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Membrane Damage in an Oxygen-Free Radical-Dependant Manner induced via Boron Deficiency and Toxicity in Maize

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ABSTRACT

Maize (*Zea mays* var. '32-A09') plants grown with variable boron (B) supply ranging from deficiency to toxicity (0.033, 0.33, 0.66, 3.3, 33 mg B L^{-1}), were investigated for the concentration of antioxidants and activities of antioxidative enzymes in leaves. Plants subjected to deficiency and excess B supply showed retarded growth and characteristic B stress symptoms. Chlorophyll *a*, *b* and carotenoids decreased under B stress at both stages i.e., 26 and 38 days after treatment. There was lipid peroxidation as indicated by the high MDA content and accumulation of H_2O_2 under B stress. The activities of SOD, APX, GR, CAT and POX increased both under deficiency and toxicity. The concentration of total non-protein thiols increased under B stress and ascorbate decreased under B deficiency. DHA concentration decreased under B deficiency and increased under B toxicity in leaves at both treatment stages. There was also an accumulation of phenols with enhanced PPO activity under B deficiency and toxicity.

Keywords: antioxidants, boron stress, oxidative stress, *Zea mays*

INTRODUCTION

Boron (B) is an essential micronutrient for higher plants. Although its requirement in plants is very low, it has been assigned important roles in many metabolic processes (Du and Wang 1999). Boron is important in crop production both from view point of its effects in deficiency and excess. The deficiencies as well as toxicities of boron occur in a wider range of crops and climatic conditions than that of any other micronutrient element (Camacho-Cristóbal *et al.* 2008). In plants, boron deficiency is the most wide spread of micronutrient deficiencies (Blevins and Lukaszewski 1998). In soil solution boron is mainly present in the form of boric acid [$B(OH)_3$], which is easily leachable under high rainfall (Yan *et al*. 2006; Camacho-Cristóbal *et al*. 2008) leading to a deficiency in plants. Contrary to this under low rain fall conditions it accumulates in the soil up to a level which becomes toxic to plants. Boron toxicity is very often observed in arid and semi arid areas (Reid 2007; Tanaka and Fujiwara 2008). Primary cell wall structure and membrane functions are closely linked to boron nutrition. The property of boron to form diester bonds with diol groups' polysaccharides and occurrence of a large proportion of boron in cell walls (O'Neill *et al*. 2004; Herrer-Rodriguez *et al*. 2010), is suggestive of a role of boron in cell walls. Boron is also required for the maintenance of the structure and functions of membranes especially plasmamembrane (Goldback *et al.* 2001; Brown *et al.* 2002; Goldbach and Wimmer 2007). Cakmak *et al.* (1995) and Wang *et al*. (1999) reported that boron deprivation alters the plasma membrane permeability for ions and other solutes. Boron is involved in metabolism of phenolics (Camacho-Christóbal *et al.* 2005). During boron deficiency PPO leads to production of quinones which are known to be highly toxic and responsible for the formation of oxygen free radicals (H_2O_2, O_2^-, and OH^-) (Cakmak and Römheld 1997). Under boron toxicity, the activities of some enzymes involved in phenolic metabolism, such as PPO peroxidase increased. These enzymes can react with flavonoids to produce semiquinones capable of detoxifying different types of ROS generated under stress (Pourcel *et al*. 2007; Cervilla *et al.* 2012).

One of the biochemical changes occurring when plants are subjected to environmental stresses is the production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals (Dat *et al.* 2000; Mittler *et al.* 2002; Halliwell 2006). These free radicals are very toxic and cause damage to the membrane proteins and lipids resulting in oxidative damage of membranes (Møller *et al*. 2007). In order to avoid the harmful effects of these reactive molecules, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes (Gill and Tuteja 2010) such as ascorbate (Asc), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascobate reductase (MDAR) dehydroascorbate reductase (DHAR) and glutathione reductase (GR).

Earlier boron stress induced oxidative damage was studied by Molassiotis *et al.* (2006) in apple rootstock EM9, Cervilla *et al.* (2007, 2012) in tomato, Gunes *et al.* (2007, 2009) in wheat and bean and Han *et al.* (2009) in *Citrus* and recently by Hajiboland *et al.* (2011) in tea and Metwally *et al.* (2012), in wheat. Although studies have been carried out on boron deficiency/toxicity in plants, these studies have been restricted to a particular stage and give no information about the antioxidant status at initiation of stress and at severe stages of boron stress in plants and its relation with phenol metabolism. Also earlier reports given by Pfeffer *et al.* (1998) under deficiency and Karable *et al.* (2003) under toxicity of boron suggested the boron involvement in the membrane damage in an oxygen free radical independent manner. Hence, it was proposed to study the effect of boron deficiency as well as toxicity on phenol metabolism in relation with membrane damage and enzymic and non-enzymic antioxidant molecules at initial and severe stages of development of boron deficiency and toxicity in maize plants.

