Examiner

IMPACT OF TEMPERATURE STRESS ON CHLOROPLAST BIOGENESIS AND PHOTOSYNTHETIC PROCESS

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CERTIFICATE

The research work embodied in this thesis entitled 'Impact of temperature Stress on Chloroplast Biogenesis & photosynthetic Process' has been carried out in the school of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full for any other degree or diploma of any other university.

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Dedicated to my family.

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ABBREVIATIONS

Chl	Chlorophyll
MS media	Murashige and Skoog media
PEA	Plant Efficiency Analyser
PAM	Pulse Amplitude Modulator
HEPES	N2, hydroxyl ethyl piperazine-N'-ethane sulphonic acid
MV	Methyl viologen
NaN_3	Sodium azide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
$MgCl_2$	Magnesium Chloride
PD	Phenyl diamine
NH_4Cl	Ammonium chloride
K_3FeCN_6	Potassium Ferricyanide
DCPIP	2,6 dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl 1) 1,1 dimethyl urea
MB	Measuring Beam
SP	Saturating Pulse
AL	Actinic Light
Ft	Fluorescence level just before applying saturating flash
F_0	Minimal fluorescence level from dark-adapted leaves
Fm	Maximal fluorescence level from dark-adapted leaves
F_v/Fm	Ratio of maximum variable to maximum total fluorescence

Q_A	Primary quinine acceptor of PS II
PS	Photosystem
OEC	Oxygen evolving complex

INTRODUCTION

Temperature is a prominent factor among the important cardinal ecological factors that determine crop growth and productivity. When plants are exposed to low or high temperature stress, various biological effects are expressed. Along with the morphological changes, growth and development of the seedlings is also affected. The latter changes are mainly because of the effect of temperature stress on early biochemical process. The most important of these processes is chloroplast development. This leads to the decreased crop productivity.

In most cases the productivity of a plant is directly related to the rate of photosynthesis. Photosynthesis is temperature dependent, like other physiological processes. So, understanding the mechanisms of heat tolerance is important if we want to improve the crop yield under stress conditions.

High temperature causes the accumulation of HSPs, which are believed to protect the biomolecules and organelles in the plant cell during high temperature stress. High temperature affects the photosynthetic functions of plants by its affect on the rate of chemical reactions and on structural organization. It also changes the excitation energy distribution by changing the structure of thylakoid membranes. Extreme high temperatures affect the functioning of the oxygen evolving complex, resulting in the release of functional manganese ions from the complex. This release may be due to the reductions of peroxides or superoxides.

Hence, temperature stress retards chloroplast development as well as affects the organization of the photosynthetic process. Rice is an important crop of India and several other countries. It is severely affected by heat-stress. Therefore, in the present investigation the rice seedlings of CSR 10 are subjected to heat-stress during chloroplast development. The impact of heat-stress on chlorophyll several photosynthetic parameters has been studied.

The parameters which have been studied are as follows:

1. Chl content at different temperatures and hours of greening.
2. Whole chain electron transport and partial photochemical reactions mediated by PS I and PS II and grana stacking.
3. Changes in the chlorophyll a fluorescence.
4. Assembly of the photosynthetic apparatus.

REVIEW OF LITERATURE

INTRODUCTION

PLANT STRESS RESPONSE

During evolution, the origin of terrestrial plants required special adaptation to the rapidly changing environmental conditions. (Levitt, 1980).

Examples for these adaptations are:

- The predominant role of the sporophyte in the life cycle of plants with the sensitive gametophyte being enclosed.
- The organization of leaves as photosynthetic organs with the active cells inside, a protective outer layer of the epidermis and cuticle and necessary gas exchange proceeding through tightly controlled apertures (stomata).
- The formation of stress resistant dormant forms (seeds) for propagation and survival of unfavorable conditions.
- The development of mechanically stabilized cormophyte allowing the generation of long lived and very big plants with systems for long distance nutrient and water transport.

These adaptations provided the basis for a very efficient, three-dimensional occupation by plants of all habitats outside water. Plants also became specialized to grow and propagate under extreme environmental conditions, e.g. under conditions of very low or very high temperatures, of high salt or very heavy metal stress or of extreme metal deficiency. Since the plants are sessile, so, they should develop the capability to rapidly respond to a multiplicity of environmental

changes from which they can not escape. Hence a network of interconnected cellular response systems is a prerequisite for plant survival and productivity.

CHLOROPHYLL FLUORESCENCE

Principle

Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates:

- It can be used to carry the photochemistry,
- Excess energy can be dissipated as heat or,
- It can be re-emitted as light. This is called chlorophyll fluorescence.

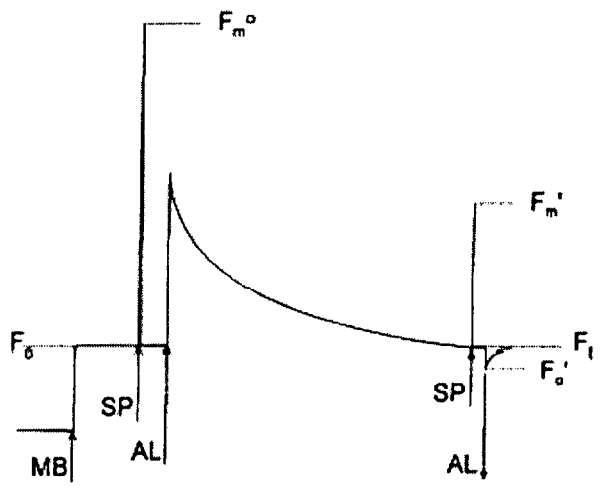
These three processes occur in competition, the increase in the efficiency of one will result in a decrease in the yield of other two. Therefore, chlorophyll fluorescence gives information about changes in the efficiency of photochemistry and heat dissipation.

In the spectrum of fluorescence the peak of fluorescence emission is of longer wavelength than that of absorption. Therefore fluorescence yield can be quantified by exposing the leaf to light of known wavelength and measuring the amount re-emitted at longer wavelengths.

Changes in the yield of chlorophyll fluorescence were first observed in 1960 by Kautsky and co-workers (Kautsky *et al.*, 1960). They found that, upon transferring photosynthetic material from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a time period of 1 s. when a leaf is transferred from darkness into light, PSII reaction centres are progressively closed. This gives rise to an increase in the yield of chlorophyll fluorescence. Following this rise the fluorescence level starts falling again for a period of few minutes. This is termed as fluorescence quenching. It is due to two reasons:

- There is an increase in the rate at which the electrons are transported away from PSII. This is due to the light-induced activation of enzymes involved in carbon metabolism and the opening of the stomata. This quenching is referred to as "photochemical quenching".

- Simultaneously there may be an increase in the efficiency with which the energy is converted into heat. This process is termed as "non-photochemical quenching" (NPQ).



Sequence of a typical fluorescence trace. A measuring light is switched on (↑MB) and the zero fluorescence level is measured (F_o). Application of a saturating flash of light (↑SP) allows measurement of the maximum fluorescence level F_m^o. A light to drive photosynthesis (↑AL) is then applied. After a period of time, another saturating light flash (↑SP) allows the maximum fluorescence in the light (F_m') to be measured. The level of fluorescence immediately before the saturating flash is termed F_t. Turning off the actinic light (AL), typically in the presence of far-red light, allows the zero level fluorescence 'in the light' to be estimated.

MECHANISMS OF DAMAGE OF THE PHOTOSYNTHETIC APPARATUS DUE TO HIGH TEMPERATURE

The damage due to heat stress includes a wide range of structural and functional changes. The effect on the growth and survival depends on the intensity and duration of heat stress.

- The primary site of damage due to heat stress is photosystem II (PSII) (Berry and Bjorkman, 1980; Mamedov *et al.*, 1993; Havaux, 1993). The PSII complex is a pigment-protein complex that utilizes light energy to drive the transport of electrons and oxidation of water to oxygen. It is believed that increasing temperature leads first to the blockage of PSII reaction centres and then to the dissociation of the antenna pigment protein complexes from the central core of the PSII (Armond *et al.*, 1978; Gounaris *et al.*, 1984; Sundby *et al.*, 1986). Separation of the light-harvesting complex II (LHC II) from the core centre induces destacking of the grana and temperature-induced migration of the reaction centre (PS IIβ) or LHC II (state transition) to the non-appressed region.

  Among partial reactions of PSII the oxygen-evolving process is most sensitive to heat (Santarius, 1975; Thomas *et al.*, 1986; Enamy *et al.*, 1994). OEC involves 4 Mn per PSII which serve as direct oxidant of water (Hansson and Wydrzynski, 1990). The three extrinsic proteins of 33, 23 and 17 kDa are associated with the luminal surface of the PSII reaction centre complex. Heat inactivation of oxygen evolution is accompanied by separation of Mn and the three extrinsic proteins from the complex (Nash *et al.*, 1985).

  It has been observed that PSI activity is much more heat stable than PSII (Pearcy *et al.*, 1977; Sayed *et al.*, 1989; Havaux, 1993). It has been found that moderately high temperature stimulates PSI activity *in vivo* and *in vitro* (Armond *et al.*, 1978; Sayed *et al.*, 1994).

- Since the photosynthetic PSII complex is integrated with thylakoid membranes, it is believed that physical property of the membranes may contribute to the thermal stability of photosynthesis (Quinn and Williams, 1985; Webb and Green, 1991). So, thermal denaturation of PSII is directly related to the major changes in the lipid phase of thylakoid membranes that occur at high temperature (Berry and Bjorkman, 1980; Yordanov *et al.*, 1986). Increasing temperature causes an increase in the fluidity of

membrane lipids (Raison *et al.*, 1982) followed by the formation of non-bilayer lipid structures (Gounaris *et al.*, 1984). These changes in the membrane structure cause destabilization of lipid-protien interactions, changing the organization and function of PSII.

- Activation of Rubisco in the light is regulated by a stromal enzyme named Rubisco Activase (Portis, 1992; Andrews *et al.*, 1995; Salvucci and Ogren, 1996). It has been shown that Rubisco activase is highly sensitive to inactivation by elevated temperatures (Crafts-Brandner *et al.*, 2004, 2002, 1997; Rokka *et al.*, 2001; Eckhardt and Portis, 1997). This causes the inactivation of Rubisco at high temperatures (Feller *et al.*, 1998).

- High temperature may cause oxidative damage to important molecules because of the imbalance between production of activated O_2 and antioxidant defenses (Foyer *et al.*, 1994). Chloroplasts are a major source of activated oxygen in plants (Asada and Takahashi, 1987; Asada *et al.*, 1998) and antioxidants, which play a critical role in preventing oxidative damage, are greatly affected by environmental stresses (Bowler *et al.*, 1994).

HEAT STRESS AND PLANT DEVELOPMENT

Plants are exposed to various environmental stresses during their life time, and the most typical kind of stress the plants receive is temperature stress. The range of temperatures experienced by plants varies both specially and temporarily at several different scales. Each plant species has its own optimum temperature for growth, and its distribution is determined to a major extent by the temperature zone in which it can survive.

High night temperatures during the growing season can have detrimental effects on reproductive development and yield of several crops (Hall, 1992). Injury may occur during vegetative or reproductive phase depending on the location and season (Kolderup, 1972). The reproductive organs of plants are vulnerable to damage at higher temperature than the vegetative organs (Sakata *et al.*, 2000). Heat stress on pollen mother cells during meiosis may lead to reduction in pollen fertility, germination and tube growth of viable pollen grains (Dane *et al.*, 1991). Damage during reproductive development can occur in two distinct stages: early flowering and pod set (Warrag and Hall, 1984; Mutters *et al.*, 1989; Ahmed *et al.*, 1992). After initiation, floral bud development is suppressed by combination of high night temperatures and long photo periods (Dow el-madina and Hall, 1986; Patel and Hall, 1990). Two or more weeks of consecutive or interrupted night temperature caused complete suppression of floral buds and prevented flowering (Ahmed and Hall, 1993). Inheritance of tolerance to heat-induced floral bud suppression was found to be consistent with the effect of a single recessive nuclear gene (Hall, 1992).

Plants experience high air and soil temperatures during periods of drought and when fields receive limited irrigation. Elevated plant temperatures that occur under these conditions influence plant health and productivity in a negative manner. High temperatures can cause changes in the metabolism. There is selective destabilization of secretory protein mRNAs in barley aleurone (Brodl and Ho, 1991, 1992), the disruption of cap and poly(A) tail function during translation (Gallie *et al.*, 1995), induction of shortening of barley primary leaves and coleoptile length (Beator *et al.*, 1992), and elevation of the level of xanthophyll lutein in dark grown pea plantlets; and by changes in other processes such as induction of circadian rhythmicity and changes in morphogenesis (Otto *et al.*, 1992).

Heat stress induced decrease of the duration of developmental phases leading to fewer organs, smaller organs, reduced light perception over the shortened life cycle and perturbation of the processes related to carbon assimilation are most significant for losses in cereal fields (Stone, 2001).

The heat-shock response is a reaction caused by exposure of an organism tissue or cell to sudden high temperature stress. It is characterized by the transient expression of heat-shock proteins (HSPs). These proteins are believed to protect the biomolecules and organelles in the plant cell during high temperature stress. HSPs are synthesized by cotton (Burke *et al.*, 1985), soyabean (Kimpel and Key, 1985) and other legumes (Hernandez and Vierling, 1993) grown in the field. The primary protein structures for HSPs are well conserved in organisms ranging from bacteria and other prokaryotes to Eukaryotes such as higher animals and plants. This suggests their involvement in the protection of the organisms against heat stress and the maintenance of homeostasis (Morimoto and Tissieres, 1994). HSPs are classified in to five classes based on their differences in molecular weight {HSP100, HSP90, HSP70, HSP60 and low-molecular weight HSPs (sm HSP)} and are located in both cytoplasm and organelles like nucleus, mitochondria, chloroplast and ER.

The induction of HSPs depends on the temperature at which the species is grown. In higher plants, HSPs are generally induced by a short exposure to a temperature of $38\text{-}40^{0}$ C. HSPs are generally characterized by their binding to structurally unstable proteins. They also function as molecular chaperones (Boston *et al.*, 1996).

In transgenic Arabidopsis, transformed with heat shock inducible antisense gene for HSP70 resulted in lowering of thermotolerance in the leaf tissue (Lee and Schoffl, 1996). HSP100 and HSP90 are suspected to function as chaperones. Studies of Arabidopsis have been reported in which a mutant, *hot 1*, lacking in HSP101, showed susceptibility to high temperatures (Hong and Vierling, 2000; 2001; Maestri *et al.*, 2002). A 104kDa protein (SAP 104) and 90kDa polypeptides accumulate in rice seedlings in response to severe abiotic stresses like high and low temperature stress, salinity stress, water stress and exogenous abscissic acid application (Pareek *et al.*, 1995; Singla *et al.*, 1998). Plant smHSPs are also known to be produced in response to several other stresses and have been shown to be expressed without heat-shock during embryo and seed development, suggesting

that smHSPs may contribute to the plants thermotolerance (Lee *et al.*, 1995). Malik *et al.*, (1999) generated transgenic carrot cell and regenerated plants in which the HSP17.7 gene was either constitutively expressed (CaS lines) or expressed as a heat inducible antisense RNA. Thermotolerance measurement demonstrated that the CaS lines were more thermotolerant that the control and the antisense lines.

Apart from heat-shock, there are also HSPs induced by osmotic and salt stress, stress from low oxygen, dinitrophenol (DNP), arsenic compounds, other chemical agents, and plant hormones such as abscissic acid and ethylene. These factors may play a specific role in the denaturation of proteins; the prevention of that denaturation, and the repair function is done by the HSPs. It has been reported that salt and drought tolerance improved when the HSP gene is over expressed in tobacco (Sugino *et al.*, 1999) and in *Arabidopsis* (Sun *et al.*, 2001).

