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Characterization of Citrus Rootstock Under Conditions of Boron Toxicity

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Abstract: Boron (B) is an essential element for an adequate development of citrus orchards. However, citrus trees are vulnerable to high B concentrations, generating morphological and physiological alterations incompatible with the proper production of citrus. In this sense, citrus rootstocks can provide valuable capabilities to citrus trees including tolerance to different stresses. The objective of this work is the characterization of 2247 × 6070–02–2 citrus rootstock using as a reference Carrizo citrange rootstock under B toxicity conditions (2.5 mM boric acid). Carrizo citrange is a diploid hybrid, and 2247 × 6070–02–2 is a novel low-HLB-sensitive tetraploid. B excess effects were analyzed after four weeks of treatment using 0.05 (control) and 2.5 mM (toxicity) H_3BO_3 concentrations, respectively, in hydroponic growth conditions. The characterization of 2247 × 6070–02–2 rootstock compared to Carrizo citrange was performed by measuring physiological parameters in leaves related to photosynthesis, stress oxidative responses, B content, and gene expression. The lower transpiration rate and, especially, the higher expression of the *CsXIP1;1* gene and the better antioxidant defense mechanisms shown by 2247 × 6070–02–2 make this rootstock more tolerant to high B content than Carrizo citrange.

Keywords: Boron (B); toxicity; citric; rootstock; tolerance antioxidant mechanisms

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1. Introduction

Boron (B) is an essential microelement for plants, with a structural and functional role that provides stability in the cell wall through diester bonds with two rhamnogalacturonan II (RG-II) monomers, among other functions [1–4]. However, excessive B concentration in soils and crops may arise from certain agricultural practices, including fertilization and the use of irrigation water from various sources [5–7]. In fact, B-containing compounds are added to fertilizers and can increase the content of this micronutrient. In addition, irrigation with groundwater from volcanic or geothermal areas, as well as irrigation with water contaminated by waste from pesticides, ceramics, detergents, glass, or by leachate from municipal landfills, could accumulate high B levels in soils that can become toxic to plants [8]. B toxicity alters different physiological processes associated with nutrient uptake, and photosynthesis processes, including electron transport rate, photosystem II (PSII) efficiency, chlorophyll concentration, transpiration rate, and stomatal conductance, among others [7,9–13]. B accumulation occurs mainly in mature leaves, and chlorotic and necrosis spots at the distal apex are typical visual symptoms of B toxicity, along with

premature abscission [14–17]. However, there are plants with higher B mobility through the phloem, and B builds up in young leaves [7,18,19]. Citrus plants belonging to the genus *Citrus* L. of the Rutaceae family are plants vulnerable to the presence of excess B, generating alterations in the cell wall and cytoskeleton, signal transduction, and changes in metabolism related to carbohydrates, nucleic acids, amino acids, lipid, proteins, and energy [7,20,21]. Despite these harmful effects that excess B provokes in plants, certain plants have developed B tolerance involving decreased transpiration and water translocation to shoots, changes in gene expression of B channels and transporters associated with B uptake, B excretion, as well as cell compartmentalization in organelles and the cell wall, and stimulation of the biosynthesis of B-chelating organic compounds like polyols and phenols, among other mechanisms [7,10,11,22–27].

