Water stress-mediated changes in antioxidant enzyme activities of mulberry (*Morus alba* L.)

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Waters stress-induced responses in the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) from three different genotypes (K-2, MR-2 and BC2-59) of mulberry (*Morus alba* L.) were determined. In response to water deficit, increases in the activities of SOD, CAT, APX and POD were observed in all the three genotypes. Progressive increase in the activities of the antioxidant enzymes was observed with decreasing leaf water potentials from −0.75 MPa to −2.25 MPa. However, BC2-59 showed significantly higher activities of all the three antioxidant enzymes under low water regimes compared to those from K-2 and MR-2. The results clearly suggest that low leaf water potentials induce the antioxidant enzymes in all the three genotypes of mulberry. Our data indicate that the genotype BC2-59 has efficient antioxidant system among the three cultivars which could protect the oxidative damage caused due to water limited conditions.

Abbreviations: AOS, active oxygen species; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; APX, ascorbate peroxidase

Key words: *Morus alba*, water stress, antioxidant enzymes

Mulberry is the base of the silk industry in India which is an exclusive source of the food material for rearing silkworms. Adaptive response in the metabolism of any plant during environmental stress such as water stress, reflects changes in the activities of enzymes or in gene expression (HOCHACHKA and SOMERO, 1973). Many of the degenerative reactions associated with several biotic, abiotic and xenobiotic stresses are mediated by toxic reactive oxygen intermediates (ALLEN, 1995). The imposition of any abiotic stress gives rise to increases in the reactive and toxic oxygen species or active oxygen species (AOS) levels (FOYER and MULLINEAUX, 1994; DANGL et al., 1996; ALSCHER et al., 1998). Plant cells have evolved defense, antioxidant mechanisms to combat the danger posed by the presence of AOS. Evaluation of the antioxidant enzymes under water-stress conditions therefore becomes very important in studying the adaptive response of several crop plants. In plant leaves, various metabolites with antioxidative function such as ascorbate, glutathione, tocopherol, polyamines and phenolics have been detected (FOYER and MULLINEAUX, 1994). Defense enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) constitute a mutually supportive team of defense in plants against AOS (ASADA, 1994; BOWLER et
al., 1994; BANDYOPADHYAY et al., 1999). SODs are metalloenzymes that scavenge the toxic superoxide radicals formed under stress conditions and catalyse the conversion of two superoxide anions into oxygen and hydrogen peroxide (SCANDALIOS, 1993; ALLEN, 1995). CAT then converts the hydrogen peroxide into water and oxygen. PODs decompose H$_2$O$_2$ by oxidation of cosubstrates such as phenolic compounds and antioxidants (CAMPA, 1991; RAO et al., 1996). PODs specific for ascorbate (APX), catalyse the first step of the H$_2$O$_2$ scavenging pathway by oxidising ascorbate (INZ and MONTAGU, 1995). Over-reduced photosystems under stressful environment can also generate oxygen radicals which then cause further damage to the photosynthetic apparatus (HURRY et al., 1998). Drought-impaired electron transport system in wheat due to water deficiency has been shown to enhance the superoxide formation (PRICE et al., 1989). It is thus imperative to presume that plants capable of more superoxide formation might have improved tolerance to stressed environments.

Tolerance to environmental stress such as drought is the major objective for mulberry crop improvement in many parts of the world. Earlier studies on drought resistance in mulberry have indicated the differential responses of various mulberry genotypes to induced-water stress (SUSHEELAMMA et al., 1990; DORCUS and VIVEKANANDAN, 1997). We are interested in studying the physiological function of different antioxidant enzymes in mulberry. In this report, we present the results on the variation in the antioxidant systems among three mulberry genotypes under water-limited conditions.

Materials and Methods

Plant material and Growth Conditions
Mulberry plants (Morus alba L., genotype K-2, MR-2 and BC2-59), obtained from the Regional Sericultural Research Station, Coonoor, Tamil Nadu, India were propagated in 30 cm pots under 12 h natural photoperiod conditions with day-night temperatures of 30°C. The plants were well watered and periodically fertilized with nutrient solution. Water stress was induced by termination of watering. Control plants were maintained under the same conditions as the water-stressed plants except that they were well watered. Measurements of leaf water potentials were made psychrometrically on leaf discs at 30°C with Plant Moisture System (Skye Instruments, UK). The third or fourth leaf from the top of the plants were collected for all extractions and estimations.