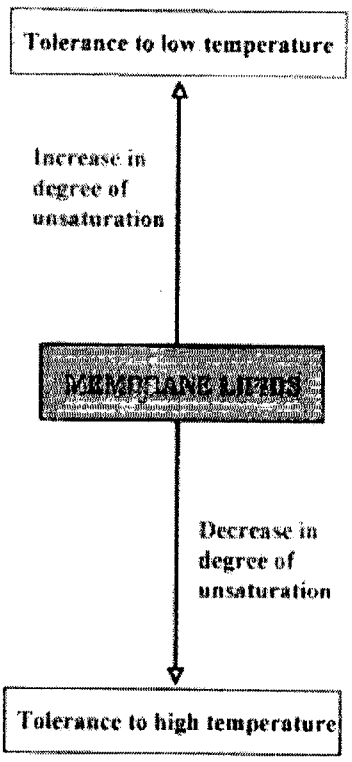
Upon exposure of plants to low temperatures, high light intensity, drought and other stresses, the amount of active oxygen species (AOS) in plants increases. The active oxygen species includes the superoxide anions (O_2^-) and hydrogen peroxide ($H_2O_2^-$) that are generated by reduction of molecular oxygen (O_2) and the hydroxyl radicals (OH) that are produced in reaction invoving H_2O_2 and $O2^-$. AOS-detoxifications systems are composed of multiple enzymes, and these are thought to function in accordance with the stress conditions in different compartments of the cell. In *Arabidopsis* subjected to low temperatures, H_2O_2 accumulates in the cells and the enzyme activities of ascorbate peroxidase and glutathione reductase increase (O'Kane *et al.*, 1996). The increase in other cytosolic AOS-detoxifying enzymes has also been described in response to high and low temperature treatment (Mittler and Zilinskas, 1992; Storozhenko *et al.*, 1998; McKersie *et al.*, 1999; 2000).

When plants are exposed to salt, drought and low temperature stress they accumulate highly soluble compounds of low molecular weight called compatible solutes. Because many plant stresses cause cell dehydration, the accumulation of these substances might play a part in increasing internal osmotic pressure and preventing loss of water from the cell. Typical compatible solutes include mannitol

and other sugar alcohols, amino acids such as proline, and amino acid derivatives such as glycinebetaine. Some of these compatible solutes, like proline, are accumulated in practically all plant species, whereas others, like glycinebetaine, are distributed only among plants with a high tolerance to salt or cold.tobacco and *Arabidopsis* transgenic to bacterial choline oxidase gene *Cod A*, the enzyme for glycinebetaine synthesis were conferred with significant resistance to low and high temperature stress (Alia Hayashi *et al.*, 1998a, b: Sakamoto *et al.*, 2000).

The fatty acid composition of membrane lipids plays a major role in the temperature acclimation of plants and cyanobacteria. Glycerolipids of thylakoid membranes serve as a major constituent of the membrane-forming bilayers and provide hydrophobic ligands to membranous proteins (Doyle and Yu, 1985). There are four abundant glycerolipids of thylakoid membranes in the chloroplasts of higher plants and in the cells of cyanobacteria playing important roles in maintaining the photosynthetic electron-transport machinery.

The degree of unsaturation of acyl residues of glycerolipids determines the physical characteristics of membranes (Chapman, 1975; Quinn, 1988; Quinn *et al.*, 1989) and consequently, the molecular motions of these lipids in the membranes. Therefore, it is believed that fatty acid unsaturation can affect various functions of membrane-bound proteins. Alterations in fatty-acid unsaturation of glycerolipids can be achieved by changing the growth temperatures of photosynthetic organisms. Pearcy (1978) and Raison *et al.*, (1982) observed that an increase in growth temperatures enhances the level of saturated fatty acids in membrane lipids and enhances the heat stability of photosynthesis. On the basis of these results, it has been concluded that the saturation of fatty acids increases heat stability. In contrast to the previous reports, Gombos *et al.*, (1994) have demonstrated that in *Synecocystis* P00 6803, unsaturation of lipid molecules does stabilize photosynthesis.



Representation of the influence of saturation levels of membrane lipids on the temperature sensitivity of plants.

HEAT STRESS AND CHLOROPLAST BIOGENESIS

Temperature, light and other environmental factors tend to affect the chloroplast developments in higher plants and algae. High temperature (32^0C) inhibits the development of normal chloroplasts in *Euglena* (Pringstein and Pringstein, 1952) and in higher plants (Feierabend and Mikus, 1976) while low temperature can inhibit the chloroplast development in chilling-sensitive plants e.g. Sorghum (Slack *et al.*, 1974) and maize (McWilliam and Naylor, 1967). During the adaptation to the temperatures, many plants show an ability to acclimate to the prevailing temperature regime during their growth. The properties and the structure of chloroplast in the primary leaf of barley seedlings were studied

when the plants were grown at constant temperatures and light intensity (Smillie *et al.*, 1978). The changes in the chloroplast activity and the structure indicate that the acclimation to temperature takes place over the entire range in which chloroplast development is permitted.

In pea, it has been observed that etiolated plantlets showed accelerated light induced accumulation of pigments and thylakoid membrane proteins after heat treatments (Otto *et al.*, 1992). Heat treatments cause morphogenetic changes in etiolated plantlets that are very similar to those known to occur in response to light. The most prominent and typical effects of heat shock on morphogenesis in barley are a reduction of the elongation of primary leaves. The circadian control of many light inducible genes and of morphogenesis by both light and temperature indicates that greening and hence chloroplast biogenesis is under circadian control after temperature / light treatment (Beator *et al.*, 1992).

High temperature affects the photosynthetic functions of the plants by its effect on the rate of chemical reactions and on the structural organization and also altering the excitation energy distribution by changing the structure of the thylakoid membranes (Berry and Bjorkman, 1980). High temperature is considered to be one of the most important environmental factors influencing photosynthetic reactions in plants (Bukhov and Mohanty, 1999). Heat shock alters photosynthetic activity due to the suppression of chloroplast electron transport and inhibition of the Calvin cycle activity (Feller *et al.*, 1998; Pastenes and Horton, 1996b). it is considered that PS II is the most sensitive component of thr photosynthetic chain (Pastenes and Horton, 1996a; Bukhov and Mohanty, 1999; Yamane *et al.*, 1998; Yamane *et al.*, 2000). Extreme high temperatures affect the functioning of the oxygen-evolving system, resulting in the release of functional manganese ions from the complex (Nash *et al.*, 1985). This release may be the result of the reductions by peroxides or superoxides (Thompson *et al.*, 1989). Other sites that are sensitive to heat stress damage include the Cyt b_6/f complex and the formation of the pH transthylakoid gradient linked to the linear electron flow (Bukhov and Mohanty, 1999).The water soluble Calvin cycle enzymes have been found to be

heat stable (McCain *et al.*, 1989) but the light activation of Rubisco is known to be one of the most heat-sensitive functions (Feller *et al.*, 1998; Weis *et al.*, 1981).

Rye seedlings grown at 32^0C showed inhibition of Chl formation parallel to the loss of 70S ribosomes. Only the tips of the leaves were light green whereas the middle and the basal parts were chlorotic. The Chl a / b ratio was 2.96 at 22^0C and 2.5 at 32^0C grown plants. When rye seedlings grown at 32^0C in light were transferred to 22^0C in light, most parts of the leaves remained irreversibly chlorotic. When plants were grown for 7 days at 32^0C in complete darkness, most of them intensively greened within a week after transfer to 22^0C in light. The amount of Chl formed at 32^0C was found to be dependent on the light intensity and decreased with higher intensities. Intact plastids isolated from completely chlorotic parts of the leaves grown at 32^0C in light contained carotenoids as the main pigments. In dark-grown leaves the higher growth temperature had no significant influence on the quantity or the composition of carotenoids (Rademacher and Feierabend, 1976).

The formation of plastidic ribosome (70 S) was selectively prevented in rye (*Secale cereale* L.) seedlinge when they were grown at higher temperature of 32^0C as compared to 22^0C, in both light and darkness (Feierabend and Schrader-Reichhardt, 1976).

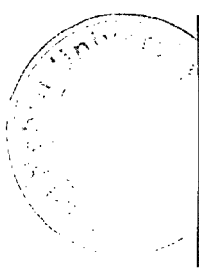
In rye seedlings grown at 32^0C, the impaired chloroplast biogenesis led to the inhibition of Chl accumulation. In higher plants when chloroplastic protein synthesis was inhibited, the accumulation of Chl stopped. Also Chl formation depended on the appearance and the amount of chloroplast rRNA (Pollack and Davies, 1970). The short intermittent exposure of rye plants growing at 32^0C to 22^0C, resulted in a significant increase of the level of chloroplast rRNA at high temperature (Feierabend, 1977).

The heat induced changes of Chl fluorescence excitation and emission properties in isolated chloroplasts of *Larrea divaricata* exhibited a fluorescence rise, suggesting the block of PSII RCs and a fluorescence decrease, caused by the functional separation of light-harvesting pigment protein complex from rest of the pigment system (Schreiber and Armond, 1978). Recently, it was shown that an

increase in temperature resulted in changes in the fluorescence parameters of nonphotochemical quenching and photochemical quenching in two bean (*Phaseolus vulgaris* L.) varietes, known to differ in their resistance to extreme high temperatures. Measurements of 77K fluorescence showed an increase in the PSI / PSII ratio with temperature, suggesting an increase in the state transitions (Pastenes and Horton, 1996a).

Also in bean (*Phaseolus vulgaris* L.) seedlings, after an increase in temperature from 30^0C to 35^0C, the supply of reducing power becomes limiting. It was suggested that the limitation in the assimilatory power was due to an oxidation of the NADPH / $NADP^+$ pool (Pastenes and Horton, 1996b).

The Chl a binding apoproteins of the antenna of photosystem II, CP47 and CP43, decline sharply during early events of bleaching but the transcription of genes encoding them, psbB and psbC, respectively, remain unchanged. This indicates that post-transcriptional events play major role in causing an irreversible loss of chloroplast function in *Euglena* at a moderately high temperature and lack of these transcripts would eventually impair the assembly of PSII in thylakoids (Thomas and Ortiz, 1995).



According to Singh and Singhal (1999), irradiation of the thylakoid membranes at 40^0C results in inhibition of PSII activity, several thylakoid proteins were lost and high molecular mass cross linking products appeared. Involvement of oxygen evolving complex (OEC) in the formation of cross linking among PSII proteins in thylakoid membrane irradiated at high temperature has been suggested (Singh and Singhal, 1999).

A change in the soluble protein pattern of plants in response to chill- and heat- stress has been reported by number of workers (Perras and Sarhan, 1989; Mohapatra *et al.*, 1987; Hernandez and Vierling, 1993; Robertson *et al.*, 1987; Lin *et al.*, 1984).

Increases in the chlorophyll fluorescence F_0 (dark level fluorescence) were studied in various higher plants. Besides the dissociation of light-harvesting chlorophyll a / b protein complexes from the reaction center complex of PSII and

the inactivation of PSII, dark reaction of Q_A via plastoquinone (PQ) seemed to be related to the F_0 increase at high temperatures (Yamane *et al.*, 2000).

SIGNALLING MECHANISM DURING HEAT STRESS

The processes involved in the temperature acclimation are initiated by the sensing of the temperature signals and transduction of these signals into the biochemical processes that finally lead to the development of the heat or freezing tolerance.

Heat stress responses have been well studied in wide range of organisms. In all species studied heat stress results in the production of specific families of proteins known as heat shock proteins (HSPs; Howarth and Ougham, 1993). These proteins have been classified into a number of families based on their molecular mass. Most of these proteins function as chaperones (Jaenicke and Creighton, 1993). All organisms produce HSPs from all of the major families (HSP90s, HSP70s and small HSPs), but plants are unique in the number of different small HSPs that they produce (Jakob and Buchner, 1994).

Despite the ubiquitous nature of the heat shock response, little is known about how the plant senses an increase in temperature or the signaling pathways resulting in HSPs. It is well known that pretreatment with a mild heating regime allows plants to tolerate higher temperatures than non-pretreated plants. These plants are termed thermotolerant (Howarth and Ougham, 1993; Burke, 2001; Sharkey *et al.*, 2001). It is known that HSPs accumulate during mild heating (Nover *et al.*, 1983) and that their appearance correlates with survival of the plant, but little is known about signaling leading to this event.

Signal transduction frequently involves the generation of second messengers and changes in protein phosphorylation. A wide range of second messengers have been implicated in signaling in response to a variety of stresses. Calcium ions (Sanders *et al.*, 1999; Knight, 2000), salicylic acid (SA; Dat *et al.*, 1998), abscisic acid (ABA; Annamalai and Yanaghiara, 1999; Gong *et al.*, 1998a,

1998b) and ethylene (Foyer *et al.*, 1997) are all involved in several stress responses.

Calcium transients in response to heat treatment have been detected using the calcium-dependent luminescent protein aequorin in tobacco (Gong *et al.*, 1998b). This suggests that calcium may have a role in heat stress signaling. It has also been shown that calcium signaling inhibitors and calmodulin inhibitors limited survival and increased electrolyte leakage from membranes after heat treatment in maize *Zea mays*; (Gong *et al.*, 1997a). However, calcium is not required for HSP production in plants despite the fact that heat stress induces uptake of calcium and the induction of some calmodulin related genes (Gong *et al.*, 1997b). This suggests the existence of some other process besides HSP induction that is required for survival of plants after heat stress. Calcium may be involved in some signaling pathway acting between the perception of heat stress and this process.

There is also some evidence that SA may be involved in heat stress responses in plants. SA is known to stabilize the trimers of heat shock transcription factors and to aid them in binding to the heat shock element in the promoter of HSP genes Jurivich *et al.*, 1992). Thermotolerance has been induced in potato plants by treatment with an acetyl-SA spray (Dat *et al.*, 1998), and the induced thermotolerance was proved to be extremely long lasting (Lopez-Delgado *et al.*, 1998). There is no evidence, however, that SA induces HSP gene transcription in plants although it results in heat shock transcription factor being bound in vivo to the transcriptional control elements of HSP70 in animal cells (Jurivich *et al.* 1992).

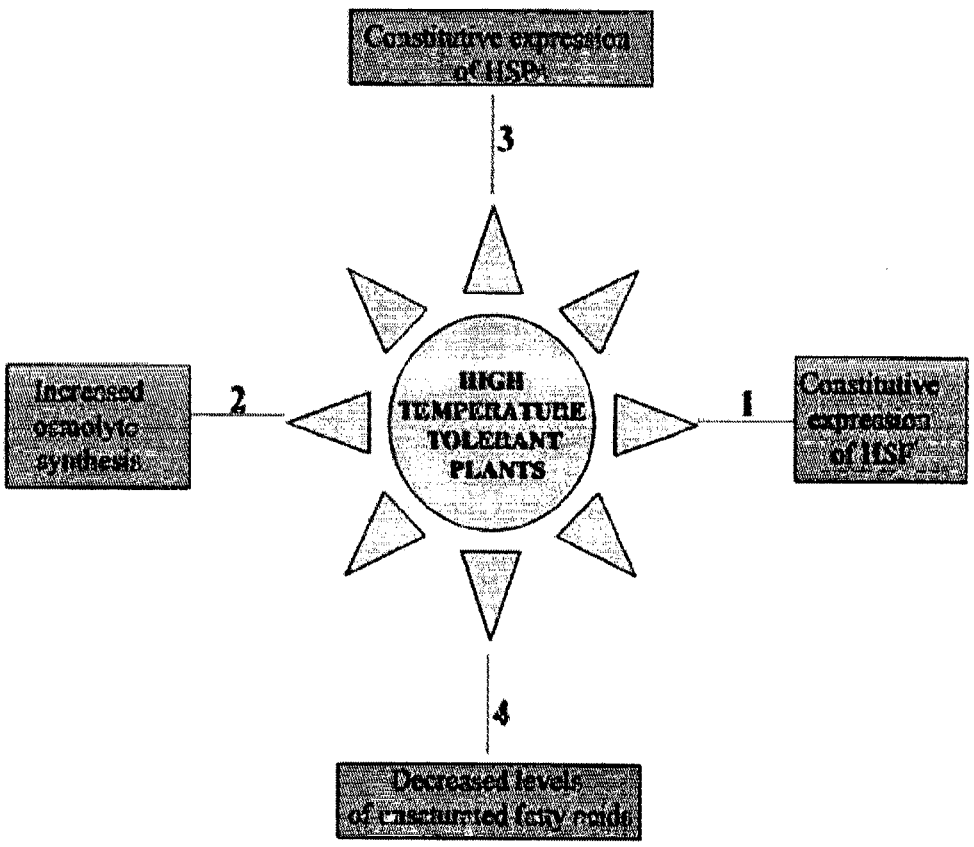
The plant hormone ABA also induces thermotolerance in maize (Gong *et al.*, 1998a) and in bromegrass (Robertson *et al.*, 1994). This suggests that ABA could also be involved in some pathway resulting in survival of heat stress in plants. ABA has been shown to induce a limited amount of HSP70 induction at ambient temperature in plants (Wu *et al.*, 1994), but no HSP90 is induced (Yabe *et al.*, 1994). ABA appears to induce chimeric genes with a small HSP promoter

from sunflower, working synergistically with heat shock transcription factor 3 (Rojas *et al.*, 1999).

