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PROTECTIVE ROLE OF LIGHT IN HEAT-INDUCED INHIBITION OF PHOTOSYNTHESIS IN ISOLATED CHLOROPLASTS

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Abstract – The effect of heat treatment in the light on the subsequent CO_2 fixation was studied with isolated spinach chloroplasts to define the role of light during heat stress. The degree of inhibition in CO_2 fixation after heat treatment at 35°C under full light intensity (600 W/m^2) was same as that in the dark. However, heat treatment of isolated chloroplasts in the light manifested thylakoidal damage, which did not occur in the dark. Under weak light (10-30 W/m^2) where no thylakoidal damage occurred, the inhibition was substantially alleviated, showing protective effect of light. The inhibition caused by heat treatment in the dark or light is prevented by the addition of a few combined compounds to the medium prior to treatment. Fructose-1,6-bisphosphate (with aldolase) and ribose-5-phosphate, known to be effective combined with oxaloacetate in preventing inhibition after heat treatment in the dark were equally effective in the light even without oxaloacetate. Addition of sugar phosphate reduces the Mehler reaction, which may occur in fast rate under high light. However, the addition of bicarbonate and catalase that would remove Mehler reaction did not provide any protection, indicating that protective role of sugar phosphate is elsewhere. Furthermore, in whole plants rapid recovery from heat stress was observed in the light. The apparently lesser or equal inhibition in spite of additional thylakoidal damage under heat stress in the light and less requirement for the protection against heat treatment suggest that the inhibitory effect of heat stress is alleviated by light treatment.

INTRODUCTION

Effects of various environmental stresses on photosynthesis have been studied under various conditions in many plants.[1-3] Although plants mostly undergo stresses in the light in natural conditions, studies were frequently done in a condition without light to avoid complications. Light is essential for the daily life of plants, but it can be hazardous to plants when excessive, which is manifested as photoinhibition. It results from the absorbed light more than being able to be dissipated by the electron transport chain, leading mostly to specific lesions to photosystem II.[2,4] Under stressed condition plants are more liable to photoinhibition since they lose some of their abilities to utilize all the incoming light fully.[5] It is well known that water stress or chilling stress is more devastating to plants in the light than in the dark due to the additional photoinhibitory effect. However, under heat-stressed condition light plays a different role that still needs to be defined.

In isolated pea and spinach chloroplasts, light harmed the electron transport chain and caused bleaching in photosynthetic pigments at high temperature.[6,7] Light intensified thermal injury of *in vivo* photosynthesis in wheat and Siratro (*Macroptilium atropurpureum*) leaves experiencing other stress.[8,9] In contrast, light was shown to provide protective effect by relieving the heat-induced inhibition of photosynthesis in both isolated chloroplasts and whole plants.[5,10,11] Heating under low light intensity (40 W/m^2) resulted in lesser inhibition of CO_2 fixation in isolated spinach chloroplasts.[11] With pea leaves, it was shown that light offered a full protection of PS II against thermal inactivation up to a medium level (100 W/m^2) of light intensity.[12] Effect of heat-stress in the dark on the photosynthesis of isolated chloroplasts was also studied by several groups revealing thermal inhibition in photosynthesis.[13-16] Fu and Gibbs reported that heat treatment of isolated spinach chloroplasts resulted in the decrease of photosynthetic activity without thermal damage to thylakoidal function.[16]

In this report, results on the effect of high temperature in the light on the subsequent photosynthesis are presented. We show that thermal lesions to chloroplastic function in the light are different from those in the dark. In addition, we present some evidences that light plays a protective role against thermal damage.

MATERIALS AND METHODS

Plant material and chloroplast isolation. Spinach (*Spinacia oleracea* L. var. Long Standing Bloomsdale) seeds were germinated and grown on pots filled with a mixture of vermiculite and soil in a ratio of 2:1 in a controlled environment chamber for 8

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† *Abbreviations:* Chl, chlorophyll; FBP, fructose-1,6-bisphosphate; FeCN, ferricyanide; IRGA, infra red gas analyzer; OAA, oxaloacetic acid; PGA, 3-phosphoglyceric acid; Pi, inorganic phosphate; R5P, ribose-5-phosphate.

