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Response of three citrus genotypes used as rootstocks grown under boron excess conditions

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ABSTRACT

In citrus, the effects of an excess of boron (B) are conditioned by the type of rootstock. In the present work, the morphological, physiological and biochemical responses of seedlings from three citrus genotypes, commonly used as rootstocks in citriculture. In particular, Citrange Carrizo (CC), *Citrus macrophylla* (CM) and sour orange (SO) seedlings were treated with an excess of B (10 mg L^{-1}) in the nutrient solution in order to determine the relative tolerance and to understand the possible mechanisms that make a rootstock more tolerant than the others. To assess these responses, different parameters were measured in plants, such as vegetative growth, B concentration in leaves, stems and roots, gas exchange and chlorophyll fluorescence, the concentration of osmolytes and the activity of enzymes related to the antioxidant system. The results showed, according to the growth parameters, that the SO rootstock was the most tolerant to an excess of B; while CC was the most sensitive. This result was due to the fact that SO plants accumulated less B in leaves, as its roots have a great capacity of restricting the uptake and transport of B towards the aerial part. Moreover, SO is suggested to diminish B toxicity risk through its antioxidant system, since it presented high activity of ascorbate peroxidase (APX) and superoxide dismutase (SOD), as well as high accumulation of quaternary ammonium compounds (QACs).

1. Introduction

Boron (B) is a micronutrient that is needed by higher plants for their main physiological functions involved in their growth and development. However, toxicity caused by an excess of this nutrient can be found in arid or semiarid regions, in which waters high in B concentration are used (Dorta-Santos et al., 2016). The addition of water from seawater desalinating plants and urban wastewater treatment plants is common in areas with a Mediterranean climate, where good-quality water resources are scarce. These non-conventional water sources can have excessive concentrations of B for sensitive crops, such as in the case of citrus plants, where a concentration above 0.3 mg L^{-1} is considered to be the threshold of toxicity (Grattan, 2013). With the use of this type of water for irrigation, phytotoxicity problems can arise, which give way to the loss of good agronomic performance of the crops (Sotiropoulos et al., 1999; Gunes and Alpaslan, 2000).

The most common symptom found in plants exposed to high concentrations of B, for crops that have a low B mobility (e.g. citrus), is the appearance of the chlorosis and/or burns on the edges and tips of the more mature leaves (Gimeno et al., 2012). Tolerance to B has been associated, among other factors, to the ability of the plant to restrict B uptake through the roots, and its subsequent transport to the aerial parts of the plant (Reid, 2010), which allows for the maintenance of a B concentration that is below its toxic values. The absorption of B through the roots is regulated by three transport mechanisms across the plasma membrane: passive diffusion of boric acid, facilitated diffusion of boric acid via channels, and export of the borate anion via passive and/or active transporters. (Yoshinari and Takano, 2017). Under boron-limiting conditions, boric acid channels and borate exporters function in the uptake and translocation of B to support growth of various plant species, while borate exporters act under conditions of excess B. Once the B enters the roots, it is transported by the transpiration stream towards the upper part of the tree (Papadakis et al., 2004a; Chatzissavvidis and Therios, 2011), so that all the factors that influence transpiration (weather conditions, genotypes, etc.) will play an important role in the crop's tolerance to B. On the other hand, besides the concentration of B that accumulates in the tissues, the different plant's tolerances to B is determined by the toxic effects this element exerts at

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the cellular, vascular, physiological and metabolic levels, as described by Princi et al. (2016). This toxicity can be dampened by different factors such as the compartmentation of B into the vacuole, the insolubilization and deposition of B in the cell wall, and/or the induction of antioxidant systems (Princi et al., 2016). When plants experience some type of stress, such as B toxicity, the creation of reactive oxygen species (ROS) can occur. The effects of ROS can be diverse, such as the inhibition of enzymes, degradation of photosynthetic pigments, lipid peroxidation of cellular membranes, and DNA fragmentation (Das and Roychoudhury, 2014). The ROS produced within cells can be eliminated by diverse antioxidant systems, through the action of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and the ascorbate-glutathione cycle (ASA-GSH). One of the parameters used to evaluate the damage caused by the oxidative stress is malondialdehyde (MDA) concentration. In addition, the compatible solutes (proline, quaternary ammonium compounds, and carbohydrates) have also an important role in the adaptation mechanism of the plants to abiotic stresses, including boron toxicity, where its functions vary from species to species and even within plants species (Siddiqui et al., 2013; Chen et al., 2012). This is due to compatible solutes can lower or balance the osmotic potential within cells and it can also act as an antioxidant and a source of energy (Marco et al., 2015; Rejeb et al., 2014).

In citrus, rootstock genotypes play a fundamental role in the tree's tolerance to abiotic stresses, including drought, cold, salinity and alkalinity. Among common commercial citrus rootstocks, sour orange (*C. aurantium* L.; SO) is considered salt and drought tolerant relative to Citrange Carrizo (*Citrus sinensis* × *Poncirus trifoliata*; CC) which is considered sensitive; while *Citrus macrophylla* (CM) is considered to have an intermediate tolerance between SO and CC (Syvertsen and Garcia-Sanchez, 2014; Ribeiro et al., 2014). The greater tolerance of SO and CM to salinity and drought as compared to CC is mainly due to the former having a high water use efficiency (WUE) and a greater capacity for restricting the entrance and transport of Cl^- and/or NA^+ from the root to the aerial part of the plant (Balal et al., 2012; Fernandez-Ballester et al., 2003; Syvertsen et al., 2010). As for the responses of these rootstocks to B, they have hardly been studied. Thus, the objective of the present study is so evaluate the morphological, physiological and biochemical responses of seedlings of the citrus rootstocks Citrange Carrizo (CC), *Citrus macrophylla* (CM) and sour orange (SO) to an excess of B (10 mg L^{-1}), in order to identify the more tolerant rootstock, as well as the mechanisms/effects that determine this tolerance. More specifically, the relationship between absorption and transport of B from the root to the aerial part of the plant and the concentration of B in the leaves will be studied, as well as the toxic effects provoked by B on the physiological and biochemical processes of these plants.

2. Materials and methods

2.1. Plant materials and experimental conditions

In this study, three-month old plants of the rootstocks Citrange Carrizo (*Poncirus trifoliata* [L.] × *Citrus sinensis* [L.]), *Citrus macrophylla* and sour orange (Citrus aurantium L.) were used, which were acquired from a commercial nursery (Viveros Torreblanca S.L.). The seedlings were grown in 7 L pots with a fine-grained universal substrate (a mix of white and black peat, coconut fiber and perlite, 5:4:1; Projar, Spain). The experiment was conducted in a multi-tunnel-type greenhouse, with the following climactic conditions: maximum photosynthetically-active radiation (PAR) of 1000 mmol m^{-2} s^{-1}, day/night temperature of 35/18 ± 3 °C, day/night relative humidity of 65/85 ± 5% and a 16-h photoperiod. The plants were watered 3 times per week, with enough water to produce a drainage of 15% of the total volume applied. The plants were irrigated with a complete nutrient solution with the following composition of macronutrients (mM): 20 N, 0.75 P, 4.2 K and 6 Ca; and micronutrients (μM): 23 B, 2 Mn, 2 Zn, 0.5 Cu, 0.5 Mo and 20 Fe. Two months after the plants were transplanted to the greenhouse pots, they were divided into two groups per rootstock. One of the groups was watered with the previously-mentioned nutrient solution. The 0.25 mg L^{-1} B concentration was considered to be the control, as this was an adequate B concentration for normal plant growth (Hoagland and Arnon, 1950). The other group of plants was watered with nutrient solutions containing 10 mg L^{-1} of B (equivalent to 925 μM). In any treatment, B was applied as boric acid (H_3BO_3). The B treatments were applied for 120 days during August–November (2015), after which, the plants were harvested. For each rootstock and B treatment, there were 12 randomly-distributed seedlings in the greenhouse, in a surface area of 30 m^2.

