹</td>
<td>ml</td>
</tr>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>101.10</td>
<td>1,000</td>
<td>101.10</td>
<td>6.0</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>236.16</td>
<td>1,000</td>
<td>236.16</td>
<td>4.0</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>115.08</td>
<td>1,000</td>
<td>115.08</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>246.48</td>
<td>1,000</td>
<td>246.48</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>25</td>
<td>1.864</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>61.83</td>
<td>12.5</td>
<td>0.773</td>
<td>2.0</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>169.01</td>
<td>1</td>
<td>0.169</td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>287.54</td>
<td>1</td>
<td>0.288</td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>249.68</td>
<td>0.25</td>
<td>0.062</td>
<td></td>
</tr>
<tr>
<td>H₂MoO₄</td>
<td>161.97</td>
<td>0.25</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>NaFeEDTA</td>
<td>558.50</td>
<td>53.7</td>
<td>30.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

3.3 Chemicals

Chemicals of analytical reagent (AR) or guaranteed grade (GR) from Merck, Himedia, Qualigens, BDH, Spectrochem and SRL companies were used. Chemicals like gluteraldehyde, potassium bromide (FT-IR grade), 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), riboflavin, methyl viologen
(MV), sodium azide (NaN₃), bovine serum albumin (BSA), lutein, zeaxanthin and β-carotene from Sigma-Aldrich Co., USA were used.

3.4 Determination of pre-growth period for imparting HL stress in rice seedlings

For the standardization of growth period in rice seedlings for imparting HL treatment, they were analyzed for better performance in terms of total chlorophyll content and Fv/Fm ratio. Thirteen varieties of rice seedlings were germinated in half strength Hoagland medium and they were analyzed for best performance after 8, 9, 10, 11 and 12 d of germination.

3.5 Experimental design and stress treatments

Two types of stress treatments in thirteen rice varieties were performed in this study: HL and UV-B treatments.

3.5.1 High intensity light treatment

Thirteen varieties of rice seeds were germinated and grown in plastic bottles containing absorbent cotton soaked with half strength Hoagland solution. After 10 d of growth, rice seedlings were exposed to HL (2000 μmolm⁻²s⁻¹), provided by 1000 W PAR64 metal halide lamps (Philips, Netherlands). A trough of transparent glass (20 cm depth) with circulating water was placed under the lamp to protect the seedlings from the heat generated by the lamp. Air was circulated around the seedlings and thus the temperature was maintained at 24±2°C (Page no. 54). Various physiological and biochemical parameters were analyzed after rice seedlings were exposed to HL stress at different time intervals (0, 2, 4, 6 and 8 h). The detailed scheme of work is illustrated in Chart 1.

3.5.2 UV-B treatment

For imparting UV-B stress, rice seedlings were exposed to UV-B radiation (280-320 nm) for one week after 4 d of germination. The seedlings
were irradiated with UV-B in the presence of continuous white fluorescent illumination of 300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) held in mobile adjustable frames over the plants in a UV chamber (ROTEK). The UV-B tubes were covered with 0.13 mm thick cellulose diacetate filters to avoid transmission of wave length below 280 nm. The rice seedlings were exposed to UV-B stress (1.93±0.29 Wm\(^{-2}\)) for 1, 2, 3 and 4 h. The intensity of 1 h UV-B irradiation was equivalent to UV-B dose of 7 kJm\(^{-2}\)d\(^{-1}\), 2 h treatment was equivalent to a dose of 14 kJm\(^{-2}\)d\(^{-1}\), 3 h UV-B irradiation was equivalent to 21 kJm\(^{-2}\)d\(^{-1}\) and 4 h treatment was equivalent to 28 kJm\(^{-2}\)d\(^{-1}\) dose of irradiation. Various morphological, physiological, biochemical and anatomical parameters were analyzed in rice seedlings after irradiating with various level of UV-B on 10 d of germination. The detailed scheme of work is illustrated in Chart 2.

Experimental set up for HL (A) and UV-B (B) treatment of rice seedlings

3.5.3 PAR (photosynthetically active radiation) and UV-B measurement

Photosynthetically active radiation in terms of light intensity at the surface of the leaves at one hour interval was measured by a solar radiation
monitor (EMCON, India). The intensity of PAR is referred as PPFD (Photosynthetic Photon Flux Density) which is measured in μmolm$^{-2}$s$^{-1}$. The UV-B radiation just above the plant inside the UV chamber at 2 h interval was measured using spectroradiometer (Solar Light Co., PMA 2200, USA) and expressed as Wm$^{-2}$.