In the present study, we described the changes in concentrations of non-enzymic antioxidants (carotenoids, ascorbate, dehydroascorbate, non protein thiols), and activity of antioxidative enzymes- SOD, CAT, APX, GR, non specific peroxidases (POX) and polyphenol oxidase (PPO) in

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the leaves of maize subjected to variable boron supply ranging from deficiency to toxicity (0.033 to 6.6 mg B L^{-1}). To find out the relation of membrane damage with antioxidative responses under boron deficiency as well as toxicity the concentrations of H_2O_2, lipid peroxidation and phenols were also studied.

MATERIALS AND METHODS

Plant culture

The seeds of maize (*Zea mays* var. '32-A09') were surface-sterilized with 5% (v/v) mercuric chloride solution and washed properly with deionised 'Manesty' stainless still water (MSW) before sowing. The composition of nutrient solution supplied was: 4 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.1 mM FeEDTA, 10 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 0.1 mM NaCl, 0.1 µM $CoSO_4$ and 0.1 µM $NiSO_4$ with varying levels of boron supply ranging from deficiency to toxicity (0.033, 0.33, 0.66, 3.3 and 6.6 mg B L^{-1}). All experimental studies were conducted in a glasshouse under controlled conditions of light, humidity and temperature. During the period in which the experiment was conducted light (PAR) ranged between 980 and 1120 µmol m^{-2} s^{-1} at 12:00 noon, relative humidity ranged between 68 and 90% at 9:30 am and maximum and minimum temperature ranged between 34 to 42°C and 25 to 28°C.

All the biochemical studies were carried out in triplicates in the young leaves (three from top) at two stages after treatment: at 26 days after treatment (DAT) when symptoms of boron treatment had initiated and at 38 DAT when symptoms became conspicuous.

Chlorophyll, MDA, H_2O_2, phenols and antioxidants

Chlorophyll (Chl) and carotenoids (Car) in leaves were extracted in 80% acetone by the method of Lichtenthaler (1987). Finely chopped leaves (1 g) were ground in pestle and mortar in 80% acetone. To prevent denaturation of the Chl, a pinch of calcium carbonate was added during grinding. The extract was centrifuged at 5000 × *g* in a refrigerated centrifuge and spectrophotometric measurements were made at 480 and 510 nm for car and 645 and 663 nm for Chl. The results were expressed as mg g^{-1} fresh weight of leaf tissue and the formula used for the determination were as given below:

$$\text{Chlorophyll a} = [12.7(A_{663}) - 2.63\ (A_{645})] \times V/1000 \times Wt\ (g)$$

$$\text{Chlorophyll b} = [22.9(A_{645}) - 4.48\ (A_{663})] \times V/1000 \times Wt\ (g)$$

$$\text{Carotenoids} = [7.6(A_{480}) - 1.49\ (A_{510})] \times V/1000 \times Wt\ (g)$$

In the above formula A is the OD at wavelength indicated and V is the total volume.

Lipid peroxidation was measured in terms of malondialdehyde (MDA) formation (Heath and Packer 1968). Fresh leaves were homogenized with 0.1% trichloroacetic acid and centrifuged at 10,000 × *g* for 5 min. The supernatant was treated with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and the mixture was incubated at 95°C in water bath for 30 min. Samples were cooled immediately in ice bath and centrifuged at 10,000 × *g* for 10 min. The absorbance was read at 532 nm and adjusted for non-specific absorbance at 600 nm. The concentration of MDA was estimated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) was estimated by method of Brennan and Frenkel (1977). Finely chopped leaves were ground in chilled pestle mortar in acetone and centrifuged at 10,000 × *g*. The pellet was discarded. To the supernatant, titanium tetrachloride was added. The precipitate formed was solubilized in chilled liquid ammonia and centrifuged. Residue was washed with acetone to remove chlorophyll. The residue was dissolved in 2N H_2SO_4 and the color intensity was read at 415 nm. The results have been expressed as µmol H_2O_2 g^{-1} fresh weight.

For the estimation of phenols, plant material was fixed in boiling 80% ethanol in the proportion of 1:10. The fixed material was crushed in a pestle and mortar. Alcohol soluble and insoluble fractions were separated by repeated centrifugation at 800 × *g*. In the alcohol soluble fractions, phenols were estimated by the method of Swain and Hillis (1959). From the alcohol soluble material pigments were removed with the help of lead acetate and potassium oxalate. Suitable aliquots were drawn from the filtrate and made to 7.5 ml volume with distilled water. The solution was then treated with 0.5 ml Folin's reagent for 3 min and 1 ml of super saturated solution of sodium carbonate. After 1 h, the colour intensity was measured. Results have been expressed as µg total phenol 100 mg^{-1} fresh weight. The standard curve was prepared using different concentrations (10-100 µg) of phenol.

Ascorbate (Asc) was assayed according to the method of Law *et al*. (1983) by extracting fresh leaf tissue in 10% TCA. After adding NaOH, the extracts were centrifuged for 5 min in a microfuge, and to the supernatant, 150 mM phosphate buffer pH 7.4 was added. The color was developed by adding 10% TCA, 44% orthophosphoric acid, 4% bipyridyl in 70% ethanol and 3% ferric chloride. The samples were incubated at 37°C for 40 min and color developed was read at 525 nm. Total ascorbate was determined in the supernatant by reduction of dehydroascorbate (DHA) to Asc by 10 mM dithiothreitol and 0.5% N-ethylmaleimide which was added after 15 min. Amount of ascorbate was determined by preparing a standard curve with L-ascorbic acid (Sigma-Aldrich, Bangalore, India).