2.2. Plant growth analysis, and boron transport and uptake

In the days 0, 45, 90 and 120 after starting the experiment, three plants per treatment were harvested, and the leaves, stem and roots were separated and weighed. The tissues were briefly rinsed with deionized water, oven-dried at 60 °C for at least 48 h, weighed and ground to a fine powder.

The dry masses of the leaves, stem and roots were used to calculate the total plant dry mass and the relative growth rate (RGR; Evans, 1972). The RGR is the increase in dry weight per unit of initial dry weight, and was calculated by the following equation (Fernandez-Ballester et al., 2003):

$$RGR = \frac{LnW_2 - LnW_1}{t_2 - t_1}$$

where W_1 and W_2 are the dry mass of the plants harvest times t_1 and t_2, respectively, expressed in g g^{-1} day^{-1}. The RGR is factored into two components: the mass-based net assimilation rate, NAR_m (mg g^{-1} day^{-1}), which is the increase in plant biomass per unit leaf mass and time, and the leaf mass fraction, LMF (g g^{-1}), which is the ratio between leaf mass and the plant dry weight.

$$\mathrm{RGR} = \mathrm{NARm} \times \mathrm{LMF} = \left(\frac{(W2-W1)(LnLw2-LnLw1)}{(t2-t1)(Lw2-Lw1)}\right)\left(\frac{\left(\frac{Lw1}{w1}\right)+\left(\frac{Lw2}{w2}\right)}{2}\right)$$

Where Lw_1 and Lw_2 are the dry mass of the leaves harvest times t_1 and t_2, respectively. The relationships between the various growth parameters and RGR were tested with GRC_x (Poorter and van der Werf, 1998). The growth response coefficients (GRC) value of a growth parameter is calculated as the ratio between the changes in that growth parameter and RGR. This is a simple way to express the relative importance of each of the underlying growth parameters (X = NAR_m or LMF) covarying with the variation in RGR.

$$\mathrm{GRCx} = \frac{Ln(X)control - Ln(X)B}{Ln(RGR)control - Ln(RGR)B}$$

GRC_x equals to 1 if a proportional change in RGR is only due to a similar proportional change in growth parameter X, and zero if X remains constant and the change in RGR is only due to changes in other growth parameter.

In all plant tissues, the B concentration was determined by inductively coupled plasma emission optical spectrometry (Iris Intrepid II, Thermo Electron Corporation, Franklin, USA) after an acid digestion in HNO_3:H_2O_2 (5:3 by volume) in a microwave that reached 190 °C in 20 min and held this temperature for 2 h (CEM Mars Xpress, North Carolina, USA). From this, net uptake rate and transport (μmol $g^{-1}_{root\ DW}$ day^{-1}) to the shoot were calculated as described by Pitman (1988):

$$Nutrient\ uptake = \left[\frac{LnR_2 - LnR_1}{R_2 - R_1}\right] \times \left[\frac{C_{T2} - C_{T1}}{t_2 - t_1}\right]$$

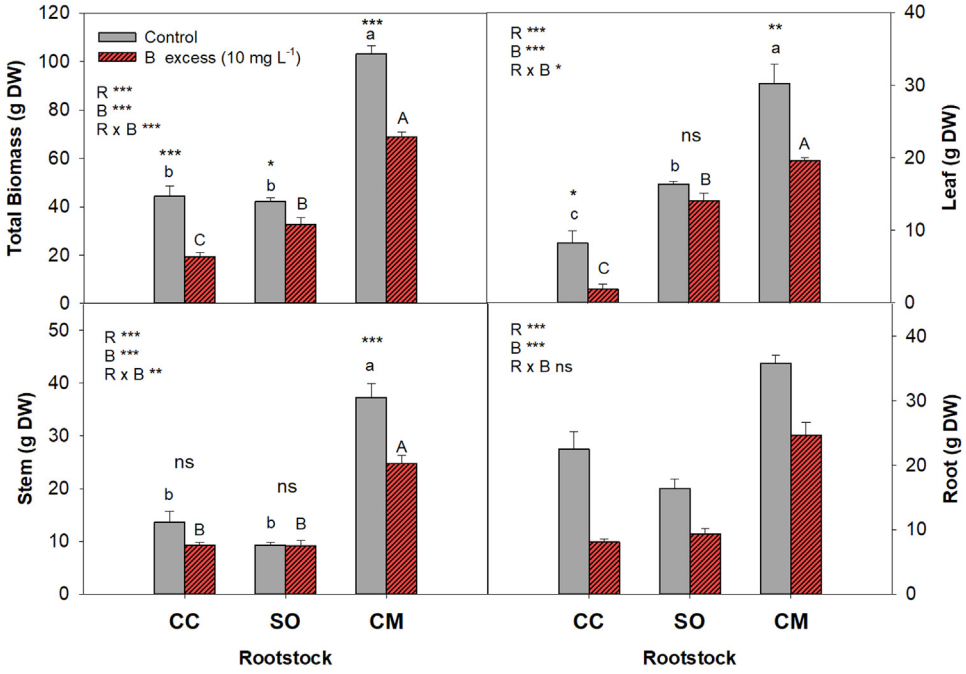

Fig. 1. Effects of an excess of B in the nutrient solution on total dry, leaf, stem and root biomass of the rootstocks Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) at 120 days of the experiment. "*ns*" indicates non-significant differences at 95%; and *, ** and *** indicates significant differences at $P < 0.05$, 0.01 and 0.001, respectively, in the two-way ANOVA Rootstock x Boron treatment, and in the single factor ANOVA for each rootstock. For each B treatment, the *lower-case letters* (control) and *upper-case* letters (B excess) indicate significant differences between rootstocks at $P < 0.05$ as established by Duncan's multiple-range test. The vertical bar indicates the standard error of the mean ($n = 4$).

$$Transport = \left[\frac{LnR_2 - LnR_1}{R_2 - R_1}\right] \times \left[\frac{C_{S2} - C_{S1}}{t_2 - t_1}\right]$$

Where R is root dry mass, C_T is total B content of the whole plant and C_S is the shoot content of the B under consideration at two consecutive harvests (t_1 and t_2).