3.6 Growth parameters

The shoot length of rice seedlings was measured using a graduated scale and was expressed in centimeters. The fresh weight of rice seedlings were recorded by weighing them immediately after harvesting using an electronic weighing balance after blotting and wrapping of seedlings separately in pre-weighed labeled aluminium foils. For dry weight measurements, the weighed seedlings were kept in a hot air oven at 100°C for one h and further kept in oven set at 60°C. On the next day, the samples were allowed to cool in a desiccator with vaccum and then weighed. Drying and weighing were repeated at regular intervals (24 h) until the values of dry weight became constant. The dry weight percentage was calculated by using the following formula:

$$\text{Dry weight percentage} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

3.7 Physiological parameters

3.7.1 Photosynthetic pigment analysis

The chlorophyll and carotenoid pigments in the leaves of rice seedlings were estimated following the method of Arnon (1949) by using 80% acetone as the extracting medium. Fresh leaves of rice seedlings were washed out thoroughly with distilled water and blotted dry with filter paper. To estimate pigment contents, two hundred milligram of fresh leaf sample was weighed, homogenized and extracted in 80% acetone (v/v). Then the homogenate was
centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was collected. 
The residue was re-extracted with the same extracting medium and centrifuged at 5000 rpm for 10 min. This process was repeated until the pellet became colourless. The final volume of the acetone extract was noted and the optical density was read at 663, 646, 750 and 470 nm against the solvent blank (80% acetone) using a UV-VIS spectrophotometer (Systronics 2201). The concentration of the total chlorophyll (Chl $a+b$) and carotenoids contents were expressed in mg chlorophyll/carotenoids g$^{-1}$ dry weight of leaf sample.

\[
Total \ chlorophyll \ (a+b) = \frac{20.12 \ (A_{646} - A_{750}) + 8.02 \ (A_{663} - A_{750})}{Fresh \ weight \ of \ the \ sample} \times Volume
\]

\[
Carotenoid = \frac{1000 \ (A_{470}) + 3.27 \ (Chl \ a - Chl \ b)}{Fresh \ weight \ of \ the \ sample \times 229} \times Volume
\]

Where,

\[
Chlorophyll \ a \ \mu g/g = \frac{12.69 \ (A_{663} - A_{750}) - 2.69 \ (A_{646} - A_{750})}{Fresh \ weight \ of \ the \ sample} \times Volume
\]

\[
Chlorophyll \ b \ \mu g/g = \frac{22.9 \ (A_{646} - A_{750}) - 4.68 \ (A_{663} - A_{750})}{Fresh \ weight \ of \ the \ sample} \times Volume
\]

3.7.2 Estimation of photosynthetic electron transport activities

The photosynthetic electron transport activities were analyzed polarographically using Oxygraph Plus oxygen electrode system (DW1/AD, Hansatech, Norfolk, UK) which consists of a highly sensitive S1 Clark Type polarographic oxygen electrode disc mounted within a DW1/AD electrode chamber and connected to the Oxygraph Plus electrode control unit (OXYG1, Hansatech). The DW1/AD electrode chamber provides a highly versatile solution to measurements of dissolved oxygen in liquid phase samples with clear cast acrylic construction providing excellent sample visibility and
uniform illumination. Precise temperature control of the sample and electrode disc can be achieved by connecting the water jacket of the DW1/AD to a thermoregulated circulating water bath. Thylakoids from rice leaves after HL and UV-B irradiation were isolated at 4°C and photosystem I (PSI) (O₂ uptake) and photosystem II (PSII) (O₂ evolution) activities were measured as described by Puthur (2000). The light dependent O₂ uptake/evolution was measured by irradiating the thylakoid suspension with white light (1800 μmolm⁻²s⁻¹) continuously, provided by a 100W halogen lamp (LS2, Hansatech). The activities of PSI and PSII was expressed in terms of μmol of O₂ consumed (PSI)/evolved (PSII) min⁻¹mg⁻¹ chlorophyll.

3.7.2.1 Preparation of thylakoid membranes

Thylakoids membranes were isolated from rice leaves according to standard method (Puthur, 2000). The fresh leaves were cut into pieces and one hundred milligram of fresh leaf tissue was gently homogenized with a chilled mortar and pestle in an ice-cold isolation buffer containing 400 mM sucrose, 20 mM tricine (pH 7.8) and 10 mM NaCl. The homogenate was filtered through 6 layers of Mira cloth to remove large debris and the filtrate was centrifuged at 5000 rpm for 6 min at 4°C. The supernatant was discarded and the thylakoid pellets were suspended in 500 μl suspension buffer (pH 7.5) containing 10 mM NaCl, 20 mM HEPES [N-(2-Hydroxyethyl) piperazine-N-(2-Ethanesulphonic acid)], 100 mM sucrose and 2 mM MgCl₂ and it was transferred to a clean chilled tube and stored on ice for hours with minimal loss of activity.

3.7.2.2 Estimation of the total chlorophyll concentration in the thylakoid suspension

To compare results obtained using different chloroplast preparations, the total chlorophyll content of the thylakoid samples was estimated
according to the method of Arnon (1949). 20 µl of the thylakoid suspension was added to the test tube containing 3 ml of 80% acetone. The tube was covered with parafilm and the contents of the tubes were mixed thoroughly using a vortex mixer to dissolve the chlorophyll and the homogenate was centrifuged at 5000 rpm for 5 min to pellet any particulate material and the supernatant was collected. The absorbance of the supernatant was measured at 645, 663 and 750 nm against the solvent blank (80% acetone). The total chlorophyll concentration was calculated from the following equation:

$$20.12 \left(A_{645} - A_{750}\right) + 8.02 \left(A_{663} - A_{750}\right) \times \text{dilution factor}$$

