



Research article

Excess boron inhibited the trifoliolate orange growth by inducing oxidative stress, alterations in cell wall structure, and accumulation of free boron

Muhammad Riaz^a, Lei Yan^a, Xiuwen Wu^a, Saddam Hussain^b, Omar Aziz^{a,d}, Zeinab El-Desouki^{a,c}, Cuncang Jiang^{a,*}

^a Microelement Research Center, College of Resources and Environment, Huazhong Agricultural University, Wuhan, Hubei, 430070, PR China

^b Department of Agronomy, University of Agriculture Faisalabad, 38040, Punjab, Pakistan

^c Department of Soil Sciences, Faculty of Agriculture, Ain Shams University, Cairo, 11241, Egypt

^d Department of Soil & Environmental Sciences, University of Agriculture Faisalabad, Sub-Campus Depalpur Okara, 56300, Punjab, Pakistan

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ABSTRACT

The boron (B) is an essential nutrient and plays an important role in the stability of the primary cell wall (CW). Due to the narrow window between B deficiency and toxicity, mismanagement practices lead to B toxicity that inhibit root growth and overall crop productivity. However, the exact cause of root growth inhibition remains unclear. The present study examined the potential causes and targets of B toxicity by studying intercellular mechanism. The trifoliolate seedlings were cultured under excess B conditions. The results indicated that plant growth was inhibited by excess B, nevertheless, the effects were prominent on roots and leaves. B toxicity exacerbated oxidative stress and root cell death. The analysis of CW functional groups, CW microstructure and B forms lead to the conclusion that alterations in CW, and accumulation of free-B and carbohydrates might cause inhibition of growth and visible symptoms of B toxicity.

1. Introduction

Boron (B) is an essential nutrient for maintaining optimal development and growth of higher plants. Plants extract B from the soil in the form of boric acid which is highly soluble and plants modulate boric acid homeostasis by uptake and efflux transporters (Dordas and Brown, 2000; Aquea et al., 2012). To date, the principal function of B is largely believed to be associated with the structure of cell wall (CW) along with a wide range of metabolic roles in the biological system of plants (Takano et al., 2008; Chormova and Fry, 2016). Dimerization of rhamnogalacturonan RG-II by B cross-linking contributes to the stable cell wall assembly and biophysical properties. Previous reports demonstrated that 90% of B is associated with the CW of plants (O'Neill et al., 2001; Chormova et al., 2014) reflecting the importance of B in the plant system. Despite the great significance of B, there exists only a narrow range between deficiency and toxicity, which is considered optimal for plants, and every plant has a different B requirement for maintaining optimal growth. Boric acid is soluble and susceptible to leaching in areas receiving high rainfall, however, if soil accumulates high concentration of B, it may cause toxicity in plants. As compared to B deficiency which can be corrected by applying chemical fertilizers, B toxicity is more difficult to manage due to the soil complex system

(Aquea et al., 2012; Lin et al., 2018). Moreover, mismanagement practices for fixing B deficiency can cause B toxicity which is quite common in the arid and semi-arid region of China and is a major limiting factor for citrus productivity. B deficiency has been extensively studied for decades in many plants, and similarly, significant advancement in biochemical and physiological data of B toxicity has also been proposed (Reid et al., 2004), but underlying mechanisms by which B is toxic to plants remain unclear.

The sites of B accumulation after taking up by roots are the old leaves, and under excess B, visible symptoms of toxicity are obvious on leaves during different period of growth (Reid et al., 2007). Plants vary in their tolerance to B toxicity, and efflux of extra B from the roots has been shown to be involved in tolerance to B toxicity by reducing free-B accumulation in above-ground parts of plants, therefore, the redistribution of excess B in leaves could be one of the mechanisms; redistributing of intracellular phase to apoplast which is less toxic than the former one (Reid et al., 2007). Therefore, studying the distribution of B forms in leaves is equally important in order to understand tolerance mechanisms in trifoliolate. Moreover, B is soluble and highly permeable to membranes.

In the plant defense system, antioxidants play a significant role in eliminating oxidative stress. The reactive oxygen species which are

* Corresponding author.

E-mail address: jcc2000@mail.hzau.edu.cn (C. Jiang).

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produced during metabolism may accumulate in high concentration and can cause oxidative injuries in plants and even cell death (Hussain et al., 2016; Riaz et al., 2018). Therefore, the antioxidant system is a major contributor in the defense system consisting of peroxidase, ascorbate peroxidase and catalase, along with a number of other enzymes.

Trifoliolate orange is regarded as the main rootstock in China and is sensitive to B deficiency and toxicity (Han et al., 2008; Jiang et al., 2009). Our recent investigation has provided new insight into these mechanisms that B deficiency-induced changes in the CW might be associated with the inhibition of plant growth that makes trifoliolate less tolerant than the citrange which is tolerant to B deficiency (Wu et al., 2017).

The objectives of this study were, to explore the underlying mechanism of B toxicity-induced growth inhibition in trifoliolate orange at the biochemical and physiological levels, to investigate how B toxicity induces alterations in the cell wall functional groups and oxidative damages by studying the antioxidant enzyme system and to explore the distribution of different B forms and find out the particular form of B which is likely to be associated with the leaf chlorosis. The study might be helpful for understanding interplay between B toxicity and its mechanism of toxicity for citrus production.

