Physiological and Nutritional Responses of ‘HB’ Pummelo [*Citrus grandis* (L.) Osbeck ‘Hirado Buntan’] to the Combined Effects of Low pH Levels and Boron Deficiency

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Abstract. Soil acidification and boron (B) starvation are two dominant abiotic stress factors impacting citrus production in the red soil region of southern China. To evaluate the combined effects of low pH and B deficiency on plant growth, gas exchange parameters, and the concentrations of B and other mineral nutrients, ‘HB’ pummelo seedlings were treated under B deficiency (0 μM H_3BO_3) or adequate B (23 μM H_3BO_3) conditions at various low pH levels (4.0, 5.0, and 6.0). The seedlings were grown with modified half-strength Hoagland’s solution under greenhouse conditions for 12 weeks. Plant biomass, leaf area, seedling height, and root traits were remarkably inhibited by low pH and B deficiency stresses, and these parameters were extremely reduced with the decrease in pH levels. After 12 weeks of treatment, typical stress symptoms associated with B deficiency in citrus leaf were observed, with more severe symptoms observed at pH 4.0 and 5.0 than at pH 6.0. Leaf gas exchange parameter measurements showed that leaf photosynthesis was significantly inhibited under both low pH and B-deficient conditions. Notably, the lower the pH level, the greater the inhibition under both normal and deficient B conditions. Further investigations of the mineral nutrient concentrations showed that under both low pH and B deficiency, the concentrations of B and other mineral nutrients were influenced remarkably, particularly at pH 4.0 and 5.0. The physiological and nutritional results of the ‘HB’ pummelo seedlings indicated that low pH can exacerbate the effects of B deficiency to a certain extent.

Abiotic stresses, such as nutrient disorder and soil acidification, are dominant soil factors that affect plant performance. It is well known that B is an essential micronutrient for vascular plants (Marschner, 1995), and it has an important role in various plant metabolic pathways (Bolaños et al., 2004; Brown et al.,

2002). However, B starvation is frequently observed in plants and is one of the major production constraints in 132 crops in many parts of the world (Shorrocks, 1997). In general, B deficiency leads to the rapid cessation of root elongation, inhibition of leaf expansion, and reduced fertility (Marschner, 1995). In woody plants, including citrus, B-deficient trees usually exhibit two key visible symptoms: necrosis of growing points (root tip, bud, flower, and young leaf) and deformity of organs (root, shoot, leaf, and fruit) (Wang et al., 2015). Further studies have suggested that the effects of B deficiency on plant growth may be realized through the B functioning in the cell wall and membrane, and that B deficiency results in damage to vascular tissues and suppression of both B and water transport (Dell and Huang, 1997; Wang et al., 2015; Wu et al., 2018).

Soil acidification is an important cause of soil degradation that seriously affects the growth and development of plants. In an agricultural system, soil acidification mainly

results from the removal of plant materials, acid deposition, and nitrogen (N) cycling in the soil (Guo et al., 2010; Zhou et al., 2014). Soil acidification causes not only soil aluminum (Al) and manganese (Mn) toxicity but also phosphorus (P), molybdenum (Mo), calcium (Ca), and magnesium (Mg) deficiency in plants (Russell, 2006; Zhang et al., 2009). During the past 30 years, Chinese agriculture has intensified and chemical fertilizer application has increased. In particular, the wide application of N fertilizer has become a major cause of soil acidification in China. Four billion hectares ($\approx 30\%$ of ice-free soils) of soil globally are acidic (Sumner and Noble, 2003). Southeast and Pacific Asia and South America are affected worst, with more than 50% of the total land in these areas harboring low pH soils. Acidic soils are mostly associated with forests ($\approx 66\%$) and pastures ($\approx 11\%$), with a relatively small proportion (5%) supporting crops. Although *Citrus* can be cultivated in soils covering a wide range of pH and are tolerant to acidic soils (Zhang et al., 2018), soil acidification has become a serious problem for citrus cultivation in China. Li et al. (2015) reported that soil acidification was a major problem in ‘Guanximiyou’ pummelo (*Citrus grandis*) orchards in Pinghe county, which is in the southern region of Fujian province in China, with an average pH of 4.34. Approximately one-third of the total area of citrus orchards in Jiangxi province (one of the main citrus-producing regions in China) suffers from acidic soil (Chen et al., 2010).