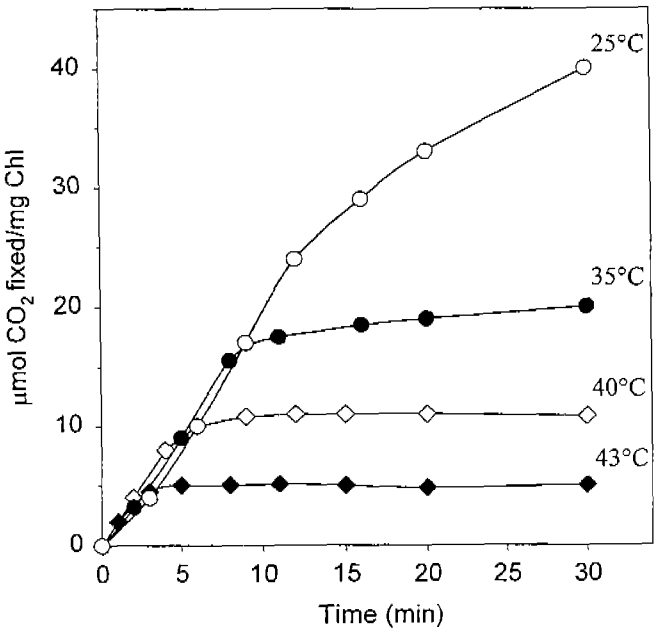

Figure 1. Time course of CO_2 fixation by isolated chloroplasts at optimal (25°C) and superoptimal temperatures (≥ 35°C). CO_2 fixation was performed at the indicated temperature from the starting point.

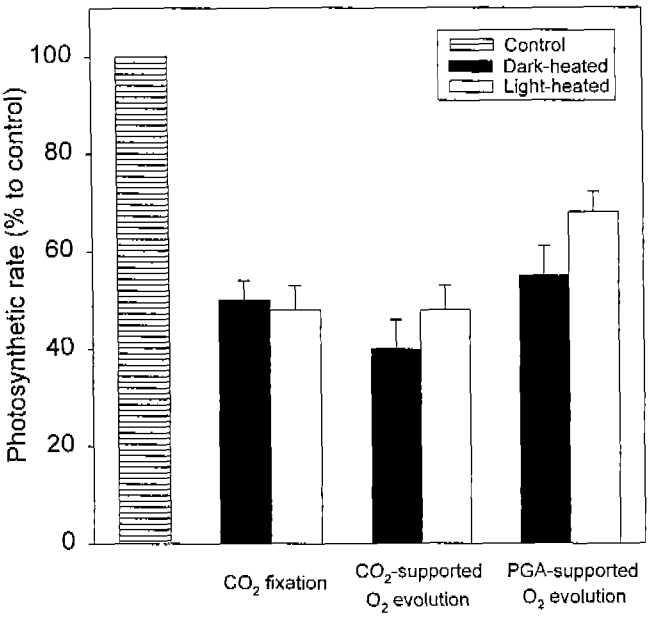

Figure 2. Effect of heat treatment (35°C, 10 min) on the subsequent photosynthetic rate. Measurement of photosynthesis was done at 25°C after heat treatment. The control rate for CO_2 fixation, CO_2-supported O_2 evolution and PGA-supported O_2 evolution was 152-191, 69-77, and 55-76 μmol/mg Chl·h, respectively. The experiment was repeated at least 3 times.