2.3. Leaf gas exchange and chlorophyll fluorescence parameters

The net assimilation of CO_2 (ACO_2) and leaf transpiration (E_{leaf}) were measured at 0, 45, 90 and 120 days in the same plants that were then harvested, using a portable photosynthesis system (model CIRAS-2, PP-System, Amesbury, MA, USA). During the measurements, the equipment was set to maintain the light intensity (PAR: μmol m^{-2} s^{-1}), and the concentration of CO_2 (400 ppm) constant in the measurement chamber. The chlorophyll fluorescence parameters were also measured with a pulse-modulated fluorometer (model FMS-2; Hansatech, King's Lynn, Norfolk, England) on leaves similar to those used for the gas exchange parameters. The chlorophyll fluorescence parameters that were measured were: the quantum efficiency of PSII, ΦPSII = (Fm′-Fs)/Fm′; the antennae efficiency of PSII, Fv′/Fm′ = (Fm′-F_0′)/Fm′; and the photochemical quenching co-efficient, qP = (Fm′-Fs)/(Fm′-F_0′), where Fs is the steady-state fluorescence yield, Fm' is the maximal value when all reaction centers are closed after a pulse of saturating light (12,000 μmol m^{-2} s^{-1} for 0.8 s), and F_0′ is the minimal fluorescence in the light-adapted state that is obtained by turning off the actinic light temporarily and applying a pulse of far-red light (735 nm) to drain the electrons from PSII.

2.4. Proline and quaternary ammonium compounds determination

At the end of the experiment, proline was extracted from dry leaf tissue with sulfosalicylic acid (3%) and quantified according to the protocol described by Bates et al. (1973). Quaternary ammonium compounds (QAC) were extracted from dry tissue with 1 M H_2SO_4 and were quantified using a glycine-betaine standard according to the method described in Grieve and Grattan (1983).

2.5. Carbohydrate determination

The carbohydrate concentration in the middle leaf was also analyzed at the end of the experimental period. The extraction of the carbohydrates was carried out using 80% ethanol with constant mixing for 30 min. A sulphuric acid assay with the anthrone reagent was conducted to measure the total soluble carbohydrates. The procedure that was used for measuring the content of starch in the plant tissues included an extraction from the pellet with MES solution and gelatinization using a heat-stable alpha amylase (Haissig and Dickson, 1979). Soluble sugars and starch were quantified using glucose as a standard.

2.6. H_2O_2 and MDA determination

At the end of the experiment, before the total harvest of the plants, leaves were frozen in liquid nitrogen to conduct the oxidative stress study. The quantification of H_2O_2 was conducted following the method described by Yang et al. (2007), and using trichloroacetic acid (TCA) as the extracting agent. Lipid peroxidation was determined by measuring malondialdehyde (MDA), using the method by Hodges et al. (1999). Lastly, the enzymatic activity of the following enzymes were measured: catalase (CAT; Chance and Maehly, 1955), ascorbate peroxidase (APX; Nakano and Asada, 1987), and superoxide dismutase (SOD; McCord and Fridovich, 1969).

2.7. Statistical analysis

The statistical analyses included a two-way analysis of variance using ANOVA (SPSS statistical package, Chicago, IL, USA) with three rootstocks (CC, CM and SO) × two B treatments (0.25 and 10 mg L^{-1} B) for each sampling date. When the variables were significant ($P < 0.05$), the treatment means were separated using Duncan's multiple range test.

3. Results

3.1. Plant growth and allometric study

At the end of the experiment, the vegetative growth parameters in control conditions showed that the CM plants had a vegetative growth

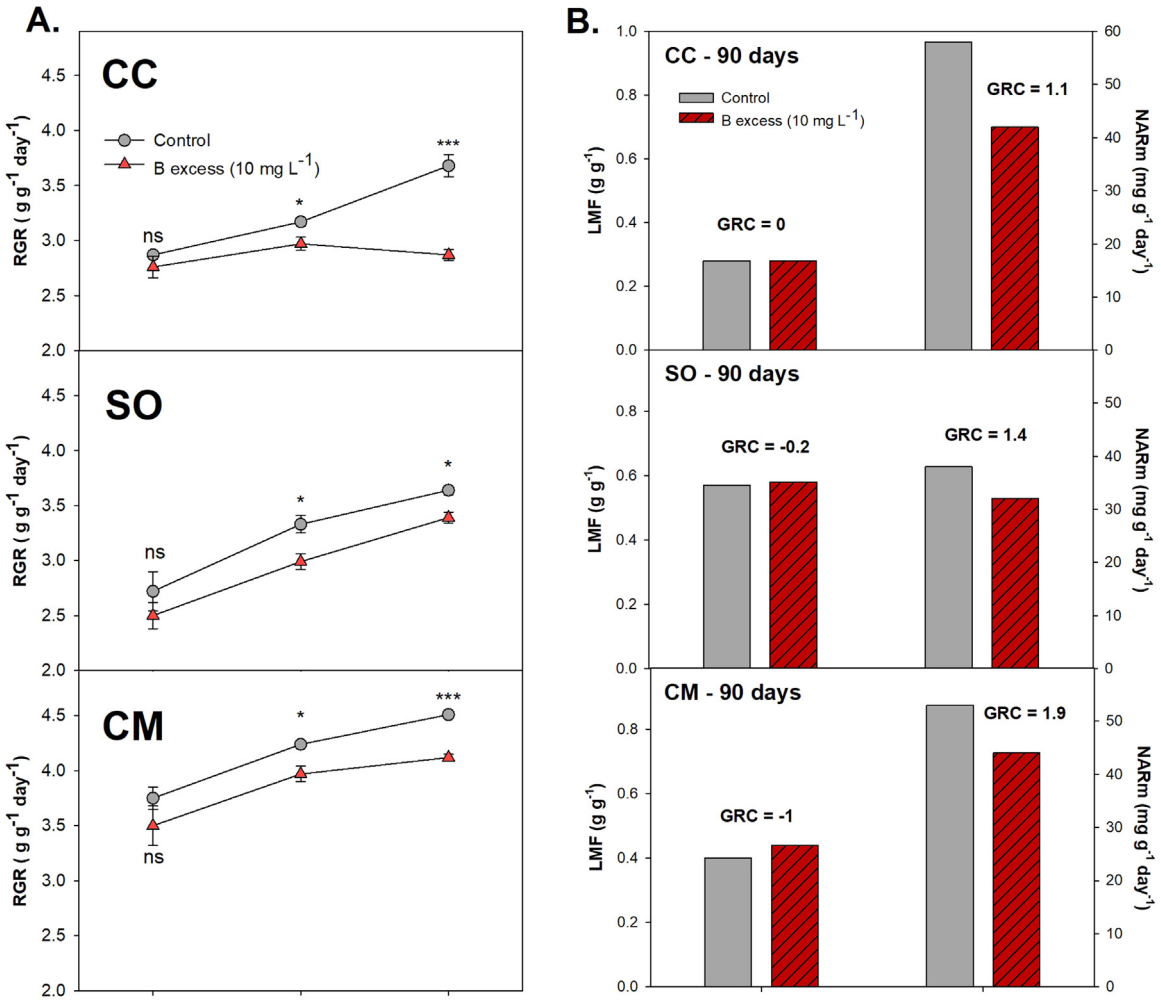

Fig. 2. A. Changes in the relative growth rate (RGR) in response to an excess of B of the rootstocks Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) during the 120 days of the experiment. "*ns*" indicates non-significant differences at 95%; * and *** indicate significant differences at $P < 0.05$ and 0.001, respectively, among the treatments. The vertical bar indicates the standard error of the mean ($n = 4$). **B.** Effect of the excess of B at 90 days of the treatment on the RGR: Leaf Mass Fraction (LMF), mass-based Net Assimilation Rate (NARm) and their growth response coefficients (GRC_{LMF} and GRC_{NARm}).