### 3.7.2.3. Assay of photosystem I and II activities

PSI and PSII activities were analyzed using Oxygraph Plus oxygen electrode system (DW1/AD, Hansatech, Norflok, UK) according to the protocol of Puthur (2000). PSI activity was measured in terms of oxygen consumption after PSII activity was blocked initially by adding DCMU to the medium. The reaction mixture consisted of reaction buffer, reduced 2,6-dichlorophenolindophenol (DCPIP) (0.1 mM), ascorbate (600 µM), MV (500 µM), NaN₃ (1 mM) and DCMU (5 µM). Thylakoid suspension equivalent to 20 µg chlorophyll was added and the volume was made up to 1 ml with reaction buffer. Electron transport to PSI was maintained by artificial electron donors, ascorbate and DCPIP in the medium. Ascorbate acted as reductant by donating electrons to DCPIP and further the electrons supplied by reduced DCPIP to plastocyanin were transferred to PSI. Electrons from PSI are bypassed to an artificial electron acceptor, MV in the reaction mixture instead of being accepted by FeS centre. Finally MV reacts with oxygen molecules in the medium and produce H₂O₂. The dissociation of H₂O₂ into oxygen and H₂O by the action of catalase in the plant tissue is arrested by NaN₃ added in the reaction mixture. Thus the oxygen consumption by activity of PSI alone is measured by oxygen electrode system.
PSII activity was measured in terms of oxygen evolution by using para-benzo quinone (pBQ) as an artificial electron acceptor and it will scavenge the electrons from plastoquinone. The transfer of electron from plastoquinone to cytochrome is terminated and so the activity of PSII alone can be measured. Splitting of water for transferring of electrons to PSII result in evolution of oxygen molecules in the medium and it was measured by Oxygraph Plus system. The reaction mixture (1 ml) in DW1/AD electrode chamber consisted of the reaction buffer, pBQ (500 μM) and isolated thylakoid suspension equivalent to 20 μg chlorophyll.

3.7.3 Assay of mitochondrial activity

3.7.3.1 Isolation of mitochondria

Mitochondrial isolation from the seedlings was carried out according to the method of Kollöffel (1967). The plant materials were gently homogenized with a chilled mortar and pestle at 4°C in ice cold 0.05 M phosphate buffer (isolation buffer, pH 7.2) containing 0.4 M sucrose and 5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was filtered through four layers of Mira cloth and the filtrate was centrifuged at 5000 rpm for 10 min. The supernatant was again centrifuged at 20,000 rpm for 15 min after removing the pellets. The resultant pellet, containing mitochondria was re-suspended in known volume of suspension buffer (0.05 M phosphate buffer with 0.2 M sucrose, pH 7.6). The protein content in the mitochondrial preparations was determined by the method of Bradford (1976).

3.7.3.2. Estimation of mitochondrial electron transport activity

Oxygen consumption by mitochondria was measured at 25°C using an Oxygraph Plus oxygen electrode system (DW1/AD, Hansatech, Norflok, UK) as per the protocol of Schmitt and Dizengremel (1989). Reaction medium contained 935 μl of assaying buffer (0.3 M sucrose, 10 mM potassium
phosphate, 10 mM Tris, 5 mM MgCl$_2$ and 10 mM KCl, pH 7.2), 40 µl mitochondrial preparations (equivalent to 0.3 mg protein) and 25 µl of 100 mM NADH. The substrate was added very last, so that oxygen uptake rate measurements were started immediately. The oxidation rate of NADH was calculated in terms of µmol O$_2$ consumed min$^{-1}$mg$^{-1}$ protein.

### 3.7.4 Chlorophyll a fluorescence parameters

Chl a fluorescence parameters were analyzed by using Plant Efficiency Analyzer (Handy PEA; Hansatech Ltd., King’s Lynn, Norfluk, UK), which is a portable fluorometer having high resolutions (Strasser et al., 2004). All measurements were performed on the upper surfaces of the first formed leaves after dark adapted for a period of 20 min using the leaf exclusion clips and then they were illuminated with continuous red light of high intensity (3000 µmolm$^{-2}$s$^{-1}$). All measurements were recorded up to 1s with a data acquisition rate of 10 µs for the first 2 ms and at 1 ms thereafter.

The various fluorescence parameters, maximal fluorescence (Fm), area above the fluorescence curve, the activity of the water-splitting complex on the donor side of the PSII (Fv/Fo), PSII structure-function-index [SFI$_{(abs)}$], relative variable fluorescence at J step (Vj), performance index [PI$_{(abs)}$], the electron transport quantum yield (Φ$_{Eo}$), the time when maximum fluorescence value is reached [Tf(max)] and the yield of electron transport per trapped exciton (Ψ$_o$) were measured. The phenomenological energy fluxes were figured as energy pipeline leaf model [RC/CSo (concentration of the active reaction centres per cross section), ABS/CSo (the number of photons absorbed per cross section), TR/CSo (the maximal trapping rate of an exciton measured per cross section), ET/CSo (the electron transport flux per cross section) and DI/CSo (the dissipation rate per cross section)]. PSII energy fluxes per reaction center (RC) [flux of absorption per reaction center
(ABS/RC), trapping flux per reaction center (TR_o/RC), electron transport flux per reaction center (ET_o/RC) and dissipated energy flux per reaction center (DI_o/RC)] was figured as specific membrane model. Data were analyzed; radar plot, energy pipeline and specific membrane models were deduced using Biolyzer HP3 software (Chl fluorescence analyzing program by Bioenergetics Laboratory, University of Geneva, Switzerland).