to 10 weeks until maturity. Chloroplasts were isolated as described before.[18] Deribbed leaves (about 10 g fresh weight) were homogenized shortly on ice in 50 ml of prechilled grinding medium containing 50 m*M* Hepes NaOH (pH 6.8), 0.33 *M* sorbitol, 2 m*M* Na_2EDTA, 1 m*M* $MgCl_2$ and 1 m*M* $MnCl_2$. The resulting homogenate was filtered through 4 layers of cheesecloth and 2 layers of Miracloth (Calbiochem, Inc, U.S.A). After centrifugation for 50 s at 750 g, the supernatant was discarded and the pellet was resuspended in 25 mL of grinding medium. The resuspended material was layered onto 15 mL of a 40% Percoll mixture which has the same composition as grinding medium, and was centrifuged in a swinging bucket for 2.5 min at 2,500 g. The resulting pellet was resuspended with reaction buffer containing 50 m*M* Tricine-NaOH (pH 8.1), 0.33 *M* sorbitol, 2 m*M* Na_2EDTA, 1 m*M* $MgCl_2$ and 1 m*M* $MnCl_2$ and Chl concentration was adjusted to be around 1 mg/mL. Intactness of isolated chloroplasts was routinely over 90% by FeCN assay. Chl concentration was determined according to Arnon.[17]

Heat treatment of chloroplasts. Isolated chloroplasts resuspended in reaction mixture were kept on ice until the initiation of the experiment, then divided into separate tubes for different treatments. When other chemicals were added before the onset of heat treatment, the additional volume was adjusted by adding the same amount of reaction buffer. Heat treatment was initiated by transferring each tube into the water chamber that was maintained at the desired temperature and terminated by applying it to ice. Subsequent measurements were performed immediately.

Measurement of $^{14}CO_2$ fixation. CO_2 fixation was measured by adding intact chloroplasts (around 50 μg Chl) to a 1 mL reaction mixture containing 50 m*M* Tricine NaOH (pH 8.1), 0.33 *M* sorbitol, 2 m*M* Na_2EDTA, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 0.25 m*M* KH_2PO_4, 10 m*M* NaH $^{14}CO_3$ (1 μCi/μmol), and 1,000 units of catalase. The reaction was done at 25°C and initiated by turning on the light at the intensity of 600 W/m^2. Radioactivity was determined using an aliquot of 50 μL taken from each reaction tube and mixed with 100 μL of 0.5 N HCl at the specified time.

Measurement of Photosynthetic O_2 Evolution. Photosynthetic O_2 evolution was carried out in a 1 mL reaction buffer containing 50 m*M* Tricine-NaOH (pH 8.1), 0.33 *M* sorbitol, 2 m*M* Na_2EDTA, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$ and 0.25 m*M* KH_2PO_4 with intact chloroplasts (containing about 30 μg Chl), using either 10 m*M* $NaHCO_3$ or 5 m*M* PGA as an electron acceptor. O_2 evolved was measured polarographically with a Clark type electrode at 25°C. Light (400 W/m^2) was provided from a slide projector filtered through a heat filter of water containing $CuSO_4$.

Reconstituted chloroplast system. The reconstituted chloroplast system was prepared as described before.[18] The intact chloroplast pellet, after Percoll cushion, was osmotically broken by adding reaction buffer containing 50 m*M* Tricine-NaOH (pH 8.1), 10 m*M* DTT (dithiothreitol), 2 m*M* Na_2EDTA, 1 m*M* $MgCl_2$, and 1 m*M* $MnCl_2$. The supernatant fraction, after centrifugation at 12,000 g for 10 min in a swinging bucket, was used as the stromal fraction, and the remaining pellet was used as the thylakoidal fraction. CO_2 fixation of reconstituted chloroplasts was performed by adding thylakoids (30 μg Chl) and 0.5 ml of the stromal fraction to a reaction mixture containing 50 m*M* Tricine-NaOH (pH 8.1), 5 m*M* $MgCl_2$, 0.2 m*M* ADP, 1 m*M* NADP, 2 m*M* PGA, 15 μ*M* spinach Fd (ferredoxin), 10 m*M* NaH$^{14}CO_3$ (1 μCi/μmol), 0.25 m*M* KH_2PO_4, and 1,000 units of catalase.