Good productivity demanded from citrus growers is associated with the use of plants tolerant to various stresses, and the choice of appropriate rootstocks can be decisive for this purpose [28,29]. Numerous breeding programs focus on obtaining novel citrus rootstocks suitable under adverse abiotic/biotic growing conditions, in addition to being suitable for good fruit production [30,31]. Thus, Carrizo citrange is a diploid hybrid (*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) commonly used for citrus growers as a reference rootstock and is considered a citrus relatively sensitive to B toxicity [32,33]. Simón-Grao et al. [32] described Carrizo citrange as more sensitive to B excess than other rootstocks, *Citrus macrophylla* and *Citrus aurantium* (sour orange). However, Carrizo citrange is also described as being more tolerant to B toxicity than the rootstocks Ziyangxiangcheng and *Poncirus trifoliata* (trifoliate orange) [33]. B toxicity tolerance usually involves lower B content in leaves and roots and improved activity of antioxidant mechanisms, observed in sour orange compared to Carrizo citrange [32,33]. Excess B induces oxidative stress in plants, leading to the generation of reactive oxygen species (ROS), which cause damage in cell membranes and biomolecules through the degradation of proteins, nucleic acids, and lipids [34]. Therefore, plants have evolved ROS scavenging systems to reduce the deleterious effects of these oxygen species [35]. Furthermore, efficient antioxidant mechanisms of tolerant plants could be activated to detoxify an increase in ROS produced in response to excess B [7,36]. Antioxidant systems include high enzyme activities such as ascorbate peroxidase (APX;EC1.11.1.11), catalase (CAT;EC1.11.1.6), glutathione reductase (GR;EC1.6.4.2) and superoxide dismutase (SOD;EC1.15.1.1), and/or increased generation of antioxidant molecules like phenols [17,36]. Tolerance to B toxicity would imply less oxidative damage, estimated by quantifying malondialdehyde (MDA) generation as an indicator of membrane lipid peroxidation [37]. In addition to the antioxidant defense, it has been described that *Citrus sinensis* rootstock increased the expression of different genes related to photosynthesis as part of a mechanism to confer tolerance to excess B. Likewise, changes in gene expression levels of microRNAs, involved at the posttranscriptional level, have also been described as contributing to stress responses [20,38,39]. In *Poncirus trifoliata*, an increase in lignin concentration was described under excess B treatment [40]. The decline in miR397 level and the resulting increase in the expression of the target gene *Laccase7* (*LAC7*) involved in lignin biosynthesis would contribute to enhance B tolerance [40]. *MiR319* and *miR171* targeting MYB and SCARECROW, respectively, were also identified concerning root growth and development in relation to the mechanisms of citrus adaptation to long-term B toxicity in *Citrus sinensis* compared to the sensitive *Citrus grandis* [41].

Furthermore, huanglongbing (HLB) disease is responsible for citrus productivity loss and damage to fruit quality, a fact that adds value to the characterization of a novel rootstock with low sensitivity to HLB, 2247 × 6070–02–2. This novel tetraploid rootstock, originated from crosses of allotetraploid somatic hybrids (*Citrus reticulata* 'Nova' + *Citrus maxima* 'Hirado Buntan' × *C. aurantium* + *P. trifoliata* '*Flying Dragon*'), has potential in enhancing stress tolerances [42–44]. This behavior is in line with the increased stress tolerance described in tetraploid citrus rootstocks [45–47]. A previous study performed under B toxicity in greenhouse growth conditions established that 2247 × 6070–02–2 had leaves with less visual symptoms, and chlorophyll values with fewer alterations due to

excess B compared to Carrizo citrange [42]. From these preliminary results, the aim of this work is to further investigate the physiological mechanisms underlying the potential tolerance to B toxicity of 2247 × 6070–02–2 rootstock in comparison with Carrizo citrange, focusing especially on the measurement of photosynthesis-related parameters, antioxidant enzyme activities (ascorbate peroxidase, catalase and glutathione reductase), phenol and B contents, and gene expression. From our results, we conclude that 2247 × 6070–02–2 is a rootstock more tolerant to high B media than Carrizo citrange.

2. Materials and Methods

2.1. Citrus Plant Material and Growth Conditions

Carrizo citrange is a diploid hybrid rootstock (*Citrus sinensis* L. Osbeck and *Poncirus trifoliate* L. Raf.) generated in the citrus breeding program of the United States Department of Agriculture (USDA) (Riverside, CA, USA). Carrizo rootstock seedlings (30–40 cm high) were supplied by the Vivergil nursery (Amposta, Tarragona, Spain). 2247 × 6070–02–2 tetraploid rootstock originated from crosses of allotetraploid somatic hybrids (*Citrus reticulata* 'Nova' + *Citrus maxima* 'Hirado Buntan' × *C. aurantium* + *P. trifoliata* '*Flying Dragon*') [48]. 2247 × 6070–02–2 seedlings (20–30 cm high) were supplied by the "Las Torres" Center of Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA-Las Torres, Alcalá del Rio, Seville, Spain). Seedlings were first obtained from in vitro culture and were provided by the Agromillora Group nursery (Subirats, Barcelona, Spain). Before the start of the hydroponic culture experiments, seedlings of both rootstocks were transplanted into 3.5-L pots with peat and acclimatized for two months in a plant growth chamber with 12/12 h light/dark regime, constant temperature of 22 °C, relative humidity of 50%, and 215 µmol m^{-2} s^{-1} of photosynthetically active radiation at plant height. The hydroponic culture was also carried out in a plant growth chamber at the same conditions used in the acclimation period in 30 L plastic containers. The composition of the hydroponic culture medium was as follows (Hoagland and Arnor, modified [49]): 2 mM NH_4NO_3, 1.5 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 4 mM KNO_3, 1 mM $MgSO_4 \cdot 7\ H_2O$, 1.25 mM KH_2PO_4, 150 µM FeNa EDTA, 0.3 µM $CoCl_2 \cdot 6\ H_2O$, 75 µM KCl, 7.5 µM $CuSO_4 \cdot 5\ H_2O$, 81.6 µM $MnSO_4 \cdot 4\ H_2O$, 1.5 µM $NaMoO_4 \cdot 2\ H_2O$, 1.5 µM $ZnSO_4 \cdot H_2O$, 0.05 mM H_3BO_3 in control condition, and 2.5 mM H_3BO_3 in B toxicity condition (pH 5.8 adjusted with KOH). Culture media were aerated by air pumps and renewed once a week. Plants grown in hydroponic conditions were maintained for 4 weeks. After measurements of photosynthesis parameters at 4 weeks of hydroponic B treatments, all leaves were harvested from each plant, lightly washed, quickly frozen in liquid nitrogen, and stored at −80 °C until further determinations. For B, phenol, and malondialdehyde determinations, RNA extraction, and enzyme activities, leaves were ground to a fine powder in a mortar pre-cooled with liquid nitrogen.