For calculating NPQ, Chl a fluorescence was measured at room temperature with a Modulated Chl fluorometer (OPTI-SCIENCES, OS1p), according to van Kooten and Snel (1990). After determining Fm, the leaf was continuously illuminated with a white actinic light of 1000 μmolm^{-2}s^{-1}. Then the steady-state value of fluorescence (Fs) was recorded after 5 min and a second saturating pulse at 8000 μmolm^{-2}s^{-1} was imposed to determine maximal fluorescence level in the light-adapted state (Fm'). Thereafter the minimal fluorescence level in the light adapted state (Fo') was determined by illuminating the leaf with a 3 s pulse of far-red light and NPQ was calculated from the following equation:

\[
NPQ = \frac{(Fm/Fm')} - 1
\]

### 3.7.5 Leaf gas exchange parameters

Leaf gas exchange parameters were analyzed by using a LI-6400 portable photosynthesis system (Infra-red gas analyzer, LI-COR, Lincoln, Nebraska, USA). Leaf surfaces were cleaned and dried using tissue paper before being enclosed in the leaf chamber for gas exchange measurements. All measurements were record on fully expanded first formed rice leaves and reading was taken between 9.00 to 10.00 am at growth temperature and ambient CO_{2} conditions. The internal light source in LI-6400 was set at an intensity of 1500 μmolm^{-2}s^{-1} to ensure a constant and uniform light across all measurements. The various leaf gas exchange parameters, net photosynthetic
rate, \( P_n \) (\( \mu \text{molm}^{-2}\text{s}^{-1} \)), stomatal conductance, \( g_s \) (\( \mu \text{molm}^{-2}\text{s}^{-1} \)) and transpiration rate, \( E \) (mmolm\(^{-2}\text{s}^{-1} \)) was measured in rice leaves after exposure with HL and UV-B irradiation. The measurements were done in the absence of HL and UV-B to avoid instantaneous effects of HL and UV-B radiation. \( P_n \), \( g_s \) and \( E \) were calculated using the equations derived by von Caemmerer and Farquhar (1981).

3.8 Biochemical parameters

3.8.1 Metabolites

3.8.1.1 Rate of lipid peroxidation

The malondialdehyde content (MDA) estimation was done according to the method of Heath and Packer (1968).

**Extraction:** Two hundred milligrams of plant tissue was weighed in triplicate and homogenized in 5 ml of 5% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12,000 rpm for 15 min. The supernatant was collected and used for the estimation of MDA.

**Estimation:** The MDA content estimation was done according to Heath and Packer (1968). Two millilitre of the supernatant was mixed with an equal aliquot of 0.5% of thiobarbituric acid (TBA) in 20% TCA and the solution was heated at 95°C for 24 min, cooled and then centrifuged at 3000 rpm for 2 min. The absorbance of the supernatant was measured at 532 and 600 nm against reagent blank using UV-VIS spectrophotometer (Systronics 2201). The absorbance value at 532 nm was corrected for non-specific turbidity by subtracting absorbance value at 600 nm. Then the MDA content was calculated using its extinction coefficient of 155 mM\(^{-1}\text{cm}^{-1} \).
3.8.1.2 Proline

Proline content in the experimental and control of rice seedlings was estimated according to the method of Bates et al. (1973).

**Extraction:** Two hundred milligrams of experimental and control plant tissues were weighed and homogenized in 10 ml of 3% (w/v) aqueous sulfosalicylic acid using a clean glass mortar and pestle. The homogenate was transferred to centrifuge tubes and centrifuged for 10 min at 10,000 rpm and the supernatant was collected and estimation of proline was done using acid ninhydrin.

**Estimation:** Two ml of supernatant was taken in test tubes in triplicate and equal volume of glacial acetic acid and 2.5% acid ninhydrin (1.25 g of ninhydrin dissolved in a mixture of 30 ml of glacial acetic acid and 20 ml of 6 M ortho phosphoric acid) were added to it. The tubes were then heated in a boiling water bath for 1 h and then the reaction was terminated by placing the tubes in ice bath. Four ml of toluene was added to the reaction mixture and stirred well using a vortex mixer. The chromophore-toluene layer was separated carefully and the optical density of the separated solution was measured at a wavelength of 520 nm using spectrophotometer (Genesis 20). L-proline was used as the standard.

3.8.1.3 Total soluble sugars

The total soluble sugar was estimated using the method proposed by Dubois et al. (1956).

**Extraction:** Two hundred milligrams of plant tissue was homogenized in 80% ethyl alcohol using a clean glass mortar and pestle. The homogenate was transferred to centrifuge tubes and then centrifuged at 10000 rpm for 10 minutes at 4°C, the supernatant was collected and re-extract the pellet using
80% alcohol. The supernatant was collected and estimation of total soluble sugars was done.

**Estimation:** From the supernatant, a known volume of aliquot was taken in the test tube and made upto 1 ml with distilled water. To this, 0.1 ml of 5% (v/v) phenol was added and mixed well. Add 5 ml of concentrated sulphuric acid was added to the tube quickly from a burette. After cooling, the optical density of the resultant solution was measured at 490 nm using a spectrophotometer. D-glucose was used as the standard.

