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Calcium-Induced Amelioration of Boron Toxicity in Radish

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Abstract Like other abiotic stresses, boron (B) toxicity is an important environmental constraint that limits crop productivity worldwide. B toxicity alters many physiological processes necessary for plant survival. The aim of the present study was to investigate the individual and combined effects of calcium (Ca) and B on morphological and physiological attributes of radish (*Raphanus sativus* L.) under normal and boron-toxicity conditions. The application of 30 mM Ca and 0.5 mM B, alone and in combination, enhanced plant growth, physiological and biochemical attributes. However, 5 mM B was detrimental to most growth and physiological parameters. The application of 30 mM Ca was most effective in alleviating the harmful effects of B toxicity by decreasing malondialdehyde and hydrogen peroxide levels and electrolyte leakage and by enhancing the activities of the antioxidant enzymes superoxide dismutase, catalase, peroxidase, glutathione reductase, and ascorbate peroxidase. Ca clearly induced plant protection mechanisms by enhancing the accumulation of proline, total soluble carbohydrates, and photosynthetic pigments in leaves.

Keywords Antioxidant systems · Boron toxicity · Calcium · Chlorophyll · Carbohydrates

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Introduction

Boron (B) plays a vital role in plant tissue growth and development, including cell wall formation and function, because it forms crosslinks between dimers of the pectin rhamnogalacturonan II (a low-molecular-mass pectic polysaccharide) (Matoh 1997; O'Neill and others 2004). However, the available literature reflects that the physiological role of B in plants is not fully understood. B is a unique essential mineral element for plants with a small window of suitable concentrations between deficiency and toxicity. The requirement for B varies among plants species and among genotypes of the same species. For example, according to Keren and Bingham (1985), sensitive plants (that is, avocado, apple, and bean) can be safely grown at concentrations of 0.3 mg B L^{-1}, semitolerant plants (that is, oat, maize, and potato) at 1–2 mg B L^{-1}, and tolerant plants (that is, carrot, alfalfa, and sugar beet) at 2–4 mg B L^{-1}. Torun and others (2006) also reported that large genetic variations were found in the tolerance of wheat to B toxicity. B-deficient plants showed altered growth and physiological and reproductive processes (Camacho-Gristobal and others 2008).

Worldwide, crop production is limited because B in the soil is either insufficient or at toxic levels (Rerkasem and others 2003). Like other nutrient deficiencies, B deficiency is very common and widespread around the world and causes large losses in crop production both quantitatively and qualitatively (Shorrocks 1997). Large areas of India, China, and the US are suffering from B deficiency (Liu and others 1980; Singh 2006; Yau and Ryan 2008). B toxicity is an especially serious threat to agriculture in arid and semiarid regions; B is frequently associated with saline soils and inland desert areas where native soils high in B coexist with low levels of rainfall and irrigation with

high-B groundwater (Gupta 1979). The main sources of elevated B are surface mining, fly ash, and industrial chemicals (Nable and others 1997). Areas of Peru, Chile, India, Israel, South Australia, West Asia, North Africa, Egypt, Iraq, Jordon, Libya, Morocco, Syria, Turkey, and the west coast of Malaysia are all suffering from B toxicity (Yau and Ryan 2008).

High B levels in the soil inhibit seed germination and polyphenol oxidase activity (Ölçer and Kocaçaliskan 2007), cell wall expansion, and photosynthetic pigment synthesis, and reduce lignin and suberin contents (Nable and others 1997; Reid 2007). B toxicity impairs nitrogen assimilation pathways by affecting key enzymes involved in those processes (Herrera-Rodríguez and others 2010) and disrupts RNA splicing (Shomron and Ast 2003; Reid 2007). Like other abiotic stressors (that is, salinity, heavy metals, drought, cold, and heat), B also causes oxidative damage induced by the formation of reactive oxygen species (ROS) such as superoxide ($O\bullet^-$), hydroxyl ($OH\bullet^-$) radicals, which are strong oxidizers of lipids, proteins, and nucleic acids (Ardic and others 2009; Cervilla and others 2007), and destabilization of cellular homeostasis (Tombuloglu and others 2011). B maintains cell membrane integrity and also improves the cellular defense mechanism (Xuan and others 2001).

Despite the obvious importance of B toxicity, the mechanisms of B tolerance and toxicity in plants are poorly understood (Reid and others 2004; Cervilla and others 2007; Reid 2007; Fitzpatrick and Reid 2009). The interrelationships among nutrients and between nutrient and plant growth regulators are very important for plant growth and development under both normal and extreme environmental conditions (Siddiqui and others 2008b, 2009b, 2010, 2011, 2012). Calcium (Ca) plays an important role in stress tolerance and also induces antioxidant enzyme activities and reduces lipid peroxidation of cell membranes under abiotic stress (Khan and others 2010; Siddiqui and others 2010). Ca also affects B availability and its utilization by plants (Kanwak and others 2008). Balanced nutrient uptake and concentrations within plant tissues are important for proper plant growth and development (Siddiqui and others 2008b). Turan and others (2009) reported that B and Ca concentrations in the soil affected the availability of and requirement for normal plant growth. Moreover, an imbalanced supply of B and Ca is toxic to the plant and impairs the growth and translocation of Ca to the shoot and fruit (Yamauchi and others 1986). However, the interactions between Ca and B are still debated (Bolaños and others 2004). Thus, studies of the interactive effects of Ca and B on plant growth are essential; Ca could alleviate the oxidative damage induced by B toxicity. Therefore, in the present experiment, the effect of B was studied in both the presence and the absence of Ca. Our main objective was to investigate the effect of Ca and/or B on the morphological and physiological attributes of radish under normal and B-toxicity conditions.