2. Materials and methods

2.1. Plant materials and experimentation

The seedlings of trifoliolate orange were collected from the fruit market of Jiangxi (Ganzhou). Before transplanting, seedlings were rinsed with tap water to remove surface contaminations, and later soaked in the deionized water for two days. The seedlings with 4–5 leaves and root length 8–10 cm long were transplanted to 6 L plastic black boxes; each box had 2 seedlings. The nutrient solution was applied in the following macro and micro concentrations; 2 mM KNO₃, 0.5 mM MgSO₄, 1.23 mM Ca(NO₃)₂, 0.32 mM NaH₂PO₄, 0.14 mM Na₂HPO₄, 4.45 μM MnCl₂, 0.18 μM Na₂MoO₄, and 28.7 μM Fe-EDTA. The present investigation consisted of three B levels; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. The treatment of 10 μM B was considered as control. The seedlings were provided with aeration system (Aerator pump, ACO-002, 40L/min) for 20 min every after 4 h intervals by an automated device that was linked to each pot (Wu et al., 2017). The seedlings were grown in a greenhouse of Huazhong Agriculture University, Wuhan, China, under natural conditions of temperature 25 °C, and humidity 68%. The pH of the nutrient solution was monitored and adjusted to 6.2 every day with 0.1 H₂SO₄ and 1 M NaOH. The culture solution was renewed every three days intervals. The reagents and chemicals used in the present study were high grades. The seedlings were harvested after completing 18 weeks in the nutrient solution until plants had visible symptoms of B toxicity on leaves and roots. The root growth of the longest primary root before and after experimental treatments was measured with a plastic ruler. The plant samples were divided into roots, stem and leaves. One part of root and leaves were stored at –80 °C for further extraction of enzymes and biochemical analysis. Rest part of the roots and leaves were oven dried at 55 °C until a constant weight for fourier transform infrared spectroscopy (FTIR) analysis and B extraction. The experiment had a complete randomized design with three treatments, and each treatment had three replications.

2.2. Determination of B forms and distribution in different plant plants

The B distribution in plant parts was determined by the curcumin method (Dible et al., 1954). Briefly, oven dried 0.2 g of plant samples (root, stem, leaves) were ground into fine powder and ashed at 500 °C in a muffle furnace for 5 h. Subsequently, samples were digested with 0.1 M of HCl and filtered, and finally, B was extracted with curcumin calorimetrically by spectrophotometer at 540 nm and B concentration

was calculated based on a standard curve.

For measurement of B forms in the leaves, the method of Xu (2002) was employed. Briefly, 2 g of old leaves were suspended into horizontal shaker at room temperature at 150 × g for 24 h, and the supernatant was collected for analyzing free-B. Then, the semi-bound B was measured in the residues suspended in 1 M of NaCl after 24 h shaking in the shaker. Finally, the remaining residue was again suspended in 1 M of HCl for 24 h and B in the supernatant was analyzed as bound B.

2.3. Transmission electron microscopy of leaves

The transmission electron microscope slides were prepared with the ultrathin sections of old leaves as described by Wu et al. (2017). Lower old leaves after rinsing with distilled water were excised into small pieces of 1 × 1 mm sizes from each replication and immediately fixed into 2.5% of glutaraldehyde solution containing phosphate buffer solution (PBS) for 12 h at 4 °C. Subsequently, samples were rinsed with 0.1 M of PBS (pH 7.4) thrice and post-fixed in 1% buffered osmium tetroxide for 2–3 h. After postfixation, samples were rinsed again with 0.1 M PBS and dehydrated stepwise in an increasing concentration of acetone 50%, 70% and 90%, and later with a mixture of 90% ethanol and 90% acetone for a time period of 15 min each time. The temperature of the whole dehydration process was maintained at 0–4 °C After embedding and solidifying with acetone and embedding solution, the sample tissue was cut into 50–60 nm thick sections using an ultramicrotome. Finally, samples were stained with 20% uranyl acetate and lead citrate, and specimens were observed and photographed by an electron microscope (JEM-100CX II).

2.4. Cell wall extraction

The method of Hu and Brown (1994) was utilized to extract root cell wall (CW). For this, 3 g of fresh roots were ground into liquid nitrogen. Then, the homogenate was washed twice with 30 mL of pre-cooled ultrapure water and centrifuged at 5000 × g for 10 min at 4 °C. The supernatant was discarded and the residue was further washed with 80% ethanol three times, methanol-chloroform (1:1 ratio) mixture once and finally once with acetone. After each centrifuging process, the supernatant was discarded, and the remaining insoluble residue was defined as the crude cell wall. The crude cell wall was dried and weighed, and the cell wall extraction rate for each treatment was calculated. The crude cell wall was employed for fourier transform infrared spectroscopy (FTIR) analysis.

2.5. FTIR analysis of roots

The root dried cell wall (CW) samples were ground and passed through 0.2 mm sieve. Subsequently, CW samples were mixed with the potassium bromide (KBr) at a mass ratio of 1:100. After mixing with an agate mortar, the mixture was transferred for tableting. A uniform, transparent tablet was prepared by Graseby-Specac Press, and then fourier transform infrared spectroscopy (FTIR) image was obtained using a VERTEX 70 fourier infrared spectrometer (Bruker, USA), with a spectral range of 4000–400 cm⁻¹, at a resolution 4 cm⁻¹, and the number of scans per sample was 32. For the accuracy of the analysis, the background sample was scanned before the experimental samples. The interference of H₂O and CO₂ was subtracted from the scan. The obtained FTIR map was subjected to baseline calibration, and OMNIC 32 software was utilized to determine the peak and absorbance values. The FTIR map was drawn using Origin 8.6.

2.6. Assay of antioxidant enzyme activities and MDA content

The roots were employed for the determination of antioxidant enzyme activities (POD and CAT) and MDA content. The 0.5 g of fresh roots were homogenized with a pre-cooled mortar and pestle by 5 mL of

phosphate buffer (pH 7.8) in ice at 4 °C and then homogenate was transferred to a centrifuge tube. The homogenate was centrifuged at 5000 × g in a cryostat for 15 min at 4 °C. The supernatant was the crude enzyme extract and was stored at 4 °C for the determination of enzyme activities. The catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm (De Azevedo Neto et al., 2006). The peroxide (POD) activity was determined by the method of Cakmak and Horst. (1991) and absorbance was recorded at 470 nm for 210 s.

Malondialdehyde (MDA) is the final decomposition product of lipid peroxidation. The MDA content was determined by the thiobarbituric acid method (Heath and Packer, 1968). The 0.3 g of roots (fresh weight) were homogenized with 3 mL of 10% trichloroacetic acid (TCA) and a small amount of quartz sand. The homogenate was mixed thoroughly and centrifuged at 5000 × g for 10 min at 4 °C. Then, 1.0 mL of the supernatant was added with 1.0 mL of 0.6% TBA solution in a test tube. The mixture was heated in boiling water for 15 min at 90 °C, then rapidly cooled in an ice water bath, and centrifuged at 5000 × g for 3 min. The absorbances were measured at 532 nm, 600 nm and 450 nm by a spectrophotometer.

