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RESEARCH PAPER

Antioxidant responses of chickpea plants subjected to boron toxicity

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ABSTRACT

This study investigated oxidative stress and the antioxidant response to boron (B) of chickpea cultivars differing in their tolerance to drought. Three-week-old chickpea seedlings were subjected to 0.05 (control), 1.6 or 6.4 mm B in the form of boric acid (H_3BO_3) for 7 days. At the end of the treatment period, shoot length, dry weight, chlorophyll fluorescence, B concentration, malondialdehyte content and the antioxidant enzymes superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were measured. The 1.6 mm B treatment did not cause significant changes in shoot length of cultivars, although shoot length increased in the drought-tolerant Gökce and decreased in the drought-sensitive Küsmen after 6.4 mm B treatment. Dry weights of both cultivars decreased with 6.4 mm B treatment. Chlorophyll fluorescence (Fv/Fm) did not change in Gökce at either B level. Nor did it change in Küsmen with 1.6 mm B but Fv/Fm decreased with 6.4 mm B. Boron concentration in the shoots of both cultivars increased significantly with increasing levels of applied B. Significant increases in total SOD activity were observed in shoots of both cultivars given 1.6 and 6.4 mm B. Shoot extracts exhibited five activity bands, two of which were identified as MnSOD and Cu/ZnSOD. In comparison to the control group, all enzyme activities (except APX and SOD) decreased with 1.6 mm B stress. GR activity decreased, while activities of CAT, POX and APX did not change with 6.4 mm B in Küsmen. On the other hand, activities of CAT, APX and SOD increased in Gökce at both B levels. In addition, lipid peroxidation was higher in Küsmen than in Gökce, indicating more damage by B to membrane lipids in the former cultivar. These results suggest that (i) Gökce is tolerant and Küsmen is sensitive to B, and (ii) B tolerance of Gökce might be closely related to increased capacity of the antioxidative system (total SOD, CAT and APX) to scavenge reactive oxygen species and thus suppress lipid peroxidation under B stress. To the best of our knowledge, this is the first report on the antioxidant response of chickpea seedlings to B toxicity.

INTRODUCTION

Boron (B) is an essential micronutrient for plant growth and development and is absorbed by plants from the soil solution in the form of boric acid (Dordas & Brown 2000). Boron frequently naturally occurs at toxic concentrations in soils that have been exposed to B-contaminated irrigation water or excess application of B-rich fertiliser, sewage sludge or fly ash, as well as from natural deposits found in semi-arid and arid zones around the world (Nable *et al.* 1997; Cervilla *et al.* 2007) including central Anatolia. Boron toxicity is an important disorder that causes negative physiological effects, including decreased shoot and root growth (Lovett & Bates 1984; Nable & Moody 1990), decreased leaf chlorophyll, inhibition of photosynthesis, lower stomatal conductance (Lovett & Bates 1984), deposition of lignin and suberin (Ghanati *et al.* 2002), increased membrane leakiness,

peroxidation of lipids and altered activities of antioxidant pathways (Karabal *et al.* 2003; Keles *et al.* 2004). Excess B also inhibits photosynthesis by causing structural damage to thylakoids and thus decreasing CO_2 uptake. These effects disrupt photosynthetic transport of electrons, favouring a condition where molecular oxygen operates as an alternative acceptor for non-utilised electrons and light energy leading to generation of reactive oxygen species (ROS) (Papadakis *et al.* 2004; Molassiotis *et al.* 2006).

Despite the importance of the above nutritional disorders, mechanisms of B tolerance and toxicity are not understood (Reid *et al.* 2004; Cervilla *et al.* 2007). It was suggested that the main tolerance mechanisms are exclusion from roots, reduced translocation to shoots and avoidance by means of shallow root systems (Paull *et al.* 1992). Genetic variations in responses to high B concentrations have prompted further investigation into the mechanisms involved. In wheat and barley cultivars, several possible tolerance mechanisms have been proposed that operate mainly by exclusion (Paull *et al.* 1992; Hayes & Reid 2004). On the other hand, it has been suggested that antioxidants and antioxidant enzymes may reduce B toxicity in some plants (Gunes *et al.* 2006; Cervilla *et al.* 2007). This antioxidant response is considered to be a critical process for protecting plants against oxidative damage caused by a wide range of environmental factors (Inze & Van Montagu 1995), including salinity, drought, heavy metals, chilling and nutritional deprivation (Mittler 2002).

During excess oxidative stress, production of reactive oxygen species (ROS), like the superoxide ($O_2^{\cdot -}$) and hydroxyl ($OH^{\cdot -}$) radicals that are strong oxidisers of lipids, proteins and nucleic acids, causes membrane damage that eventually leads to cell death (Del Rio *et al.* 2003). Plants possess enzymatic and non-enzymatic antioxidant defence mechanisms to combat the oxidative effects of ROS. Among these, superoxide dismutase (SOD) decomposes $O_2^{\cdot -}$ to $O_2^{\cdot}$ and H_2O_2 which are oxidised to molecular oxygen and H_2O by peroxidases (POX), catalase (CAT) and ascorbate–glutathione pathway enzymes, such as ascorbate peroxidase (APX) and glutathione reductase (GR) (Mathews *et al.* 1984). It has been shown that tolerant genotypes have better ability to cope with abiotic stress by inducing antioxidant defence systems. This indicates a correlation between antioxidant defence systems and stress tolerance (Bor *et al.* 2003; Demiral & Türkan 2004).

