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STIMULATION OF STRESS-RELATED ANTIOXIDATIVE ENZYMES IN COMBATING OXIDATIVE STRESS IN *CASSIA* SEEDLINGS

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SUMMARY

The role of antioxidant systems in protection against water and UV-B stresses was studied in *Cassia angustifolia* Vahl. and *Cassia auriculata* Linn seedlings at 5 and 7 days after sowing (DAS). Water and UV-B stresses increased superoxide dismutase (SOD) activity compared to control plants except at 5 DAS under water stress in *C. angustifolia*, where the SOD activity decreased. Peroxidase (POX) and polyphenol oxidase (PPO) activities increased in seedlings under both stresses at the two stages. Catalase (CAT) activity increased under both stresses except in *C. angustifolia* under UV-B stress, where the activity decreased 5 DAS. *C. angustifolia* seedlings exhibited high H_2O_2 and low ascorbate (ASA), thiobarbituric acid reactive substances (TBARS) and proline contents in comparison to *C. auriculata* seedlings. It is apparent that not only superoxide dismutase but H_2O_2 scavenging systems as represented by catalase and peroxidase, is also equally important in preventing oxidative stress induced by water and UV-B stresses. The high activity of polyphenol oxidase might also participate in the enhanced tolerance to oxidative stress.

Key words **: Antioxidants, *C. angustifolia*, *C. auriculata*, oxidative stress, UV-B stress, water stress.**

INTRODUCTION

Plants are exposed to different kinds of stresses throughout their life span. In addition to growth, various metabolic processes are affected at the advent of stress, albeit the magnitude of effects varies (Levitt 1972). Both abiotic and biotic stresses are known to induce production of reactive oxygen species (ROS) that cause damage to the cell and/or signal the start of physiological defense responses (Dat *et.al.* 2000).

Increasing evidence suggest that water stress and UV-B exposure induced the production of reactive oxygen species such as superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). The plants inturn enhance the activities of enzymatic and non-enzymatic antioxidants viz. superoxide dismutase, ascorbate peroxidase, guaiacol-peroxidase, catalase and metabolites like ascorbic acid, glutathione, ∝-tocopherol and carotenoids etc. to combat the oxidative stress injury under these stresses (Bowler *et al.* 1992, Scandalios 1993).

The objective of the present study was to investigate the role of various antioxidant enzymes and metabolites under water and UV-B stresses in two *Cassia* species, *C. angustifolia* Vahl. and *C. auriculata* Linn in order to evaluate the importance of these antioxidative systems in controlling the levels of hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS) in plants.

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MATERIALS AND METHODS

Seeds of *Cassia angustifolia* Vahl. (V_1) and *Cassia auriculata* Linn (V_2) were surface sterilized by treating with 0.1% mercuric chloride solution for five minutes and then thoroughly washed with double distilled water. Thirty sterilized seeds were placed in petridish lined with double layer of *Whatman* filter paper. Seeds were germinated at 28±2°C under laboratory conditions. The petridishes containing seeds were exposed to two stress conditions, water-stress and UV-B exposure. Water-stress was induced at two stages of seedling development 5 and 7 days after sowing (DAS) by replacing the wet filter paper with dry filter paper for 24 h before analysis. Seeds for UV-B treatment exposure (280-320 nm) were germinated in complete darkness in petridishes lined with double layer of *Whatman* filter paper. Seed coats were removed in dark under green safe lamp (Philips 25 W lamp covered with 8 layers of green cellophane). Irradiation at the level of seedling was 2.66 mw cm^{-2} sec^{-1}. Seedlings on 5 and 7 DAS were exposed to UV-B radiation for 45 min before analysis.

Enzyme extract for superoxide dismutase (SOD) was prepared by grinding 0.2 g seedlings with 5 ml of chilled phosphate buffer (0.1 m, pH 7.8) while extract for catalase (CAT), peroxidase (POX) and polyphenol exidase (PPO) were prepared by grinding 0.2g seedlings with 10.0 ml of chilled phosphate buffer (0.1 M, pH 6.8). The homogenate was centrifuged at 10,000g for 20 min. The supernatant served as enzyme extract. All operations were carried out at 4°C.