Citrus is an important fruit crop produced throughout tropical and subtropical regions of the world. However, citrus production can be seriously affected by B deficiency (Chen et al., 2012; Sheng et al., 2008). Although citrus plants are not classified as the most sensitive species to B deficiency, the occurrence of B deficiency has been reported in the major citrus-producing countries of the world, such as Spain, the United States, Brazil, and China (Sheng et al., 2009; Shorrocks, 1997). East and south China are important citrus-growing regions, but the soil B level is low (hot water extraction B < 0.25 $\text{mg}\cdot\text{kg}^{-1}$) (Sheng et al., 2008). In these regions, corky split veins in the leaves are frequently visible in pummelo (*Citrus Grandis*). Based on 1400 soil samples from these regions, the soil pH is generally neutral to acidic (4.0–7.0), and the average pH is 4.5 (Liang et al., 2010). The pH of a soil solution is known to affect B adsorption (Goldberg et al., 2000) and B availability in plants (Marschner, 1995). Soil pH changes affect B speciation [i.e., $\text{B}(\text{OH})_3$ vs. $\text{B}(\text{OH})_4^-$] and soil adsorption processes, ultimately affecting B absorption by plants (Goldberg et al., 2000). As pH increases, the rate of B uptake is dramatically reduced, particularly when the pH exceeds 8 (Läuchli and Grattan, 2012; Marschner, 1995), at which point the speciation of B starts to progressively shift from boric acid $\text{B}(\text{OH})_3$ to anionic borate $\text{B}(\text{OH})_4^-$ (equal activities of the two species occur at pH 9.3). The effect of liming on reducing B

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Fig. 1. Symptoms in the leaf of 'HB' pummelo seedlings caused by low pH and boron (B) deficiency. All the plants were grown under sand culture conditions and treated for 12 weeks. (A) Control, normal B, pH 6.0. (B) Normal B, pH 5.0. (C) Normal B, pH 4.0. (D) B deficiency, pH 6.0. (E) B deficiency, pH 5.0. (F) B deficiency, pH 4.0.

availability in plants is well-known for agricultural crops, and it is at least partly caused by the increased absorption of B in the soil as the pH increases (Gupta et al., 1985). Therefore, it is important to clarify the mechanisms of B absorption and distribution in citrus under different acidic soil pH conditions. Our study aimed to determine the combined effects of low pH and B deficiency on the performance of 'HB' pummelo seedlings, including plant growth, gas exchange, and mineral nutrient accumulation.

Materials and Methods

Plant materials and treatment. 'HB' pummelo [*Citrus grandis* (L.) Osbeck 'Hirado Buntan'] seedlings were used in this experiment. The seed germination and seedling culture of 'HB' pummelo were conducted according to Zhou et al. (2014). Thirty days after planting, a total of 108 seedlings of uniform size were selected. According to published methods (Papadakis et al., 2004; Sheng et al., 2009), the seedlings were washed with deionized water to remove surface contaminants and then transplanted to 5-L black pots containing B-free medium composed of quartz sand:perlite (1:1, v/v). Experiments were performed in a growth chamber with a light/dark regime of 14/10 h, 28/22 °C, 75% relative humidity, and light intensity of 800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of PAR. The plants were precultured with half-strength Hoagland's No. 2 nutrient solution for 2 to 3 weeks until the appearance of the new leaf. Then, they were irrigated twice per week with a modified Hoagland's No. 2 nutrient solution with 0 μM H_3BO_3 [B deficiency treatment (BD)] or 23 μM H_3BO_3 [control plants (CK)], and the pH of these nutrient solutions was adjusted to 4.0 (BD4 and CK4), 5.0 (BD5 and CK5), and 6.0 (BD6 and CK6)

with 0.1 M HCl or 0.1 M KOH. The treatments started on 25 May 2017 and ended on 16 Aug. 2017, when the visible symptom (corky split vein) of B deficiency had appeared. The modified B-free full-strength Hoagland's No. 2 nutrient solution contained 6 mM KNO_3 , 4 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 2 mM MgSO_4 , 9 μM MnCl_2 , 0.8 μM ZnSO_4 , 0.3 μM CuSO_4 , 0.01 μM H_2MoO_4 , and 50 μM Fe-EDTA (Hoagland and Arnon, 1950).

Sampling and plant growth parameter measurements. At the end of the experiment, nine plants per treatment were randomly harvested, rinsed in deionized water, and blotted carefully with tissue paper. The materials were then divided into the leaf, stem, and root. Leaf area (cm^2) was determined using a leaf area meter (Li-3100C; LI-COR Biosciences Inc., Lincoln, NE). The fresh materials were placed in a forced air oven at 105 °C for 15 min, followed by 75 °C until constant weights were reached to determine their dry weights (mg) for the calculation of the shoot dry weight/root dry weight (S/R) ratio. Then, all the dried samples were ground into a fine powder for the determination of B and other mineral nutrient concentrations in the tissues. Seedling height (cm) and taproot length (cm) were measured using a scaled ruler.