The Arabidopsis gene also encodes a HSP, *APX1* (defined as such because of the presence of a heat shock transcription factor binding site in its promoter; Storozhenko *et al.*, 1998) induced by ethephon, a mimic of the plant hormone ethylene (Wu *et al.*, 1994). Ethylene has been implicated in a number of stress-induced pathways, many of which also include molecules such as SA and calcium ions Foyer *et al.*, 1997).

Phosphorylation of specific pre-existing proteins plays a role in both cold (Komatsu and Kato, 1997; Monroy *et al.*, 1993) and heat (Krishnan and Pueppke, 1987) signaling in plants. Phosphorylation level of the given protein is determined by the equilibrium between the activities of the related protein kinase and protein phosphatase. Sangwan *et al.*, (2002) have reported a heat shock – activated MAPK (HAMK) immunologically related to ERK (Extracellular signal-related kinase) superfamily of protein kinases. Link *et al.*, (2002) have also reported the activation of 50 kDa MAP kinase by heat stress in tomato, further providing an evidence of the involvement of MAP kinase in the regulation of heat response by phosphorylating a heat stress transcription factor.

STRATEGIES EMPLOYED FOR PRODUCING HIGH TEMPERATURE-TOLERANT TRANSGENIC PLANTS



Altered levels of enzymes, membrane structure, photosynthetic activities and protein metabolism represent principal components of plant heat shock response (Singla *et al.*, 1997). The rate of photosynthesis is ascribed to protein denaturation, loss of membrane integrity, photoinhibition and ion imbalance. High temperature

(a) induces photosynthetic ion imbalance,

(b) affects chloroplast biogenesis and senescence,

(c) causes disintegration of grana,

(d) brings about disruption of the structure of membrane proteins,

(e) influences protein–lipid interactions,

(f) affects electron transport activities and

(g) substantially decreases RuBP carboxylase enzyme activity (Singla *et al.*, 1997).

Due to heat shock many ultrastructural changes occur in plants in the nucleus, endoplasmic reticulum, mitochondria and plastids (Pareek *et al.*, 1997).

In 1995, a group in Germany (Lee *et al.*, 1995) succeeded in producing high temperature stress tolerant transgenic *Arabidopsis* plants by altering the level of expression of heat shock proteins (HSPs) through change in the expression levels of *Arabidopsis* heat shock transcription factor (AtHSF). In the transgenic AtHSF of *Arabidopsis* was constitutively expressed but its activity for DNA binding, trimer formation and transcriptional activation of Hsp genes is repressed at normal temperature. They were able to derepress the HSF function by experimental means which led to constitutive expression of HSPs at normal temperature and increased basal thermotolerance.

Malik *et al.* (1999) have reported increase in thermotolerance in transgenic carrot cell lines and plants by constitutive expression of carrot hsp17.7 gene (driven by CaMV 35S promoter). Another group of workers from Japan (Alia *et al.*, 1998 b) raised high temperature-tolerant transgenic plants through increased osmolyte synthesis. Earlier workers have documented that *codA* gene obtained from soil bacterium *Arthrobacter globiformis* (encoding for choline oxidase enzyme) is responsible for choline to glycinebetaine conversion and high glycinebetaine level is important in providing protection against water stress, salt stress and low temperature stress. In the study undertaken by Alia *et al.*, transgenic *Arabidopsis* plants over-expressing *codA* gene and overproducing exhibit high temperature tolerance as well.

The Arabidopsis gene also encodes a HSP, *APX1* (defined as such because of the presence of a heat shock transcription factor binding site in its promoter; Storozhenko *et al.*, 1998) induced by ethephon, a mimic of the plant hormone ethylene (Wu *et al.*, 1994). Ethylene has been implicated in a number of stress-induced pathways, many of which also include molecules such as SA and calcium ions Foyer *et al.*, 1997).

Phosphorylation of specific pre-existing proteins plays a role in both cold (Komatsu and Kato, 1997; Monroy *et al.*, 1993) and heat (Krishnan and Pueppke, 1987) signaling in plants. Phosphorylation level of the given protein is determined by the equilibrium between the activities of the related protein kinase and protein phosphatase. Sangwan *et al.*, (2002) have reported a heat shock – activated MAPK (HAMK) immunologically related to ERK (Extracellular signal-related kinase) superfamily of protein kinases. Link *et al.*, (2002) have also reported the activation of 50 kDa MAP kinase by heat stress in tomato, further providing an evidence of the involvement of MAP kinase in the regulation of heat response by phosphorylating a heat stress transcription factor.

MATERIAL AND METHODS

Plant Material

Two cultivars, CSR 10 and Pusa Basmati 1 (PB 1) of rice (*Oryza sativa* L.) seeds were used as experimental material. Seeds of CSR 10 were obtained from Central Soil Salinty Research Institute (C.S.S.R.I.), Karnal and that of PB 1 were obtained from Indian Agricultural Research Institute (I.A.R.I.), New Delhi

Chemicals

Chemicals like sodium azide, L-ascorbic acid, P-phenyldiamine, methyl vilogen, 2-6-dichlorophenyl-indophenyl were purchased from Sigma and other chemicals were purchased from Amersham chemical company, Qualigens, BDH, S.d.fine and SRL.

Plant Growth Conditions

The seeds were treated with 0.1% $HgCl_2$ solution for two minutes, washed with tap water several times. Seeds were grown in vermiculite using half strength Murashige and Skoog (MS) liquid media having no agar and vitamins as nutrient solution. Seeds were grown first in complete darkness for six days at 28^0C before subjecting to heat-stress. After 6-days one set of seedlings was transferred to plant growth chamber at 28^0C (control) under cool white fluorescent light of constant intensity of 80 μ moles m^{-2} s^{-1}, and 75% humidity. The other sets of seedlings were subjected to heat-stress in the incubators with temperature set at 35^0C, 40^0C and 45^0C. Three replicates from control and treated were taken for doing the experiments.

Nutrient Solution Used for Growth of Rice Seedlings:

For the growth of rice seedlings Murashige and Skoog medium was prepared.

Major Salt Solution:

g/500ml (20x)	
NH_4NO_3	16.5g
KNO_3	19g
$CaCl_2.2H_2O$	4.4g
$MgSO_4.7H_2O$	3.7g
KH_2PO_4:	1.7g

Minor Salt Solution:

g/500ml (200x)	
KI	0.083g
H_3BO_3	0.62g
$MnSO_4.4H_2O$	1.69g
$ZnSO_4.7H_2O$	0.86g
$CuSO_4.5H_2O$	0.0025g
$CoCl_2.6H_2O$	0.0025g

Iron Source:

250ml	
$FeSO_4.7H_2O$	0.695g
$Na_2EDTA.2H_2O$	0.93g

For 1 litre of M.S. media 50 ml of major salt solution is mixed with 5ml of minor salt solution and 10 ml of iron source and rest of the volume is made by double distilled water. Media was without any vitamins and agar.

Chlorophyll Estimation

Materials

90% Ammoniacal acetone.

Method

6-day old etiolated rice seedlings grown at 28^0C were transferred to 28^0C, 35^0C, 40^0C, 45^0C and were exposed to light (80 $\mu molesm^{-2}s^{-1}$) for up to 72h. At different time periods (12-72h), about 50mg of leaves were taken out and homogenized in 10ml of 90% ammoniacal acetone in a pre-chilled mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 min at 4^0C and the supernatant was taken for pigment estimation. Absorbance was taken at 663nm, 645nm, 470nm. Reference cuvette contained 90% ammoniacal acetone. Chlorophyll was calculated as described by Porra *et al* (1989).

Chl (a+b) = (9.05 X OD 663 + 22.2 X OD 645) V / W

Chlorophyll a Fluorescence Measurements:

Plant Efficiency Analyzer (PEA) Measurements:

Chl a fluorescence induction (the Kautsky effect), that is the OJIPS transient (O for the initial or minimal level; P for the peak or the maximal level; J and I for intermediates interactions; and S for the semi-steady state fluorescence; Strasser et al. 1995), was measured using a shutter-less fluorimeter, plant efficiency analyzer (PEA; Hansatech, Kings Lynn, UK). Data accumulation began at 70 ms and lasted for 60 s, with the fluorescence signal at 70 ms considered as F_0. Chl a fluorescence transient was measured using a 650-nm excitation light at irradiance of 2500 μmoles photons m^{-2} s^{-1}. A leaf area of 12.5 mm^2 was illuminated by an array of six high intensity light emitting diodes (LEDS). A high performance pin photodiode, associated with an amplifier circuit, was used to collect fluorescence signal. This signal was digitized in the control box using an

analogue/digital converter. The initial fast rise in fluorescence was digitized at the rate of 100000 readings s^{-1} to ensure accurate determination of F_0. After 2 ms, a data acquisition rate of 1000 readings s^{-1} was used until 1s had elapsed. Finally, only ten readings s^{-1} were taken for slow decline of fluorescence from the peak (P) to the semi steady state (S) or the terminal level (L). (Govindjee and Paul Spilotro, 2002).

6-day old etiolated rice seedlings grown at 28^0C were transferred to 28^0C, 35^0C, 40^0C, 45^0C and were exposed to light (80 $\mu molesm^{-2}s^{-1}$) for up to 72h. At different time periods (24-72h), three replicates from each set of seedlings were taken. The control (28^0C) and heat-stressed (35^0C, 40^0C, 45^0C) leaves were subjected to the dark-adaptation for 20 min. before carrying out the experiment.

Pulse Amplitude Modulation (PAM) Measurements

F_0 and F_v/F_m, non-photochemical quenching, photochemical quenching, electron transport rate and quantum yield of photosystem II were measured at room temperature by a PAM-2100 Chl fluorimeter (Walz, Germany). Red actinic illumination (wavelength, 655 nm) provided by five LEDS (H3000); Stanley, Irvine, CA, USA) focused onto the leaf surface (79 mm2). Two other H-3000 LEDS, that emit 650-nm pulses , were used as measuring light. Leaf clip holder 2030-B, equipped with a microquantum sensor, monitored photosynthetically active radiation. An additional spacer was added to the clip in order to avoid pinching of the leaf. Chl fluorescence was detected by a photodiode (BYP 12; Siemens, Munich, Germany) that was shielded by a long-pass far-red filter (RG9; Schott, Southbidge MA, USA) and a heat filter. All the PAM-2100 fluorescence data was recorded in a time span of 5 min. and 20 s.

6-day old etiolated rice plantlets grown at 28^0C were transferred to 28^0C (control), 35^0C, 40^0C, 45^0C (heat-stress) and were exposed to light (80 $\mu molesm^{-2}s^{-1}$) for up to 72h.The control (28^0C) and heat-stressed (35^0C, 40^0C, 45^0C) leaves were subjected to the dark-adaptation for 20 min. before carrying out experiment. Three replicates were taken for each experiment at 24 h, 48 h and 72 h.

The F_v / F_m measurements were made using the intact leaves with the help of PAM whereas F_0 was measured with the help of PEA and PAM both.

Estimation of oxygen evolution, uptake in whole chain, PS I and PS II electron transport

Materials

Isolation Buffer

20 mM HEPES/NaOH buffer, pH 7.6

10 mM NaCl

0.4 M sucrose

0.5% BSA, pH 7.6

Suspension / Reaction Buffer

50 mM HEPES/NaOH buffer, pH 7.3

10 mM NaCl

4 mM $MgCl_2$

Whole Chain solutions

0.5 mM MV

1 mM NaN_3

2 mM NH_4Cl

PS II solutions

5 mM PD

1 mM K_3FeCN_6

PSI solutions

1 mM Sodium Ascorbate

100 μM DCPIP

40 μM DCMU

0.5 mM MV

1 mM NaN_3

2 mM NH_4Cl

Method

6-day old etiolated rice seedlings grown at 28^0C were transferred to 28^0C (control), 35^0C, 40^0C, 45^0C (heat-stress) and were exposed to light (80 μmolesm^{-2}s^{-1}) for up to 72h. After 72 h, leaves of the control (28^0C) and the treated, (35^0C, 40^0C, 45^0C) seedlings were homogenized very gently in isolation buffer. The homogenized solution was filtered through eight layers of cheese cloth and one layer of mira cloth. The filtrate was centrifuged at 5000 rpm at 4^0C for 5min. The supernatant was discarded and the pellets were dissolved in minimum volume of isolation buffer. The Chlorophyll estimation was done in 80% ammonical acetone from this suspension of thylakoids. The spectrophotometric calculation was used to measure the total chlorophyll content and the chloroplast suspension was made containing 1mg chlorophyll per ml.

Whole Chain

1ml of suspension buffer was added in oxygen monitor (Hansatech) along with the whole chain solutions. Chloroplast suspension equivalent to 20 μg of Chlorophyll was added. The recorder plot was used to obtain the curve according to the oxygen consumption.

PSII

PSII chemicals were added instead of whole chain chemicals. The recorder plot was used to obtain the curve for oxygen evolution.

PSI

PSI chemicals were added along with whole chain chemicals. The recorder plot was used to obtain the curve for oxygen consumption.

Calculation: One small division = 15.56 μM oxygen/mg chlorophyll/h

Measurement of oxygen evolution in PS II and uptake in PS I in the presence and absence of Mg^{++}

Materials

5 mM HEPES buffer, pH 7.6

5 mM HEPES + 4 mM $MgCl_2$ buffer, pH 7.3

PSI and PSII electron transport were measured as described above but in the presence and absence of Mg^{++} in the reaction buffer.

Room Temperature Spectra

The chloroplast suspension was prepared as mentioned above. Then the chloroplast suspension was suspended in 5mM HEPES buffer making the final concentration as 3μg chl / ml. The fluorescence spectra were recorded in the presence and absence of Mg^{++} in both control and the treated plants (45^0C).

The spectra were recorded with SLM Amnico spectrofluorimeter having photon-counting device and were connected with photomultiplier tube sensitivity. Rhodamine B was used in the reference channel as a quantum counter. A tetraphenlbutadiene (TPD) block was used to adjust voltage in both the channels (i.e samples as well as reference channel) to 20,000 counts per second at excitation and emission wavelengths of 390 and 750 nm respectively. The emission spectra were recorded at excitation and emission bandwidths of 4nm.The excitation spectra was measured at 440nm. The data was stored in microcomputer IBM PC 30 diskettes.

RESULTS

Plants growth:

Two cultivars Rice (*Oryza sativa* L) i.e., CSR10 and Pusa Basmati (PB1) were taken for study. 6-d-old etiolated rice seedlings of CSR 10 were transferred to 28^0 C (Control), 35^0 C, 40^0 C, 45^0 C (heat-stress) and of PB 1 were transferred to 25^0 C (Control), 42^0 C, 50^0 C (heat-stress). The seedlings were subjected to cool white fluorescent light (80 μmol m^{-2} s^{-1}). Half-strength MS media was given to the seedlings as the nutrient media. The rice seedlings were exposed to heat stress for up to 72 hours.

CSR10 control seedlings (Fig 1) looked greener than those seedlings which were subjected to heat-stress. Growth of seedlings was reduced upon increase in the temperature of growth of the seedlings. Seedlings shown in Fig. 1 are after 72 hours of greening at different temperatures.

Chlorophyll Contents:

6-d-old etiolated control and heat-stressed rice seedlings of two cultivars were transferred to different temperatures and cool white fluorescent incandescent light (80 μmol m^{-2} s^{-1}). Leaves were harvested after 12, 24, 48, 72, hours of greening and their Chl content was estimated.

Total Chlorophyll

The total chlorophyll content of CSR10 and PB1 is shown in Fig 2 and Fig 3 respectively. CSR10 cultivar had maximum Chl biosynthesis within 48 hours of greening and there was almost no further increase in Chl contents after that (Fig 2). In PB1 genotype chlorophyll biosynthesis continued up to 72 h of greening (Fig 3). The total chlorophyll content was more in CSR10 than that of PB1. As compared to their respective controls, the chlorophyll content (corrected for the loss of moisture during heat-stress) decreased in response to heat-stress in both the cultivars. As compared to the control, the chlorophyll biosynthesis, in the seedlings grown at 35^0 C, increased by 18.05% after 72 h whereas in the seedlings

grown at 40^0 C and 45^0 C the chlorophyll biosynthesis was inhibited by 32.01% and 91.19% respectively after 72 h (Fig. 2).

