Scientia Horticulturae 338 (2024) 113660

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Effects of high boron concentration in irrigation water on the relative tolerance and metabolic responses of different citrus varieties: Lemon, orange and mandarin

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ARTICLE INFO

Keywords:
Climate change
NMR
Tolerance
Metabolites

ABSTRACT

Climate change is causing many crops, such as citrus, to grow in adverse environmental conditions, in which the lack of water has resulted in the use of non-conventional water sources for irrigation. However, these types of waters can lead to boron toxicity problems, especially if they come from seawater desalination plants, in sensitive crops such as citrus. To deal with this new reality, there is a need to know which varieties are the most tolerant within a given species, and which physiological and biochemical processes are negatively affected by the excess of boron. This information would allow growers to design agronomic strategies to palliate this problem. Thus, tools such as -omic sciences, and within them, the study of primary metabolites, could help us better understand how this crop is affected by this type of stress. In this context, a study was conducted to classify the relative tolerance of different citrus cultivars to boron stress. A metabolomic study, through the use of the Nuclear Magnetic Resonance (NMR) technique, was also conducted to determine the metabolic profiles of each of these cultivars. In total, 17 varieties of citrus were assayed: 3 commercial varieties of lemon (Eureka, Fino 49, and Verna), 7 varieties of mandarin (Satsuma Owari, Satsuma Iwasaki, Clementina Hernandina, Murcot, Clemenules, Nova and Ortanique) and 7 oranges (Washington Navel, Midknight, Valencia Late, Lane Late, Navelina, Sanguinelli and Navelate). For each crop specie, the plants were irrigated with water containing a boron concentration of 5 mg L^{-1}. The most important results are: i) for each crop, the different levels of tolerance to boron toxicity were strongly influenced by the variety; ii) the level of boron tolerance was not directly related with the concentration of boron accumulated in the leaves; iii) the stress due to boron toxicity affected the main primary metabolism routes in the citrus trees, with a generalized response found in the routes involving the metabolites acetate, citrate, malate, proline, GABA, and alanine, with a significant reduction in their concentration as a result of this stress; and iv) the concentration of aspartate significantly increased in the citrus varieties considered to be more tolerant, such as Eureka, Murcot, Nova, Navelate, Valencia Late and Midknight.

1. Introduction

Boron (B) is an essential micronutrient for plants, although the need for it is less than for the rest of the nutrients, except for molybdenum and copper (Chatzissavvidis and Antonopoulou, 2020). B is involved in many metabolic pathways, including carbohydrate and phenol metabolism, lignification, and it also plays important physiological functions, as it confers integrity to the plasma membrane of cells, as part of the cell wall (Cakmak et al., 1995). However, despite its essential nature, it is damaging to plants when it is accumulated in excess in their tissues.

Although B toxicity is less common than deficiency, as of today, this toxicity has become an important problem that can limit the agronomical performance of citrus and other crops, especially in soils located in arid and semi-arid regions worldwide, such as Spain, Egypt, Morocco, Turkey, Iraq, Jordan, Syria, Chile, the Unites States, and Southern Australia (Ayvaz et al., 2013; Landi et al., 2012; Miwa et al., 2007). In the last few years, agricultural production in these areas has become gravely threatened by climate change, as in this scenario, higher

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https://doi.org/10.1016/j.scienta.2024.113660
Received 10 July 2024; Received in revised form 6 September 2024; Accepted 13 September 2024
Available online 20 September 2024
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temperatures and longer and more intense periods of drought are observed. Faced with the panorama of a lack of good quality water, the agricultural sector, in order to be able to continue irrigating their crops, have begun to rely on the use of non-conventional water sources, mainly from marine desalination plants, which tend to have a high content of B (Dionysiou et al., 2006). For example, in some areas in the north of Greece, well water with a high concentration of B has been used to irrigate olive and kiwi orchards (Chatzissavvidis et al., 2004; Sotiropoulos et al., 2006), and in areas in Eastern Spain, the use of water from desalination plants has started to create toxicity problems in crops such as citrus, as it can reach concentrations higher than 1 mg L^{-1} (Simón-Grao et al., 2018).