2.2. Total B Determination

For the determination of total B content [µmol B g^{-1} Fresh Weight (FW)], 200–250 mg of finely crushed leaves was transferred to porcelain crucibles. First, they were completely dried at 80 °C, and second, turned to ash by heating at 550 °C for 6 h. Once they reached room temperature in a desiccator, the ash samples were dissolved with 1.0 mL of 0.1 M HCl. B was determined by the azomethine-H method according to Beato et al. [50] and de Andrade et al. [51], with the following modifications. Samples derived from the toxicity treatment were diluted 50-fold and twice for control samples, in a total volume of 400 µL with 0.1 M HCl, adding 400 µL of masking solution and 200 µL of azomethine-H reagent (Sigma Aldrich Merck KGaA, Darmstadt, Germany). After vigorous vortexing, the samples were kept at room temperature for 1 h, centrifuged (13,000× *g*, 5 min), and the absorbance measured by spectrophotometry at 410 nm.

2.3. Photosynthetic Parameters and Chlorophyll Determination

Net photosynthetic CO_2 assimilation (PN, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and maximum photochemical efficiency (Fv'/Fm') were measured

on fully expanded leaves of light-adapted plants using a portable infrared gas analyzer (Li 6400, Li-Cor Inc., Lincoln, NE, USA) at the fourth week of B treatment in hydroponic media equipped with a leaf chamber fluorometer (6400–40, Li-Cor Inc., Lincoln, NE, USA). Airflow rate, CO_2 concentration, and irradiance (photosynthetically active radiation) were adjusted to 200 µmol s^{-1}, 410 ppm, and 215 µmol m^{-2} s^{-1}, respectively.

Chlorophyll determination (µg Chl g^{-1} FW) was carried out using a mortar pre-cooled with liquid nitrogen and 80% (*v*/*v*) acetone at 4 °C to extract pigments from a fine powder leaf material. Extraction was repeated at least four times and, between each, the acetone extract was vortexed for 10 s and centrifuged at 4 °C (13,000× *g*, 2 min). The collected supernatants were combined and, subsequently, the acetone extracts were diluted with 80% (*v*/*v*) acetone and immediately analyzed by spectrophotometry. Their chlorophyll concentrations were calculated as Lichtenthaler [52].

2.4. Quantitative Real-Time PCR Expression Analyses

Total RNA was obtained using a Tri-Reagent ®RNA/DNA/Protein isolation reagent (Molecular Research Center Inc., Cincinnati, OH, USA), and RNA Clean and Concentrator columns (Zymo Research Corporation, Irvine, CA, USA). cDNA was synthesized from two micrograms of DNase-treated total RNA by reverse transcription with M-MLV reverse transcriptase (New England Biolabs Inc., Ipswich, MA, USA) and oligo (dT)18 primers (Meridiam Bioscience, Memphis, TE, USA). Gene expression was determined by quantitative RT-PCR (MyiQ real-time PCR detection system, Bio-Rad, Hercules, CA, USA) using gene-specific primers (Table S1) and SensiFAST SYBR & Fluorescein Kit (Meridian Bioscience, Memphis, TE, USA), following the manufacturer's instructions. The gene expression levels (relative units) of *Citrus sinensis* X Intrinsic Proteins (*CsXIP1;1;* NCBI gene ID: 102628667) were measured using the *Citrus sinensis* ubiquitin–60S ribosomal protein L40 (*CsUB*) as a housekeeping gene (Table S1). The method used to determine gene expression was the Pfaffl Method. Two experimental replicates were performed using between three and five independent RNA extractions in each replicate.