Total non-protein thiols were estimated by the method of Boyne and Ellman (1972). Finely chopped fresh leaf tissues were ground in chilled medium with 5% sulphosalicylic acid and homogenate was centrifuged at 10,000 rpm for 10 min. The reaction was carried out with the use of 10 mM DTNB and 0.1 mM GSH (glutathione reduced). The colour intensity of extract was measured in a spectrophotometer at 412 nm within 15 min.

Enzymes

Polyphenol oxidase (EC 1.14.18.1) (DOPA oxidase) was assayed by the method of Shenshi and Noguchi (1975). The enzyme was extracted in 0.01M phosphate buffer pH 7.5 containing 0.2 M KCl, 0.001 M EDTA, 0.01 M sodium ascorbate and 1 g of polvinylpyrrolidone (PVP 30). The material was ground in a pestle mortar in an ice bath and was centrifuged at 10000 × g for 20 min at 4°C. The supernatant was used for the enzyme assay. The assay mixture for polyphenol oxidase contained 0.1 M phosphate buffer pH 6.5 and suitably diluted enzyme extract. The reaction was initiated by the addition of 0.01 M DL-DOPA (3,4-dihydroxy 1-phenol alanine). The reaction was allowed to proceed for 30 min at 30°C and 0.25 M lead acetate was added to stop the reaction. The contents were centrifuged and optical density of the supernatant was measured in a Perkin Elmer UV/Vis Lambda Bio 20 spectrophotometer (Rotkreuz, Switzerland) at 470 nm.

The activities of enzyme catalase (CAT) and peroxidase (POX) were assayed in the fresh leaf tissue extracts (10%) prepared in glass distilled water. Catalase (EC 1.11.1.6) was assayed by an adaptation of the permanganate titration method described by Pandey and Sharma (2002). Peroxidase (EC 1.11.1.7) was assayed by addition of suitable enzyme extract to a reaction mixture containing 0.1 M phosphate buffer pH 6.0, 0.01% H_2O_2 and 0.5% *p*-phenylene diamine. Reaction was stopped by adding 4 N H_2SO_4. The colour intensity was read at 485 nm.

For assay of antioxidant enzymes, fresh leaves (were homogenized with 150 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA and 2% PVP (for APX 1 mM of ascorbate was also added) for assaying SOD, APX and GR. The homogenate was centrifuged at 15,000 × *g* for 10 min and the supernatant was used as the enzyme preparation. All enzyme preparations were made at 4°C.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by measuring the ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in a reaction mixture containing 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0 to 50 µl enzyme extract. Riboflavin was added last and tubes were illuminated for 10 min. Blanks were not illuminated and the above reaction mixture without the enzyme extract developed the maximum color at 560 nm. One unit of SOD represents the amount that inhibits the NBT reduction by 50% (Beauchamp and Fridovich 1971). Ascorbate peroxidase (EC 1.11.1.11) was determined as per the method

of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.5 mM ascorbate and 0.1 mM hydrogen peroxide. Oxidation of ascorbate was followed as fall in absorbance per min. at 290 nm after adding hydrogen peroxide. The amount of ascorbate oxidized was calculated by using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. The glutathione reductase (EC 1.6.4.2) assay was performed in a reaction mixture containing 100 mM phosphate buffer pH 7.0, 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH and 25 to 50 µl of the enzyme extract. The oxidation of NADPH was followed by monitoring the decrease in absorbance per min. at 340 nm. The amount of NADPH oxidized was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1} (Madamanchi *et al*. 1992).

Statistical analysis

Data was analyzed in triplicate. Standard analyses of variance (ANOVA) were used to assess the significance of treatment means. The data are presented as mean values ± standard error (SE, n=3). Differences between treatments means were compared using LSD at the 0.05 probability level.

RESULTS

Plants grown under deficient and toxic supply of boron showed growth retardation and characteristic boron deficiency and toxicity symptoms. The appearance of white translucent streaks in the middle of the leaf lamina, failure of young emerging leaf to unroll, short and thick internodes and cessation of apical growth were observed under boron deficiency whereas chlorosis and irregular white patches in the laminar portion were observed under toxic boron supply. The severity of the toxic symptoms increased with increase in boron supply.

Concentration of Chl *a*, *b* and Car decreased at both stages under boron deficiency and toxicity. The decreased concentration of photosynthetic pigments was more pronounced at later stage of treatment especially under severe boron deficiency (0.033 mg B L^{-1}) (**Fig. 1**).

Both at 26 and 38 DAT lipid peroxidation was observed in plants supplied deficient and toxic boron. This was evident from the accumulation of MDA content in the leaves of plants grown at deficient (0.033 and 0.33 mg B L^{-1}) and toxic (3.3 and 6.6 mg B L^{-1}) levels of boron. Accumulation of MDA content was found to be more under severe toxicity of boron (6.6 mg B L^{-1}) supply at 38 DAT (**Fig. 2**).

