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Mulberry leaf metabolism under high temperature stress

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Abstract

Effects of high temperature on the activity of photosynthetic enzymes and leaf proteins were studied in mulberry (*Morus alba* L. cv. BC2-59). A series of experiments were conducted at regular intervals (120, 240 and 360 min) to characterize changes in activities of ribulose-1,5-bisphosphate carboxylase (RuBPC) and sucrose phosphate synthase (SPS), photosystem 2 (PS 2) activity, chlorophyll (Chl), carotenoid (Car), starch, sucrose (Suc), amino acid, free proline, protein and nucleic acid contents in leaves under high temperature (40 °C) treatments. High temperature markedly reduced the activities of RuBPC and SPS in leaf extracts. Chl content and PS 2 activity in isolated chloroplasts were also affected by high temperature, particularly over 360 min treatment. Increased leaf temperature affected sugar metabolism through reductions in leaf starch content and sucrose-starch balance. While total soluble protein content decreased under heat, total amino acid content increased. Proline accumulation (1.5-fold) was noticed in high temperature-stressed leaves. A reduction in the contents of foliar nitrogen and nucleic acids (DNA and RNA) was also noticed. SDS-PAGE protein profile showed few additional proteins (68 and 85 kDa) in mulberry plants under heat stress compared to control plants. Our results clearly suggest that mulberry plants are very sensitive to high temperature with particular reference to the photosynthetic carbon metabolism.

Additional key words: chlorophyll, *Morus alba*, nucleic acids, photosynthesis, photosystem 2, proteins, RuBP carboxylase, sucrose phosphate synthase.

Introduction

Field grown plants are often subjected to fluctuating temperature that has a profound effect on the plant metabolism. Photosynthesis is particularly sensitive to thermal stress and inhibition of photosynthetic carbon dioxide fixation by high temperature has been well documented in many species (for review see Berry and Björkman 1980). Several components of the photosynthetic apparatus and associated metabolic processes are heat labile. Export of photoassimilates is another metabolic process that is sensitive to inhibition by high temperature (Law and Crafts-Bradner 1999). Temperature above 30 °C has been shown to cause disorientation of the lamellar system of chloroplasts (Singla *et al.* 1997). Leaf temperatures above some threshold (40 - 50 °C) have been shown in some plants to cause reductions in the rate of photosynthesis and other metabolic activities (Björkman and Powells 1984, Ludlow and Björkman 1984). Accumulation of sugars and free amino acids like proline, facilitates the recovery of the plant undergoing stress phenomenon (Chauhan *et al.* 1995). Labate and Leegood (1988) have noted the importance of Pi recycling during starch and sucrose synthesis under temperature stress. Also, increase in the activity of the hydrolytic enzymes promote the rate of ion transport under stressful conditions (Singla *et al.* 1997).

Mulberry is one of the most important cash crops in south India. Exposure to elevated temperatures and drought are major abiotic factors influencing growth and

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Abbreviations: DTT - dithiothreitol; G6P - glucose-6-phosphate; F6P - fructose-6-phosphate; HSP - heat shock proteins; Pi - inorganic phosphate; PS 2 - photosystem 2; PVP - polyvinylpyrrolidone; RuBP - ribulosebisphosphate; RuBPCO - RuBP carboxylase/oxygenase; SPS - sucrose phosphate synthase; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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metabolism of the mulberry. We have previously reported the changes in mulberry leaf metabolism in response to water stress (Barathi *et al.* 2001). In the present study, we analysed the influence of high (40 °C) temperature on the physiological state and acclimation ability of mulberry plants, and thus to determine its photosynthetic performance.

Materials and methods

Chemicals: The biochemicals and enzymes were from *Sigma* (St. Louis, USA) and radiochemical $NaH^{14}CO_3$ (specific activity 16.5 kBq μmol^{-1}) was obtained from Bhaba Atomic Research Centre, Mumbai, India. All other reagents were purchased from commercial sources and were of analytical grade.