Materials and Methods

Plant Cultures and Ca and B Treatments

Experiment was performed under glasshouse conditions using radish (*Raphanus sativus* L. cv. Prolsy Kafrelsheikh) obtained from a local market in Riyadh, Saudi Arabia. Healthy seeds were surface sterilized with 1 % sodium hypochlorite for 10 min then vigorously rinsed with sterilized double-distilled water (DDW) before sowing. The seeds were sown in plastic pots (25 cm diameter, 25 cm height) filled with perlite and supplied with Raukura's nutrient solution (Smith and others 1983). The pots were arranged in a simple randomized design in the greenhouse with a single factor and four replicates. The pots were covered with black plastic to reduce evaporation. One week after sowing, seedlings were thinned so that each pot contained healthy plants of uniform size. Pots were irrigated every 2 days with DDW (100 mL) to keep the perlite moist. Ca and B treatments were initiated 15 days after germination as follows [the concentration (in mM) of each element is indicated as a subscript]: (1) $Ca_0 + B_0$ (control), (2) $Ca_{20} + B_0$, (3) $Ca_{30} + B_0$, (4) $Ca_{40} + B_0$, (5) $Ca_0 + B_{0.5}$, (6) $Ca_0 + B_5$, (7) $Ca_{20} + B_{0.5}$, (8) $Ca_{20} + B_5$, (9) $Ca_{30} + B_{0.5}$, (10) $Ca_{30} + B_5$, (11) $Ca_{40} + B_{0.5}$, and (12) $Ca_{40} + B_5$. The range of concentrations of B (that is, 0.05–5 mM) selected for this study was based on earlier experiments (Ardic and others 2009). The sources of Ca and B were calcium chloride and boric acid, respectively. Plants were sampled on the seventh day after treatment to assess their growth characteristics [plant height (PH), fresh weight (FW), and dry weight (DW); leaf area (LA); and leaf area index (LAI)] and physiological attributes [concentrations of B, Ca, photosynthetic pigments [chlorophyll (Chl) *a* and Chl *b*], total soluble carbohydrates (TSC), proline (Pro), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA); electrolyte leakage; and activity levels of carbonic anhydrase (CA) and the antioxidants catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX)].

Plant Growth Characteristics

Plant height was measured using a meter scale after removal of plants from the pots. After recording the FW with a balance, plants were placed in a 60 °C oven for 48 h and then were weighed for DW. LA was measured using a

LI-3000 Portable Leaf Area Meter (LI-COR, Lincoln, NE, USA). LAI was calculated according to the method of Watson (1958).

Physiological and Biochemical Parameters

Chemical Content of Leaves

All the chemical reagents used in this procedure were of analytical grade. Absorbances were determined using a UV–Vis spectrophotometer, unless otherwise specified.

Chlorophyll (Chl) was extracted from fresh leaves using the DMSO method of Barnes and others (1992). Chl *a* and Chl *b* concentrations were calculated based on the absorbance of the extract at 663.8 and 646.8 nm.

To determine B and Ca concentrations, we followed the digestion approach of Hseu (2004). A leaf sample (0.5 g) was placed in a 250 mL digestion tube, and 10 mL of 2:1 concentrated nitric acid:perchloric acid was added. Samples were heated for 45 min at 90 °C, then the temperature was increased to 150 °C and boiled for 2–3 h until a clear solution was obtained. At intervals, 5 mL of concentrated nitric acid:perchloric acid with hydrogen peroxide was added to the sample (at least three times), and the digestion continued until the volume was reduced to about 1 mL. The interior walls of the tube were washed down with a little double distilled water (DDW) and the tubes were swirled throughout the digestion to keep the walls clean and prevent loss of the samples. After cooling, 5 mL of 1 % HNO_3 was added to each sample. Thereafter, the solution was filtered through Whatman No. 42 filter paper and smaller than 0.45 μm Millipore filter paper. The filtrate was diluted to a total of 25 mL with distilled water. After dilution, the concentrations of Ca and B were determined with an atomic absorption spectrometer (Model 300, Perkin-Elmer, Waltham, MA, USA) and an inductively coupled plasma optical emission spectroscope (Model iCAP6000, Thermo-Scientific, Thermo-Fisher Scientific, Waltham, MA, USA).

Proline concentration was determined spectrophotometrically by adopting the ninhydrin method of Bates and others (1973). We first homogenized 300 mg of fresh leaf samples in sulphosalicylic acid, then added 2 mL each of acid ninhydrin and glacial acetic acid. The samples were heated at 100 °C. The mixture was extracted with toluene and the free toluene was quantified at 528 nm using L-proline as a standard.

Total soluble carbohydrate concentration (TSC) was estimated by the absorbance at 490 nm as described by Dubois and others (1956), using glucose as a standard. TSC was expressed as mg g^{-1} DW.

Malondialdehyde (MDA) content was determined according to the method of Heath and Packer (1968). Leaves were weighed and homogenates containing 10 % trichloroacetic acid (TCA) and 0.65 % 2-thiobarbituric acid were heated at 95 °C for 60 min then cooled to room temperature and centrifuged at 10,000×*g* for 10 min. The absorbance of the supernatant was read at 532 and 600 nm against a reagent blank.

Hydrogen peroxide (H_2O_2) content was measured as described by Velikova and others (2000). Fresh leaf samples (0.5 g) were homogenized in 5 mL of 0.1 % (w/v) TCA. The homogenate was centrifuged at 12,000 rpm for 15 min and the supernatant was added to 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide. The absorbance of the supernatant was recorded at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve plotted using known concentrations of H_2O_2.

Electrolyte Leakage

Electrolyte leakage was used to assess membrane permeability, as described by Lutts and others (1995). Samples were washed three times with DDW to remove surface contamination. Leaf discs were cut from young leaves and placed in sealed vials containing 10 mL of DDW and incubated on a rotatory shaker for 24 h; subsequently, the electrical conductivity of the solution (EC_1) was determined. Samples were then autoclaved at 120 °C for 20 min and the electrical conductivity was measured again (EC_2) after cooling the solution to room temperature. Electrolyte leakage (%) was calculated as (EC_1/EC_2) × 100 %.

