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Changes in the Antioxidant Enzymes and Lipid Peroxidation in Betel Vine (*Piper betel* L.) Subjected to Water Stress

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ABSTRACT

An experiment was conducted to study the effect of water deficit stress on the antioxidant enzyme activity in betel vine (*Piper betel* L.) cv. 'SGM1'. There was a significant increase in the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) in the leaves and roots subjected to water stress. The increased activity was higher in the leaves than the roots collected both on the 5th and 10th day after water deficit treatment. The activity of catalase was much higher in the roots than leaves suggesting that catalase activity was the major stress-coping mechanism in roots. With increasing stress levels and duration of stress, membrane damage (lipid peroxidation) increased.

Keywords: catalase, leaf, peroxidase, root, SOD

INTRODUCTION

Betel vine (*Piper betel* L.) belongs to the dicotyledonous family Piperaceae. It is a perennial dioecious plant. The family is of considerable economic importance because of the presence of essential oils in fruits and leaves of many species. Betelvine is second to *Piper nigrum* L. in economic importance. There are about 100 varieties of betel vine in the world, of which about 40 are found in India and 30 in West Bengal. The betel vines (usually the male plants) are cultivated throughout India except for the dry northwestern parts in about 55,000 ha of land and about 66% of production is contributed by the state of West Bengal. It grows best under shaded, tropical forest ecological conditions with a rainfall of about 2250-4750 mm, relative humidity of 40-80% and temperature ranging from 15-40°C (Guha 2006). The oil of betel vine leaf is made up of a mixture of about 21 different compounds whose chief constituent is eugenol making up about 29.5% of the oils. About 15-20 million people consume betel vine leaves in India on a regular basis and taking into account those in other countries around the world, the total may exceed 2 billion consumers (Jeng *et al*. 2002). Leaves worth about Rs 30-40 million are exported from India to countries like Bahrain, Canada, Great Britain, Hong Kong, Italy, Kuwait, Nepal, Pakistan, Saudi Arab and many other European countries (Guha 2006). Betel vine leaf is traditionally used for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries and the root is known for its female contraceptive effects (Chopra *et al*. 1956). The leaves are an antiseptic and carminative, curing cough, cold, pains and sores in the throat and chest. The leaves also cure indigestion, stomach ache, diarrhoea, cholera, tuberculosis, scales, burns, swelling, bruises, respiratory disorders, and leukemia (Jana 2006).

The deficit of water is the single and most important factor limiting plant growth and yield. Plants subjected to water stress imposed by water deficit, i.e. drought, undergo increased exposure to activated forms of oxygen and accumulation of free radicals associated with damage to the membrane and lipid peroxidation. Water stress leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals which are highly reactive and may cause cellular damage through the oxidation of lipids, proteins and nucleic acids (McKersie and Leshem 1994; Pastori and Foyer 2002; Apel and Hirt 2004; Costa *et al*. 2005). ROS interact with a wide range of molecules causing pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and DNA mutation (Mittler 2002). To minimize the effect of oxidative stress, plant cells have evolved a complex antioxidant system, which is composed of low-molecular mass antioxidants (glutathione, ascorbate, carotenoids and α-tocopherol) as well as ROS-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) (Sairam *et al*. 2000; Alscher *et al*. 2002; Apel and Hirt 2004).

MATERIALS AND METHODS

Betel vine cv. 'SGM1' cuttings 12-15 cm long and 8-10 mm in diameter with 3 to 4 active nodal buds were obtained from the Sugarcane Research Institute (ICAR), Sirugamani, Tiruchirappalli and maintained in earthen pots containing 4 kg of air dried soil and farmyard manure (3:1). The pots were maintained in a greenhouse at 16/8 hr light/dark provided by fluorescent tubes (Phillips, India) of 200 μmol/m^2/s, 25 ± 2°C/15 ± 2°C day/night temperatures and 60 ± 5% relative humidity. After five weeks of culture, the pots were divided into five sets, each of which contained three replicates. One set was maintained as control, which was irrigated with 100% of the water holding capacity of the soil once daily. The treatments made up of 75, 50, 25 and 12.5% of water quantity of the control water supply were named as mild, moderate, severe and very severe treatments. The treatment pots were irrigated once every two days with the mentioned quantity of water for respective treatments. The pots were maintained under control and water deficit stress conditions for a period of 10 days and analyzed for the antioxidant enzymes and for lipid peroxidation at the 5th and 10th day.