The SOD activity was determined according to the method of Beauchamp and Fridovich, (1971) with some modification (Giannopolitis and Ries 1977). The 3.5 ml reaction mixture contained 63 µM nitroblue tetrazolium chloride (NBT), 13 mM methionine, 0.1mM EDTA, 13 µM riboflavin, 0.05 M sodium carbonate pH 10.2 and 0.4 ml enzyme. Reaction was started by placing tubes below two 15W fluorescent lamps for 10 min. Reaction was stopped by keeping the tubes in dark for 10 min. Absorbance was recorded at 560 mm.

Catalase, peroxidase and polyphenol oxidase activities were assayed according to Chance and Maehly (1955) with modifications. The reaction mixture for CAT contained 2.0 ml enzyme, 1.0 ml of 100 µmol H_2O_2 that was incubated at room temperature for 5 minutes. The reaction was stopped by adding 1.0 ml of 12% H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N $KMNO_4$ until a faint purple color persisted for 30 s. On unit of CAT activity is defined as that amount of enzyme which breaks down 1 µmol of H_2O_2 in 1 min under the assay conditions described. The 7.0 ml reaction mixture for POX contained 2.0 ml enzyme, 2.5 ml of 125 µmol phosphate buffer, pH 6.8, 1.0 ml of 50 µmol pyrogallol and 1.0 ml of 50 µmol H_2O_2. The tubes were incubated for 5 min at room temperature after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm. The reaction mixture for PPO was same as that for POX except that H_2O_2 was not added. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm.

For the determination of ascorbate (Mukherjee and Choudhuri 1983) the seedlings (0.25g) were homogenized in a cold mortar placed on ice using 10 ml of 6% trichloroacetic acid. To 4.0 ml of the extract 2.0 ml of 2% dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% thiourea (in 70% ethanol) was added. The mixture was kept in boiling water bath for 15 min and after cooling at room temperature 5 ml of 80% (v/v) H_2SO_4 was added to the mixture at 0°C. The absorbance at 530 nm was recorded. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

For measurement of TBARS content, the seedlings (0.2 g), were homogenized in 10 ml of 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 g for 20 min. To 4.0 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added a 1.0 ml aliquot of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifuging at 10,000 g for 10 min, the absorbance of the supernatant was read at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of TBARS was calculated using TBARS extinction coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol g^{-1} FW following the method of Heath and Packer (1968).

Hydrogen peroxide was estimated with titanium reagent as described by Teranishi *et al.* (1974). Sample

preparation for H_2O_2 estimation was done as described by Mukherjee and Choudhuri (1983). The proline content was determined using the method of Bates *et al.* (1973).

RESULTS AND DISCUSSION

The role of antioxidants in protecting the two *Cassia* species from the deleterious effects of oxidative stress induced by water and UV-B stresses was investigated. Interactions of the antioxidant enzymes SOD, CAT, POX and PPO and their involvement in scavenging ROS are very complex. The effects of the ROS on membrane and cellular damages are likely to differ depending on the stress imposed on them.

Activity of SOD (Fig. 1 A) varied with the stress imposed on the seedlings of the two species. Under water stress, the activity decreased in V_1 and increased in V_2 on 5 DAS. However, the activity increased significantly in both species on 7 DAS. Under UV-B exposure the SOD activity again increased in the two species above control values at both the stages. The increased activity of SOD