Chickpea (*Cicer arietinum* L.), the most widely grown grain legume in the world after beans and soybean, is an important source of human and animal nutrition, in particular in semi-arid and arid zones, including central Anatolia in Turkey, one of the largest global producers of boron (Katerji *et al.* 2001; Simsek *et al.* 2003). The B content of central Anatolian soils varies between 0.86 and 4.86 mM per kg and B toxicity has been observed in chickpea and other grain legumes growing in the region.

Although antioxidant responses of chickpea under abiotic stress conditions such as salt (Eyıdogan & Oz 2007), drought (Moinuddin & Imas 2007) and chilling (Nayyar *et al.* 2005) have been reported, no data are available on the antioxidant system of this plant under B toxicity. Therefore, we investigated the impact of excess B on the growth, lipid peroxidation and antioxidant enzymes of two chickpea cultivars differing in their sensitivity to drought.

MATERIAL AND METHODS

Plant material and boron stress applications

Seeds of chickpea (*Cicer arietinum* cv. Gökce and cv. Küsmen) were provided by the Anatolia Agricultural Research Institute in Eskisehir. Gökce is drought tolerant and Küsmen is drought sensitive (Anonymous 2000; Gunes *et al.* 2008). Seeds were surface sterilised with sodium hypochlorite and washed thoroughly with sterile water. Plants were germinated on perlite in a climate room under a constant day/night temperature of 20 °C and 16/8 h light/dark, with a light intensity of 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a relative humidity of 65%. Seedlings were grown for 21 days in half-strength Hoagland solution (Hoagland & Arnon 1950) containing 3.0 mM $Ca(NO_3)_2$, 10.0 mM KNO_3, 2.0 mM $MgSO_4.7H_2O$, 2.0 mM $NH_4H_2PO_4$, 0.05 mM H_3BO_3, 0.01 mM $MnCl_2.4H_2O$, 0.1 M $CuSO_4.5H_2O$, 0.1 M $ZnSO_4.7H_2O$, 0.1 M MoO_3, 0.18 M $FeSO_4.7H_2O$ and 0.27 M tartaric acid. Preliminary studies using B concentrations from 0.8 to 6.4 mM and two time periods (day 7 and 14) showed that Gökce could withstand 6.4 mM B while Küsmen showed significant damage leading to plant death after 14 days with 6.4 mM B. Therefore, after 21 days on normal growth medium, B treatment was initiated with half-strength Hoagland solution containing 0.05, 1.6 or 6.4 mM B. Plants were harvested on the seventh day after B treatment. After 0 and 7 days of B treatment, 20 plants from each group [0.05 (control), 1.6 and 6.4 mM B] were taken at random and divided into shoot and root fractions. The material (shoot) was rinsed three times in distilled water and then blotted on filter paper. Prior to these harvests, the fourth, fifth and sixth leaves (counting from bottom) from one shoot of each treatment were sampled. All samples were stored at −80 °C until analyses. Dead leaves were removed from shoots and not used in determination of B concentration or antioxidant enzyme activity.

Growth parameters

After 0 and 7 days of B treatment, 20 plants from each group [0.05 (control), 1.6 and 6.4 mM B] were taken at random and divided into shoot and root fractions. Shoot lengths and dry weights of the 7-day control and treated plants were measured. Shoots were oven-dried at 60 °C for 72 h and their dry weights were recorded.

Chlorophyll fluorescence

On day 0 and day 7 of B treatment, chlorophyll fluorescence parameters (PSII maximum efficiency, Fv/Fm) of leaves of 10 plants from each group were measured with a Plant Efficiency Analyser (Hansatech, UK).

Boron determination

To measure B concentration in shoots, the curcumin method was used and absorbance was read with a spectrophotometer (Jasco V-530 UV/VIS) at 540 nm (Dible *et al.* 1954).

Lipid peroxidation

Lipid peroxidation in shoot samples was determined in terms of malondialdehyte (MDA) content according to the method of Madhava Rao & Sresty (2000). The MDA content, an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$.

Enzyme extraction and assay

All operations were performed at 4 °C. For protein and enzyme extractions, 0.5 g shoot samples were homogenised with 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA Na_2 (ethylenediaminetetra aceticacid disodium salt) and 2% (w/v) polyvinylpolypyrrolidone (PVPP). For determination of APX activity, 2 mM ascorbate was added to the homogenisation buffer. Samples were centrifuged at 20,159 *g* for 40 min, and supernatants were used for determination of protein content and enzyme activity. Total soluble protein content of the enzyme extracts was determined according to Bradford (1976) using bovine serum albumin as standard. All spectrophotometric analyses were conducted in a Shimadzu spectrophotometer (UV 1600).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by its ability to inhibit photochemical reduction of NBT (nitrotetrazolium blue chloride) at 560 nm (Beauchamp & Fridovich 1971). The assays were carried out at 25 °C and the reaction mixture (3 ml) contained 0.033 mM NBT, 10 mM L-methionine, 0.66 mM EDTA Na_2 and 0.0033 mM riboflavin in 0.05 mM sodium phosphate buffer (pH 7.8). Riboflavin was added last and the test tubes containing the reaction mixture were incubated for 10 min under 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 25 °C. The reaction mixture without added enzyme developed maximum colour due to maximum reduction of NBT. Non-irradiated reaction mixture was used as control as it did not develop colour. One unit of SOD activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT and the specific enzyme activity was expressed as units·mg^{-1} protein.