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Original Research Paper

Leaf and root samples (0.5 g) collected from both control and treatment conditions were washed in distilled water. The samples were homogenized with 10 ml of ice cold 100 mM phosphate buffer (pH 7.5) containing 0.5 mM EDTA with a pre-chilled pestle and mortar. The homogenized samples were centrifuged for 15 min at 4°C with 15,000 × *g*. The supernatant was collected and stored at 4°C until used for the assay of enzymes. For APX extraction, 2 mM ascorbate and 5% polyvinyl pyrrolidine (PVP) were added to the phosphate buffer and EDTA at the same concentration which was used for the extraction of other enzymes. SOD activity was analyzed as per the method of Sen Gupta *et al*. (1993). In a test tube 3 ml of the reaction mixture was added to 0.1 ml of 60 µM riboflavin and the tubes were exposed to 15W fluorescent lamps for 15 min. The reaction mixture consists of 0.1 ml of 200 mM methionine, 0.01 ml of 2.25 mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml of distilled water and 0.05 ml enzyme extract. After 15 min, the reaction was stopped by covering the tubes with black cloth. The non-irradiated samples served as blank. Absorbance was taken at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking the enzymes. CAT activity was measured according to Aebi (1984). Three ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH 7), 0.5 ml of 75 mM H_2O_2, 0.05 ml of enzyme extract, and 0.95 ml of distilled water was maintained for 1 min and the absorbance was read at 240 nm immediately after the addition of H_2O_2 and 1 min later. The amount of H_2O_2 decomposed was used for calculating the enzyme activity. APX activity was measured as per the method of Yoshimura *et al*. (2000). Three ml of reaction mixture containing 25 mM phosphate buffer (pH 7), 0.5 ml of 0.1 mM EDTA, 0.1 mM H_2O_2, 0.25 mM ascorbate and 0.05 ml enzyme mixture was used. The reaction was started with the addition of 0.1 mM H_2O_2. Decrease in absorbance for a period of 30 sec was done at 290 nm. Enzyme activity is expressed by the decrease in ascorbic acid content by comparing with a standard curve drawn with known concentrations of ascorbic acid. GR activity was analyzed as per the method of Sarin *et al*. (2002). The reaction mixture containing 1 ml of 0.2 M phosphate buffer (pH 7.5), 1 ml of 0.1 mM EDTA, 0.5 ml of 3 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and 0.2 ml distilled water. The reaction mixture was added to 0.1 ml of 2 mM oxidized glutathione (GSSG). The absorbance was measured at 412 nm at 25°C immediately and after 1 min after the addition of oxidized glutathione. Melanodialdehyde (MDA) amount was measured as per the method of Stewart and Bewley (1980). The leaf and root samples (0.5 g) were homogenized in 5 ml of distilled water. With the homogenate 5 ml of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added and the mixture was incubated at 95°C for 30 min the reaction was stopped by putting the tubes in cold water. The samples were centrifuged at 10,000 × g for 30 min the supernatant was taken for measuring the absorbance at 532 nm and the amount of non-specific absorption at 600 nm value was subtracted from this value.

For the analysis of enzyme activity and MDA assay, triplicates were maintained to avoid experimental errors.