3.8.1.4 Total protein

Total protein content of the plant material was estimated using Folin-Ciocalteau reagent according the method of Lowry et al., (1951).

**Extraction:** Two hundred mg of leaf tissue was homogenized in 5 ml of phosphate buffer using pre-chilled glass mortar and pestle. A known volume of the homogenate was pipetted in to a centrifuge tube and equal volume of 10% TCA was added. This mixture was kept in a refrigerator (4°C) for 1 h for flocculation. The protein precipitate was collected by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was decanted off. The residue was washed twice with cold 2% TCA followed by washing with 30% perchloric acid to remove starch. Diethyl ether was used to extract lipids and 80% acetone to remove the pigments.

**Estimation:** The pellet obtained after centrifugation was dried and later digested in 5 ml 0.1 N sodium hydroxide by heating in a water bath for 10 min. After cooling the suspension was cleared by centrifugation (5000 rpm for 10 min at 4°C) and the supernatant was collected. Known volume of aliquot was pipetted and made up to 1 ml with distilled water. To the aliquots, 5 ml of alkaline copper reagent was added and shaken well. After 10 min, 0.5 ml of 1 N Folin-Ciocalteu’s phenol reagent was added and
shaken well immediately. The tubes were kept for 30 min for colour
development. The optical density of the solution was read at 700 nm using a
UV-VIS spectrophotometer (Systronics 2201). BSA fraction V powder was
used as standard. Total protein was expressed as mg of protein g$^{-1}$ dry weight
of plant tissue.

3.8.1.5 Total free amino acids

Total free amino acids were determined by following the method of
Moore and Stein (1948).

*Extraction:* Five hundred milligrams of fresh samples were homogenized in a
clean mortar and pestle with 80% (v/v) ethanol. The extract was centrifuged
at 10,000 rpm for 15 min at 4°C and the supernatant was made up to 10 ml
with 80% ethanol.

*Estimation:* One ml of the resultant supernatant was mixed with 1 ml of
ninhydrin reagent in a test tube. Tubes were kept in boiling water bath for 20
min and further 5 ml of diluent (equal volume of water and n-propanol) was
added to it. This mixture was incubated at room temperature for 15 min and
absorbance was read at 570 nm using a UV-VIS Spectrophotometer
(Systronics 2201) against a reagent blank and the results were expressed as
mg g$^{-1}$ sample. Standard curve was plotted by using leucine in 0.1 M citrate
buffer at pH 5.

*Preparation of reagent:* Reagent solution was prepared by dissolving 20 g of
ninhydrin and 3 g of hydindantin in 750 ml of methyl cellosolve. The solution
was stirred carefully to avoid air bubbles into the solution. 250 ml of sodium
acetate buffer (pH 5.5) was added to this solution and the resulting reddish
reagent solution was immediately transferred to a 1 L dark glass bottle. The
reagent was used freshly without storage.
3.8.2 UV-B absorbing compounds

Estimation of UV-B absorbing or screening compounds, anthocyanin and flavonoid content, in the leaf material of rice seedlings were done spectrophotometrically.

Anthocyanin content was determined according to the method of Mancinelli et al. (1975) with some modifications. Fresh rice leaf samples (0.2 g) were homogenized and extracted in 5 ml of acidified methanol (1: 99, HCl: methanol, v/v) using a mortar and pestle. The extract was kept at 4°C for 24 h and the content was made up to 10 ml. The amount of anthocyanin was estimated from the absorbance at 530 nm using UV-VIS spectrophotometer (Systronics 2201). Anthocyanin content was expressed as µmolg⁻¹ DW and the concentration of anthocyanin was calculated using its extinction coefficient of 33 mM⁻¹ cm⁻¹.

Flavonoids were extracted and measured according to the method of Mirecki and Teramura (1984). Two hundred mg of fresh leaf samples were homogenized in a clean mortar and pestle with 5 ml of solvent containing acidified methanol:HCl:H₂O (79:1:20) and UV-B absorbing compounds were extracted after keeping the homogenate for 24 h at room temperature. The flavonoid content was determined from the absorbance of the supernatant at 315 nm using UV-VIS spectrophotometer (Systronics 2201). Flavonoid content was expressed as µmolg⁻¹ DW and the concentration of flavonoids was calculated using its extinction coefficient of 33 mM⁻¹ cm⁻¹.

3.8.3 Phenylalanine ammonia lyase (PAL, EC 4.3.1.24)

PAL activity in the fresh plant samples was determined in accordance to the methodology of Zucker (1965).
**Extraction:** For PAL enzyme analysis, 0.2 g of fresh leaves were homogenised in 3 ml borate buffer (pH 8.8) containing 23 μl of mercaptoethanol. Extraction was performed at 2°C. The homogenate was centrifuged at 8,500 rpm for 20 min at 4°C in refrigerated centrifuge (Thermo scientific X1R). The supernatant was transferred to a clean test tube and stored in an ice bath and used for enzyme assay.