Enzyme Activity

The activity of CA (EC 4.2.1.1) was determined by the method of Dwivedi and Randhawa (1974). Leaf samples were cut into small pieces and suspended in cysteine hydrochloride solution. The samples were incubated at 40 °C for 20 min. The pieces were blotted and transferred to test tubes containing phosphate buffer (pH 6.8), then alkaline bicarbonate solution and bromothymol blue indicator were added. The test tubes were incubated at 50 °C for 20 min. After the addition of 0.2 mL of methyl red indicator, the reaction mixture was titrated against 0.05 N HCl. The results were expressed as μmol CO_2 kg^{-1} FW s^{-1}.

To determine the enzymatic activities of the antioxidant proteins, a crude enzyme extract was prepared by homogenizing 500 mg of leaf tissue in extraction buffer containing 0.5 % Triton X-100 and 1 % polyvinylpyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged at 15,000 *g* for 20 min at 4 °C. The supernatant was used for the enzymatic assays described below.

For APX, the extraction buffer was supplemented with 2 mM ascorbate. All enzyme activities were expressed as mg^{-1} protein min^{-1}.

We used the method of Chance and Maehly (1955) to determine POD (EC 1.11.1.7) activity by using 5 mL of an assay mixture containing phosphate buffer (pH 6.8), 50 M of pyrogallol, 50 mM of H_2O_2, and 1 mL of the enzyme extract diluted 20 times. This was incubated for 5 min at 25 °C, 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 measuring absorbance at 420 nm. A unit of peroxidase activity was the amount of purpurogallin formed mg^{-1} protein min^{-1}.

Aebi's (1984) method was used to measure CAT (EC 1.11.1.6) activity. The decomposition of H_2O_2 was monitored by the decrease in absorbance at 240 nm. For the assay, a 50 mM phosphate buffer (pH 7.8) and 10 mM H_2O_2 were used.

The activity of SOD (EC 1.15.1.1) was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the methods of Giannopolitis and Ries (1977). The reaction solution (3 mL) contained 50 µmol NBT, 1.3 µmol riboflavin, 13 mmol methionine, 75 nmol EDTA, 50 mmol phosphate buffer (pH 7.8), and 20–50 µL enzyme extract. The reaction solution was irradiated under a bank of fluorescent lights at 75 µmol m^{-2} s^{-1} for 15 min. The absorbance at 560 nm was read against a blank (nonirradiated reaction solution). One unit of SOD activity was defined as the amount of enzyme that inhibited 50 % of NBT photoreduction.

Glutathione reductase (EC 1.6.4.2) activity was assayed as described by Foyer and Halliwell (1976), with minor modifications. The assay mixture consisted of 50 µL of the enzyme extract, 100 mM phosphate buffer (pH 7.8), 0.1 µM EDTA, 0.05 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and 3.0 mM oxidized glutathione in a total volume of 1.0 mL. The NADPH oxidation rate was monitored by reading the absorbance at 340 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (A_{340}) was divided by the NADPH molar extinction coefficient (6.22 mM cm^{-1}) and the enzyme activity expressed as 1 µmol of NADPH mg^{-1} protein min^{-1}.

We measured APX (EC 1.11.1.11) activity following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.5 mM ascorbate. The reaction was started by adding the sample solution to the reaction buffer solution. The H_2O_2-dependent oxidation of ascorbate was tracked by the decrease in absorbance at 290 nm (absorbance coefficient at 2.8 mM^{-1} cm^{-1}).

Statistical Analysis

Each pot was treated as one replicate and all the treatments were repeated four times. The data were analyzed statistically with SPSS v17 statistical software (SPSS Inc., Chicago, IL, USA). Means were statistically compared by Duncan's multiple-range test (DMRT) at the $p < 0.05$ % level.

Results

Boron toxicity was assessed on the basis of plant growth and physiological and biochemical characteristics of *R. sativus* L.

Plant Growth Characteristics

We measured five plant growth characteristics (PH, FW, DW, LA, and LAI; Table 1). The application of $B_{0.5}$ significantly enhanced all five traits relative to the control ($Ca_0 + B_0$). However, at B_5, FW was significantly reduced; PH, LA, and LAI were lower but not significantly so; and DW was unchanged. Calcium at C_{30} enhanced all five growth attributes relative to the control, and other concentrations generally improved growth traits, but not always to a significant level.

The combined treatments of $Ca_{20} + B_{0.5}$ and $Ca_{30} + B_{0.5}$ significantly improved all growth traits relative to the control, although $Ca_{30} + B_{0.5}$ yielded larger plants by all measured parameters. $Ca_{40} + B_{0.5}$ significantly enhanced all traits except DW relative to the control. The application of $Ca_{20} + B_5$ and $Ca_{30} + B_5$ significantly ameliorated PH and FW compared to the application of B_5 alone, whereas the other plant traits were generally improved, although not always statistically so. $Ca_{40} + B_5$ was harmful to plants according to all of our measured traits (although the decrease in FW was not statistically significant).

Physiological and Biochemical Parameters

All concentrations of Ca alone (that is, Ca_{20}, Ca_{30}, and Ca_{40}) significantly increased leaf concentrations of Chl *a* and Chl *b*, as did $B_{0.5}$ (Fig. 1a). Ca_{30} induced the synthesis of significantly more Chl *a* than any other Ca treatment, although its effects on Chl *b* were statistically indistinguishable from those of Ca_{20}. Plants treated with B_5 had significantly lower concentrations of both chlorophylls than the control. The addition of Ca_{20} and Ca_{30} to the $B_{0.5}$ and B_5 treatments significantly improved the concentrations of both chlorophylls relative to boron alone, and

Table 1 Effect of Ca and/or B on the growth performance of *Raphanus sativus* L. (mean of four replicates)

