



The role of calcium, silicon and salicylic acid treatment in protection of canola plants against boron toxicity stress

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Abstract

Boron (B) toxicity often limits crop yield and the quality of production in agricultural areas. Here, we investigated the effects of calcium (Ca), silicon (Si) and salicylic acid (SA) on development of B toxicity, B allocation in canola (*Brassica napus* cultivar Sarw 4) and its role in non-enzymatic antioxidants in relation to yield of this cultivar under B toxicity. Canola seedlings were subjected to four B levels induced by boric acid in the absence or presence of Ca, Si and SA. The results showed that Ca, Si and SA addition ameliorated the inhibition in canola growth, water content (WC), and improved siliqua number, siliqua weight and seed index. The B content in shoots and roots and total B accumulation in the whole plant were increased in control plants under B-toxicity-stress, and these parameters were significantly decreased by addition of Ca, Si and SA. The shoot ascorbate pool (ascorbate, AsA, and dehydroascorbate, DHA), α -tocopherol and phenolics (free and bound) were increased under B toxicity, and were significantly decreased in most cases by addition of Ca, Si and SA, except α -tocopherol, which increased at low B levels (0, 25 and 50 mg kg soil⁻¹). The glutathione content did not obviously change by B stress, while added Ca, Si and SA inhibited its accumulation under B stress. In addition, B toxicity reduced the shoot flavonoids content; however, this reduction was not alleviated by the use of Ca, Si and SA treatments. It could be concluded that growth and yield of canola plants grown under high B concentration improved after external application of Ca, Si or SA.

Keywords Boron toxicity · Canola (*Brassica napus* L. cultivar Serw 4) · Calcium · Salicylic acid · Silicon

Introduction

Boron (B) is an essential micronutrient for growth and development of vascular plants, but excessive B in the soil or in irrigation water occurs in many dry areas of the world, including Egypt, Turkey, Iraq, Jordan, Syria, Morocco, Chile, USA, Spain and South Australia, and causes B toxicity (Ayvaz et al. 2013; Landi et al. 2012). Boron toxicity

often limits crop yield and the quality of production in agricultural areas (Miwa et al. 2007). Even for boron-tolerant plants, boosting soil conditions by adding amendments is a needful step of reclamation (Kayama 2010).

Most of the ionic stresses including B trigger the formation of reactive oxygen species (ROS), which lead to the establishment of oxidative stress in plants. Plants with high levels of antioxidants have a great resistance to this oxidative damage (Foyer and Shigeoka 2011). Non-enzymatic antioxidants may act directly in the detoxification of ROS and radicals, or they can reduce substrates for antioxidant enzymes (Mittler 2002). Non-enzymatic components include the major cellular redox buffers ascorbate (AsA) and glutathione (GSH) as well as tocopherol, carotenoids, and phenolic compounds (Gratão et al. 2005; Mittler et al. 2004; Yıldırım and Uylaş 2016). AsA is a crucial component of the detoxification of ROS in the aqueous phase due to the ability to donate electrons in enzymatic and non-enzymatic reactions (Caverzan et al. 2016). Glutathione is oxidized by ROS to form oxidized glutathione (GSSG), which is present in all cellular compartments and along with its oxidized

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form maintains the redox balance in cellular compartments (Caverzan et al. 2016). Tocopherols is a group of lipophilic antioxidants (Sharma et al. 2012) and the α -tocopherol present in the membrane of chloroplasts protects them against photooxidative damage (Blokhina et al. 2003). Phenolic compounds are abundantly found in plant tissues and possess antioxidant properties (Grace and Logan 2000). The antioxidant activity of flavonoids is attributed to their reaction with free radicals as a hydrogen donor (Zhang et al. 2017). Few studies are available on the antioxidant response of plants submitted to toxic levels of B (Cervilla et al. 2012). This prompted us to conduct a broader study on non-enzymatic antioxidant protection of canola plants under high B stress.

Calcium (Ca) is an essential element that plays an important role in stress tolerance (Tuteja 2009). Also, Ca is an important signaling compound, regulates cellular metabolism, moreover, is important for energy production within mitochondria and chloroplasts (Batistič and Kudla 2010). Previous studies showed a major role of Ca in alleviating the negative effects of B (Kanwak et al. 2008; Turan et al. 2009). The interactions between Ca and B are still being discussed (Bolaños et al. 2004). Thus, studies of the interactive effects of Ca and B on plant growth are essential.

Silicon (Si) is the second most abundant element in the earth's crust (Zhu and Gong 2014) and is taken up in the form of silicic acid, $\text{Si}(\text{OH})_4$, by plants. It is ultimately irreversibly precipitated throughout the plant as amorphous silica and is the only nutrient element that is not detrimental when accumulated excessively in plants (Ma et al. 2001). The positive roles of Si have been confirmed against biotic (Fauteux et al. 2006; Fallah 2012; Liang 1999) and abiotic stresses (Shi et al. 2016; Van Bockhaven et al. 2013; Zhu and Gong 2014). Si also protects the plant by other processes, which can boost the defense mechanisms, including the accumulation of lignin, phenolic compounds, and phytoalexins (Epstein 1999; Fawe et al. 2001; Ma and Yamaji 2006). Formation of B-Si (boron-silicate) complexes in the soil leads to lower B availability and consequently lowers tissue B accumulation (Inal et al. 2009). A limited number of investigations have been carried out with Si supplied to plants suffering from B toxicity. Therefore, the present study aimed to study the metabolic effect of Si in counteracting canola growth retardations induced under excess B toxicity.

Salicylic acid (SA) is a plant hormone that regulates many aspects of plant growth and development, as well as resistance to (a)biotic stress (Dempsey and Klessig 2017). The role of the SA in defense mechanisms under both biotic and abiotic stresses suggests that it also alleviates salt stress, B and cadmium toxicity in plants (Al-Hakimi and Hamada 2001; El-Feky et al. 2012, 2014; Eraslan et al. 2007; Metwally et al. 2003; Mimouni et al. 2016; Radi et al. 2014). Exogenous application of SA enhanced growth, physiological process and antioxidant activity and increase plant

tolerance to the abiotic stress (Eraslan et al. 2007; He et al. 2002). In lower concentrations (less than 1 mM), it has been reported to be beneficial for plant growth (Rivas-San Vicente and Plasencia 2011). Recently, Yıldırım (2017) concluded that endogenous increase in SA attributed to lower B uptake, accumulation and higher B toxicity tolerance in *Populus alba*.