The monoamine oxidase (MAO), xanthine oxidase (XOD) and total antioxidant capability (T-AOC) were measured in roots by the commercially available kits, following the manufacturer guidelines.

2.7. Assessment of loss of plasma membrane integrity

Cell death was measured by staining solution of Evans blue based on the loss of membrane integrity as described by Yamamoto et al. (2001). The root tips (0–10 mm) were exercised from each treatment and stained in 0.05% of Evans blue solution for 15 min at room temperature and subsequently rinsed with phosphate buffer saline (PBS). The root samples were observed under a fluorescence microscope (Olympus IX81). Each sample was scanned for at least three times. To extract the Evans blue dye trapped by roots, the method of Baker and Mock. (1994) was employed. Briefly, roots (0.1 g) were homogenized by pestle and mortar in 1 mL of 1% SDS. After centrifuging at 5000 × g for 5 min, the supernatant was used for the quantification of Evans blue uptake at 597 nm by a spectrophotometer.

2.8. Statistical analysis

The data obtained were statistically analyzed by the analysis of variance (ANOVA) with Statistix 8.1 computer-based software. The statistical differences among treatments were separated by the least significant difference (LSD) test at $p < 0.05$. Principle component analysis was performed with Rstudio and graphs were generated by SigmaPlot 12.5.

3. Results

3.1. Influence of B toxicity on plant growth parameters

Boron toxicity has been reported to inhibit root and plant growth parameters. In the present study, the trifoliolate orange seedlings exposed to different concentrations of B stress led to severe inhibition of primary root growth irrespective of B concentrations (Fig. 1), and the lowest root growth was recorded with the B2 treatment indicating that B stress could induce some of the toxicity symptoms on roots by inhibiting main and lateral root growth (Fig. 2). At the end of the experiment, B1 and B2 treatments decreased the total dry biomass accumulation by 20% and 33.79%, and total root growth by 19.67% and 44%, respectively (Fig. 1). The symptoms of B toxicity were visible on underground and aboveground parts of seedlings; the leaves showed severe signs of toxicity with discoloration spots on old leaves, later changed into necrotic patches both in leaf tips and margins, progressively extended back along the leaf and ended up drying of leaf tissues at the edges (Fig. 2).

3.2. Distribution of B contents and B forms in plant parts

The B contents in roots, stem and leaves were increased as B supply increased in the nutrient solution and there were significant differences with the B contents in different parts of the plant (Fig. 3). In root, stem and leaves, B accumulation was increased by 2.13, 3.27, and 4 fold by B1 treatment, and it was increased by 3.09, 4.63 and 6.89 fold by B2 treatment, respectively as compared with the control treatment. It should be noted that irrespective of B levels, B contents was the highest in leaves particularly in B2 treatment which was regarded as the extreme stress condition. These results indicate that B primarily accumulates in the old leaves. Although with the increase of B supply, roots and stem also accumulated a significant amount of B, however, the magnitude of accumulation was lower than leaves (Fig. 3 and Supplementary Fig. 1).

The experiment results showed that the degree of free-B and bound-B were greater than semi-bound B in leaves. The free-B was increased by 8.03, 12.34 fold, and semi-bound by 7.16, 8.99 fold and bound-B by 7.73, 9.3 fold by B1 and B2 treatments, respectively, relevant to the control treatment. There was no significant difference between bound-B under B1 and B2. The free-B was the highest among all forms of B and was more pronounced at B2 level (Fig. 3).

3.3. Subcellular cell wall (CW) and plasma membrane (PM) microstructure

The cell wall is a major site of B accumulation and toxicity. To examine the influence B stress-induced changes on the CW, transmission electron microscopy of leaves was carried out (Fig. 4). Both treatments of B-stress induced changes in the CW and plasma membrane, however, significant alterations were more prominent under B2 treatment when compared with control plants. Micrographs showed that microstructure of plasma membrane of the epidermis cell was remarkably convoluted and these negative effects were exaggerated with the increase of B supply. Moreover, B stress accumulated starch grains, plastoglobulus, and caused thickened cell wall (Fig. 4).

3.4. Activities of antioxidant enzymes, and MDA contents

The imbalance between reactive oxygen species and antioxidant enzymes may result in oxidative stress and subsequently induce lipid peroxidation of membranes. In order to investigate the accumulation of reactive oxygen species, we carried out quantitative and qualitative measurements in the root tips. The results showed that B toxicity significantly influenced ROS concentration in the roots and was much higher in B2 treatment compared with normal B treated seedlings. B stress increased the peroxidase (POD) activity. In contrast, catalase (CAT) activity was slightly increased by B1 and then significantly reduced by B2 (Fig. 5).

The MDA (malondialdehyde) is the end product of lipid peroxidation and is a good indicator of membrane peroxidation. Results showed that B stress significantly increased MDA contents in roots and were more pronounced by B2. MDA contents were increased by 60.12% and 96.83% by B1 and B2 treatments, respectively compared with the control treatment (Fig. 5).

3.5. Activities of monoamine oxidase, xanthine oxidase and T-AOC

The activities of monoamine oxidase (MAO) and xanthine oxidase (XOD) were significantly affected with B toxicity (Fig. 5), maximum activities were observed in B2 treatment. The B toxicity triggered the activities of MAO and XOD; a remarkable high concentration was recorded in the treatment of B2 (Fig. 5). B toxicity increased MAO by 20.62%, 42.23%, and XOD by 34.79%, 21.62% whereas reduced the total antioxidant enzyme capability (T-AOC) by 16.04%, 31.58% under B1 and B2 treatments when compared with control treatment (Fig. 5).

