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Induction of Phenolic Compounds is Affected by Boron Supply in Marshmallow (*Althaea officinalis* L.) Cells

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ABSTRACT

Boron (B) is a non-metal micronutrient which is essential for plants growth and development. Formation of boron complex with cell wall matrix and phenolic compounds is a definite influence of boron in physiological process. It has been suggested that B-toxicity and deficiency may induce excess production of reactive oxygen species thereby promote defense responses by antioxidant enzymes or non-enzymatic compounds e. g. phenolics. Marshmallow (*Althaea officinalis* L.) is a plant whose range of boron requirement has not been reported yet. On the other hand this plant contains valuable flavonoid glycosides, phenolic acids, tannins and flavonoids and has exhibited strong total antioxidant activity as well. The present study was therefore undertaken in order to investigate ~~on~~ the effects of different concentrations of boron on phenolic compounds of suspension-cultured marshmallow cells. The cells were grown in a modified MS medium without glycine and boron was supplied in the form of H_3BO_3 with the concentrations of 0.1, 0.01, and 1mM as control, deficiency and excess concentrations, respectively. Deficiency and excess boron supply increased the amount of pectin-bound cinnamic acid, ferulic acid, benzoic acid and tannic acid. Boron in 1mM concentration increased H_2O_2 content of the cells, but had no effect on H_2O_2 content in deficient concentration, compared to the control group. The flavonoid content of the cells treated with 1 and 0.01 mM B was also higher than of the cell under control condition, but both B- deficiency and excess B led to a similar decrease in anthocyanin content.

Keywords: *Althaea officinalis*, Anthocyanin, Boron, Flavonoid, Marshmallow, Phenolic compounds

Introduction

Boron (B) is a non-metal micronutrient which is essential for plants growth and development. Unlike other micronutrients, predominant form of boron in soil with neutral pH is a non-ion form of H_3BO_3 and at higher pH, in form of $B(OH)_4$ (Yang and Gu, 2004). Boron enters plant cells, in part, by passive diffusion through the lipid bilayer of the plasma membrane and, in part, through proteinaceous water channels (Dordas *et al.,* 2000). The primary function of B is extracellular and largely related to its capacity to form borate complex with hydroxyl groups of sugars and some phenolics (e. g., caffeic acid and hydroxy frulic acid) (Dugger, 1983; Monday and Munshi, 1993). Formation of boron complex with cell wall materials and phenolic compounds is a definite influence of boron in physiological process. Phenolic metabolites represent considerable part of plant organic matter therefore their responses to almost all environmental

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factors may be assumed. They are triggered in plants in response to biotic and abiotic stresses. Cell wall phenolics have several important roles in providing mechanical strength, regulation of growth and morphogenesis, and responses to stresses and pathogens. Cross-linking of phenolic monomers has been associated with reduction of extensibility and growth (Ghanati *et al.,* 2002). Accumulation of phenols was reported in B-deficiency as a result of the insufficient formation of complex between B and free phenols (Marschner, 1995). Boron deficiency also resulted in the accumulation of phenolic compounds in cell walls of *Linum usitatissimum* L. roots, while high concentrations of B resulted in the decrease of lignin content and wall-bound phenols under aluminum stress, thereby ameliorated Al toxicity (Dahajipour Heidarabadi *et al.,* 2011). Phenolic compounds usually possess different antioxidant activity potentials and their hydroxyl group can act as a hydrogen or electron donor. These compounds are well known potential natural antioxidants and scavengers of reactivation oxygen species (ROS) (Dai and Mumper, 2010).