Canola quality rapeseed is one of the main oil crops world-wide but canola is a new crop in Egypt. Traditionally, *Brassica napus* is unsuitable as a source of food for either humans or animals due to the presence of two naturally occurring toxicants, erucic acid and glucosinolates. However, in the 1970s, very intensive breeding programs in several countries including Australia produced high quality varieties that were significantly lower in these two toxicants. Those cultivars must yield oil low in erucic acid (below 2%) and meal low in glucosinolates (total glucosinolates of 30 $\mu\text{moles/g}$ toasted oil free meal), and are often referred to as “double low” varieties. Due to the strong competition between canola and other strategic winter season crops on the limited arable land in Nile Valley and Delta the cultivated area of canola in Egypt is relatively small (Mekki 2013). Ghallab and Sharaan (2002) reported that the cultivation of canola in Egypt may provide an opportunity to overcome some of, the local deficit of edible vegetable oil production. Particularly, it could be successfully grown during winter season in newly reclaimed land outside the Nile Valley soils to get around the competition with other crops engaged the old cultivated area. Since, 1978 some spring types of *Brassica napus* L. have been introduced from Europe and evaluated under Egyptian conditions and the crop has great promise as an oilseed crop in the winter season in Egypt (Mekki 2007; Sharaan and Ghallab 2002). Some canola genotypes were evaluated under newly reclaimed sandy soil conditions in Egypt and were found that Serw 4 had the highest seed yield, out of six, test cultivars (Mekki 2013).

Canola has a higher requirement for B than do cereals (Grant and Bailey 1993). B is essential to development of both male and female reproductive organs in canola (Asad et al. 2002; Dell and Huang 1997) and is an important factor in the fertilization process of flowers. B-deficient plants may grow normally but seed yield may be severely reduced (Gupta 1993). Application of B to canola has been found to significantly increase grain oil content (Pageau et al. 1999). However, if soluble B fertilizer placed close to seed and on sands that have limited B sorption capacity it may cause toxicity in crops (Moraghan and Mascagni 1991). Hughes-Games (1991) reported larger variations in tolerance of canola to B toxicity than barley and wheat. Tolerance was suggested to be related to the origin of a cultivar (Du et al. 2002).

Boron toxicity is an important nutritional disorder that can limit plant growth and productivity in arid and semi-arid

environments. Elbehiry et al. (2017) found that B toxicity representing more than 50% of northern western part of Egypt Nile Delta profiles. This area is dominated by fine texture, irrigated by mixed and contaminated water, and has low content of organic matter, which contributes as a contamination source of B. Thus, in this investigation it seemed necessary to consider some physiological and biochemical responses of canola cultivar Serw 4 and how far these responses are correlated with the B-tolerance mechanisms at different B levels. Particular attention was focused to investigate the role of Ca, Si, and SA in counteracting the adverse effects of B toxicity on canola plants at vegetative growth and productivity stages, and consequently to cultivate these plants in the newly reclaimed Egyptian soil.

Materials and methods

Two experiments were carried out in a greenhouse of Faculty of Science, Assiut University (27°11'00"N 31°10'00"E) with day/night temperature of 25–30/12–16 °C, relative humidity of 42–48% and daily photon flux density of about 900–1100 $\mu\text{mol m}^{-2} \text{S}$ in order to assess the possible involvement of 15 mM Ca as CaCl_2 (this concentration was adjusted from preliminary experiments), 2 mM Si as Na_2SiO_3 (Eraslan et al. 2008), and 0.5 mM SA as sodium salicylate (Metwally et al. 2003), in adaptation of canola plant to B toxicity. The seeds of canola (*Brassica napus* L.) cultivar Sarw 4, commonly used in Egypt, were kindly supplied by the Agricultural Research Center, Giza, Egypt.

Experiment 1: short duration treatment (vegetative stage)

The seeds of canola were planted in plastic pots lined with polyethylene bags and filled with 2 kg of soil. Experimental soil was loam in texture (46% sand, 24% silt and clay 30%), containing 7.95% CaCO_3 and had a pH (1: 1 water extract) of 7.8, 3.86 ds m^{-1} EC (1: 1 soil: water), 1.3 mg kg^{-1} B, 3412.5 mg kg^{-1} $\text{CO}_3^{2-} + \text{HCO}_3^-$, 816.5 mg kg^{-1} Cl^- , 360 mg kg^{-1} Ca^{2+} , and 144 mg kg^{-1} Mg^{2+} mg kg^{-1} . Treatments, with six replicates, consisted of four groups. The first group was B only in final concentration of: 0 (control), 25, 50 and 100 mg kg^{-1} soil B as boric acid. Ca group was Ca (15 mM) combined with B levels as: 0 + Ca, 25 + Ca, 50 + Ca and 100 + Ca. Si group was Si (2 mM) combined with B levels as: 0 + Si, 25 + Si, 50 + Si and 100 + Si. SA group was SA (0.5 mM) combined with B levels as: 0 + SA, 25 + SA, 50 + SA, and 100 + SA. All these treatments were applied and incorporated into the soil before seed sowing with the basal fertilization of 120 mg N kg soil^{-1} , 100 mg P kg soil^{-1} , and 50 mg K kg soil^{-1} , and the pH was buffered

to 5.7. Canola seeds were sown at the rate of 15 seeds to each pot. After a good stand of plants developed, they were thinned to 10 plants per pot. Soil was kept at approximately 90% field capacity by watering with tap water under the above mentioned condition.

Harvesting and plant growth yield

At the end of the experimental period (1 month), plants were fractionated into roots and shoots. Plant height and root length were measured. The roots were only briefly rinsed with deionized water, in order to avoid washing out of freshly acquired B, blotted gently with filter paper. The shoots and roots were quickly weighed separately for fresh weight (FW) determination, immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Another fraction of freshly harvested roots and shoots were oven-dried at 60°C for 48 h in order to determine the dry weight (DW), relative water content and to follow some analysis.

$$\text{Relative water content} = (\text{FW} - \text{DW})/\text{DW}.$$

Experiment 2: long duration treatment (fruiting stage)

Canola plants were grown under the growth conditions mentioned above. Treatments, with six replicates, consisted of following combinations: six final B levels, 0, 10, 20, 30, 40 and 50 mg kg soil^{-1} . One Ca^{2+} level (15 mM) combined with B levels (mg kg soil^{-1}), 0 + Ca, 10 + Ca, 20 + Ca, 30 + Ca, 40 + Ca and 50 + Ca. One Si level (2 mM) combined with B levels, 0 + Si, 10 + Si, 20 + Si, 30 + Si, 40 + Si and 50 + Si. One SA level (0.5 mM) combined with B levels, 0 + SA, 10 + SA, 20 + SA, 30 + SA, 40 + SA and 50 + SA. At the end of the experimental period (4 months) a number of parameters such as number of siliqua, siliqua weight, seed index [100 seed weight (g)] were determined.

