

Boron toxicity is alleviated by hydrogen sulfide in cucumber (*Cucumis sativus* L.) seedlings

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Abstract Boron (B) is an essential micronutrient for plants, which when occurs in excess in the growth medium, becomes toxic to plants. Rapid inhibition of root elongation is one of the most distinct symptoms of B toxicity. Hydrogen sulfide (H₂S) is emerging as a potential messenger molecule involved in modulation of physiological processes in plants. In the present study, we investigated the role of H₂S in B toxicity in cucumber (*Cucumis sativus*) seedlings. Root elongation was significantly inhibited by exposure of cucumber seedlings to solutions containing 5 mM B. The inhibitory effect of B on root elongation was substantially alleviated by treatment with H₂S donor sodium hydrosulfide (NaHS). There was an increase in the activity of pectin methylesterase (PME) and up-regulated expression of genes encoding PME (*CsPME*) and expansin (*CsExp*) on exposure to high B concentration. The increase in PME activity and up-regulation of expression of *CsPME* and *CsExp* induced by high B concentration were markedly reduced in the presence of H₂S donor. There was a rapid increase in soluble B concentrations in roots on exposure to high concentration B solutions. Treatment with H₂S donor led to a transient reduction in soluble B concentration in roots such that no differences in soluble B concentrations in roots in the absence and presence of NaHS were found after 8 h exposure to the high concentration B solutions. These findings suggest that increases in activities of PME and expansin may underlie the inhibition of root elongation by toxic B, and that H₂S plays an ameliorative role in protection of plants from B toxicity by counteracting B-induced

up-regulation of cell wall-associated proteins of PME and expansins.

Keywords Boron toxicity · Cucumber (*Cucumis sativus* L.) · Expansin · Hydrogen sulfide (H₂S) · Pectin methylesterase · Root elongation

Abbreviations

B	Boron
MDA	Malondialdehyde
PME	Pectin methylesterases
RGII	Rhamnogalacturonan II

Introduction

Boron (B) is an essential micronutrient for plant growth, which when occurs in excess in the soil due to low rainfall, irrigation and pollution (Roessner et al. 2006), becomes toxic to plants. A number of physiological processes have been shown to be altered by B toxicity. These include, disruption of cell wall development, metabolic disruption by binding to the ribose moieties of ATP, NADH and NADPH, and inhibition of cell division and elongation (Stangoulis and Reid 2002; Reid et al. 2004). In addition, plants suffering from B toxicity also exhibit increases in contents of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂), resulting in oxidative stress and membrane peroxidation (Cervilla et al. 2007, 2009; Ardic et al. 2009). One of the main symptoms of B toxicity is rapid inhibition of root elongation (Nable 1988; Holloway and Alston 1992; Chantachume et al. 1995; Reid et al. 2004; Choi et al. 2007). It has been shown that the root apex is the critical site for sensing and expressing B toxicity (Reid et al. 2004). In plants, B plays an important role as a component of

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structure in the primary cell wall (O'Neill et al. 2004). Recent studies revealed that B cross-links the pectic polysaccharide and the network, thus affecting the physical and biochemical properties of cell walls (O'Neill et al. 2001, 2004). It was reported that the cell wall dysfunctions under conditions of B deficiency (O'Neill et al. 2004; Dell and Huang 1997). In contrast, little information is available on the effect of B toxicity on plant cells.

Pectin methylesterases (PMEs), which catalyze the specific demethylesterification of pectic polysaccharide in plant cell walls and concurrently release methanol and protons, are involved in diverse physiological processes in plants (Pelloux et al. 2007). For instance, it has been reported that PME is involved in aluminum (Al) toxicity in plants (Blamey 2001; Schmohl et al. 2000; Yang et al. 2008). Plants suffering from Al toxicity and B toxicity display similar symptoms such that both Al and B toxicity rapidly inhibit root elongation and the root apex is a critical site for sensing and expression of symptoms associated with Al (Ryan et al. 1993) and B toxicity (Reid et al. 2004; Choi et al. 2007). However, in contrast to Al phytotoxicity, there has been no detailed study to evaluate the role of PME in B toxicity.

Increasing evidence demonstrates that hydrogen sulfide (H_2S) acts as an important signaling molecule to regulate many physiological processes in mammalian cells (Wang 2002; Lefer 2007). It has been shown that plants can emit H_2S when exposed to excess sulfur (Sekiya et al. 1982; Hällgren and Fredriksson 1982), but there is limited information on the regulatory role of H_2S in mediating physiological processes in plants. Very recently, it was reported that H_2S is involved in mediating copper toxicity (Zhang et al. 2008) and seed germination under osmotic stress by protecting these plants from oxidative damage (Zhang et al. 2009).