Measurement of Leaf Photosynthesis. Leaf photosynthesis was measured by CO_2 uptake in photosynthetic gas exchange

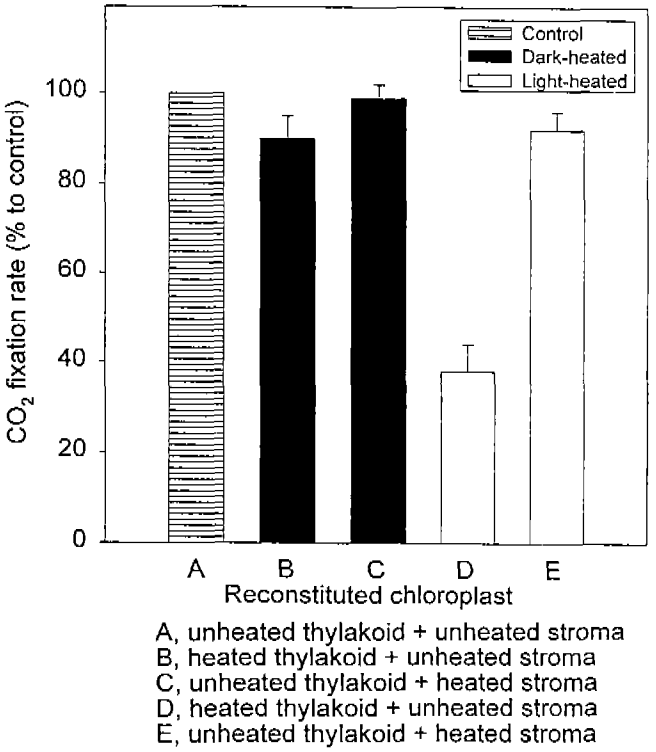

Figure 3. CO_2 fixation by reconstituted chloroplasts made of thylakoids and stroma isolated from heated (35°C, 10 min) or unheated chloroplasts. CO_2 fixation was performed at 25°C. The control rate was 68-85 μmol/mg Chl·h. The experiment was repeated 3 times.

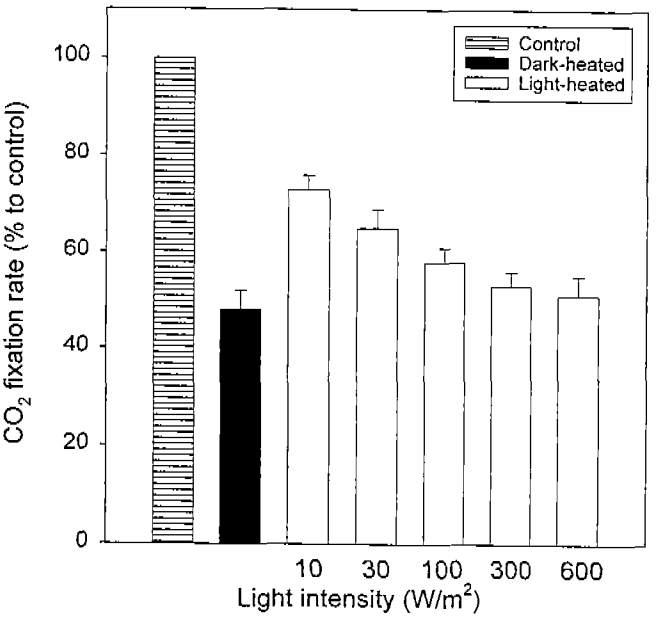

Figure 4. CO_2 fixation by isolated chloroplasts after heat treatment (35°C, 10 min) under various light conditions. CO_2 fixation was performed at 25°C. The control rate was 129-153 μmol/mg Chl·h. The experiment was repeated 3 times.

using an ADC type 225 Mk.III IRGA as described by Harris *et.al.*[19] Randomly selected leaves were taken near the bottom of the spinach stem with a razor blade. The upper part of the leaf was attached to the wall of the leaf chamber with Scotch tape and the rest was tightly sealed into the chamber by placing two halves of the chamber together with wing nuts while the stem of the leaf was immersed into water. Gas was provided via tanks of 1,000 μl/L CO_2, 20% O_2 and balance N_2, and was humidified by passage through two wash bottles containing water at room temperature. The CO_2-IRGA was calibrated from standard CO_2 tanks and H_2O-IRGA was calibrated by means of an ADC water vapor generator. Leaf temperature was measured by a fine thermocouple placed against the leaf in the gas chamber, which was connected to a recorder. Light was provided from a slide projector at the intensity of 400 W/m^2. CO_2 and water vapor exchange was monitored until they reached a maximal steady-state.