Catalase (CAT; EC 1.11.1.6) activity was estimated according to Bergmeyer (1970), by measuring the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture contained 0.05 M Na-phosphate buffer (pH 7.0) with 0.1 mM EDTA and 3% H_2O_2. The decrease in absorption was followed for 3 min, and 1 µmol H_2O_2 destroyed per min is defined as one unit of CAT.

Peroxidase (POX; EC 1.11.1.7) activity was based on the method described by Herzog & Fahimi (1973). The reaction mixture contained 3,3′-diaminobenzidine-tetra hydrochloride dihydrate (DAB) solution with 0.1% (w/v) gelatin, 150 mM Na-phosphate-citrate buffer (pH 4.4) and 0.6% H_2O_2. The increase in absorbance at 465 nm was followed for 3 min. A unit of POX activity is defined as $\mu mol \cdot ml^{-1}$ H_2O_2 decomposed per min.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano & Asada (1981). The assay depends on a decrease in absorbance at 290 nm as ascorbate is oxidised. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA Na_2, 1.2 mM H_2O_2 and 0.1 ml enzyme extract in a final assay volume of 1 ml. The concentration of oxidised ascorbate was calculated using an extinction coefficient of 2.8 $mM^{-1} \cdot cm^{-1}$. A unit of APX activity is defined as $\mu mol \cdot ml^{-1}$ oxidised ascorbate per min.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured according to Foyer & Halliwell (1976). The assay medium contained 0.025 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH Na_4 and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient of NADPH (6.2 $mM^{-1} \cdot cm^{-1}$). One GR enzyme unit is defined as $\mu mol \cdot ml^{-1}$ oxidised GSSG per min. All specific enzyme activities assayed are expressed as units·mg^{-1} protein.

Electrophoretic SOD separation

Before loading on gels, fresh leaves of the B-treated drought-resistant Gökce and drought-sensitive Küsmen were homogenised with 9 mM Tris HCl (pH 6.8) and 13.6% (v/v) glycerol at 4 °C. Homogenates were centrifuged at 20,159 *g* for 5 min at 4 °C and the supernatants were used for determination of enzyme activity. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard. Samples containing equal amounts of protein (80 µg per well) were subjected to non-denaturating PAGE (polyacrylamide gel electrophoresis) as described in Laemmli (1970) except that SDS was omitted. Extracts were subjected to electrophoresis at 4 °C in 5% stacking and 12% separating gels under a constant current (120 mA). SOD activity was detected by staining with riboflavin and nitroblue tetrazolium as described by Beauchamp & Fridovich (1973). Quantitative evaluation of SOD isozyme activity in four independent gels was carried out by densitometric

Table 1. Densitometric analyses of SOD isozymes in shoot of Gökce and Küsmen subjected to boron stress.

	Gökce			Küsmen		
	control	1.6 mM	6.4 mM	control	1.6 mM	6.4 mM
MnSOD-1	100	128 ± 5*	126 ± 8*	100	189 ± 6*	123 ± 7*
MnSOD-2	100	124 ± 7*	123 ± 8*	100	186 ± 5*	139 ± 5*
MnSOD-3	100	99 ± 6	96 ± 6	100	107 ± 8*	98 ± 8
Cu/ZnSOD-1	100	132 ± 4*	110 ± 3*	100	172 ± 4*	102 ± 1
Cu/ZnSOD-2	ND	100*	ND	ND	100*	ND

ND = not detected.
Gels stained for SOD activity were documented and analysed with the Bio-Profil V99 software package (Vilber Lourmat). SOD activity of non-stressed plants was taken as 100% and the percentages in terms of the control values for each treatment are shown. Tabulated values are average of data (%) from four independent gels ± SEM.
*Significant difference with respect to control at $P < 0.05$.

Table 2. Results of one-way ANOVA of boron treatment on shoot length, dry weight, Fv/Fm, boron content, lipid peroxidation (MDA) and SOD, CAT, POX, APX and GR activities.

	mean square	F
shoot length	584.394	268.140*
shoot dry weight	2.66×10^{-2}	164.896*
Fv/Fm	1.030	1956.195*
boron content	87600.959	113.513*
MDA	8458.307	113.513*
SOD	104038.053	166.862*
CAT	22104.735	258.051*
POX	28.853	287.792*
APX	50628.701	1460.240*
GR	8699.347	395.175*

F = between treatments mean square/residual mean square.
*$P < 0.001$.

analysis using the BIO-PROFIL V99 software program of the Vilber Lourmart imaging system (Marne la Vallee, France) (Table 1).

Statistical analysis

All analyses were done using a completely randomised design. All data obtained were subjected to one-way analyses of variance (ANOVA) and the mean differences were compared with a lowest standard deviation (LSD) test. Each data point was the mean of six replicates ($n = 6$) and P-values < 0.05 were considered significantly different. In all figures, the spread of values is shown with error bars representing standard errors of the means (Table 2).

RESULTS

Plant growth

Effects of B toxicity on shoot length of the two cultivars are shown in Table 3. In both cultivars, 1.6 mM B did not cause a significant difference in shoot length ($P > 0.05$). However, while 6.4 mM B caused a significant decrease in shoot length of Küsmen, it induced shoot elongation in Gökce as compared to the controls. The shoot dry weight of Küsmen was 188 mg in the control and dropped to 167 and 130 mg after 1.6 and 6.4 mM B treatment, respectively. Growth with 1.6 mM B did not cause a significant difference in shoot dry weight of Gökce ($P > 0.05$) but growth with 6.4 mM caused a significant decrease (40%).