To understand the role of H_2S in physiological processes in plants, we investigated the effect of H_2S on B toxicity-induced inhibition of root elongation in cucumber seedlings. Our results indicated that B toxicity rapidly inhibited root elongation and this inhibitory effect was significantly alleviated by an exogenous supply of H_2S donor. We further demonstrated that treatment with H_2S donor reduced high B concentration-induced increase in the activity of PME and up-regulated expression of genes encoding PME (*CsPME*) and expansin (*CsExp*).

Materials and methods

Plant growth

Seeds of cucumber (*Cucumis sativus* L. cv. Zhongnong 8, supplied by the Chinese Academy of Agricultural Sciences) were sterilized in 5% (v/v) sodium hypochlorite solution

for 10 min and germinated on filter paper. The germinated seedlings were pre-cultured hydroponically in aerated one-fourth Hoagland's solution (pH 6.0) in controlled environment with a light/dark regime of 14/10 h, temperature of 20/26°C and a light intensity of 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After the pre-culture, the seedlings were incubated in 1/4 Hoagland's solution supplemented with 5 mM H_3BO_3 in the absence and presence of 200 μM H_2S donor NaHS for varying periods. Root elongation was measured after seedlings were grown under varying treatments for 24 and 48 h.

Effect of H_2S on root elongation under B toxicity

Three-day-old seedlings were incubated in 1/4 Hoagland's solution, defined as control solution (CK), or solution supplemented with 5 mM H_3BO_3 (+B), 5 mM of H_3BO_3 + 200 μM NaHS (+B +S) and 200 μM NaHS (+S) for 24 and 48 h. To test whether the B toxicity-induced inhibition of root elongation was related to H_2S , the effect of 200 μM of NaCl on root elongation in the absence and presence of 5 mM B was also investigated.

Determination of H_2O_2 and MDA

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in roots were measured to assess the effect of B on oxidative stress and membrane lipid peroxidation. Hydrogen peroxide was measured according to Alexieva et al. (2001) with some modification. Briefly, 1 g root sample was ground with 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000g for 20 min at 4°C. The supernatant was used and hydrogen peroxide measured spectrophotometrically. The reaction mixture consisted of 1 mL of the extracted supernatant, 1 mL of K phosphate buffer and 2 mL of 1 M KI. The reaction was developed for 1 h in darkness and absorbance measured at 390 nm.

For the measurement of MDA, the root sample was homogenized in 5 mL of 10% trichloroacetic acid (TCA) solution containing 0.25% thiobarbituric acid (TBA). The mixture was incubated in water at 95°C for 30 min and the reaction was terminated in an ice bath. The mixture was centrifuged at 10,000g for 20 min, and the supernatant absorbance was measured at 532 nm. The value of non-specific absorption at 600 nm was subtracted.

Measurement of PME activity

The PME activity was assayed following the method of Richard et al. (1994). Briefly, about 1 g of root sample was homogenized in liquid N_2 , and PME was extracted in a buffer solution containing 0.1 M citric acid, 0.2 M Na_2HPO_4 and 1 M NaCl for 1 h. The extraction was centrifuged at 15,000g for 20 min at 4°C, and the supernatant

was used to detect the PME activity. As much as 2 μ l of extraction sample was added to 1 mL reaction solution containing 0.5% (w/v) citrus pectin, 0.2 M NaCl and 0.15% (w/v) methyl red (pH 6.8). Pectin de-esterification decreased the pH, leading to the color change from yellow to red. This color change was measured spectrophotometrically at 525 nm and was used to calculate PME activity.

Expression patterns of genes encoding PME and expansins

Quantitative PCR (qPCR) was used to investigate the effect of H₂S on the expression patterns of genes encoding PMEs (*CsPME1*, *CsPME2*, *CsPME3* and *CsPME7*) and expansins (*CsExp1* and *CsExp2*) in cucumber seedlings in the absence and presence of 5 mM B and NaHS. Total RNAs were extracted from cucumber roots with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free NDase I (Promega, Madison, WI, USA). The total RNAs were reverse-transcribed into first-strand cDNA in a 20- μ L volume with M-MLV reverse transcriptase (Promega). The samples that were diluted to 100 μ L with water and 5 μ L of each sample (\sim 8 ng RNA equivalent) were PCR amplified using SYBR GreenER™ qPCR SuperMix Universal (Invitrogen) in a 25- μ L reaction, containing 5 μ L diluted cDNA, 12.5 μ L SYBR GreenER™ qPCR SuperMix Universal, 0.5 μ L Rox Reference Dye, 1 μ L of 10 μ M forward primer, 1 μ L of 10 μ M reverse primer and 5 μ L water. The Mx3000P™ Real-Time PCR System (Agilent Technology, Santa Clara, CA, USA) was used to run quantitative RT-PCR with the following six primer pair combinations:

CsExp1 5'-TCTTCTTTGTCTTCACCTTCGCTG-3' and 5'-ACCCCTGGCTGTATAAATCCCA-3', *CsExp2* 5'-CTTGCTCTATCCAATTCTTCTTCCT-3' and 5'-TTGTTGCTGTTATCGTCACTGATG-3', *CsPME1* 5'-TCGTAGTTCTCATCGCCGTAATAATC-3' and 5'-TTCGCTTCAGTCTCTTTCGATAGCTT-3', *CsPME2* 5'-GATAGGAAAGTTCGTAAGGCGTT-3' and 5'-GCCATCCATCACTGTCTAAGTCGC-3', *CsPME3* 5'-CAACGAAAAACGAACTCTATCCCC-3' and 5'-CAAGTCTCCTGGTTGGTAATGGC-3', *CsPME7* 5'-CTCAACACAGCCAAAGAGATTCGTCAT-3' and 5'-ACCATTACACGGAGGGTAACAACCTG-3'.

In addition, a housekeeping gene, *CsActin11*, was employed as a control: 5'-GTGTGAAGAAGAAGTAGCCGCAT-3' and 5'-TCTCCATATCATCCCAGTTTGTGA-3'.

Primers were designed across exon–exon junctions of cDNA to avoid potential problems due to contaminating genomic DNA. Amplification efficiency for each primer pair was calculated using serial cDNA dilutions. After correcting the cycle threshold values according to the amplification efficiency, the expression values of the six genes were normalized to the corresponding controls.

Determination of root-soluble B contents

Because of the high permeability of B, cucumber roots exposed to varying solutions were simply blotted for determination of soluble B in roots. Soluble B of the roots was measured in hot water extracts by a colorimetric method based on the reaction between B and azomethine-H (Gaines and Mitchell 1979). To compare root B contents with B concentrations in the external solutions, B concentrations in roots were expressed in millimolar on the basis of root water content.

Statistical analysis

The analysis of variance was conducted between different treatments. The significant differences between treatments were evaluated by LSD multiple range tests ($P < 0.05$) using the SAS statistical software.

Results

H₂S donor alleviated the B toxicity-induced inhibition of root elongation

To examine the sensitivity of cucumber seedlings to B toxicity, the effect of high B concentration (5 mM) on root elongation of cucumber seedlings was investigated. Exposure of cucumber seedlings to high B concentration led to a significant inhibition of root elongation (Fig. 1a). For instance, root elongation was inhibited by 40% after exposure of cucumber seedlings to 5 mM B for 24 h compared with those grown in control solution. At 24 h after the addition of 200 μ M H₂S donor NaHS to high concentration B solutions, the high B concentration-induced inhibition of root elongation was not affected (Fig. 1a). However, the inhibitory effect of high B concentration on root elongation was significantly alleviated 48 h after the addition of NaHS to high concentration B solutions (Fig. 1a). In addition to inhibition of root elongation by high B concentration, we also observed that roots exhibited distinct curvature when grown in high B solutions and that this change in root morphology was effectively abolished in the presence of NaHS after 48 h (Fig. 1b). Root elongation was insensitive to NaHS in the control solution (Fig. 1a). These results indicate that H₂S is capable of alleviating high B concentration-induced root elongation and root curvature. To test whether the ameliorative effect of NaHS on B-dependent root elongation is due to Na⁺, the effect of 200 μ M of NaCl on root elongation in the absence and presence of 5 mM B was also investigated. In contrast to NaHS, addition of 200 μ M NaCl had no effect on root elongation in high B solution for 24 and 48 h (Fig. 1a), suggesting that the observed mitigating