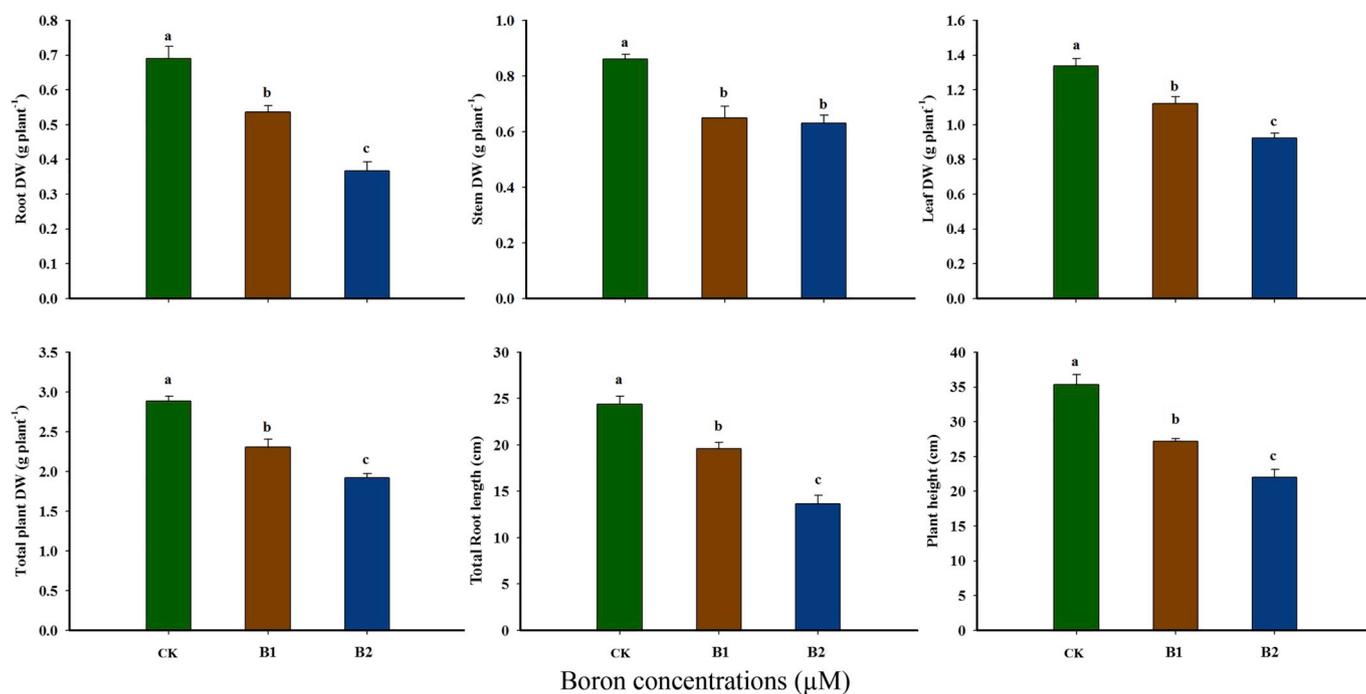


Fig. 1. Effect of B toxicity on plant growth parameters of trifoliate orange. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. Values are means of three replicates ± S.E. Lowercase letters (a, b, c) above the bars indicate a significant difference between treatments by LSD test at $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Plasma membrane integrity by Evans blue uptake

Plasma membrane integrity of roots was observed by Evans blue. The treatment of B toxicity increased uptake of Evans blue dye in root segments; starting from the root tips and extending back to mature zone with diffuse staining in the apical meristem zone of the elongation zone (Fig. 6 and Supplementary Fig. 2). The uptake of Evans blue showed that B stress had a serious negative effect on membrane integrity by inducing cell death.

3.7. FTIR analysis of roots

The FTIR analysis were performed to investigate the effect of B stress on the functional groups of the cell wall (CW) structure. The data of main absorption vibrations bands showed that excess B neither created any extra wavelength nor disappeared, only changed the

magnitude of peaks, indicating that B stress can affect CW properties by affecting main structural components of the CW and accumulation of certain compounds in the CW (Fig. 7). The excess B had main effects on pectin (3417, 2925, and 1735 cm^{-1}), carbohydrates (3417, 1157, and 1024 cm^{-1}) and proteins (1635, 1543, and 1247 cm^{-1}) (Fig. 7) by increasing their peak values either accumulation of these compounds or damaging their structure (Abidi et al., 2014; Tatulian, 2013; Liu et al., 2014). The assignments of major cm^{-1} functional groups corresponding to their peak values are presented in Supplementary Table 1.

3.8. Principal component analysis

The principal component analysis (PCA) was carried out to investigate the main factors contributing to B toxicity in trifoliate orange. The principal component analysis showed that PC1 had 78.56% scores and PC2 19.50% scores indicating a clear separation between

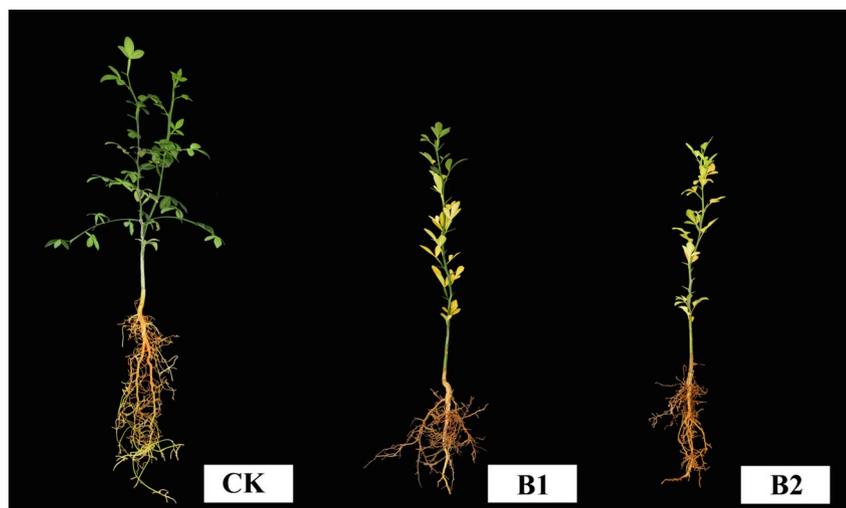


Fig. 2. Plant growth and visible symptoms of toxicity under B toxicity. The picture was collected at the end of the experiment. The seedlings were harvested after completing 18 weeks in the nutrient solution until plants had visible symptoms of B toxicity on leaves and roots. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B.