Boron concentration

Shoot B concentration increased significantly with increasing levels of applied B in both cultivars ($P < 0.001$). In

Table 3. Effect of boron toxicity on shoot length (cm), DW (mg) and chlorophyll fluorescence (Fv/Fm) of shoots of Gökce and Küsmen chickpea cultivars.

	day 0	day 7		
cultivars		control	1.6 mM	6.4 mM
Gökçe				
shoot length	17.33 ± 1.92	19.72 ± 1.75	18.90 ± 1.86	24.90 ± 2.07
DW	83.3 ± 12.10	110 ± 21.60	122 ± 16.40	65 ± 5.80
Fv/Fm	0.852 ± 0.01	0.856 ± 0.01	0.856 ± 0.01	0.826 ± 0.01
Küsmen				
shoot length	15.75 ± 1.66	20.4 ± 1.25	20.30 ± 0.46	18.82 ± 1.35
DW	96 ± 20.10	188 ± 18.90	167.5 ± 9.60	130 ± 7.10
Fv/Fm	0.863 ± 0.00	0.851 ± 0.00	0.840 ± 0.01	0.766 ± 0.01

DW = dry weight.

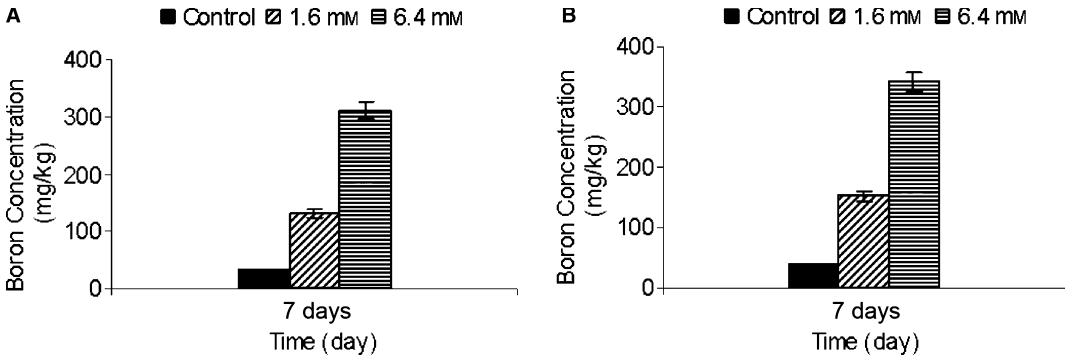

Fig. 1. Effect of increasing levels of exogenous applied boron [0.05 (control), 1.6 and 6.4 mM] on the B concentration in shoots of Gökce (A) and Küsmen (B). The values are means of six replicates ±SE.

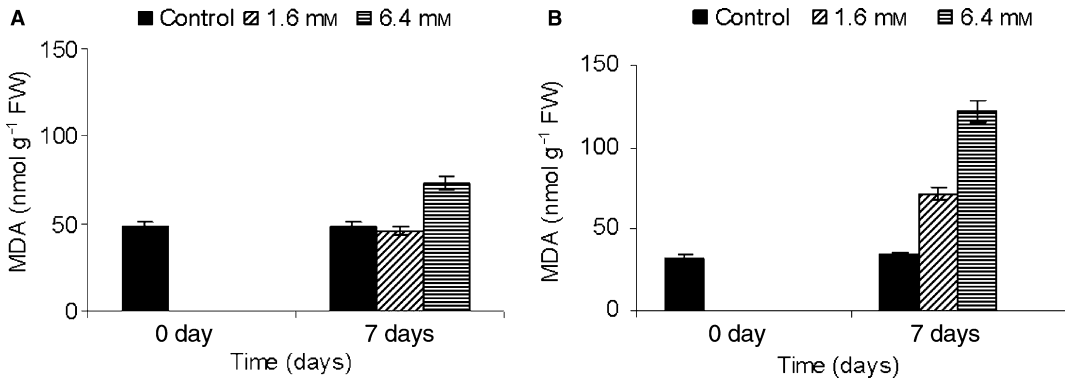

Fig. 2. Boron stress-induced changes in malondialdehyde (MDA) content (nmol·g^{-1} FW) in shoots of Gökce (A) and Küsmen (B).

both cultivars treated with 6.4 mM B, the B concentration increased almost ninefold compared to the control. However, B concentration was higher in Küsmen than in Gökce throughout the experimental period because of the lower constitutive level of B in Gökce (Fig. 1).

Chlorophyll fluorescence

Chlorophyll fluorescence (Fv/Fm) of the drought-tolerant Gökce was not significantly affected by B treatment, in comparison to the control ($P > 0.05$). However, the Fv/Fm of Küsmen decreased by 10% after 6.4 mM B treatment (Table 3).

Lipid peroxidation

The effect of B on lipid peroxidation was determined by evaluating the MDA content of both cultivars (Fig. 2). There was a significant dose-dependent effect of B toxicity in terms of MDA content of shoots in Küsmen (Fig. 2), with a two- or threefold increase after 1.6 or 6.4 mM B, respectively. However, treatment of Gökce with 1.6 mM B had no significant effect on MDA content, but 6.4 mM B increased (5%) MDA content in comparison to the control values.