Effects of B deficiency including loss of membrane integrity, accumulation ofphenolics and induction of imbalance between production and scavenging of reactive oxygen species have been shown in turnip plants (Hajiboland and Farhanghi, 2010). Boron deficiency may also induce production of ROS by impairing ascorbate metabolism (Marschner, 1995; Lukaszewski and Blevins, 1996). B-toxicity also has been suggested to induce excess production of ROS and oxidative burst (Gunes *et al.*, 2006), but there is a very narrow range between deficiency and toxicity of B and the optimum B level for one species could be either toxic or insufficient for other species (Blevins and Lukaszewski, 1998; Yau and Rayan, 2008). Marshmallow (*Althaea officinalis* L.) is a plant whose range of boron requirement has not been reported yet. On the other hand this plant contains valuable flavonoid glycosides, phenolic acids, tannins and flavonoids and has exhibited strong total antioxidant activity as well (Elmastas *et al.,* 2004; Komissarenko and Kovalev 1992). Therefore the present study was undertaken in order to evaluate the effects of different concentrations of B on the contents of cell wall phenols, and certain soluble phenolic compounds, i. e., flavonoids and anthocyanins. Regarding to role of hydrogen peroxide as a very dangerous ROS which can pass through cellular membranes (Wojtazsek, 1997) and initiate protective responses to limit or repair oxidative damage (Veal and Day, 2011), a potential relationship between phenolic compounds and concentration of hydrogen peroxide is discussed as well.

Materials And Method

Cell culture, treatments, and viability

Calli of marshmallow were established from seed explants on modified MS medium without glycine, containing 3% sucrose and supplemented with 3 mg/L IAA, 4.5 mg/L NAA, and 0.2 mg/L kinetin, at pH 5.8 (Ghanati *et al.,* 2002). The cells were grown in aforesaid media in the darkness at 25 °C and were renewed every 10 days. Reaching to a line with stable growth (after 11 subcultures), the cells were transferred to liquid media and renewed every week. Boron was supplied in the form of H_3BO_3 with the concentration of 0.01, 0.1, and 1mM as deficient, normal (control), and excess concentrations, respectively. Viability of the cells was determined with aquatic solution (0.25%, w/v) of Evans blue (Gahan, 1984).

Extraction and assay of wall-bound phenolics

For preparation of walls the cells were homogenized in water with a mortar and pestle. After centrifugation at 1000 × g, the pellet was sequentially washed with 10 volumes (v/w) of 0.5mM $CaCl_2$, EtOH, $CHCl_3$–MeOH (2:1, v/v) and acetone followed by air-drying. Air-dried materials were desired as wall preparation. Phenolics were liberated from the wall preparations of 6 days treated samples with ammonium oxalate (20 mM, 70 °C) and then with 0.1M NaOH, under $N_2$for 24 h. After acidification of both fractions to approximately pH 3.0 with HCl, phenolics were extracted three times with EtOAc, air-dried and re-dissolved in 50% MeOH before determination by HPLC on an ODS-80 Ts column (4.6 mm × 250 mm, Tosoh, Japan). Phenolics were eluted at a flow rate of 0.5 mL/min with a linear gradient of 30–80% MeOH containing 0.1% HOAc and were detected at 280 nm. The amounts of cinnamic acid, ferulic acid, benzoic acid and tannic acid were determined using were commercially available authentic standards (Ghanati *et al.*, 2002).

Determination of H_2O_2

The cells were homogenized with 2 mL of 0.1% (w/v) TCA on ice bath. The homogenate was centrifuged at 12000×g for 15 min and 0.5 mL of suspension was added to 0.5 mL of 10 mM potassium phosphate buffer and 1 mL 1M KI. The content of H_2O_2 was determined using a spectrophotometer (Cintra 6, GBC, Australia) at 390 nm using standards of 0-30 ng/ mL (Velikova *et al.,* 2000).

Flavonoid and anthocyanin content

The flavonoid content of the cells was measured as described by krizek *et al.* (1998). In brief, the cells were homogenized with 3 mL ethanol containing 1% HCl and then centrifuged at 12000×g for 10 min. The supernatant was heated at 80 °C for 10 min and after cooling the absorbance was measured at 270, 300, and 330 nm. Flavonoid content in each wavelength was measured considering an extinction coefficient of 33, 000 /cm M. For anthocyanin assay, the cells were homogenized in 3 mL of methanol containing 1% HCl followed by centrifugation at 12000 × g for 10 min. The supernatant was kept at the dark overnight and then its absorbance was read at 550 nm and total anthocyanin content was calculated using an extinction coefficient of 33, 000 /cm M. (Wagner, 1979).

Statistical analysis

All observations and 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 expressed as significant at a level of $p \leq 0.05$.