RESULTS AND DISCUSSION

Effect of heat treatment in the light

The time course of CO_2 fixation at optimal temperature (25°C) with isolated chloroplasts typically follows three stages: the lag, the linear and the late phase.[20] Depending on the physiological status of chloroplasts, the rate and duration of each phase are changed. The late phase arrives with lesion in chloroplastic function, mostly with thylakoidal damage.[20] At superoptimal temperatures (≥ 35°C), the lag period disappears and the linear phase is shortened progressively with no change in the rate as temperature goes up (Fig. 1). After cessation of the linear phase chloroplasts were shown to undergo irreversible damage since there was little CO_2 fixation even if chloroplasts after this point were returned to optimal temperature.[21] This point of "no return" is about the 10th min at 35°C, the 4-5th min at 40°C, and the 2-3rd min at 43°C (Fig. 1). Therefore, it can be said that the onset in malfunctioning of chloroplasts is accelerated at higher temperature due to thermal damage in the light.

The heat treatment of isolated chloroplasts in the dark at 35°C for 10 min at pH 8.1 was shown to result in an approximate 50% reduction in subsequent CO_2 fixation without any loss in thylakoidal function.[16] The inhibitory effect of heat treatment was magnified as the temperature and time became higher and longer or the pH in the medium was lowered with concomitant damage to thylakoids.[16,18] As shown in Fig. 2, heat treatment of isolated chloroplasts in the light at 35°C for 10 min at pH 8.1 resulted in comparable inhibition in subsequent CO_2 fixation or CO_2-supported O_2 evolution to that in the dark. Apparently, the inhibitory effect of heat treatment at 35°C seemed to be same regardless of light although lesser inhibition is observed in PGA-supported O_2 evolution (Fig. 2).

The isolated chloroplasts tend to lose their activity gradually during photosynthesis even at optimal temperature due to the loss in thylakoidal function.[20] In view of this, the thylakoidal function was tested after heat

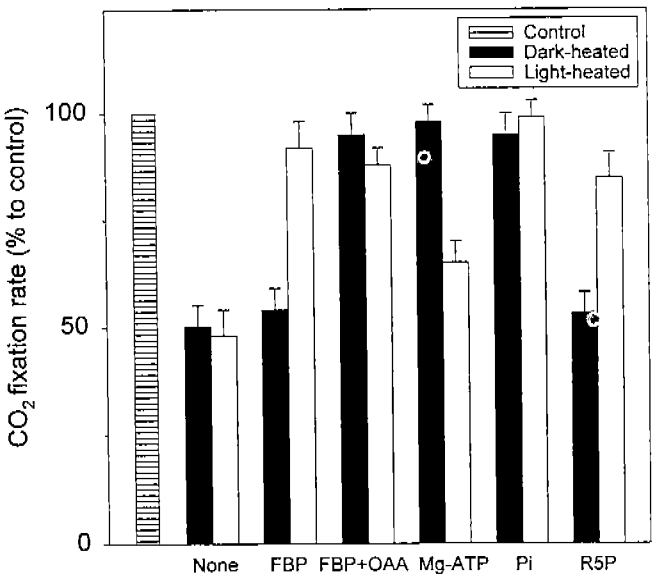

Figure 5. CO_2 fixation by isolated chloroplast after heat treatment (35°C, 10 min) in the presence of 5 mM FBP, R5P, Mg-ATP, FBP and OAA, or 10 mM Pi. These compounds were added prior to heat treatment and CO_2 fixation was performed at 25°C. The control rate was 178-242 μmol/mg Chl·h. The experiment was repeated at least 3 times.