of 2.3 times higher than CC and SO (Fig. 1). The excess of B decreased the dry biomass of all three rootstocks, although this decrease was greater in CC plants (56% as compared to the control plants) than SO (23%) and CM (33%). In the case of the CC rootstock, the reduction of the total dry biomass was due to a decrease in leaf (77%) and root (64%) biomass, while the stem was not affected by the excess of B. For CM plants, this reduction was due to the decrease in growth of the aerial part (leaves and stem, 34%) as well as the root (31%). For SO, the plants were only significantly affected in the root growth (43%). These results evidenced the different susceptibilities to an excess of B shown by the different plants and their different plant tissues as well. It also showed that the three rootstocks studied had different tolerances to an excess of B.

The harvesting of the plants at different dates during the experimental period allowed for the calculation of the relative growth rate (RGR) and its components net assimilation rate (NARm) and leaf mass fraction (LMF). Throughout the experiment, the control plants (0.25 mg L^{-1}) and those treated with an excess of B (10 mg L^{-1}), increased their RGR as compared to the value recorded at 45 days (Fig. 2. A). However, the RGR values obtained at days 90 and 120 were more significant for the control plants than for the plants treated with and excess of B, with the greatest differences between treatments observed at 120 days. The plants that were more affected were CC plants, followed by the CM ones. The SO plants had a RGR that was less affected by the treatment with B. At 90 days, the NARm, LMF and GRC parameters were also calculated, which indicated that the reduction of RGR in the three rootstocks were due to the reduction of NARm, more than the changes in LMF (Fig. 2. B).

3.2. Concentration and distribution of B in plant tissues

The use of the nutrient solution with an excess of B (10 mg L^{-1}) for irrigation led to a gradual increase of the B concentration in leaves throughout the experiment, independent of the rootstock (Fig. 3. A). The CC plants also had the greatest concentration of B, except at the end of the experiment (120 d), where the values for SO and CM were equalized. At 90 days, the three rootstocks had the greatest concentration, with CC having the highest value (626.5 mg B kg^{-1} dw). There were no significant differences between SO and CM in any sampling date.

As for the B concentration in stems at the end of the experiment, (120 d), it was observed that all the rootstocks accumulated B in this tissue, due to the excess of B in the nutrient solution; with CM being the rootstock that accumulated the most, followed by CC and SO (Fig. 3. B). Lastly, the concentration of B in roots (B_{root}) results at the end of the experiment showed that SO and CM had an increase of this element in this tissue that was almost 4 times higher than their respective controls, while CC had a concentration that was almost 3 times higher (Fig. 3. B).

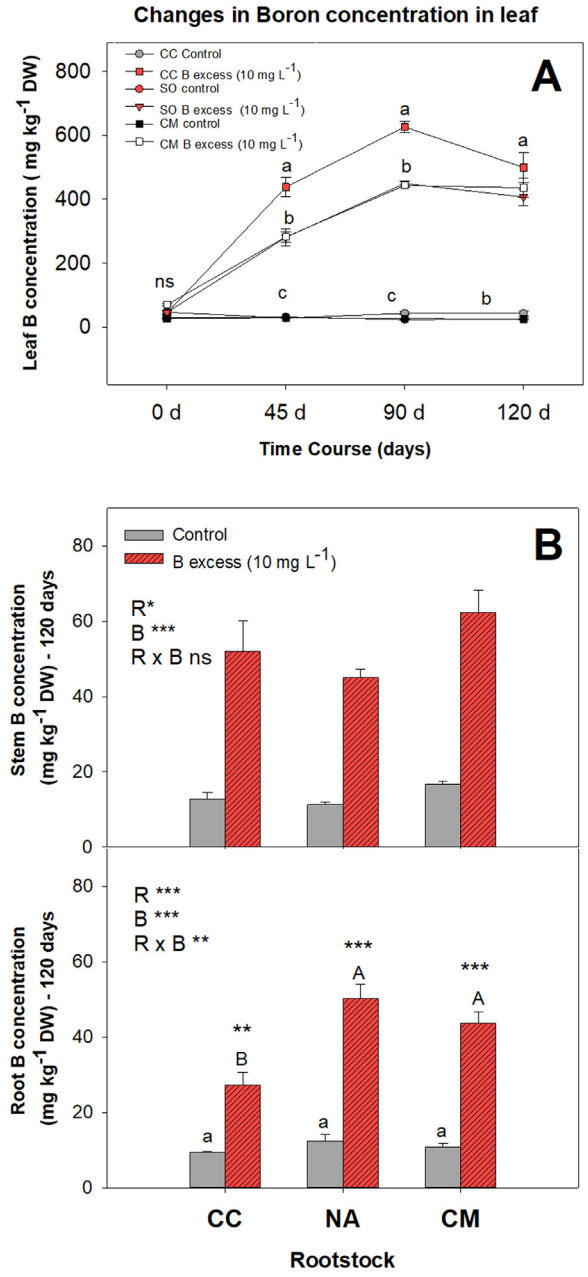

Fig. 3. A. Changes in the concentration of B_{leaf} as a response to an excess of B in the rootstocks Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) during the 120 days of the experiment. "*ns*" indicates non-significant differences at 95%; for each sampling date, the different *lower-case letters* indicate significant differences among rootstocks at $P < 0.05$ as established by Duncan's multiple-range test. The vertical bar indicates the standard error of the mean ($n = 4$). **B:** Effects of the excess of B on the concentration of B in stems and roots of Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) during the 120 days of the experiment. "*ns*" indicates non-significant differences at 95%; while *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, in the two-way ANOVA rootstock x Boron treatment, and in the one-way ANOVA of each rootstock. For each B treatment, the *lower-case letters* (control) and *upper-case letters* (B excess) indicate significant differences between the rootstocks for $P < 0.05$ as established by Duncan's multiple-range test. The vertical bar indicates the standard error of the mean ($n = 4$).

3.3. Boron uptake and transport in the plant

With respect to the absorption of B by the roots and the subsequent transport of this nutrient from the root to the aerial part, Fig. 4 shows the data from the intervals 0–45 and 45–90 days. The data from the period 90–120 days are not shown due to drastic defoliation suffered by CC (defoliation of approximately 50%), and due to the decrease in growth of the aerial part of CM. In the period comprised by 0–45 days, the results of the control plants showed that SO had the greatest absorption and transport of B was SO (4.52 and 4.01 μmol g^{-1} ps_{root} day^{-1}, respectively), followed by CC and CM, which did not show significant differences between them for any of the two parameters. Excess of B in the nutrient solution led to significant increase in the absorption and transport of B in the three rootstocks with the greatest increase found in the CC followed by CM and lastly, by SO (Fig. 4). In the 45–90 day interval of the experiment, there was a similar response as the period 0–45 days in the treatment with excess of B; while in this sampling date, in the control treatment, the greatest absorption as well as the transport was observed in CC plants (Fig. 4).