Plants and high temperature treatments: Mulberry (*Morus alba* L. cv. BC2-59) plants, obtained from Regional Sericultural Research Station, Coonoor, Tamil Nadu, India, were grown in 30-cm pots under natural photoperiod [irradiance (400-700 nm) of 1600 - 1800 $\mu mol\ m^{-2}\ s^{-1}$]. Mean maximum day/night temperature was 30/23 °C and approximate air humidity 60 %. High temperature (40 °C) treatment was given for 120, 240 and 360 min in a plant growth chamber (*Labline*, USA). Young and fully expanded leaves were harvested at regular intervals and used for all the physiological and biochemical measurements.

Chloroplast isolation and estimation of PS 2 activity: Chloroplasts were isolated according to the method of Leegood and Walker (1993). Freshly harvested leaves were illuminated for 15 min to degrade the accumulated foliar starch. 5 g of leaves were cut into strips and homogenized at 4 °C in 15 cm^3 of cold extraction medium which consisted of 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 1 % PVP-40, 0.5 mM DTT and 2 mM sodium ascorbate, pH 6.5. The crude extract was squeezed through two layers of cheesecloth and the filtrate centrifuged at 250 *g* for 5 min to remove cell debris. The supernatant was then centrifuged at 2 500 *g* for 10 min. The pellet was suspended in a cold medium consisting of 0.33 M sorbitol, 2 mM EDTA, 1 mM $MnCl_2$ and 50 mM HEPES (pH 7.6). A portion of this chloroplast preparation was layered on to a sucrose gradient comprising 1.5, 1.0 and 0.75 M sucrose in 10 mM Tricine-KOH (pH 7.6) and centrifuged at 2 500 *g* for 15 min. The chloroplasts at the interface between 1.0 and 1.5 M sucrose were diluted with a suspension medium consisting of 0.33 M sorbitol, 50 mM HEPES (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. This suspension was centrifuged at 5 000 *g* for 5 min to yield a pellet of intact purified chloroplasts. The intactness of the purified chloroplasts was in the range of 80 to 85 % according to Lilley *et al.* (1975). The PS 2 activity of isolated chloroplasts were determined spectrophotometrically as described by Raghavendra and Das (1976).

Extraction and assay of enzymes: All extractions were performed at 4 °C. The leaf blades (10 g) were homogenized in 50 cm^3 of 100 mM Tris-HCl buffer (pH 7.8) containing 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM magnesium acetate and 1.5 % PVP-40. The homogenate was squeezed through four layers of cheesecloth and then centrifuged at 10 000 *g* for 10 min. The supernatant was precipitated with 750 g dm^{-3} $(NH_4)_2SO_4$ and spun at 30 000 *g* for 30 min and the precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.0) which contained 1 mM DTT and 0.2 mM NADPH.

RuBP carboxylase (RuBPC) activity was assayed at 30 °C by the incorporation of $^{14}CO_2$ into acid stable products (Lorimer *et al.* 1977) and the radioactivity was measured in liquid scintillation counter. Sucrose phosphate synthase (SPS) was assayed at 30 °C by measuring the production of UDP (Huber 1981). Chlorophyll (Chl) contents were determined in 80 % acetone extract (Arnon 1949) and carotenoid (Car) contents according to the method of Ikan (1969).

Contents of starch and sucrose in the leaf tissue were estimated enzymatically according to the method of Ramachandra Reddy *et al.* (1996). The total sugar contents in the 80 % ethanolic extract was determined using the anthrone method (Dubois *et al.* 1956). The contents of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were estimated as described by Leegood (1993).

Total amino acid, free proline and protein contents: Total amino acids were quantified using ninhydrin according to the method of Moore and Stein (1954). The amino acid contents of a sample were determined with the help of a standard curve prepared for glycine. Free proline was extracted from leaves with 3 % aqueous sulphosalicylic acid and estimated using ninhydrin according to the method of Bates *et al.* (1973). Total protein content was measured by the dye-method (Bradford 1976).