Antioxidant enzyme activity

Figure 3 shows changes in SOD activity after exposure to varying B concentrations. SOD activity in control Gökce (drought tolerant) and Küsmen (drought sensitive) was unchanged throughout the experiment. However, treatment with 1.6 or 6.4 mM B increased SOD activity of both cultivars ($P < 0.001$). The increase in SOD activity was higher in Küsmen (67%) than in Gökce (50%) with 1.6 mM B. Moreover, SOD activity in shoots of both cultivars was stimulated more by 1.6 mM B than 6.4 mM B.

To better understand the significance of SOD stimulation by excess B, we performed a native PAGE analysis of SOD isoform patterns (Fig. 4). Five SOD activity bands were identified in shoots of both cultivars. These isoenzymes were characterised using KCN (potassium cyanide) to inhibit both Cu/ZnSOD and H_2O_2 and Cu/ZnSOD and FeSOD, whereas MnSOD is known to be resistant to these treatments (Beauchamp & Fridovich 1973). The

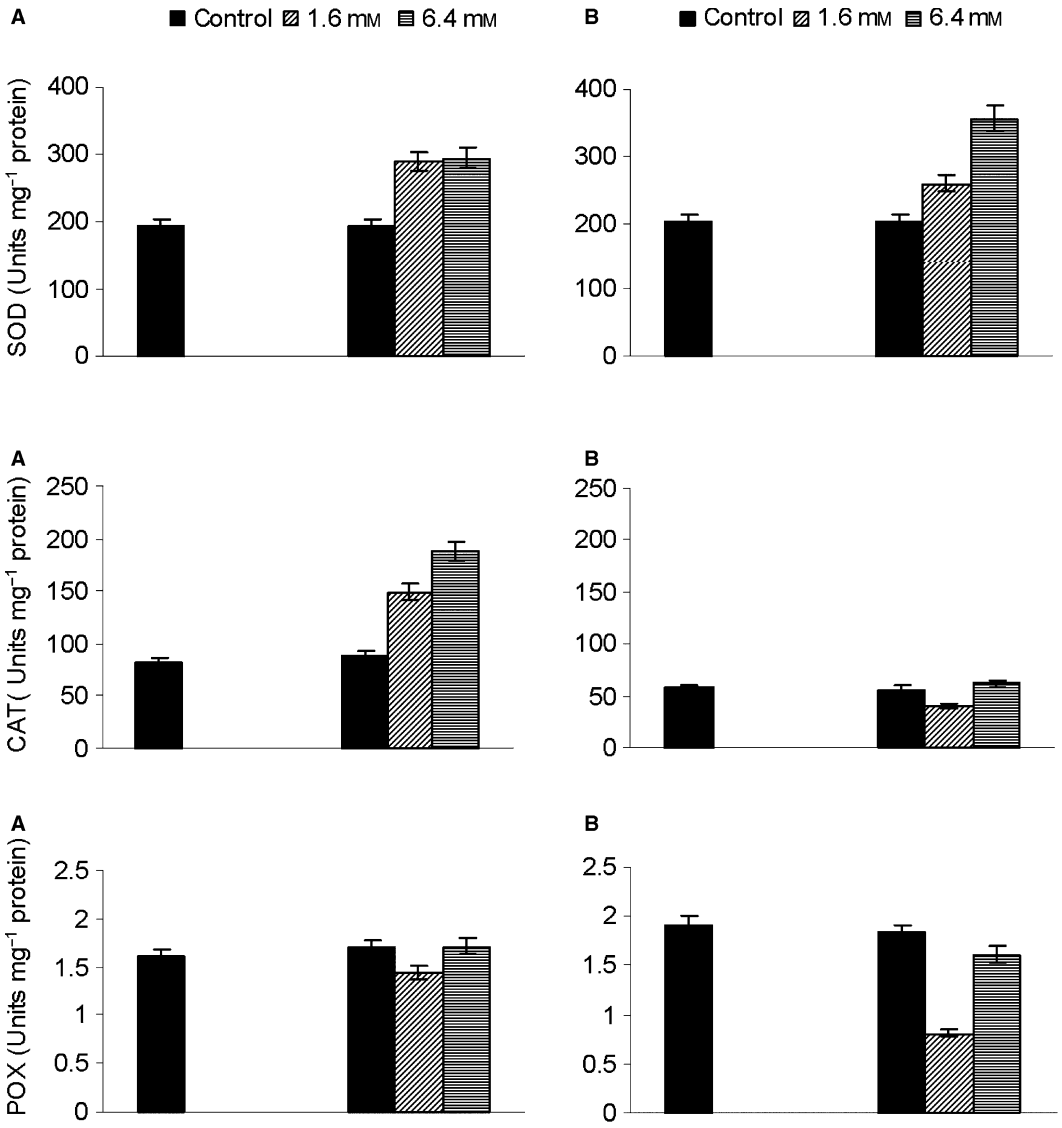

Fig. 3. Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activity in shoots of Gökce (A) and Küsmen (B) before (day 0) and after (day 7) exposure to boron stress.

isozymes were identified as Cu/ZnSOD1 and II, and MnSOD1, II and III. While Cu/ZnSODI activity was observed in all groups of both cultivars, the Cu/ZnSODII isozyme was expressed only after 1.6 mM B treatment in the two cultivars. Moreover, the intensity of Cu/ZnSODI isozymes from both cultivars after 1.6 mM B stress was higher than that of other treatments (Table 1). While the intensities of MnSODI and MnSODII isoforms increased, the intensity of mitochondrial MnSODIII did not change in the shoots of either cultivar exposed to 1.6 or 6.4 mM B. These changes in SOD isoform patterns explain the increase in SOD activity described above. No FeSOD activity was detected in the gels.

