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Boron effects on wall polysaccharide composition of marshmallow cells

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Abstract

Marshmallow is a medicinal plant containing mucilage polysaccharides and various phenolic acids. Boron (B) is an essential micronutrient whose necessity for plant growth and development has been attributed to its role in cell wall pectin network and maintenance of integrity and performance of membranes. The present study was aimed to investigate the effects of different concentrations of B (0.01, 0.1, and 1 mM respectively as deficient, sufficient or control, and excess concentrations) on cell wall polysaccharides of suspension-cultured *Althaea officinalis* cells in a modified LS medium. The results showed that under B deficiency higher ratio of cell production was devoted to produce wall materials (4.4% of fresh weight), compared with normal and excess B supply (2.3% and 1.8% of fresh weights, respectively). Moreover, B deficiency drastically reduced relative contents of hemicellulose A and cellulose (78% and 72%) compared to those of the control cells. No significant change appeared in the relative amount of pectin in cell walls of deficient and excess B treated cells, in comparison with normal concentration of B. Boron deficiency significantly increased hemicellulose B (157% of the control). This may help the cells with increasing sites for B adsorption under insufficient B supply, while improving its health benefit, since hemicellulose contributes to lowering cholesterol and increasing gut bacteria.

Keywords*:* Boron; marshmallow; medicinal plant; mucilage polysaccharides; tissue culture

1. Introduction

Boron (B) is a micronutrient essential for normal plants growth and development. B-deficiency limits crop production, on the other hand excess B is also toxic to plants. A narrow B concentration range exists between deficient and toxic levels for plants (Bonilla et al., 2010). Possible involvement of B in signaling processes and cytoskeleton-mediated trafficking have been recently argued, however cross-linking with rhamnogalacturonan-II in pectic polysaccharide has been widely accepted as the main role of B that is essential for maintenance of the cell wall structure and plasticity (O'Neill et al., 2004; Miwa et al., 2013). Plants absorb boron in the form of boric acid. It has long been believed that boron uptake is a passive process; that is, boron transport rate is in proportion to the concentration gradients. In view of dependence on energy, carriers, inhibitors, temperature, and other environmental factors, however, a part of B uptake and translocation have been considered to be active, metabolic-dependent, and carrier-mediated. The identification of B transporters has suggested that plants sense and respond to the B conditions and regulate transporters to maintain B homeostasis (Miwa and Fujiwara, 2010, Leaungthitikanchana et al., 2014, Uraguchi et al., 2014). Boron takes part in many physiological processes such as sugar transport, cell wall synthesis, carbohydrate and phenol metabolism, cytoskeleton features, and membrane integrity (Caffall and Mohnen, 2009; Alves et al., 2011). Metabolic regulation by boron occurs by virtue of its ability to complex with compounds rich in hydroxyl groups in the cis-configuration (Camacho-Cristobal et al., 2008). Boron-polysaccharide complex was firstly isolated from radish root cell walls and subsequently the sugar-moiety of the complex was identified as a pectic polysaccharide ramnogalacturonan II. Two molecule of RGII are cross-linked by borate cis-diol ester bound to form a B-dimeric RGII complex (Kobayashi et al., 1996). Hu and Brown (1994) showed that under B deficient conditions the greatest portion of absorbed B (95-98%) is accumulated in cell wall and more than 70% of wall-bound B was associated with pectin. Role of B in maintaining membrane integrity through binding with certain glycoproteins (such as arabinogalactan proteins), has been suggested as well (Goldbach and Wimmer, 2007; Ghanati and Dahajipour Heidarabadi, 2013). Marshmallow (*Althaea*

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officinalis L.) is a medicinal plant containing therapeutic polysaccharide mucilage composed of galacturomannan, arabinan, and arabinogalactans (Basch et al., 2003; Sutovska et al., 2009). Aqueous extract of this plant contains polysaccharides which has been traditionally used as bio adhesive to protect epithelial cells from irritation (Schmidgall and Hensel, 2002). The objective of the present study was to determine the effect of deficiency and excess B nutrition on cell wall polysaccharides of callus-cultured marshmallow cells.