Leaf gas exchange measurements. After 6 weeks of treatment, gas exchange parameters, including the photosynthetic rate (P_n ; $\mu\text{mol CO}_2\text{ m}^{-2}\cdot\text{s}^{-1}$), stomatal conductance (g_s ; $\text{mmol m}^{-2}\cdot\text{s}^{-1}$), intercellular CO_2 concentration (C_i ; $\mu\text{mol}\cdot\text{mol}^{-1}$), and transpiration rate (E ; $\text{mmol H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$), of all plants were measured using a Li-6400 device (LI-COR Biosciences Inc.) between 9:30 and 11:30 AM on a clear day. During all of these measurements, the leaf temperature and relative humidity were 28.9 ± 1.4 °C and

$71.6 \pm 2.8\%$, respectively. The controlled light intensity was $1000 \pm 8.7 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and the controlled CO_2 concentration was $400 \mu\text{mol}\cdot\text{mol}^{-1}$. Measurements were performed according to the method described by Sheng et al. (2009).

Root morphology analysis. Nine seedlings were randomly sampled in each treatment group and rinsed with deionized water. For the root morphology analysis, the root samples were scanned using an Epson digital scanner (Expression 10000XL 1.0; Epson Inc., Nagano, Japan), and the image was analyzed using WinRhizo Pro (S) v. 2009c (Regent Instruments Inc., Quebec, Canada) software. The determined traits included total root length, root surface area, root volume, and root number.

Determination of mineral nutrients. The mineral nutrient concentrations of P, potassium (K), Ca, Mg, iron (Fe), Mn, zinc (Zn), and B in the different plant tissues were determined following the method described by Storey and Treeby (2000). Briefly, 0.50 g of each sample was dry-ashed in a muffle furnace at 500 °C for 6 h, followed by dissolution in 0.1 N HCl. The mineral nutrients concentration was then determined using inductively coupled plasma mass spectrometry 7900 (ICP-MS7900; Agilent Technologies Inc., Santa Clara, CA).

Experimental design and statistical analysis. The experiment was set up in a completely randomized 3×2 factorial design with three nutrient solution pH values (pH = 4.0, 5.0, and 6.0) and two B treatments (0 and 23 μM of H_3BO_3). The values are presented as the means $\pm$ SE of nine seedlings (three plants in each pot, three repeats). The data were subjected to an analysis of variance using SAS (SAS 8.1; SAS Institute Inc., Cary, NC), and the differences were compared using Duncan's test with a significance level of $P < 0.05$.

Results

Visible symptoms and plant growth. After 12 weeks of low pH and B deficiency treatment, visible symptoms appeared on the leaves of the 'HB' pummelo seedlings under B deficiency at all pH levels, whereas no remarkable symptom was observed under normal B conditions (Fig. 1). The main visible symptom under B deficiency was vein swelling or cracking (corky split vein), which are typical symptoms of citrus suffering from B deficiency stress. This visible symptom was initially observed in the adult leaves, but not in the young leaves. However, as the treatment duration progressed, the vein swelling or cracking symptoms appeared in the young leaves, and the mature leaves started yellowing under B deficiency. In addition, more severe leaf symptoms were observed at pH 5.0 than at 6.0 (Fig. 1D and E). Interestingly, the leaf symptoms at pH 4.0 were not as severe as those at pH 5.0 or 6.0 (Fig. 1D–F).

Compared with the control plants (normal B and pH 6.0), B deficiency treatment

Table 1. Combined effects of low pH and boron (B) deficiency on plant growth, biomass, and the root/shoot (R/S) ratio of 'HB' pummelo seedlings.

Treatments	pH	Seedling ht (cm)	Leaf area (cm ² /plant)	Biomass (g/plant DW)	R/S ratio
Normal B (0.25 mg·L ⁻¹)	6.0	38.17 ± 3.09 A a	655.12 ± 79.53 A a	9.79 ± 1.22 A a	0.34 ± 0.02 C a
	5.0	32.73 ± 1.68 A ab	507.37 ± 67.30 A b	8.04 ± 0.62 B b	0.31 ± 0.23 C a
	4.0	28.73 ± 1.24 A b	416.76 ± 32.94 B c	7.44 ± 0.53 B b	0.28 ± 0.02 C b
Deficient B (0 mg·L ⁻¹)	6.0	16.23 ± 1.13 B a	203.60 ± 30.67 D a	5.25 ± 0.72 C a	0.41 ± 0.04 B b
	5.0	15.27 ± 0.54 B ab	171.50 ± 10.56 D a	4.48 ± 0.43 C b	0.44 ± 0.04 B b
	4.0	14.72 ± 0.79 B b	101.48 ± 12.23 E b	3.62 ± 0.19 D c	0.58 ± 0.04 A a
<i>F</i> values					
pH levels (P)		15.99***	13.34***	18.17***	1.77 ^{NS}
B treatment (B)		404.71***	186.62***	218.27***	41.67***
P × B		8.00**	2.48 ^{NS}	1.40 ^{NS}	6.61**

F values for the pH levels, B treatment, and their interactions are shown. Significance of analysis of variance: *P < 0.05; **P < 0.01; ***P < 0.001; NS = nonsignificant.