To investigate changes in H_2O_2 scavenging enzymes, the activities of CAT, APX and POX were measured. In control groups of both cultivars, no changes occurred in CAT activity during the experiment period (Fig. 3). As compared with Küsmen, higher constitutive CAT activity was observed in shoots of Gökce. Moreover, CAT activity was significantly higher in Gökce than in Küsmen at both B levels ($P < 0.001$). As compared to the controls, in Gökce, CAT activity increased significant by 70% and 115% under 1.6 and 6.4 mM B, respectively. On the other hand, in Küsmen, 1.6 mM B treatment caused a significant decrease in CAT activity ($P < 0.01$) that did not change with 6.4 mM B.

Growth with 6.4 mM B did not have a significant effect on POX activity of the two cultivars. POX activity in shoots decreased after 1.6 mM B treatment, and the rate of this decrease was higher in Küsmen than in Gökce (Fig. 3).

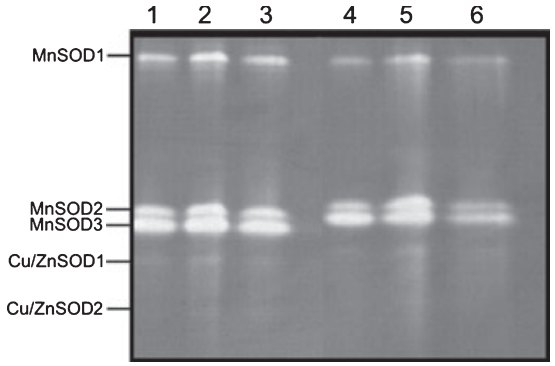

Fig. 4. Native PAGE and activity staining for SOD activity in shoots of boron-treated chickpea seedlings. Lanes 1, 2 and 3 are SOD isozyme activity in shoots of Gökce treated with 0.05 mM (control), 1.6 mM and 6.4 mM B, respectively. Lanes 4, 5 and 6 show the activity in Küsmen treated with 0.05 mM (control), 1.6 mM and 6.4 mM B.

APX activity of both cultivars did not change significantly in controls during the experiment (Fig. 5) but increased by 143% in Gökce and 53% in Küsmen in 1.6 mM B treatments. However, at the highest B concentration (6.4 mM), APX activity of Gökce increased but that of Küsmen decreased.

Treatment with B did not affect GR activity of shoots of Gökce ($P > 0.05$) but decreased GR activity of Küsmen by 35% (1.6 mM B) and 20% (6.4 mM B), in comparison to the controls (Fig. 5).

DISCUSSION

Growth

The shoot lengths of seedlings of the two chickpea cultivars were affected differently by growth in 1.6 or 6.4 mM B (Table 3). While growth in 6.4 mM B caused an increase in shoot length of Gökce, it decreased it in Küsmen. Increasing concentrations of B improved shoot length in Gökce, but decreased shoot length of Küsmen. Although there are a number of processes with different sensitivities that contribute to the overall response to high B, it has been shown that toxicity is correlated with intracellular B concentration (Reid *et al.* 2004). Hence, improved growth of Gökce with excess B might be related to ability to reduce the intracellular concentration of B by active efflux from the roots, as also reported by Hayes & Reid (2004) for barley. Inhibited growth of Küsmen seems likely to be due to accumulation of higher B concentrations than in Gökce. Analysis of B concentration in seedlings of the two chickpea cultivars showed a higher concentration in