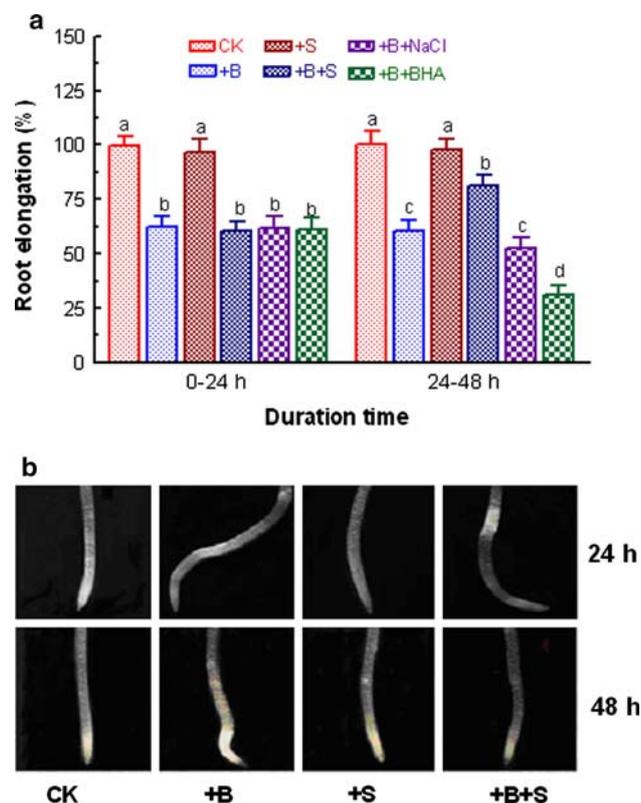


Fig. 1 Effect of H₂S donor of NaHS on root elongation (**a**) and root morphology (**b**) of cucumber under boron toxicity condition. After preculture, cucumber seedlings were treated in 1/4 Hoagland's nutrient solution supplemented with 5 mM boric acid (+B), 200 μM NaHS (+S), 5 mM boric acid plus 200 μM NaHS (+B+S), 5 mM boric acid plus 200 μM NaCl (+B+NaCl) and 5 mM boric acid plus 100 μM BHA (+B+BHA) for 24 and 48 h. The control solution (CK) was 1/4 Hoagland's solution. After treatments, root length or morphology was recorded. Data are mean ± SE of 16 replicates for root length. Means with different letters are significantly different ($P < 0.05$) with regard to treatments. **b** Root curvature in high B solutions was abolished in the presence of NaHS after 48 h

the effect of NaHS on the inhibitory effect of high B concentration is likely to result from H₂S. In animal cells, H₂S can act as an antioxidant (Kimura and Kimura 2004). To test whether the observed effect of H₂S on root elongation in the presence of 5 mM B results from its antioxidant capacity, the effect of butylated hydroxyanisole (BHA), a lipophilic antioxidant, on the B-dependent inhibition of the root elongation was also investigated. In contrast to H₂S, the high B concentration-induced inhibition of root elongation was not recovered by 100 μM of BHA (Fig. 1a).

Effect of B toxicity and NaHS on H₂O₂ and MDA production

Previous studies have shown that plants suffer from oxidative stress and membrane peroxidation under B toxicity, as evidenced by accumulation of H₂O₂ and MDA (Molassiotis

et al. 2006; Gunes et al. 2006; Cervilla et al. 2007). To examine whether the alleviating effect of NaHS on cucumber seedlings suffering from B toxicity was related to oxidative stress and membrane peroxidation, we measured H₂O₂ and MDA contents in roots of cucumber seedlings exposed to 5 mM B in the absence and presence of NaHS. There was an increase in accumulation of H₂O₂ in response to treatment with 5 mM B for 48 h, and this increase in H₂O₂ production was not affected by NaHS (Table 1). In contrast to H₂O₂, there were no changes in MDA contents in cucumber seedlings exposed to high B concentration for 48 h (Table 1). No effect of NaHS on MDA contents was found in cucumber roots in both control and high B solutions (Table 1). These results are indicative that the inhibition of root elongation by high B concentration is unlikely to be caused by membrane peroxidation.

PME activity was enhanced by B toxicity

To investigate the mechanism by which H₂S alleviated the high B concentration-induced inhibition of root elongation, the effect of high concentration of B and NaHS on PME activity in cucumber roots was measured. There was no change in PME activity in cucumber roots after exposure to 5 mM B for 24 h (Fig. 2). In contrast, a significant increase in PME activity was observed after 48 h exposure of cucumber seedlings to high B concentration (Fig. 2). The high B concentration-induced increase in PME activity was abolished by the addition of NaHS (Fig. 2). In addition, we found that treatment of cucumber seedlings with NaHS for both 24 and 48 h did not affect PME activity in the control solutions.

Excess B up-regulated the expression of PME and *Exp* genes

To further evaluate the role of PME in high B concentration-induced root elongation, the responses of genes encoding

Table 1 Effect of high B concentrations (5 mM) on hydrogen peroxide (H₂O₂) and MDA content in cucumber roots in the absence and presence of 200 μM of NaHS

Treatment	H ₂ O ₂ (ng g ⁻¹ root FW)	MDA (nM g ⁻¹ root FW)
CK	0.57 ± 0.08b	9.71 ± 0.77a
+S	0.55 ± 0.07b	9.83 ± 0.68a
+B	1.06 ± 0.28a	9.56 ± 0.71a
+B+S	0.92 ± 0.23a	9.81 ± 0.70a