3.4. Leaf gas exchange parameters and chlorophyll fluorescence parameters

Starting at 45 days of the experiment, a decrease of ACO_2 can be observed in all three rootstocks watered with 10 mg L^{-1} B, as compared to the control plants, with the greatest decrease found in CM as compared to the other two rootstocks (Fig. 5). At 90 days of the experiment, a slight recovery could be observed in all the rootstocks, but these values dropped again at 120 days, except for the CM rootstock, so that the decrease in ACO_2 was 82% in CC and 50% in the other two rootstocks. The E_{leaf} data showed a decrease through time with excess of B in CC, a decrease only at 120 days for SO, and a progressive decrease of CM until day 90, at which time, E_{leaf} stabilized. This means that at the end of the experiment, the CC plants had the greatest decrease (90%), followed by CM (70%) and SO (50%).

As for chlorophyll fluorescence recordings, B toxicity significantly decreased the quantum efficiency of PSII (Φ_{PSII}) in CC plants (Table 1), and this decrease was due to the decrease in Fv'/Fm' (efficiency of the antennas in the reaction center), while qP (quenching photochemistry) was not affected. In the other two rootstocks, B did not affect these two chlorophyll fluorescence parameters.

3.5. Leaf proline, quaternary ammonium compounds and carbohydrate

At the end of the experiment (120 d), the concentration of proline in all three rootstocks decreased significantly and similarly when facing an excess of B; although independently of the B treatment, the CM plants had the highest concentration (Table 1). In CC plants, the concentration of QAC decreased with the excess of B, while it increased in SO and CM plants, with the latter genotype being the one that had the highest concentration. The total soluble sugars (SS_{leaf}) results showed that there was a significant and similar decrease in concentration in all three rootstocks due to the excess of B; with the CM rootstock being the one with the highest concentration. There was a decrease in the concentration of starch due to B excess in SO plants; however, the other two rootstocks did not show significant differences with respect to the controls.

3.6. Lipid peroxidation, H_2O_2, antioxidant enzymes

The MDA concentration in the plants treated with excess B (10 mg L^{-1}) increased in the CC and CM rootstocks, but not in SO. However, the H_2O_2 concentration increased in all three rootstocks. For both compounds, the highest concentration was found in CM plants treated with an excess of B (Fig. 6. A).

With respect to the enzymatic activities, it was observed that the activity of CAT increased in CC and CM plants treated with high B, reaching values of 2.6 and 2.4 U g^{-1} fw, respectively; on its part, the SO rootstock did not show significant differences as compared to its control (Fig. 6. B). For APX, CC and SO plants watered with excess B showed an increase in the enzyme's activity, being more drastic in the SO plants,

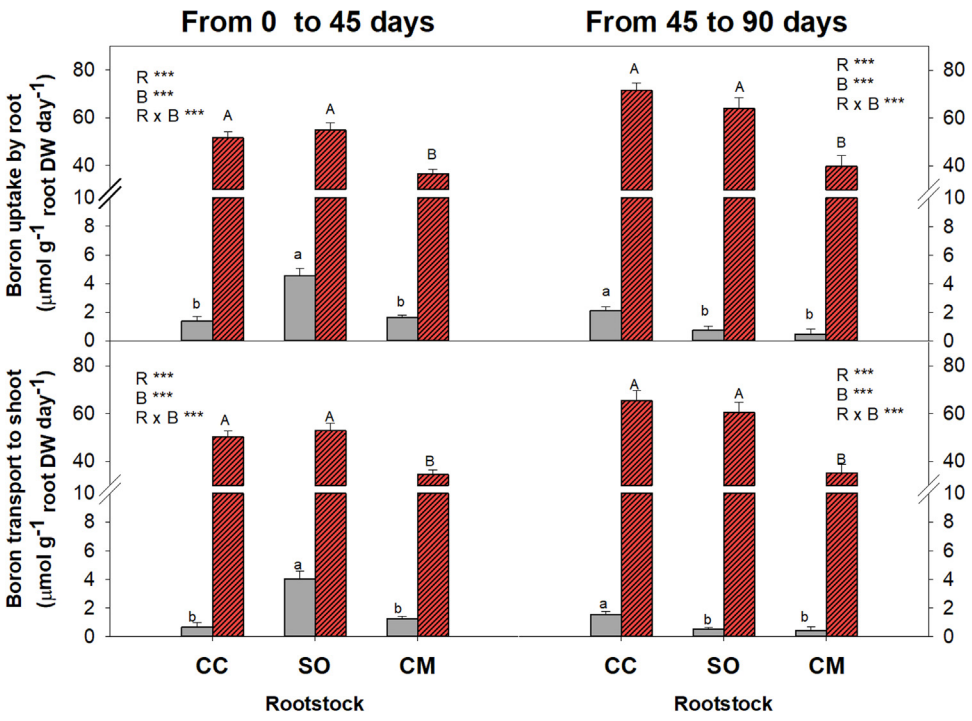

Fig. 4. Effects of an excess of B on the absorption and transport of B in Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) in the period comprised between 0 and 45 and 45–90 days of the experiment. In the ANOVA, "*ns*" indicates non-significant differences at 95%; *** indicates significant differences at $P < 0.001$. For each B treatment, the *lower-case letters* (control) and *upper-case letters* (B excess) indicate significant differences between the rootstocks for $P < 0.05$ as established by Duncan's multiple-range test. The vertical bar indicates the standard error of the mean ($n = 4$).

which increased up to 12 times as compared to its control (Fig. 6. B). The results of the SOD activity showed that the excess of B on the CC and SO rootstocks led to a doubling of its activity as compared to control plants, while CM plants did not show significant differences as compared to its control (Fig. 6. B).

4. Discussion

4.1. SO rootstock plants are more tolerant to an excess of B than CM and CC

To establish the different tolerances to the excess of B in irrigation water among the rootstocks, it was calculated the percentage of reduction of the total dry weight of the rootstocks treated with 10 mg $^{-1}$ with respect to the control treatments (without an excess of B). And, with this data, it can be concluded that the relative tolerance of the rootstocks used follows the order SO > CM > CC, as the reduction in growth was 23%, 33%, and 56%, respectively. This classification follows an order that is similar to that observed with these rootstocks for other stresses, such as salinity and drought (Cámara et al., 2004; Fernandez-Ballester et al., 2003), evidencing the weaknesses possessed by the CC plants for adapting to adverse environmental conditions. On the other hand, this differential tolerance among rootstocks is of great interest, as it allows for the study of physiological and biochemical mechanisms that could be related to said tolerance in citrus, highlighting that this tolerance will depend on two factors: i) accumulation of B in the different plant tissues and ii) toxicity of B in the physiological processes.