Treatments	Plant height (cm)	Shoot FW (g)	Shoot DW (g)	Leaf area (cm^2)	Leaf area index
Control	20.33 ± 1.2 f	3.58 ± 0.18 de	0.26 ± 0.02 cd	32.25 ± 4.53 ef	4.79 ± 0.09 e
Ca_{20}	32.67 ± 1.2 de	4.20 ± 0.31 cd	0.37 ± 0.03 bc	53.78 ± 4.64 d	7.21 ± 0.19 d
Ca_{30}	41.67 ± 2.0 c	5.03 ± 0.16 c	0.40 ± 0.01 b	66.61 ± 4.73 c	7.94 ± 0.46 c
Ca_{40}	28.00 ± 2.1 e	3.34 ± 0.11 e	0.29 ± 0.05 bc	49.76 ± 1.82 d	7.07 ± 0.22 d
$B_{0.5}$	41.33 ± 2.6 c	4.67 ± 0.15 c	0.43 ± 0.09 b	54.13 ± 1.57 d	7.30 ± 0.34 d
B_5	17.33 ± 1.2 f	2.39 ± 0.26 f	0.26 ± 0.03 cd	26.13 ± 3.62 ef	3.59 ± 0.14 e
$Ca_{20}B_{0.5}$	52.37 ± 1.5 b	5.87 ± 0.33 b	0.76 ± 0.04 a	89.52 ± 1.48 b	9.79 ± 0.21 b
$Ca_{30}B_{0.5}$	70.00 ± 1.7 a	7.34 ± 0.40 a	0.84 ± 0.05 a	106.82 ± 5.37 a	12.92 ± 0.33 a
$Ca_{40}B_{0.5}$	48.00 ± 1.7 b	4.59 ± 0.20 c	0.36 ± 0.04 bc	65.51 ± 1.80 c	8.69 ± 0.23 c
$Ca_{20}B_5$	32.00 ± 2.1 de	3.55 ± 0.15 de	0.23 ± 0.02 cd	33.94 ± 2.26 e	4.85 ± 0.32 e
$Ca_{30}B_5$	35.00 ± 1.7 d	4.26 ± 0.22 cd	0.33 ± 0.03 bc	49.40 ± 1.54 d	7.06 ± 0.22 d
$Ca_{40}B_5$	18.00 ± 1.2 f	2.02 ± 0.37 f	0.15 ± 0.01 d	23.25 ± 1.57 f	3.32 ± 0.06 e

Same letters show no statistical difference at $p < 0.05$ (Duncan multiple-range test)

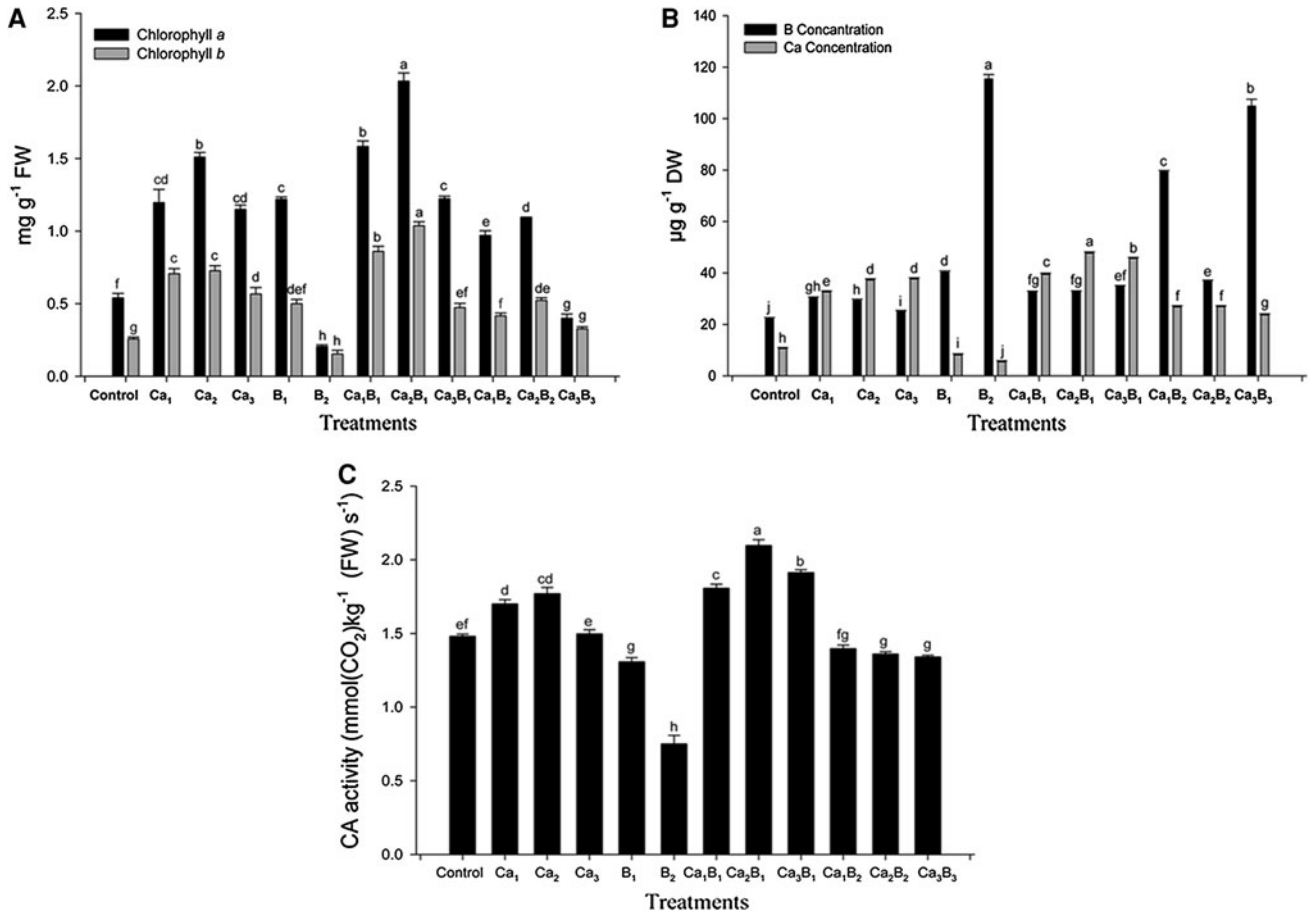

Fig. 1 Effect of Ca and/or B on the concentration of Chl *a* and Chl *b* (**a**), Ca and B (**b**), and activity of CA (**c**) in leaves of *Raphanus sativus* L. (mean of four replicates). *Bars followed by the same letters* show no statistical difference at $p < 0.05$ (Duncan's multiple-range test). Average of four determinations are presented *with bars* indicating SE. $Ca_1 = 20$ mM, $Ca_2 = 30$ mM, $Ca_3 = 40$ mM, $B_1 = 0.5$ mM, $B_2 = 5$ mM

plants exposed to $Ca_{30} + B_{0.5}$ had significantly higher concentrations of both Chl *a* and Chl *b* than any other treatment.