Physiological and biochemical analysis

Total flavonoid content

Total flavonoid content was measured as described in Moreno et al. (2000) with a slight modification, and the results were expressed as quercetin equivalents. Methanol extract was added to test tubes containing 0.1 ml of 10% aluminum nitrate, 0.1 ml of a 1 M potassium acetate solution and 3.8 ml of methanol. After 40 min at room temperature, the absorbance was measured at 415 nm. Quercetin was used as a standard. The results were expressed as mg g FW^{-1} .

Determination of ascorbate and dehydroascorbate

Ascorbate (AsA) and dehydroascorbate (DHA) were measured according to Kampfenkel et al. (1995) with minor modifications. Briefly, total ascorbate (TA) (AsA + DHA) was determined after reduction of DHA to AsA with dithiothreitol (DTT), and the concentration of DHA was estimated from the difference between the total ascorbate pool (TA) and AsA. Shoot samples (0.3 g) were homogenized in 6% trichloroacetic acid (TCA) pre-chilled on ice. The homogenate was then centrifuged at 12,000g for 10 min and the resulting supernatant was used for the determination of TA and AsA. The reaction mixture for the TA pool contained 0.1 ml aliquot of the supernatant, 0.25 ml of 50 mM phosphate buffer (pH 7.5) containing 2.5 mM ethylenediamine tetraacetic acid (EDTA), and 0.05 ml of 10 mM DTT. After incubation for 10 min at room temperature, 0.05 ml of 0.5% *N*-ethylmaleimide was added to remove excess DTT. AsA was determined in a similar reaction mixture, except that 0.1 ml of H₂O was added rather than DTT and *N*-ethylmaleimide. Color was developed in both reaction mixtures after the addition of the following reagents: 0.2 ml of 10% TCA, 0.2 ml of 44% ortho-phosphoric acid, 0.2 ml of 4% *a,a'*-dipyridyl in 70% ethanol, and 0.3% (w/v) FeCl₃. After vortexing, the mixture was incubated at 40 °C for 40 min. Then, TA and AsA were determined when monitored at 525 nm using a spectrophotometer (Unico UV-2100 spectrophotometer). The results were expressed as mg g FW⁻¹.

Glutathione

Total glutathione (GSH + GSSG) levels were determined according to the method of Griffith and Meister (1979). The results were expressed as μmol min⁻¹ g FW⁻¹.

Determination of α-tocopherol (vitamin E) content

Fresh shoots were harvested from each treatment (0.5 g) and stored in liquid nitrogen at –80 °C. Plant α-tocopherol was extracted and purified by following procedure as described by Schmieden and Wild (1994).

HPLC analysis was carried out using Agilent HPLC 1200 (Santa Clara, CA) system consisting of degasser, quaternary pump, and a fluorescence detector (FLD). HPLC chemstation software (Agilent) was used for instrument control, data acquisition, and data analysis. α-Tocopherol was separated on Zorbax Eclipse -C18 column 4.6 × 150 mm, 5 μm particle size. The column temperature was maintained at 30 °C. The injection volume was 20 μl and the flow rate was 1 ml/min. Each analysis was carried out in triplicate. The elution system consisted of solvent A: methanol and B: acetonitrile with a flow rate as following: 0–4 min, 10–14 min 70% B at a flow rate 1 ml/min and 4–10 min 0% B at a flow rate

1.2 min. The eluates were monitored by FLD at 295 nm, and emission wavelength of 330 nm. α-Tocopherol was calculated from a standard curve prepared with α-tocopherol.

Determination of free and cell wall-bound phenolics

Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth (1995). Phenolic concentration in the extract was determined from standard curve prepared with gallic acid. The results were expressed as μg g FW⁻¹.

Determination of boron

Shoot and root samples (0.01 g DW) were dry ashed in a muffle furnace at 500 °C for 6 h by a method described by Gaines and Mitchell (1979). The ash was then dissolved in 0.1 N HCl. For B concentration measurement, B was determined using flame atomic absorption spectrometry (AAS).

Total accumulation in whole plant (%)

$$= (\text{B concentration in shoot and root}) / \text{B in soil} \times 100.$$

Statistical analysis

Each pot contained ten plants in the short duration treatments (vegetative stage), and 5 plants in the long duration treatments (fruiting stage). Each treatment contained six replicates pots. Two way ANOVA was performed on the data with six replicates of six measurements from two independent experiments. Analysis of two-way ANOVA was performed using the SPSS statistical 22.0 package.

For comparison of the means, a posthoc test (Tukey's multiple range tests) ($P < 0.05$) were used for significant differences. Analysis of correlation (Pearson's correlation) was performed to obtain the relation between mean values of different parameters of canola cultivar under boron and Ca, Si or SA treatments.

Results

Development of B toxicity in canola

Results of the first experiment revealed that plant height and root length did not exhibit any significant reduction at low concentration of B (25 mg kg soil⁻¹), as compared to control (experiment 1; Fig. 1, Fig. S1 (at 15-day), Tables S1, S2). On the other hand, higher concentrations (50 and 100 mg kg soil⁻¹) caused a 47 and 66% reduction in the height of the tested plants, respectively. For the same respective B

