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Research article

Different metabolite profile and metabolic pathway with leaves and roots in response to boron deficiency at the initial stage of citrus rootstock growth

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ABSTRACT

Boron (B) is a microelement required for higher plants, and B deficiency has serious negative effect on metabolic processes. We concentrated on the changes in metabolite profiles of trifoliate orange leaves and roots as a consequence of B deficiency at the initial stage of growth by gas chromatography-mass spectrometry (GC-MS)-based metabolomics. Enlargement and browning of root tips were observed in B-deficient plants, while any obvious symptom was not recorded in the leaves after 30 days of B deprivation. The distinct patterns of alterations in metabolites observed in leaves and roots due to B deficiency suggest the presence of specific organ responses to B starvation. The accumulation of soluble sugars was occurred in leaves, which may be attributed to down-regulated pentose phosphate pathway (PPP) and amino acid biosynthesis under B deficiency, while the amount of most amino acids in roots was increased, indicating that the effects of B deficiency on amino acids metabolism in trifoliate orange may be a consequence of disruptions in root tissues and decreased protein biosynthesis. Several important products of shikimate pathway were also significantly affected by B deficiency, which may be related to abnormal growth of roots induced by B deficiency. Conclusively, our results revealed a global perspective of the discriminative metabolism responses appearing between B-deprived leaves and roots and provided new insight into the relationship between B deficiency symptom in roots and the altered amino acids profiling and shikimate pathway induced by B deficiency during seedling establishment.

1. Introduction

It is well known that boron (B) is a crucial micronutrient required for vascular plants growth and development (Loomis and Durst, 1992). Boron deficiency normally brings about sudden root growth suspension and twisting of leaves in plants mainly due to disordered cell wall structure (Dell and Huang, 1997). Studies show that B performs its function in plant cell walls by formation of diester bridges between rhamnogalacturonan-II molecules (Kobayashi et al., 1996; O'Neill et al., 2001). On the other hand, this participation does not appear to explain every one of the issues induced by boron deprivation and there are in reality some different aspects of B nutrition in plants that are further away from plant cell wall structure (Alves et al., 2011a). Till now, it has been documented that B is required in many physiological functions including cell wall formation and structure, membrane integrity and function, nitrogen metabolism, transport and distribution of carbohydrates, RNA metabolism and so on (Brown et al., 2002; Matas et al., 2009; Beato et al., 2010).

Boron functions in many plant metabolites have been extensively documented and its deficiency can cause changes in a wide range of metabolites such as carbohydrate, amino acids and phenolic compounds (Bolaños et al., 2004; Dordas and Brown, 2005; Beato et al., 2010). There is still much dispute about the roles of B in these metabolites and furthermore, the principle of B involved in numerous metabolic pathways is still not completely understood. Meanwhile, the changes that occur in the metabolome overall due to B deprivation are not adequately reported. The utilization of metabolomics innovations for a more detailed cellular metabolite examination offers a discriminating way to deal with

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the above doubts. In recent years, the metabolomics analysis has been generally applied to explore the changes in various distinctive metabolites caused by abiotic stress (Armengaud and Gibon, 2009; Takahashi et al., 2012; Foito et al., 2013; Sung et al., 2015). Alves et al. (2011a) investigated the effects of B deficiency on the changes in the metabolites in several organs of *Lupinus albus* plants and indicated B deficiency altered amino acid profiles by using metabolomics technique. Beato et al. (2011) reported that metabolomic profiling revealed that the levels of several organic acids, amino acids, and phenolics increased in tobacco roots due to 24 h of B starvation. Metabolomics procedure based on gas chromatography–mass spectrometry (GC-MS) can be utilized to comprehend the metabolic systems inside plants, which allows the unbiased, sensitive, rapid and synchronized examination of metabolites in a complex extract (Kueger et al., 2012). Wu et al. (2013) compared metabolic changes between cultivated and wild barleys in response to salt stress using GC-MS.

Citrus, in China, is ranked as a chiefly significant profitable fruit crops. Recently, boron deficiency is often observed in orchards of citrus and a major factor of extraordinary loss of profitability, quality and productivity (Han et al., 2008; Jiang et al., 2009). Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is considered an important species of rootstock resource, being extensively grown in China. In our laboratory using the GC-MS approach, it has been shown that there were differences of metabolite profiles between the leaves from different location (upper and lower leaves) in plants of navel orange under long-term B deficiency (Liu et al., 2015a). However, it is understood that the most rapid response to B deficiency is the cessation or inhibition of root (Wang et al., 2013). This prompted us to further explore the different effects of B deficiency on metabolite profiles between roots and shoots. In the present study, therefore, we focused on the impacts of B starvation on metabolite profiles in roots and leaves at the initial growth stage of trifoliate orange seedlings. The goal of this study was to understand the differences of B-deficiency-induced changes in most important metabolites and their metabolic pattern between leaves and roots by using GC- MS.