2.5. Antioxidant Enzyme Activities

The measurement of ascorbate peroxidase (APX) (µmol H_2O_2 min^{-1} mg^{-1} protein), catalase (CAT) (µmol H_2O_2 min^{-1} mg^{-1} protein), glutathione reductase (GR) (µmol DTNB red min^{-1} mg^{-1} protein), and antioxidant enzymes activities was performed according to Elavarthi and Martin [53] using 200 mg of finely crushed leaf tissue from both rootstocks, 2247 × 6070–02–2 and Carrizo citrange, obtained from both B treatments. The extraction buffer used for the determination of antioxidant enzyme activities contained 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA).

Enzyme activities were referred to the protein content. The soluble protein was quantified employing 0.01 mL of extract and 1 mL of Coomassie brilliant blue G–250 (5 × diluted in water) (BioRad, Hong Kong), and the absorbance was recorded at 595 nm. A calibration line was prepared using bovine serum albumin (BSA) as a standard for proteins.

2.6. Phenol Determination

Total soluble content (µmol phenol g^{-1} FW) was determined similarly to that described by Ainsworth and MGillespie [54]. Phenol content was measured from a plant extract prepared in a 1:5 *w*/*v* ratio with 95% (*v*/*v*) methanol. Once the extract was centrifuged, measurements were carried out using 100 µL of the extract, to which 200 µL of the 50% (*v*/*v*) Folin-Ciocalteu reagent (PanReac Applichem ITW Reagent, Darmstadt, Germany) and 1.1 mL of 2% Na_2CO_3 (*w*/*v*) were added. The mixture was incubated in the dark for 2.5 h at 35 °C. After this incubation, the mixture was centrifuged at 13,000× *g* for 2 min and, finally, the absorbance was measured at 765 nm. A calibration line was prepared using caffeic acid as a standard for soluble phenolic compounds.

2.7. Malondialdehyde Determination

The generation of malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS) is significant for the existence of damage due to peroxidation of polyunsaturated fatty acids, and it is widely used as a marker of oxidative stress. The MDA production estimation assay (nmol g^{-1} FW) employs thiobarbituric acid (TBA), according to Hodges et al. [55]. Each extract was made using 2 mL of 80% (*v*/*v*) ethanol and 50 mg of finely crushed leaf, which was subsequently centrifuged at 13,000× *g* at 4 °C for 5 min. In the assay, 180 µL of the ethanolic extract obtained reacted with 420 µL of a solution containing 20% (*v*/*v*) trichloroacetic acid and 0.01% (*w*/*v*) PVPP. In parallel, another 180 µL of the obtained extract reacted with 420 µL of another solution containing 20% (*v*/*v*) trichloroacetic acid, 0.01% (*w*/*v*) PVPP, and 0.65% (*w*/*v*) thiobarbituric acid. Both mixtures were mixed by shaking and incubated at 95 °C for 30 min; once cooled, they were centrifuged at 13,000× *g* for 5 min at 4 °C. The absorbance values of both reaction samples were measured at 440, 532, and 600 nm, and calculations were carried out as indicated by the authors.

2.8. Statistical Analyses

All analytical determinations were carried out on leaves from three or five separate plants harvested randomly. Obtained data were subjected to two-way analysis of variance (ANOVA) using the SPSS v.29.0.1.0 software. Differences among means were evaluated using Tukey's Honestly Significant Difference test ($p < 0.05$). Data were obtained from a representative experiment that was repeated twice with very similar results.

3. Results

3.1. Boron Content and Expression Studies of CsXIP1;1 Gene in 2247 × 6070–02–2 and Carrizo Citrange Rootstocks Growth Under B Toxicity Conditions

Leaf B content was determined in 2247 × 6070–02–2 and Carrizo citrange rootstocks under both B treatments, sufficient (0.05 mM) and excess (2.5 mM) H_3BO_3. The lower leaf B concentration described in species tolerant to excess B under toxic conditions corresponds to that observed in 2247 × 6070–02–2 rootstock compared to Carrizo citrange (Figure 1A). Chlorotic spots at the distal apex in leaves were evident in Carrizo citrange plants after treatment with 2.5 mM B in contrast to 2247 × 6070–02–2 (Figure S1) [14–17]. This effect correlates with the higher B accumulation in Carrizo citrange under B toxicity (Figure 1A). Furthermore, expression levels of the *Citrus sinensis* X Intrinsic Proteins (*CsXIP1;1*) gene were analyzed in both rootstocks growing under B-sufficient and B-toxic conditions. 2247 × 6070–02–2 rootstock had higher expression levels of the *CsXIP1;1* gene in both B treatments compared to Carrizo (Figure 1B).