Upon illumination, in the control seedlings (grown at 25^0 C) of PB 1 the chlorophyll content increased gradually. As compared to the control, after 72 h the chlorophyll biosynthesis was inhibited by 67.85% and 97.44% in the heat-stressed seedlings grown at 42^0 C and 50^0 C respectively (Fig. 3).

F_0 and F_v/F_m

6-d-old etiolated control and heat-stressed rice seedlings of CSR 10 were transferred to different temperatures and cool white fluorescent incandescent light (80 μmol m^{-2} s^{-1}). Leaves were harvested after 24, 48, 72, hours of greening and their F_0 and F_v/F_m were measured as described in material and methods.

As compared to the control, the F_0 values decreased in the experiments done with PAM (Fig. 4) and PEA (Fig. 5) in the seedlings grown at 45^0 C. There was an increase in the F_0 values with the increasing temperatures in the experiments done with PEA (Fig. 5) The rise was most prominent in case of the seedlings grown at 45^0 C.

As compared to the control, F_v/F_m values decreased in the heat-stressed seedlings with the increasing temperatures and hours of greening (Fig. 6).

Photochemical parameters:

Photochemical parameters i.e., electron transport rate (ETR), quantum yield of photosystemII (ΦPSII), photochemical quenching (qP) and non-photochemical quenching (qN) were measured by PAM fluorimeter (PAM 2100).WC, PS I and PS II activities were measured with the help of oxygen monitor (Hansatech). 6-d-old etiolated control and heat-stressed rice seedlings of CSR 10 were transferred to different temperatures i.e. 28^0 C (control), 35^0 C, 40^0 C, 45^0 C (heat-stress) under cool white light (80 μmol m^{-2} s^{-1}) as described in material and methods. Measurements were done after 24, 48, and 72 hours of greening in the control and heat-stressed seedlings.

Fig 1 A.

Fig 1 B.

Fig 1. Photographs of control (28^0 C) and heat-stressed (35^0 C, 40^0 C, 45^0 C) rice seedlings. Fig 1A. shows rice seedlings of control and 35^0 C rice seedlings after 48 h of greening. Fig 2 B. shows rice seedlings of control and 40^0 C, 45^0 C after 72 h of greening.6-d-old etiolated rice seedlings grown at 28^0 C were transferred to 28^0 C, 35^0 C, 40^0 C and 45^0 C for 72 h in light (80 μ moles m^{-2} s^{-1}).

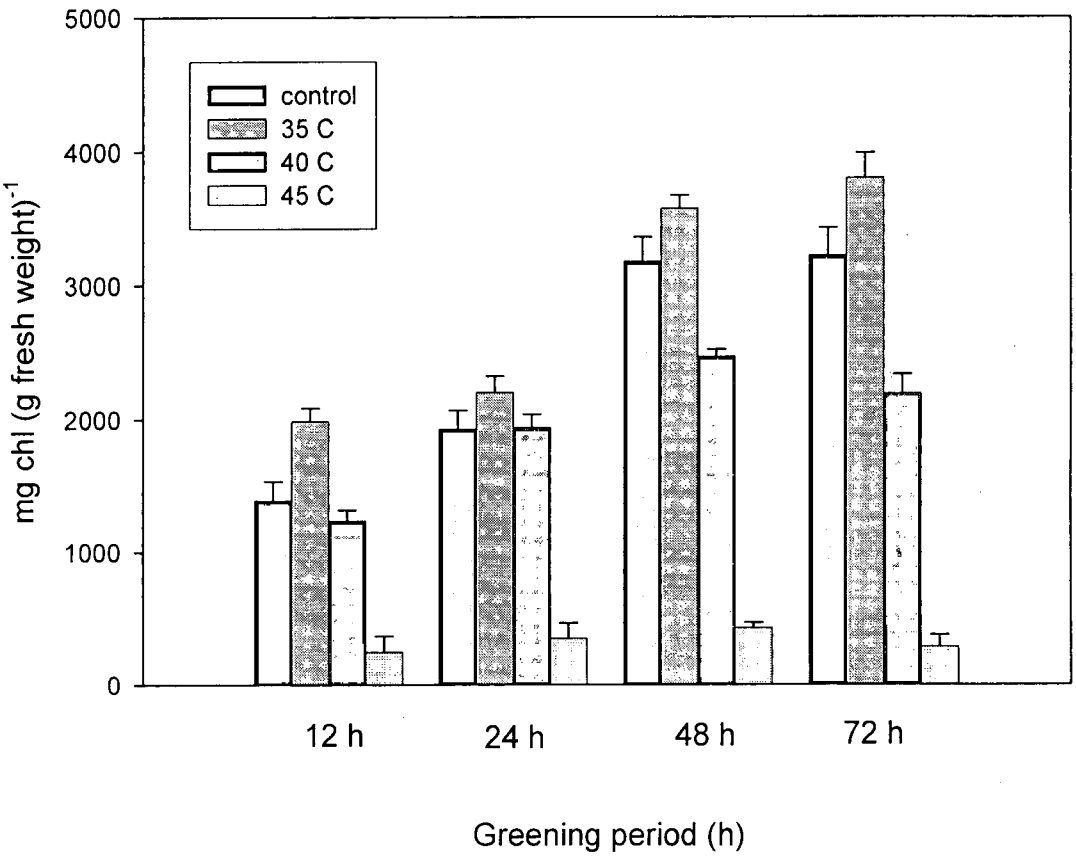


Fig 2. Chlorophyll contents of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h. Chlorophyll content was measured at specific time points as described in material and methods. Each data is average of three replicates. The error bar represents SD.

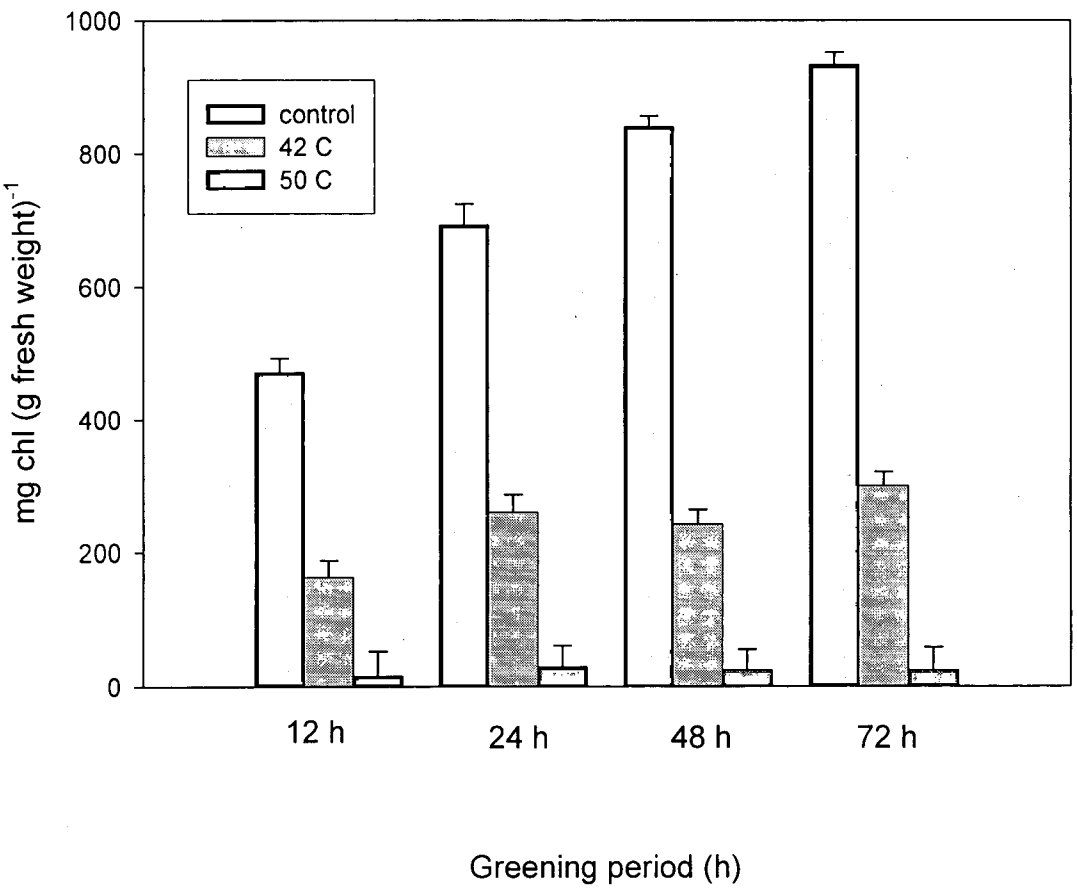


Fig 3. Chlorophyll contents of control (28^0 C) and heat-stressed PB-1 seedlings (42^0 C, 50^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 42^0 C and 50^0 C for a period of 72h. Chlorophyll content was measured at specific time points as described in material and methods. Each data is average of three replicates. The error bar represents SD.

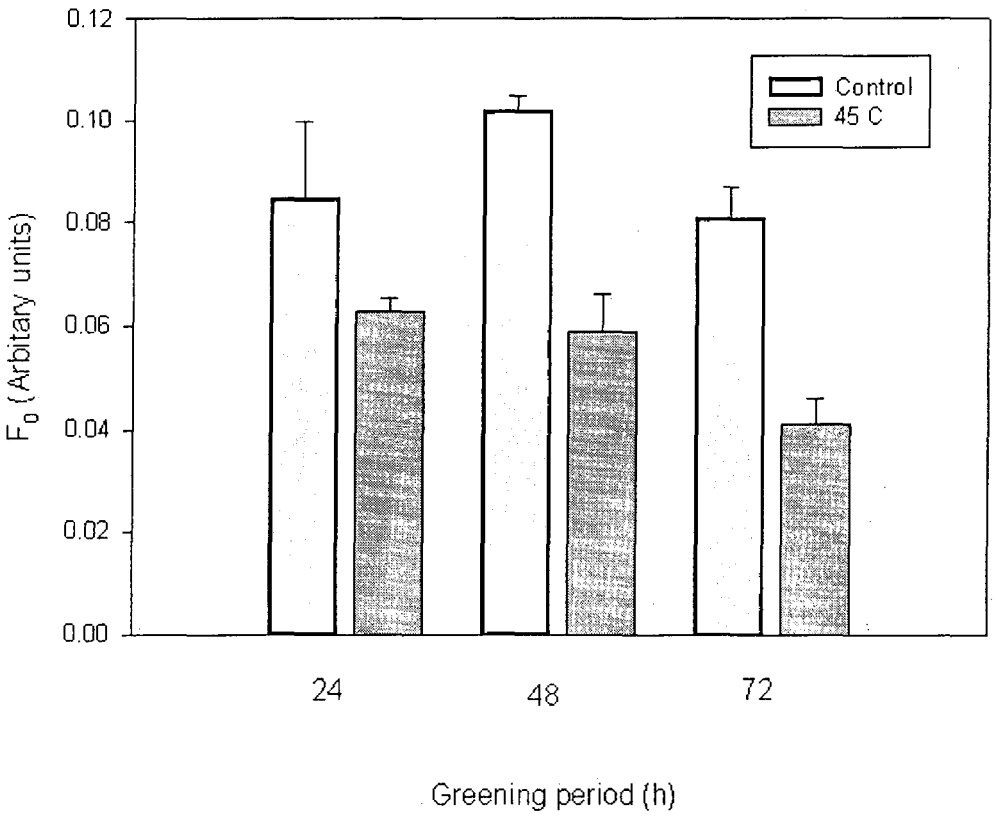


Fig 4. F_0 values of control (28^0 C) and heat-stressed seedlings (45^0 C) as measured by PAM.6-d-old etiolated rice seedlings grown at 28^0 C were transferred to cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C and 45^0 C for a period of 72h. F_0 was measured at specific time points as described in material and methods. Each data is average of three replicates. The error bar represents SD.

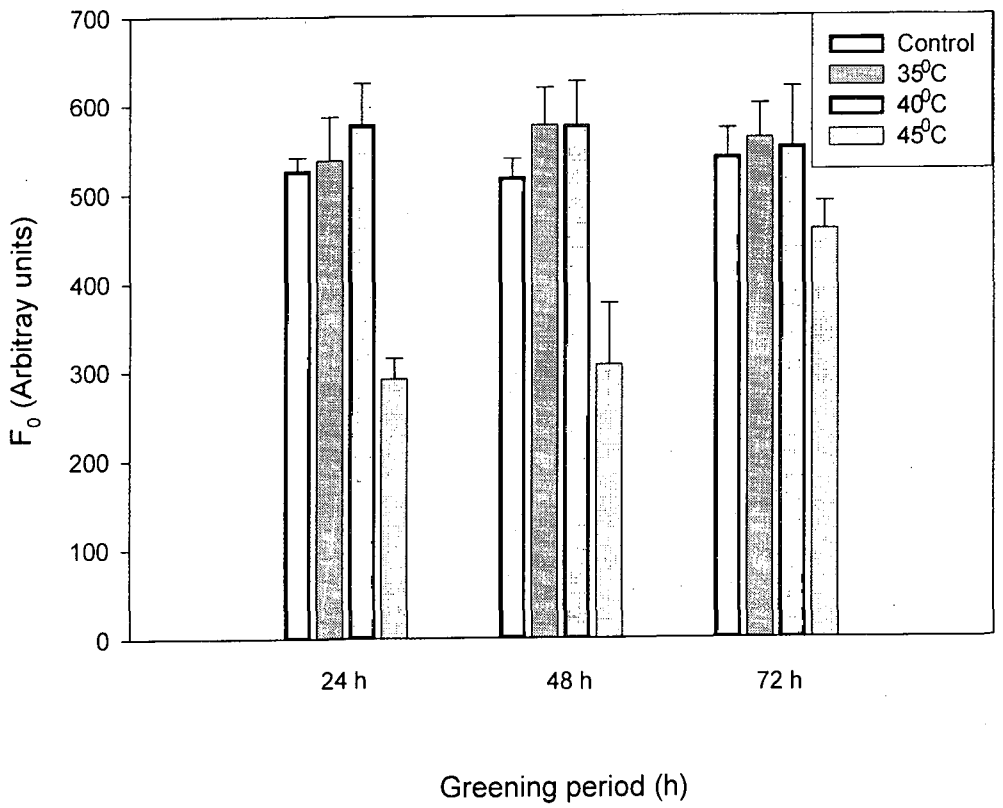


Fig 5. F_0 values of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PEA. 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h. F_0 was measured at specific time points as described in material and methods. Each data is average of three replicates. The error bar represents SD.

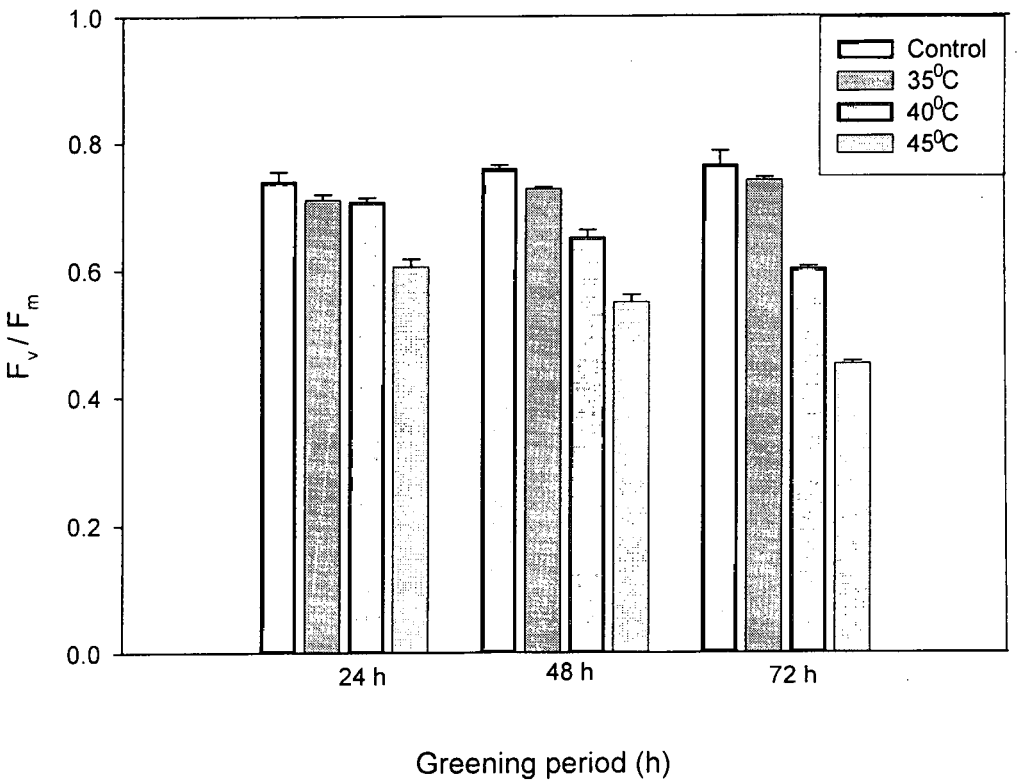


Fig 6. F_v / F_m values of control (28^0 C) and heat-stressed seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PAM. 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C and 45^0 C for a period of 72h. F_v / F_m was measured at specific time points as described in material and methods. Each data is average of three replicates. The error bar represents SD.