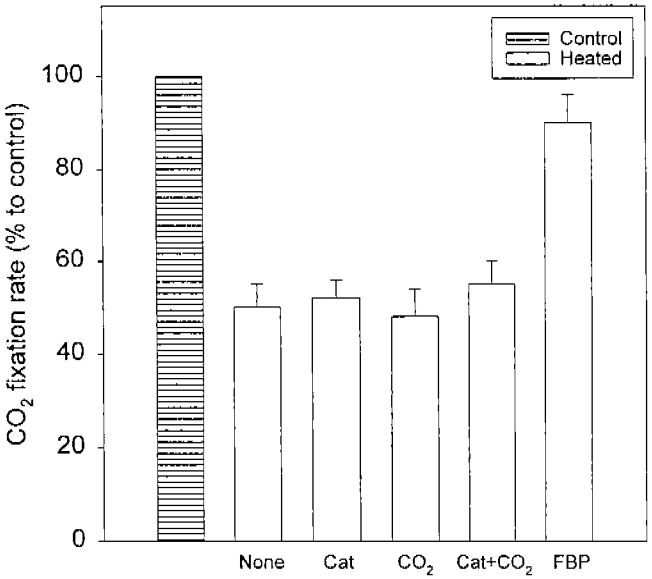

Figure 6. CO_2 fixation by isolated chloroplast after heat treatment (35°C, 10 min) in the presence of CO_2 (10 m*M* bicarbonate), Cat (1,000 U of catalase) or both which were added prior to heat treatment. CO_2 fixation was performed at 25°C. The control rate was 190-217 μmol/mg Chl·h The experiment was repeated at least 3 times.

treatment in the light by the reconstituted chloroplast system. Heat treatment in the light elicited thylakoidal damage, which did not occur after heat treatment in the dark (Fig. 3). With additional thylakoidal damage, it is expected of higher inhibition in the light than in the dark since it was shown earlier that heat treatment in the dark at 35°C at lower pH resulted in much greater inhibition with resultant thylakoidal damage.[16,18] Presumably, light plays a certain role on reducing the inhibitory effect of heat treatment or affects different sites.

Protective role of light against heat treatment

Thylakoidal damage occurring during heat treatment in the light would be reduced or prevented at lower light intensity as no thylakoidal damage was observed after heat treatment in the dark. Consequently, the effect of heat treatment was compared at varying light intensities. At all light intensities tested, heat treatment in the light showed lesser inhibition than that in the dark (Fig. 4). Especially, at lower light intensities (10 and 30 W/m^2) where thylakoidal damage is not likely to occur, inhibition by heat treatment was significantly reduced exhibiting protective role of light. Weis previously reported the protective effect of light by showing that heating in the light (40 W/m^2) took a higher temperature than that in the dark (40 vs. 35°C) to get 50% inhibition in CO_2 fixation even with thylakoidal malfunction.[11] He attributed the beneficial effect of light to the light-generated alkalization of the stroma. Similarly, protective effect of light was observed up to 100 W/m^2 in pea leaves.[12] How light generates a protective effect during heat-treatment is only speculative. It is possible that stromal alkalization as suggested by Weis is responsible for protection as indicated by the pH-dependence of heat-treatment and disappearance of protective effect of light at lower pH.[16,18] Heat treatment in the dark brought in the decline of ATP and ADP content in the chloroplasts (S-S. Jun, unpublished). Therefore, it is also speculated that supplementation of ATP through photophosphorylation may be the reason. Even the low rate of photophosphorylation at low light intensity may be enough to substitute for the loss of adenine nucleotide during heat-treatment.

Effect of protective agents against heat treatment in the light

Addition of Mg-ATP or some sugar-P in the CO_2 fixation medium changes the pattern of CO_2 fixation favorably.[22-25] FBP (with aldolase), R5P or Mg-ATP was helpful in delaying the loss of photosynthetic activity in the late phase and FBP was more effective at higher temperature (data not shown). The decline in CO_2 fixation of isolated chloroplasts due to heat-treatment was shown to be irreversible.[15] However, the addition of Mg-ATP, Mg-ADP, FBP (with aldolase) plus OAA, or R5P plus OAA prior to heat-treatment prevented the resulting decline in CO_2 fixation of the heated chloroplasts.[16] OAA was proposed to replenish the reduced adenine nucleotide content with sugar phosphate by chloroplast respiration.[16] Mourioux and Douce reported that addition of Pi (10 m*M*) to the suspending medium stabilized the gradual loss in the rate of CO_2- dependent O_2 evolution in spinach chloroplasts.[26]