3.2. Analysis of the B Toxicity Effect on Photosynthesis-Related Parameters in 2247 × 6070–02–2 and Carrizo Citrange Rootstocks

Photosynthetic and fluorescence parameters were measured in 2247 × 6070–02–2 and Carrizo citrange rootstocks under B-toxic conditions compared to those grown in control medium. The higher value of net photosynthesis in 2247 × 6070–02–2 rootstock with respect to Carrizo citrange rootstock under toxicity conditions is noteworthy (Figure 2A). No statistically significant differences were found in transpiration rates between Carrizo citrange and 2247 × 6070–02–2 rootstock under toxicity conditions (Figure 2B). In addition, a decrease in the maximum efficiency of PSII (Fv'/Fm') was observed in Carrizo citrange with 2.5 mM B, while the values remained high and similar to the control condition in the 2247 × 6070–02–2 rootstock (Figure 2C). These results would agree with the chlorophyll values obtained in conditions of excess B, where chlorophyll contents under B toxicity were higher in 2247 × 6070–02–2 with respect to Carrizo citrange (Figure 2D–F).

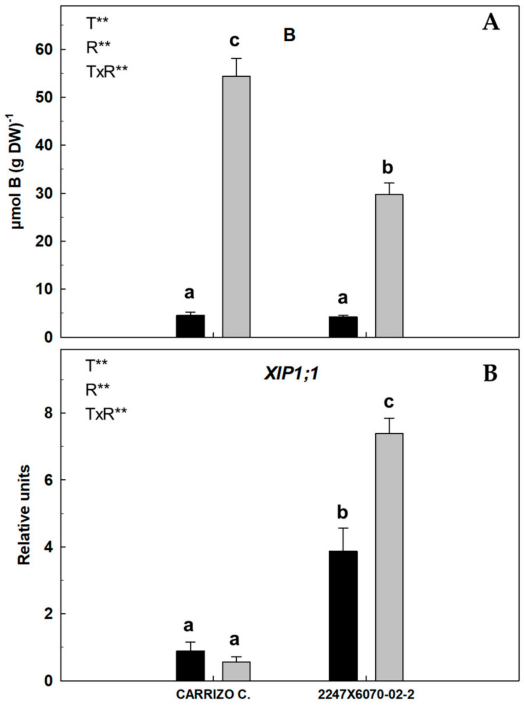

Figure 1. Total B determination and quantitative real-time PCR expression analysis of *CsXIP1;1* transcript levels. (**A**) Boron content and (**B**) quantitative real-time PCR expression analysis of *CsXIP1;1* transcript levels were measured in leaves of rootstock plants in control treatment (0.05 mM H_3BO_3, black bars) and toxicity treatment (2.5 mM H_3BO_3, grey bars) after four weeks. For more details, see Materials and Methods. Results are presented as means ± SD (n = 3–5 separate plants) and are from a representative experiment that was repeated twice with very similar results. Different letters indicate statistically significant differences between rootstock and B treatments according to Tukey's HSD test ($p < 0.05$). Significant code: ** = $p < 0.001$; T: boron treatment, R: citrus rootstock; T × R: interaction between boron treatment and citrus rootstock.