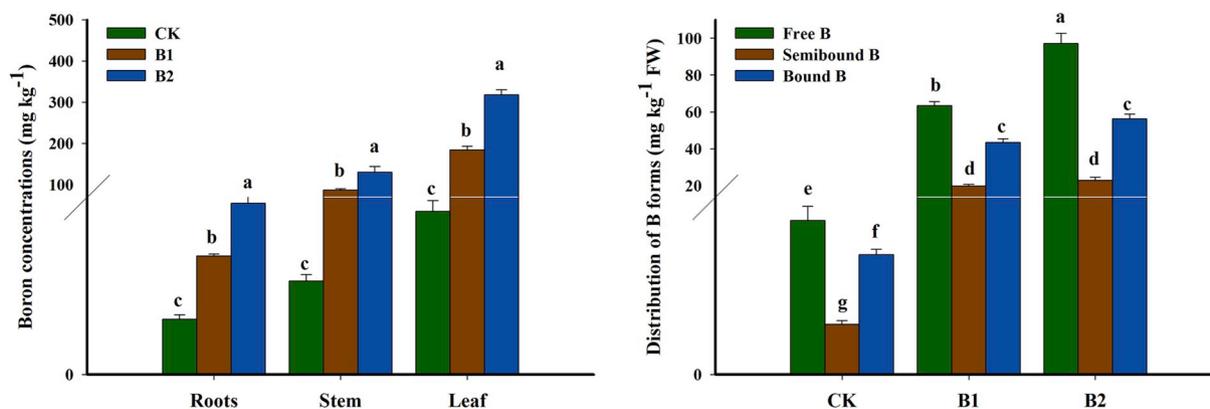


Fig. 3. Distribution of B concentrations and different forms of B in various parts of plants. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. Values are means of three replicates ± S.E. Lowercase letters above the bars indicate the significant difference between values by LSD test at $p \leq 0.05$.

treatments and components (Fig. 8). The biplot overlapping scoring plot and loading plot showed that in PC1, the contributions of MDA, free-B, POD and MAO were highest, indicating these factors greatly measure the total root length, total DW and T.AOC of the plant.

In order to evaluate a graphical correlation, we plotted the correlation matrix. The blue colour indicates perfect positive correlation and red colour perfect negative correlation. The size and intensity of colour are proportional to the correlation coefficient. The correlation plot indicates that plant growth parameters including total DW, T.AOC and total root length had negative correlated with free-B, MDA, MAO and XOD and positive correlation with T.AOC and total root length (Fig. 8).

4. Discussion

Boron (B) is indispensable micronutrient for normal growth and development of higher plants. Boron toxicity is one of the major limiting factors for the growth of plants due to imbalance application of chemical fertilizers, and trifoliolate orange rootstock (important rootstock in China) is sensitive to both B deficiency and toxicity (Riaz et al., 2018; Reid et al., 2004). However, mechanisms of B toxicity-induced inhibition of plant growth are not well elucidated especially in the citrus plantations (Kayihan et al., 2016). Therefore, in the current study, the mechanisms underlying B stress-induced inhibition of root and plant growth parameters, leaf chlorosis and alteration in the chemical composition of the cell wall, were explored to find out the association of different forms of B with the inhibition of growth as well as antioxidant response to B toxicity. Consistent with previous studies (Wang et al., 2016), B toxicity in the present study remarkably hampered total root growth and accumulation of total plant dry biomass, and these effects were visible on the root growth characteristics and leaves in the previous studies (Shah et al., 2017; Wu et al., 2018). Roots are the main

parts of the plant that actively absorb nutrients from the soil, and the response of plants to B toxicity is not same between sensitive and tolerant plants. The deeper analysis of root by Princi et al. (2016) study showed that B tolerant variety of tomato increased the root length ratio (RLR, root length/whole plant dry weight) that indicates how much of plant biomass is used to develop roots under stress condition. In the present study, B toxicity (200 and 400 μM) resulted in oxidative stress that could be associated with imbalance antioxidant enzyme system (Fig. 4).

One of the most critical symptoms of B toxicity is on leaves with necrotic spots along the leaf margins, and these signs of B toxicity have been extensively studied as a typical disorder of B toxicity (Sutton et al., 2007). It has been proposed that B toxicity symptoms on old leaves are directly associated with B contents in leaves, therefore, leaf B content has been comprehensively investigated than shoot B, however, B contents in different plant species vary greatly in various parts as well as growth stages (Reid, 2009). The results showed that increase of B supply in the nutrient solution increased the uptake of B from root to leaf, and a significant amount B was accumulated in the leaves indicating B mobility in citrus as also validated by the translocation factor (Supplementary Fig. 1). B exists as boric acid in solution and has pKa of 9.2, therefore, it is present in an uncharged form at this pH (Dordas et al., 2000). The high accumulation of B in leaves rises a question about B forms that could be the possible cause of visible symptoms of B toxicity on leaves. The study results showed that free-B was most dominant that might be associated with B stress symptoms. Interesting, bound-B was similar at both B levels, this could be due to limited binding sites on cell wall by rhamnogalacturonan RG-II. With the increase of B concentrations in the nutrient solution, the cell wall could not adsorb extra B to detoxify excess B (Dannel et al., 1998). Only a small amount of bound-B was increased under B2, indicating apiose or

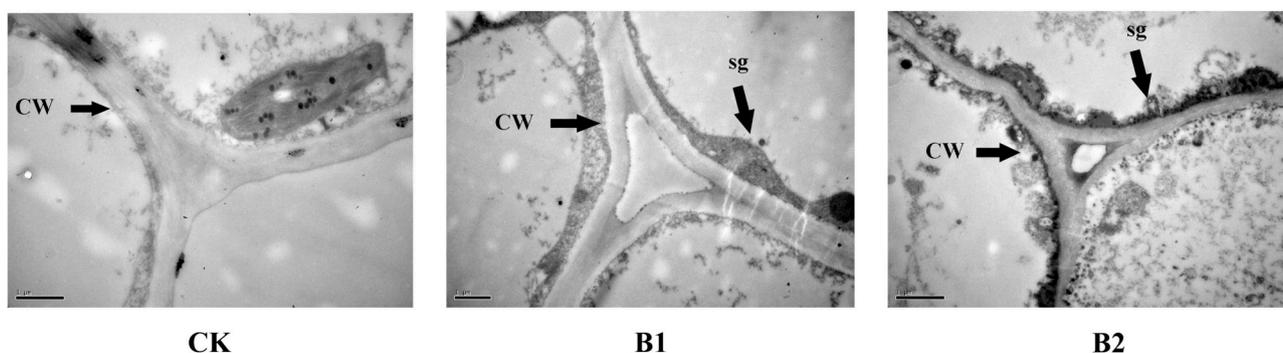


Fig. 4. Electron microscopy of leaf microstructure where CW; cell wall, PM; plasma membrane, sg; starch. The scale; 1 μM. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B.