2. Materials and methods

2.1. Cell culture

Calli were established from leaf explants of marshmallow on solidified LS medium (Linsmaier and Skoog, 1965) containing 0.1 mM B, 3% sucrose, 0.2mg/L kinetin, 3 mg/L IAA, 4.5 mg/L NAA. The pH of medium was adjusted at 5.8. After obtaining homogenous calli, suspension cultures were established by introducing 0.5 gram cell from callus to conical flasks containing 30 mL of liquid media as above, without agar. The flasks were maintained in dark at 25°C, shaken at 110 rpm, and sub-cultured weekly. After several subcultures, the cells were treated with different concentrations of B (0.01, 0.1, and 1mMas deficient, sufficient or control, and excess concentrations, respectively). Boron was added in the form of H_3BO_3. After 7 days, the cells were harvested, frozen in liquid N_2, and kept at -80 ºC for further analytical experiments.

2.2. Extraction of cell wall and its polysaccharide components

Frozen samples were homogenized in 4 volumes (v/w) of EtOH using a mortar and pestle and then filtered on nylon mesh (42 µm) under reduced pressure. The filtered cake was washed successively by suspension and filtration in 10volumes (v/w) of EtOH (1 h), $CHCl_3$: MeOH (2:1, v/v, overnight), and acetone (1 h). The residue (cell wall) was dried under a fuming hood and then weighed. Pectin was subsequently extracted from the dried residue with ammonium oxalate20 mM (70ºC), and then 0.1M NaOH. The two extracts were combined and dialyzed thoroughly (MW cut-off 12000) against deionized water, freeze dried, and then weighed. Hemicellulose was dissociated with 0.02% $NaBH_4$ in 17.5% NaOH and then neutralized with the same volume of glacial acetic acid. Further centrifugation (10,000 g) resulted in the separation of hemicellulose B as supernatant and hemicelluloses A as precipitate. The amount of hemicellulose A was also determined gravimetrically. Hemicellulose B was dialyzed thoroughly against deionized water, lyophilized, and weighed (Ghanati and Dahajipour Heidarabadi, 2013). Uronic acid was measured by a modified m-hydroxybiphenyl spectrophotometric method using galacturonic acid as a standard (Blumentkrantz and Asboe-Hansen, 1973). The amount of B absorbed by the cells was determined by Azometine-H method as described by Lohse (1982). Total sugars were assayed by the phenol-sulfuric acid method (Dobios et al., 1956).

2.3. Statistical analysis

All experiments were repeated at least 3 times with 3 independent replicates. Statistical analysis was performed using the Student's T-test, and the differences between the treatments were considered significant at $p \leq 0.05$.

3. Results

Figure 1 shows the amounts of B taken up by marshmallow cells in media with deficient, sufficient, and excess concentrations of B (0.01, 0.1, 1 mM of B, respectively). As shown in this figure, B absorption by marshmallow cells was proportional to the concentration of B supply, so that B content of the cells in deficient condition was 57% of the controls. Under excess B supply however, B uptake increased to 142% of control condition (Fig. 1).

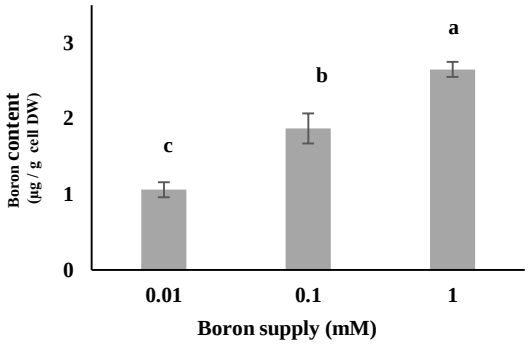


Fig. 1. Boron content of marshmallow cells treated with different concentrations of boron. Data are presented as the means± SD with n = 3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test

B deficiency significantly reduced cell fresh weight, compared to the control group (Table 1). In contrast, higher B supplies improved fresh weight of marshmallow cells (131% of the control). However, no significant difference was observed between dry weights of the cells under different concentrations of B (Table 1). Deficient

concentration of B resulted in increase of their cell wall content (41 µg of dry weight), while cell wall contents of excess B-treated cells was identical to the control group (Table 1). Among different wall polysaccharide components, relative amounts of pectin was stable either in B deficient or excess B-supplied cells (Table 2). Relative amounts of hemicellulose A and cellulose drastically reduced by both B deficiency and excess B concentrations (Table 2). Relative amount of hemicellulose B however, remarkably increased under B deficiency, compared to the normal and excess B supplied cells (Table 2).