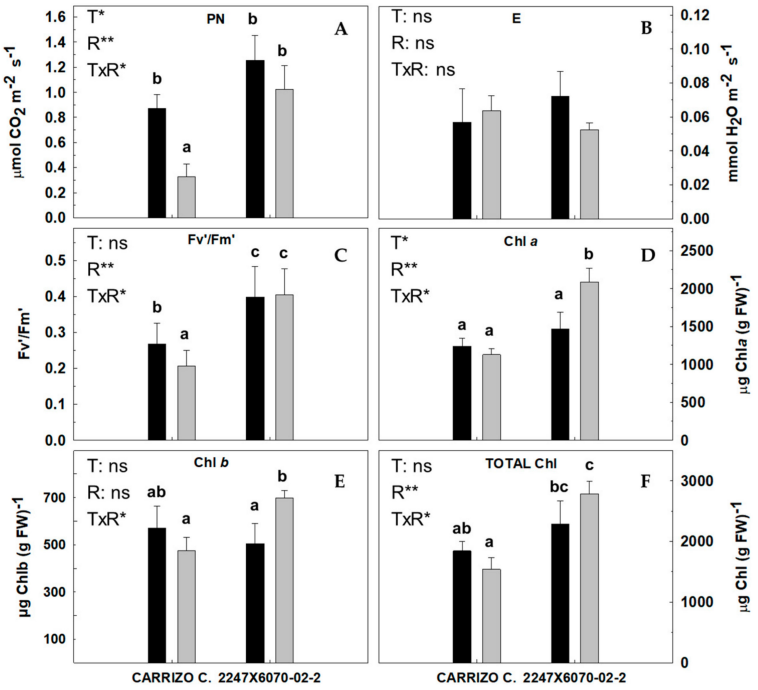

Figure 2. Photosynthetic parameters and chlorophyll determination. Net photosynthetic CO_2 assimilation, PN, µmol CO_2 m^{-2} s^{-1} (**A**); transpiration rate, E, mmol H_2O m^{-2} s^{-1} (**B**); maximum

photochemical efficiency Fv′/Fm′ (**C**); chlorophyll a, µg *Chla* g^{-1} FW (**D**), chlorophyll b, µg *Chlb* g^{-1} FW (**E**), and total chlorophyll, µg *Chl* g^{-1} FW (**F**) contents were measured in leaves of rootstock plants in control treatment (0.05 mM H_3BO_3, black bars) and toxicity treatment (2.5 mM H_3BO_3, grey bars) after four weeks. For more details, see Materials and Methods. Results are presented as means ± SD (n = 3–5 separate plants) and are from a representative experiment that was repeated twice with very similar results. Different letters indicate statistically significant differences between rootstock and B treatments according to Tukey's HSD test ($p < 0.05$). Significant code: ** = $p < 0.001$, * = $p < 0.05$ and ns = no significant; T: boron treatment, R: citrus rootstock; T × R: interaction between boron treatment and citrus rootstock.

3.3. Evaluation of Antioxidant Protective Mechanisms in 2247 × 6070–02–2 and Carrizo Citrange Rootstocks Grown Under B Toxicity Conditions

B toxicity leads to increased ROS levels that generate the activation of antioxidant mechanisms, largely in tolerant plants. Detoxification mechanisms of ROS include the participation of antioxidant molecules, such as phenols, as well as antioxidant enzymes [7,56]. The increase in phenolic content under conditions of B toxicity is characteristic in tolerant species. Leaf values obtained in 2247 × 6070–02–2 rootstock were higher than those of Carrizo citrange (Figure 3A). In addition, malondialdehyde (MDA) was also measured as an indicator of membrane lipid peroxidation. The higher phenol concentrations obtained in excess B conditions had a good correspondence with the lower MDA values in 2247 × 6070–02–2 rootstock under excess B condition compared with Carrizo citrange (Figure 3B).

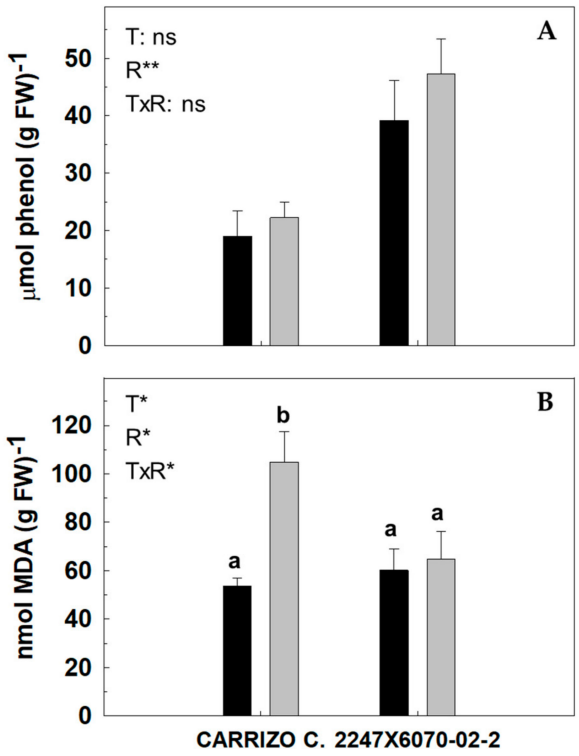

Figure 3. Phenol and malondialdehyde (MDA) determination. Phenol (**A**) and malondialdehyde (MDA) (**B**) contents were measured in leaves of rootstock plants in control treatment (0.05 mM H_3BO_3, black bars) and toxicity treatment (2.5 mM H_3BO_3, grey bars) after four weeks. For more details, see Materials and Methods. Results are presented as means ± SD (n = 3–5 separate plants) and are from a representative experiment that was repeated twice with very similar results. Different letters indicate statistically significant differences between rootstock and B treatments according to Tukey's HSD test ($p < 0.05$). Significant code: ** = $p < 0.001$, * = $p < 0.05$ and ns = no significant; T: boron treatment, R: citrus rootstock; T × R: interaction between boron treatment and citrus rootstock.