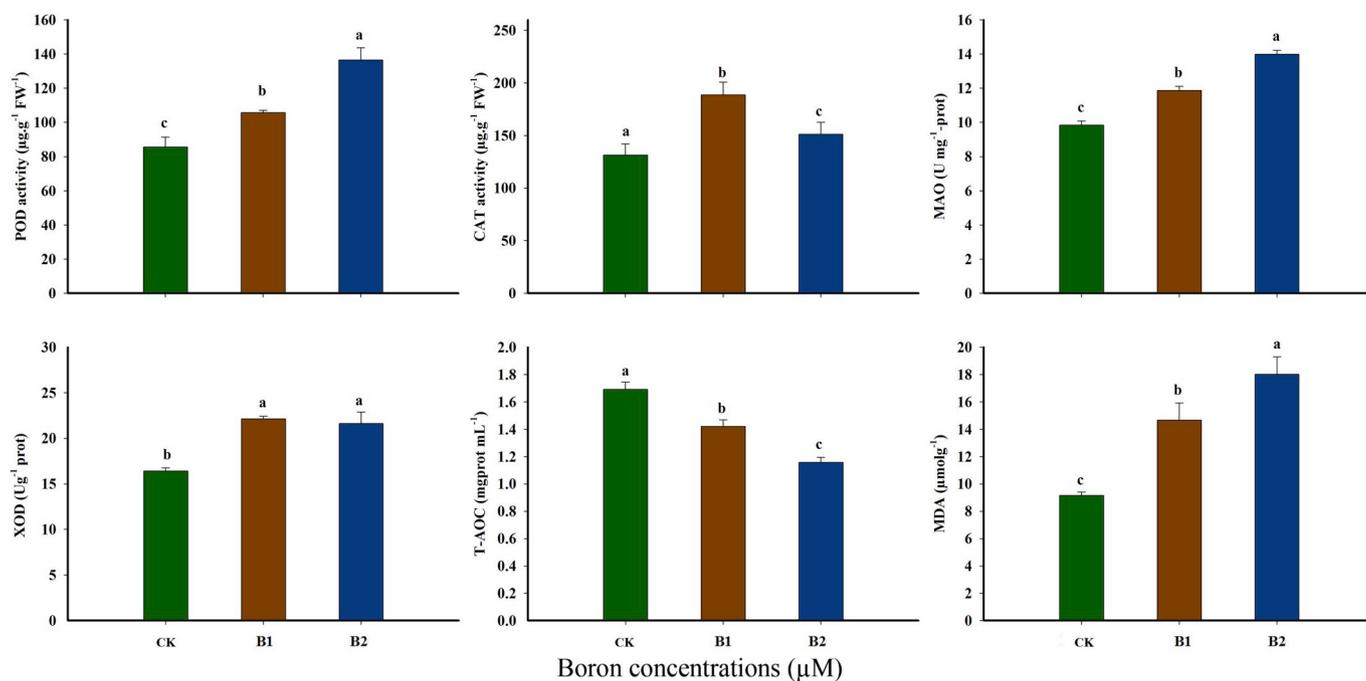


Fig. 5. Enzyme activities, MDA contents, MAO and XOD activities under different B levels. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. Values are means of three replicates ± S.E. Lowercase letters above the bars indicate the significant difference between values by LSD test at $p \leq 0.05$.

ribose might have provided extra sites for this slight increase in the bound-B either by free sugars or component of RNA, ATP or NADHP. Therefore, extra B binding on the cell wall may further disrupt the cell development processes (Reid, 2007). B is usually present in the cell in three main forms; free-B, semi-bound B and bound-B. Semi-bound B is mainly involved in the synthesis of the cell wall in the cytoplasm and bound B in cell wall synthesis by binding rhamnogalacturan II (Hu and Brown, 1994). The free-B was likely associated with the leaf chlorosis (Wang et al., 2014), and the reduced amount of free-B could be associated with the tolerance of plants to B toxicity (Landi et al., 2015). In addition, in leaves, high B accumulation at the end of transpiration could also induce osmotic imbalance and may induce inhibition of specific nutrients uptake and their distribution (Reid et al., 2004; Rivero et al., 2018). B is mainly involved in the formation of cell wall structure by cross-linking pectic polysaccharides rhamnogalacturan RG-II together by diester bond. This bond formation gives stable and

mechanical structure to the cell wall (O'Neill et al., 2004). Previous study from our group (Wu et al., 2017) involving the B deficiency-induced inhibition of trifoliate orange growth showed that B deprivation mainly targeted cell wall and induced changes in the cell wall structure and architecture, and resulted in thickened cell wall indicating the failure of cross-linking of RG-II together by B. Therefore, alteration on distribution patterns and properties of pectin in the cell wall may destroy the integrity of cell wall in trifoliate orange roots. Herein, we found that cell wall has a limited site for B adsorption, and extra B accumulated as free B, that might block the usage of sugar, starch and other molecules by the cell. This free B could be the main reason of B toxicity for leaf chlorosis, while in B deficiency, the leaf chlorosis was not prominent. Therefore, changes in the cell wall structure may result in the abnormal growth of plants by affecting cell wall structure, cell membranes and uptake of essential nutrients across the membranes. The reason for alterations in the cell wall and lipid peroxidation may

