Changes in mulberry leaf metabolism in response to water stress

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Abstract

A series of experiments were conducted to characterize the water stress-induced changes in the activities of RuBP carboxylase (RuBPCO) and sucrose phosphate synthase (SPS), photosystem 2 activity, and contents of chlorophylls, carotenoids, starch, sucrose, amino acids, free proline, proteins and nucleic acids in mulberry (*Morus alba* L. cv. K-2) leaves. Water stress progressively reduced the activities of RuBPCO and SPS in the leaf extracts, the chlorophyll content, and PS2 activity in isolated chloroplasts. Plants exposed to drought showed lower content of starch and sucrose but higher total sugar content than control plants. While the soluble protein content decreased under water stress, the amino acid content increased. Proline accumulation (2.5-fold) was noticed in stressed leaves. A reduction in the contents of DNA and RNA was observed. Reduced nitrogen content was associated with the reduction in nitrate reductase activity. SDS-PAGE protein profile showed few additional proteins (78 and 92 kDa) in the water stressed plants compared to control plants.

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

Introduction

Water stress elicits in plants a complex of responses beginning with stress perception, which initiates a signal transduction pathway(s) and causes multifaceted changes at the cellular, physiological and developmental levels (Bray 1997). Photosynthetic inhibition is one of the primary repercussions of water stress (Berkowitz 1998). Numerous studies suggested that water deficit affects not only stomatal conductance and so CO_2 supply but also the chloroplast machinery (Dickson and Isebrands 1991). Various photochemical activities of chloroplasts are inhibited under water stress, including electron transport. Stress inhibition of protein synthesis could be causal to depressed activity of the key enzymes involved in photosynthesis. It has also been shown that low leaf water potential brings major fluctuations in the content of photosynthates (Kameli and Losel 1993) and their translocation and consequently alter leaf carbon balance (Schulze 1991). Accumulation of compatible, low molecular mass osmolytes such as sugar alcohols, special amino acids and glycine betaine has been suggested as a major mechanism in adaptation or tolerance of plants to water stress (Veeranjaneyulu and Ranjita Kumari 1989, Bohnert and Jensen 1996, Bray 1997). Decline in nitrate reductase (NR) activity has often been shown to decline when water status is lowered (Smirnoff *et al.* 1985, Kaiser and Forster 1989) which could increase the nitrate content in the cell and contribute to osmotic adjustment (Cornic 1994).

Mulberry is one of the most important cash crops in south India. The problem of drought has presented a challenge to growing mulberry in dry regions. Studies on the response of various mulberry genotypes to induced-water stress would be highly significant for understanding the drought tolerance mechanism. The present investigation was undertaken to evaluate the primary leaf metabolism in mulberry under water stress. In addition, we also provide data on the extent of osmotic adjustment.

Received 30 March 2000, *accepted* 19 June 2000.
Acknowledgements: Research in the laboratory of ARR was supported by grants from Department of Biotechnology (#BT/R&D/08/018/95), New Delhi, ICFRE (#38-69/97-ICFRE), Dehradun, and University Grants Commission, New Delhi. DS acknowledges DBT for the award of Junior Research Fellowship.
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Materials and methods

Mulberry (*Morus alba* L. cv. K-2) plants, obtained from Regional Sericultural Research Station, Coonoor, Tamil Nadu, India, were grown in pots under natural photoperiod [irradiance (400-700 nm) of 1600 - 1800 μmol m^{-2} s^{-1}]. Mean maximum day/night temperature was approximately 30/23 °C and relative humidity was about 60 %. Young and fully expanded leaves were used in all the experiments. Water stress was induced by termination of watering to the plants. Control plants were maintained well watered. Leaf water potential was measured psychrometrically on the leaf discs at 30 °C using a pressure chamber (*SKPM 1400*, *Skye Instruments*, Powys, Wales, UK).

Before chloroplast isolation freshly harvested leaves were illuminated for 15 min. The leaves were cut into strips and homogenized in a semi-frozen grinding medium which consisted of 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 1 % PVPP, 0.5 mM DTT and 2 mM sodium arsenate. 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 onto 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 used in the present study was in the range of 80 to 85 % according to Lilley *et al.* (1975). The photochemical activity in the isolated chloroplasts were determined spectrophotometrically as described by Raghavendra and Das (1976).

Extraction of enzymes was performed at 4 °C. The leaf blades (10 g) were homogenized with 50 cm^3 of 100 mM Tris-HCl (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 solution was filtered off to remove the cellulose and washed thrice with the extraction medium. The protein was precipitated with 75 % (m/v) ammonium sulphate 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. The elutes were collected at room temperature.

RuBP carboxylase activity was assayed at 30 °C by the incorporation of $^{14}CO_2$ into acid stable products (Lorimer *et al.* 1977). 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 content was determined in 80 % acetone extracts (Arnon 1949) and carotenoid content according to the method of Ikan (1969).

The contents of starch and sucrose in the leaf tissues were estimated enzymatically according to the method of Ramachandra Reddy *et al.* (1996). The total sugar content in the 80 % ethanolic extract was determined using the anthrone method (Dubois *et al.* 1956).

