Article

Evaluation of Three New Citrus Rootstocks under Boron Toxicity Conditions

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Abstract: Boron (B) toxicity is a common limiting factor both in arid and semiarid regions, such as the Mediterranean Basin. Citrus trees are sensitive to B-toxicity, which generates a negative impact in citrus orchards. In this work, two promising citrus rootstocks (UFR-6 and 2247 x 6070-02-2) have been assessed against B-toxicity and compared with Carrizo citrange, a common commercial citrus rootstock in Mediterranean Basin. Three B concentration treatments (Control, 1 and 2.5 mM H₃BO₃) were established, irrigating the plants three times per week for 21 days under greenhouse conditions. During the assay, above-ground symptoms, and chlorophyll index (SPAD) were recorded. At the end of the experiment, stomatal conductance, relative water content, and B concentration in leaves and roots were determined. The increasing B concentration in plants generates visual damage in leaves for all citrus rootstocks assayed. Carrizo citrange displayed the greatest visual symptoms, decreased its chlorophyll index (SPAD), and stomatal conductance throughout the B-treatment. However, UFR-6 and 2247 x 6070-02-2 displayed less symptoms than Carrizo citrange and only reduced its parameters under the 2.5 mM H₃BO₃ treatment. These results can aid citrus grower rootstock planting decisions with under B-toxicity conditions.

Keywords: B-excess; HLB; stomatal conductance; RWC; UFR-6

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1. Introduction

The Mediterranean Basin is the second largest citrus producing region in the world, with an overall production more than 26 million tons [1]. However, numerous factors are threatening Mediterranean citriculture. Desertification and climate change create a hostile environment for citrus crops [2–4].

Boron (B) is an essential element for plants; this micronutrient is necessary for regular growth and development [5,6]. However, B excess can easily occur due to small differences between optimal levels and toxicity [7], after the application of fertilizers with excess of this element and/or the continual irrigation with water of high B concentration [8]. Thus, B-toxicity is more frequent in arid and semiarid regions, such as the Mediterranean Basin, in which citrus are a major crop [8,9]. Citrus trees are considered sensitive to B excess; thus, a B concentration above of 0.3 mg L^{−1} can result in phytotoxicity and reduced yields [10,11]. High B levels in citrus leaves lead to visual symptoms in mature, or even young, leaves and also produce premature abscission [9,12,13]. As a plant micronutrient, B is involved in several biochemical and physiological essential processes, which can be altered in case of excess. Thus, B-toxicity has an effect on plant growth [14], uptake of other micro- and macro- elements [15], photosynthesis [16,17], and chlorophyll and carotenoid levels [16,18].

In this context, there is a necessity to find sustainable methods for Mediterranean citriculture that can solve abiotic disorders, which limit citrus crops, such as boron toxicity. Diversification and accurate choice of citrus rootstocks can be a significant method to reduce abiotic stress [19,20]; even this methodology can improve the citrus industry [21]. Thus, suitable citrus rootstocks selection has a positive impact on field performance, improving and/or maintaining plant growth and fruit production and quality [22]. Nevertheless, the Mediterranean Basin citriculture is often a monoculture orchard, where 61% of citrus crops in Spain used Carrizo citrange [23]. Carrizo citrange is boron excess sensitive [5]. Consequently, the University of Florida Citrus and Research Education Center (CREC) is obtaining new citrus rootstocks with tolerance against abiotic and biotic stress from their breeding programs. These new citrus rootstocks require characterization under abiotic and biotic limiting factors outside of Florida for Mediterranean Basin growers. The focus of this work was an evaluation of two new promising citrus rootstocks against boron toxicity.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Two new citrus rootstocks recently obtained from the CREC breeding program, UFR-6 ('Changsha' mandarin + Trifoliate orange 50–7), a commercial rootstock with small tree size, notable fruit quality and low HLB sensitivity in the field [24] and 2247 × 6070-02-2 ['Nova' + HBP × Sour orange + *Poncirus trifoliata* (var. *Monstrosa*)], an unreleased selection that has displayed low HLB sensitivity in the field (F. G. Gmitter Jr. and J. W. Grosser personal communication), were assayed against boron toxicity conditions. Carrizo citrange (*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) was used as the reference rootstock due to its prevalence in Mediterranean Basin orchards. Six-month-old citrus plants belonging to these three citrus rootstocks were first obtained from in vitro culture and provided by Agromillora Group nursery (Subirats, Barcelona, Spain).