All plants were grown in sand culture conditions and treated for 12 weeks. Data are presented as mean of three replicates (n = 3); samples from three plants were collected for each replicate. Different small and capital letters behind the mean values indicate significant differences (*P* < 0.05) between the different pH levels and different B concentration treatments, respectively.

Fig. 2. The combined effects of low pH and boron (B) deficiency on the (A) photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), (B) stomatal conductance ($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), (C) intercellular CO_2 concentration ($\mu\text{mol} \cdot \text{mol}^{-1}$), and (D) transpiration rate ($\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) of 'HB' pummelo seedlings. All the plants were grown under sand culture conditions and treated for 12 weeks. Data are presented as means $\pm$ SE of nine replicates (n = 9; one plant for each replicate). Different small letters above the bars indicate significant differences (*P* < 0.05) between the different pH levels and different boron concentration treatments. DB = deficient B concentration; NB = normal boron concentration.

significantly inhibited the seedling height, leaf area, and biomass of the seedlings at all pH levels. Conversely, the R/S (root/shoot) ratios were significantly increased by B deficiency treatment (Table 1). Similarly, low pH also influenced the plant growth. Under both normal and deficient B conditions, the seedling height, leaf area, and biomass were more significantly inhibited at pH 4.0 and 5.0 than at pH 6.0. The R/S ratios were significantly decreased at pH 4.0 under normal B conditions, whereas no remarkable differences

were found at pH 5.0 in comparison with the control plants. However, the highest R/S ratio was found at pH 4.0 under B deficiency, and furthermore, no remarkable difference was detected between pH 5.0 and 6.0 (Table 1).

Leaf gas exchange parameters. As shown in Fig. 2, the leaf gas exchange parameters were greatly influenced by B deficiency at all pH levels. Under the normal B concentration, P_n , g_s , and E were higher at pH 6.0 than at pH 4.0 and 5.0. However, for C_i , the highest

value was at pH 4.0, and no significant difference was found between the other two pH values. Under B deficiency, P_n , g_s , and E were significantly decreased at all pH levels compared with the control at the same pH, whereas C_i was significantly increased (Table 2).

Root morphology traits. To further investigate the effects of low pH and B deficiency on seedling roots, the root morphological traits were examined. Under the normal B concentration, the primary root length, root volume, and root number at pH 4.0 and 5.0 were inhibited significantly compared with the control plants at pH 6.0; however, no remarkable difference was observed between pH 4.0 and 5.0. Root total length and root surface area decreased dramatically at pH 4.0, whereas no significant decline was found at pH 5.0 compared with pH 6.0. Under B deficiency conditions, except the primary root length, other root morphological traits such as root total length, root surface area, root volume, and root number of the seedlings were significantly decreased at all pH levels, whereas the root density was increased under the same conditions. Moreover, no significant differences were found in all root-related morphological traits of all pH levels (Fig. 3; Table 3).

Mineral nutrient concentration. For the main micronutrients (Fe, Mn, B, and Zn), the B concentration decreased significantly in all organs of the seedlings under B deficiency. The B concentration also decreased at pH 4.0 in the leaf and root under normal B conditions, but not under deficient B conditions (Fig. 4A–C). Fe concentration decreased significantly under B deficiency stress in all organs, except in the leaf and stem at pH 4.0. The leaf and stem Fe concentration also decreased under low pH levels, especially at pH 4.0 (Fig. 4D–F). The impact of B deficiency stress on Mn tissue concentration was only detected at pH 6.0 in the leaf and root. Furthermore, the Mn concentration in both the leaf and root was also decreased at pH 4.0 under normal B conditions, but only in the root under B deficiency (Fig. 4G–I). The concentration of Zn decreased significantly in all organs at pH 4.0 and 5.0 under both normal and B-deficient conditions, with the exception of the stem under B-deficient

Table 2. *F* values and levels of significance for pH levels, boron (B) deficiency treatment, and their interactions according to the analysis of variance (ANOVA) on leaf gas exchange parameters.

Variance source	df	Leaf gas exchange parameters			
		P_n	g_s	C_i	<i>E</i>
pH levels (P)	2	69.68***	30.82***	56.59***	47.01***
B treatments (B)	1	703.58***	145.41***	247.31***	409.66***
P × B	2	6.35**	2.26 ^{NS}	7.73**	0.29 ^{NS}

Significance of ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS = nonsignificant.

P_n = photosynthetic rate; g_s = stomatal conductance.