Results

The morphology of marshmallow callus after 11 subcultures is shown in Fig. 1. The calli were friable and felt apart easily to generate cell suspension cultures. Viability of the cells in suspension culture was evaluated by Evans blue staining by subtracting the blue-colored cells (dead cells) from a total of 100 counted cells (Fig. 2). Neither B deficiency (0.01 mM) nor its excess (1mM) had significant effects on the viability of marshmallow cells, compared to the control conditions (Fig. 3). The contents of phenolic acids esterified to pectin in the walls of marshmallow cells treated with different concentrations of B are shown in Table1. Boron deficiency (0.01 mM B)

remarkably increased total amounts of wall-bound phenols, and the amount of almost all detected phenolic acids were 1.5-2 times higher than those of the sufficient concentration of B. Among wall-bound phenolics of B-deficient cells, increase of Ferulic acid was noticeable, suggesting stiffening of cell walls in these conditions. In excess B conditions (1 mM) also total amounts of wall-bound phenolic acids were remarkably higher than that of sufficient B conditions. Among wall-bound phenolics of excess B-supplied cells increase of tannic acid was the most pronounced ones (Table 1).

Figure 1. Morphology of marshmallow calli. The calli were bright and friable and easily separated in suspension cultures.

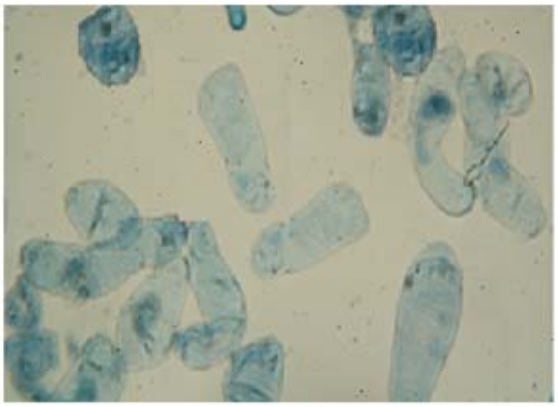

Figure 2. Staining of suspensio n-cultured marshmallow cells by Evans Blue. Dead cells stained blue while live ones did not stain. Scale bar represents 60 µm.

Total contents of flavonoids of the cells treated with 1 and 0.01 mM B were significantly higher than that of the control cells (Fig. 4). However in comparison with B deficiency, excess B brought a higher increase in flavonoid contents of the cells (Fig. 4). The content of anthocyanin of marshmallow cells decreased by both deficiency and excess B, compared to the control cells (Fig. 5).

The H_2O_2 concentrations were measured in cells as an indicator of oxidative stress. This parameter significantly increased under excess B, but did not change under B deficiency, compared to control conditions (Fig. 6).

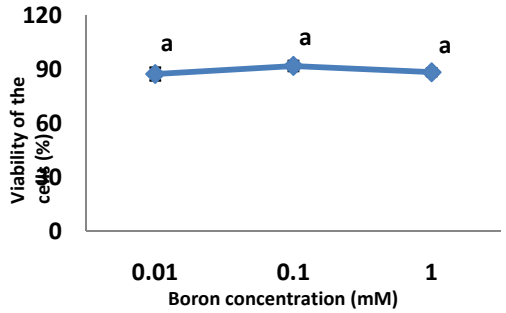

Figure 3. Evaluation of viability of the cells supplied with different concentrations of boron. Data are presented as the means ± SD (vertical bars), n = 3. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's t-test.

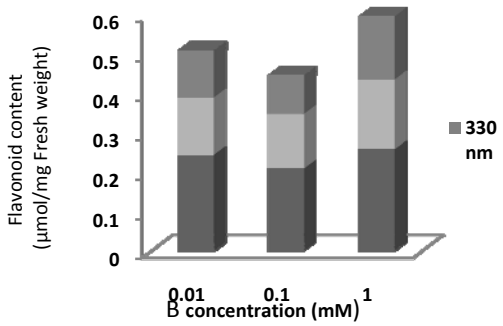

Figure 4. Falvonoid contents of marshmallow cells treated with different concentrations of boron.