Total amino acids were quantified using ninhydrin method of Moore and Stein (1954). The amino acid contents of the sample were determined with the help of a standard curve prepared for glycine. Free proline from leaves was extracted in 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).

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

Nitrogen content was estimated by Kjeldahl method using the *KJEL PLUS* System (Pelican, India). The leaf powder was digested with concentrated sulphuric acid in the presence of a salt mixture of copper sulphate, potassium sulphate and selenium. This digested solution was distilled in the presence of 40 % NaOH and the ammonia liberated was collected as ammonium borate which was titrated against 0.1 M HCl. The NR activity was assayed using the leaf disc method of Jaworski (1971).

SDS-PAGE on 12 % (m/v) polyacrylamide gels was conducted according to Laemmli (1970). Leaf samples were solubilized in 2X-SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 5 % (m/v) SDS, 1 mM PMSF, 2 % (v/v) 2-mercaptoethanol, 10 % sucrose and 0.01 % (m/v) 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 on the gel after SDS-PAGE were visualized by Coomassie Brilliant Blue following standard protocol (Sambrook *et al.* 1989).

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. Reagents were purchased from commercial sources and were of analytical grade.

Results and discussion

The activities of RuBPCO and SPS were about 40 % less in water-stressed (leaf water potential -2.25 MPa) than in the control plants (Fig. 1*A,B*). Under water stress, the chlorophyll and carotenoid contents, and the PS2 activity decreased in all plants (Fig. 1*C,D,E*). Water stress also caused a decrease in the starch and sucrose content, while total sugar content increased (Fig. 1*F,G,H*). Marked increase (2.5-fold) in the free proline content was noticed (Fig. 2*C*). N content (Fig. 2*D*) was about 50 % less in water stressed plants than in control plants and the nitrate reductase activity was also significantly reduced under drought (Fig. 2*E*). Polypeptides of 78 and 92 kDa accumulated under water stress (Fig. 3) while few others (14, 43 and 68 kDa) were not detected in water-stressed leaves.

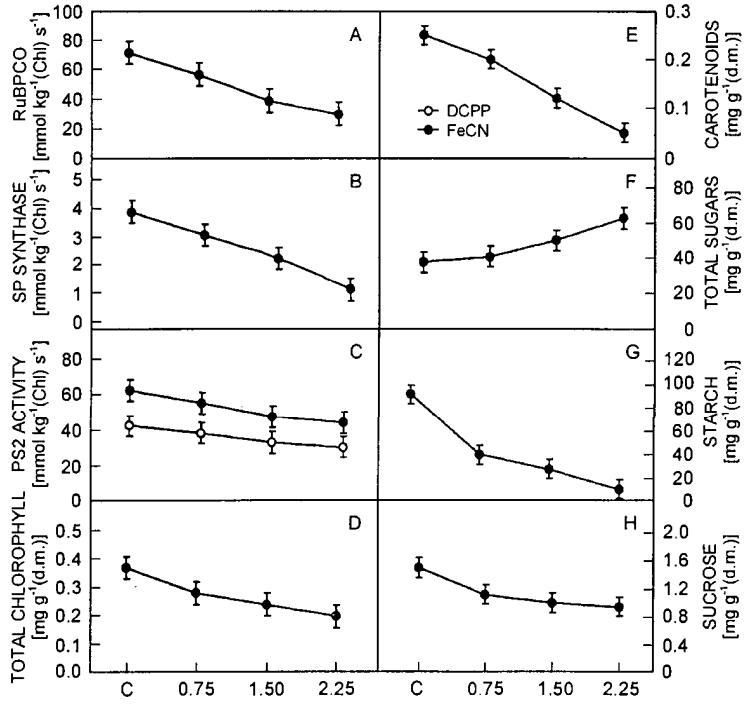

Fig. 1. Effect of decreasing leaf water potential on the activity of RuBP carboxylase (*A*), activity of sucrose phosphate synthase (*B*), PS 2 activity [photoreduction of DCPIP (*open circles*) or FeCN (*closed circles*) in isolated chloroplasts] (*C*), chlorophyll *a+b* content (*D*), carotenoid content (*E*), total sugar content (*F*), starch content (*G*), and the content of sucrose (H) in mulberry leaves. The leaf water potential in control plants (C) was -0.5 MPa. Means of 5 independent determinations ± SE.

Water stress is one of the primary stresses limiting growth, productivity and distribution of plants (Boyer 1983, Jones and Corlett 1992). The effect of water stress is believed to be a secondary effect mediated by decreased CO_2 partial pressure inside the leaf due to water stress mediated-stomatal closure. Mild drought stress usually does not appreciably affect RuBPCO and SPS activities, although exceptions to this have been reported for some plants, including cotton and bean (Jones 1973, O'Toole *et al.* 1977). The stressed mulberry plants exhibited less RuBPCO and SPS activities with decreasing leaf water potentials which is presumed to be partially a consequence of decreased CO_2 concentration inside the leaf due to stomatal closure. It was previously reported that inhibition of the activities of RuBPCO and SPS caused by water stress can be reversed by high CO_2 treatment of water stressed plants without relief of the water stress (Vassey *et al.* 1991). Also, isolated chloroplasts from stressed mulberry leaves showed significantly lower activities of PS 2. The direct effects of more severe water stress on the biochemistry of photosynthesis appears to result from changes in the photosynthetic enzymes.