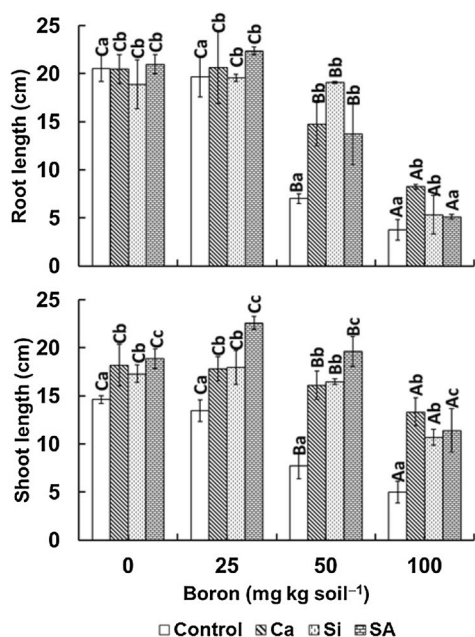
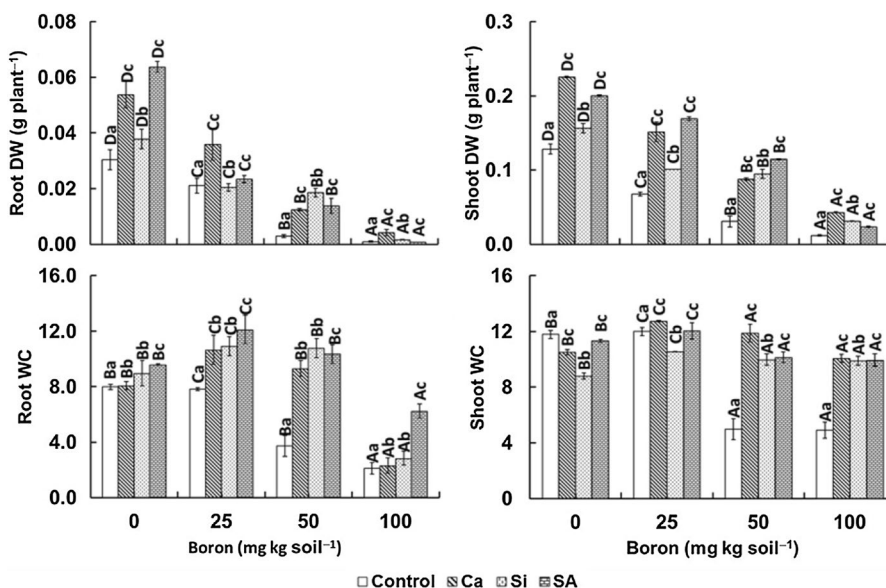


Fig. 1 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on root and shoot lengths of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)

treatments, inhibition in root elongation was measured as 66 and 88%, compared to control.

Treatments with Ca, Si or SA significantly increased the height and root length of canola plants more than those of stressed plants. B toxicity-dependent reductions in the height of canola at the higher B concentrations (50–100 mg kg soil⁻¹) were measured as 8.8, 26.87 and 22.09% for Ca, Si and SA treatments, respectively.

Fig. 2 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on root and shoot dry weight (DW) and relative water content (WC) of root and shoot of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)



Similar improvement in Ca, Si and SA applications were also recorded for root growth at higher B toxicity treatments. The reductions in root length at 100 mg kg soil⁻¹ B treatment for each respective application was 59.86, 74.05 and 75.02%, compared to control.

The successive increase in B concentration induced an inhibitory effect on the shoots and roots dry weight and relative water content of canola plants (experiment 1; Fig. 2, Tables S1, S2). Compared to control, the highest reduction in dry weight of the shoots (90.77%) and roots (96.87%) were recorded at 100 mg kg soil⁻¹ B treatment. Similarly, relative water content of shoots and roots severely reduced at the same B toxicity and reached to 58.43 and 73.56% reductions, respectively.

Application of Ca, Si or SA to B-stressed plants reduced the negative effect of low and moderate B levels (25 and 50 mg kg soil⁻¹) on shoots and roots dry weight and relative water content. Although Ca, Si, and SA applications had significant improvement in shoot dry weight and relative water content, their effects on root dry weight and root relative water content were found to be insignificant, compared to 100 mg kg soil⁻¹ B treated plants. Compared to control plants, reduction in shoot dry weight with application of Ca, Si and SA were recorded to be 66.52, 75.53 and 81.68%, while the decrease in shoot relative water content for each respective applications was 14.67, 16.09 and 15.72% at 100 mg kg soil⁻¹ B treatment.

The results of this study indicated that shoot (-0.79) and root (-0.94) relative water content represented a strong negative correlation with increase in B concentration in the soil. As it was expected, Ca and SA applications also exhibited a similar but smaller negative correlations between shoot (-0.39) relative water content and increase in soil-B concentration. Interestingly, Si application

conversely revealed a significantly positive correlation (0.24) between the same parameters.

B allocation in plants

Results indicated that the plant shoots contain a higher concentration of B than roots. These results confirmed by B accumulation in shoots, which recorded its highest value (sixfold) at 100 mg kg soil⁻¹, compared to their B toxicity treated roots (experiment 1; Fig. 3, Tables S1, S2). Also, B in roots (3.3-fold) and total accumulation in whole plant (1.8-fold) were increased with increasing B concentration in the medium.

Ca supply reduced B accumulation in both shoots (56.09%) and roots (46.03%), compared to B-treated plants at 100 mg kg soil⁻¹ B concentration (Fig. 3). At the same B toxicity treatment, 54.68% reduction in total B accumulation

were also recorded with Ca application in comparison to B-treated plants.

Similar to Ca application, Si also reduced total B uploading to the entire plant (34.13%) and to the shoots (41.48%), compared to B treated plants at 100 mg kg soil⁻¹ B treatment (Fig. 3). Contrary to shoots, B accumulation at the same treatment increased 10.73% in the roots, compared to B-treated plants.

Application of SA also caused a reduction in B accumulation in shoots (67.85%) and roots (23.35%) as well as total accumulation in whole plant (61.59%) of the 100 mg kg soil⁻¹ B-stressed plants, compared to their B-toxicity-treated controls (Fig. 3).

Non-enzymatic antioxidants

As B was transported and accumulated in shoot system, all the studied parameters were done on shoots of canola plants.

Ascorbate pool (vit. C)

The data presented in Fig. 4 and Table S1 and S2 (experiment 1) showed that B with or without Ca, Si and SA did not enhance AsA content (reduced form) over their corresponding absolute control of canola shoots; only the highest level (100 mg kg soil⁻¹) without Ca, Si or SA enhanced it.