**Enzyme Assay:** The PAL assay system contained 1 ml of the supernatant, 1 ml of buffer, 1 ml of 0.05 M L-phenylalanine as substrate and it was incubated at 37°C for 1 h. The reaction was stopped with 30% trichloroacetic acid and absorbance of the formed trans-cinnamic acid was measured at 290 nm wavelength. The spectrophotometric determination of PAL activity in rice leaves is based on changes of optical density at 290 nm compared to the resulting mixture with stopped enzyme reaction at the beginning by heating, which was taken as blank. Standard curve was prepared using trans-cinnamic acid and PAL activity was expressed as μmoles of trans-cinnamic acid formed min$^{-1}$ mg$^{-1}$ of protein. Protein content in the PAL enzyme extract was determined by the method of Bradford (1976).

3.8.4 ROS scavenging mechanism

3.8.4.1 Non-enzymatic antioxidants

3.8.4.1.1 Ascorbate (AsA) content

For the estimation of AsA content, the method of Chen and Wang (2002) was adopted.

**Extraction:** Two hundred mg of plant tissue was weighed using an electronic balance and homogenized with 5 ml 5% (w/v) TCA. The homogenate was transferred to centrifuge tube and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and used for the estimation of AsA content.
**Estimation:** An aliquot of 0.1 ml of the supernatant was mixed well with 0.3 ml of 200 mM NaH$_2$PO$_4$. To this mixture, 0.5 ml of 10% (v/v) TCA, 0.4 ml of 42% (v/v) H$_3$PO$_4$, 0.4 ml of 4% (w/v) bipyridyl (dissolved in 70% alcohol) and 0.2 ml of 3% FeCl$_3$ (w/v) was added. The mixture was incubated at 42°C for 15 min. The absorbance was measured immediately after incubation at 524 nm and AsA content was calculated from a standard curve prepared using different concentrations of AsA.

### 3.8.4.1.2 Glutathione (GSH) content

The GSH content estimation was done as per the protocol of Chen and Wang (2002).

**Extraction:** Two hundred mg of plant tissue was weighed using an electronic balance and homogenized in 5 ml of 5% TCA (w/v). The homogenate was filtered through a filter paper and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and used for the estimation of reduced glutathione content.

**Estimation:** To an aliquot of 0.5 ml of the supernatant, 2.6 ml of 150 mM NaH$_2$PO$_4$ buffer (pH 6.8) and 0.18 ml of 3 mM 5,5-dithio-bis(2nitrobenzoic acid) (DTNB) were added (DTNB was dissolved in 100 mM phosphate buffer, pH 6.8) and kept for 5 min. Then the absorbance was read at 412 nm and GSH content was calculated from a standard curve using varying concentrations of reduced glutathione.

### 3.8.4.1.3 Total phenolics

Total phenolic was estimated using Folin-Denis reagent according to the method of Folin and Denis (1915).

**Extraction:** One hundred mg of fresh tissue was weighed using an electronic balance and homogenized in 80% ethanol (v/v) in a clean mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20 min and the
supernatant was collected. The residue was reextracted with 80% ethanol. The homogenate was again centrifuged and supernatant was pooled. Pooled supernatant was then dried in an oven and the residue was dissolved in 5 ml of distilled water.

**Estimation:** Aliquots of 50 μl in triplicate were pipetted out and made up to 2 ml with distilled water. Equal volume of Folin-Denis reagent was added to it. The contents were thoroughly mixed and after 3 min, 2 ml of 1N sodium carbonate was added. This mixture was kept for 1 h after thorough mixing for colour development. The optical density of the resultant solution was measured at 700 nm and total phenolic content in the plant tissue was calculated using tannic acid as standard.

3.8.4.2 Enzymatic antioxidant system assay

3.8.4.2.1 Superoxide dismutase (SOD, EC 1.15.1.1)

Estimation of SOD activity in the rice seedlings was done as per the modified protocol of Giannopolitis and Ries (1977).

**Extraction:** Five hundred mg of leaf tissue was weighed and homogenized gently in 50 mM phosphate buffer of pH 7.8 with pre-chilled mortar and pestle. The homogenate was centrifuged at 16,000 rpm for 15 min in refrigerated centrifuge (REMI C-24 BL) at 4ºC. The supernatant was used for enzyme assay.

**Enzyme Assay:** SOD activity was carried out by monitoring the ability of SOD to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT). The reaction mixture consisted of 0.1 ml of 1.5 M sodium carbonate, 0.3 ml of 0.13 M methionine, 0.3 ml of 10 μM EDTA, 0.3 ml of 13 μM riboflavin and 0.3 ml of 0.63 mM NBT and 0.1 ml enzyme extract. The reaction mixture was made up to 3.0 ml using phosphate buffer (50 mM, pH 7.8). Different assay systems were set, viz. dark-control, light-control and test samples. Test tubes containing
only assay mixture without enzyme extract were illuminated under fluorescent lamp for 30 min (light-controls). Test samples (tubes containing assay mixtures with enzyme extract) were also illuminated and other set (tubes containing assay mixtures with enzyme extract) was kept in dark (dark-control). The formazan accumulation in different tubes was quantified using UV-VIS spectrophotometer (Systronics 2201) by recording the absorbance of the developed blue colour at 560 nm against the blank (reaction mixture without NBT). Results were expressed as units SOD mg⁻¹ protein⁻¹. One unit of SOD was defined as the enzyme activity that inhibited the photo reduction of NBT to blue formazan by 50%.

