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Research article

Boron alleviates the aluminum toxicity in trifoliate orange by regulating antioxidant defense system and reducing root cell injury

Muhammad Riaz [a], Lei Yan [a], Xiuwen Wu [a], Saddam Hussain [b], Omar Aziz [a], Yuhan Wang [a], Muhammad Imran [a], 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

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ABSTRACT

Aluminium (Al) toxicity is the most important soil constraint for plant growth and development in acid soils (pH < 5.5) globally in agricultural regions. Boron (B) is an essential micronutrient for the growth and development of higher plants. The results of previous studies propose that B might ameliorate Al toxicity; however, none of the studies have been conducted on trifoliate orange to study this effect. Thus, a study was carried out in hydroponics comprising of two different Al concentrations, 0 and 400 μM. For every concentration, two B treatments (0 and 10 μM as H_3BO_3) were applied to investigate the B-induced alleviation of Al toxicity and exploring the underneath mechanisms. The results revealed that Al toxicity under B deficiency severely hampered the root growth and physiology of plant, caused oxidative stress and membrane damage, leading to severe root injury and damage. However, application of B under Al toxicity improved the root elongation and photosynthesis, while reduced Al uptake and mobilization into plant parts. Moreover, B supply regulated the activities of antioxidant enzymes, proline, secondary metabolites (phenylalanine ammonia lyase and polyphenol oxidase) contents, and stabilized integrity of proteins. Our study results imply that B supply promoted root growth as well as defense system by reducing reactive oxygen species (ROS) and Al concentrations in plant parts thus B induced alleviation of Al toxicity; a fact that might be significant for higher productivity of agricultural plants grown in acidic conditions.

1. Introduction

It has been estimated that soils of 30% of the ice-free land area of the world are acid, where crop productivity is limited by a range of growth-limiting factors related to soil acidity (Horst et al., 1999). Aluminium (Al) toxicity is the most important soil constraint for plant growth and development in acid soils, and Al is the third most abundant element in the earth's crust. When presented as less hazardous alumino-silicates or Al oxides. Aluminum can easily be soluble under acidic conditions and may induce toxicity problems in plants (Poschenrieder et al., 2008). Because of its solubility in the acidic condition, Al^{3+} can easily be absorbed by numerous susceptible plants (George et al., 2012). It has long been reported by various studies that Al toxicity could result in inhibition of root growth and cell division (Lenoble et al., 1996b,a), cell death, imbalanced nutrient uptake and mobilization, and accumulation of reactive oxygen species (ROS) (Corrales et al., 2008). Al-induced inhibition of root growth is the first symptom and is apparent within minutes of its exposure, even at a negligible concentration. However, the exact mechanism of root growth inhibition has not been elucidated yet. The root growth process implies a complex system consisting of cell integrity, cell division, and expansion (Kochian, 1995; Pineros and Kochian, 2001; Barlow, 2002; Doncheva et al., 2005).

Boron (B) is an essential micronutrient (H_3BO_3, boron acid) for the development and growth of higher plants. The role of B has been reported to aid in the formation of primary cell walls by cross-linking pectic poly pectic polysaccharide RG-II (rhamnogalacturonan II). This cross-linked RG-II gives rise to a stable and complex cell wall with reduced pore spaces (O'neill et al., 2004; Corrales et al., 2008). B deficiency leads to deformities in plants; B deficiency and Al toxicity both induce inhibition of root growth (Shorrocks, 1997). Moreover, the $Al(OH)_3$ is structurally similar to $B(OH)_3$ (Kochian, 1995). Due to the structural similarity of the

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

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molecule and the sharing of a common effect of root growth inhibition, a strong interaction between B and Al is expected. Likewise, mitigation of Al toxicity in different plants by B has been well reported by many researchers (Blevins and Lukaszewski, 1998; Stass et al., 2007; Yu et al., 2009). These authors have advocated that B possibly restricted the Al binding sites, ultimately alleviating the consequences of Al toxicity. Various studies have shown the alleviation of Al by B in pea (Yu et al., 2009; Li et al., 2017), apple (Wojcik, 2003), *Citrus grandis* (Jiang et al., 2009a), sunflower (Ruiz et al., 2006), wheat (Hossain et al., 2004), *Phaseolus vulgaris* (Stass et al., 2007), and *Linum usitatissimum* (Heidarabadi et al., 2011).

Intercellular mechanisms during normal cell metabolism produce ROS (reactive oxygen species) as a byproduct of aerobic metabolism, however, their quantities are varied (Kochian et al., 2004). The ROS are produced under stress condition and can induce oxidative stress, and ultimately result in the death of cells if ROS are not properly scavenged by ROS scavenging system (both enzymatic and non-enzymatic antioxidants). The damage caused by ROS is often indicated by lipid peroxidation. The antioxidant enzymes being a first line defense system work as ROS scavengers (Yamamoto et al., 2003; Apel and Hirt, 2004; Meriga et al., 2010; Hussain et al., 2016).

Phenolic metabolites contribute to a greater part of plant organic matter; hence, they are supposed to be triggered in response to biotic and abiotic stresses, including Al (Barcelo and Poschenrieder, 2002). The PAL (phenylalanine ammonia lyase) is a key enzyme in the plant defense system that transforms L-phenylalanine into *trans*-cinnamic acid. As a primary intermediary in the biosynthesis of phenolics, the PAL enzyme is an indicator of environmental stress (Levine et al., 1994). Under Al stress conditions, an increase of the PAL activity has been reported (Heidarabadi et al., 2011; Hajiboland et al., 2015). The accumulation of PAL and other related antioxidant enzymes are first defense systems in response to a stress condition (Cakmak and Römheld, 1997).