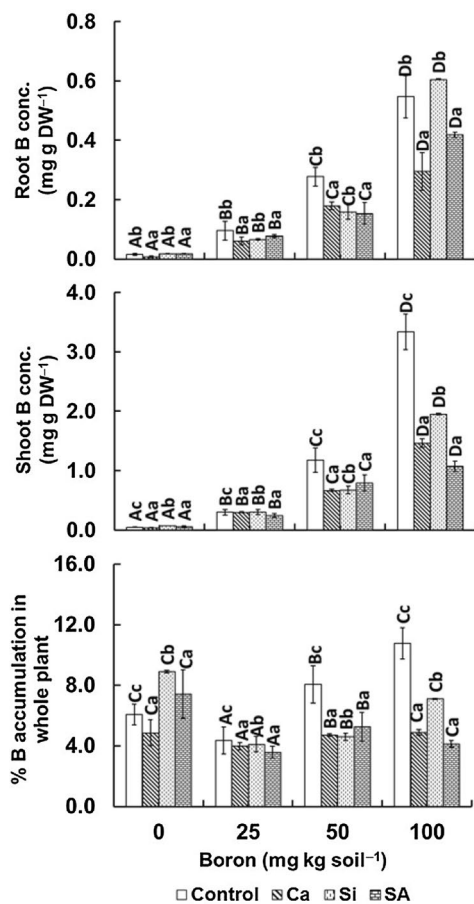


Fig. 3 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on root and shoot boron concentrations and % boron accumulation in whole plant of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)

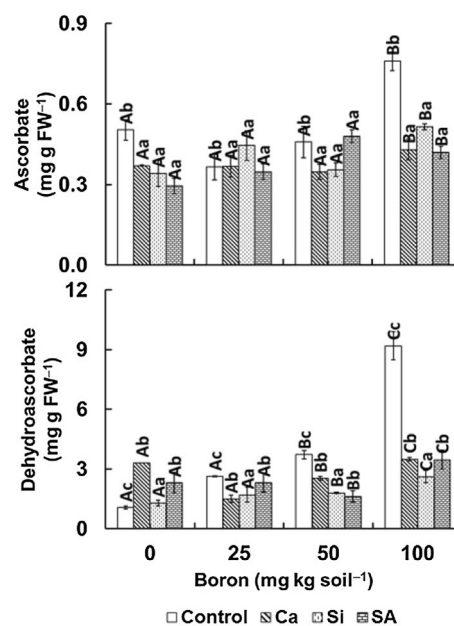


Fig. 4 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on shoot ascorbate and dehydro-ascorbate of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)

On the other side, DHA (oxidized form) of canola plant increased gradually with the rise of B level (Fig. 4). The highest DHA content in shoots was consistently found in plants grown at the highest B level. However, application of Ca, Si and SA caused an inhibition in DHA content in B-stressed plants as evidenced by 61.95, 71.52 and 62.29%, respectively, compared to stressed controls.

Tocopherol (Vit. E)

B toxicity had stimulatory effects on shoot α -tocopherol content at the highest B level (100 mg kg soil⁻¹), while 25 and 50 mg kg soil⁻¹ B failed to induce the same response, when compared with their absolute control (experiment 1; Fig. 5, Tables S1, S2). Overall, Ca, Si and SA in this investigation had significant stimulatory effects on α -tocopherol content in canola shoots under both B toxicity and non-B toxicity conditions, compared to absolute controls. The most

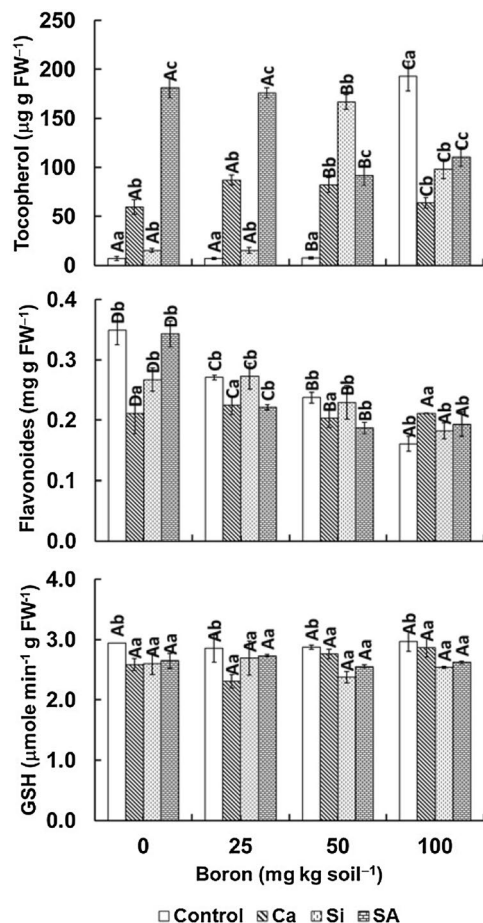


Fig. 5 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on shoot alpha-tocopherol, flavonoids and glutathione (GSH) of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD- post-hoc)

effective role was played by SA under both B toxicity (in most cases) and non-B toxicity conditions. In the presence of SA, the highest stimulation in α -tocopherol content was found to be 2494.59% over the control without B toxicity and 2422.96% at 25 mg kg soil⁻¹ B treatment, as compared to B-treated plants.

Flavonoids

Increasing B level from 25 to 100 mg kg soil⁻¹ reduced the biosynthesis of flavonoids in shoots of the tested plant (experiment 1; Fig. 5, Tables S1, S2). However, this reduction was not alleviated by the use of Ca, Si and SA treatments. In this respect, under non-B toxicity conditions, Ca and Si decreased flavonoids content in shoots of canola plants, compared to absolute control.

Glutathione

The results of the current study (experiment 1; Fig. 5, Tables S1, S2) revealed that increase in soil B (25–100 mg kg soil⁻¹) concentrations did not induce any significant alteration in glutathione accumulation in shoots of canola plants ($P \leq 0.1686$). On the other hand, application of Ca, Si and SA caused an inhibition (3.30, 14.45 and 11.62%, respectively) in glutathione content of the 100 mg kg soil⁻¹ B-stressed plants, compared to their B toxicity treated controls.

Free phenolics

Boron toxicity has a major effect on free phenolic compounds content. The data in Fig. 6 and supplementary Tables 1 and 2 (experiment 1) clearly demonstrated that free phenolics compounds accumulation in shoots of canola plants was stimulated by all the B levels. In addition, free phenolics compounds accumulation increased gradually with the rise of B level. The highest free phenolics compounds content in shoots was consistently found in plants grown at the highest B level (100 mg kg soil⁻¹). Application of Ca, Si and SA significantly controlled the rise of free phenolics accumulation in B toxicity applied plants. As can be seen Fig. 6, especially Ca and SA had the most significant effect on the reduction of free phenolics accumulation in B toxicity treated canola shoot.