After preculture in control solution (CK), cucumber seedlings were transferred to solutions containing 5 mM B, 5 mM B plus 200 μM NaHS or 200 μM NaHS for 48 h. The control solution (CK) was 1/4 Hoagland's solution. Data are mean ± SE of four replicates. Means with different letters within a column are significantly different ($P < 0.05$) with regard to treatments

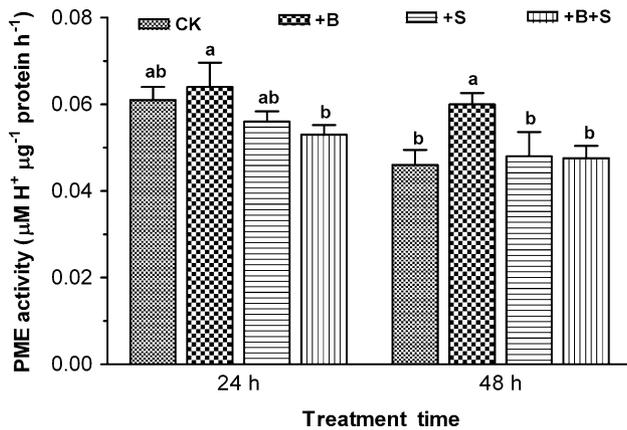


Fig. 2 Effect of H₂S donor of NaHS on PME activity in cucumber root under B toxicity conditions. After pre-culture, cucumber seedlings were treated in 1/4 Hoagland’s nutrient solution containing 5 mM B, 200 µM NaHS, 5 mM boric acid plus 200 µM NaHS for 48 h. The control solution (CK) was 1/4 Hoagland’s solution. Data are mean ± SE of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatments

PME to the high B concentration at the transcriptional level using quantitative RT-PCR (q-PCR) technique were investigated. In *Arabidopsis thaliana*, PME is encoded by 67 PME-related genes (Micheli 2001). In cucumber, four genes encoding PME were identified (*CsPME1*, *CsPME2*, *CsPME3* and *CsPME7*) and their responses to high B concentration in the absence and presence of NaHS were studied. Exposure of cucumber seedlings to high concentration B solutions led to marked increases in transcripts for the four PME genes, and the increases in PME transcripts displayed transient characteristics such that the transcripts were greater after 24 than 48 h exposure to high B solution. More importantly, we found that the high B concentration-induced up-regulation of *CsPMEs* expression was significantly reduced by NaHS (Fig. 3). In contrast, NaHS had no effect on *CsPMEs* expression for cucumber seedlings grown in the control solutions (Fig. 3). In addition to *CsPME*, the effect of high B concentration and NaSH on the expression of genes encoding expansins, which are important cell wall proteins responsible for cell elongation, was also investigated. Similar to *CsPMEs*, there were significant increases in *CsExp1* and *CsExp2* transcripts when the seedlings were challenged with high B concentration (Fig. 3). However, unlike the expression of *CsPMEs*, the enhanced expression of both *CsExp1* and *CsExp2* by high B concentration was greater after exposure to the high concentration B solutions for 48 than 24 h (Fig. 3). Moreover, the up-regulated expression of *CsExp1* and *CsExp2* genes by high B concentration was substantially attenuated by NaHS (Fig. 3), whereas NaHS had no effect on the transcripts of *CsExp1* and *CsExp2* in the control solutions (Fig. 3).

Effect of NaHS on root B concentrations

To examine the effect of NaHS on B accumulation in roots, we measured soluble B concentrations in cucumber roots exposed to high B concentrations in the absence and presence of H₂S donor, NaHS. As shown in Fig. 4, soluble B concentrations in cucumber roots increased rapidly when exposed to solutions containing 5 mM B. For instance, the soluble B concentrations were increased from 0.34 to 6.4 and 9.2 mM after 1 and 4 h of exposure to 5 mM B, respectively. The soluble B concentrations in roots became relatively constant, ranging from 7.5 to 9.8 mM, after exposure to high B solutions for up to 48 h. Addition of NaHS to the high concentration B solutions reduced root-soluble B contents in roots after 1 and 4 h exposure to high B solutions by approximately 20% (Fig. 4). There were no differences in soluble B concentrations in roots exposed to high B solutions in the absence and presence of NaHS after 8 h exposure to the high concentration B solutions.