3.8.4.2.2 Guaiacol peroxidase (GPOX, EC 1.11.1.7)

GPOX activity in the fresh samples was measured by adopting the method of Gaspar et al. (1975).

Extraction: Five hundred mg of fresh plant tissue was weighed and gently homogenized in 50 mM Tris-HCl buffer (pH 7.5) using a chilled mortar and pestle. The extract was filtered through two layered muslin cloth. The filtrate was transferred to centrifuge tube and centrifuged at 15,000 rpm for 15 min at 4°C in refrigerated centrifuge (Thermo scientific X1R). The supernatant was transferred to a test tube and stored in an ice bath and used for enzyme assay.

Enzyme Assay: GPOX activity was measured following the H₂O₂ dependent oxidation of guaiacol (extinction coefficient 26.6 mM⁻¹ cm⁻¹) at 420 nm. Three ml assay mixture consisted of 2.86 ml 100 mM phosphate buffer (pH 7.8), 30 μL 1% guaiacol and 100 μl enzyme extract. The blank was prepared by adding 50 mM Tris-HCl (pH 7.5) to the reaction mixture instead of enzyme extract. All the components were mixed thoroughly and 12 μL of H₂O₂ was added to initiate the enzyme activity. Immediately after the addition of H₂O₂, the increase in absorbance due to oxidation of guaiacol was measured at 420
nm using a UV-VIS spectrophotometer (Systronics 2201) for 3 min at intervals of 30 sec. One unit of GPOX activity was defined as the amount of enzyme that caused the formation of 1 μM of tetraguaiacol per min.

3.8.4.2.3 Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity in the fresh samples was assayed by following the method of Nakano and Asada (1981).

**Extraction:** Leaf extraction for enzyme assay was prepared as per the protocol of Zhang and Kirkham (1996). Five hundred mg of fresh plant tissue was ground with a pre-chilled mortar and pestle in 10 ml of extraction medium. The extraction buffer consisted of 50 mM sodium phosphate buffer (pH 7.0), containing 0.33 M sorbitol, 1 mM MgCl₂, 2 mM EDTA, 10 mM NaCl, 0.5 mM KH₂PO₄ and 1 mM ascorbate. The homogenate was filtered through two layers of cheese cloth and centrifuged at 4°C for 4 min at 2000 rpm. The pellet was discarded and the supernatant was centrifuged again at 5000 rpm for 15 min at 4°C and the supernatant was saved. It was again centrifuged at 15000 rpm for 15 min at 4°C and the resulting supernatant was used as the cytosolic fraction for APX assay.

**Enzyme Assay:** Cytosolic APX activity was assayed by monitoring the decrease in absorbance at 290 nm due to AsA oxidation. The 3 ml assay system consisted of 0.5 mM AsA, 0.1 mM EDTA in 50 mM sodium phosphate buffer (pH 7.0). Twenty μL of cytosolic enzyme extract was added to the buffer and the enzyme reaction was initiated by adding 10 μL of 100 mM H₂O₂ to reach a concentration of 0.1 mM H₂O₂ in the final reaction mixture. H₂O₂ dependent oxidation of AsA ($\varepsilon=2.8 \text{ mM}^{-1} \text{cm}^{-1}$) was followed by monitoring the decrease in absorbance at 290 nm. One unit was defined as the amount of enzyme that oxidized one μmol of AsA min⁻¹ at room temperature under the above conditions.
3.9 Foliar micromorphology and stomatal characteristics

The foliar micromorphology of rice seedlings was examined and the stomatal characteristics such as stomatal length and width of stomata on both abaxial and adaxial epidermis of the leaf blade were studied using a scanning electron microscopy (SEM). Modified method of Li et al. (2014) was primarily used for this microscopic technique. Samples of the middle region of young rice leaves treated with HL or UV-B were collected for SEM analyses. Leaf segments of control and treated plants were fixed in 2.5% gluteraldehyde, prepared in 0.1 M phosphate buffer (pH 7.4) for 12 h. Fixed specimens were washed twice with 0.1 M phosphate buffer and dehydrated by passing through an ascending acetone series. Ten minutes incubation time was provided in each acetone series. Dehydrated leaf samples were mounted on to grooves cut on aluminium stubs using double side adhesive conducting carbon tapes to expose the sections. Then the specimens were sputter coated with gold palladium and further photomicrographs were taken using the photographic attachment of the SEM (Make: JEOL Model JSM-6390LV, magnification: 5x to 300,000 x, probe current: 1 pA to 1 mA).