Nitrogen content: N content was estimated by Kjeldahl method using the *KJEL PLUS System* (Pelican, India). The leaf powder (500 mg) was digested with concentrated sulphuric acid (10 cm^3) in the presence of a salt mixture of copper sulphate (5 g), potassium sulphate (25 g) and selenium (0.5 g). This digested solution was distilled in the presence of 400 g dm^{-3} NaOH and the ammonia liberated was collected as ammonium borate which was titrated with 0.1 M HCl.

DNA and RNA contents: The nucleic acids were extracted from the mulberry leaf tissues (Schneider 1945) and the levels of DNA (Burton 1956) and RNA (Rawal *et al.* 1977) were quantified following standard protocols.

SDS-PAGE analysis on 120 g dm^{-3} polyacrylamide gels was conducted according to the procedure described by Laemmli (1970). Samples were solubilized in 2X-SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 50 g dm^{-3} SDS, 1 mM PMSF, 20 g dm^{-3} 2-mercapto-ethanol, 100 g dm^{-3} sucrose and 1 g dm^{-3} bromophenol blue and heated at 70 °C for 3 min. The apparent molecular masses of proteins were estimated by comparison with the mobility of standard proteins (*Bangalore Genei*, Bangalore, India). Proteins after SDS-PAGE were visualized by Coomassie Brilliant Blue (R-250) staining following standard protocols (Sambrook *et al.* 1989).

Results and discussion

Relative to the plants grown under normal temperature conditions, mulberry plants subjected to high temperature exhibited significant decrease in the carboxylating activity of RuBPCO (RuBPC) (Fig. 1*A*) and the SPS activity (Fig. 1*B*). The activities of RuBPC and SPS were about 30 % lower in mulberry plants subjected to high temperature treatment than in control plants. The activity of PS 2, and Chl and Car contents also decreased in high temperature treated plants (Fig. 1*C,D,E*). There was a considerable decrease in the content of total sugars, starch and sucrose under heat stress (Fig. 1*F,G,H*). The decrease in protein content in high temperature-treated mulberry

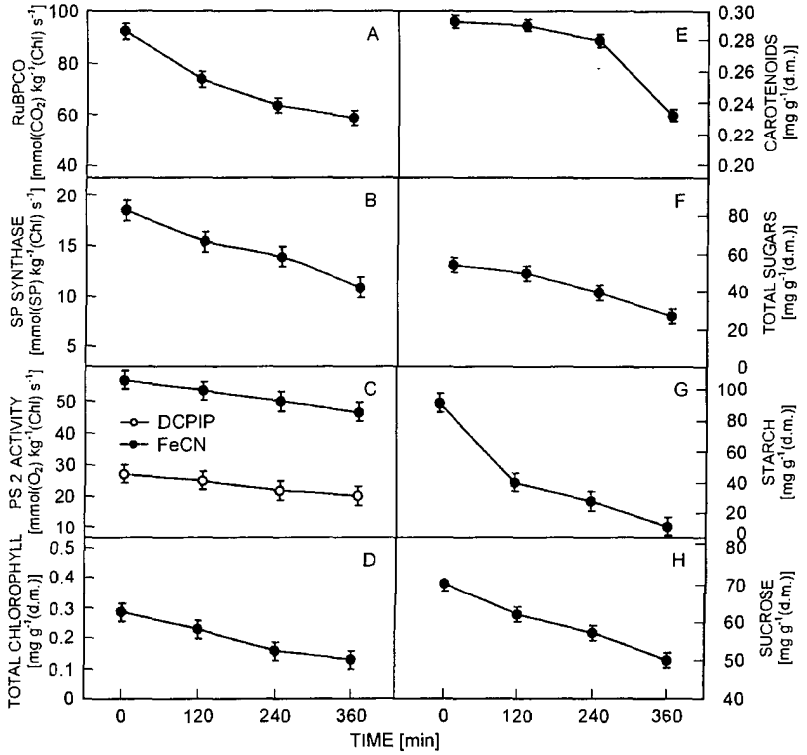

Fig. 1. Effect of high temperature (40 °C) on the activities of RuBPC (*A*), SPS (*B*), and PS 2 (photoreduction of DCPIP - *open circles*, FeCN - *closed circles*) (*C*), Chl contents (*D*), Car contents (*E*), total sugar contents (*F*), starch content (*G*) and the content of sucrose (*H*) in mulberry leaves. Values are means of 5 independent determinations ± SE.