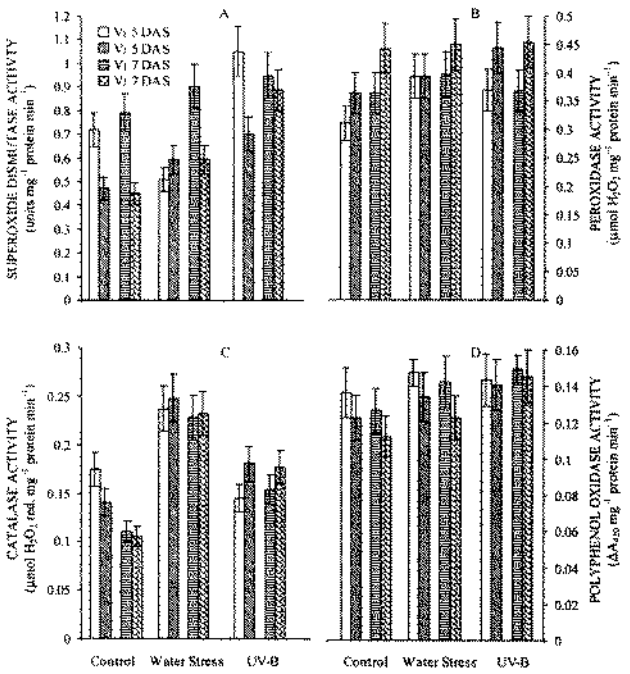

Fig. 1. Effect of water stress and UV-B stress on SOD (A), POX (B), CAT (C) and PPO (D) activities in seedlings of *C. angustifolia* (V_1) and *C. auriculata* (V_2). Vertical bars indicate ± SD values of 3 replicates from 2 independent experiments.

might protect plants from oxidative injury and would definitely not favour accumulation of (O_2^-). SOD catalyzes the dismutation of (O_2^-) to H_2O_2 and plays a key role in the quenching of active oxygen. Varied responses of SOD activity to water stress have been reported. Water stress did not influence SOD activity in sorghum (Zhang and Kirkham 1996) and wheat (Sairam *et al.* 1998, Sairam and Srivastava 2001), decreased with osmotic stress in upland rice (*Oryza sativa* L.) (Reddy and Vajranabhaiah 1993) and increased in maize (Jagtap and Bhargava 1995), wheat (Sgherri *et al.* 2000) and leafy spurge (Davies and Swanson 2001). Activity of SOD increased in cucumber seedlings exposed to 6 h of UV-B radiation (Noriaki and Mika 2000) and in wheat (Dawar *et al.* 1998).

Peroxidase (POX) decomposes H_2O_2 by oxidation of phenolic compounds. The POX activity (Fig. 1B) showed significant increase under water-stress and UV-B exposure in V_1 and V_2 on 5 DAS. On 7 DAS the POX activity increased in both species under water-stress and UV-B compared to control but the effect was not significant for V_1 under UV-B and for V_2 under water-stress. Other investigators have reported increases in maize (Zhang *et al.* 1995), grasses (Fu and Huang 2001) and *Cucumis sativus* var. long Green (Tekchandani and Guru-prasad 1998), decrease in sunflower and sorghum seedlings (Zhang and Kirkham 1996) and no change in wheat (Fangmeier *et al.*1994) in POX activity in response to water-stress, while Noriaki and Mika (2000) have reported increase in POX activity in cucumber seedlings under UV-B stress.

Catalase (CAT) scavenges H_2O_2 by breaking it down directly to form water and oxygen and an increase in its activity is related with increase in stress tolerance (Kraus *et al.* 1995). CAT activity (Fig. 1 C) increased in both seedlings under water-stress at both stages but under UV-B stress CAT activity decreased in V_1 and increased in V_2 on 5 DAS and in both species at 7 DAS. Previous studies have shown that response of CAT activity to water-stress may be varied. CAT activity was not affected by mild drought in sorghum (Zhang and Kirkham 1996) and cool-season grasses (Fu and Huang 2001), decreased in pea (Moran *et al.* 1994) leafy spurge (Davis and Swanson 2001) and increased in wheat genotype C 306 (Sairam and Srivastava 2001). Dawar *et al.* (1998) have reported increased activities of CAT and SOD during first hours of

UV-B exposure in wheat. The increased levels of H_2O_2 presumably stimulate CAT activity.

Polyphenol oxidase and peroxidase are the two major enzymes responsible for oxidation of phenolic compounds. Some studies have reported that these enzyme activities increase in response to different types of stresses, both biotic and abiotic (Rivero *et al.* 2001). PPO activity increased in V_1 and V_2 at both stages (Fig. 1D). We hypothesized that an increase in the activity of these enzymes under water and UV-B stresses could be indicative of an increased production of ROS and a build-up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants (Scalet *et al.* 1995).

Ascorbic acid (ASA) participates in the removal of H_2O_2 as a substrate of ascorbate peroxidase, directly reduces superoxide, quench singlet oxygen and regenerate reduced ∝- tocopherol. Reduction in ascorbate content in response to drought was reported in *Vigna catjang* (Mukherjee and Choudhuri 1983) sorghum (Zhang and Kirkham 1996) and wheat leaves (Bartoli *et al.* 1999). The increased ascorbate content in V_1 and V_2 seedlings (Fig. 2A) could be postulated as a key factor to control the oxidation at the membrane level, limiting the increase in hydrogen peroxide and lipid radical content.