The total sugars content in mulberry leaves 2.3-fold increased in stressed leaves (at leaf water potential -2.25 MPa). The accumulation of total sugars during drought stress corresponds to reductions in starch concentrations. Total soluble protein and nucleic acid contents also dropped in drought stressed plants as compared with unstressed plants. Stress inhibition of protein synthesis could be the cause to this decline. The total amino acid and free proline contents increased. Proline accumulation in water-stressed plants is due to enhanced synthesis and decreased oxidation (Hanson and Hitz 1982, Choudhary

et al. 1996). It is believed that the enhanced amino acid and specially the proline content mediate osmotic adjustment, stabilizes sub-cellular structures and scavenges free-radicals. The reduced nitrogen content and

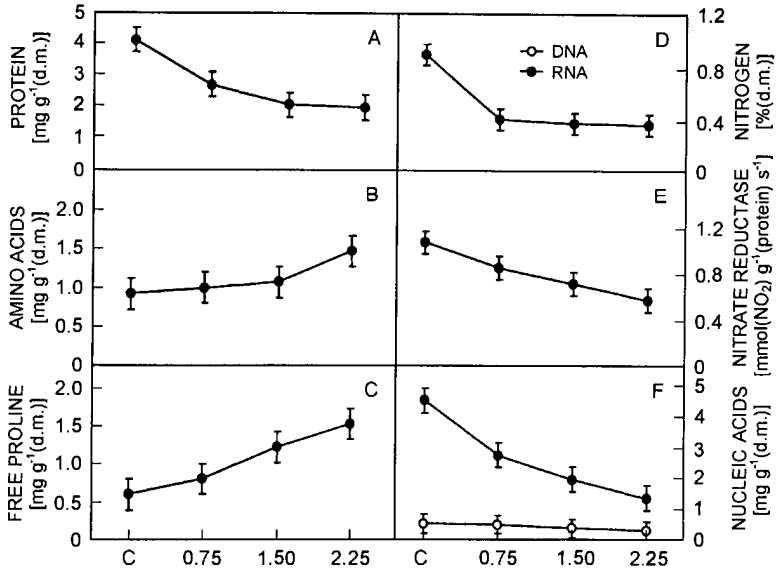

Fig. 2. Effect of decreasing leaf water potential on soluble protein content (*A*), amino acid content (*B*), free proline content (*C*), nitrogen content (*D*), nitrate reductase activity (*E*), and the nucleic acid [DNA (*open circles*) or RNA (*closed circles*)] content (*F*) in mulberry leaves. The leaf water potential in control plants (C) was -0.5 MPa. Means of 5 independent determinations ± SE.

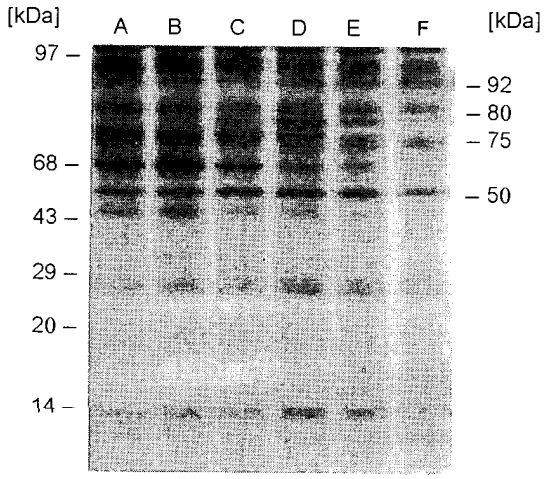

Fig. 3. Effect of decreasing leaf water potential (-0.75 to -2.25 MPa) on the protein profile in mulberry leaves. Proteins were analysed using 12 % SDS-PAGE as described in Materials and methods. *Lane A* - control plants; *lane B* to *F* - leaf water potential -0.75, -1.0, -1.5, -2.0, and -2.25 MPa, repectively.

NR activity in water stressed leaves can be attributed to the decreased nitrogen metabolism (Campbell 1999).

In this study, we have also made attempts to highlight the stress-induced protein alterations (which represent products of altered gene expression) at different leaf water potentials (Fig. 3). The protein alterations ranged in molecular masses from as low as 14 kDa to as high as 92 kDa under drought. Alterations in the contents of certain specific polypeptides were found to be unique to a given water status in the plants. Specific polypeptides were detected at either the early stage or the late stage of water stress. For instance, accumulation of 78 and 92 kDa polypeptides were noted only at leaf water potential -1.0, -1.5 and -2.0 MPa. Polypeptides of 14, 43 and 68 kDa were not detected in leaves at water potential -2.25 MPa. From the data presented, it is clear that stress-responsive alterations in protein pattern show a clear water-status-dependent response. Future studies most focus on the precise biochemistry and identity of various stress proteins.

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