3.10 Ultrastructure of chloroplasts

The ultrastructure of the chloroplasts was observed as described by Wang et al. (2014) using transmission electron microscope (TEM). Samples of the middle region of young rice leaves treated with HL or UV-B radiation were collected for TEM analyses. Fresh leaf segments were cut into pieces of approximately 1-2 mm² and fixed in 2.5% gluteraldehyde, prepared in 0.1 M phosphate buffer (pH 7.4) for 12 h (primary fixation). Leaf samples were then washed in phosphate buffer (pH7.4) for two times and post-fixed in 1% osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.4) for 12 h at the room temperature (secondary fixation). The fixed leaf samples were washed twice with 0.1 M phosphate buffer and dehydrated in an ascending series of
acetone (50%, 60%, 70%, 80%, 90% and 100%; 10 min each). After dehydration, leaf samples were embedded in epoxy resin (Epon 812) and allowed to harden by heat treatments (50°C for overnight, then at 60 °C for two days), ultra-thin sections were cut using ultra microtome (Leica, UC6), double stained with uranium acetate and lead citrate in series, and examined under a Jeol/JEM 2100 TEM (Make: Jeol Ltd., USA, magnification 2000x-150000x, voltage 200 kV).

3.11 Functional group analysis of cuticular wax deposition

Fourier transform infrared spectrometry (FT-IR) analysis was used to analyze the important functional group in the chloroform extract of cuticular wax deposition with the help of IR spectrometer.

**Epicuticular wax extraction and wax content determination:** For extraction of cuticular wax from the leaf blades of rice seedlings, the method of Walton (1990) was adopted with minor modifications. Fresh leaf samples were washed and 0.5 g of leaf tissue was weighed and cut into pieces. The leaves were immersed two times repeatedly for 30 s each in a test tube with 25 ml chloroform for extraction at room temperature. During each extraction, the solvent was agitated for 30 s by pumping with a Pasteur pipette. Wax extracts from leaves were pooled and were left to dry overnight under hood. After evaporation of the chloroform, the test tubes were weighed using precision balance (Sartorius, Germany). The wax content was calculated by subtracting the initial weight of the test tube from its final weight and expressed as mg g⁻¹ dry weight of rice leaves. All extraction experiments were carried out as entirely independent triplicates, results are given as mean values.

**Infra-red analysis of the epicuticular wax deposition:** The waxy deposition, accurately weighed and it was mixed with potassium bromide powder to make a pellet at high pressure. The disk was placed in instrument beam to
measure the solid state spectrum in a FT-IR (JASCO-4100). Infra-red analysis of the wax extracts were recorded in the 400 to 4000 cm\(^{-1}\) range with 2 cm\(^{-1}\) resolution.

3.12 Analysis of xanthophyll cycle pigments

High performance liquid chromatography (HPLC) analysis of xanthophylls cycle pigments was done by the method of Gopalakrishnan and Annamalainathan (2016).

**Extraction:** Fresh leaf samples were weighed (1 g) and processed immediately with liquid nitrogen. Samples were homogenized in 6 ml of 100% ice cold acetone containing 0.1% butylated hydroxytoluene (w/v) by using a pre-cold mortar and pestle. The homogenate was centrifuged for 25 min at 4\(^{\circ}\)C for 8000 rpm and the supernatant was collected. The pellets were re-extracted with 6 ml of acetone and the supernatant was pooled. The extracts were analyzed on the same day to reduce loss of pigments during analysis. All analytical procedures were carried out under dim light.

**Analysis:** Identification and separation of pigments were done on a reverse phase column, Waters Spherisorb ODS-5 µm column (250x4.6 mm) in HPLC. Samples were injected with a Rheodyne 7010 injector, with a 20 µl loop at 30\(^{\circ}\)C for 60 min. The solvent system consisted of acetonitrile:methanol:water (solvent A, 84:14:2) and methanol:ethyl acetate (solvent B, 68:32). The mobile phases were pumped with a Waters 600 high pressure pump at a flow rate of 0.5 ml per min. The column was equilibrated with mobile phase prior to injecting each sample and the analysis done in gradient mode. The gradient used was 0-20.5 min in 100% solvent A, 20.5-25.5 min decreasing to 0% solvent A, 25.5-40 min 100% solvent B, 40.0-40.1 min increasing to 100% solvent A and finally from 40.1-60 min 100% solvent A. Peaks were detected at 450 nm with waters 996 photodiode array detector
and integrated using Empower software and peaks were identified using standard methods. Analysis was done in triplicates.

Standards of xanthophylls (lutein, zeaxanthin and β-carotene) from M/s. Sigma Aldrich were used for the study. Pigments were identified by comparing their absorption spectra and retention time with standards. Standard curves for quantification of pigments were made by plotting concentration against absorbance responses. Lutein, zeaxanthin and β-carotene were quantified according to their respective standards. The response factor of lutein standard was used for quantifying the pigments viz. neoxanthin, violaxanthin and antheraxanthin. The concentration of pigment was determined using linear regression and expressed as μgg\(^{-1}\) fresh weight. Level of de-epoxidation and epoxidation state of xanthophyll cycle pigments was calculated as \(\frac{Z + A}{V + A + Z}\) and \(\frac{V + A}{V + A + Z}\), respectively.

Where, Z- zeaxanthin
A- antheraxanthin
V- violaxanthin

3.13 Statistical analysis

Statistical analyses were carried out according to Tukey’s studentized range (HSD) test at 5% probability level. One-way ANOVA were applied using the SPSS software (Version 16.0, SPSS Inc., Chicago, USA) to analyze the consequences of HL and UV-B irradiation in rice varieties. The data is an average of recordings from three independent experiments each with three replicates (\(i.e. n=9\)). The data represent mean±standard error (SE).