Fig. 3. The combined effects of low pH and boron (B) deficiency on the root morphology of the 'HB' pummelo seedlings. All plants were grown under sand culture conditions and treated for 12 weeks. Data are presented as means $\pm$ SE of nine replicates ($n = 9$; one plant for each replicate). Different small letters above the bars indicate significant differences ($P < 0.05$) between the different pH levels and different B concentration treatments. DB = deficient B concentration; NB = normal boron concentration.

conditions. The leaf and stem Zn concentrations under normal B conditions were remarkably higher than those under B-deficient conditions (Fig. 4J–L).

The concentrations of mineral macronutrients (P, K, Ca, and Mg) in the leaves, stems, and roots of the seedlings are indicated in Fig. 5. The P concentrations decreased

significantly in all organs at all pH levels under B-deficient conditions. The P concentrations also decreased significantly in the leaf, but not in the stem or the root, at pH 4.0 and 5.0 under normal B conditions in comparison with the control plants. However, the low pH levels did not influence all organs under B-deficient conditions. The concentrations of K decreased with the decrease in pH in the root, whereas no significant difference was detected in the stem under both normal and deficient B conditions. There was no remarkable change in leaf K concentration among the different pH levels under B-deficient conditions, whereas a significant decrease was observed at pH 4.0 under normal conditions. However, the Ca concentrations did not change dramatically under the different pH levels, except in the root at pH 4.0. Additionally, the Ca concentration decreased significantly under B deficiency at pH 6.0 in the leaf, at pH 5.0 and 6.0 in the root, and at all pH levels in the stem. The Mg concentrations also decreased under B deficiency in the leaf at all pH levels and in the root at pH 5.0 and 6.0. Low pH levels also decreased the Mg concentrations in the leaf and root under normal B conditions, but not under deficient B conditions.

Discussion

Physiological responses of citrus to low pH and B deficiency stress. Both B and pH are dominant soil factors that affect plant growth and development, and plant performance is significantly inhibited under low pH and/or B-deficient soil conditions. Therefore, it is important to understand the interaction of low pH and B deficiency and how plants respond to these abiotic stresses in field environments (Läuchli and Grattan, 2012; Smith et al., 2013).

In this study, the interactions of low pH and B deficiency were assessed to investigate their effects on 'HB' pummelo seedlings. At the end of our experiment, corky split veins were observed under B-deficient conditions (Fig. 1). Corky split vein is a typical symptom of citrus that have been proved in various citrus while suffering from B starvation stress (Han et al., 2008; Liu et al., 2011b; Yang et al., 2013, 2015; Zhou et al., 2014, 2015). Other symptoms were also observed under B deficiency at different pH values, with more severe symptoms observed at pH 4.0 and pH 5.0 than at pH 6.0 (Fig. 1). We also found that these symptoms appeared earlier at pH 5.0 than at pH 4.0 and pH 6.0 (data not shown). These results suggest that citrus plants could

Table 3. *F* values and levels of significance for pH levels, boron (B) deficiency treatment, and their interactions according to the analysis of variance (ANOVA) on root morphological traits.

Variance source	df	Root morphological traits					
		Primary root length	Total root length	Root surface area	Root volume	Root number	Root density
pH levels (P)	2	4.65*	14.49***	4.92*	22.71***	3.99*	0.72 ^{NS}
B treatments (B)	1	0.62 ^{NS}	251.72***	134.93***	185.85***	56.02***	54.58***
P × B	2	0.523 ^{NS}	7.55**	3.50*	14.34***	2.51 ^{NS}	1.41 ^{NS}

Significance of ANOVA: *P < 0.05; **P < 0.01; ***P < 0.001; ^{NS} = nonsignificant.

Fig. 4. The combined effects of low pH and boron (B) deficiency on the micronutrients of 'HB' pummelo seedlings. All plants were grown under sand culture conditions and treated for 12 weeks. Data are presented as means $\pm$ SE of nine replicates (*n* = 9; one plant for each replicate). Different small and capital letters above the vertical bars indicate significant differences (*P* < 0.05) between the different pH levels and different B concentration treatments, respectively. DB = deficient B concentration; NB = normal boron concentration.

be more sensitive to B deficiency stress at low soil pH, but not at severe low soil pH. Several field surveys have typically observed the corky split vein symptoms of B deficiency in 'Gannan' navel orange production regions of China, where the soil suffers from both acidification and B starvation (Chen et al., 2010; Liang et al., 2010; Sheng et al., 2009). Interestingly, our results showed that there was no significant difference in the B concentration among all pH values in all organs under B deficiency conditions (Fig. 4).