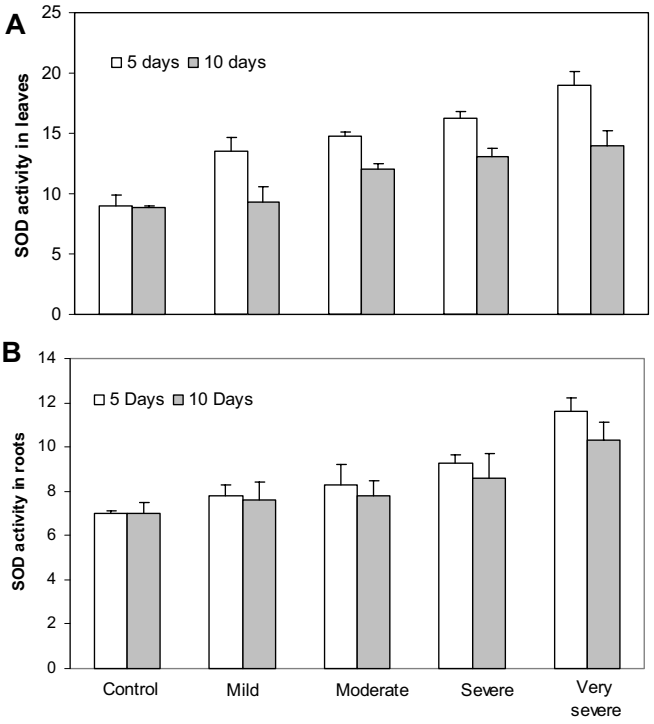

Fig. 1 Effect of water stress on superoxide dismutase (SOD) activity (Units: min^{-1}. mg^{-1} protein) in the leaves (**A**) and roots (**B**) of betelvine (*Piper betel* L.) cv. 'SGM 1' after 5 and 10 days of treatment. Values represent mean ± Standard Error (SE).

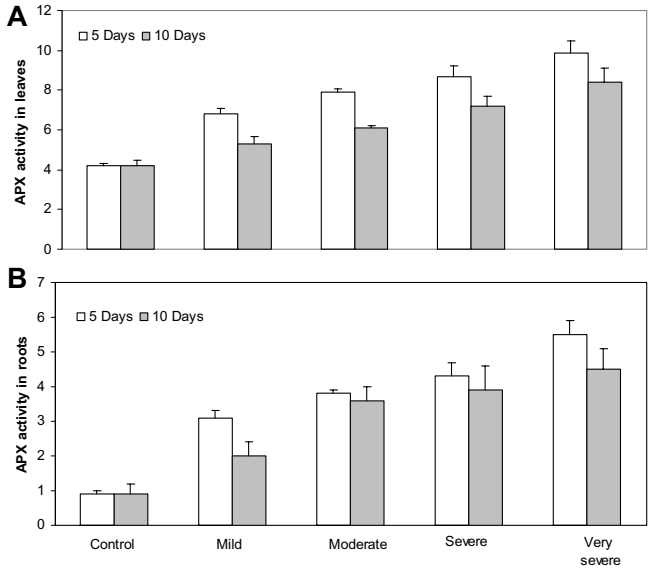

Fig. 2 Effect of water stress on ascorbate peroxidase (APX) activity (µmol Ascorbate oxidized min^{-1}. mg^{-1} protein) in the leaves (**A**) and roots (**B**) of betelvine (*Piper betel* L.) cv. 'SGM 1' after 5 and 10 days of treatment. Values represent mean ± Standard Error (SE).

Statistical analysis

The experiment was laid out in a complete randomized block design with three replications and the experiment was repeated three times each in three different periods at four month interval. Each data point represents the mean of three replicates analyzed thrice and each value is therefore the mean of nine estimations (n = 9) and then subjected to statistical analysis (Standard Error calculated at P = 0.2).

RESULTS

The leaf-SOD activity was comparatively high than the root-SOD activity at both five and ten days after stress exposure (**Figs 1A, 1B**). SOD activity decreased with an increase in the duration of stress exposure even with the same amount of stress. Activity of catalse was observed high in the root system than in the leaf, suggesting that catalase expression may be the important stress coping mechanism in the root. APX and GR activities were also increased with increase in the stress at 5 days exposure (**Figs 3, 4**).