The experiment was carried out with a total of 72 citrus plants for the 2020 summer season and under greenhouse conditions with environmental control [27 °C average temperature, 56% average humidity, and 12:12 h (L:D) photoperiod]. This greenhouse is located in "Las Torres" Center of Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), in the municipality of Alcalá del Río, Seville, Spain (37°30'43.3'' N; 5°57'47.4'' W). Once all plants were received, each citrus rootstock was transferred to a 1.6-litre pot with silica sand. Plants were acclimated for two weeks and irrigated three times a week, with minor modifications for citrus plants of Hoagland and Arnon solution [25] (3 mM of KNO₃, 3 mM Ca(NO₃), 1 mM MgSO₄ 7H₂O, 1.2 mM H₃PO₃ 85%, 20 µM Fe-EDDAH, 54.4 µM MnSO₄ H₂O, 7.64 µM ZnSO₄ 7H₂O, 0.5 µM, CuSO₄ 5H₂O, 46.25 µM H₃BO₃, 0.55 µM MoO₃). To prepare a stock solution, all nutritive reagents were weighted using a digital scale (COBOS precision, CB-3000C, L'Hospitalet de Llobregat, Barcelona, Spain) and dissolved in a tank in the laboratory; lastly, this primary solution was diluted in the greenhouse before the irrigation process.

2.2. Treatments and Experimental Design

Three B-toxicity treatments were established in this experiment (Control, 1 and 2.5 mM H₃BO₃) under a factorial experimental design with a random distribution of four block repetitions constituted by an eight-plant elemental plot ($n = 8$). The experiment began after acclimation on the first day (D1) of treatment application, using eight plants per rootstock and treatment. Each plant was irrigated with 500 mL of Hoagland and Arnon solution Monday, Wednesday, and Friday during the assay. The three treatments were prepared separately in a specific tank using nutritive solution amended with each boron concentration above.

2.3. Evaluation of Plant Symptoms Caused by Boron Toxicity

For each rootstock/plant and treatment, symptoms in above-ground leaves were evaluated to estimate the effect of the different boron treatments using a symptoms scale of 0–4: plants without symptoms = 0; plants with 25% leaves affected by chlorosis = 1; 50% leaves affected by chlorosis = 2; over 50% leaves affected by chlorosis = 3; and fully desiccated and dead plants = 4. This evaluation process was carried out on days 1, 10, 15 and 21, starting from the first B treatment application until the end of the assay (when the plants started the defoliation process). The values obtained from this evaluation process were used to calculate the standardized area under the abiotic stress progress curve (SAUASPC, [26]), which increases in the same proportion as the symptom scale.

2.4. Chlorophyll Index (SPAD)

The leaf chlorophyll index was measured in all plants assayed by a SPAD chlorophyll meter (Minolta Co., Osaka, Japan). Two expanded leaves per plant were measured on four different evaluation days (1, 10, 15 and 21).

2.5. Water Status

2.5.1. Stomatal Conductance

Stomatal conductance was studied at the end of the experiment (D21) in a total of four plants per each citrus rootstock and treatment. A Leaf Porometer SC-1 (Decagon Devices, Pullman, WA, USA) was used to perform this evaluation process [27].

2.5.2. Relative Water Content

As stated above, relative water content (RWC) was estimated at the end of experiment (D21). For this process, two mature and fully expanded leaves per plant were selected, with a total of four plants per rootstock and treatment. Then, two discs of 1 cm in diameter per selected leaf were cut and taken. All four discs obtained per each plant were weighed using a precision digital scale (METTLER TOLEDO AJ100, Columbus, OH, USA). Next, each four-disc group was covered with distilled water for 4 h at room temperature and weighed again. After this process, each group of discs was placed in labeled paper enveloped, dried in a heater for 24 h at 80 °C, and finally weighted. The RWC was calculated according to Morgan (1984) [28] with the following equation:

$$RWC (\%) = \frac{(W - DW)}{(TW - DW)} \times 100$$

where W = fresh weight of the four discs in each citrus rootstock and treatment, TW = discs turgent weight (after 4 h in distilled water) and DW = dry weight discs.

2.6. Boron Concentration in Leaves and Roots

To obtain the micronutrient concentration, three leaves and three roots for each citrus rootstock and treatment were used for boron study. This analysis was carried out by a commercially certified specific laboratory for agriculture (Laboratorio Agrama S.L., La Rinconada, Seville, Spain). The methodology analysis technique used for boron was inductively coupled plasma optical emission spectroscopy (ICP-OES).