This result would indicate less damage from the peroxidation of membrane lipids in 2247 × 6070–02–2 rootstock. Increased ROS generated under B toxicity would also be detoxified by the involvement of antioxidant systems including different enzymes, such as ascorbate peroxidase, catalase, or glutathione reductase. Interestingly, the activities of ascorbate peroxidase and glutathione reductase were higher in the 2247 × 6070–02–2 rootstock under B toxicity and showed the same trend for catalase and glutathione reductase, although without significant statistical differences (Figure 4A–C). The greater activity of these enzymes would give 2247 × 6070–02–2 better tolerance to B toxicity by having a greater capacity to scavenge ROS.

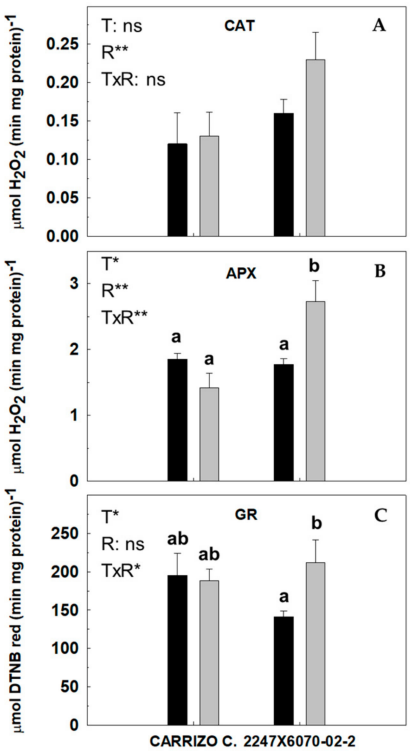

Figure 4. Antioxidant enzyme activities. Ascorbate peroxidase (APX) (**A**), catalase (CAT) (**B**), and glutathione reductase (GR) (**C**) activities were measured in leaves of rootstock plants in control treatment (0.05 mM H_3BO_3, black bars) and toxicity treatment (2.5 mM H_3BO_3, grey bars) after four weeks. For more details, see Materials and Methods. Results are presented as means ± SD (n = 3–5 separate plants) and are from a representative experiment that was repeated twice with very similar results. Different letters indicate statistically significant differences between rootstock and B treatments according to Tukey's HSD test ($p < 0.05$). Significant code: ** = $p < 0.001$, * = $p < 0.05$ and ns = no significant; T: boron treatment, R: citrus rootstock; T × R: interaction between boron treatment and citrus rootstock.

4. Discussion

Transpiration drives B movement through the xylem, generating B accumulation in leaves. Moreover, B mobility through the phloem can occur by forming B-sugar alcohol complexes, as has been described in "Newhall" navel oranges (*Citrus sinensis*) grafted on trifoliate orange (*Poncirus trifoliata*) plants [7,19,34]. It has been described that a decrease in transpiration rate under B toxicity leads to lower leaf B accumulation [11]. In agreement with this, a likely trend (although not statistically significant) of lower transpiration rate was observed in 2247 × 6070–02–2 compared to Carrizo citrange rootstock under the B excess condition (Figure 2B), which would contribute to explain the lower leaf B concentration in 2247 × 6070–02–2 (Figure 1A). These results are consistent with those described by other authors [12,32,57]. This decrease in B accumulation would also result from changes in the appropriate translocation of B [26,58]. Thus, the *CsXIP1;1* gene in

