



Original Research Article

Role of CaCl₂ and salicylic acid on metabolic activities and productivity of boron stressed barley (*Hordium vulgare* L.)

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A B S T R A C T

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The role of salicylic acid and calcium chloride in ameliorating boron toxicity on barley growth was studied. Three sets of treatments were applied: plants treated with 3 mg/L boron; plants treated with 3 mg/L boron in addition to 5 mM calcium chloride; plants treated with 3 mg/L boron with the addition of 1 mM salicylic acid. Either calcium chloride or salicylic acid alleviates boron toxicity as observed in increasing CO₂ consumption, protein content, chlorophyll (a+b), and carbohydrate content compared to samples treated only with 3mg/L boron. The addition of salicylic acid or calcium chloride resulted in increased protease activity and decreased α - and β -amylase, catalase, peroxidase and superoxide dismutase. Salicylic acid or calcium chloride reduced grain contents of boron, nitrogen, phosphorus and potassium. Grains carbohydrate content was increased with salicylic acid and calcium chloride. The present data showed that calcium chloride was better than salicylic acid in alleviating boron toxicity.

Introduction

Since the discovery of boron as an essential plant nutrient, its importance as an agricultural chemical has grown very rapidly and its availability in soil and irrigation water has become an important determinant of agricultural production (Saleem et al., 2011). Boron has many functions on plant growth and metabolism. It has a role in sugar translocation (Blevins

and Lukaszewski, 1998), cell wall synthesis and structure (Loomis and Durst, 1991), auxin biosynthesis and metabolism (Lovatt, 1985; Li and Liang, 1997), lignification (Kobayashi et al., 1999), ascorbate metabolism (Lukaszewski and Blevins, 1996), nitrogen metabolism (Ruiz et al., 1998), membrane function and ion uptake (Schon et al., 1991). In addition, boron appears to be necessary for tip

growth such as occurs in apical meristems, root hairs, and pollen tubes; this locally specific high demand for B is probably the result of the need for B for the secretion of cell wall material (Goldbach and Wimmer, 2007).

Boron deficiency is the most common and widespread micronutrient deficiency problem, which impairs plant growth and reduces yield. Normal healthy plant growth requires a continuous supply of B, once it is taken up and used in the plant; it is not translocated from old to new tissue. That is why deficiency symptoms start with the youngest growing tissues. Therefore, adequate B supply is necessary for obtaining high yields and good quality of agriculture crops (Saleem et al., 2011). Boron deficiency affects diverse processes in vascular plants such as root elongation, indoleacetic acid oxidase activity, sugar translocation, carbohydrate metabolism, nucleic acid synthesis, and pollen tube growth (Blevins and Lukaszewski, 1998; Goldbach and Wimmer, 2007; Saleem et al., 2011).

Boron is a plant nutrient which has a critical limit between deficiency and toxicity. When B is present at high concentrations in the soil or ground water, plant growth and reproduction can be affected by its toxicity. Soils with insufficient or toxic levels of B are widespread in agricultural areas throughout the world, limiting crop productivity (Nable et al., 1997; Stangoulis and Reid, 2002; Rerkasem et al., 2004). Whereas B deficiency can be resolved by application of B-enriched fertilizers, toxicity is a more difficult problem to manage. Tolerance to B toxicity varies between plant types and between cultivars of the same species (Nable, 1988).

Several authors (e.g. Camacho-Cristóbal *et al.*, 2008; Reid, 2007; Reid *et al.*, 2004, Stangoulis and Reid, 2002) have abstracted that there appear to be three main candidate sites for B toxicity: (1) disruption of cell wall development; (2) metabolic disruption by binding to the ribose moieties of ATP, NADH or NADPH; and (3) disruption of cell division and development by binding to ribose, either as the free sugar or within RNA). Additionally in leaves, accumulation of high concentrations of B at the end of the transpiration stream might lead to osmotic imbalances.

The most important source of B, which leads to toxicity problems, is the irrigation water treated with municipal wastewaters or B-enriched river waters. A typical example of such source can be found in the Northern part of Nile Delta region in Egypt, where the shortage of fresh water directs reuse of drainage water. A potential limitation to use this water for agricultural production is the extent by which boron affects the growth and yield of crops in the reuse system (Abo-Waly et al., 1997). The role played by many growth modulators in alleviating the retardations induced under boron and other abiotic stresses on plant growth and productivity is clearly evident in many investigations (Wassif et al., 1999; Gunes et al., 2000; Gunes et al., 2007a and b).