Thus, as B is mainly uptaked through the transpiration stream, high temperatures induce a high accumulation of this nutrient in leaves, reaching toxic concentrations in different crops, including citrus trees. One of the strategies that is utilized to decrease the concentration of B in citrus leaves is the use of different rootstocks: thus, Gimeno et al. (2012) observed that in Verna lemons, the trees that were more tolerant to B excess in the irrigation water were those that were grafted on *Citrus macrophylla* and Cleopatra mandarin, while those that were grafted onto Carrizo citrange or bitter orange were more sensitive. These same authors showed that the relative tolerance between these four rootstocks was not closely related to the accumulation of B in the leaves, pointing to the idea of the involvement of different physiological and/or biochemical mechanisms in this tolerance. Other studies have also pointed to the weakness of the Carrizo citrange rootstock to excess B with respect to new rootstocks such as the rootstocks UFR-6 and 2247×6070–02–2, created by the CREC (*Citrus Research and Education Center*; Aparicio-Duran et al., 2021) improvement program. However, and despite these studies, there is little scientific knowledge on the relative tolerance to B of varieties of the same species. These genetic improvement programs have provided the citrus market with a great variety of cultivars, but little is known with respect to their behavior when dealing with some type of stress. Thus, the aim of the present work is to elucidate the relative tolerance to B excess of the main cultivars of lemon, orange, and mandarin, and between these three citrus groups.

In the last decade, in order to better understand the adaptive mechanisms of citrus plants to excess B, many research studies have been conducted at the molecular level of plants. The evidence shows that the expression levels of genes related with B absorption and translocation, energy and carbohydrate metabolism, cell wall and membranes, N metabolism, nucleic acid metabolism, signal transduction, etc., are altered by this type of stress (Wang et al., 2017; Yang et al., 2021). Despite the research conducted in the last few decades improving our understanding of B toxicity, the basic mechanisms behind the tolerance of some cultivars over others is still unknown, especially which routes of primary metabolism are more affected or modified by this toxicity (Chatzissavvidis and Therios, 2010). In this sense, metabolic analysis techniques are –omic tools that allow us to obtain global knowledge on primary metabolism and its metabolic pathways. As opposed to the knowledge obtained from the analysis of genes and proteins, metabolites act as direct indicators of biochemical activity, and can therefore be useful for describing what is occurring in metabolic processes (Sheth et al., 2014). Despite the technical approaches that have been used to decipher plant-environment interactions, very few metabolic studies relate B tolerance with the biochemical portion. Under this context, the objective of the present study was to determine the relative tolerance to B toxicity of different varieties of citrus trees within the lemon, mandarin, and orange groups, by determining the leaf B concentration, and characterizing the metabolic profile (amino acids, organic acids, sugars, among other compounds) of the different varieties, to better understand how these latter parameters are related with tolerance to B excess among the cultivars of the same species and between different species. To characterize the metabolic state of the different varieties in these conditions, the Nuclear Magnetic Resonance (NMR) technique was used. This is a fast and highly reproducible spectroscopy technique that is based on the energy of absorption and re-emission of the atomic nuclei, which allow establishing metabolic responses of the crops to abiotic stresses.