leaves was associated with increase in amino acid content (Fig. 2*A,B*). Marked increase (1.5-fold) in the free proline content was noticed in high temperature-treated leaves (Fig. 2*C*). Foliar nitrogen content (Fig. 2*D*) was about 30 % lower in plants under heat stress. Chloroplast metabolite content (G6P and F6P) was also affected in leaves treated with high temperature (Fig. 2*E*). Marked decrease in the contents of both DNA and RNA was observed in high temperature-treated mulberry leaves (Fig. 2*F*). SDS-PAGE of leaf proteins showed the accumulation of two new polypeptides (68 and 85 kDa) in the high temperature-treated leaves (Fig. 3).

High temperature-treated mulberry leaves exhibited lower RuBPC and SPS activities with increasing duration of high-temperature-treatment. There are several ways the activities of photosynthetic enzymes could be

decreased in plants subjected to leaf temperature stress, including the differences in activation states, a decrease in total soluble protein content per leaf area, or specific transcriptional or translational control of synthesis of specific proteins (Maroco *et al.* 1999). This is believed to be a secondary effect mediated by decreased CO_2 partial pressure inside the leaf due to stomatal closure (Makino *et al.* 1994, Law and Crafts-Bradner 1999). Also isolated chloroplasts from stressed mulberry leaves showed significantly lower activities of PS 2. Temperature above 30 °C has been shown to cause disorientation of the lamellar system of the chloroplasts and under elevated leaf temperature, lateral migration of the light harvesting complex of PS 2 (LHC 2) has been noted (Singla *et al.* 1997). We presume this phenomenon could possibly be important in preventing the overexcitation of PS 2. Recent study indicates that heat stress induces changes in RuBPCO structure in such a way that its affinity for CO_2 is hampered and reduced RuBPC capacity at high temperature limits photosynthesis (Law and Crafts-Bradner 1999).

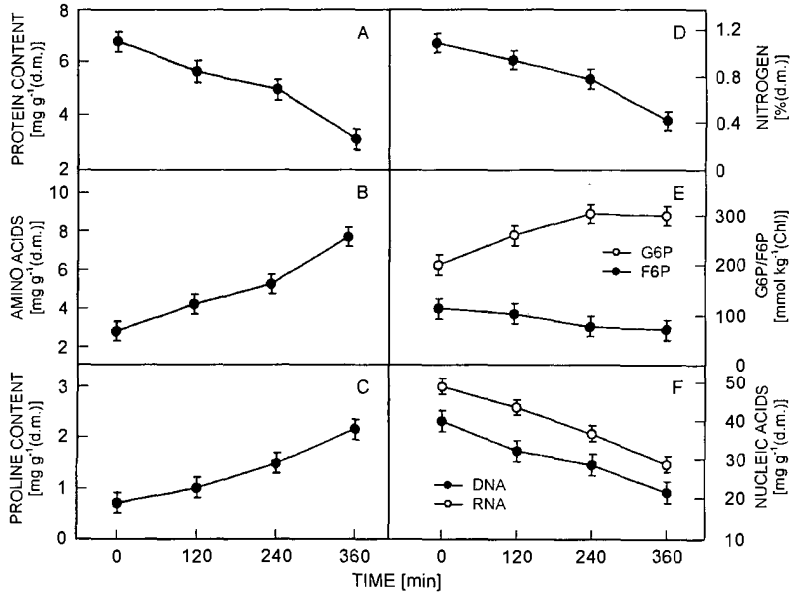

Fig. 2. Effect of high temperature (40 °C) on soluble protein content (*A*), amino acid content (*B*), free proline content (*C*), nitrogen content (*D*), G6P and F6P contents (*E*), and DNA (*closed circles*) or RNA (*open circles*) content (*F*) in mulberry leaves.