The concentration of H_2O_2 in leaves at 26 DAT increased in plants subjected to deficient (0.033 and 0.33 mg B L^{-1}) and toxic (3.3 and 6.6 mg B L^{-1}) B supply and was more pronounced under severe boron deficiency (0.033 mg B L^{-1}) and toxicity (6.6 mg B L^{-1}). At 38 DAT the H_2O_2 concentration decreased under deficient and toxic supply (**Fig. 2**).

The total concentration of phenolic compounds in the leaves of the plants was found to be increased both under deficiency and toxicity of boron. The increase was more pronounced under deficiency of boron than toxicity at both 26 and 38 DATs, but the accumulation was more at 26 DAT (**Fig. 2**).

The concentration of ascorbate decreased significantly in leaves of plants supplied with deficient B supply (0.033 and 0.33 mg B L^{-1}) both at 26 and 38 DAT. However, the ascorbate concentration in plants supplied toxic boron decreased at 26 DAT but increased at 38 DAT (**Fig. 3**). The concentration of DHA decreased under deficiency (0.033 and 0.33 mg B L^{-1}) but increased under toxicity (3.3 and 6.6 mg B L^{-1}) at both stages of treatment (**Fig. 3**). Total non-protein thiols were found to be increased under boron deficiency and toxicity at both stages and this increase was more at 38 DAT (**Fig. 3**).

Increased CAT activity was observed both under deficiency and toxicity of boron and increase was more pronounced at 38 DAT under boron deficiency. Increased activity of POX under deficiency and toxicity of boron was more pronounced at 26 DAT. The activity of PPO increased

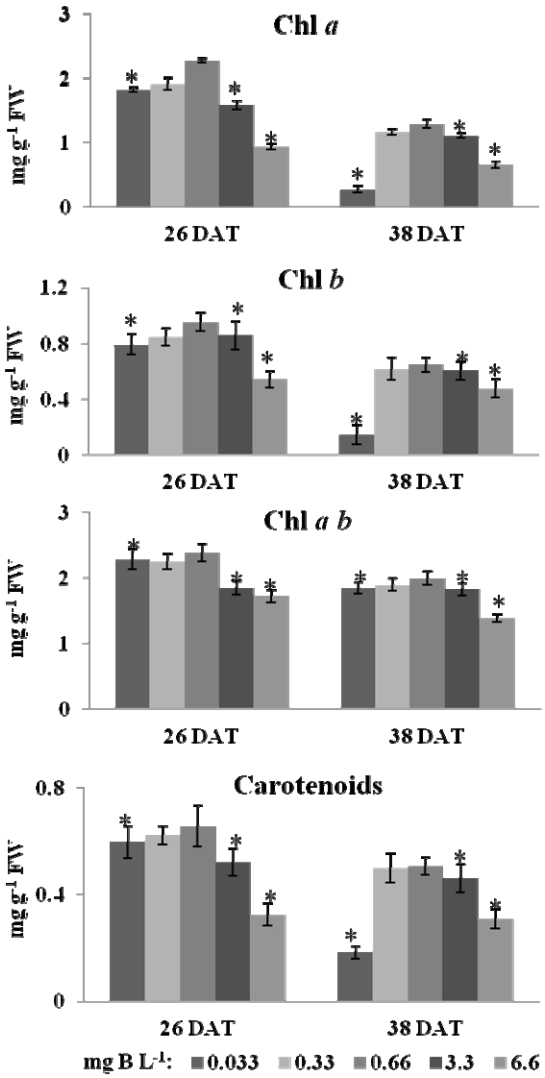

Fig. 1 Effect of boron stress on the concentration of photosynthetic pigments in maize (*Zea mays* var. '32-A09') leaves. Asterisks indicate significant difference at $P \leq 0.05$ from control (0.66 mg B L^{-1}) supply. Vertical bars indicate ±SE (n=3).

both at 26 and 38 DAT under deficient and toxic boron supply and was more pronounced under deficiency (**Fig. 4**).

The activity of SOD was found to be increased both under deficiency and toxicity of boron. This increase in activity was more or less similar at both days. Increased activity of APX was observed at both days and this increase in activity was more pronounced under severe deficiency and toxicity at earlier stage. GR activity was also found to be increased under deficient and toxic boron treatment and increase in this enzyme was more marked under deficiency at 26 DAT (**Fig. 5**).

DISCUSSION

The observed growth retardation and visual symptoms of B deficiency and toxicity indicated that the 0.66 mg B L^{-1} supply was optimum for growth of maize plants. Severe retardation in growth and visual deficiency effects was obtained in maize plants raised at deficient levels of B supply (below 0.66 mg BL^{-1}, particularly at 0.033 mg BL^{-1}). Besides reduction in growth, some of the effects of B deficiency in maize observed were, the appearance of white translucent streaks in the middle of the leaf lamina, failure of young emerging leaf to unroll, short and thick internodes and cessation of apical growth (Pandey and Archana 2009). Boron toxicity symptoms observed in maize plants - such as yellowing of veins and irregular white patches in leaves are similar to the earlier report of Krantz and Helsted (1964). The deficiency symptoms were found to appear in the