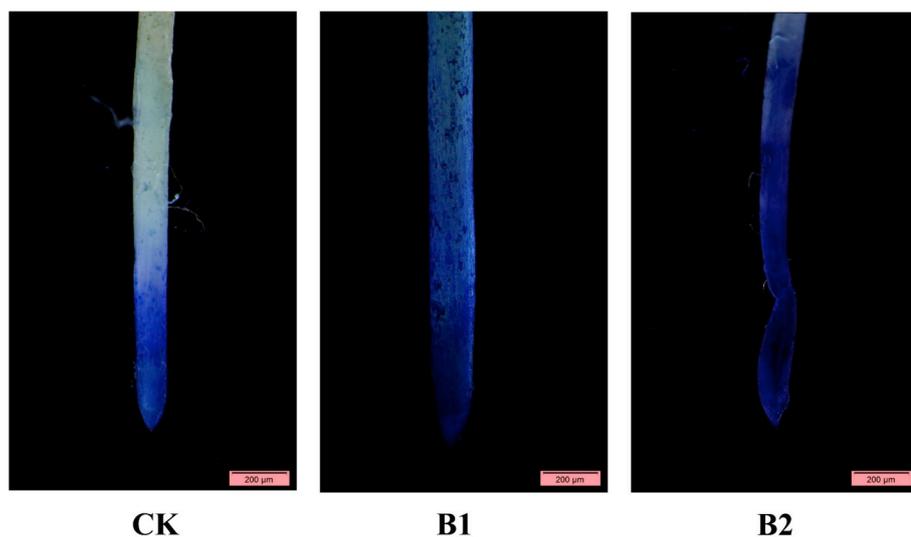


Fig. 6. Histochemical analysis of root tip integrity by Evans blue staining. The scale; 200 μm. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

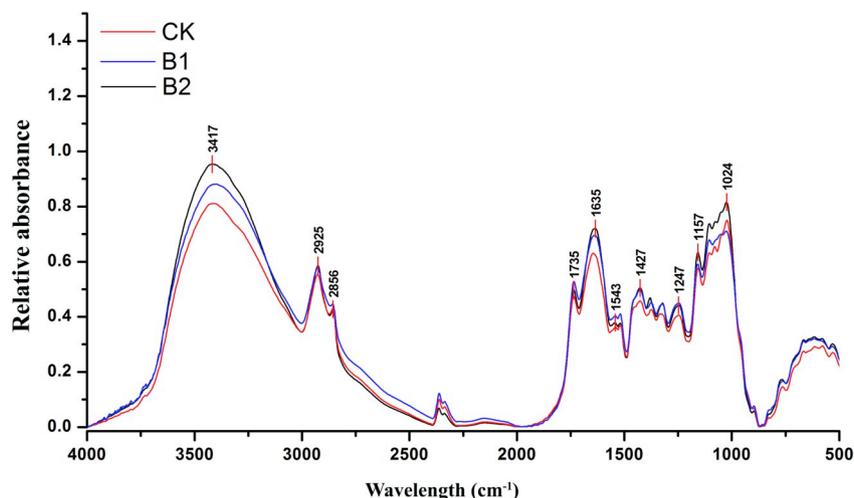


Fig. 7. FTIR analysis of root cell wall functional groups. Values are means of three replicates. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B.

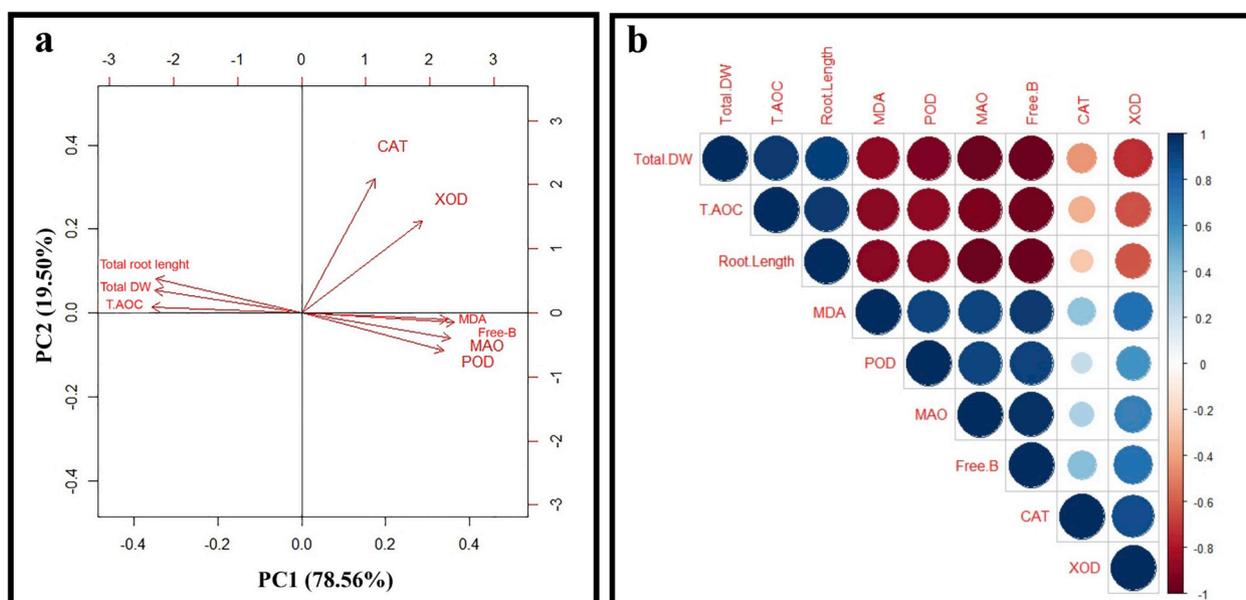


Fig. 8. Principal component analysis of major plant growth factors. a) biplot overlapping scoring plot and loading plot, b) correlation matrix. The plants were exposed to three B levels under hydroponics for 18 weeks; CK (10 μM as optimal), B2 and B3 as 200 μM and 400 μM B representing excess B. Plot of the first two PC scores (PC1 and PC2 loading) were obtained by exploratory PCA of individual spectra. The PCA separated the CK and excess B treatments on PC1 and PC2 accounting for 78.56% and 19.50% respectively of the total variation. PCA also clearly isolated essential factors on the PC1 and PC2.

ultimately interrupt the transport of ions by changing the plasma membrane characteristics. Metal stress may exert negative effects on the cell wall by disintegrating the mechanical and structural properties (Kochian et al., 2004) resulting in the decrease of cell wall extensibility and increase of rigidity. Another study of Morita & Yokota (2002) suggested that in cultured tobacco cells, B toxicity increased the suberin and lignin which might induce cell wall stiffness.