Citrus belongs to evergreen subtropical fruit trees cultivated in humid and subhumid tropical, subtropical and temperate regions of the world mainly on acidic soils. In China, citrus is an important fruit tree, and high Al and low B are common in citrus plantations (Han et al., 2008). The high rainfall washes out alkaline and alkaline earth elements from root zones, creating acidic conditions and thus causing severe B deficiency and Al toxicity problems (Jiang et al., 2009b; Zhou et al., 2015). Liming is the traditional method of alleviating Al toxicity in acid soils to inactivate toxic Al ions by increasing the soil pH (Cassel, 1980). However, liming may also induce adverse effects on plant growth by decreasing the availability of essential elements (Pavan et al., 1983) and is not always economically feasible. An alternative approach for increasing agricultural productivity in acid soils is to select Al-tolerant crops (Foy, 1992) but this practice has not been widely used because not enough highly Al-tolerant crops have been developed. In some reports, it was suggested that B may alleviate the toxic effects of Al on plant growth and improve the plant performance in acid soils (Lenoble et al., 1996a,b; Yang and Zhang, 1998). Till date, none of the studies, however, have been investigated the ameliorative role of B against Al toxicity on trifoliate orange. Therefore, the present study was conducted on citrus (trifoliate) to investigate the role of B on root and plant growth, and activation of antioxidative defense system under Al toxicity.

2. Materials and methods

2.1. Plant material, growth conditions, and treatments

The present study was carried out at Huazhong Agriculture University (HZAU), Wuhan, China. Six-month-old seedlings of '**trifoliate orange**' were bought from the Fruit Bureau of Ganzhou, Jiangxi Province, China. All the selected plants consisted of only one main shoot with 7–11 leaves, which were not fully expanded at the beginning of the experiment. All the plants were washed with distilled water to remove surface contaminants followed by a transplantation to 4 L black pots. The seedlings were then grown in a growth chamber with a light/dark regime of 14/10 h, 22/18 °C, and 70% relative humidity for 22 weeks. After transplanting, seedlings were supplied with modified Hoagland and Arnon nutrient solution containing two B (0 and 10 μM H_3BO_3) and two Al (0 and 400 μM $AlCl_3 \cdot 6H_2O$) levels at pH 4.0. For the convenience of discussion, the treatments were divided into 4 groups including B-Al- (0 μM B and 0 μM Al), B-Al+ (0 μM B and 400 μM Al), B+Al- (10 μM B and 0 μM Al as a control), and B+Al+ (10 μM B combined with 400 μM Al). The treatment comprising of sufficient B but without Al (B+Al-, 10 μM B and 0 μM Al) was considered as a control for comparison.

2.2. FDA-PI double staining, hematoxylin and morin staining

The cell viability of roots was observed by the double staining method as described by Jones and Senft (1985) using FDA (fluorescein diacetate) and PI (propidium iodide) dye. Briefly, trifoliate orange root apices were stained with FDA-PI staining working solution (50 μL FDA & 15 μL PI solution) at room temperature for 15 min in dark place. The cell viability intensity was observed (green for viable and red for dead cells) under a fluorescence microscope (OLYMPUS SZX16, X-Cite, series 120Q). Hematoxylin staining was carried out according to the method of Yu et al. (2009). The randomly collected roots from different treatments were stained with hematoxylin and observed under stereoscopy and photographed with a digital camera. The morin staining was conducted according to the method described by Zhu et al. (2013). The free-hand sections were prepared by sharp razor blades and were studied under a microscope equipped with fluorescence.

2.3. Antioxidant enzyme assay, and determination of MDA, H_2O_2 content

For the estimation of enzyme activities (after completing 22 weeks), new and young aerial leaves from each replication were collected and washed with deionized water. Finally, 0.5 g of fresh leaves were homogenized into liquid nitrogen with chilled pestle and mortar in phosphate buffer (pH 7.8). The resultant homogenate was subjected to centrifugation process at 12000×*g* for 15 min at 4 °C, and the supernatant was collected into another tube for the assay of enzyme activities. The peroxidase (POD) activity was assayed according to Cakmak and Horst (1991) method by employing 0.2% guaiacol solution, 50 mM phosphate buffer solution (pH 7.0), 30% H_2O_2 and 50 μL enzyme extract. The absorbance was recorded at 470 nm by spectrophotometer (Hitachi UV-3100 UV/VIS; TECHCOMP, Shanghai, China) for 3 min. The catalase (CAT) activity was observed as described by Aebi (1983). The total 3 mL reaction solution contained 0.3% H_2O_2, 2.55 mL of deionized water and 50 μL of enzyme extract. The decomposition of hydrogen peroxide was estimated at 240 nm by a spectrophotometer. The ascorbate peroxidase (APX) activity was quantified by Nakano and Asada (1981) method. The 3 mL of reaction solution contained 50 mM (pH 7.0) phosphate buffer solution, 0.25 mM ascorbic acid, 0.1 mM EDTA, 1 mM H_2O_2 and 200 μL enzyme extract. The decrease in absorbance was measured at 290 nm spectrophotometrically for 1 min and extinction coefficient 2.8 mM cm^{-1} was used in the final calculation.

For MDA (malondialdehyde) content, 0.5 g of fresh leaves