All Ca and B treatments significantly increased the levels of B in leaves relative to the control (Fig. 1b). The highest concentration of B was recorded at B_5, whereas

the addition of Ca to B_5 significantly lowered the levels of B in leaves, in the order $Ca_{40} + B_5$, $Ca_{20} + B_5$, then $Ca_{30} + B_5$. Both $B_{0.5}$ and B_5 significantly lowered the leaf Ca concentration relative to the control, with B_5 having the larger effect (Fig. 1b). All other treatments significantly enhanced Ca levels in leaves relative to the control and to either the $B_{0.5}$ or the B_5 treatment. The maximum value of Ca in leaves was found with the $Ca_{30} + B_{0.5}$ treatment.

The activity of CA relative to the control was significantly inhibited by the addition of boron alone, especially at B_5 (Fig. 1c). The application of Ca alone significantly increased the activity of CA relative to the control at Ca_{20} and Ca_{30}, and all concentrations of Ca in combination with $B_{0.5}$ or B_5 significantly improved CA activity compared to boron alone. $Ca_{30} + B_{0.5}$ enhanced CA activity significantly more than any other treatment.

The level of lipid peroxidation was indicated by the MDA concentration in leaves, and membrane damage and alteration was assessed by H_2O_2 content and electrolyte leakage (Fig. 2a–c). In all cases, B_5 was significantly more damaging to plants than any other treatment. However, the addition of Ca_{20} and Ca_{30} to $B_{0.5}$ significantly improved all parameters relative to $B_{0.5}$ alone, and all three concentrations of Ca combined with B_5 significantly ameliorated damage to plants. The combined application of $Ca_{30} + B_{0.5}$ yielded the lowest values of MDA, H_2O_2, and electrolyte leakage.

All treatments significantly increased the concentration of Pro in leaves relative to the control, although the effects were most notable with B_5 alone and when Ca and B were combined (Fig. 3a). The highest value occurred with the $Ca_{30} + B_{0.5}$ treatment. Levels of TSC were higher than the control in all treatments, although the differences were not significant for Ca_{40} or $B_{0.5}$ (Fig. 3b). Values significantly higher than all others were seen with the $Ca_{30} + B_{0.5}$ and $Ca_{30} + B_5$ treatments.

The application of Ca and B significantly influenced the activities of antioxidant enzymes (Fig. 4a–c). The application of all concentrations of Ca and B, alone and in combination, significantly increased relative to the control the activities of SOD, CAT, POD, GR, and APX (Fig. 4a–c).