The activities of scavenging enzymes (CAT, APX, SOD and GR) increased than the control when stress treatment was given for ten days, but compared to five days treatment, the activity of all the enzymes decreased (**Figs. 1-4**). This decreased activity of antioxidant enzymes associated with an increase in lipid peroxidation in both the leaf and root at ten days stress treatment. Lipid peroxidation rate was high in the root than in the leaf, moreover, increased lipid peroxidation was observed with severity of stress and long expo-

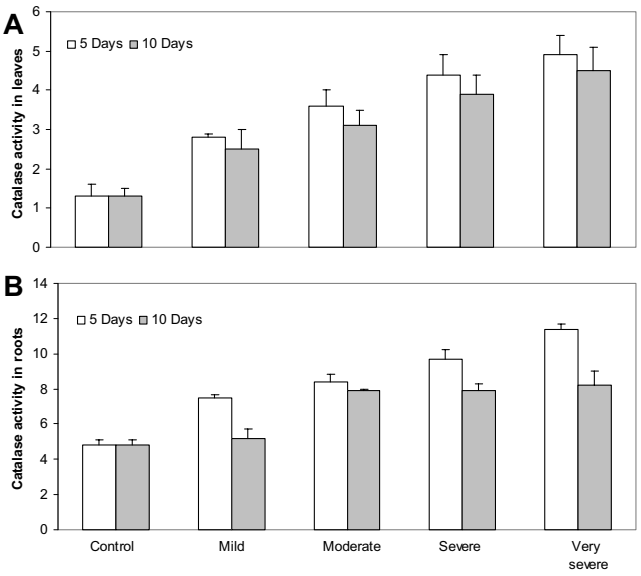

Fig. 3 Effect of water stress on catalase activity (µmol H_2O_2 reduced min^{-1}. mg^{-1} protein) in the leaves (**A**) and roots (**B**) of betelvine (*Piper betel* L.) cv. 'SGM 1' after 5 and 10 days of treatment. Values represent mean ± Standard Error (SE).

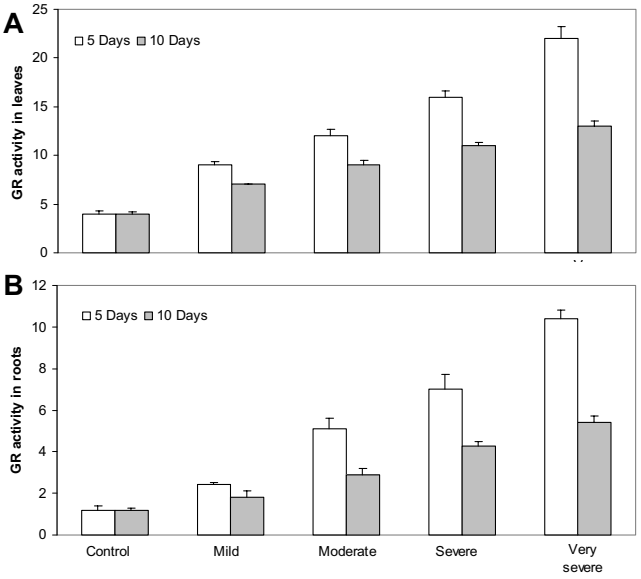

Fig. 4 Effect of water stress on glutathione reductase (GR) activity (µmol NADPH reduced min^{-1}. mg^{-1} protein) in the leaves (**A**) and roots (**B**) of betelvine (*Piper betel* L.) cv. 'SGM 1' after 5 and 10 days of treatment. Values represent mean ± Standard Error (SE).

sure duration (10 days) (**Fig. 5**).

DISCUSSION

The oxidative damage to the cellular components is limited under normal growing conditions due to efficient processing of ROS through a well coordinated and rapidly responsive antioxidant system consisting of several enzymes and redox metabolites. Under various abiotic stresses the extent of ROS production exceeds the antioxidant defense capability of the cell, resulting in cellular damage (Almeselmani *et al*. 2006). Water stress like other abiotic stresses produce ROS, which was converted into H_2O_2 by the activity of SOD (Alscher *et al*. 2002) and H_2O_2 was converted into water by catalases (Fridivich 1989) and peroxidases (Yoshimura *et al*. 2000; Beak and Skinner 2003; Gara *et al*. 2003; Rizhsky *et al*. 2003). Enhancement of SOD and APX activity will increase oxidative stress tolerance (Sen Gupta *et al*. 1993). Higher activity of APX diminished the lipid peroxidation level (Candan and Tarhan 2003). MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes which increase with environmental stresses. Lipid peroxidation is linked to the activity of antioxidant enzymes, i.e., with an increase of SOD, APX, GPX, CAT, etc., oxidative stress tolerance is enhanced and MDA is decreased (Esfandiari *et al*. 2007). There were negative relationships between SOD activity and lipid peroxidation or MDA level. As indicated in figures, when SOD activity was high, ROS, especially superoxide radical scavenging was done properly and oxidative stress decreased, thus tolerance to oxidative stress increased. Water stress increased the superoxide level in cells, if this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler 2002; Esfandiari *et al*. 2007).