Enzymes

Extraction
All extractions were performed at 4°C. The leaf blades (10g) were homogenized with 50 volumes of 100 mM Tris-HCl (pH 7.5) containing 5 mM DTT, 10 mM MgCl$_2$, 1mM EDTA, 5 mM magnesium acetate and 1.5% PVP-40. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 10,000g for 10 min. The solution was filtered off to remove the cellulose and washed thrice with extraction medium. The protein was precipitated with 75% (w/v) ammonium sulphate and spun at 30,000g for 30 min and the precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.8) containing 1mM DTT and 2 mM EDTA. The preparation was applied to a column of Sephadex G-25, equilibrated with 10 mM Tris-HCl (pH 8.0) which contained 1 mM DTT, 10 mM NaHCO$_3$, 20mM MgCl$_2$ and 0.2 mM NADPH. The elutes were collected at room temperature. Protein content was estimated according to Bradford (1976) with BSA as standard.

Superoxide dismutase (SOD, EC 1.15.1.1)
SOD was determined essentially as described by BEAUCHAMP and FRIDOVICH (1971) as modified by DHINDSA and MATOWE (1981), which measured the inhibition in the photochemical reduction of nitroblue tetrazolium. In the spectrophotometric assay, 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75µM nitroblue tetrazolium (NTB), 2 µM riboflavin and 100 µl of the enzyme supernatant. Riboflavin was
added at last and the reaction was initiated by placing the tubes under two 15-W fluorescent lamps. The reaction was terminated after 10 min by removal from the light source. Nonilluminated and illuminated reactions without supernatant served as calibration standards. Reaction product was measured at 560 nm. The volume of the supernatant corresponding to 50% inhibition of the reaction was assigned a value of 1 enzyme unit.

Catalase (CAT, EC 1.11.1.6)
A modified method of Luck (1974) was employed for the assay of CAT. 50μl of the enzyme extract was added to 3 ml of hydrogen peroxide-phosphate buffer (pH 7.0). The time required for decrease in absorbance from 0.45 to 0.40 was noted. The enzyme solution which contained hydrogen peroxide-free phosphate buffer was used as control.

Ascorbate peroxidase (APX, EC 1.11.1.11)
Ascorbate peroxidase was spectrophotometrically assayed following a decrease in absorbance at 265 nm (Asada, 1994). The assay mixture contained 0.25 M ascorbate and 1 mM H₂O₂ in 50 mM phosphate buffer (pH 7.0) with 37.5μl of enzyme extract. Corrections were made for low rates of ascorbate disappearance due to non-enzymatic and H₂O₂-independent oxidation. Rate of ascorbate disappearance was determined during the linear phase of the reaction.

Peroxidase (POD, EC 1.11.1.7)
A 0.1 ml of the enzyme extract was added to the reaction containing 0.05 ml guaiacol solution, 0.03 ml hydrogen peroxide solution in 3 ml phosphate buffer solution (pH 7.0). The solution was then mixed well and waited until the absorbance at 436 nm read 0.05 in the spectrophotometer. Time was then noted for the absorbance to increase by 0.1. The enzyme activity was calculated using the extinction coefficient of guaiacol dehydrogenation product under the conditions specified (Putter, 1974).

Results and Discussion

In this study, all the three mulberry genotypes are shown to possess increased activities of the antioxidant enzymes produced due to water stress (Figs. 1-4). The effect of water stress on total foliar activities of active oxygen species-scavenging enzymes (SOD, CAT, APX and POD) was followed over the time course of the experiment by following the decreased leaf water potential to assess the performance of the mulberry plants under water limited conditions. We also wanted to ascertain the drought-tolerant mulberry variety in terms of its antioxidant defense enzyme activities. Interestingly, the magnitude of increase in the enzyme activities differs among the three genotypes (Figs. 1-4). Water stress treatment enhanced the SOD activity by 47% (Fig. 1) in the leaves of BC2-59 at −2.25 MPa (115 units mg⁻¹ protein) compared to control plants (78 units mg⁻¹ protein). Similarly, water stress treatment also enhanced CAT, APX and POD by approximately two-fold at −2.25
Fig. 2. Effects of water stress on the activity of catalase (CAT) in the leaves of three mulberry genotypes (K-2, MR-2 and BC2-59). Each point is an average of at least four independent determinations.

Fig. 3. Activity of ascorbate peroxidase (APX) in the leaves of three mulberry genotypes (K-2, MR-2 and BC2-59) in response to low leaf water potentials. Each point is an average of at least four independent determinations.

Fig. 4. Foliar peroxidase (POD) activity in three different mulberry genotypes (K-2, MR-2 and BC2-59) in response to different leaf water potentials. Each point is an average of at least four independent determinations.