Salicylic acid (o-hydroxybenzoic acid) is among a group of plant growth modulator which is considered as a hormone-like substance playing an important role in the regulation of plant growth and development (Klessig and Malamy, 1994). A considerable interest has been aroused by the ability of salicylic acid (SA) to produce a protective effect on plants under different abiotic stresses (Lopez- Delgado

et al., 1998; Gunes et al., 2005; Sahu et al., 2010).

In plants, calcium ions are ubiquitous signaling second messengers in living organisms. There are a number of external stimuli lead to changes in cytosolic calcium concentrations which in turn regulate a wide variety of responses and several physiological processes (Bush, 1995) Both these facts emphasize the role of calcium ions as one of the most important messengers involved in signal response coupling.

In a previous study (Elfeky et al. 2012); we studied the effect of elevated boron concentrations (0, 0.5, 1.5, 3.0 and 6.0 mg/L as boric acid) on barley growth and productivity. We demonstrated that lower concentrations of B (0.5 and 1.5 mg/L) stimulated barley growth parameters (shoot and root fresh and dry weights) by 5%, leaf area by 4.5% and 7%, Chl a and Chl b contents by 3% and 7% at vegetative and flowering stages, respectively, and yield by 5.5%, compared to non-boron treated barley (Elfeky et al. 2012).

On the other hand, the toxicity of boron on barley growth started at the concentration of 3.0 mg/L, causing decrease in all the measured parameters. In the same study, different compounds were applied that alleviated B toxicity at 3 mg/L. Interestingly, treatment with 1.0 mM salicylic acid or 5.0 mM calcium chloride induced almost the same ameliorative effect on growth of B treated barley. Therefore, the present study aims to study the metabolic effect of SA and CaCl₂ in counteracting barley growth retardations induced under excess B toxicity at 3.0 mg/L B to improve its tolerance to B stress.

Materials and Methods

Plant material and treatments:

Grains of barley (*Hordeum vulgare* L. var. Giza 123) were obtained from Sakha research Station, Kafr El-Shaikh, Egypt. Barley grains were sterilized and germinated in pots filled with sandy soil (pH 7.5 and B concentration 0.4 mg/L) and was irrigated with distilled water for the first two weeks. Three sets of treatments were prepared for the different measurements. The set I (control) included plants irrigated twice a week with Hoagland solution (2.5 mM Ca(NO₃)₂, 3.0 mM KNO₃, 0.17 mM KH₂PO₄, 1.5 mM MgSO₄, 50 µM Fe as (Na Fe DTPA), µM MnSO₄, 0.4 µM ZnSO₄, 0.2 µM CuSO₄ and 0.1 µM H₂ MoO₄) containing 3 mg/L B. Set II included plants irrigated with Hoagland solution containing 3mg/L B with the addition of 5 mM CaCl₂ and set III plants irrigated with Hoagland solution with the addition of 1 mM salicylic acid in presence of 3.0 mg/L B. Barley plants were cultivated and left to grow till the end of season and samples were collected at seedling (14 d old), vegetative (22 d old), and harvesting (92-d old) stages.

Experimental design and Data analysis

Each experiment was set up as randomized complete block (RCBD) with 3 replicates each containing a row of each treatment. Samples from each treatment were removed and subjected to the different measurements. Data was statistically analyzed using Analysis of variance (ANOVA) for RCBD followed by the computation of least significant difference (LSD) according to Cochran and Cox (1960).

Determination of CO₂ consumed:

The rate of photosynthesis was measured as CO₂ consumed using a closed gas-analytical system LI 6000 (Li-Cor, USA), portable measuring device. The experiment was carried out on the youngest fully developed leaves from randomly selected seedlings, at a concentration of 400 µmol/L CO₂, 21% O₂ and 50% relative humidity and 29-32°C.

Carbohydrate content

Carbohydrate content was determined in plant samples (shoots and grains) according to Naguib (1962). The carbohydrate content (mg g DW⁻¹) was determined from a standard curve using glucose sugar.

Proline content

Proline was determined in plant shoots according to Bates et al. (1973). The amount of proline expressed as g 100g DW⁻¹ in the tested samples was determined from a standard curve.

Total protein content

Total soluble proteins were estimated in plant shoots and grains according to Lowry et al. (1951). The protein concentration (g 100g DW⁻¹) was calculated by using a calibration curve.

Enzymes assays

The activity of enzymes was assayed using seedling shoots (14-d old). Protease activity was estimated calorimetrically using the method described by Ong and Gaucher (1973). One unit of protease was chosen to equal 1.0 Δ O.D 660/60 min at 37 °C. The assay of α and β-amylase

activities was carried out according to a modification of the method of Das and Sen-Mandi (1992).