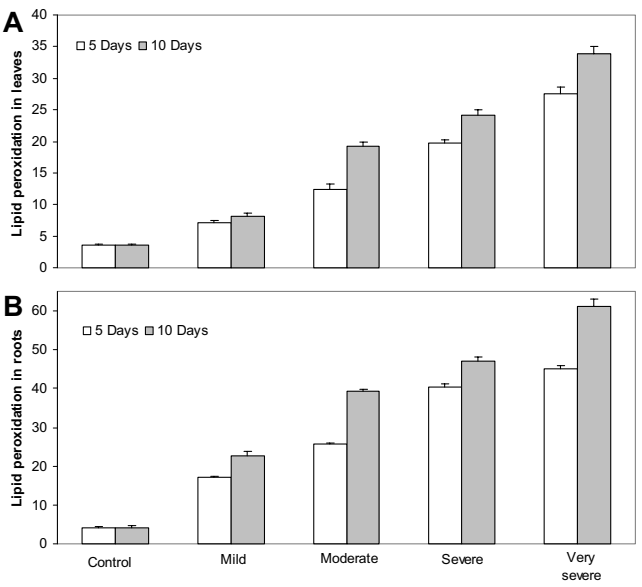

Fig. 5 Effect of water stress on lipid peroxidation (nmol MDA. g^{-1} fresh weight) in the leaves (**A**) and roots (**B**) of betelvine (*Piper betle* L.) cv. 'SGM 1' after 5 and 10 days of treatment. Values represent mean ± Standard Error (SE).

Lipid peroxidation has been used as an indicator of oxidative stress as reported in mulberry (Sudhakar *et al*. 2001), *Lycopersicon pennellii* (Mittova *et al*. 2002), sugar beet and wild beet (Bor *et al*. 2003) subjected to salinity stress, in wheat (Shao *et al*. 2005; Esfandiari *et al*. 2007) and *Arabidopsis* (Munne-Bosch and Alegre 2002) subjected to water stress, in rice (Verma and Dubey 2003) subjected to heavy metal stress and in wheat (Sairam *et al*. 2002; Almeselmani *et al*. 2006), bentgrass (Liu and Huang 2000) and rice (Uchida *et al*. 2002) subjected to heat stress. Mohan *et al*. (1990) reported that GR plays an important role in the protection of plant from both high and low temperature stresses by preventing the oxidation of enzymes and membranes. The coordinate function of antioxidant enzymes such as SOD, APX, catalase and GR helps in procession of ROS and regeneration of redox ascorbate and glutathione metabolites (Wise 1995; Foyer and Nector 2000).

Relatively higher activities of ROS-scavenging enzymes have been reported in tolerant genotypes when compared susceptible ones, suggesting that the antioxidant system plays an important role in plant tolerance against environmental stress (Costa *et al*. 2005). In the present study, we observed increased antioxidant scavenging enzymes with increase in water stress but we found reduction in the activity of SOD and CAT under prolonged exposure to water stress. In addition to the SOD and CAT activity, the activity of APX and GR were also affected by the water stress in betelvine. Here also we found the similar trend i.e., the activity of these enzymes increased with increase in stress level during five days of exposure whereas at ten days exposure the plant root and leaf were affected with reduced activity of ROS-scavenging enzymes as indicated by MDA level. Similar to our results, salt stress induced increased the acti-

vity of APX and GR in sugar beet and wild beet (Bor *et al*. 2003) and in mulberry (Harinasut *et al*. 2003). Similarly, under heat stress, levels of catalase have been shown to drop in wide species (Dat *et al*. 2000; Jiang and Huang 2001) and the activity of SOD also decreased in bentgrass under high temperature stress (Liu and Huang 2000).

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