Electron transport rate (ETR)

Electron transport rate of CSR10 at 24, 48 and 72 hours of greening are shown in Fig. 7. Electron transport rate of control increased gradually in response to PAR (μmol photons m^{-2} s^{-1}) at different hours of greening. The ETR of heat-stressed seedlings decreased as compared to the control with increase in the temperature as well as the greening period. The maximum decline in ETR was observed after 72 h of greening in the seedlings exposed to 45^0 C.

The ETR reduced to 72.47%, 41.03% and 12.2% of control in the treated seedlings (35^0 C, 40^0 C, 45^0 C) after 72 h of treatment (Fig. 7).

Photochemical quenching

Photochemical quenching (qP) of CSR10 at 24, 48, and 72 hours of greening is shown in Fig. 8. Photochemical quenching in CSR10 decreased in response to increase in AR (μmol m^{-2} s^{-1}). The photochemical quenching (qP) also decreased with increase in temperature, as compared to control. After 72 h the values of qP of the leaves subjected to 35^0 C, 40^0 C, 45^0 C reduced to 66.25%, 48.32%, 16.84% of control.

Non-photochemical quenching

The qN of CSR10 at 24, 48, and 72 hours of greening are shown in Fig 9. In control the non-photochemical quenching (qN) increased in response to PAR (μmol m^{-2} s^{-1}). The qN decreased in response to heat-stress. The qN of plants at 45^0 C reduced by 51.03% as compared to control after 72 h of greening .

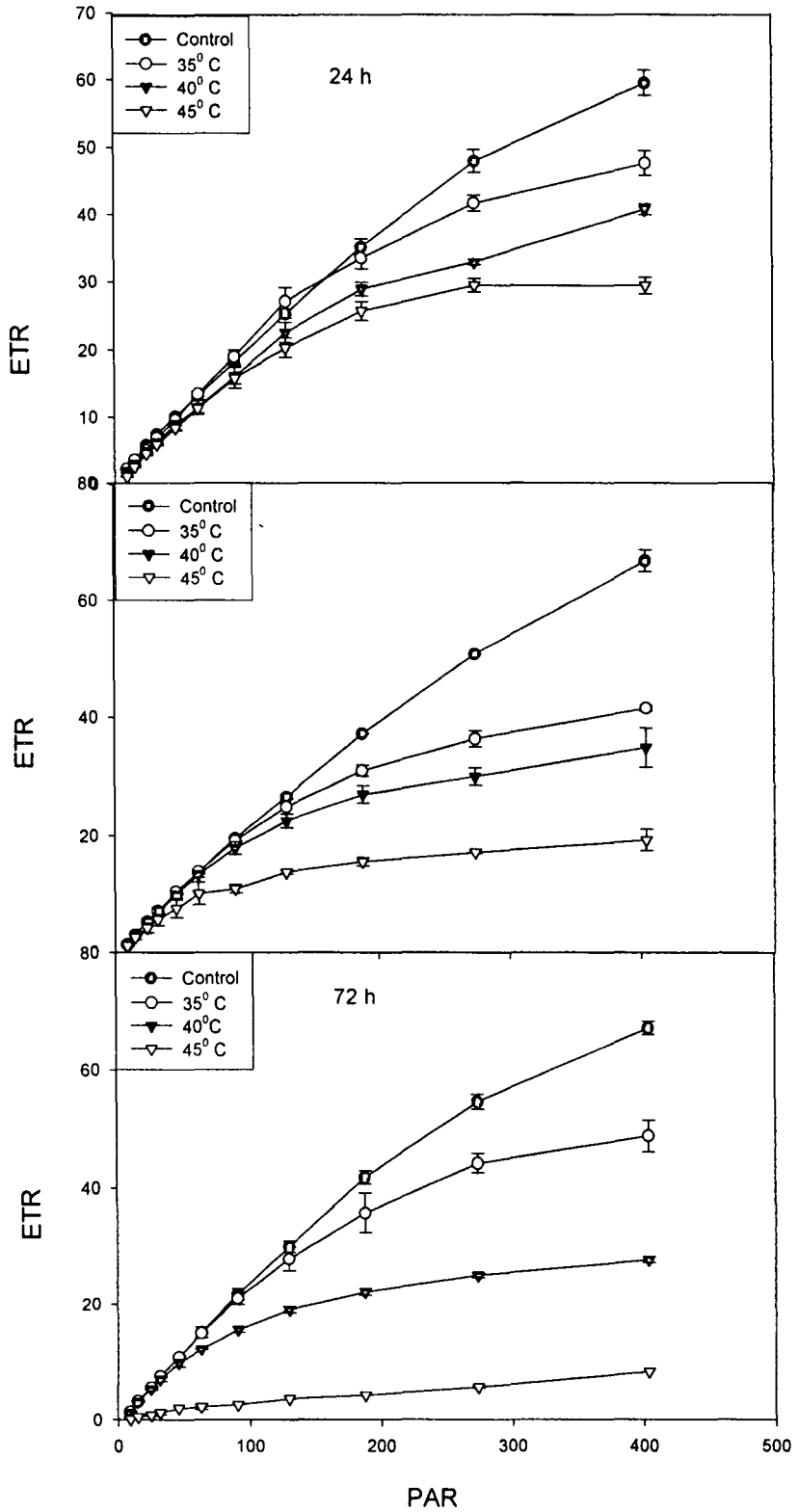


Fig 7. ETR values of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PAM at different time periods.

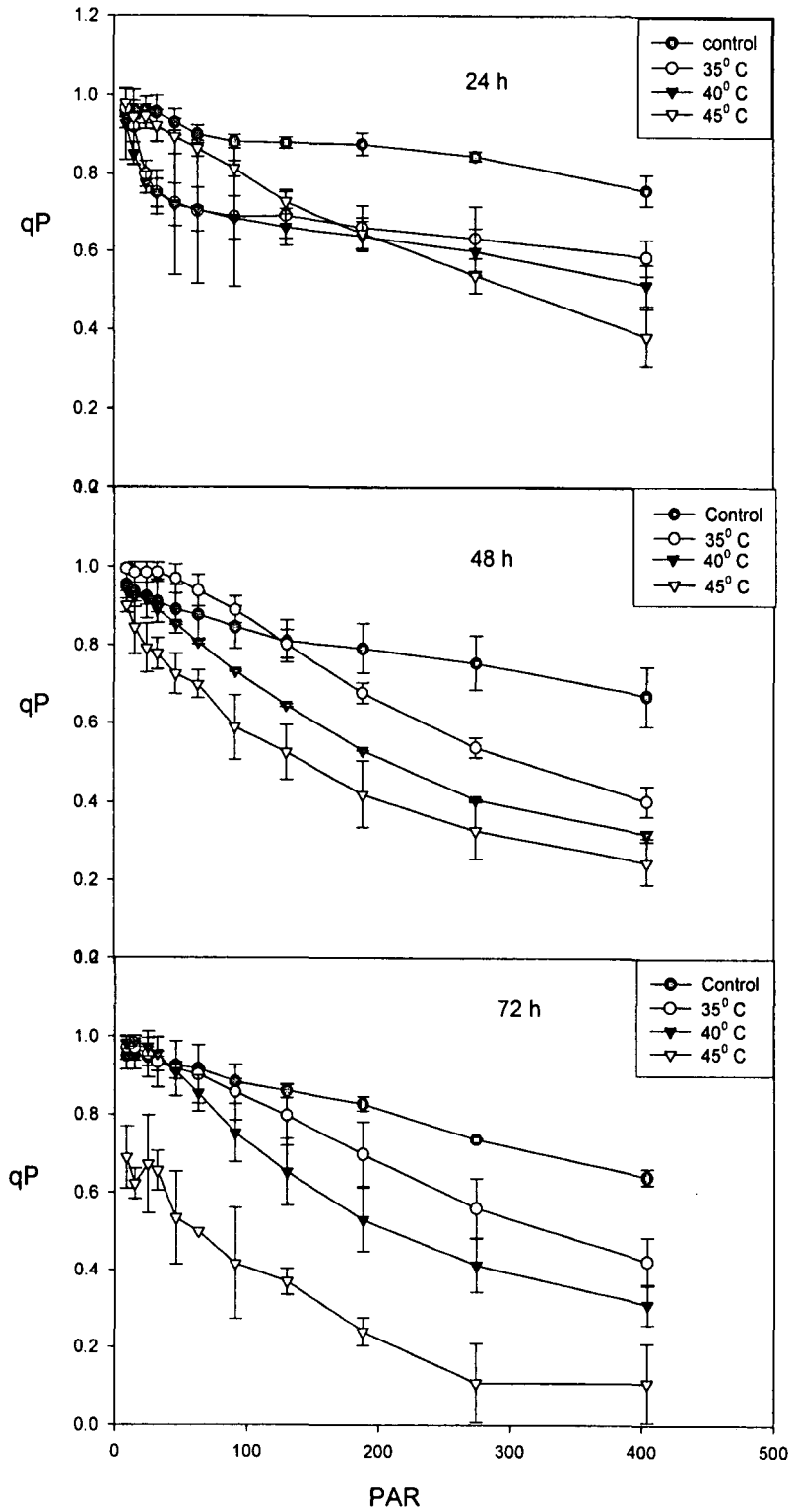


Fig 8. qP values of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PAM at regular periods. The error bar represents SD.

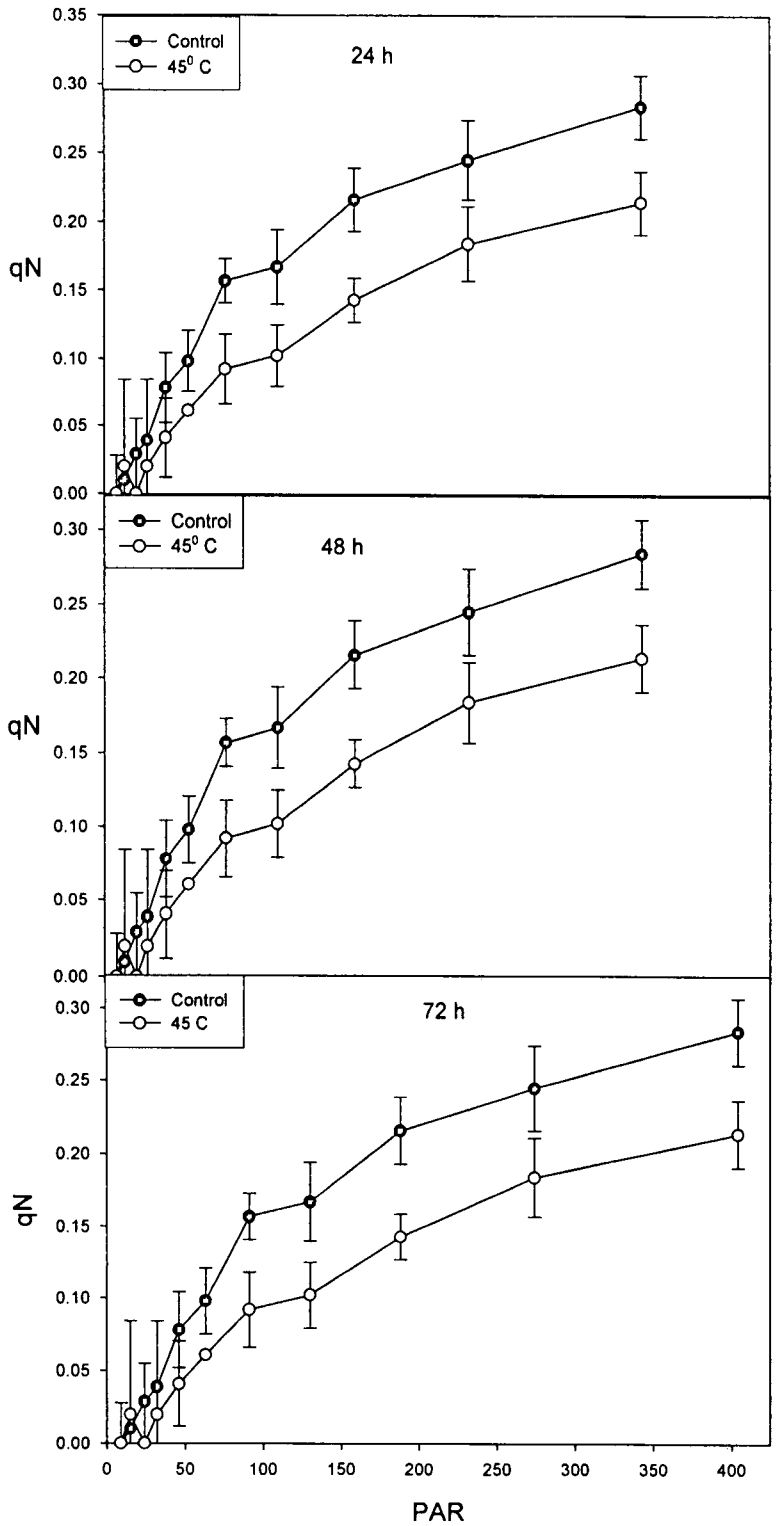


Fig 9. qN values of control (28^0 C) and heat-stressed rice seedlings (45^0 C) as measured by PAM at regular periods. The error bar represents SD.

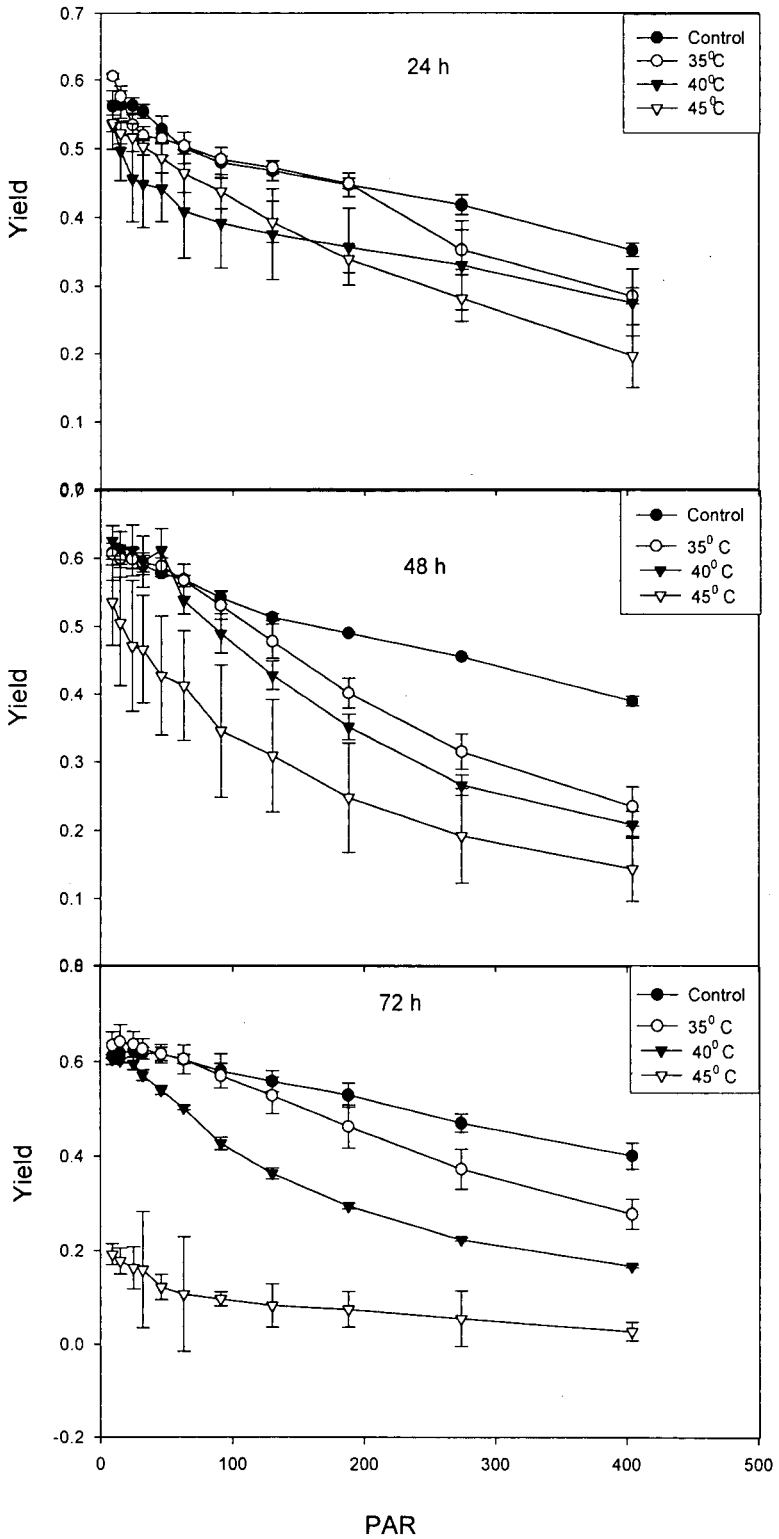


Fig 10. Yield values of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PAM at regular periods. The error bar represents SD.