The effects of low pH and B deficiency stress on the performance of seedlings are typically assessed by changes in biomass. In our work, plant biomass decreased significantly under B-deficient conditions, especially at pH 4.0 and 5.0. Moreover, plant biomass decreased with decreasing pH under normal B conditions (Table 1). As an essential micronutrient for vascular plants,

B has an important role in many metabolic pathways, and B deficiency will inhibit leaf expansion and root elongation by limiting cell enlargement and cell division (Dell and Huang, 1997; Marschner, 1995). Several previous studies have demonstrated that citrus plant biomass is also significantly inhibited by B deficiency (Han et al., 2012; Liu et al., 2011a; Mei et al., 2016; Zhou et al., 2014), and the results of this work corroborate those reported in the literature. The photosynthesis determination results showed that P_n , C_i , g_s , and E were inhibited under low pH and B deficiency conditions (Fig. 2). These results suggested that the reduced biomass of the seedlings could have been caused by the increased photosynthetic efficiency, which corroborates similar research of other citrus fruits (Han et al., 2008, 2009; Long et al., 2017; Lu et al., 2014).

The underground parts of the plant are also influenced by low pH and/or B deficiency. Because the roots are the primary organs that penetrate the soil, they are directly affected by soil pH and nutrient starvation stress. To further investigate the effects of low pH and B deficiency on the root growth and development of the 'HB' pummelo seedlings, root morphological traits were determined. Our results showed that root growth and development were significantly inhibited under low pH and B deficiency conditions (Fig. 3). It has been proven that plant roots are affected by B deficiency as a result of the role of B in cell wall construction (Dell and Huang, 1997; Marschner, 1995). Similar results have also been reported for the roots of other citrus (Li et al., 2016; Mei et al., 2016; Zhou et al., 2014, 2015; Wu et al., 2018). Further plant anatomical investigations of the roots of citrus (Li et al., 2016; Mei et al., 2016; Zhou et al., 2015) suffering from B starvation stress have been performed, and these results indicated that the root cell and vessel walls were thickened under B deficiency conditions. Further research at the molecular level suggested that the expression of several genes coding for enzymes involved in cell wall metabolism were significantly altered in the roots of *Arabidopsis* (Camacho Cristóbal et al., 2008) and *Citrus* (Zhou et al., 2015) plants, such as xyloglucan endotransglycosylase/hydrolases, expansins, pectin methylesterases, and polygalacturonases. Therefore, based on the aforementioned research results, the inhibited root growth and development by B deficiency stress may be explained.

A dramatic morphological difference was also observed in the roots of the seedlings at low pH under both normal and deficient B conditions (Fig. 3). Several previous studies have shown that the plant root system architecture, which is evaluated based on root length, root surface area, root volume, and root tip, is significantly influenced by low soil pH (Zhou et al., 2018). The root system architecture is believed to have an important role in plant growth performance by adjusting the nutrient uptake from soil (Forde and Lorenzo, 2001; Hodge et al., 2009). Therefore, the root system architecture directly influenced the growth of the seedlings in this work (Fig. 3). Biochar amendment has recently been demonstrated to significantly increase the total absorptive surface area of the root system in citrus (Guo et al., 2016), and it can significantly increase the soil pH and organic matter (Major et al., 2010; Van Zwieten et al., 2010).

Fig. 5. The combined effects of low pH and boron (B) deficiency on macronutrients in 'HB' pummelo seedlings. All the plants were grown under sand culture conditions and treated for 12 weeks. Data are presented as means $\pm$ SE of nine replicates ($n = 9$; one plant for each replicate). Different small and capital letters above the vertical bars indicate significant differences ($P < 0.05$) between the different pH levels and different B concentration treatments, respectively. DB = deficient B concentration; NB = normal boron concentration.

Nutritional responses of citrus to low pH. Low pH not only affects the availability of soil mineral nutrients but also impacts the absorption of mineral nutrients by the roots. Previous studies have shown that soil acidification affected the availability of soil N, P, Ca, Mg, S, B, Cu, and Zn, and the levels of organic matter and cation exchange capacity, thereby inducing soil and leaf nutrient imbalances in 'Guanximiyu' pummelo (*Citrus grandis*) seedlings (Li et al., 2015). In this work, nutrient solution with low pH (pH 4.0, 5.0, and 6.0) culture was used for the 'HB' pummelo seedlings. The two effects of low pH were separated, and the work focused on investigating the effects of low pH on the absorption of mineral nutrients by the roots. The same method has also been used for researching rose (Roosta and Rezaei, 2014). B mainly exists as boric acid in the soil; therefore, its uptake by the root cells is significantly affected by various environmental factors such as soil pH, soil clay content, and soil organic matter.