MPa compared to those of control plants (Figs. 2–4). Water stress is thus believed to generate active oxygen species and this response of plants to water stress seems to be a complex phenomenon (BOWLER et al., 1992). The data clearly show that BC2-59 showed significantly higher activities of all the three antioxidant systems compared to K-2 and MR-2. In plants, various environmental perturbations like high light, drought and temperature stress can cause excessive reaction oxygen species (AOS), overwhelming the system and necessitating additional defenses (SCANDIOLIOS, 1993). Plant cells have capacity to respond to the oxidative stress and to marshal and maintain antioxidant defense systems at levels that correspond to the altered environmental conditions (FOYER, 1993). The acclimation of plants to survive under extreme stress conditions have been well correlated with their ability to scavenge the AOS (BRIDGER et al., 1994; MCKERSIE et al., 1996). Antioxidant levels and the activities of oxygen free radical-scavenging enzymes (SOD, CAT, APX and POD) have been correlated with the tolerance to several different stresses (MCKERSIE et al., 1996). An inevitable result of chloroplast, mitochondria and plasmalemma-linked electron transport is the leaking of electrons on to molecular oxygen in plant cells, with the resultant production of reactive, toxic oxygen species (ASADA, 1999). The AOS can attack vital cell components like polyunsaturated fatty acids, proteins
and nucleic acids. To a lesser extent, carbohydrates are also the targets of AOS (Noctor and Foyer, 1998).

We presume that the metabolism of active oxygen species under stressful environment is dependent on different functionally interrelated antioxidant enzymes. Although environmental stress has been shown to induce the antioxidant enzymes, for the first time in this study, we report concerning the various responses of different antioxidant enzymes among three different mulberry genotypes subjected to water-deficit. This investigation provides an evidence that different mulberry genotypes have varying capacity to synthesize the antioxidant enzymes. However, water-stress induced changes in the antioxidant enzymes in all the three genotypes appeared to be dependent on the duration of water stress as indicated by the leaf water potentials (Figs. 1–4). Both APX and POD are known to metabolise H₂O₂ to H₂O through a metabolic cycle widely known as the ascorbate-glutathione cycle or Halliwell-Asada pathway (Noctor and Foyer, 1998). Certain POD isomers utilize phenolic compounds and H₂O₂ to initiate the biosynthesis of several secondary metabolites required for plant growth, development and differentiation (Gasper et al., 1991). The enhanced activities of POD as observed in this study indicate that mulberry is capable of scavenging the oxygen species produced during water stress for the biosynthesis of certain beneficial secondary metabolites to sustain during adverse environmental regimes. APX activity in BC2-59 was significantly higher under severe water stress compared to those in the other two genotypes (Fig. 3) indicating a more efficient antioxidant defense mechanism in this genotype. Also on the other hand, our data suggest that water-deficit regime in mulberry may prompt an overall efficient SOD/ascorbate/glutathione cycle in BC2-59 providing an efficient drought adaptive mechanism in the crop. The ability of higher plants to maintain a high redox state of ascorbate and glutathione under stressful conditions has been attributed to the coordination between SOD that generates H₂O₂ and GR-APX that metabolises H₂O₂ (Foyer, 1993). From this study, it is clear that water-deficit regime is capable of inflicting oxidative damage in mulberry and there are variations among the mulberry genotypes in the mechanism of antioxidative adaptability. Although the responses of antioxidant enzymes is variable among the three mulberry genotypes, high antioxidant capacity in response to low leaf water potentials is expected to improve mulberry plant protection and hence it would be useful to identify the superior mulberry genotypes which could tolerate water stress. The present study clearly shows that the genotype BC2-59 is superior with respect to its antioxidant defense systems and should be more tolerant than the other two genotypes due to the higher AOS-scavenging systems. Such studies can be used in mulberry breeding programmes or transgenic mulberry research to generate the plants with elevated activities of antioxidant systems for improved tolerance to low water regimes. Additional experimentation is in progress to determine the differential role of these antioxidant enzymes in different genotypes subjected to low water regimes.

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References


A. Ramachandra Reddy·K.V. Chaitanya·D. Sundar 水分ストレスによりもたらされるクワの抗酸化酵素活性の変化

水分ストレスにより誘起される superoxide dimutase (SOD), catalase (CAT), ascorbate peroxidase (APX) および peroxidase (POD) の活性変化を 3 種の異なる遺伝子型をもつクワ (K-2, MR-2 および BC2-59) で調べた。SOD, CAT, APX および POD の活性は、いずれも水分制限することによって上昇することが、すべての遺伝子型のクワにおいて観察された。これらの抗酸化酵素は、葉の水ポテンシャルが -0.75 MPa から -2.25 MPa に低下するとにしたがって漸減した。低水分条件下における、BC2-59 の抗酸化酵素活性は、どの抗酸化酵素においても、K-2 と MR-2 のそれと比較して有意に高かった。これらの結果は、いずれの遺伝子型のクワにおいても、葉の水ポテンシャルが低下すると、抗酸化酵素が誘導されることを示唆しており、かつ 3 種の遺伝子型のクワのなかでは、BC2-59 が水分制限下における酸化傷害から保護するための最も効果的な装置を備えていることを示している。