2.7. Data Analysis

All data obtained were subjected to analysis of variance (ANOVA) using the STATISTICA 10 software (StatSoft, Palo Alto, CA, USA). Means separation were obtained using Fisher's test ($p < 0.05$). Normality and homogeneity assumptions were tested before ANOVA. Two-way ANOVA analysis was carried out for SAUASPC values and one-way ANOVA for the remaining results obtained. Data of Carrizo citrange under 2.5 mM H_3BO_3 treatment is not available for stomatal conductance, RWC and B concentration in leaves due to the lack of plant material.

3. Results

3.1. Visual Symptoms Caused by Boron Toxicity

The highest effect on visual symptoms were more visible at the end of the trial (D21). Control plants from all citrus rootstocks did not display any visual symptoms of damage. Only chlorosis symptoms were found in all citrus rootstocks under 1 mM H_3BO_3 , but leaves defoliation were not detected in any citrus at this treatment. On the other hand, several leaves defoliation were found in Carrizo citrange at the highest boron concentration (2.5 mM H_3BO_3); whereas UFR-6 and 2247 x 6070-02-2 showed chlorosis symptoms at this highest concentration, and very slight defoliation symptoms were found for these both citrus rootstocks (Figure 1).

Figure 1. Plant symptoms of the three citrus rootstocks (Carrizo citrange, UFR-6 and 2247 x 6070-02-2) under three boron treatments (Control, 1 mM H_3BO_3 and 2.5 mM H_3BO_3) at the end of the experiment (21 days).

All citrus rootstocks displayed different response. Hence, the highest significant response of SAUASPC was obtained with Carrizo citrange plants under a 2.5 mM H_3BO_3 treatment. The SAUASPC response was similar for UFR-6 and 2247 x 6070-02-2 under the highest toxicity concentration and was statistically lower compared with the Carrizo citrange and the same treatment. For 1 mM H_3BO_3 , SAUASPC response in all citrus rootstocks was significantly lower compared with the results at 2.5 mM H_3BO_3 . Carrizo citrange showed the highest value for 1 mM H_3BO_3 , with statistical differences compared with the two other citrus rootstocks under the same concentration and with its control. However, UFR-6 and 2247 x 6070-02-2 displayed similar SAUASPC values without significant differences against control treatments (Figure 2).

Figure 2. Mean standardized area under the abiotic stress progress curve (SAUASPC) under three toxicity boron treatments (Control, 1 mM H₃BO₃, and 2.5 mM H₃BO₃) on three citrus rootstocks (Carrizo citrange; UFR-6 and 2247 x 6070-02-2) leaves over twenty-one days of treatments. Values in columns with different letters are statistical differences among treatments and rootstock by Fisher LSD test ($p < 0.05$). Significant codes: *** = $p < 0.001$; T: treatment, R: citrus rootstock.

3.2. Chlorophyll Index (SPAD)

Carrizo citrange showed similar SPAD values at the first assessment timing for all treatments. However, the 2.5 mM H₃BO₃ treatment showed a slight reduction at the D10, decreasing with the lowest SPAD values down to D15, whereas boron treatment of 1 mM H₃BO₃ displayed a similar SPAD response to Control until D15. At D21, plants from 1 mM H₃BO₃ treatment had reduced SPAD values, and plants treated with 2.5 mM H₃BO₃ had slightly increased SPAD values with a similar response to 1 mM. In the case of UFR-6, a similar SPAD response was found for all treatments until D10. Plants of this citrus rootstock decreased their SPAD values after D15 with the lowest response at D21. Treatment 1 mM H₃BO₃ maintained a similar response to Control plants throughout the assay. Lastly, 2247 x 6070-02-2 displayed a similar SPAD response for all treatments from D1 to 15. Plants of this citrus rootstock had only reduced SPAD response at D21 with the highest concentration treatment (Figure 3).

Figure 3. Time course for the mean of chlorophyll index values (SPAD) $\pm$ standard error (SE) with three treatments (Control, 1 mM H₃BO₃ and 2.5 mM H₃BO₃) on three different citrus rootstocks plants (Carrizo citrange, UFR-6 and 2247 x 6070-02-2) during twenty-one days of assessment.