Antioxidant enzymes

Catalase activity was assayed according to Kato and Shimizu (1987). The activity was expressed in units of µM of the substrate converted per minute per gram fresh weight. Peroxidase activity was measured according to Kato and Shimizu (1987). Activity was calculated using the extinction coefficient (26.6 mM⁻¹ cm⁻¹ at 470 nm) for tetra guaiacol. Enzyme activity was expressed in units of µM of the substrate converted per min per gram fresh weight. Superoxide dismutase was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the initial rate of the reaction in the absence of enzyme.

Results and Discussion

Data presented in Table 1 showed that both SA and CaCl₂ increased net photosynthesis by 37.5% with SA and 44% with CaCl₂ compared to control (3 mg/L B treated plants) while both decreased Chl a/b ratio with 14% and protein content with 34%. Chlorophyll (a+b) was increased with 20% and carbohydrate content was stimulated with 25% with SA and 26% with CaCl₂. Besides, proline insignificantly increased with 3% with SA and 6% with CaCl₂ compared to control samples (plants treated with 3mg/L boron). Statistical analysis revealed a highly significant effect (at P ≤ 0.05 and 0.01) of 1.0 mM salicylic acid and 5.0 mM CaCl₂ treatments on the photosynthetic

parameters compared to those estimated in 3.0 mg/L B treated barley leaves.

Data in Table 2 showed that the presence of 1.0 mM SA or 5.0 mM CaCl₂ in the nutrient significantly increased protease activity with about 78% compared to control (B treated barley seedlings). Activities of α - and β -amylases were significantly decreased by about 15% and 11% respectively. The decrease of antioxidant enzymes was 21% for catalase, 36% for peroxidase and 34% for superoxide dismutase. Statistical analysis showed the highly significant effect (at $P \leq 0.05$ and 0.01) of 1.0 mM salicylic acid and 5.0 mM CaCl₂ on the estimated enzyme activities compared to those 3.0 mg/L B treated barley..

Data in Table 3 showed that treatment of barley with 1.0 mM SA or 5.0 mM CaCl₂ reduced grain contents of B by about 30%, N with 34% P with 37% and K with 35%. In addition, carbohydrate content was increased in grains with 25% with SA and 66% with CaCl₂. Statistical analysis showed the highly significant effect (at $P \leq 0.05$ and 0.01) of 1.0 mM salicylic acid and 5.0 mM CaCl₂ on yield parameters of 3.0 mg/L boron treated barley.

We previously reported the ameliorative impact of both salicylic acid (1 mM) and calcium chloride (5 mM) on the growth and development of 3 mg/L B stressed barley (Elfeky et al 2012). This stimulates the investigation of the metabolic effect of SA and CaCl₂ in counteracting retardations induced under this excess B concentration.

The present results indicated that the presence of either SA or CaCl₂ in the nutrient media resulted in an increase in the contents of Chla and Chlb compared to

control (3.0 mg/L B treated plants) leading to increased Chla/Chlb ratio. Besides, the addition of SA or CaCl₂ increased net photosynthesis (measured as consumed CO₂) associated with increase in the carbohydrate contents (Table 1). The addition of SA was found to increase net CO₂ assimilation rate as an indicator for net photosynthesis by Habibi (2012) in drought stressed barley and Ying et al (2013) in water stressed red berry. In addition, an increase in total soluble carbohydrates and photosynthetic pigments in leaves of boron treated radish were observed subsequent to addition of 30 mM Ca (Siddiqui et al 2013).

Aydin and Sevinc (2006) indicated that excess B induced a significant decline of photosynthetic rate which could be mainly assigned to changes in carboxylation efficiency, chlorophyll concentration. In addition, high B levels reduced chlorophyll content of tomato plants compared to control plants (Kaya et al., 2009). Since B stress decreases plant CO₂ assimilation, less of the absorbed photon energy captured by the light harvesting system is used in electron transport (Kocabek et al., 2009). The ability of reactive oxygen species to cause photooxidative damage in organic molecules could probably explain the structural damages in the chloroplasts, and the reduction of leaf chlorophyll. Among roles of SA in plants is that it plays an important role in activation of redox changes in components of the signal transduction pathway (Idrees et al 2013, Wang et al 2013). Since light and dark reactions of photosynthesis are tightly coupled (Lawlor, 1987), it can be expected that the inhibitory effect of B stress concomitant with the inhibition of dark reactions and *vice versa* which leads to the

Table.1 CO₂ consumed (mg m⁻² s⁻¹), Chl a+b, ratio of Chla/Chlb, protein (g 100 g DW⁻¹), proline content (g 100 g DW⁻¹) and total carbohydrate content (mg g DW⁻¹) of shoots of 3.0 mg/L B treated barley in presence of SA or CaCl₂ at vegetative stage.