Quantum yield of photosystem II (PSII)

Quantum yield of photosystem II of CSR10 at 24, 48 and 72 hours of greening are shown in Fig. 10. Quantum yield of PS II of CSR10 decreased with increase in PAR values. As compared to respective controls, the yield decreased in the treated seedlings with increase in temperature, hours of greening and PAR values.

The yield reduced to 69.49%, 41.59% and 6.97% of control in the treated plants (35^0 C, 40^0 C, 45^0 C) after 72 h of treatment.

Whole chain electron transport

Whole chain (WC) oxygen uptake activity of CSR10 was measured after 72 hours of greening (Fig. 11). The whole chain electron transport was measured in the thylakoids as oxygen uptake from water to MV. The WC oxygen uptake activity decreased with the increasing temperature of the seedlings. As compared to the control, there was a reduction in the activity by 5%, 43.73% and 62.58% in the seedlings grown at 35^0 C, 40^0 C and 45^0 C respectively.

PS II

PS II oxygen evolution activity of CSR10 after 72 hours of greening is shown in Fig. 12. PD supported and PS II mediated oxygen evolution was measured as an index of PS II activity in the thylakoid membrane isolated from control (28^0 C) and heat-stressed seedlings (35^0 C, 40^0 C, 45^0 C). The PS II activity decreased with the increasing temperature of the seedlings. As compared to the control, the thylakoid membranes isolated from the heat-stressed seedlings (35^0 C, 40^0 C, 45^0 C) exhibited reduced activity by 12.2%, 39.7% and 84.1% respectively.

PS I

PS I oxygen uptake activity of CSR10 at 72 hours of greening is shown in Fig. 13. PS I activity measured in thylakoid membrane isolated from control (28^0 C) and heat-stressed seedlings (35^0 C, 40^0 C, 45^0 C) was monitored as oxygen uptake with $DCPIPH_2$ as electron donor and MV as electron acceptor. As

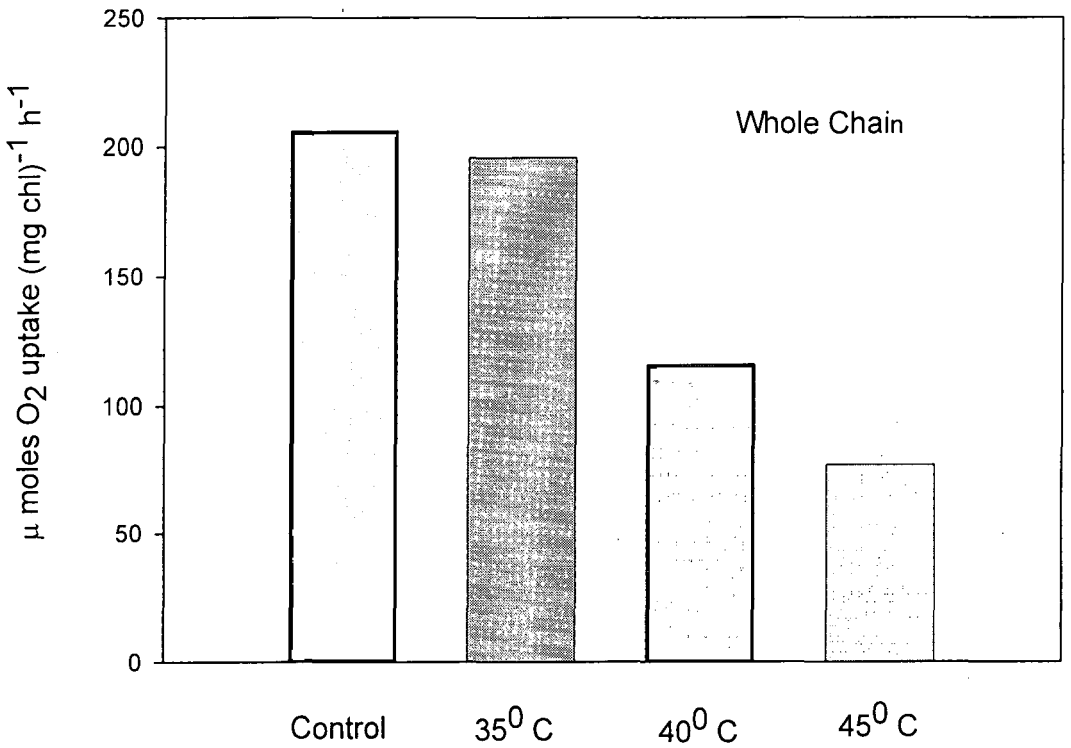


Fig 11. Whole Chain electron transport was monitored as H_2O^{--} > MV in the thylakoids of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h. After 72h WC electron transport rate of the harvested leaves of control and heat-stressed rice was monitored in terms of oxygen evolution at 25^0 C by oxygen monitor as described in material and methods. Each data is average of three replicates

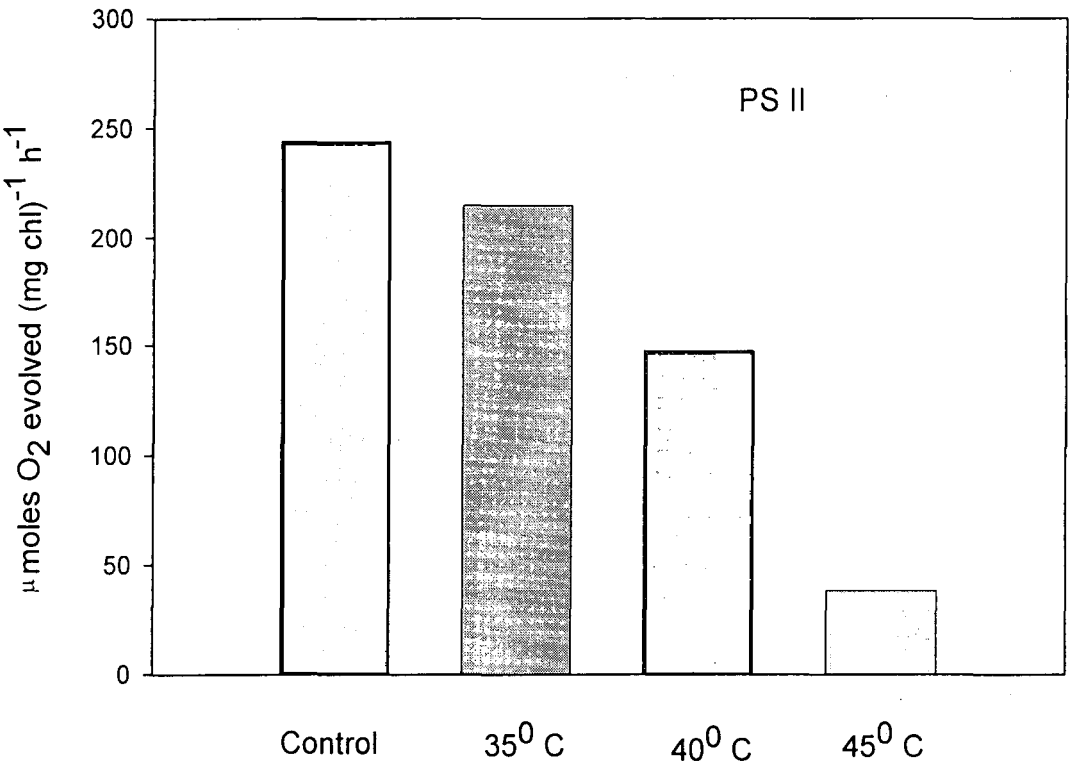


Fig 12. PSII mediated electron transport rate was assayed as H_2O >PD^{--} >$K_3Fe(CN)_6$ in the thylakoids of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^{0}C, 35^0 C, 40^{0}C and 45^0 C for a period of 72h. After 72h PSII electron transport rate of the harvested leaves of control and heat-stressed rice was monitored in terms of oxygen evolution at 25^0 C by oxygen monitor as described in material and methods. Each data is average of three replicates.

compared to the control, a decrease in the PS I activity was observed in the seedlings grown at 35^0 C and 40^0 C by 9.98% and 8.43% respectively. On the other hand, there was an increase in the PS I activity by 39.4% in the seedlings grown at 45^0 C.

Photochemical Reactions of PS II and PS I in the Presence and Absence of Mg^{++}

To understand the grana stacking of control (28^0 C) and treated (45^0 C) plants, thylakoid membranes were suspended in low salt medium to unstack the grana to individual membranes. Both PS I and PS II are uniformly distributed in the thylakoid membrane and LHCP II distribute energy almost equally to both PS II and PS I. After addition of magnesium ions, thylakoids stack and form grana which consequently results in non uniform distribution of PS II and PS I in thylakoid membrane where LHCP II remains close to PS II and transfers its energy to PS II.

In the presence of Mg^{++}, PS II activity increased by 24.6% in control (28^0 C) and 13.33% in the treated (45^0 C) seedlings (Fig. 14) whereas PS I activity decreased by 21.54% in control (28^0 C) and 10.96% in the treated seedlings (45^0 C, Fig. 15).

OJIP Transient

6-d-old etiolated control and heat-stressed rice seedlings of CSR 10 were transferred to different temperatures i.e. 28^0 C (control), 35^0 C, 40^0 C, 45^0 C (heat-stress) under cool white fluorescent incandescent light (80 μmol m^{-2} s^{-1}) as described in material and methods.. Measurements were done after 24, 48, and 72 hours of greening in the control and heat-stressed seedlings at 2500 μmol m^{-2} s^{-1}.

The fluorescence curves and radar plots of CSR 10 at 24 h, 48 h and 72 h of greening and 2500 μmol m^{-2} s^{-1} light intensity are shown in Fig 16, 17, 18, 19, 20 and 21. The maximal fluorescence (Fm) declined in rice seedlings exposed to

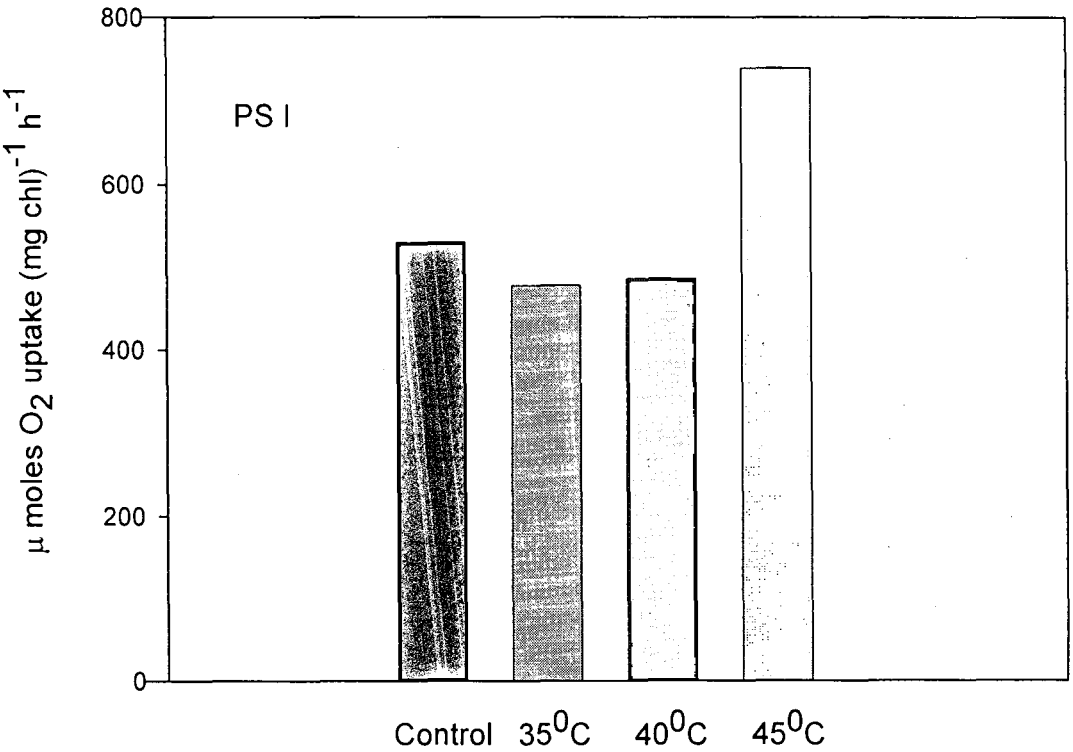


Fig 13. PSI mediated electron transport rate with $DCPIPH_2$ as electron donor and MV as electron acceptor in the thylakoids of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) . 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h. After 72h PSI electron transport rate of the harvested leaves of control and heat-stressed rice was measured as described in material and methods. Each data is average of three replicates.