2247 × 6070–02–2 rootstock had an increased expression under conditions of excess B, and not in Carrizo citrange (Figure 1B). This gene belonging to the XIP subfamily is the least characterized of the Major Intrinsic Proteins (MIPs) family and its physiological function is not well defined [59]. NtXIP1;1 was the first protein characterized as a boric acid channel with plasma membrane localization, and expressed in both roots and leaves in *Nicotiana tabacum* [58,60]. The higher expression of the *CsXIP1;1* gene found in 2247 × 6070–02–2 rootstock in B excess (Figure 1B) correlates with the upregulation pattern of *CsXIP1;1* in leaves of sweet oranges (*Citrus sinensis* L. Osb.) under drought and salt stresses [56]. Blast analyses of *CsXIP1;1* showed 76.18% and 68.61% identity values compared to another *CsXIP* gene (*CsXIP1;2*) [61] and *NtXIP1;1* [58,60], respectively (Table S2, Figure S2). Recently, a member of the XIP family, *AmXIP1;2*, has been described in *Avicennia marina*. The transformation of yeasts with this gene led to increased sensitivity to boric acid, suggesting its potential role as a boric acid channel [62]. In addition, overexpression studies of *XIP1;1* in *Nicotiana tabacum* and Arabidopsis generated effects of B deficiency in plants and lower B content in leaves compared to control plants [58]. Furthermore, the B concentration in the xylem of *NtXIP1;1*-overexpressing plants was lower as a consequence of a possible impairment in B translocation [58]. These authors proposed that NtXIP1;1 may play a role in the extrusion of B or its redistribution at the periphery of the stem or young leaves, or in the subcellular partitioning of B between the cytoplasm and the apoplast [58]. Similar to what was described in *NtXIP1;1*-overexpressing plants, higher *CsXIP1;1* expression and lower B content in leaves were also observed in the 2247 × 6070–02–2 rootstock under conditions of B toxicity (Figure 1A,B). Therefore, it could be hypothesized that the deregulation of *CsXIP1;1* gene expression in 2247 × 6070–02–2 rootstock compared with Carrizo citrange would significantly modify B translocation and accumulation, promoting B's exit through the leaves (Figure 1A,B). Hence, CsXIP1;1 together with the lower transpiration rate (Figures 1B and 2B) would act as part of the protective mechanisms against excess B in the 2247 × 6070–02–2 rootstock.

Generally, B toxicity affects the photosynthesis process and causes damage in chlorophylls and chloroplasts [7,16,63]. Under B toxicity, Simón-Grao et al. [32] reported a lower value of maximum PSII efficiency Fv'/Fm' in the Carrizo citrange rootstock compared to other rootstocks, such as sour orange or *Citrus macrophylla*. Under toxicity conditions, the higher values of net photosynthesis, Fv'/Fm', and contents of chlorophyll a and b (Figure 2A,C–E) of the 2247 × 6070–02–2 rootstock with respect to Carrizo citrange would indicate a higher B toxicity tolerance of this rootstock similar to that proposed in citrus [32] and maize [12]. In addition, toxic B levels produce reactive oxygen species (ROS) derived from the excess of photons that have not been used in the photosynthetic electron transport chain [56]. Antioxidants control the excess of ROS generated in response to B stress, and stress tolerance may be the result of an enhanced antioxidant defense that depends on the activity of certain antioxidant enzymes and the action of different antioxidant compounds, such as phenols [7,36]. Thus, 2247 × 6070–02–2 rootstock had higher activity of the antioxidant enzymes under B toxicity (Figure 4). The improved antioxidant defense in 2247 × 6070–02–2 rootstock would also be supported by a higher phenol content in comparison with Carrizo citrange (Figure 3A). Malondialdehyde content was hardly increased by B toxicity in 2247 × 6070–02–2 rootstock with respect to Carrizo citrange, indicating less peroxidation damage (Figure 3B) [53,64]. These facts suggest that 2247 × 6070–02–2 rootstock improved its tolerance to B toxicity by having a greater capacity to scavenge ROS.

5. Conclusions

In conclusion, the novel 2247 × 6070–02–2 rootstock, described as not very sensitive to HLB, presents better characteristics of tolerance to B toxicity than Carrizo citrange. The lower B content, higher expression of the *CsXIP1;1* gene, enhanced net photosynthesis, Fv'/Fm', and chlorophyll values, along with the lower transpiration rate and improved antioxidant defense mechanisms shown by 2247 × 6070–02–2, would support that this rootstock is suitable for growing on high B media.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14112741/s1, Figure S1: Representative images of citrus rootstock plants grown under hydroponic conditions after 4 weeks of B treatment; Figure S2: Alignments of sequence of CsXIP1;1 (XM_052432608.1), CsXIP1;2 (MK084820.1), NtXIP1;1 (HM475295.1) transcript genes. Point indicates highly conserved nucleotides with CsXIP1;1 (XM_052432608.1). The multiple alignments were carried out using the NCBI BLASTN program; Table S1: List of primers used for quantitative real-time PCR analyses. Citrus primers used in quantitative RT-PCR analyses are listed; Table S2: Results of BLASTN analysis. Blast analysis was performed using the sequence of *Citrus sinensis* CsXIP1;1 gene (XM_052432608.1) as a query sequence using nucleotide collection (nt/nr) database (https://www.ncbi.nlm.nih.gov; accessed on 8 May 2024).

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