Treatment	3.0 mg/L B	+ 1.0 mM SA	+ 5.0 mM CaCl ₂	L.S.D _{0.05}	L.S.D _{0.01}
CO ₂ consumed	0.16 ± 0.00	0.22 ± 0.00 **	0.23 ± 0.00 **	0.01	0.01
Chl (a+b)	59.5	71.4	71.4		
Chl a /Chl b)	2.1	1.8	1.8		
Total carbohydrate	240 ± 12	300 ± 9.0**	302 ± 8.0**	1	6
Protein	30.7 ± 2.0	20.4 ± 1.9 **	20.3 ± 2.0 **	0.85	0.58
Proline	33.6 ± 1.42	34.5 ± 2.02 ns	35.5 ± 1.85 ns	0.3	0.7

** Results significantly different from control at (P< 0.01).

Table.2 Enzymes activities of 3.0 mg/L B treated barley seedlings in presence of SA or CaCl₂. Unit of enzyme: amount of enzyme (μl) causing 50% inhibition of the initial rate of the reaction in absence of the enzyme.

Enzyme	3.0 mg/L B	+ 1.0 mM SA	+ 5.0 mM CaCl ₂	L.S.D _{0.05}	L.S.D _{0.01}
Protease (mg ml ⁻¹)	0.18 ± 0.01	0.32 ± 0.01 **	0.32 ± 0.002 **	0.01	0.02
α-amylase (Optical density)	0.34 ± 0.01	0.29 ± 0.00 **	0.28 ± 0.002 **	0.001	0.002
β-amylase (Optical density)	0.37 ± 0.00	0.33 ± 0.00 **	0.33 ± 0.002 **	0.001	0.002
Catalase (μM substrate reduced g FW ⁻¹ min ⁻¹)	0.84 ± 0.07	0.66 ± 0.03 **	0.65 ± 0.04 **	0.02	0.12
Peroxidase (μM substrate reduced g FW ⁻¹ min ⁻¹)	1.36 ± 0.12	0.87 ± 0.03 **	0.85 ± 0.02 **	0.11	0.17
Superoxide dismutase *(unit of enzyme)	5.80± 0.26	3.80 ± 0.17 **	3.60 ± 0.2 **	0.29	0.43

** Results significantly different from control at (P< 0.01).

Table.3 Yield grain contents of 3.0 mg/L B treated barley in presence of SA or CaCl₂.

Yield parameters	3.0 mg/L B	+ 1.0 mM SA	+ 5.0 mM CaCl ₂	L.S.D _{0.05}	L.S.D _{0.01}
B (mg kg DW ⁻¹)	0.46 ± 0.04	0.33 ± 0.03 **	0.32 ± 0.03 **	0.06	0.09
N (%)	4.60 ± 0.40	3.00 ± 0.32 **	3.00 ± 0.31 **	0.57	0.86
P (%)	0.94 ± 0.03	0.60 ± 0.02**	0.59 ± 0.02**	0.04	0.06
K (%)	0.62 ± 0.03	0.41 ± 0.02 **	0.40 ± 0.02 **	0.04	0.06
Carbohydrate(mg/g DW)	120 ± 4.0	150 ± 2.0 **	200 ± 2.0 **	8.00	1.00

** Results significantly different from control at (P< 0.01).

observed reduction in carbohydrate contents in B-treated barley shoots compared to SA and CaCl₂. Such improvement in growth and yield of barley by salicylic acid or calcium chloride was reported in many plant species (Romero-Aranda et al., 2006). Ameliorative effect of SA on growth of crop plants under abiotic stress conditions may have been due to its role in nutrient uptake (Glass, 1974), water relations (Barkosky and Einhelling, 1993), stomatal regulation (Arfan et al., 2007), photosynthesis and plant growth and development (Khan et al., 2013).

Results showed that shoots of barley treated with 3.0 mg/L B only contained a significant high protein content and a non significant low proline content in comparison to those detected when treated with SA and CaCl₂ (Table1) reflecting the alterations in N metabolism. These data are in agreement with those obtained by Wimmer et al. (2003) and Reguera et al. (2010). Our experimental data demonstrated also that the increased soluble protein concentrations detected in barley seedlings treated with 3.0 mg/L B only was coupled with a lower protease activity (Table 2). This further supports the possible involvement of B toxicity in inducing alterations in N metabolism. Boron induced alterations on plant proteins could be also explained on the basis that B is an essential component of the cell wall. In addition, B toxicity is known to induce more NO₃-N accumulation in the sap sugar (Papadakis et al., 2004; Molassiotis et al., 2006).