Bound phenolics

Based on the data presented in Fig. 6 and Table S1 and S2 (experiment 1), it can be observed that the increase in B supply, in the range studied, had a considerable stimulatory effect on bound phenolics compounds accumulation in shoots of canola plants. The highest bound phenolics

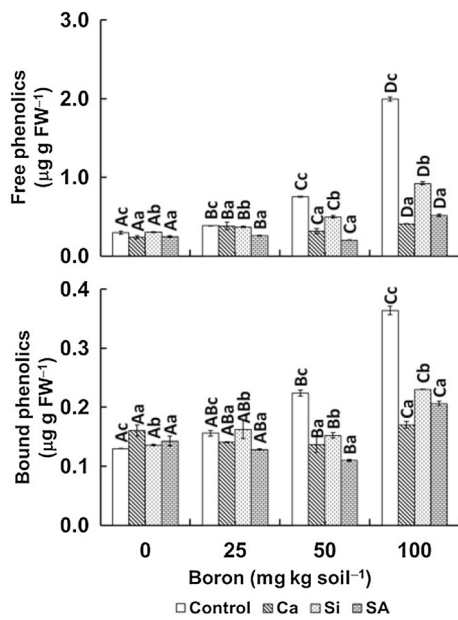


Fig. 6 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on shoot free and bound phenolics of 30-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)

compounds accumulation was consistently found in plants grown in the highest soil B concentration.

Ca, Si and SA in this investigation had a noticeable effect on bound phenolics content. At 50 and 100 mg kg soil⁻¹ B, Ca, Si and SA treatments showed a uniform pattern in their response through reducing the bound phenolics content under B toxicity stresses to a significant level, compared to their corresponding controls. The minimum values were recorded in canola for plants treated

with Ca and SA and grown under B toxicity conditions, respectively.

Crop yield

Siliqua number

Under B toxic conditions, external application of Ca, Si and SA significantly alleviated the inhibitory effect of B toxicity on siliqua number up to 40 mg kg soil⁻¹ B concentration (experiment 2; Fig. 7, Tables S1, S2). At the highest B level (50 mg kg soil⁻¹), the most effective alleviation treatments were Ca, and SA, respectively. For instance, at 50 mg kg soil⁻¹ B with Ca and SA treatments the siliqua number increased to 134.61 and 63.46%, respectively, than that of respective B-stressed control, which decreased 56.70% than control.

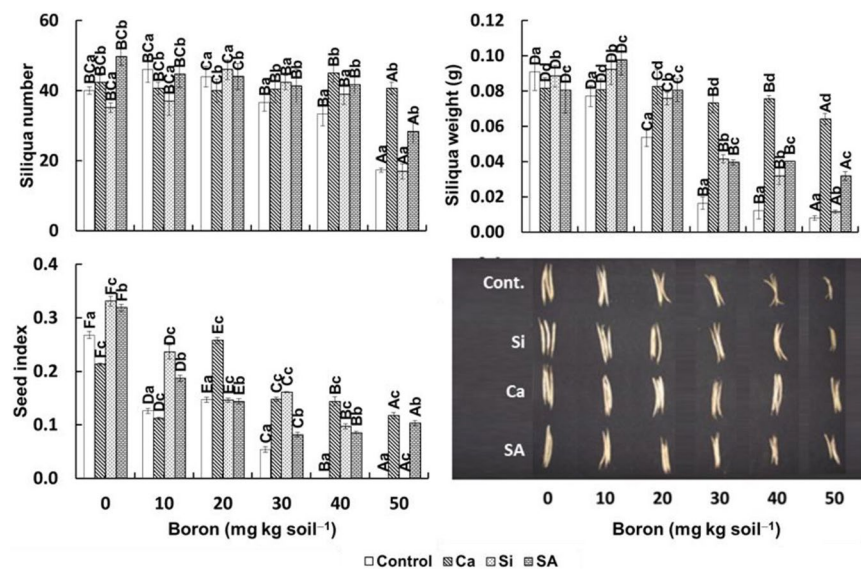
Siliqua weight

From the data in Fig. 7 and Tables S1 and S2 (experiment 2), it can be observed that B toxicity stress caused a significant reduction in siliqua weight of canola plants, reaching to 91.28%, as compared to control. Application of Ca, Si and SA alleviated the inhibitory effect of B toxicity up to 40 mg kg soil⁻¹ concentration. Only Ca and SA were able to alleviate the negative effect of B toxicity on siliqua weight at 50 mg kg soil⁻¹ concentration (29.51 and 64.98%, respectively, less than control).

Seed index

The results depicted in Fig. 7 and Table S1 and S2 (experiment 2) showed that application of Ca, Si and SA succeeded

Fig. 7 Effect of calcium (Ca), silicon (Si) or salicylic acid (SA) on siliqua number, siliqua weight, seed index and siliqua size of 120-day-old canola plants under different boron concentration. The data are means \pm SD ($n=6$). Different letters, capital for B treatments and small for Ca, Si and SA, indicate significant (two way ANOVA; Tukey HSD-post-hoc)



in alleviating the inhibitory effect of B toxicity on crop yield (seed weight) of canola plants. Only, Ca and SA were found to be effective in enhancing crop yield under B toxicity conditions, where they increased the seed weight. Specifically, the seed index was decreased to 56.34 and 61.47% at 50 mg kg soil⁻¹ B with Ca and SA treatments, respectively, of control. In addition to inhibition of crop production under B toxicity stress, the highest B concentration (50 mg kg soil⁻¹) with or without Si treatment led to collapse in plant yield.

Discussion

In this study, the impacts of Ca, Si and SA as an agriculturally effective fertilizer element on mitigation of B toxicity were studied in canola.

Development of B toxicity in canola

In the current study, canola did not show great visible shoot injury symptoms (Fig. S 1). The symptoms restricted as an injured zone all around the margin accompanied by leaf curling, while the adverse effects of B excess were alleviated by Ca, Si, and SA treatments. In certain species, however, yield reductions may occur without visible shoot injury symptoms (Francois 1986).

Our results showed that B treatments significantly decreased the growth as well as shoot and root relative water content. A similar adverse effect of B toxicity on shoot growth was previously reported in canola (Koohkan and Maftoun 2016). The strong negative correlation between water content and B concentration in shoots and roots confirms earlier reports of Pandey (2013) who concluded that excess B resulted in poor water uptake leading to water stress in brassica.