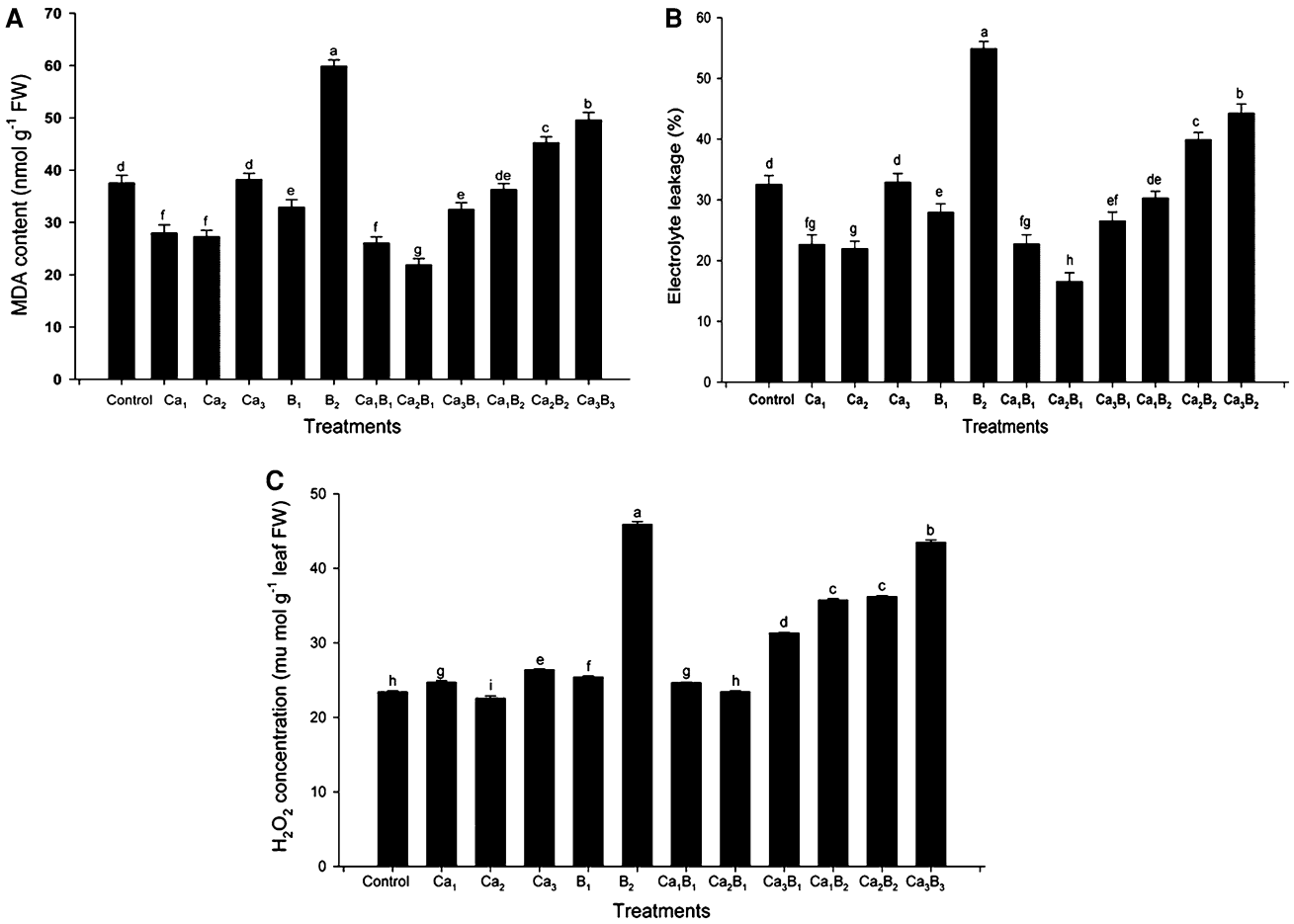

Fig. 2 Effect of Ca and/or B on content of MDA (**a**), electrolyte leakage (**b**), and concentration of H_2O_2 (**c**) of *Raphanus sativus* L. *Bars followed by the same letters* show no statistical difference at $p < 0.05$ (Duncan's multiple-range test). Average of four determinations are presented *with bars* indicating SE. $Ca_1 = 20$ mM, $Ca_2 = 30$ mM, $Ca_3 = 40$ mM, $B_1 = 0.5$ mM, $B_2 = 5$ mM

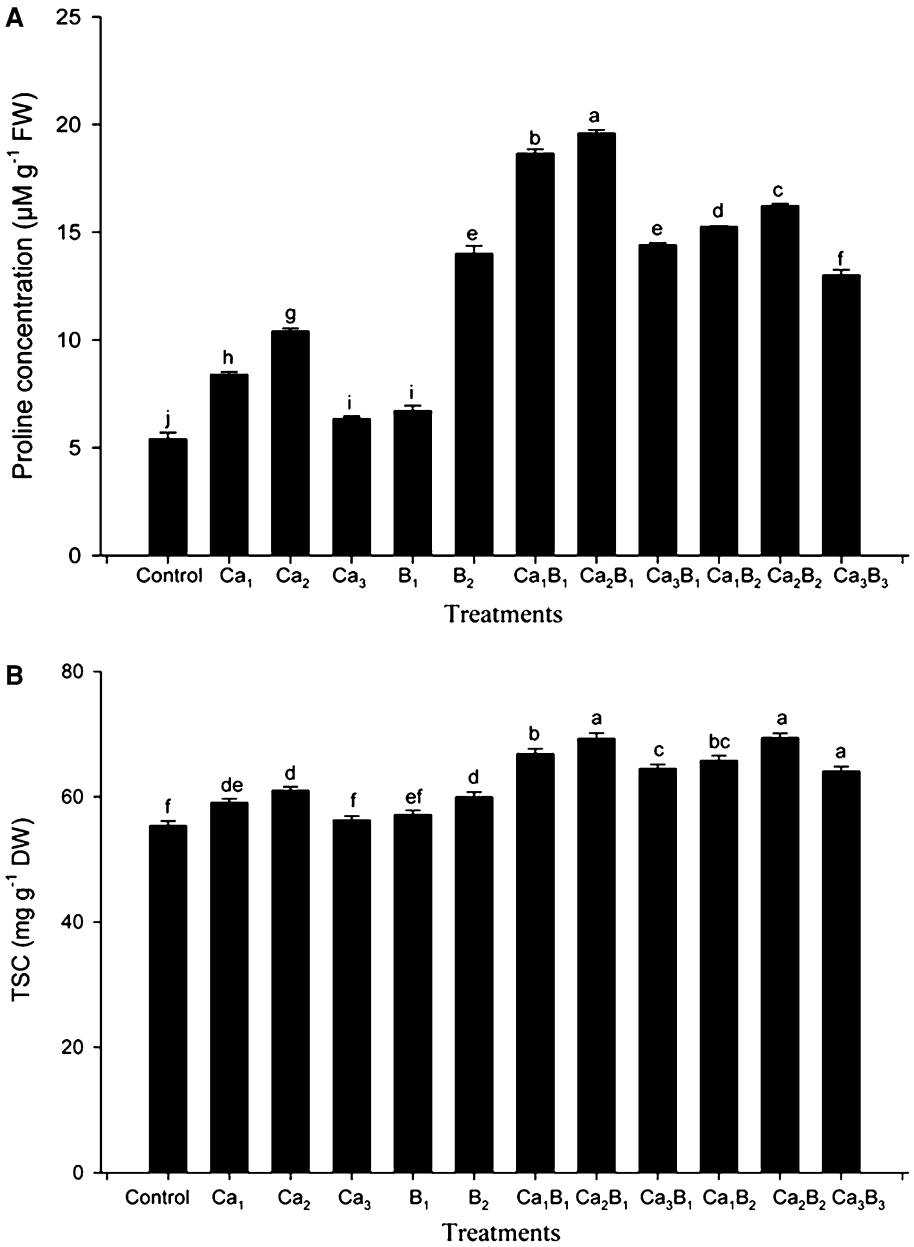

Fig. 3 Effect of Ca and/or B on the concentration of Pro (**a**) and TSC (**b**) in leaves of *Raphanus sativus* L. *Bars followed by the same letters* show no statistical difference at $p < 0.05$ (Duncan's multiple-range test). Average of four determinations are presented *with bars* indicating SE. $Ca_1 = 20$ mM, $Ca_2 = 30$ mM, $Ca_3 = 40$ mM, $B_1 = 0.5$ mM, $B_2 = 5$ mM

The activities of CAT and POD were significantly higher than any other treatment at $Ca_{20} + B_{0.5}$, whereas the activities of SOD, GR, and APX were significantly maximized at $Ca_{30} + B_5$.