Environmental stresses increase the formation of reactive oxygen species that consequently oxidize membrane lipids, proteins and nucleic acids inducing growth inhibition in plants (Gong et al., 2005). The increase in the activities of

antioxidant enzymes in B-stressed plants was evident to be closely related to the induction of lipid peroxidation (Karabal et al., 2003). In our experiments, superoxide dismutase, peroxidase and catalase activities measured in 3.0 mg/L B only treated barley seedlings were increased compared to those measured in seedlings treated with SA and CaCl₂ (Table3). This leads to the conclusion that the oxidative stress may be an influential component of B stress in barley as recommended by Kaya et al. (2009).

Altered activities of these antioxidant enzymes have been commonly reported, and are used frequently as indicators of oxidative stress in plants (Mittler, 2002). The reactive oxygen species accumulation has been reported in apple rootstock (Molassiotis et al., 2006) under conditions of B toxicity. Barley growth retardations induced under excess B stress in our work were significantly alleviated by SA or CaCl₂ supplement. 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. (Gil'vanova et al, 2012) The protection role of SA is represented mainly in regulating reactive oxygen species (ROS) and alternation of activities of antioxidant enzymes *in vivo* (Molina et al., 2002; Wang et al., 2004; Klessig and Malamy, 1994). Elwan and El-Hamahmy (2009) found that salicylic acid treatment caused a reduction in peroxidase of pepper leaves grown in a moderately salt stressed

greenhouse. Dolatabadian *et al.* (2009) studied the effect of salicylic acid and salt on wheat seed germination and stated that salt stress significantly increased the activity of the antioxidative enzymes while salicylic acid reduced their activities as stress signal molecules. In contrast to 3.0 mg/L B treated barley, presence of either 1.0 mM SA or 5.0 mM CaCl₂ decreased the accumulation of B, N, P and K in shoot tissues implying a possible recovery of the plasma membrane integrity of barley tissues. In addition, the impairment of photosynthetic rate initiated under excess B toxicity was also improved. Our data showed also that barley grains treated with both modulators contained a lower content of B, N, K and P and higher carbohydrate content compared to those treated with B only.

In addition, weight of straw and grains as well as 100-grains were all increased in grains of barley treated with SA or CaCl₂. Beneficial effect of Ca is consistent with its role on the maintenance of cell membrane integrity and its cooperative role with B in building the plant cell wall (Sotiropoulos *et al.*, 2002). However, the Ca/B ratio of leaves may be a good index for diagnosing B toxicity as recommended by Gupta (1972). Turan *et al.* (2009) showed that growing wheat plants under excess B with application of high levels of Ca partially alleviated the B toxicity symptom by reducing B absorption. Several studies have shown that salicylic acid is an essential component of the plant resistance to pathogens and participates in the plant response to adverse environmental conditions. The effect of salicylic acid on plant resistance to the different environmental stresses could be resulted from increasing the level of cell division within the apical meristems of seedling roots which caused an increase in

plant growth as reported by Sakhabutdinova *et al.* (2003).

The protective salicylic acid action includes the development of anti-stress programs and acceleration of normalization of growth process after removal of stress factors. Salicylic acid participates in signal regulation of genes expression in the course of leaf senescence (Morris *et al.*, 2000) inhibition of fruit ripening (Srivastava and Dwivedi, 2000) and many other processes.

The protective role of calcium recorded on barley growth and yield recorded in our work could be explained on the basis that calcium is one of the signal molecules involved in the responses of plant to abiotic stress as shown in many plant species. Calcium concentration in cytosol is increased under environmental stress (Krol *et al.*, 2006) which regulates gene expression and the relative physiological and biochemical reactions (Bush 1995; Monroy and Dhindsa, 1995; Gong *et al.*, 1998a). Calcium chloride treatment increased antioxidant enzyme activities and heat resistance in many plants under heat stress as reported by Jiang and Huang (2001) and Larkindale and Huang (2004).

The role played by salicylic acid or calcium chloride added in the nutrient solution in counteracting the damage induced under B stress was evident by the improvement of barley growth and yield criteria. The alleviation was significantly shown by the reversibility of B-mediated growth inhibition measured by ion accumulation, photosynthetic activity, soluble protein and oxidative stress. Therefore, it is suggested that both salicylic acid and calcium chloride may participate in the maintenance of the membrane integrity and stability of cell

wall structure of barley tissues by counteracting B toxicity. Moreover, both may have a role in abiotic stress tolerance by protecting barley tissues against oxygen radicals induced under B stress as they act as signal molecules involved in responses of plants under abiotic stress.

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