Discussion

Inhibition of root elongation is one of the earliest and distinct symptoms of plant B toxicity (Chantachume et al. 1995; Reid et al. 2004). In the present study, we found that exposure of cucumber seedlings to 5 mM B rapidly inhibited root elongation and led to root curvature (Fig. 1a). We further demonstrated that the B toxicity-induced inhibitory effect on root elongation and changes in root morphology were closely related to enhanced activity of PME due to up-regulation of *CsPMEs*. More importantly, we found that an H₂S donor (NaSH) can alleviate B toxicity syndromes such that NaSH abolished B-induced increase in PME activity and substantially alleviated the inhibitory effect on root elongation. Therefore, these observations indicate that alteration of PME activity is likely to underlie B toxicity-induced inhibition of root elongation, and that H₂S plays a regulatory role in alleviating the inhibitory effect of B on root elongation by targeting PME.

A rapid inhibition of root elongation was observed on exposure of cucumber seedlings to 5 mM of B (Fig. 1). This finding is consistent with that reported in literature. For instance, Reid et al. (2004) demonstrated that root elongation was inhibited by 70% after exposure of wheat seedlings to 10 mM B for 27 h. In addition to inhibition of root elongation, we also found that cucumber roots exhibited distinct curvature when exposed to high B concentration (Fig. 1b). The high concentration B-induced root curvature, which is an interesting observation and warrants further investigation, may result from alterations in gravitropic properties of roots by possibly disrupting distribution of hormones such as auxin and ethylene in roots. Boron is an

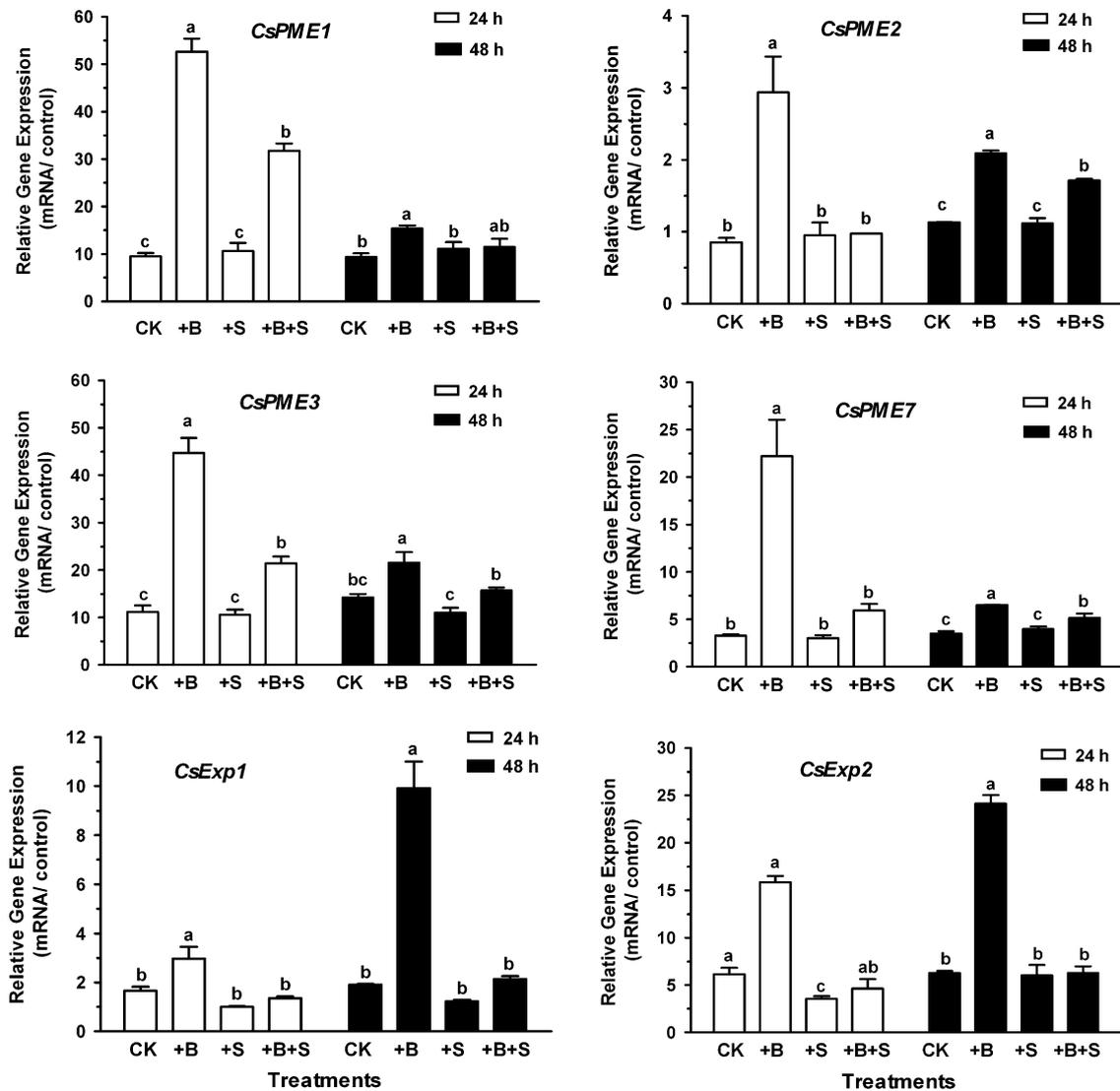


Fig. 3 Effect of boron toxicity on expression of *PME* and *Exp* of cucumber root. After preculture, cucumber seedlings were treated in 1/4 Hoagland's nutrient solution containing 200 μ M NaHS for 48 h.