Soil pH is considered one of the most important factors affecting the soil B supply, but the soil B supply capacity does not increase with the increase in the pH value. Our results showed that the B concentration

decreased significantly in all organs of the seedlings, and that no remarkable difference was observed at all pH values under B-deficient conditions. However, the B concentrations decreased dramatically in the leaf and root at pH 4.0 compared with the other pH values under normal B conditions (Fig. 4A–C). Similarly, a previous study also showed that the shoot B concentration increased with increased B and was greater at pH 6.0 than at pH 8.0 in broccoli (*Brassica oleracea* L.) (Smith et al., 2013). In this research, boric acid (H_3BO_3) was used in the control nutrient solutions, and no H_3BO_3 was added to the B-deficient nutrient solutions. Results obtained from model plants have shown that B transport is a process mediated not only by passive diffusion but also by transporters whose activity is regulated in response to B conditions (Miwa and Fujiwara, 2010). Under normal B conditions, H_3BO_3 is absorbed into the root cell and transported to the xylem via passive diffusion. In contrast, under limited B conditions, H_3BO_3 is absorbed into the root cell and xylem loading by B transporters, such as BOR1, PIP1;1, NIP5;1, NIP6;1, and other aquaporin proteins in vascular plants, including citrus, occurs (An et al., 2012; Takano et al., 2001, 2002, 2006; Tanaka et al., 2008).

Interestingly, no remarkable differences were observed among all pH values under B deficiency conditions (Fig. 4A–C), which might be attributed to the lack of H_3BO_3 in the B-deficient nutrient solutions.

The uptake of macronutrients (P, K, Ca, and Mg), which was detected in the leaf, stem, and root of the 'HB' pummelo seedlings, was also influenced by low pH stress under both normal and deficient B conditions (Fig. 5). P is a nutrient that is sensitive to soil pH, and its optimum pH range for absorption ranges from 6.0 to 7.0. Therefore, its uptake by the roots is inhibited under both high and low pH (Marschner, 1995). P is absorbable in the form of initial orthophosphate ($H_2PO_4^-$), but the ratio of $H_2PO_4^-/HPO_4^{2-}$ differs under different pH values, and the absorption efficiency also varies in the root cells (Shekofteh, 2009). In our work, the P concentration increased with the increase in pH from 4.0 to 6.0 in the leaf under normal B conditions, whereas no significant change was observed in the stem and root (Fig. 5A–C). These results corroborate those of previous studies of rose (Roosta and Rezaei, 2014).

K, Ca, and Mg are absorbed in the form of K^+ , Ca^{2+} , and Mg^{2+} by the plant root, respectively. In this work, no significant differences in these nutrients were observed between pH 4.0 and 5.0 in the roots under normal B conditions, but the concentrations under the former (pH 4.0 and 5.0) treatment were remarkably lower than they were at pH 6.0. However, no significant differences were detected in the leaf and stem, which corroborates the results of previous research of trifoliate orange (Zhou et al., 2014). At the cellular level, a high H^+ concentration (low pH) not only competes for binding sites with K^+ but also decreases the efficiency of the H^+ current in the plasma membrane of the root cell. Additionally, internal and external pH have a role in the regulation of K^+ secretion into the xylem sap. Previous studies have shown that the activity of K channels in plant root cells is also affected by internal and external pH (Lancombe et al., 2000). As a result, the absorption of K is inhibited in the root under low pH conditions. Under normal B conditions, the root Ca concentration decreased sharply at pH 4.0 compared with that at pH 6.0 in this work (Fig. 5G–I). The same tendency was found in Mg concentrations under normal B conditions (Fig. 5G–I). This was mainly attributed to the inhibition of the cation exchange capacity of the root cells by high H^+ concentrations under low pH conditions. Ca is primarily absorbed by the root apices where the Casparian band is not yet created. Therefore, the decreased root Ca concentrations may be caused by the inhibition of root development under low pH stress (Fig. 3). Recent studies have confirmed that low pH affected reactive oxygen species and methylglyoxal metabolisms more in roots than in the leaves of 'Xuegan' (*Citrus sinensis*) and 'Sour pummelo' (*Citrus grandis*). The most seriously impaired ascorbate metabolism in roots was suggested to have a role

in low pH-induced root death and growth inhibition (Long et al., 2019).

Micronutrient (except B) absorption in the rhizosphere is influenced by a variety of complex factors, such as soil properties (including soil pH), plant properties, and interactions of the roots with microorganisms. In this work, we focused on the Fe, Mn, and Zn uptake under low pH. Previous studies have shown that the root rhizosphere pH significantly influences the Fe, Mn, and Zn uptake (Chen et al., 2002; Marschner, 1995). In this study, leaf and stem Fe concentrations were decreased significantly at pH 4.0 under both normal and deficient B conditions (Fig. 4). High pH, even in neutral conditions, can affect Fe uptake and translocation into the plant. Interestingly, at pH 4.0, the Fe concentration was also decreased significantly under both normal and deficient B conditions. This result may be caused by the inhibition of root growth and development at low pH (Fig. 3).