Lipid peroxidation measured as TBARS content indicates the dominant presence of free radical reactions in tissues. The content of TBARS was found to increase after prolonged drying and caused lipid peroxidation which could be attributed to the decreases in SOD and CAT activities. These decreased activities induced by severe drought stress favour accumulation of O_2^- and H_2O_2 which can result in lipid peroxidation (Fu and Huang 2001). In this study, V_1 showed high TBARS content in control (Fig. 2B). V_2 showed low TBARS content in control and high TBARS content under treatments. The low/high TBARS and H_2O_2 contents coincide with high SOD, POX, CAT and PPO activities. This indicates that at an early stage of drought (24h) lipid peroxidation does not occur and there is lack of accumulation of O_2^- and H_2O_2 and membrane damage because SOD scavenges O_2^- radicals and catalase eliminates H_2O_2 breaking it into water and oxygen.

H_2O_2 is a toxic compound produced as a result of scavenging of superoxide radical, and its higher concentration is injurious to cell/plant resulting in lipid peroxidation and membrane injury. Sairam *et al.* (1998) report low H_2O_2 content and TBARS content in genotype C 306 under water-stress in comparison to susceptible HD 2329. In this study V_1 seedlings exhibited high H_2O_2 (Fig. 2C) and low TBARS content in comparison to V_2 seedlings which had low H_2O_2 and high TBARS content (Fig. 2B).

Increased proline content was reported in water-stressed plants (Kramarov *et al.* 1999). The proline content (Fig. 2D) was found to increase under both stresses in V_1 and V_2 seedlings at both stages. However, the proline content of V_2 plants at both stages was significantly higher than V_1 plants.

In conclusion the *Cassia* species were able to adapt themselves to water and UV-B stresses by increasing the activities of antioxidant enzymes. A co-ordianted response involving the high activities of SOD, CAT, POX, PPO

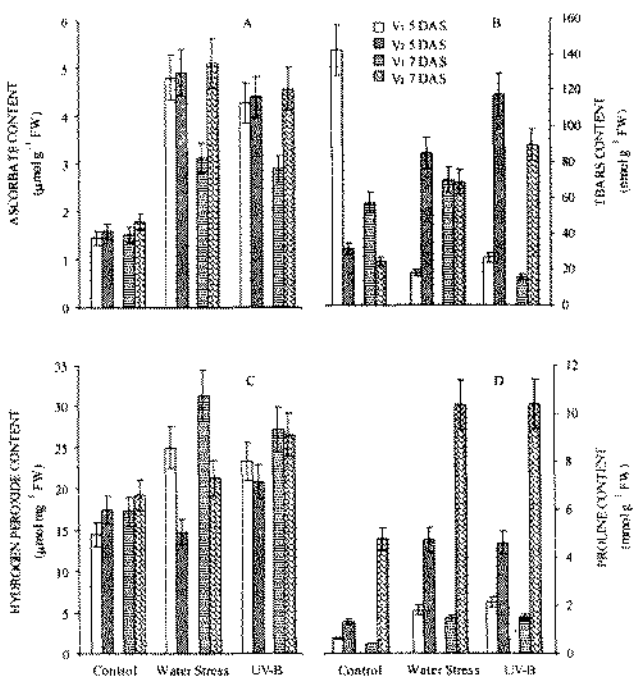

Fig. 2. Effect of water stress and UV-B stress on ascorbate (A), TBARS (B), hydrogen peroxide (C) and proline (D) contents in seedlings of *C. angustifolia* (V_1) and *C. auriculata* (V_2). Vertical bars indicate ± SD values of 3 replicates from 2 independent experiments.

and ASA is triggered in V_1 and V_2 seedlings to prevent oxidative stress. It is apparent that not only superoxide radical scavenging enzyme SOD is important but also the H_2O_2 scavenging enzymes CAT and POX are also equally important in imparting tolerance against drought and UV-B stress induced oxidative stress. The high activity of PPO might also participate in the enhanced tolerance to oxidative stress.

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