important constituent of primary cell wall, and excess B disrupts the cell wall synthesis (Reid et al. 2004). Pectin is the main component of plant cell wall. PME, which catalyzes the demethylesterification of cell wall polygalacturonans, is closely associated with cell wall developments and cell elongation (Pelloux et al. 2007). It has been shown that up to 90% of the cellular B is localized in the cell wall fraction (Bevins and Lukaszewski 1998). Several reports demonstrate that cell wall development is disrupted by B deficiency (Bevins and Lukaszewski 1998; O'Neill et al. 2004). In contrast to B deficiency, little is known of the response of cell wall components to B toxicity. Ghanati et al. (2002) reported that the contents of suberin and lignin in cultured tobacco cells are enhanced when challenged with 10 mM B, leading to a stiffening of the cell wall

The control solution (CK) was 1/4 Hoagland's solution. The relative mRNA level was normalized based on the mRNA in roots grown in CK solutions. Data are mean \pm SE of three replicates

matrix. Pollen tube growth is inhibited by high external B concentrations (16 mM), and the inhibitory effect may result from disruption of cross-link between B and pectin chains, leading to a rigidification of the cell wall (Holdaway-Clarke et al. 2003). Similar explanations may also account for the inhibitory effect of high B on root elongation in cucumber seedlings.

In the present study, we demonstrated that B toxicity elicited an increase in PME activity and enhanced expression of *CsPMEs* (Fig. 3). These results resemble the effect of Al on root growth. For example, Yang et al. (2008) found that Al significantly stimulates the PME activity of rice roots, especially in Al-sensitive cultivar, and that the PME activity is negatively correlated with Al-induced inhibition of root elongation. The enhanced PME activity

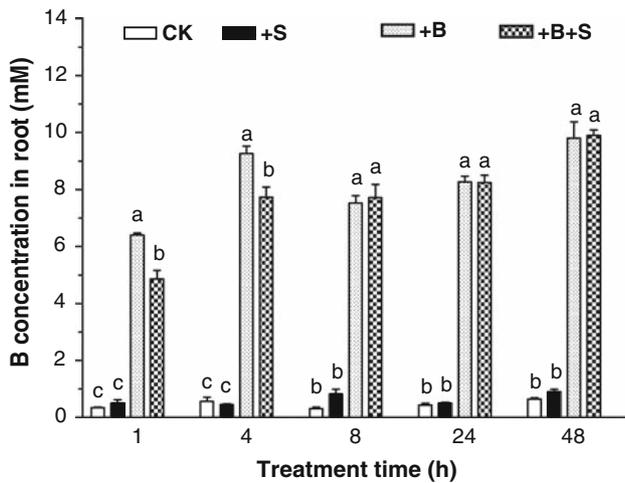


Fig. 4 Root-soluble B concentrations in control (CK) and high B solutions in the absence and presence of NaHS. Cucumber seedlings pre-grown in the CK solutions were transferred to the treatment solutions containing 5 mM B for varying periods (1, 4, 8, 24, 48 h) in the absence and presence of 200 μM of NaHS. Data are mean ± SE of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatments

would cause a higher degree of demethylesterification in cell wall pectin under B toxicity, which in turn may stiffen the cell wall by disrupting pectin gelation status, leading to the observed root elongation.

We also found that genes encoding expansins were up-regulated on exposure of cucumber seedlings to high concentration B solutions (Fig. 3). Expansins are important proteins that mediate primary wall loosening and activate the secondary wall loosening factors, leading to turgor-driven wall extension (Cosgrove 2005). Boric acid and borate are capable of forming complexes with a number of biological compounds containing two hydroxyl groups in *cis*-configuration. The complex formed between borate esters and apiose residues of rhamnogalacturonan II (RG-II) plays an important role in controlling cell wall porosity and tensile strength (Ryden et al. 2003; O’Neill et al. 2004) and is involved in B deficiency-induced changes in cell wall structure (Matoh 1997; Ishii et al. 2001). In contrast to B deficiency, little is known of whether the RG-II complex is involved in the B toxicity syndrome. There has been a report demonstrating that genes encodings expansins are down-regulated by B deprivation in Arabidopsis roots (Camacho-Cristóbal et al. 2008). In contrast to B deficiency, we found that there were increases in the transcripts of *CsExp1* and *CsExp2* on exposure of cucumber seedlings to high B concentration. The up-regulation of *CsExp1* and *CsExp2* may alter expansin-dependent wall loosening, thus contributing to the observed inhibition of root elongation induced by high B concentration. The observations that B toxicity up-regulated the expression of both *CsPMEs* and *CsExps* (Fig. 3) and inhibited root elongation (Fig. 1) may