3.3. Water Status

3.3.1. Stomatal Conductance

Stomatal conductance response differed among treatments applied for each citrus rootstock. Carrizo citrange displayed a significant reduction in stomatal conductance when boron toxicity increased. Thus, this citrus rootstock displayed significant differences between control treatments and 2.5 mM H_3BO_3 treatment. In contrast, neither new citrus rootstock displayed significant differences compared with their respective control plants under 1 mM H_3BO_3 and 2.5 mM H_3BO_3 treatment. UFR-6 displayed similar values for Control and 1 mM treatment and a slightly higher value in the 2.5 mM treatment, while 2247 x 6070-02-2 showed similar values for control and 1 mM H_3BO_3 plants, and slightly lower at 2.5 mM H_3BO_3 treatment (Figure 4).

Figure 4. Mean stomatal conductance ($\text{mmol}/\text{m}^2\text{s}$) at the end of the experiment with three boron toxicity treatments (Control, 1 mM H_3BO_3 and 2.5 mM H_3BO_3) and using three citrus rootstocks (Carrizo citrange, UFR-6 and 2247 x 6070-02-2). Values in columns with different letters means statistical differences among the treatments per each rootstock by Fisher LSD test ($p < 0.05$). Significant codes: * = $p < 0.05$; ns: non-significant differences.

3.3.2. Relative Water Content

RWC did not display high differences among treatments for each citrus rootstock studied. RWC results for Carrizo citrange plants showed a similar behavior between control and 1 mM H_3BO_3 treatments without significant differences. In the case of UFR-6, there were no significant differences between control and 1 mM H_3BO_3 treatment; however, a slight lower significant RWC was found at 2.5 mM H_3BO_3 concentration in this citrus rootstock. Lastly, 2247 x 6070-02-2 showed the highest significant RWC values in control treatment, with 1 mM H_3BO_3 and 2.5 mM H_3BO_3 being significantly lower than control but similar between them (Figure 5).

Figure 5. Relative Water Content (RWC) at day 21 of the assay in three citrus rootstocks studied (Carrizo citrange, UFR-6 and 2247 x 6070-02-2) and under three boron toxicity treatments (Control, 1 mM H₃BO₃, and 2.5 mM H₃BO₃). Values in columns with different letters means statistical differences among treatments for each citrus rootstock by Fisher LSD test ($p < 0.05$). 2.5 mM H₃BO₃ treatment of Carrizo citrange: data not available. Significant codes: * = $p < 0.05$, and *** = $p < 0.001$; ns: non-significant differences.

3.4. Boron Concentration in Plants

The increase in B concentration in leaves for all citrus rootstocks was directly proportional to B toxicity treatments. Carrizo citrange displayed the highest B concentration values in leaves at 1 mM H₃BO₃ treatment with statistical differences compared with its control treatment. UFR-6 displayed the highest B concentration at 2.5 mM H₃BO₃ treatment, with significant differences among 1 mM H₃BO₃ and Control treatment. 2247 x 6070-02-2 displayed the highest B concentration in leaves at 2.5 mM H₃BO₃ treatment, followed by 1 mM H₃BO₃ and Control, with significant differences among all treatments assayed (Figure 6A).

As with leaves, B concentration in roots increased when the treatment concentration applied was higher. Carrizo citrange displayed highest B concentrations in roots at 2.5 mM H₃BO₃, followed by 1 mM H₃BO₃ and Control, with significant differences for each treatment. UFR-6 showed its highest B concentration at 2.5 mM H₃BO₃, having significant differences compared with control, and 1 mM H₃BO₃. Finally, 2247 x 6070-02-2 displayed the highest B concentration in roots at the highest dosage applied with significant differences compared with the control and 1 mM H₃BO₃ (Figure 6B).

Figure 6. Mean B concentration (mg/kg) ± standard error (SE) after twenty-one days for the three rootstocks (Carrizo citrange, UFR-6 and 2247 x 6070-02-2) under three concentrations of boron treatments applied (Control, 1 and 2.5 mM of H₃BO₃). (A) Mean B concentration in leaves. (B) Mean B concentration in roots. Values in columns with different letters mean statistical differences among treatments per each rootstock by Fisher's test ($p < 0.05$). 2.5 mM H₃BO₃ treatment of Carrizo citrange leaves (A): data not available. Significant codes: *** = $p < 0.001$; ns: non-significant differences.