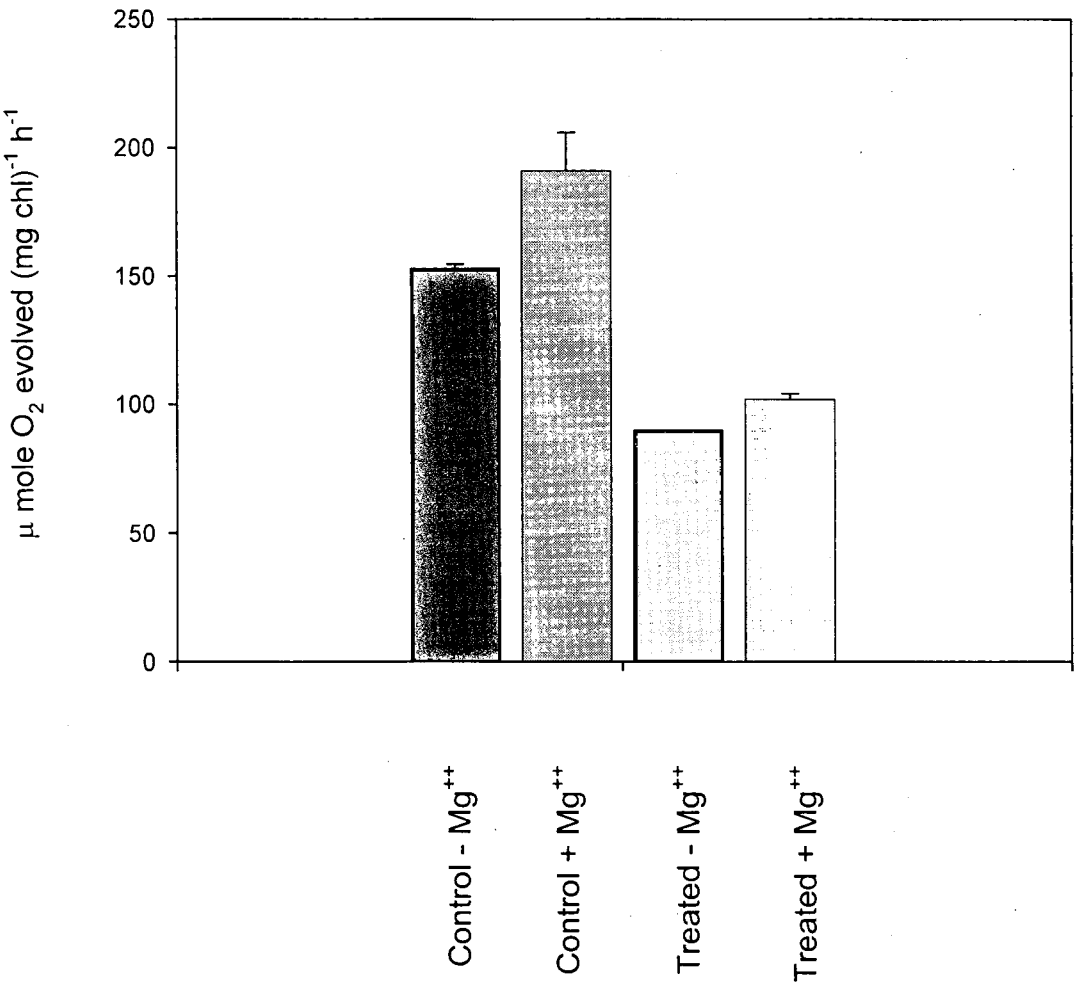


Fig 14. PSII mediated electron transport rate was assayed as H_2O >PD^{--} >$K_3Fe(CN)_6$ in the thylakoids of control (28^0 C) and heat-stressed rice seedlings (45^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C and 45^0 C for a period of 72h. After 72h PSII electron transport rate of the harvested leaves of control and heat-stressed rice was monitored in terms of oxygen evolution at 25^0 C by oxygen monitor as described in material and methods. Each data is average of three replicates.

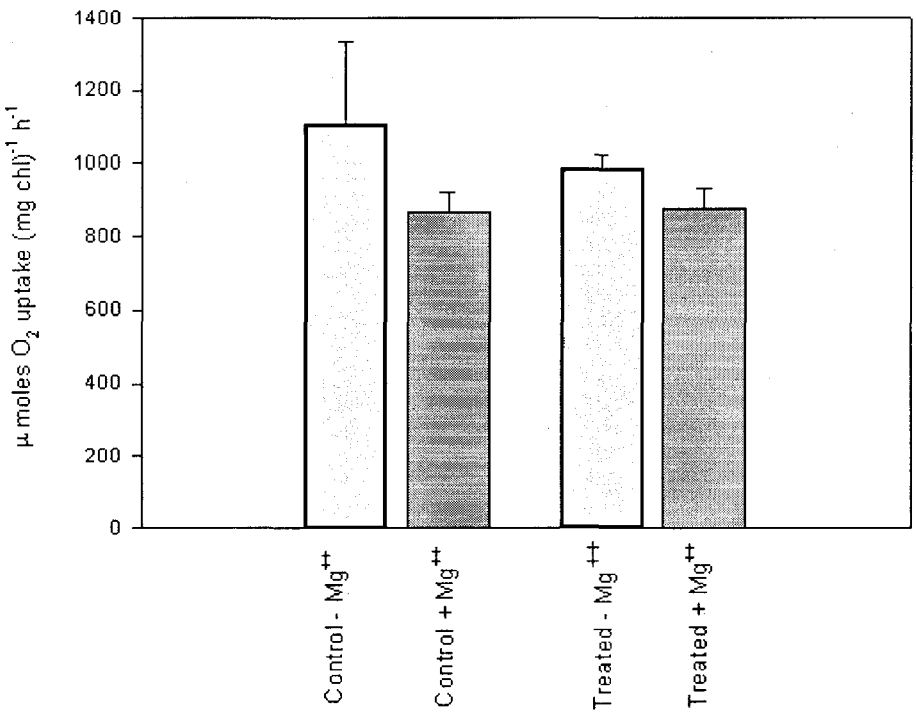


Fig 15. PSI mediated electron transport rate with $DCPIPH_2$ as electron donor and MV as electron acceptor in the thylakoids of control (28^0 C) and heat-stressed rice seedlings (45^0 C). 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C and 45^0 C for a period of 72h. After 72h PSI electron transport rate of the harvested leaves of control and heat-stressed rice was measured in presence and absence of Mg^{++} as described in material and methods. Each data is average of three replicates.

45^0 C even after 24 h of greening though Fm increased gradually with the increasing period of greening. As compared to the control, after 72 h of greening there was a 41% and 45.3% decrease in the Fm of the seedlings grown at 35^0 C and 45^0 C respectively, whereas the Fm of the seedlings subjected to 40^0 C was almost equal to that of the control.

Room Temperature Spectra

6-d-old etiolated control and heat-stressed rice seedlings of CSR 10 were transferred to 28^0 C (control) and 45^0 C (heat-stress) under cool white fluorescent incandescent light (80 μmol m^{-2} s^{-1}) as described in material and methods. Measurements were done after 72 hours of greening in the control and heat-stressed seedlings.

The room temperature spectra of the leaves of control (28^0 C) and treated (45^0 C) seedlings is shown in Fig 22. The leaves of the control (28^0 C) and treated (45^0 C) seedlings were taken after 72 h of illumination and processed as described in material and methods. As seen in the Fig. 19 and 20, Mg^{++} induced increase in fluorescence was higher in case of the control (28^0 C) seedlings as compared to that of the heat-stressed (45^0 C) samples.

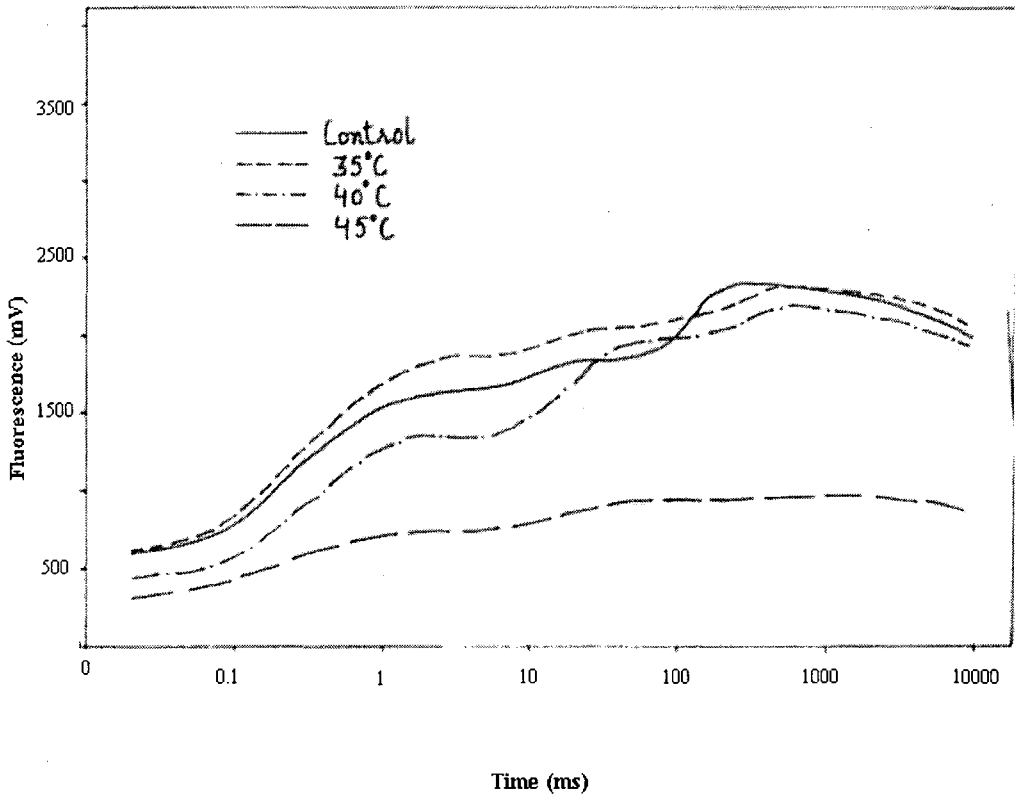


Fig 16. Chlorophyll a fluorescence of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PEA after 24 h at 2500μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 25^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

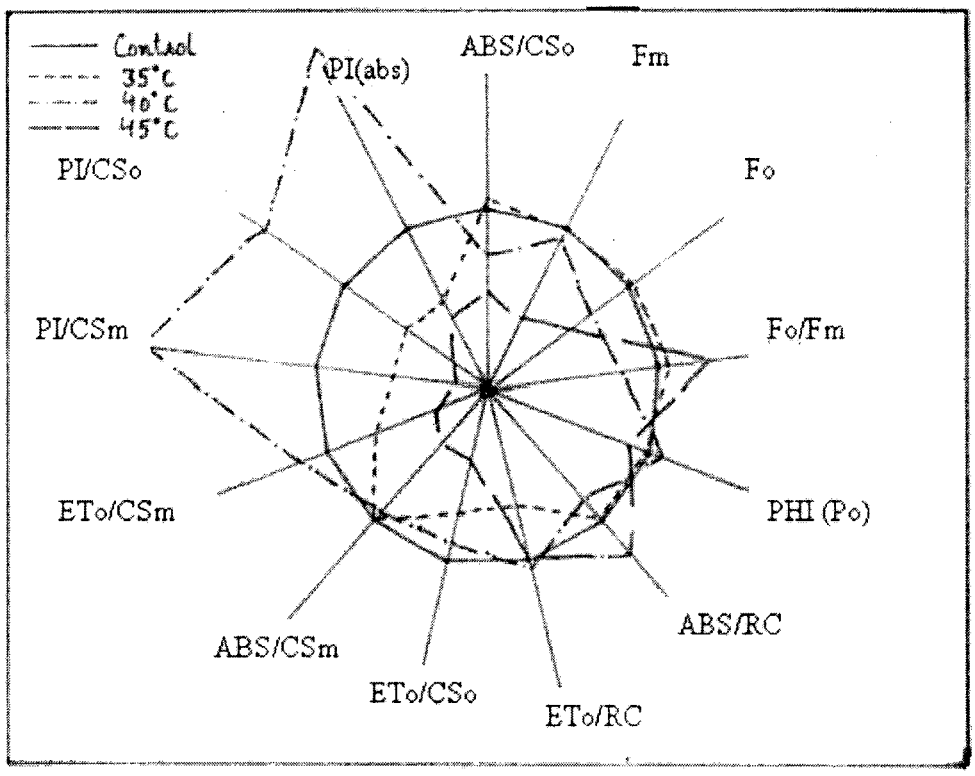


Fig 17. Radar plot of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C and 45^0 C) as measured by PEA after 24 h at 2500μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

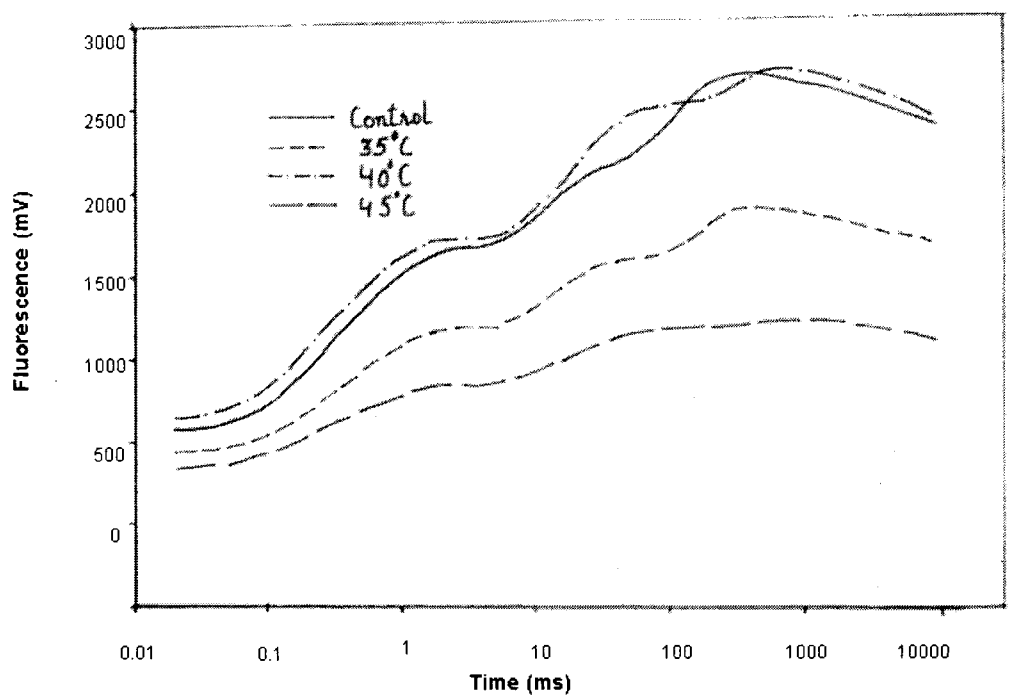


Fig 18. Chlorophyll a fluorescence of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PEA after 48 h at 2500μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 25^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

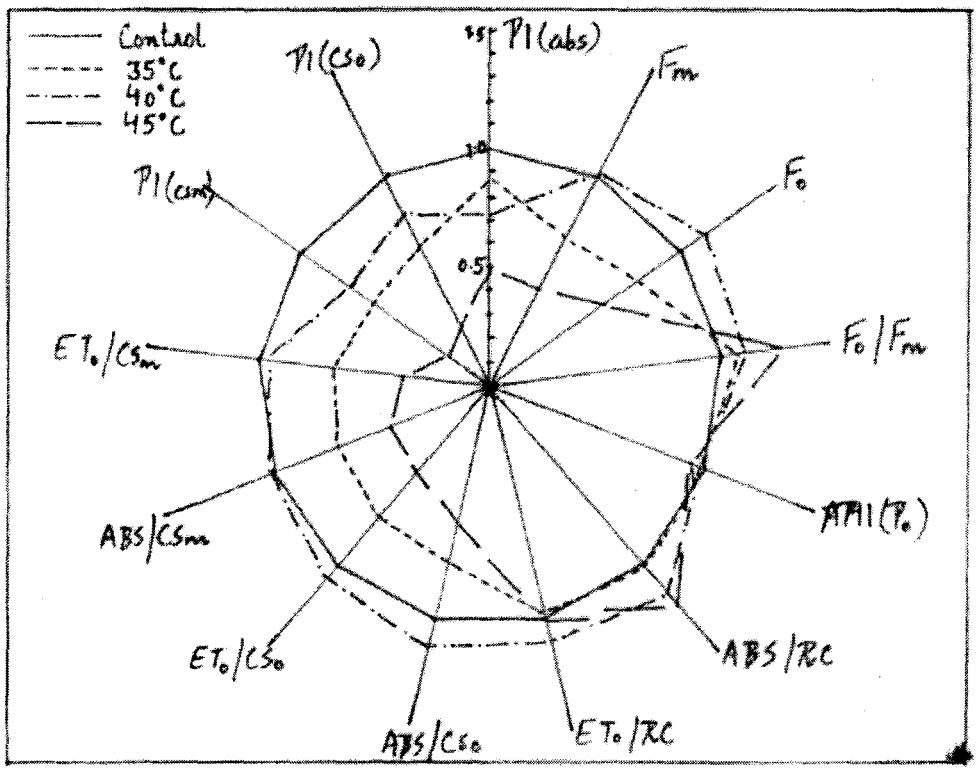


Fig 19. Radar plot of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C and 45^0 C) as measured by PEA after 48 h at 2500μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