suggest that both PME and expansins are associated with B toxicity in cucumber seedlings. The interactions between PME and expansins in modulation of root growth under B toxicity remains unknown and warrants further investigation.

Plants suffering from B toxicity often exhibit symptoms associated with oxidative stress and membrane peroxidation as evidenced by enhanced accumulation of H_2O_2 and MDA (Choi et al. 2007). In the present study, we found that exposure of cucumber seedlings to 5 mM B significantly increased H_2O_2 contents in roots, but MDA contents were not affected by the treatment (cf. Table 1). The reported effect of excess B on H_2O_2 and MDA contents in roots varies in literature, including increases in both H_2O_2 and MDA contents (Gunes et al. 2006; Molassiotis et al. 2006; Cervilla et al. 2007), and no changes in H_2O_2 (Karabal et al. 2003) and MDA contents (Molassiotis et al. 2006; Ardic et al. 2009) in response to high B treatment. The discrepancy in changes in H_2O_2 and MDA contents among different studies may result from differences in plant species, growth conditions, B concentrations used to treat plants and duration of treatment with high B concentration. The inhibitory effect of high B concentration on root elongation was alleviated by NaSH, but there was no effect of NaSH on the high B concentration-induced accumulation of H_2O_2 (Table 1). These findings suggest that accumulation of H_2O_2 may not be a cause for inhibition of root elongation induced by high B concentrations.

Previous studies have shown that H_2S acts as a signaling molecule in animal cells (Wang 2002; Lefer 2007). Given that plants can also produce H_2S , it is informative to examine whether H_2S also plays a role in modulation of physiological processes in plants. In the present study, we found that treatment with H_2S donor mitigated the B toxicity-induced inhibition of root elongation. More importantly, we found that the high B concentration-induced increases in PME activity and transcripts of *CsPMEs* and *CsEXPs* were also markedly reduced by the treatment with H_2S donor (Fig. 3). These observations corroborate that changes in PMEs and expansins may underlie the B toxicity-induced inhibition of root elongation, and that the ameliorative effect of exogenous H_2S on inhibition of root elongation may result from its action on PMEs and expansins. Recently, it was demonstrated that H_2S may act as an antioxidant to counteract oxidative stress induced by copper toxicity (Zhang et al. 2008) and osmotic stress during wheat seed germination and sweet potato seedling growth (Zhang et al. 2009). In contrast to these studies, we found B toxicity-induced inhibition of root elongation was not correlated with the accumulation of H_2O_2 and MDA contents. Thus, the H_2S donor alleviated high B concentration-induced inhibition of root elongation and curvature (Fig. 1), but did not affect H_2O_2 and MDA contents (Table 1).

Therefore, the ameliorative role of H₂S played in B toxicity symptoms seems unlikely to result from its effect on oxidative stress.

There was a rapid accumulation of soluble B in roots on exposure of cucumber seedlings to high B solutions (Fig. 4). In barley, B concentrations in roots can be rapidly equilibrated with the external B concentrations in the B-sensitive genotype, while for the B-tolerant genotype root B concentrations are lower than those in the external solutions due to operation of a B efflux transporter (Hayes and Reid 2004; Reid 2007). In the present study, we found that B concentrations in cucumber roots were greater than in the external solutions, suggesting lack of effective B efflux transporters in cucumber plants used in the present study. As the alleviating effect of H₂S on root elongation was observed after 24 h of exposure to high B solutions (Fig. 1), the observations that H₂S donor only marginally inhibited B concentrations in roots in the first 4 h of exposure to high B solutions (Fig. 4) indicate that the alleviating effect of H₂S on high B-induced inhibition of root elongation is unlikely to directly result from its impact on B accumulation in roots.

In summary, we found that high external B concentrations suppressed root elongation, stimulated PME activity and up-regulated expression of genes encoding PME and expansins of cucumber seedlings. The inhibition of root elongation, increases in PME activity and up-regulation of PME and EXP expression were reversed by treatment with H₂S donor. These findings highlight that H₂S plays a regulatory role in mediation of root elongation under conditions of B toxicity by possibly targeting cell wall-related PME and expansins.

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