Ca application alleviated B toxicity, as manifested by increases in shoot and root lengths, shoot and root dry weight and water content of shoot and root, compared to B-stressed plants. These results suggest that Ca plays an important role in alleviating the damage to canola incurred under B toxicity conditions. Similar alleviation activity by applied CaCl₂ has been reported for different plant species under B toxicity stress conditions (El-Feky et al. 2012; Siddiqui et al. 2013; Turan et al. 2009). The negative weak and strong correlations between water content and B concentration in shoots and roots, respectively, confirmed that maintaining critical Ca levels may be required to keep the water channels open through gating processes such as phosphorylation (Azad et al. 2004; Martínez-Ballesta et al. 2008). As most plant Ca is stored in leaves and root to shoot transfer of Ca²⁺ has been elaborated at length (Gilliham et al. 2011). The results of this study suggest that Ca alleviates B toxicity by improving plant-water status.

Data reported here showed beneficial effects of Si on canola plant growth under B toxicity conditions, and corroborate the beneficial effects of Si observed on barley plant under 20 mg B kg soil⁻¹ stress (Inal et al. 2009). Furthermore, Hossain et al. (2002) and Hattori et al. (2003) suggested that Si increased plant growth by increasing the cell wall extensibility in the plant roots and shoots. According to the weak and strong correlations between water content and B concentration in shoots and roots, respectively, we speculate the improved water status in shoot was due to silicate crystals deposited in the epidermal cells form a barrier that reduces water loss through the cuticle and improve water relations in plant tissues (Romero-Ananda et al. 2006). Under salt stress, Si can improve plant tolerance through enhancing root water uptake, which contributes to the regulation of aquaporin activity and gene expression (Liu et al. 2015; Zhu et al. 2015). These results suggest that the Si-improved plant-water status could be the major reason for the increased growth parameters under the B- toxicity condition. Accordingly, Akcay and Erkan (2016) found that *PIP1* type aquaporin upregulation in B, Si, and B + Si treated barley shoots at day 1 might have provided the tissues with water and CO₂ to cope with stress and helped their adjustments to changing environmental conditions.

In the present study, SA application significantly inhibited the decrease in growth under B-toxicity condition, and water content was also alleviated in SA-treated plants compared with SA-untreated plants, indicating that SA application alleviated the B- toxicity. The present result is consistent with the previous findings of El-Feky et al. (2012). The improvement of water content of stressed plants by SA could be another explanation for growth enhancement in B-stressed plants. In this respect, Boursiac et al. (2008) suggested that SA can regulate root water transport as a regulator of aquaporin activity.

B allocation in plants

In our study, an increase of B supply concentration resulted in a correspondent increase in the B concentration of all plant organs. B accumulated principally in the shoots (six-fold) than roots, which reveal that the distribution of B significantly correlated with the increase of B levels. Moreover, total B accumulation in whole plant was increased by increasing B supply, these results have also been observed in previous studies (Koohkan and Maftoun 2016).

The application of Ca reduced accumulation of B in both root and shoot, distribution and total B accumulation in whole plant in the present study at high B concentration (50 and 100 mg kg soil⁻¹). This might be explained as a protective effect resulting from the essential role of Ca in the physiological process i.e. Ca plays an important role in the

cell wall and controls the translocation of root-B to leaves. Whereas under B toxicity, the aquaporins nodulin 26-like intrinsic proteins (NIPs), the tonoplast intrinsic proteins (TIPs), and the plasma membrane intrinsic proteins (PIPs) are involved in reducing the accumulation of toxic boric acid levels in plant tissues (Wang et al. 2016). In the response of plants to environmental stimuli, the functions of aquaporin could be regulated by Ca^{2+} via reversible phosphorylation (Wang et al. 2016). Similar alleviation by applied Ca has been reported for wheat, barley and radish species under B toxicity (El-Feky et al. 2012; Siddiqui et al. 2013; Turan et al. 2009). Gupta and Macleod (1981) and Taban et al. (1995) also concluded that the application of Ca could reduce the availability of B, resulting in decreased uptake of B. Moreover, Turan et al. (2009) concluded that localization of Ca in cell wall leads to decreased cell wall B permeability.

In the present study, the B content in root and shoot, B distribution and total B accumulation in whole plant were lower in plants treated with Si than in untreated ones, except at 100 mg kg soil⁻¹ the B content in root was unaffected by Si treatment. The application of Si could reduce the availability of B, resulting in decreased uptake of B. Supplementation of Si could lead to thick lignified/suberized exodermis, which could directly delay the entry of B into the roots. This suggestion was confirmed by the result of Fleck et al. (2011) who concluded that Si nutrition of rice plants reduced the oxidation power of roots and enhanced the development of casparian bands in the exodermis and endodermis, as well as lignin depositions in the sclerenchyma. Also, the result of Cheng et al. (2014) who concluded that the relatively higher metal (Pb, Zn and Cu) tolerances in rhizophoraceous mangrove species are ascribed to their thick lignified/suberized exodermis, which could directly delay the entry of metals into the roots. Both Si and B are classified as metalloids. They both form uncharged acidic compounds at neutral pH and were absorbed into plant tissues in nonionic form, which is uncommon with other nutrients (Reid 2010). Akcay and Erkan (2016) showed that the presence of Si under B stress reduces shoot and root B levels, when compared to B accumulation under B stress alone. They also suggested that some form of competition through a channel protein and preference of transporter protein in favor of Si intake might be another likely explanation. In accordance with these results Farooq et al. (2015) suggested that the positive effect of Si application in decreasing B uptake might be attributed to the formation of B-silicate complexes in the soil. Gunes et al. (2007) also found that supplemental Si reduced B translocation from the roots to shoots of spinach plants and this effect could be related to Si being irreversibly precipitated as amorphous silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) in the cell walls and lumens (Epstein 1999).

From our results, it was clear that SA reduced the B-toxicity stress condition by reducing the accumulation of B

inside the plant organs. Also, SA mediated the reduction in the root-to-shoot B distribution. These results are in agreement with the findings of El-Feky et al. (2012, 2014) who found that SA decreased B content of barley tissues under B toxicity. Recently, Yıldırım (2017) reported that lower B accumulation, better growth performance and higher chlorophyll content of *Populus alba* was attributed to endogenous increase in SA production and its contribution on gene regulation under B toxicity. We speculated that SA may work through some special mechanism, such as diminishing the uptake, or activating the efflux from the roots, i.e. by mechanisms leading to lower cytoplasmic B content. However, the role of exogenously applied SA under B stress in plants is not yet clear and needs further investigations.