Discussion

The application of various concentrations of Ca and a low concentration of boron ($B_{0.5}$), individually as well as in combination, to radish seedling substrate generally improved growth (Table 1), physiological characteristics, and biochemical processes (Figs. 1, 2, 3, 4); however, high levels of boron alone (B_5) were generally harmful.

We found that supplying Ca and low levels of B to radish plants improved plant growth. This effect may be explained by their physiological roles; both are required for the formation of cell walls and membranes (Cakmak and Römheld 1997). The role of Ca is very similar to that of a hormone; it regulates the protein pumps that take up nutrients into roots and move those nutrients among cells within the plant (White and Broadley 2003). Boron forms an important structural component of the peptic rhamnogalacturon II complex in cell walls (Matoh 1997). High levels of B (B_5) suppressed plant growth, which might be due to its toxic effects on root cell division, cell wall expansion, chlorophyll content, and photosynthetic rate (Liu and Yang 2000; Herrera-Rodriguez and others 2010).

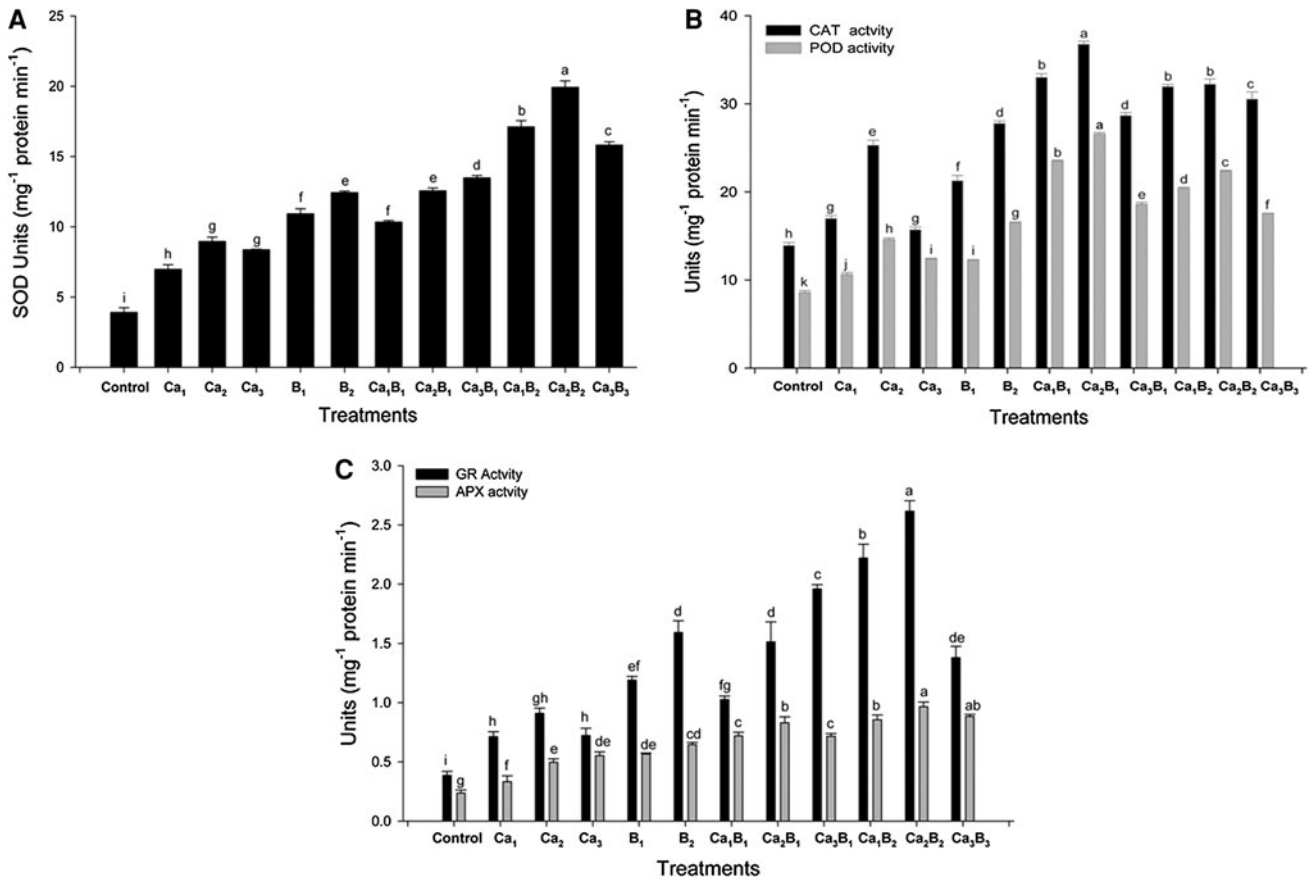

Fig. 4 Effect of Ca and/or B on the activity of SOD (**a**), CAT and POD (**b**), and GR and APX (**c**) of *Raphanus sativus* L. *Bars followed by the same letters* show no statistical difference at $p < 0.05$ (Duncan's multiple-range test). Average of four determinations are presented *with bars* indicating SE. $Ca_1 = 20$ mM, $Ca_2 = 30$ mM, $Ca_3 = 40$ mM, $B_1 = 0.5$ mM, $B_2 = 5$ mM

Combining Ca and B was most effective at improving PH, FW, DW, LA, and LAI, especially at $C_{30} + B_{0.5}$ concentrations.

In the present study, the synthesis of both chlorophyll *a* and *b* was severely affected by the higher dose of B_5 (Fig. 1a). This inhibition of photosynthetic pigments might be due to lipid peroxidation and hydrogen peroxide accumulation, which were both significantly affected by B_5 (Fig. 2a–c) and could damage reaction centers (Kyle 1987). In other studies, oxidative damage in apple and grape (Gunes and others 2006) and photooxidation damage to organic molecules in orange plants were induced by B toxicity (Cervilla and others 2007). Boron toxicity caused the inhibition of protein synthesis through the formation of borate esters with ribose (Reid 2007) and also altered the activities of several enzymes, and consequently the plant metabolism (Herrera-Rodrigues and others 2010). Interestingly, we found that the application of Ca in combination with B notably improved the levels of photosynthetic pigments as well as CA enzyme activity, and Ca application even helped to alleviate the toxic effects of B on these traits (Fig. 1a, c). Supplying Ca to the plants may have reduced the loss of photosynthetic pigments by reducing the photooxidation of organic molecules (Wise and Naylor 1987) or by maintaining membrane integrity (Hirschi 2004). Improved CA activity might have improved various physiological processes such as ion exchange, acid-base balance, inorganic carbon diffusion within cells and between them and their environments, and reversible hydrogenation of CO_2, thus maintaining its constant supply to RuBisCO. All these scenarios may explain the improved tolerance of radish to B toxicity.