The plant defensive system plays a vital part against stress-induced oxidative damages resulting from over-accumulation of reactive oxygen species (ROS) (Hussain et al., 2016). In a normal plant system, ROS are produced during plant metabolism and there is always a balance between ROS and ROS-scavenging antioxidant enzymes (Eraslan et al., 2008). However, due to stress of metal toxicity including B, ROS accumulates and may induce oxidative stress if they are not scavenged by ROS scavengers even can cause cell death (Mittler, 2002; Molassiotis et al., 2006). Root cell death due to B toxicity induces root injuries which could be directly related to the cessation of root growth

(Sakamoto et al., 2011; Riaz et al., 2018). The results exhibited that B toxicity has caused severe accumulations of ROS that are a potent cause of oxidative damages, root injuries and rupture of plasma membrane as shown by the higher uptake of Evan blue (Supplementary Fig. 2) (Kinraide et al., 1998; Ardic et al. 2009). The plasma membrane (PM) is present at the margin of the root cell, enriched with phospholipids and phosphates (Kinraide et al., 1998). In order to maintain normal functions, superoxidase dismutase (SOD) catalyzes the dismutation of superoxide to H_2O_2 and O_2 , that is further regulated by catalase (CAT) and peroxidase (POD) enzymes. The results indicate that B induces changes in the POD and CAT antioxidant enzymes that are predominantly involved in the stress response against B toxicity. CAT is one of the important antioxidant enzymes in the plant defense system, and B toxicity significantly reduced its activity (Han et al., 2008), similar findings were also observed by Keles et al. (2004) under high B stress in citrus. There was a small increment in POD activity to eliminate the oxidative stress but their concentration was not enough to

completely detoxify free radicals (Dube et al., 2000). Moreover, MDA is an indicator of membrane peroxidation under stress conditions. The significant increase of MDA contents caused by B toxicity represents peroxidation of membranes, a modification of membrane fluidity and membrane leakage. The MAO being a flavoprotein is present at the outer membrane of mitochondria and modulates the oxidation of deamination of aromatic compounds (amines), generates H_2O_2 thus contributes to the additional buildup of ROS (Cadenas and Davies, 2000). Similarly, XOD also produces H_2O_2 and $O_2^{\cdot-}$, and their high concentration may cause cell death as well as membrane damages (Hussain et al., 2016). The high level of B significantly decreased the total antioxidant enzyme capabilities. By contrast, the activities of ROS producing intermediates (MAO and XOD) were significantly enhanced with the increase of B levels. B toxicity induced imbalance of antioxidant enzymes due to over generation of ROS, and for the survival of plants against extreme conditions, these antioxidant enzymes act synergistically to provide better protection. PCA analysis and correlation plot validated that most of important plant growth factors had a significant correlation with the antioxidant system (Fig. 7).

The cell wall is the major binding sites of B, and 30–90% of cellular B is associated with the cell wall while the failure of RG-II, leads to the number of changes in the cell wall properties and inhibition of root growth (O'Neill et al., 2004). It could be said that in the cell wall, B toxicity enforces adverse effects on root system likely by decreasing uptake of essential nutrient by roots, and influencing cell wall extensibility. The accumulation of carbohydrates (sugars) indicates limited usage by plant metabolism. Carbohydrates are essential for normal growth of plants (Han et al., 2008). Additionally, other metabolic pathways also utilize sugars to reduce the stress and oxidative damages caused by B toxicity (Yokota and Konishi, 1990; Keles et al., 2004) and

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.05.021>.

Contributions

M.R and C.J designed and supervised this study; M. R conducted the experiments, performed data interpretation, and drafted the manuscript; X, W helped in determining cell wall components; LY, and A.O helped in replacing nutrition solution in the experiment and determining B and Al concentration; S.H and Z.D helped to revise the manuscript grammatically. All authors read and approved the final manuscript.

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Supplementary Table 1

Assignments of major cell wall components corresponding to their peak values under different B treatment in trifoliolate root cell wall

Absorption	Assignment	Comments
3417	-OH, -NH stretching	Protein, carbohydrate (cellulose, hemicellulose)
2935, 2856	C-H stretching	Protein, cellulose, pectin
1735	C = O stretching from -COOR	Pectin
1635	-C = O stretching from -CO-NH;	Amide I
1543	N-H deformation and C-N stretching	Amide II
1427	-COO- stretching	Pectin
1157	Interaction between C-N stretching and C-N-H in-plane bending of protein	Amide III
1024	C-C, C-O stretching or - CH bending	Carbohydrate chain from cellulose

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work as a precursor of ascorbate metabolism in plants (Cervilla et al., 2007). In the present study, high accumulation of carbohydrates might be associated with inhibition of root growth as also demonstrated by FTIR analysis (Fig. 6). The B toxicity increased the peak intensities (3417, 2925, and 1735 cm^{-1}) related to the pectin structure and also affected the peaks (1635, 1543, and 1247 cm^{-1}) corresponding to amide, amide II, and amide III of proteins, respectively indicating destruction of a number of protein structures by B-toxicity. Additionally, the higher peak intensities of polysaccharides (3417, 1157, and 1024 cm^{-1}) indicating that B toxicity had changed the cellulose and carbohydrate contents as well as the structure.

5. Conclusions

The study results indicate that excess B resulted in a decrease of root growth and plant growth parameters with the visible symptom of chlorosis on leaves. The increase of B contents in leaves indicate that this element was absorbed and transported from root to shoots representing mobility of B in citrus. The high amount of free-B in leaves could be a major cause of leaf chlorosis as bound-B was not enhanced by increasing B levels, might be due to limited binding sites; once saturated no further binding occurs. The excess B caused significant changes in the structure of the root cell wall as well as leaf microstructure. The imbalance of antioxidant enzyme activities could not completely detoxify the reactive oxygen species and ultimately resulted in the cell death and membrane peroxidation that may be one of the causes of root growth inhibition. The combination of these results provides compelling evidence that may be helpful in understating the response of trifoliolate to B toxicity.

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