4.2. CC plants accumulate more B in the leaves than SO and CM plants

Species and genotypes sensitive to an excess of B generally contain higher concentrations in leaves and shoots than the tolerant genotypes (Nable et al., 1997; Camacho-Cristóbal et al., 2008). The results of our experiment with citrus rootstocks also showed that the least-tolerant rootstock (CC) had the highest concentration of B in its leaves. Thus, at day 45 of the experiment, although all of the rootstocks had exceeded the toxicity threshold value (250 mg kg^{-1} dw) established for citrus by Embleton et al. (1973), CC had already reached a concentration of 400 mg kg^{-1} dw, while the other had not yet reached 300 mg kg^{-1} dw. This greater B accumulation in the leaves of CC plants with respect with those from SO and CM could be explained by the data on the absorption of B by the root and its transport from the root to the aerial part, and by the morphological architecture of the plants. Thus, the CM plants showed a greater capacity for restricting the uptake of B by the plant and its transport from the root towards the aerial part, as shown in Fig. 4. These data on B absorption and transport are the final result of a series of mechanisms that include molecular factors as well as physiological ones, in which B transporters, transpiration and the efficient use of water play an important role. For example, in CM plants have been reported a decrease of the B transporters responsibles of B influx to root cells (NIP5 and PIP5), and the activation of the BOR4 gene which codes for trassporters related to the efflux of B which are responsible for the extrusion of B from the cells inside (Martínez-Cuenca et al., 2015). Also, the CM plants had a greater transpiration and efficient use of water than CC plants, and these parameters are very related to the accumulation of B in the leaves, as a lesser transpiration rate decreases the entrance of B into the plants (Mesquita et al., 2016). On the other hand, although in our experiment the absorption and transport of B were similar in CC and SO, the greatest accumulation in the former could be due, among other factors, to the smaller leaf/root ratio (0.8 CC and 1.7 SO). For the same absorption capacity of B per gram of root, the CC rootstock had a great amount of root as compared to the leaf biomass, so that there is a concentration effect of B that reached the leaves as compared to SO plants. Also, SO distributes less biomass to the stem than CC and CM (17%, 43% and 37%, respectively). As the stem hardly accumulates B, it makes it so that everything that has been transported to the aerial part goes to the leaves, where it is concentrated more in those rootstocks that had the least leaf biomass, as in the case of CC.

4.3. Toxicity of B in the physiological and morphological processes of plants

Boron toxicity caused a decrease in vegetative growth in the three rootstocks. The RGR analysis and its components showed that the reduction of RGR was mostly due to the reduction of the "efficiency" of the leaf biomass (NARm), more so than the quantity of the leaf biomass (LMF) (Fig. 2. B). The changes in the NAR are associated to changes in the net assimilation of CO_2 as well as respiration (Munns, 1993;

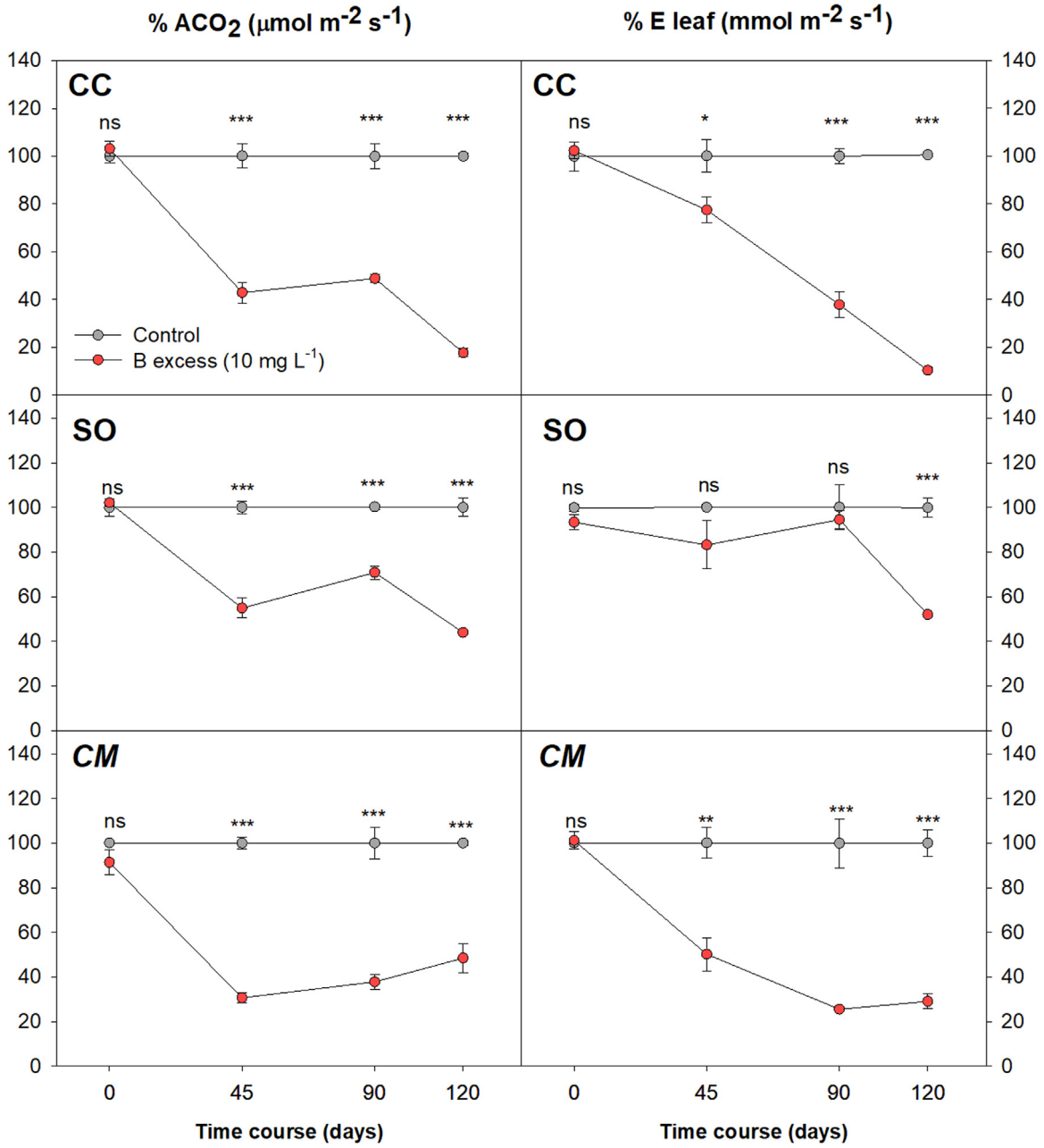

Fig. 5. Changes in the net assimilation rate of CO_2 (ACO_2) and leaf transpiration (E_{leaf}) as a response to an excess of B in Citrange Carrizo (CC), sour orange (SO) y *Citrus macrophylla* (CM) plants during the 120 days of the experiment. The data are expressed as a percentage relative to the data from the control treatment for each rootstock (ACO_2, µmol m^{-2} s^{-1}, CC = 10.5, SO = 9.75, CM = 9.75; E_{leaf}, mmol m^{-2} s^{-1}, CC = 2.70; SO = 2.38; CM = 2.41). "*ns*" indicates non-significant differences at 95%; while *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, among the treatments. The vertical bar indicates the standard error of the mean ($n = 4$).