Nutritional responses of citrus to B deficiency. As shown in Figs. 4 and 5, both B and other mineral nutrient concentrations were influenced significantly by B deficiency stress in 'HB' pummelo seedlings. Similar results have also been reported in other citrus species, such as 'Newhall' navel orange (*C. sinensis*), trifoliate orange (*Poncirus trifoliata*), carrizo citrange (*C. sinensis* × *P. trifoliata*), red tangerine (*C. reticulata*), cleopatra mandarin (*C. reshni*), fragrant citrus (*C. junos*), and sour orange (*C. aurantium*) (Liu et al., 2011a; Mei et al., 2016; Zhou et al., 2014).

Previous studies have shown that not only is P concentration significantly affected by B status (deficiency or toxicity), but B concentration is also dramatically influenced by P availability (Wang et al., 2018; Zhou et al., 2014). In this study, the P concentration significantly decreased in all organs of the seedlings under B-deficient conditions, particularly in the leaf at pH 6.0 (Fig. 5A–C). Furthermore, the growth and development of the roots were inhibited dramatically by B deficiency stress (Fig. 3). P is believed to be an essential mineral nutrient for plant growth, cell energy homeostasis (ATP), nucleic acid formation, and reversible protein phosphorylation (Maathuis, 2009); furthermore, P deficiency can induce root growth and development (Lisa et al., 2001; López-Bucio et al., 2003). Previous studies have suggested that the growth and development of citrus roots are significantly inhibited under B deficiency stress via decreased P concentration (Zhou et al., 2014). Our findings are in agreement with those of previous studies.

Ca is a main chemical component of the cell wall, and more than 90% of B in plants exist in the cell wall. Some reports indicate that Ca and B promote each other (Ramon et al., 2000), whereas other studies suggest that a mutual antagonism exists between them. Several Ca_2^+ channel/transporter genes were upregulated in response to short-term B deficiency in *Arabidopsis* roots

(Quiles-Pando et al., 2013). It was recently shown that B-deprived tobacco BY-2 cells took-up more Ca_2^+ than the control cells (Koshiba et al., 2010). Another study showed that B deficiency increased the levels of cytosolic Ca_2^+ in *Arabidopsis* roots (Quiles-Pando et al., 2013). All these results suggest that the Ca concentration could be increased by B deficiency. Ca is a crucial secondary intracellular messenger that has a major role in the plant response to stress. Moreover, Ca_2^+ can bind to Ca_2^+ sensors, such as calmodulins (CaMs) and calmodulin-like proteins (CMLs) (Sanders et al., 2002). Therefore, the lack of decrease in Ca concentration in the seedling might be associated with the response to B deficiency.

In this study, Fe and Mn, which are key micronutrients for photosynthesis in higher plants, were also decreased by B deficiency stress (Fig. 4). These results are similar to the findings of current research of other citrus (Mei et al., 2016; Zhou et al., 2014). Previous studies also suggested that the inhibition of leaf photosynthesis could be caused by decreased concentrations of Mg, Fe, and Mn in the citrus leaves (Zhou et al., 2014). It is worth noting that the effect of B deficiency on the Zn concentration is different in the leaves of 'Newhall' navel orange grafted on two different rootstocks under B deficiency conditions (Liu et al., 2011b). The Zn concentration was decreased significantly under B deficiency in both old and new leaves of a citrus plant grafted on carrizo citrange, but not in trifoliate orange. This result might indicate that carrizo citrange is more tolerant than trifoliate orange to B deficiency stress (Zhou et al., 2015). Our results also corroborate those of previous studies (Fig. 4).

Conclusion

In conclusion, low pH and B deficiency stress not only affected plant growth and development in both the aboveground and belowground parts of the 'HB' pummelo seedlings but also impacted the concentrations of B and other mineral nutrients. Plant biomass, leaf area, seedling height, and root traits were remarkably decreased by low pH and B deficiency stresses, and these parameters were extremely reduced with the decrease in pH level. After 12 weeks of treatment, typical B deficiency symptoms of citrus leaf were observed, with more severe symptoms observed at pH 5.0 than at pH 6.0. The leaf gas exchange parameter measurements showed that leaf photosynthesis was significantly inhibited under both low pH and B deficiency conditions. It is worth noting that the lower the pH level, the greater the inhibition under both normal and deficient B conditions. Further investigations of the mineral nutrient concentration results showed that under both low pH and B deficiency conditions, not only the B concentration but also the other mineral nutrient concentrations were influenced dramatically, especially at pH 4.0 and 5.0. Based on the physiological and nutritional performances of the 'HB'

pummelo seedlings, these results indicate that low pH can alleviate the effects of B deficiency to a certain extent.

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