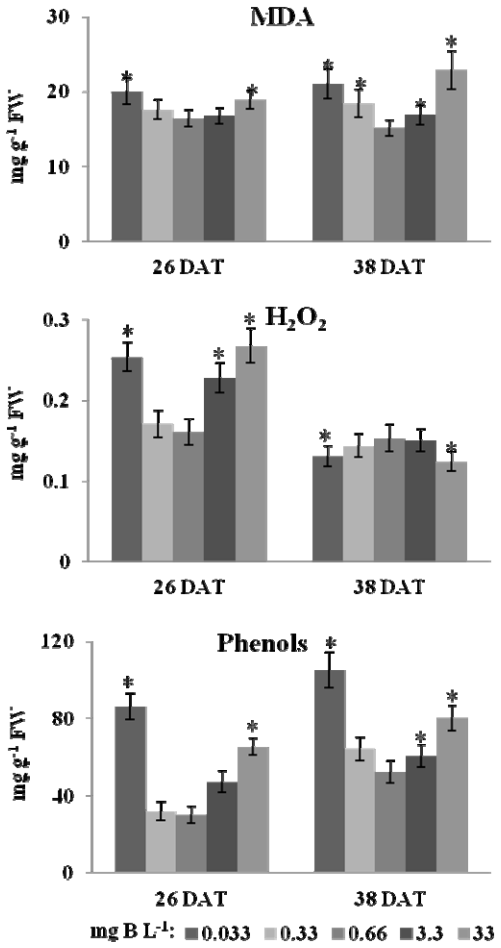

Fig. 2 Effect of boron stress on the concentration of MDA, H_2O_2 and phenols in maize (*Zea mays* var. '32-A09') leaves. Asterisks indicate significant difference at $P \leq 0.05$ from control (0.66 mg B L^{-1}) supply. Vertical bars indicate ±SE (n=3).

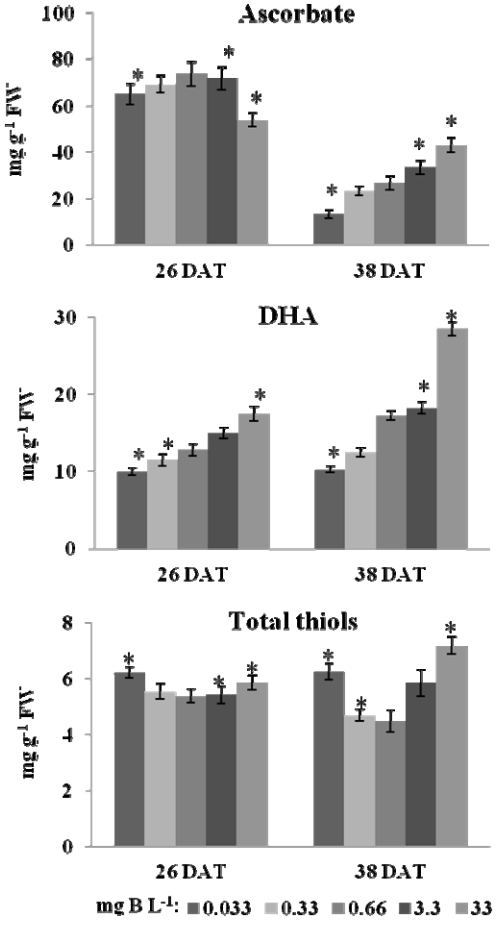

Fig. 3 Effect of boron stress on the concentration of Asc, DHA and total thiols in maize (*Zea mays* var. '32-A09') leaves. Asterisks indicate significant difference at $P \leq 0.05$ from control (0.66 mg B L^{-1}) supply. Vertical bars indicate ±SE (n=3).

younger leaves first where as toxicity symptoms appeared in the older leaves which suggested the immobile mode of translocation of boron in maize plants.

There was decrease in Chl *a* and Chl *b* pigments under boron deficiency and toxicity. A decrease in Chl *a*/*b* ratio observed both under deficiency and toxicity, indicated that Chl *a* concentration is more affected than Chl *b*. This might be due to a preferential degradation of Chl *a* over Chl *b*, probably because of closer association of Chl *a* with light harvesting reaction centres (LHC), which may cause Chl *a* to be destroyed to a larger extent than Chl *b* (Yamamoto and Bassi 1996). Wang *et al*. (2011) reported that under boron toxicity conditions, the amount of photosynthetic pigment in pear leaves was significantly reduced and the CO_2 assimilation rate appeared to reduce with the increase in the intercellular CO_2 concentration indicating the reduced photosynthetic capacity of pear leaves. Recently Metwally *et al*. (2012) also reported a gradual fall in photosynthetic pigments concentration (Chl *a*, *b* and Car) in wheat cultivar leaves grown under boron toxicity. Under boron deficient conditions Hajiboland *et al*. (2011) reported that reduction of photosynthesis in the young leaves of tea plants occurred mostly due to stomatal limitation whereas in older leaves it occurs mainly through reduction of leaf Chl *a*/*b* ratio.