Poorter, 2002). Thus research on the toxicity of B should be focused on how the accumulation of this nutrient affects the correct functioning of the physiological and biochemical processes of plants.

As previously observed, the greatest accumulation of B in the leaves of the CC rootstock could be the cause of this rootstock being less tolerant. However, in the case of the other rootstocks, SO and CM, the accumulation of B was more similar, but SO was more tolerant. This could indicate that for the same concentration of B, this was more toxic for CM than in SO, as indicated by the physiological study conducted in this experiment. Thus, the high concentration of B in the leaves resulted in a decrease of ACO_2 in all three rootstocks (Fig. 5), but the percentage of reduction was depended on the genotype. The CC plants accumulated B the most, and were the ones that had the greatest percentage of growth reduction, and this is in agreement with the ACO_2 data, as a greater reduction was observed in these plants (60% in days 45% and 90%, and 85% in day 120). In the other two rootstocks, although they had the same leaf concentration of B between them, the SO plants reduced ACO_2 to a lesser degree, which backs our hypothesis in that the leaves of these rootstocks have a greater sensitivity to B toxicity than the CM leaves.

The study on chlorophyll fluorescence conducted in this experiment did not detect any significant changes with the excess of B in SO and CM plants, indicating that the different susceptibility of $ACO_2$to B is not due to mechanisms related to the capture and use of light. But in this study, it was shown that the CC leaves had damage to its photosynthetic machinery, as in these plants, there was a decrease in the proportion of

Table 1
Effects of the excess of B on the concentration of proline, quaternary ammonium compounds (QAC), total soluble sugars (SS), leaf starch, photochemical efficiency of PSII (Φ_{PSII}), the efficiency of the antennas from PSII (Fv'/Fm') and the "Photochemical Quenching" (qP) coefficient in leaves from Citrange Carrizo (CC), sour orange (SO) and Citrus macrophylla (CM) at 120 days of the experiment. "ns" indicates non-significant differences at 95%; while *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, in the two-way ANOVA rootstock x Boron treatment, and in the one-way ANOVA of each rootstock. "γ" indicates significant differences at 95% between Boron treatments in each rootstock. For each B treatment, the lower-case letters (control) and upper-case letters (B excess) indicate significant differences between the rootstocks for $P < 0.05$ as established by Duncan's multiple-range test. The values are the mean of 4 repetitions.

Main factor		$Proline_{leaf}$	QAC_{leaf}	SS_{leaf}	$Starch_{leaf}$	ΦPSII	Fv'/Fm'	qP
Rootstock	CC	20.18 b	1.84 a	38.16c	13.52 b	0.527 b	0.811 b	0.708
	SO	20.56 b	0.56 b	60.94 b	18.00 a	0.538 b	0.795 b	0.696
	CM	25.46 a	1.73 a	91.10 a	12.67 b	0.627 a	0.897 a	0.711
Boron	Control	24.06	1.32	68.16	16.04	0.586	0.832	0.709
	B excess	20.10	1.43	58.64	13.42	0.542	0.837	0.701
Rootstock × Boron								
Carrizo Citrange	Control	21.99$^{\gamma}$	2.25$^{\gamma}$ a	43.56$^{\gamma}$	14.46 ^{ns} b	0.642$^{\gamma}$ a	0.873$^{\gamma}$ a	0.727
	B excess	18.37	1.42 B	32.74	12.59 A	0.412 B	0.749 B	0.689
Sour Orange	Control	22.79$^{\gamma}$	0.43$^{\gamma}$ c	66.59$^{\gamma}$	21.23$^{\gamma}$ a	0.496 ^{ns} b	0.725 ^{ns} b	0.685
	B excess	18.40	0.69C	55.31	14.76 A	0.580 A	0.865 A	0.707
C. Macrophylla	Control	27.39$^{\gamma}$	1.28$^{\gamma}$ b	94.33$^{\gamma}$	12.43 ^{ns} b	0.619 ^{ns} a	0.898 ^{ns} a	0.714
	B excess	23.51	2.18 A	87.87	12.92 A	0.635 A	0.896 A	0.707
Rootstock		* **	* **	* **	*	*	ns	
Boron		* **	ns	*	*	ns	ns	ns
R × B		ns	* **	ns	*	* **	*	ns

photochemical energy absorbed by photosystem II in the chloroplasts (Φ_{PSII}). The reduction of Φ_{PSII} could be due to changes in qP and/or Fv'/Fm' (Maxwell and Johnson, 2000). In this experiment, the changes in Φ_{PSII} of the CC leaves were due not only to the changes that were produced in the Fv'/Fm' parameter (Table 1); which suggests that the loss of quantum efficiency by PSII in this rootstock was the result of the collection complex being damaged (Guerfel et al., 2009). This could be attributed to a damage of the structure of chloroplast and/or other photosynthetic pigments (Papadakis et al., 2004a, 2004b; Paparnakis et al., 2013).

4.4. Plant protection mechanisms against B toxicity

One of the most common responses to stress in plants is the overproduction of different types of organic solutes. Among these solutes, proline, quaternary ammonium compounds (QAC) and sugars are included and highlighted (Serraj and Sinclair, 2002; Rivero et al., 2004; Parvaiz and Satyawati, 2008). In our assay, a reduction of proline, total sugars and starch was observed, as well as an increase in quaternary ammonium compounds, which indicates that in citrus, only the synthesis of quaternary ammonium compounds (QAC) plays a role in the adaptation of these plants to B toxicity. Thus, the results showed that SO and CM had an increase in the concentration of QACs in leaves when they were watered with high concentrations of B; while in CC, the concentration of this solute was reduced (Table 1). The fact that QAC is highly important for the protection of thylakoid membranes (Robinson and Jones, 1986; Genard et al., 1991), could explain CM and SO maintaining their photosynthetic efficiency (Φ_{PSII}; Table 1) to levels similar to control plants despite having an excess of B in their leaves, while in CC, the decrease in concentration of QAC could have contributed even more to the incorrect functioning of the light gathering complexes, as shown by the chlorophyll fluorescence values Fv'/Fm' (Table 1). The proline data in our experiment confirmed that in fruit trees such as citrus, B produces a decrease in concentration of this osmolyte (Molassiotis et al., 2006; Gunes et al., 2006), although the mechanisms behind this have yet to be described. On the other hand, the reduction in total soluble sugars could be due to a decrease of the net assimilation rate of CO_2 (ACO_2) that resulted from B toxicity in leaves, as there is a clear dependency between ACO_2 and total soluble sugars. However, the decrease in starch of the SO rootstock could indicate that the degradation of this reserve compound could provide carbohydrates to meet the plant's needs when faced with the possible inhibition of synthesis due to the excess of B in the leaves. Sang et al. (2015) observed that the proteins involved in the metabolism of energy and photosynthesis had a better adaptation to an excess of B in the citrus species that were more tolerant.