2. Material and methods

2.1. Plant material and treatments

The trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seeds, after being soaked in water at 50 °C for 2 h and thoroughly rinsed with distilled water, were transferred to glass petridishes with moistened gauze. For proper germination they were kept in an incubator at 28 °C. Both the glass petridish and gauze were treated under high temperature sterilization beforehand. Germinated seeds after that were transplanted to black plastic pots containing distilled water. After sprouting of 1–2 new leaves, some homogeneous size seedlings were chosen to plant in barrel (four seedlings per barrel), each containing 8-L of nutrient solution. The seedlings were fixed on a black foam board with holes. Prior to the experiment, both the black pots and foam boards were immersed in 1 mol L^{-1} HCl and washed with distilled water to remove contaminants. The composition and salt contents of the basal culture solution modified from Hoagland and Arnon (1950) was: 2 mM KNO_3, 1.23 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.14 mM Na_2HPO_4, 0.32 mM NaH_2PO_4, 4.45 μM $MnCl_2$, 0.8 μM $ZnSO_4$, 0.16 μM $CuSO_4$, 0.18 μM Na_2MoO_4, and 28.7 μM Fe-EDTA. One of eight nutrient solution was provided to growing plants, in the start, the solution concentration was 1/4, 1/2, then finally, in the 2nd week to full strength. Two B levels were applied to these plants: 5 μM H_3BO_3 (control treatment, CK) and 0 μM H_3BO_3 (B deficiency treatment, B0). Every day, the nutrient solutions pH was maintained to 5.8–6.2 by adding 0.5 M H_2SO_4 or 1 M NaOH, and the solution was adjusted for 20 min with 4 h intervals to ensure proper aeration. The solution was renewed once per week.

The experiment had two B treatments, were replicated four times in a completely randomized design (CRD) and each replication had two plants. The average value of the four replications was used in this study. This experiment was conducted in an illuminated greenhouse with a light/dark regime of 14/10 h, 28/22 °C, 60%–80% relative humidity and 300–320 μmol m^{-2} s^{-1} photosynthetic photon flux density at Huazhong Agricultural University, Wuhan, China. The duration of experiment was 30 days from April 3th, 2015 to May 3th, 2015.

2.2. Plant sampling

The entire seedlings were first rinsed with deionized water and afterward separated into their different parts (leaves, stems and roots) after harvest. The leaves and roots were then divided into two equal parts; one part was stored at −80 °C for later extraction of metabolites, and the other part was dried in the oven to a consistent weight at 70 °C and employed for B analysis. The dry samples were ground into fine powder and then ashed at 500 °C for 4 h. The ashes were dissolved and suspended in 0.1 mol L^{-1} HCl, and after that the solution was used to determine B concentration by employing the curcumin colorimetric method (HitachiUV-3100 UV/VIS; TECHCOMP, Shanghai, China) (Dible et al., 1954).

In addition, the microstructure of trifoliate orange seedlings root tips was studied by the method of tissue paraffin section using safranine-fast green as stains (Liu et al., 2015b). Root tips tissues were immediately fixed in formalin/glacial acetic acid (FAA). The samples were dehydrated in xylene and alcohol series (75, 85, 90 95 and 100%), and then and embedded in paraffin wax. Four-micrometer-thick sections were cut and stained with safranine (1%)-fast green (0.5%), for further histological examinations.

2.3. Metabolite analysis

Metabolites from the leaves and roots of trifoliate orange (100 mg of fresh weight) were extracted according to the method described by Lisec et al. (2006) with minor modifications. The frozen leaves and roots at −80 °C were placed into the 2 ml Eppendorf (EP) tubes and extracted with 400 μl of 100% methanol-chloroform (3:1, v/v)(pre-cooled at −20 °C). As an internal quantitative standard, twenty milliliter of Adonitol (0.2 mg/ml store in dH_2O) was then added and vortex mixed for 10 s, followed by homogenizing in ball mill for 3 min at 65 Hz. The tubes were centrifuged for 15 min by 12,000 rpm at 4 °C and the supernatant was transferred into a new 2 ml GC/MS glass vial. The extracts at 37 °C were then dried without heating for 2 h in a vacuum concentrator. After adding 80 μl Methoxylamine hydrochloride (dissolved in pyridine, final concentration of 20 mg/ml), the dried metabolites were incubated at 80 °C for 20 min in an oven. A hundred microliter N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (v/v) was then added into each sample and continued to do that at 70 °C for an hour. After cooling to the room temperature, ten microliter of a standard blend of fatty acid methyl esters (FAMEs) in chloroform (i.e. C8-C16: 1 mg/ml; C18-C24: 0.5 mg/ml) was added into the mixed sample. The samples after the above reactions were analyzed for the determination of metabolite contents by the gas chromatograph system (Agilent 7890) combined with mass spectrometer (Pegasus HT time-of-flight, GC-TOF-MS). This system had an Rxi-5Sil MS column with 30 m × 250 μm inner diameter and 0.25 μm film thickness (Restek, USA). The analyte with 1 μl aliquot was infused in split less mode. The Helium gas was used as the transporter, the front inlet had 3 ml