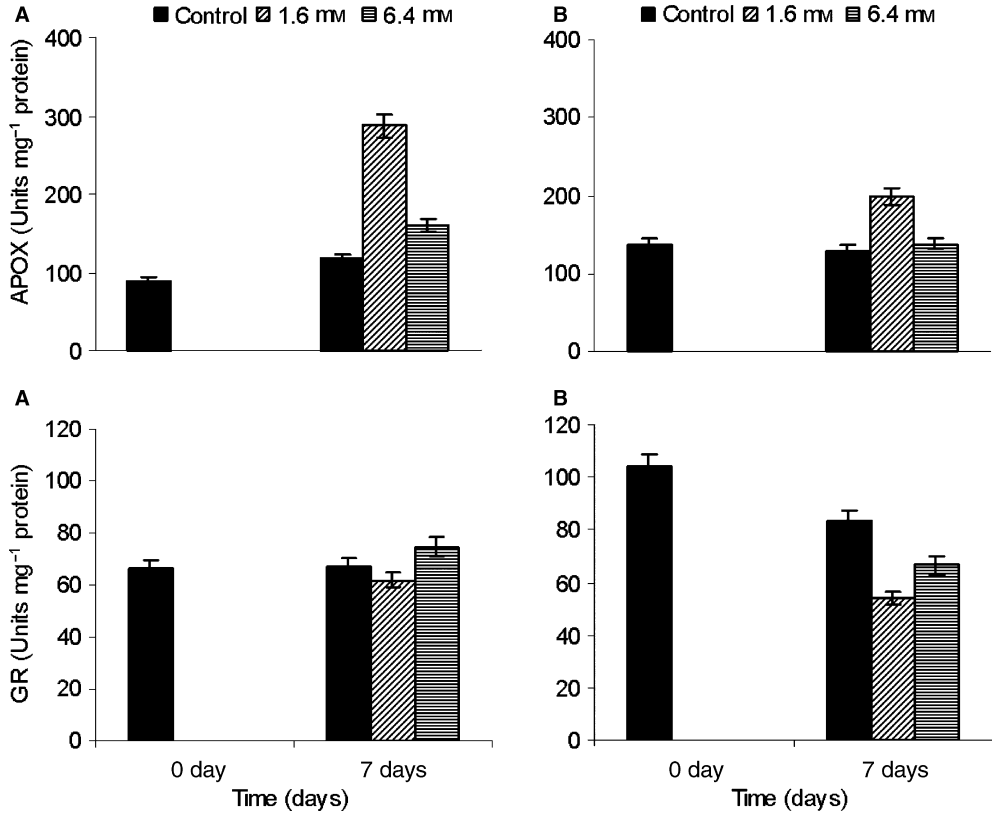

Fig. 5. Ascorbate peroxidase (APX) and glutathione reductase (GR) activity in shoots of Gökce (A) and Küsmen (B) before (day 0) and after (day 7) exposure to boron stress.

Küsmen than in Gökce, even at high B concentrations in the medium (Fig. 1). This result also validates findings that species and genotypes susceptible to B toxicity generally have higher concentrations of B in their leaves and shoots than tolerant genotypes (Nable *et al.* 1997). Therefore, our results are in accordance with previous findings of an inverse relationship between endogenous B concentrations and B tolerance in different species of tomato (Toledo & Spurr 1984), *Prunus* rootstocks (El-Motaium *et al.* 1994) and wheat (Kalaycı *et al.* 1998).

Chlorophyll fluorescence

To understand the effect of excess B on PSII machinery, we measured the chlorophyll fluorescence parameter Fv/Fm (Table 3). Fv/Fm (maximum quantum yield of PSII) is the most frequently used fluorescence parameter (Björkman & Demming 1987). In our study, maximum quantum yield of PSII was more dramatically affected by both levels of B in the drought-sensitive Küsmen than in the drought-tolerant Gökce (Table 3), indicating more serious damage to PSII machinery in the former cultivar. Similarly, Papadakis *et al.* (2004) observed a significant decrease in Fv/Fm in leaves of Navelina orange plants grown under excess B concentrations. This decrease in chlorophyll fluorescence can be attributed to oxidation of chlorophyll and chloroplastic membranes, which might be exacerbated by excess B, as reported in hot pepper (Lee 2006) and apple rootstocks (Sotiropoulos *et al.* 2006).

Boron concentration

The uptake of B differs among plant species and cultivars (Hu & Brown 1997). Shelp *et al.* (1995) showed that B is immobile in phloem and that remobilisation of B from the leaves to other organs is limited. In addition, plants take up and transport B through the transpiration stream (Marschner 1995). However, B is mobile in the phloem of all species that utilise polyols as a primary photosynthetic metabolite (Brown & Hu 1998; Reid 2007), moves with the transpiration stream and, once it enters a leaf, it tends to remain. In our experiment, both cultivars showed an increase in total B concentrations. An increase in B concentration in plants leading to B toxicity has previously been observed in sunflower (Ruiz *et al.* 2003), barley (Karabal *et al.* 2003) and tomato (Cervilla *et al.* 2007). Moreover, B accumulation in the shoot was highest in 6.4 mM B treated shoots of Küsmen on day 7. In all treatment groups, Küsmen had higher B accumulation than Gökce. Similarly, Kaur *et al.* (2006) found that both shoot B uptake and B concentration in *Brassica rapa* were considerably lower in tolerant genotypes than in sensitive genotypes.

Lipid peroxidation

Boron is known to play important roles in the structure of cell walls, membranes and membrane-associated reactions (Power & Woods 1997; Brown *et al.* 2002). Liu & Yang (2000) reported that the level of membrane permeability, which is associated with increased MDA content, of soybean increased under B deficiency. In our study, B toxicity also resulted in increased MDA content (Fig. 2). Compared with the drought-tolerant cultivar (Gökce), more damage was observed in the sensitive cultivar (Küsmen) because of an inadequate response of the antioxidant system and higher accumulation of B. Others have also found that excess B increased MDA concentrations in apple rootstock (Molassiotis *et al.* 2006), grape (Gunes *et al.* 2006) and tomato (Cervilla *et al.* 2007). This suggests a possible role of membrane stability in tolerance mechanisms to B toxicity.

Antioxidant enzymes

Superoxide dismutase, the first line of defence against ROS, catalyses the dismutation of superoxide to $O_2^{\cdot}$ and H_2O_2. SOD activity increases in cells in response to environmental stress. Mittler (2002) reported that increased SOD activity might be considered as circumstantial evidence for enhanced ROS production. In this study, total SOD activity increased significantly in both cultivars subjected to B stress, in comparison to the controls (Fig. 3). Our results corroborate previous reports indicating an increase in total SOD activity in response to excess B (Garcia *et al.* 2001; Karabal *et al.* 2003; Molassiotis *et al.* 2006; Sotiropoulos *et al.* 2006; Cervilla *et al.* 2007).