Table 1. Effects of different concentrations of B on biomass and cell wall content of suspension-cultured marshmallow cells

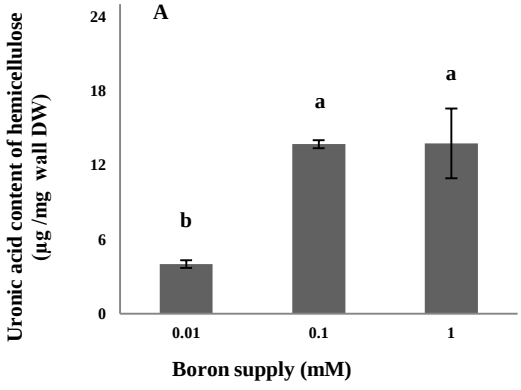
B supply (mM)	FW (mg)	DW (mg)	Cell wall content (µg/g cell DW)
0.01	0.93 ± 0.1^{c}	0.11 ± 0.02^{a}	41 ± 3^{a}
0.1	1.05 ± 0.1^{b}	0.10 ± 0.02^{a}	24 ± 2^{b}
1	1.37 ± 0.1^{a}	0.13 ± 0.01^{a}	25 ± 1^{b}

Data are presented as the means± SD with n = 3. Different letters indicate significant differences at $p\leq0.05$, according to the Student's t-test. FW: fresh weight, DW: dry weight

Table 2. The amount of cell wall polysaccharide components of suspension-cultured marshmallow cells treated with different concentrations of B

B supply	**Cellulose**	**HA**	**HB**	**Pectin**
	% of cell wall dry weight			
(mM)	6.65 ± 0.2^{b}	2.66 ± 0.1^{a}	11.42 ± 0.1^{a}	16.71 ± 0.1^{a}
0.01	23.61 ± 0.2^{a}	12.19 ± 0.4^{b}	7.29 ± 0.3^{b}	18.8 ± 0.2^{a}
0.1	16.11 ± 0.1^{b}	1.94 ± 0.1	7.83 ± 0.6^{b}	17.6 ± 0.1^{a}

Data are presented as the means± SD with n = 3. Different letters indicate significant differences at $p\leq0.05$, according to the Student's t-test. HA: Hemicellulose A, HB: Hemicellulose B



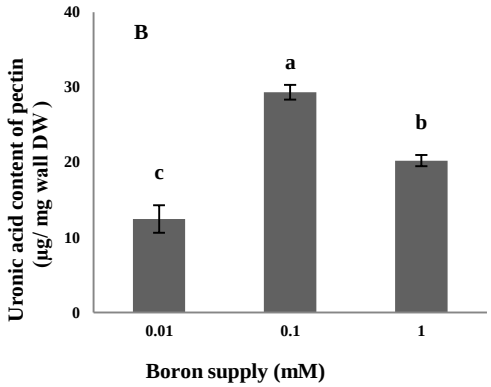


Fig. 2. Comparison of the uronic acid content of hemicelloluse (A) and pectin (B) in marshmallow cells treated with different concentrations of B. Data are presented as the means± SD with n = 3. Bars with different letters are significantly different at $p\leq0.05$, according to the Student's t-test

The uronic acid content of hemicellulose B in 1mM B-treated cells was identical to that of the control group (Fig. 2A). Uronic acid moiety of hemicelluloses B in 0.01mM B-treated cells however, remarkably reduced to 30% of it in control conditions (Fig. 2A). Boron deficient condition, resulted in decrease of uronic acid content of pectin, compared to that of the normal concentration of B (Fig. 2B). Excess B concentrations also reduced the content of uronic acid of pectin, compared to normal B concentration; nonetheless it was still higher than those of B-deficient cells (Fig. 2B). Figure 3 shows total sugar content of cell walls of marshmallow cells in different concentrations of B. As shown in this figure, the wall total sugar content of B-deficient cells was identical to that of the cells in control conditions, while the cell wall total sugar content of excess B-supplied cells was lower than that of the control cells.