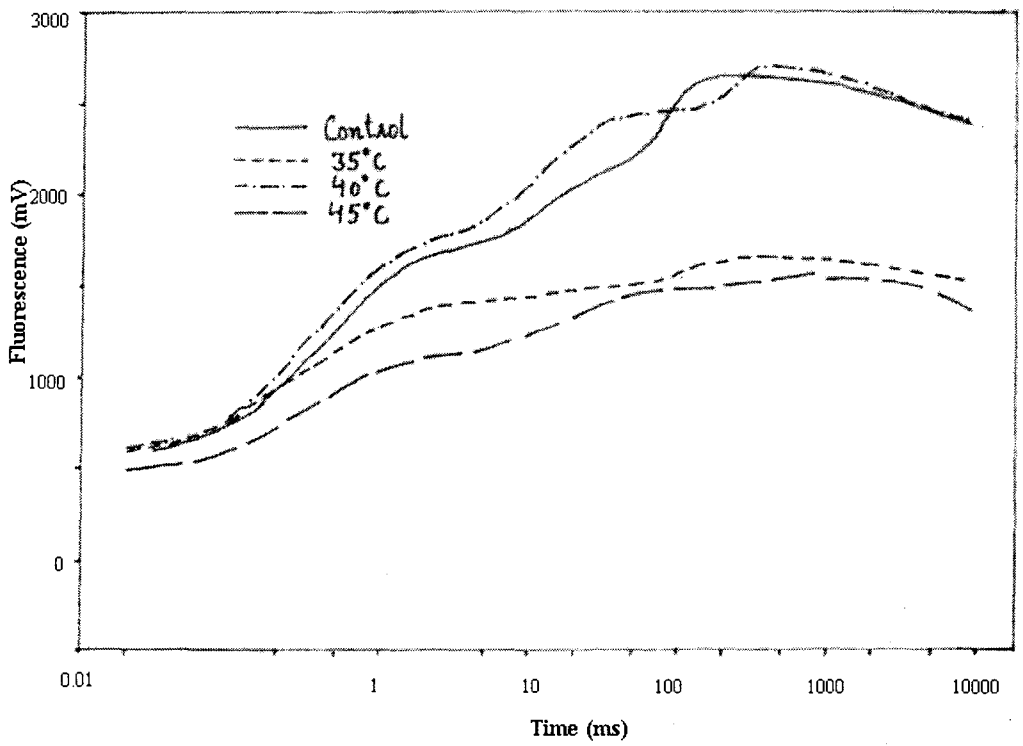


Fig 20. Chlorophyll a fluorescence of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C, 45^0 C) as measured by PEA after **72 h at 2500**μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 25^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

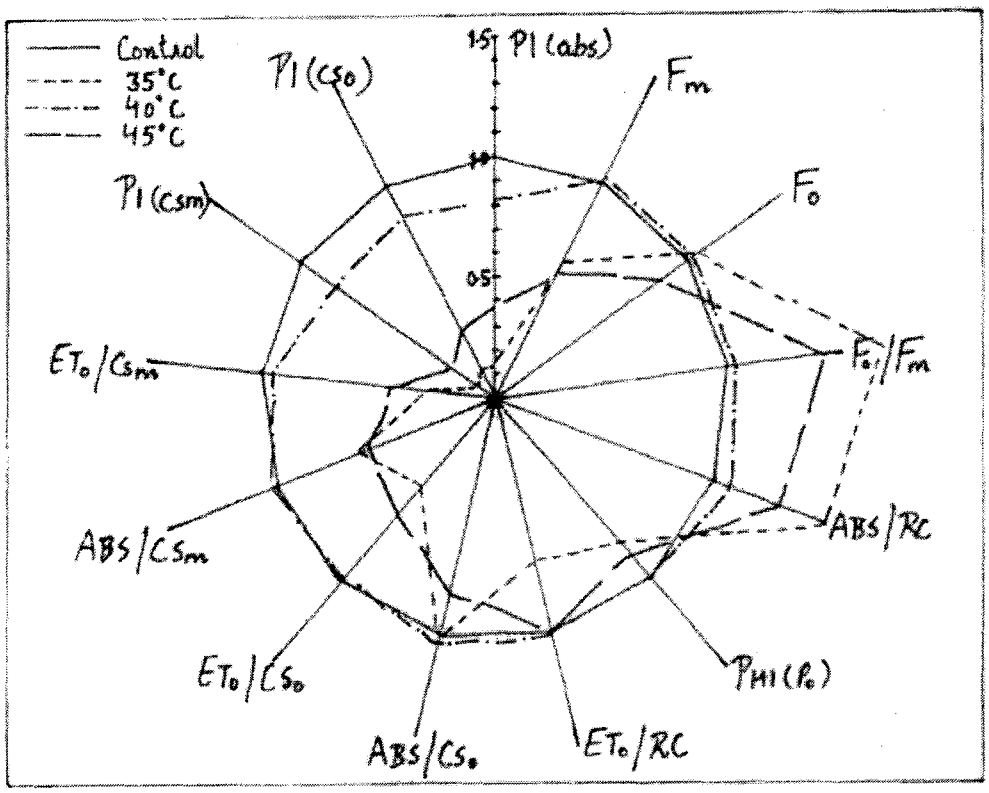


Fig 21. Radar plot of control (28^0 C) and heat-stressed rice seedlings (35^0 C, 40^0 C and 45^0 C) as measured by PEA after 48 h at 2500μ moles m^{-2} s^{-1} of light intensity. 6-d-old etiolated rice seedlings grown at 28^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 28^0 C, 35^0 C, 40^0 C and 45^0 C for a period of 72h.

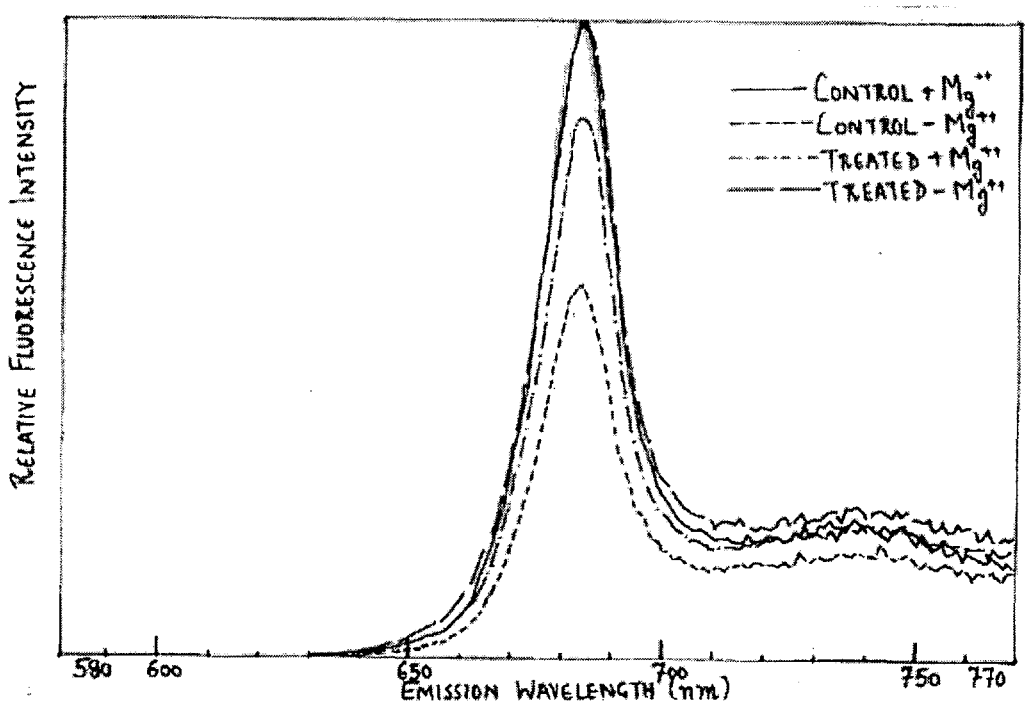


Fig 22. Room temperature fluorescence emission spectra (E440) of thylakoid membranes in presence and absence of Mg^{++} isolated from control (28^0 C) and treated (45^0 C) rice seedlings exposed to light for 72 h. 6-d-old etiolated rice seedlings grown at 25^0 C were transferred to continuous cool white fluorescent light (80 μ moles m^{-2} s^{-1}) at 45^0 C for a period of 72h. Spectra were recorded at excitation and emission slit widths of 4 nm. Other experimental details are as in material and methods.

DISCUSSION

Two cultivars of rice (*Oryza sativa* L.), CSR10 and Pusa Basmati (PB1) were taken for study. 6-d-old etiolated control and heat-stressed rice seedlings of two cultivars were subjected to heat-stress with cool white fluorescent incandescent light (80 μmol m^{-2} s^{-1}). CSR10 and PB 1 both accumulate more chlorophyll than the heat-stressed seedlings. Growth of seedlings is reduced with increase in temperature in both the varieties as shown in Fig 1 (Pollack and Davies, 1970; Rademascher and Feierabend, 1976).Seedlings shown in figure are after 72 hours of greening .

Total chlorophyll content increases with increase of greening period in both the varieties. CSR10 shows its maximum increase in 48 hours of greening and there is almost no further increase (Fig 2). PB1 shows increase in chlorophyll content up to 72 hours of greening (Fig 3). Total chlorophyll content is more in CSR10 than PB1 at same time of greening. Chlorophyll content decreases with increase in temperature in both the cultivars as compared to control. However, CSR10 shows relatively less decrease. In 45^0 C, % chlorophyll of control is much more in CSR10 than PB1 (Fig 2 and Fig 3). So CSR10 variety is more tolerant to heat-stress than PB1.

Chlorophyll biogenesis and photosynthesis are complementary to each other. Photosynthesis is highly affected by heat-stress (Berry and Bjorkman, 1980; Armond *et al*, 2000). Photosystem II (PSII) is a good indicator of photosynthesis. So the changes in PSII ultimately affect photosynthesis (Baker, 1991).

The F_0 values increased with the increasing hours of greening in the experiments done with PEA. There are reports that F_0 increases when the plants are subjected to heat-stress (Schreiber and Berry, 1977; Georgieva and Yordanov, 1993). But as compared to the control, there was a decrease in the F_0 values in the experiments done with PEA as well as PAM. The rise was most prominent in case of the seedlings grown at 45^0 C with the. The F_v/F_m values decreased in the heat-stressed seedlings as compared to the control (Salvucci and Crafts-Brandner, 2004).

Electron transport rate of the CSR 10 variety increases with increase in PAR (μmol photons m^{-2} s^{-1}). Also ETR of the heat-stressed seedlings decreases as compared to control with increase in temperature and greening period (Fig 6). Percent ETR of heat-stressed seedlings was found to be minimum in seedlings grown at 45^0 C at different hours of greening (Guo *et al*, 2003).

Quantum yield of PSII is affected by heat-stress. Quantum yield of photosystem II of CSR10 decreases with increase in PAR values and hours of greening. Also the yield of all the seedlings decreases as compared to control with increase in temperature, hours of greening and PAR values. Percent yield of control is minimum in seedlings grown at 45^0 C after 72 hours of greening period (Fig). Photochemical quenching of CSR10 decreases with increase in greening hours and

PAR (μmol m^{-2} s^{-1}) (Fig 8). Also the photochemical quenching (qP) decreases with increase in temperature as compared to control (Georgieva and Yordanov, 1994). Relative decrease in % qP of control in heat-stress is minimum in seedlings grown at 45^0 C after 72 hours of greening (Fig 8).

Non-photochemical quenching (NPQ) of CSR10 increases with increase in Greening period and PAR (μmol m^{-2} s^{-1}) values (Fig 9). But with increase in heat-stress NPQ decreases as comparative to control. The % control NPQ of heat-stressed seedlings was found to be minimum in seedlings grown at 45^0 C at different hours of greening (Georgieva and Yordanov, 1994).

WC, PSII and PSI activities are also affected by heat-stress (Bukhov *et al*, 2001). WC oxygen uptake activity decreases gradually with increasing temperature with the maximum reduction occurring in the seedlings grown at 45^0 C. The same trend was repeated in case of PS II oxygen evolution. After 72 h the activity decreased by 84% in the seedlings grown at 45^0 C (Pastenes and Horton, 1996a; Bukhov and Mohanty, 1999; Yamane *et al.*, 1998; Yamane *et al.*, 2000; Vani *et al*, 2001). The PS I activity did not show a specific trend. While there was a decrease in the PS I activity in the seedlings grown at 35^0 C, 40^0 C, the seedlings grown at 45^0 C experienced a 39.4% increase (Pearcy *et al.*, 1977; Sayed *et al.*, 1989; Havaux, 1993; Pastenes and Horton, 2002).

To understand the grana stacking of control (28^0 C) and treated (45^0 C) plants, thylakoid membranes were suspended in low salt medium to unstack the grana to individual membranes. Both PS I and PS II are uniformly distributed in the thylakoid membrane and LHCP II distribute energy almost equally to both PS II and PS I. After addition of magnesium ions, thylakoids stack and form grana which consequently results in non uniform distribution of PS II and PS I in thylakoid membrane where LHCP II remains close to PS II and transfers its energy to PS II (Mohanty *et al*, 2002).

In the presence of Mg^{++}, increase in the PS II activity was greater in the control (28^0 C) seedlings than the treated (45^0 C) samples (Fig. 14) and PS I activity decreased more in control (28^0 C) than in the treated seedlings (45^0 C, Fig. 15).

Upon sudden illumination of a dark-adapted leaf, PS II fluorescence yield increases following a triphasic kinetics (O-J, J-I, I-P, Strasser *et al*; 1995). The three phases have been interpreted as follows.

The (O-J) phase corresponds to a complete reduction of the primary electron acceptor QA of PS II, the release of fluorescence quenching during the (J-I) phase is controlled by the PS II donor side (water splitting activity) and the third phase (I-P) corresponds to the release of fluorescence quenching by the oxidized plastoquinone pool (Neubauer and Schreiber, 1987).

Chlorophyll fluorescence is a good measure of the photosynthetic activities (Govindjee and Satoh, 1980; Krause and Weis, 1981; Govindjee, 1995). An increase in the F_0 level has been reported in response to the increasing temperature (Schreiber and Berry, 1977; Georgieva and Yordanov, 1993). Schreiber and Armond (1978) suggested that perturbation of the thylakoid membranes lead to the separation of LHC II from the PS II core complex and a block of PS II reaction center, which results in the F_m decrease and F_0 increase.

Both the values of Fm and F_0 decreased in the seedlings grown at 45^0 C as compared to the control. There was a gradual F_m and F_0 increase in the seedlings grown at 45^0 C with the increasing hours of greening (Fig. 16, 17, 18, 19, 20 and

21). Also J-I phase of the seedlings grown at 45^0 C indicates a decrease in the efficiency of the water-splitting of the seedlings.

In the room temperature spectrum, Mg^{++} induced increase in fluorescence was higher for the control (28^0 C) seedlings than that of heat-stressed (45^0 C) samples (Fig.22).

SUMMARY

Heat stress has a great impact on chloroplast biogenesis. It reduces photosynthesis in plants. Various photochemical parameters are reported to change with the plant genotype, the system used and the time period of the stress imposed.

In the present investigation, effect of heat stress on the chloroplast biogenesis and photosynthetic process has been studied. 6-d-day old etiolated seedlings of CSR 10 and PB 1 were taken. In CSR 10 the control was transferred to 28^0 C whereas the other sets of the seedlings were transferred to 35^0 C, 40^0 C and 45^0 C (heat-stress). In PB 1 the control was transferred to 25^0 C whereas the other sets of the seedlings were transferred to 42^0 C and 50^0 C (heat-stress). Total Chlorophyll content decreased in the heat-stressed seedlings in both the varieties. CSR 10 accumulates more chlorophyll than in PB 1 both in the control as well as in the heat-stressed seedlings.

The F_0 values were measured by PEA and PAM. In both the cases different trends in the value of F_0 were observed. F_v / F_m values decreased with the increasing temperature and hours of greening.

Photochemical parameters like electron transport, quantum yield of PS II, photochemical quenching and non-photochemical quenching also changed with the heat-stress, greening period and PAR (μmol m^{-2} s^{-1}). All the four parameters decreased with the increasing temperature and hours of greening.

WC oxygen uptake and PS II oxygen evolution decreased with the increasing temperature whereas there was an increase in the PS I oxygen uptake activity in the seedlings grown at temperature 45^0 C as compared to the control.

In the presence of Mg^{++}, increase in the PS II activity was greater in the control (28^0 C) seedlings than in the treated (45^0 C) samples. PS I activity decreased more in control (28^0 C) than in the treated seedlings (45^0 C).

Both the values of Fm and F_0 decreased in the seedlings grown at 45^0 C as compared to the control. There was a gradual F_m increase and F_0 increase in the seedlings grown at 45^0 C with the increasing hours of greening.

In the room temperature spectrum, Mg^{++} induced increase in fluorescence was higher for the control (28^0 C) seedlings than that of heat-stressed (45^0 C) samples.

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