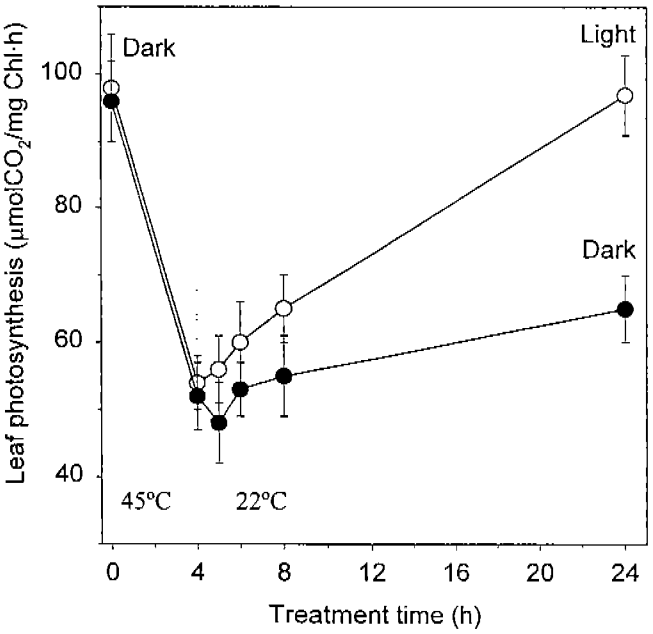

Figure 7. Leaf photosynthesis after heat treatment and during recovery. Heat treatment was done at 45°C in the dark and recovery was allowed at 22°C in the dark or light. Leaf photosynthesis was measured at 25°C using IRGA. The experiment was repeated at least 3 times.

To this end, we tested the effectiveness of several compounds known to be beneficial to chloroplasts for protection against heat treatment in the light or in the dark. As shown in Fig. 5, the effectiveness of some compounds was not identical in the light and dark. All compounds but Mg-ATP tested are more effective or at least equally effective in the light than in the dark. Moreover, while OAA is required with FBP or R5P for the protection in the dark, it is dispensable in the light. The degree of protection is concentration-dependent, saturating at 5 m*M* (data not shown). The effectiveness of FBP or R5P may come from the suppression of Mehler reaction occurring excessively under high light.[27-29] In a similar study, Barenyi and Krause reported more than 50% reduction in CO_2 fixation and electron transport after 10 min incubation at 20°C in the light (850 W/m^2) with protective effect of bicarbonate.[30] However, inclusion of bicarbonate (10 m*M* $NaHCO_3$) and catalase in the medium did not give enough protection as FBP (Fig. 6) ruling out the above proposition. Pi provides 85-100% protection in the dark, but always-perfect protection in the light. Requirement of lesser elements and higher effectiveness for the protection in the light suggest that light is indeed beneficial rather than harmful during heat treatment in the light.

Faster recovery in the light

The inhibitory effect of heat treatment also occurred in whole plants, but only above 45°C. As shown in Fig. 7, incubation of whole plants at 45°C for 4 h resulted in approximately 50% reduction in leaf photosynthesis. However, in contrast to isolated chloroplasts where the inhibitory effect of heat treatment was not reversible, whole plants were gradually recovered from the thermal inhibition once they were moved to a normal temperature whether in the dark or light, but recovery was faster in the light. After a day of recovery period, leaf photosynthesis was returned to normal level in the light, but only 60-70% of normal in the dark (Fig. 7). In the dark, it took several days for the complete recovery (data not shown). The accelerated recovery in the light provides an additional evidence for the beneficial role of light under high temperature stress.

CONCLUSIONS

In the present report, we have tried to define the role of light under the heat-stressed condition. Mostly light exacerbates various stress elicitors including drought and chilling. Contrastingly, we present here three lines of evidences to support that light alleviates the inhibitory effect of heat-stress on photosynthesis: First, the heat-induced inhibition of CO_2 fixation is significantly alleviated in the weak light. Secondly, the components for protection are less (no requirement for OAA) in spite of additional thylakoidal damage. Finally, faster recovery from thermal inhibition is occurred in the light than in the dark in whole plants.

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