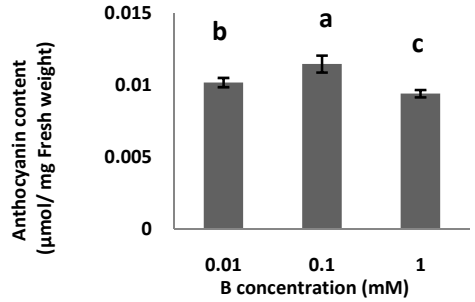

Figure 5. Anthocyanin content of marshmallow cells treated with different concentrations of boron. Data are presented as the means ± SD (vertical bars), n = 3. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's t-test.

Discussion

It has been suggested that increase of B supply induces the producti on of ROS. Among reactive oxygen species, H_2O_2 can be used as a substrate for biosynthesis of lignin and production of phenolic compounds (Karabal *et al.*, 2003). Concentration of H_2O_2 has been introduced as an indicative for oxidative stress induced by excess B in tomato, apple, and grape (Cervilla, 2007; Molassiotis *et al.,* 2006; Gunes *et al.,* 2006). Similarly excess B supply in the present study increased H_2O_2 content of marshmallow cells. B deficiency however, did not significantly change amounts of H_2O_2 of marshmallow cells, coincident with Karabal and Yucel (2003) report on barley cultivars.

Increase of phenolics has been well defined in B deficient tissues, particularly in those plant species which have a higher B requirement (Marschner 1995; shkolnik 1984). Boron complexes with 6-phosphoglucoronic acid and thus, inhibits 6-phosphogluconate dehydrogenase. If B regulate pentose shunt catabolic pathway by this complexing, phenolic compound do not accumulate. On the other hand B deficiency would result in uninhibited dehydrogenase and excess phenolic caid would be formed (Dugger, 1983; Loomis and Durst, 1991; Marschner 1995). In B deficient plants hydroxycinnamic acid derivatives i. e, caffeic acid, cholorogenic acid, ferulic acid, vanilic acid and some coumarins accumulated (Perkin and Aronoff 1965; Rajartnam and Lowry 1974). It is suspected t hat hydroxycinnamic acids are very important components not only for the biology of the cell wall. They can be coupled by peroxidase-mediated oxidative coupling and creation of these cross-links has also been postulated to have a number of other important roles, such as in controlling cell wall extensibility.

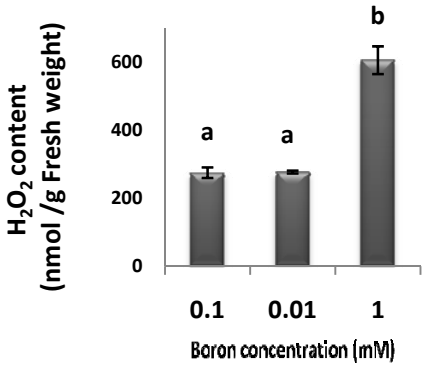

Figure 6. Comparison of H_2O_2 content of marshmallow cells supplied with in different concentrations of boron. Data are presented as the means ± SD (vertical bars), n = 3. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's t-test.

In B deficient condition increase of ferulic acid was noticeable. Ferulic acid has been reported to exhibit a wide range of important biological and therapeutic properties i. e., anti-inflammatory, anti-bacterial, anti diabetic, anti-carcinogenic, anti-aging, and neuro-protective effects, which can be attributed to its antioxidant capacity because of its phenolic nucleus and extended side chain conjugation (Buana fina 2009). Therefore, it seems that B deficient

condition, although limiting wall loosening and growth of marshmallow cells, may be beneficial regards to improve its medical properties. Accumulation of phenols has been reported tobacco at both minimum and maximum levels of supply (Ruiz *et al.*, 1998). The pentose pathway provides main precursor for shikimic acid pathway, the primary part of phenolics i. e., monophenols, lignin, flavonoids, and anthocyanin metabolism route.

Table 1. Wall-bound phenolic acids extracted from marshmallow cells treated with different concentration of Boron.