The results revealed that the content of B in leaves of plants was significantly and negatively correlated with the tolerance of plants to B toxicity. We found the highest accumulation of B in leaves at the highest applied dose of B (B_5) (Fig. 1b). However, applying Ca with B significantly decreased the B content of leaves. Paull (1990) reported that B tolerance was correlated with a reduced accumulation of B in some wheat and barley genotypes. In our study, the growth medium containing $B_{0.5}$ increased Ca uptake, but the growth medium containing B_5 decreased Ca

content in leaves (Fig. 1b). These results suggested that Ca and B have interrelated metabolic roles. This interrelationship could be responsible for the improvements in PH seen, giving plants better leaf positioning for harvesting solar energy as well as facilitating leaf expansion, leading to larger LA. Thus, the addition of both Ca and B to the growth medium was more effective at improving plant growth than B alone (Table 1). An imbalanced supply of B and Ca is toxic to plants and impairs the growth and translocation of Ca and B within plants (Yamauchi and others 1986).

Lipid peroxidation, electrolyte leakage, and H_2O_2 content have been used as indicators of oxidative damage induced by different environmental stresses. The increases in oxidative damage as measured by MDA, H_2O_2, and electrolyte leakage in plants at B_5 (Fig. 2a–c) indicate that B toxicity may have caused cellular dysfunction by increasing lipid peroxidation. These results corroborate previous findings of increases in MDA and H_2O_2 content and electrolyte leakage in response to B toxicity (Ardic and others 2009; Cervilla and others 2007; Guimarães and others 2011). It is well established that Ca, an important nutrient, helps plants to protect against lipid peroxidation of the membrane by stabilizing the plasmalemma against lipolytic enzymes (Hirschi 2004; Siddiqui and others 2011). A high concentration of Ca in the cytosol of plants enhances physiological processes by triggering several enzymes, by affecting oxidative signal transduction, and by regulating antioxidant enzymes under different environmental conditions (Khan and others 2010; Siddiqui and others 2011). Thus, we postulate that the application of Ca protected the plants from B toxicity.

The functions of osmolytes in osmotic adjustment vary from species to species and even within plants species (Siddiqui and others 2008a, 2009a). Compatible solutes can lower or balance the osmotic potential within cells. In our study, both Pro and TSC levels increased relative to the control when plants received a high concentration of B (Fig. 3a, b). Both Pro and TSC were maximized with a combined application of $C_{30} + B_{0.5}$. Khan and others (2010) reported similar results. Pro, a universal osmoprotectant, acts as an antioxidant and a source of energy (Matysik and others 2002) and regulates gene expression for osmotic adjustment (Iyer and Caplan 1998). TSCs are the major soluble constituents that help plant cells in maintaining osmotic balance. This work indicates that alleviation of B toxicity is associated with increases in the concentrations of Pro and TSC caused by the addition of Ca to the growth medium.

The generation of ROS is metabolically induced by abiotic and biotic stresses in plant cells (Foyer and Noctor 2000). ROS are harmful to plants because they react with a large variety of biomolecules, including DNA, proteins, and carbohydrates. Plants must limit the production of ROS, detoxify them once formed, and repair damage caused by ROS. The levels of MDA, H_2O_2, and electrolyte leakage were substantially higher in plants subjected to B_5 (Fig. 2a–c). MDA and H_2O_2 are considered indicators of oxidative damage. Mittler (2002) suggested that membrane damage might be caused by high levels of H_2O_2, which could trigger the Haber–Weiss reaction, resulting in hydroxyl radical formation and thus lipid peroxidation. However, application of Ca may have lowered these factors by enhancing antioxidant systems (Fig. 4a–c). ROS scavenging is associated with antioxidant enzymes such as POD, SOD, CAT, APX, and GR (Meloni and others 2004; Siddiqui and others 2009a). In the present study, the activities of SOD, GR, and APX were highest in plants receiving $Ca_{30} + B_5$. These results substantiate the findings of Khan and others (2010) and Siddiqui and others (2011). The balance among SOD, GR, and APX activities in the cell could be crucial for determining the steady-state levels of O_2^- and H_2O_2. The accumulated Ca may have been responsible for the decreased content of MDA and H_2O_2; the unique importance of Ca for stabilizing membranes is well known (Marschner 1995; Hirschi 2004). The application of $Ca_{30} + B_{0.5}$ enhanced POD and CAT activities, suggesting that Ca alone did not affect these two enzymes. SOD constitutes the first line of defense against superoxide and ROS, and GR also plays an important role in maintaining the ratio of cellular antioxidants and prooxidants. Thus, enhancing the antioxidant systems of plants by applying Ca could induce plant defenses against oxidative damage by H_2O_2 under B toxicity.

Conclusions

In conclusion, application of Ca had a significant synergistic effect on plant growth when applied in conjunction with a lower level of B. A decrease in accumulation of B was recorded in plants supplied with Ca. In the present study, out data revealed that MDA and H_2O_2 contents and electrolyte leakage increased on treatment with a high dose of B. However, Ca significantly reduced the adverse effects of B toxicity by mitigating cellular oxidative damage through improved free radical scavenging by antioxidant enzymes. Calcium clearly induced protective mechanisms by enhancing the accumulation of Pro, TSC, and photosynthetic pigments in leaves. This experiment therefore provides information that supplemental irrigation with Ca is an effective crop management practice for reducing B toxicity.

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