4. Discussion

The Mediterranean Basin is suffering a desertification process which can lead a B excess, as other arid and semiarid regions in the world. B can be naturally found in groundwater, and the evaporation of this water resource available for plants is increasing in these regions, which generates B-toxicity in the topsoil where crops are grown [2,29–31]. Citrus rootstocks selection can help address the most limiting abiotic and biotic factors, such as B-toxicity. This work provides the citrus industry and breeding programs with information to select the correct citrus rootstock to address B toxicity problems. To our knowledge, these two new citrus rootstocks (UFR-6 and 2247 x 6070-02-2) have never

been studied under boron toxicity conditions. Citrus plants are known to be sensitive to B-toxicity from soils, irrigation water and fertilization [32], although a different response has been reported depending on the citrus rootstock genotype [33].

B treatment generated visual symptoms in all citrus rootstocks assayed, but leaf symptoms more intense in Carrizo citrange than in UFR-6 and 2247 × 6070-02-2 [34]. SPAD is an indirect measure of chlorophyll index [35]; its assessments provide a numerical measurement of chlorosis [36]. In this study, Carrizo citrange plants displayed the lowest SPAD response after both B toxicity treatments (1 and 2.5 mM H₃BO₃); additionally, Carrizo citrange exhibited reduced chlorophyll in an early assessment, compared with UFR-6 and 2247 × 6070-02-2. These citrus rootstocks showed a similar chlorophyll index (SPAD) under 1 mM H₃BO₃ and reducing SPAD under 2.5 mM H₃BO₃ treatment. Similar with other reports, the visual symptoms are in accordance with the response of chlorophyll index obtained in this work [37].

Different studies have reported that B-toxicity generates an imbalance in water plant status [38,39]. Hence, stomatal closure is an hormonal response to avoid water loss [12], and this plant process is regulated by abscisic acid (ABA) [40–42]. Under B excess conditions stomatal conductance is reduced in summer squash leaves (*Cucurbita pepo* L.) [43]. Furthermore, according to prior works under B-toxicity the stomatal closure genotype-dependent where citrange plants are known to close their stomates [9,44]. Thus, neither of these promising citrus rootstocks (UFR-6 and 2247 × 6070-02-2) showed reduced stomatal conductance under B-toxicity. RWC indicates reactions and the ability of plants to maintain an optimal water status under stress conditions [45]. According to other studies, salts excess reduces RWC in leaf tissue, as well as B-toxicity in our study [46–48]. Thus, UFR-6 was affected in this parameter at concentration of 2.5 mM H₃BO₃ treatment, whereas 2247 × 6070-02-2 responded negatively compared with control in both boron treatment (1 and 2.5 mM H₃BO₃).

B-toxicity in plants is generated by its accumulation in leaves because of high B concentrations applied and/or long-time exposure. B-toxicity sensitive citrus rootstocks tend to accumulate high concentrations of this microelement in leaves [8]. One characteristic of citrus-tolerant rootstocks against B-toxicity has been reported as the capacity to exclude this compound from leaves; thus, tolerant citrus rootstocks display a lower B concentration in leaves than those susceptible [49]. In our results, UFR-6 and 2247 × 6070-02-2 displayed lower concentration of Boron in leaves than Carrizo citrange under the same concentration of Boron treatment (1 mM H₃BO₃). B leaf concentration is not always correlated with other parameters or tolerance responses [50,51]. According to Huang et al. [17], citrus rootstocks with a similar B concentration in leaves and roots could have a sensitive or tolerant response depending on the location where the boron is accumulated. Thus, in our result all citrus rootstocks showed similar response of boron concentration in roots compared with same treatment. 2247 × 6070-02-2 displayed the highest concentration response of boron concentration in roots; nevertheless, this citrus rootstock as well UFR-6 displayed only chlorosis symptoms in leaves. This response could be accounted for by the low presence of B in cytoplasm and its movement to the cell wall where B is less toxic [17,50].

5. Conclusions

Our results can be useful for better citrus rootstock selection in those regions suffering B toxicity problems. Clearly, UFR-6 and 2247 × 6070-02-2 exhibited better responses compared with Carrizo citrange for most of parameters analysed. These two new promising citrus rootstocks tested (UFR-6 and 2247 × 6070-02-2) could be useful for citrus growers to combat and prevent this abiotic factor in those areas with the above issues. Thus, further research will involve long-term field effect under permanent boron toxicity conditions to confirm these positive results.

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