Metwally *et al*. (2012) reported that excess boron concentration in the culture medium was found to induce inhibitory effects on carotenoids content in the sensitive cultivars of wheat compared with the tolerant one. This might be responsible for protection against oxidative damage, as carotenoids have ability to accept excitation energy from triplet chlorophyll and prevent singlet oxygen formation which in turn prevents photooxidative damage. Carotenoids have an important role in preventing the initiation of lipid peroxidation by ROS. A low content of carotenoids under boron deficiency and toxicity in leaves of maize is indicative of lipid peroxidation.

The concentration of H_2O_2 increased in the leaves of maize plants subjected to boron deficiency and toxicity at earlier stage but decreased at later stage. Mittler (2002) had proposed that membrane damage might be caused by high H_2O_2 levels, which could accelerate the Haber-Weiss reaction, resulting in hydroxyl radical (OH^-) formation. Hydroxyl radicals are most reactive species and able to react indiscriminately to cause lipid peroxidation, denaturation of proteins and mutation of DNA. Lipid peroxidation is the most common indicator of oxidative stress and is potentially harmful because the uncontrolled self-enhancing process causes disruption of membrane lipids and other cell components (Halliwell 2006). The lipid peroxidation, showed marked increase in maize plants investigated for boron deficiency and toxicity. Our findings are in accordance with Molassiostis *et al.* (2006) and Cervilla *et al.* (2007) who reported significant increase in MDA concentration in *in vitro* culture studies of apple and tomato respectively under boron toxicity. Han *et al*. (2009) also observed accumulation of TBA reactive contents in *Citrus* both under boron deficiency and toxicity. The observed increase in MDA and H_2O_2 concentration under boron deficiency and toxicity in maize is in accordance with increased antioxidative responses particularly increased total SOD activity that suggests increased generation of superoxide ion ($O_2^{\bullet-}$) in boron-stressed plants. Contrary to our results Karabal *et al.* (2003) found no relationship between the H_2O_2 concentration and lipid peroxidation in barley cultivars subjected to high boron levels and suggested that boron toxicity induced membrane damage in an oxidative stress

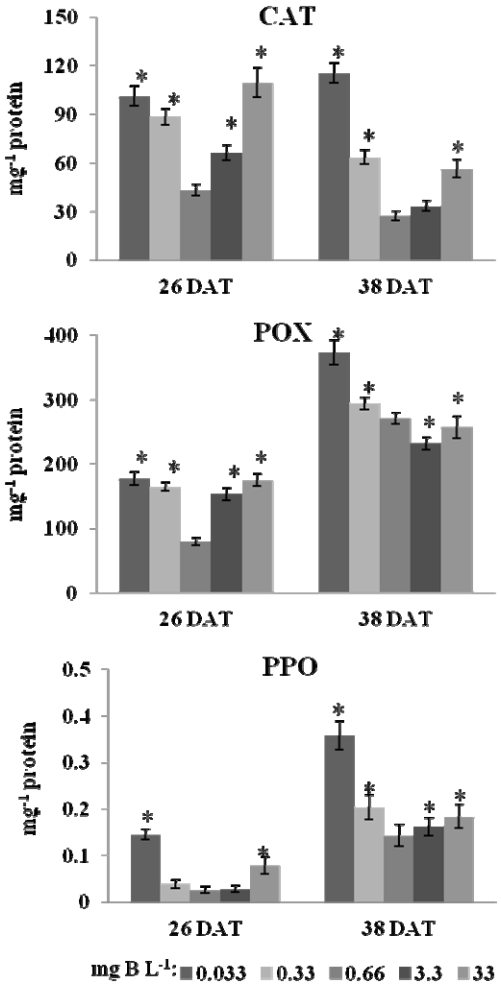

Fig. 4 Effect of boron stress on the activity of CAT, POX and PPO in maize (*Zea mays* var. '32-A09') leaves. Asterisks indicate significant difference at $P \leq 0.05$ from control (0.66 mg B L^{-1}) supply. Vertical bars indicate ±SE (n=3).

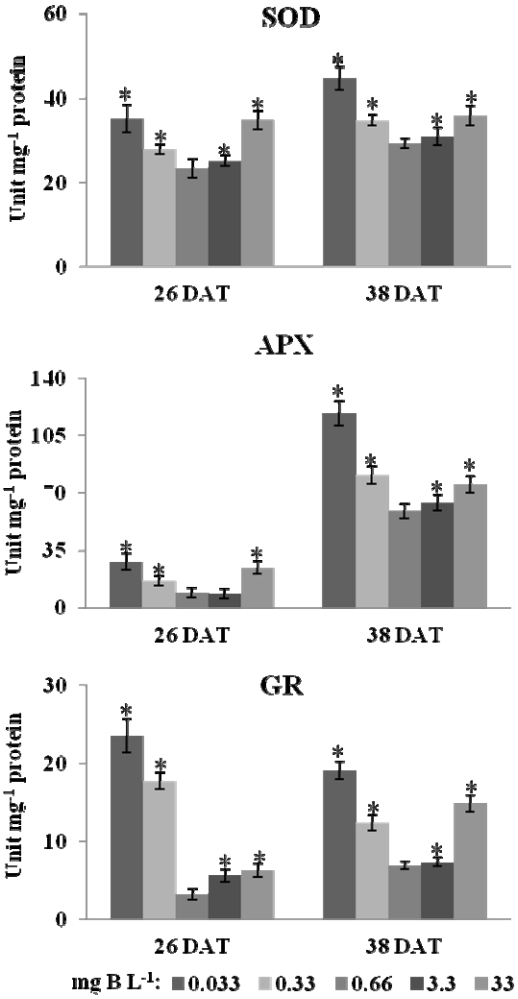

Fig. 5 Effect of boron stress on the activity of SOD, APX and GR in maize (*Zea mays* var. '32-A09') leaves. Asterisks indicate significant difference at $P \leq 0.05$ from control (0.66 mg B L^{-1}) supply. Vertical bars indicate ±SE (n=3).