B supply (mM)	Phenolics (µg/mg FW)				
	Tannic acid	Benzoic acid	Cinnamic acid	Ferulic acid	Total
0. 01	0. 292±0. 008	0. 359±0. 03a	1. 80±0. 09a	1. 396±0. 14a	3. 83±0. 26a
0. 1	0. 187±0. 04c	0. 168±0. 04b	1. 06±0. 11b	0. 586±0. 12b	1. 98±0. 31b
1	0. 433±0. 05a	0. 284±0. 01a	1. 63±0. 23a	1. 51±0. 06a	3. 85±0. 35a

Flavonoids are a large group of naturally phenolics compounds ubiquitously distributed in the plant kingdom. According with increased phenols bound to pectin in deficiency and excess B conditions, soluble phenols such as flavonoids in B deficiency and excess B increased in marshmallow cells. Increase of flavonoids e.g. flavenol, flavenones, and flavenol-3-glucosides has been reported in boron deficient tomato leaves (Dugger, 1983). Flavonoids involved in many biological processes. Fixation of boron with cross-linkable flavonoids and tannins has been recently proven and used for hardening of wood in industry (Tondi *et al.*, 2012). Treatment of marshmallow cells with excess B resulted in increase of flavonoids as well as of tannic acids. Therefore, it is plausible that in excess B supply conditions a part of absorbed B is bound to flavonoid and tannins. Cross linking of phenolic acids to each other to produce polyphenols as well as to wall polysaccharides is, in part, peroxidase-mediated oxidative coupling. The optimum precursor for peroxidase is H_2O_2 as an electron donor. Increase of hydrogen peroxide in excess B-supplied marshmallow cells provided the cells with higher precursor for production of more flavonoids. Among different properties of flavonoids most interest has been devoted to their antioxidant activity, which is due to their ability to reduce formation of free radicals. Flavonoids like many other polyphenols are excellent free radical scavengers because they are highly reactive as hydrogen or electron donors (Seyoum *et al.*, 2006). Increase of flavonoids in marshmallow cells under excess or deficient B supply may provides the cells with higher ability to withstand against stress conditions. In this relation however, anthocyanin apparently do not have a crucial role.

References

1. Blevins DG, Lukaszewski KM (1998) Boron in plant structure and function. Annu Rev Plant Physiol Plant Mol Biol 49, 481-500.
2. Bowler C, Montagu MV, Inze D (1992) Superoxidedismutase and stress tolerance.

Annu Rev Plant Physiol Plant Mol Biol 43, 83-116.

3. Buanafina MMO (2009) Feruloylation in grasses: current and future perspectives. Molecular Plant 2, 861-872.
4. Cervilla LM, Blasco CB (2007) Oxidative stress and antioxidant in Tomato (Solanum lycopersicum) plants subjected to Boron toxicity. Ann Bot 100, 747-756.
5. Dai J, Mumper RJ (2010) Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313-7352.
6. Dordas C, Chrispeels MJ, Brown PH (2000) Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots. Plant Physiol 124, 1349-1361.
7. Dugger WM (1983) Boron in plant metabolism. In: Encyclopedia of Plant Physiology, A Lauchli, RL Bieleski eds. (Springer-Verlag, New York), pp 626-650.
8. Elmastas M, Ozturk L, Gokce I, Erenler R, Aboul-Enein HY (2004) Determination of Antioxidant Activity of Marshmallow Flower (*Althaea officinalis* L.) Anal Lett 37, 1859-1869.
9. Gahan PB (1984) Plant Histochemistry and Cytochemistry (Academic Press, London), P. 234.
10. Ghanati F, Morita A, Yokota H (2002) Induction of suberin and increase of lignin content by excess boron in tobacco cells. Soil Sci Plant Nutr 48, 357-364.
11. Gunes A (2006) Antioxidant and stomatal responses of grapevine (Vitis vinifera L.) to boron toxicity. Sci Hortic 110, 279-284.
12. Hajiboland R, Farhanghi F (2010) Remobilization of boron, photosynthesis, phenolic metabolism and antioxidant defense capacity in boron deficient turnip (*Brassica rapa* L.) plants. Soil Sci Plant Nutr 56, 427-437.
13. Han SH, Tang N, Jiang HX, Yang LT, Lie Y, Chen LS (2009) CO_2 assimilation, photosystem II photochemistry, carbohydrate metabolism and antioxidant system of citrus leaves in response to boron stress. Plant Science 176, 143-153.
14. Hu H, Brown PH (1994) Localization of boron in cell walls of squash and tobacco and its association with pectin. Plant Physiol 105, 681-689.
15. Hu H, Brown PH, Labavitch JH (1996) Species variability in boron requirement is correlated with cell wall pectin. J Exp Bot 47, 227-232.
16. Inze D, Van Montagu M (1995) Oxidative stress in plants. Curr Opin Biotech 6, 153-158.
17. Karabal F, Yucel M (2003) Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. Plant Sci 164, 925-933.
18. Komissarenko SN, Kovalev VN (1992) Coumarins of *Althaea officinalis* and *A. armenica*. Chem Nat Compd 28, 243-244.
19. Krizek DT, Britz S J, R M (1998) Inhibitory effects of ambient levels of solar UV-A and UV-B radiation and growth on Cv. New red fire lettuce. Physiol Plant 103, 1-7.
20. Loomis WD, Durst RW (1991) Boron and cell walls. In DD Randall, DG Blevins, CD Miles, eds, Curr TPLB 10, 149-178.
21. Lukaszewski K M, Blevins DG (1996) Root growth inhibition in boron-deficient or aluminum-stressed squash may be a result of impaired ascorbate metabolism. Plant Physiol 112, 1135-1140.
22. Marschner H (1995) Mineral nutrition of higher plants. 2nd Ed. Academic Press. London. 379-396.
23. Molassiotis A, Sotiropoulos T, Tanou G, Diamantidis G, Therios I (2006) Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (Malus domestica Borkh), Environ Exp Bot 56, 54-62.
24. Mondy NI, Munshi CB (1993) Effect of Boron on enzymatic discoloration and