In environmental stress conditions, it could be the case that the energy absorbed by the photosynthetic apparatus cannot be completely channeled towards CO_2 assimilation, thus, the excess of energy could produce damage due to the creation of reactive oxygen species (ROS) if the plants are not able to deactivate these compounds. One of the parameters used to estimate if the plant is overproducing ROS, is the ACO_2/Φ_{PSII} ratio (Cakmak and Römheld, 1997). In our experiment, it was verified that an excess of B decreased the ACO_2/Φ_{PSII} ratio (*data not shown*), so that reactive oxygen species were created, and these plants responded to this increase by increasing the enzymatic activities related to the antioxidant systems. Thus, in CC, there was a significant increase in the activities of SOD, APX and CAT; in SO there was an increase in SOD and APX, while in CM only CAT increased. In citrus trees, this is not always the case. For example, it has been shown that toxicity due to heavy metals can inhibit the proper functioning of these enzymes (Balal et al., 2017). The fact that in the SO leaves the concentration of MDA (Fig. 6. A) was not affected, provides support to our previous hypothesis that B is less toxic for these plants, and suggests that this rootstock has a potent and efficient antioxidant system, being able to deal with the ROS. Conversely, it was found that the CM plants had a high concentration of MDA, and this could be due to the fact that in this rootstock, only the concentration of CAT increased with the high concentration of B. In CC, although it accumulated a high concentration of B, the concentration of MDA was not too high, due to the increase in activity of SOD, CAT and APX.

5. Conclusions

Of the three rootstocks studied, sour orange (SO) was the most tolerant to an excess of boron (B), while the least tolerant was Citrange Carrizo (CC). The tolerance of SO as compared to the other two rootstocks could be that this rootstock accumulates a smaller concentration of B in its leaves as compared to CC, and its leaves were less sensitive to B toxicity than *Citrus macrophylla* (CM) ones. This lesser accumulation of B in the leaves observed in the leaves of SO could be related, among other factors, in that its roots had a low rate of absorption and transport of B to the aerial part of the plants. The different sensitivity to toxicity due to B between *Citrus macrophylla* and Citrange Carrizo with respect to sour orange could be that the latter rootstock had a combination of different responses: i) it has a very potent antioxidant system that is

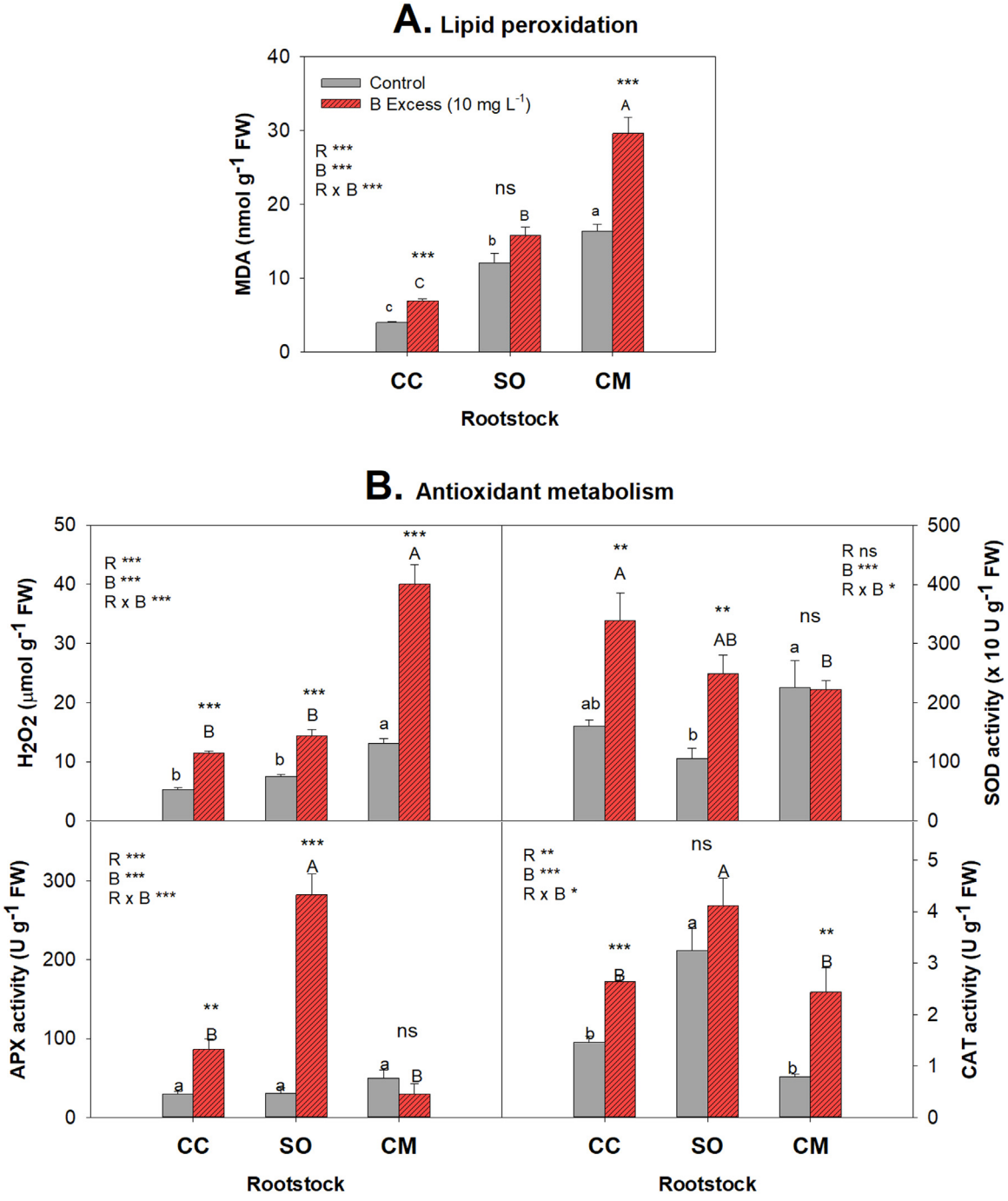

Fig. 6. Effects of the excess of B on the concentration of malondialdehyde (MDA; **A. Lipid peroxidation**) and hydrogen peroxide (H_2O_2; **B. Antioxidant metabolism**) and on the activity of antioxidant enzymes (SOD, APX and CAT; **B. Antioxidant metabolism**) in leaves from Citrange Carrizo (CC), sour orange (SO) and *Citrus macrophylla* (CM) at 120 days of the experiment. "*ns*" indicates non-significant differences at 95%; while *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively. For each B treatment, the *lower-case letters* (control) and *upper-case letters* (B excess) indicate significant differences between the rootstocks for $P < 0.05$ as established by Duncan's multiple-range test. The vertical bar indicates the standard error of the mean ($n = 4$).

based on the high activity of the enzymes superoxide dismutase, ascorbate peroxidase and catalase; and ii) the over-production of quaternary ammonium compounds could contribute, to a certain degree, to the avoidance of cellular damage in its photosynthetic machinery. The responses reported in the present work are about rootstock genotypes un-grafted. Therefore, it is necessary to carry out additional studies with citrus varieties grafted on these rootstocks to contrast the behaviors described in this work.

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