In order to determine whether the increase in SOD activity was due to induction of new isoforms or to an increase in activity of the constitutive isoenzymes, tissue extracts were subjected to native PAGE analysis. Five SOD activity bands were identified in shoots of both cultivars, and B treatment differentially affected SOD isozyme activity. In shoots of both cultivars, the isozymes were identified as MnSOD (I, II and III) and Cu/ZnSOD (I and II). The rate of increase in intensity of the Cu/ZnSODI isozyme with 1.6 mM B in Küsmen was higher than that for Gökçe. While Cu/ZnSODII was only expressed in 1.6 mM B-treated groups of both cultivars, other isozymes were found in all groups. The increase in intensity of MnSODI and II in Küsmen was higher than in Gökçe at both B concentrations. On the other hand, intensities of MnSODIII isozymes did not change in either cultivar under B stress. Therefore, the appearance of additional Cu/ZnSOD or an increase in intensity of these isoforms is the most likely explanation for the increase in SOD activity in shoots. These results may support the hypothesis that SOD is tightly controlled in response to excess B. Similar to our results, B toxicity was shown to induce SOD activity in tobacco leaves (Garcia *et al.* 2001) and barley roots (Karabal *et al.* 2003).

The regulation of expression and activity of different SOD isoforms is complex, and the genes involved respond differently to environmental cues (Sen Gupta *et al.* 1993; Sreenivasulu *et al.* 2000). Hence, compartment-specifically induced SOD isoforms are essential for the tolerance mechanism in Gökce in response to B stress. Increased

cytoplasmic Cu/ZnSOD activity in Gökce can most likely be explained by up-regulation of genes encoding this enzyme, which correlates with the total protein/enzyme activity of the gene products. Zgallai *et al.* (2006) also observed an increase in the steady-state level of cytosolic Cu/Zn-SOD transcripts during PEG (polyethylenglycol) treatment.

To test for variations in H_2O_2 scavenging enzymes, we measured the activities of CAT, POX and APX in shoots of both cultivars. In comparison to control groups, CAT activity increased with increasing B stress in Gökce, but in Küsmen, activity either decreased or showed no change with 1.6 or 6.4 mM B, respectively (Fig. 3). Like Gökce, an induction of CAT activity was also reported in B stressed sunflower (Dube *et al.* 2000), apple rootstocks (Sotiropoulos *et al.* 2006) and tomato (Cervilla *et al.* 2007). Moreover, Garcia *et al.* (2001) reported that foliar application of boric acid together with fungicide induced SOD, CAT and APX activity. In contrast, Keles *et al.* (2004) observed a decreased in CAT in citrus leaves due to B toxicity. Increased levels of CAT in Gökce clearly indicate higher efficiency of detoxification of H_2O_2 produced in peroxisomes exposed to excess B.

Unlike CAT and APX activity, POX activity in both cultivars exhibited a significant decrease after 1.6 mM B treatment. Similarly, Palavan-Unsal *et al.* (2000) and Dube *et al.* (2000) observed a significant decrease in POX activity in whole shoots and leaves, respectively, of *Helianthus annuus* under B toxicity. However, compared with the 1.6 mM B-treated groups, POX activity increased in shoots of both chickpea cultivars after 6.4 mM B stress. An increase in POX activity has been reported to be an early response to several stressors (Castillo 1992) and may provide cells with tolerance to H_2O_2 formed when plants are exposed to stressors.

To investigate changes in enzymes involved in the ascorbate–glutathione cycle, the activities of APX and GR (important in removal of H_2O_2) were measured (Asada & Takahashi 1987). In our study, APX activity increased with 1.6 mM B in shoots of both cultivars, with a higher increase in Gökce. However, there was no significant change in APX activity in shoots of Küsmen and Gökce with 6.4 mM B. These results suggest that the H_2O_2 produced by SOD activity was removed by higher activity of the H_2O_2 detoxifying enzyme APX in the drought-tolerant cultivar Gökce (Fig. 3). Induction of APX activity was also reported in B stressed tobacco (Garcia *et al.* 2001) and hot pepper (Lee 2006). Boron treatment did not affect GR activity in shoots of Gökce, corroborating previous reports indicating no change in total GR activity in response to excess B (Karabal *et al.* 2003). However, a significant decrease in GR activity was observed in Küsmen. As reported by Karabal *et al.* (2003) for barley, the drought-tolerant chickpea cultivar (Gökce) has higher constitutive GR activity.

In conclusion, our results demonstrate that B toxicity mediates changes in activities of antioxidant enzymes in a cultivar-dependent manner. We observed increases in growth accompanied by higher constitutive and induced activity of the antioxidant enzymes SOD, CAT and APX and a decrease in lipid peroxidation in Gökce with excess B, in comparison to Küsmen. Although Küsmen exhibited similar behaviour in terms of enhanced SOD and APX activity, peroxidation of lipid membranes in Küsmen was not prevented.

These results clearly indicate that (i) excess B induces oxidative stress damage, at least in Küsmen, and (ii) while higher antioxidant activity in Gökce contributes to B stress tolerance, higher free radical scavenging capacity and the protection mechanism of this cultivar against B stress was also revealed by lower levels of lipid peroxidation. Since there is no information on the antioxidant response of chickpea under B stress, we hope that this study provides a basis for understanding the role of antioxidant enzymes in the response to excess B.

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