Non-enzymatic antioxidants

In this respect, Mittler (2002) concluded that non-enzymatic antioxidant activity is represented by a series of antioxidant molecules that the plant uses against ROS formation. In the current study, AsA and α -tocopherol significantly increased in shoots under the highest B level, while, DHA and phenolics (free and bound) of canola shoot increased with the rise of B level. Our results, further revealed that B levels used failed to change the glutathione content in shoots ($P \leq 0.1686$) and the correlation between B in shoot was weak (0.233), however increasing B level reduced flavonoids content in canola shoots. These antioxidants could be important in protecting against oxidative stress triggered by high B concentrations. Molassiotis et al. (2006) concluded that excess B stimulates the non-enzymatic antioxidant mechanism in the apple rootstock EM 9 (*Malus domestica* Borkh) explants. In tomato, Cervilla et al. (2007) suggested that oxidative stress caused by B toxicity both increases the AsA pool and increases its oxidation. According to De Gara et al. (2000), DHA accumulation is generally considered a negative event for the cell metabolism. In contrast, Keles et al. (2004) found that the excessive B concentration inhibits the synthesis of α -tocopherol in the leaves of the orange plant. Munné-Bosch et al. (1999) also recorded that the increases in α -tocopherol observed in water-stressed plants might prevent chlorophyll photooxidation. Regarding glutathione, Yadav (2010) suggested that the availability of GSH is linked to the production of phytochelatin, which are used to alleviate the toxicity by nutrients and heavy metals.

In this context, Yıldırım (2017) suggested that higher up regulation of glutathione S-transferase, heavy metal-associated isoprenylated plant protein and ABC transporters in *Populus nigra* associated to internal detoxification of excess B under toxic condition. Cervilla et al. (2007) also showed that GSH amount increased in tomato specie (Kosaco), while no significant change was observed in the other (Josefina). Our result reveals that there might be no relationship

between B content of canola and GSH accumulation. In dissimilarity with our result, Cervilla et al. (2012) recorded an increase in flavonoids content in tomato plants under the treatment with 2 mM of B. Our result suggested that the reduction of flavonoids resulted in impairment of B tolerance. Similar to our result, Cervilla et al. (2012) found that total concentration of phenols significantly increased under B treatment in tomato plants, this result suggesting a role of these secondary metabolites in the defence mechanisms against B stress.

Ca, Si and SA caused an inhibition in accumulation of AsA, DHA, tocopherol, phenolics and glutathione however did not alleviate the reduction of flavonoids in 100 mg kg soil⁻¹ B-stressed plants, compared to corresponding stressed controls. Decreases in AsA, an antioxidant molecule capable of directly quenching ROS (Salin 1988) and H₂O₂ (Noctor and Foyer 1998), might be attributed to the reduction in B uptake and accumulation.

Ca considerably protected B-induced oxidative injury in canola shoots, and this protection might be related to the avoidance of ROS generation and the reduction of B uptake. These results ask for further investigation.

We speculated that Si-mediated alleviation of oxidative damage by strengthening the structural integrity of cell membranes, particularly during B stress that lead to hampered B uptake. In this respect, Liang et al. (2015) suggested that Si might help to maintain membrane integrity and decrease permeability. Ma et al. (2016) also suggested that Si supplement wheat plant showed lower lipid peroxidation. Further study is needed to elucidate how Si initiates these responses.

Similarly, the effect of SA on non-enzymatic antioxidant in SA-treated plants could be responsible for the lower accumulation of H₂O₂ and MDA at SA application, which suggests that oxidative damage induced by B stress is alleviated by the addition of SA.

Crop yield

Our results showed that excess B induced a significant reduction in yield components of canola plants, however external application of Ca, Si and SA alleviated these inhibitory effects to varying degrees. In this respect, the most effective alleviation treatment was Ca followed by SA and Si. The results of the current study suggested that the inhibition of canola plant yield by B toxicity stress was associated with reduced seedling growth, water content and increased B ions. Boron treatments in our earlier studies were found to cause a reduction in tiller number of two wheat cultivars (Metwally et al. 2017). Kaya et al. (2009) observed that B toxicity caused a remarkable reduction in fruit yield and growth in tomato plants. Canola seed yield

varied significantly among the cultivars and B application decreased the seed yield (Öztürk et al. 2010).

The pronounced effects of Ca, Si and SA on the improvement of yield under B stress may also be due to its role in enhancement of the water uptake, which was impaired by B stress. Results of the present study indicated a significant decrease in the uptake of the water by B stress in both shoots and roots of canola plants. However, application of Ca, Si and SA remarkably alleviated the negative effect of B on the water uptake. Also, when Ca, Si and SA were co-applied with B, a significant decrease in B accumulation was observed in B-stressed canola plants, resulting in a significant increase of the growth parameters of the stressed plants as well as yield.

Our results are in agreement with the results reported in other plant species Sotiropoulos et al. (2002) reported that Ca partially protected kiwifruit plants against the harmful effects of B excess. Moreover, El-Feky et al. (2014) recorded an increase in weight of straw, grains and 100-grains of B-stressed (3.0 mg l⁻¹ B) barley treated with 5 mM CaCl₂.

Si enhances growth, yield and crop quality in response to abiotic and biotic stresses (for review, see Coskun et al. 2016). Little is known about the mechanism underlying Si-increased crop yield under B toxicity in plants. The role played by Si in counteracting the damage on yield induced under B stress was evident in our results. Similarly, Ahmed et al. (2016) reported that yield parameters such as plant height, spike length, a hundred grain weight, biological and grain yield were significantly improved by adding Si through seed priming under water deficit conditions.

Enhancement in yield productivity by application of SA was reported in other plants. El-Feky et al. (2014) found an increase in weight of straw, grains and 100-grains of B-stressed barley treated with 1 mM SA.

Conclusion

From the previous discussions, it could be concluded that growth and yield of canola plants improved after external application of Ca, Si or SA. The reversal of inhibitory effect of excess B by Ca, Si and SA treatments was conferred by preventing growth inhibition or various forms of reducing B uptake and translocation to shoot system. In addition, adding treatments might enhance the non-enzymatic antioxidant and protect plants. Finally, the increase in grains number by treatment with Ca, Si and SA could be attributed to enhancing water content and other physiological activities.

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