independent manner. Similarly, Pfeffer *et al.* (1998) also reported no correlation of membrane damage with reactive oxygen species. Hajiboland *et al*. (2011) also reported increased MDA content in tea (*Camellia sinensis* L.) leaves with no significant changes in H_2O_2 concentration under boron deficiency. In accordance with the present results, others have found that excess boron increased both MDA and H_2O_2 concentrations in apple root stock (*Malus domestica* Borkh) (Molassiostis *et al*. 2006), grape (*Vitis vinifera* L.) (Gunes *et al.* 2006) tomato (*Solanum lycopersicum*) (Cervilla *et al.* 2007, 2012), pear (*Pyrus pyrifolia*) (Wang *et al*. 2011) and wheat cultivars (*Triticum aestivum* L.) (Metwally *et al*. 2012).

Ascorbate and reduced glutathione (GSH) are the main antioxidant components of the Halliwell-Asada cycle that operates in most plant cell organelles like mitochondria, chloroplasts and peroxisomes (Perl-Treves and Perl 2002). Ascorbate is an essential metabolite and performs multiple functions in plants. It acts as a primary antioxidant and reacts directly with hydroxyl radicals, superoxides and singlet oxygen and indirectly with H_2O_2 via the APX reaction. In addition, it also acts as a secondary antioxidant and reduces the oxidized form of membrane bound antioxidant α-tocopherol (Noctor and Foyer 1998). We observed a decreased concentration of ascorbate and DHA under boron deficiency. This diminished ascorbate pool might be responsible for over expression of $OH^{\bullet}$, $O_2^{\bullet-}$ and H_2O_2 which in turn cause oxidative stress under boron deficiency.

Increased ascorbate along with DHA concentration in plants is suggestive of an important role for this antioxidant against oxidative stress caused by boron toxicity. Cervilla *et al.* (2007) also observed an increase in DHA concentration along with increase of reduced ascorbate concentration in tomato subjected to boron toxicity. Increased ascorbate pool at later stage under toxicity in maize leaves suggested the scavenging capacity of plants over boron toxicity.

Total thiol (GSH+GSSG) concentration increased under deficient and excess boron supply which is in consonance with the observation by Han *et al.* (2009). On the contrary, Ruiz *et al.* (2003) reported decrease in thiol contents under excess boron supply. Cakmak and Römheld (1997) also reported the decreased concentration of GSH in boron-deficient plants, which is contrary to our findings. The increased concentration of total thiols under boron stress condition might be due to the overexpression of GR in the chloroplasts (Foyer *et al.* 1995; Han *et al.* 2009) which caused increase in the concentration of leaf GSH.

The non-enzymatic antioxidants – ascorbate and GSH – are the important components of the free radical scavenging system in plants. Wang *et al*. (2011) reported that with increasing concentrations of boron, the level of ascorbate and GSH was first increased and then decreased with further increase in boron concentration in culture medium in pear leaves.

Increased activity of SOD may be taken as index of enhanced generation of superoxide ($O_2^{\bullet-}$) in B stressed plants. The enhanced leakage of electrons responsible for production of superoxide ions ($O_2^{\bullet-}$) results from the electron transport system (ETS) depleted of its component. Leaves of maize plants showed enhanced SOD activity under boron deficiency which is in consonance with reports of Cakmak and Römheld (1997), Han *et al.* (2009) and

Hajiboland *et al*. (2011). The increase in activity of SOD under boron toxicity was earlier observed by Keles *et al.* (2004), Sotiropoulos *et al.* (2006), Gunes *et al.* (2006), Crevilla *et al.* (2007), Ardic *et al*. (2009) and Wang *et al*. (2011) but is contradictory to reports of Karabal *et al.* (2003) and Keles *et al.* (2004) who reported almost no change in leaf SOD activity under boron toxicity.