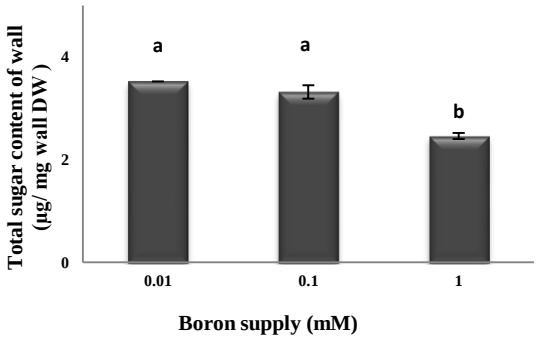


Fig. 3. Total sugar content of walls of marshmallow cells treated with different concentrations of B. Data are presented as the means± SD with n=3. Bars with different letters are significantly different at $p\leq0.05$, according to the Student's t-test

4. Discussion

Whole B content of marshmallow cells followed the concentrations of B supply in the medium, however similar to other reports (Ghanati et al., 2001) the relationship between whole cell B content and B supply was not linear. When B supply was 10 times lower than control conditions, cell B content showed only a reduction of 43%, implying that B-deficient cells served their full ability to actively uptake low available B (Uraguchi et al., 2014). Similarly under B supply 10 times higher than control, only an increase of 42% occurred in whole cell B content, implying that plants sense and respond to the B conditions and regulate transporters to maintain B homeostasis (Miwa and Fujiwara, 2010; Leaungthitikanchana et al., 2014). Boron deficiency and toxicity severely limit crop production worldwide (Camacho-Cristobalet al., 2008). Although the range of B requirement by marshmallow has not been reported yet, identical dry weights of the cells under 0.01 and 1 mM of B to that of the control group, suggest that the range between B deficiency and toxicity for marshmallow is not as tight as other plants. Much of the literature has shown a close relationship between boron and plant cell walls and high concentrations of B usually results in reduced total cell wall production (Fleischer et al., 1998; Kakegawa et al., 2000; Ghanati and Dahajipour Heidarabadi, 2013; Liu et al., 2014).In accordance with other reports (Yang et al., 2002), in the present study the highest production of cell wall materials by marshmallow cells occurred in deficient concentration of B. Among different cell wall polysaccharides, there has been considerable debate on the role of pectin in determination of B requirements. It is usually said that the level of B requirements by a given plant is related to the amount of its pectin, since particularly under low B supply the most cellular B is associated with pectin (Kobayashi et al., 1996; Mengel and Kirkby, 2001; Yu et al., 2002). Relative amount of pectin to total cell wall dry weight of marshmallow cells under B deficiency however, was identical to those of B sufficient, and excess B treated cells. Uronic acid contents of pectin of marshmallow cells were remarkably lowered by B deficiency. Kakegawa and his coworkers (2000) found that galactoronic acid content of *populus alba* significantly reduced under B deficiency condition. On the other hand decreasing uronic acid content of marshmallow cells was accompanied by increase of total content of neutral sugars, suggesting that under B deficiency a cell wall structure rich in neutral sugar moieties is preferable, as it probably provides cells with more sites for borate binding with cis diol-bearing sugars. Based on the results presented here it is more likely that the mere amount of pectin is not decisive for B deficiency symptoms, but rather structural changes of it are important (Liu et al., 2014). Drastic reduction of cellulose in B-deficient marshmallow cells coincided with other reports (Goldbach and Wimmer, 2007), although it may not be caused directly by B deficiency but by a cascade of events catalyzed by the lack of B (Marschner, 1995). Remarkable increase in the relative amount of hemicellulose B in B-deficient marshmallow cells was in accordance with Rajartnam and Lowry's report (1971) on oil palm. Similar results were presented by Zehirov and Georgiev (2003) under B starvation in soybean. Like pectin, hemicellulose is a polyhydroxyl compound with adjacent cis-diol configurations required for formation complex with B (Marschner, 1995). Increase of relative amount of hemicellulose under B deficiency conditions may provide marshmallow cells with higher ability to absorb boron. Moreover, some health benefits of marshmallow are attributed to its wall soluble fibers (particularly hemicellulose B). Hemicellulose directly binds cholesterol in the gut, preventing cholesterol absorption and promoting its excretion. Moreover hemicellulose digestion by beneficial gut bacteria increases their number and creates short-chain fatty acids which colon cells use as fuel and reduces cholesterol (Murray et al., 2005).

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