phenolic and ascorbic acid contents of potatoes. J Agric food chem 41, 554-558.

25. Perkins HJ, Aronoff S (1956) Identification of the blue-fluorescent compounds in boron-deficient plants. Arch Biochem Biophys 64, 506–507.
26. Rajarathnam JA, Lowry JB, (1974) The role of boron in the oil-palm (Elaeis guinensis). Ann Bot 38, 193-200.
27. Ruiz JM, Bretones G, Baghour M, Ragala L, Belakbir A, Romero L (1998) Relationship between boron and phenolic metabolism in tobacco leaves. Phytochem 48, 269-272.
28. Shkol'nik MY (1984) Trace Elements in Plants. Developments in Crop Science (6), Elsevier, New York. pp. 68–109.
29. Sotiropoulos TE, Molassiotis A, Almaliotis D, Mouhtaridou G, Dimassi K, Therios I, Diamantidis G (2006). Growth, nutritional status, chlorophyll content, and antioxidant responses of the apple rootstock MM 111 shoots cultured under high boron concentrations in vitro. J Plant Nutr 29, 575-583.
30. Seyoum A, Asres K, Kandeel F (2006) Structure–radical scavenging activity relationships of flavonoids. Phytochem 6, 2058-2070.
31. Tondi G, Wieland S, Wimmer T, Thevenon MF, Pizzi A, Petutschnigg A (2012) Tannin-boron preservatives for wood buildings: mechanical and fire properties Eur J Wood Prod DOI 10.1007/s00107-012-0603-1.
32. Veal E, Day A (2011) Hydrogen peroxide as a signaling molecule. Antioxid Redox Signal 15, 147-151.
33. Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant system in acid rain- treated bean plants protective role of exogenous polyamine. Plant Sci 151, 59-66.
34. Wagner G (1979) Content and vacuole/extravacuole distribution of neutral sugar, free amino acids and anthocyanin in protoplast. Plant physiol 64, 88-93.
35. Wakabayashi K, Takayuki H, Kamisaka S (1997) Osmotic stress suppresses cell wall stiffening and the increase in cell wall-bound ferulic and differulic acids in wheat coleoptiles. Plant Physiol 113, 967-973.
36. Wojtazsek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem. J. 322, 681-692.
37. Yang YH, Gu HJ (2004) Effects of boron on aluminum toxicity on seedlings of two soybean cultivars. Water Air Soil Poll 1, 1-10.
38. Yau SK, Ryan J (2008) Boron toxicity tolerance in crops: a viable alternative to soil Amelioration. Crop Sci 48, 854-865.