CAT, a H_2O_2 scavenging enzyme with relatively low affinity for H_2O_2 showed an increase in activity in boron deficient plants as reported by Agarwala *et al.* (1991) and contrary to the findings of Han *et al*. (2009). In our study the activity of CAT was also increased in boron toxic plants which contradicts the results of Keles *et al.* (2004), Molassiotis *et al.* (2006) and Gunes *et al.* (2006) who observed a decrease in CAT activity under boron toxicity. The activity of non-specific peroxidase (POX) a group of enzyme with Fe as a co-factor and concerned with different activities like detoxification of H_2O_2 and lignification of cell walls showed an increase in boron stressed plants. The observed increase in the activity of POX due to boron stress has also been reported by Molassiotis *et al.* (2006), Sotiropoulos *et al*. (2006), Ardic *et al.* (2009) and Hajiboland *et al*. (2011). Like CAT, APX is an important antioxidant enzyme involved in scavenging of H_2O_2. The specific activity of APX increased both under deficiency and toxicity of boron supply especially at later stages. The increased APX activity has been reported by Karabal *et al.* (2003) in barley genotypes and Cervilla *et al.* (2007) in tomato (*Solanum lycopersicum*) under B toxicity and by Hajiboland *et al*. (2011) in tea leaves under boron deficiency. APX can eliminate H_2O_2 and $O_2^{\bullet-}$ through the Asa-GSH-NADPH catalytic oxidation pathway. The oxidized Asa undergoes reduction by a GSH-mediated non-enzymatic reaction. GR promotes the reduction of oxidized glutathione (GSSG) to GSH. Thus, SOD, CAT, APX, GR, Asa and GSH play important roles in scavenging ROS in plant cells (Wang *et al.* 2011).

Increased activities of CAT, POX and APX resulted in low concentration of H_2O_2 at later stage of growth in boron deficient and toxic plants. However, in spite of detoxification of H_2O_2 by increase in the activity of these antioxidant enzymes, increase in lipid peroxidation suggests that there is membrane damage and oxidative stress in deficient and toxic plants of maize. This is probably due to accumulation of highly reactive oxygen species (ROS) due to phenolic compounds especially in boron deficient plants.

GR is another important antioxidant enzyme concerned with reduction of oxidized glutathione in the chloroplast and cytosol and regeneration of ascorbic acid. Glutathione acts as a disulphide reagent to protect thiol groups on enzymes and reacts with singlet oxygen and hydroxyl radicals. The GR activity was significantly enhanced under boron deficiency which is in accordance with the findings of Han *et al*. (2009) and contradictory with the findings of Cakmak and Römheld (1997). The increased GR activity was also observed under boron toxicity as earlier reported by Karabal *et al.* (2003) in barley and Cervilla *et al.* (2007), in tomato. Contrary to our results Keles *et al.* (2004) reported a decrease in GR in *Citrus* plants. Ardic *et al*. (2009) reported that a drought tolerant cultivar showed enhanced GR activity but a decrease was observed in the drought-sensitive one. They observed that drought tolerant cultivar are better protected from boron-stress induced oxidative damage due to enhanced SOD, CAT, POX and GR activities under high boron levels.

Higher concentration of phenols and enhanced activity of PPO enzyme under boron deficiency probably enhances the oxidative damage in boron deficient plants more than the boron toxic plants. Accumulation of phenolic compounds particularly caffeic acid and quinones are highly reactive and leads to enhanced generation of superoxide ions ($O_2^{\bullet-}$), which are known to cause peroxidative damage to cellular membranes (Cakmak and Römheld 1997). Accumulation of phenolics in boron stressed plants can activate enzymes that use phenolics as substrate. The enhanced PPO activity along with enhanced accumulation of phenolics results in the production of quinones which are highly toxic. Accumulation of phenolic compounds was found to be less at later stages of treatment compared to earlier treatment. This might be due to upregulation of antioxidative system in plants. Recently, Cervilla *et al*. (2012) also reported accumulation of phenolic compounds with increased PPO activity in tomato leaves grown under boron toxicity.

The observed changes in the lipid peroxidation, H_2O_2, phenol accumulation and altered enzymic and non-enzymic activities are suggestive of oxidative stress in the boron deficient plants. Responses of enzymic and non enzymic components of the antioxidative system along with contents of MDA and H_2O_2 in response to variable boron supply in maize leaves showed that deficiency as well as toxicity appears to result in membrane damage and induce a general response of oxidative stress. On the basis of the results obtained in maize plants at initial and severe stages of boron deficiency and toxicity we concluded that oxidative damage in boron deficient and toxic plants at initial stages may be due to accumulation of ROS as is evident from the accumulation of H_2O_2. However, although H_2O_2 production in boron deficient and toxic plants is reduced at later stage due to upregulation of antioxidative system but high lipid peroxidation suggests that membrane damage may be due to the enhanced accumulation of toxic quinones leading to accumulation of MDA which is suggestive of involvement of boron in membrane damage in an oxygen free radical dependent manner. Earlier the involvement of boron in membrane damage was suggested to be in an oxygen free radical independent manner by Pfeffer *et al*. (1998) and Karabal *et al*. (2003). Han *et al*. (2009) has reported that boron toxic leaves were damaged more severely by oxidative stress due to greater accumulation of MDA contents, because of having lower ability to scavenge ROS compounds than deficient leaves. In our study we found increased antioxidative enzymes with increased non-protein thiol contents in boron deficient and toxic plants. However, inspite of having higher scavenging capacity for ROS these plants experience oxidative damage more so in deficient plants than toxic. This is due to the decrease in Asc and greater accumulation of highly reactive and toxic phenolic compounds due to enhanced activity of PPO enzyme which leads to production of more toxic quinones in boron deficient leaves.

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