Increased leaf temperature affected the sugar metabolism. The lowered contents of sugars, especially starch, in mulberry leaves might be due to down regulation of photosynthesis under high temperature. However, the basis for the actual decrease in the sugar content under high temperature is uncertain. But we believe that under high temperature treatment, a decrease in the capacity of triose phosphate utilization via sucrose synthesis may reduce the overall carbohydrate levels in mulberry leaves. High temperature adversely affected the sucrose-starch balance which could be due to the altered UDP sucrose synthase at elevated temperatures (Leonardos *et al.* 1996). Consistent with this hypothesis, high temperature treatment of mulberry plants appeared to strongly affect the photosynthetic metabolite content as indicated by the changes in the contents of G6P or F6P (Fig. 2*E*). G6P increased with increase in temperature treatments while a slight reduction was noticed in F6P content. The total soluble protein and nucleic acid contents also dropped. This could be due to protein denaturation and inhibition of the protein synthesis at higher temperatures. In contrast, the total amino acid and free proline amounts increased. Proline accumulation in high temperature stressed plants is due to its enhanced

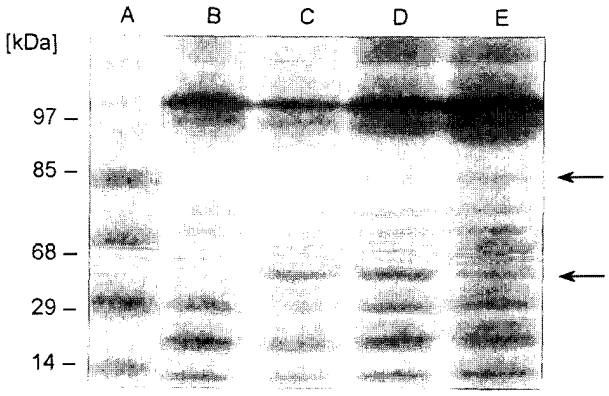

Fig. 3. Effect of high temperature (40 °C) on the foliar protein profile in mulberry. Proteins were analysed using 12 % SDS-PAGE. *Lane A* - molecular mass standards, *lane B* - control plants, *lane C* - 120 min of heat treatment, *lane D* - 240 min of heat treatment, and *lane E* - 360 min of heat treatment. New polypeptides are marked by *arrows*.

synthesis and decreased oxidation. It is reported that the enhanced amino acid, especially the proline content mediate osmotic adjustment, stabilize sub-cellular structures and scavenges free radicals (Bohnert *et al.* 1995). We also examined the levels of nitrogen in heat stress-treated plants. A big difference was noted in the total nitrogen content of heat stressed mulberry leaves compared to control plants. The decreased foliar nitrogen in heat stressed leaves might be attributed to decreased nitrogen metabolism (Makino *et al.* 1994).

Low and high molecular mass heat shock proteins (HSP) are known to express at detectable levels in plants grown under high temperature. But to our knowledge, studies on HSPs in mulberry is not established. In this study, mulberry plants responded to heat stress by the synthesis of new polypeptides (68 and 85 kDa) as evidenced on the SDS-PAGE gel. However, induction of heat shock proteins (HSP) may not be applicable to mulberry plants growing in the field because in our experimental conditions HSPs were shown to be synthesized under rapid exposure to heat shock. Less information is available regarding the synthesis and assembly of HSPs in plants grown in the field under constant high temperature conditions. It is well known that accumulation of HSPs is important for protection against thermal killing. Further studies are in progress in mulberry to assess the precise biochemistry and identification of the HSPs in laboratory as well as in field conditions. More complete characterization of HSPs, including intracellular localization and biochemical properties of HSPs in different mulberry cultivars could provide us an evidence for the efficiency in the thermotolerance among mulberry cultivars which could be used in plant breeding programs as well as in creation of mutant or transgenic mulberry plants for superior biomass yields.

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