2. Materials and methods

2.1. Plant material and growing conditions

For the present study, 17 different citrus species were utilized (Viveros Sevilla S.A.; Brenes, Sevilla): 3 cultivars of lemon (*Citrus lemon*; Eureka, Fino 49, and Verna) grafted onto *Citrus macrophylla*; 7 mandarins (*Citrus reticulata*; Satsuma Owari, Satsuma Iwasaki, Clementina Hernandina, Murcot, Clemenules, Nova, and Ortanique) grafted on Carrizo citrange; and 7 oranges (*Citrus sinensis*; Washington Navel, Midknight, Valencia Late, Lane Late, Navelina, Sanguinelli, and Navelate) grafted on Carrizo citrange. The plants were transplanted to 9 L pots, and the experiment was conducted during the months of March to September 2022, in a multi-tunnel greenhouse at the experimental field of the CEBAS (Santomera, Murcia, Spain). The greenhouse was equipped with everything necessary to maintain the temperature below 35 °C during the period in the day of maximum heat (a cooling system and 'aluminet' shading nets). The plants were divided into two groups; one of them was irrigated with a Hoagland nutrient solution (4 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM NaH_2PO_4, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM Na_2MoO_4, 125 μM Fe-EDDHA) complemented with B at a concentration of 5 mg L^{-1} (applied as boric acid, H_3BO_3), while the other group of plants was irrigated with a Hoagland nutrient solution with a standard concentration of B (0.25 mg L^{-1}). These treatments were applied for a period of five months, at the end of which the morphological, nutritional, and metabolic studies took place.

2.2. Parameters analyzed

2.2.1. Parameters of growth, percentage in the reduction of growth, and the level of tolerance to stress

At the end of the experiment, the shoot of each of the cultivars was sampled and weighted, separating the leaves from the stem (grams of fresh weight; g FW), after which they were dried in an oven at 60 °C for at least 48 h. After this, the samples were weighted again to obtain their dry weight (grams of dry weigh; g DW). To determine the level of relative tolerance of each of the citrus cultivars, B toxicity was calculated. Table 1 shows the relative tolerance indices used to classify the cultivars. These indices were calculated as: 100 x (biomass of plants irrigated with B excess / biomass of plants irrigated without excess B). Values close to 100 indicate tolerance, and values close to 0 sensitivity. Also, the leaves were photographed to determine the leaf area (cm^2) through an image analysis protocol using digital pictures and the ImageJ® software (Martin et al., 2020); In addition, leaves were sampled for their processing as dry and fresh samples for further analyses.

2.2.2. Quantification of the relative content of chlorophylls and the percentage of chlorosis

To quantify the relative content of chlorophylls in both completely developed old and medium-age leaves and sprouts, the measurement

Table 1
Relative tolerance index established based on the percentage of shoot biomass of the plants irrigated with B (g DW) with respect to the treatment without excess of B.

Level of tolerance	% Shoot biomass with respect to the control treatment
Tolerant	100–90
Semi-tolerant	89–70
Sensitive	69–0 %

was performed with a CL-01 portable measurement device (Hansatech, PP System). This device determines the relative chlorophyll concentration of the leaf samples through dual-wavelength (610 and 940 mm) optical absorbance. The calibration is automatic, and the temperature is compensated in all the measurements, so that measurements can be taken in environments at different temperatures. To quantify the phytotoxic effects generated by B toxicity, the ImageJ® software was used (Fig. 1). Using this program, the 'chlorosis index' was calculated as the ratio of the surface affected by yellow chlorosis in leaves from plants irrigated with B (5 mg L^{-1}) with respect to its total area; the values close to 100 indicate a high degree of chlorosis, and values close to 1 indicate a low degree of chlorosis.

2.2.3. Mineral analysis: quantification of B accumulated in leaf tissues

The concentration of B was determined for each cultivar and treatment with dried and ground leaf tissue, using the inductively-coupled plasma technique (ICP, Iris Intrepid II, Thermo Electron Corporation, Franklin, USA), after digestion with HNO_3:H_2O_2 (5:3 v:v; CEM Mars Xpress microwave, North Carolina, USA), with a temperature gradient up to 200 °C.

2.2.4. Metabolomic study of leaf tissue

A metabolic analysis was performed on fresh leaf samples, which were ground using liquid nitrogen, lyophilized, and prepared as per the protocol by Van der Sar et al. (2013). The study utilized a 500 MHz Bruker NMR spectrometer equipped with a 5 mm broadband cryoprobe, with measurements taken at 300.1 ± 0.1 K under defined acquisition parameters. Data were gathered using the NOESY pre-saturation pulse sequence with water suppression. The 1H—NMR spectra were processed using the Chenomx NMR Suite for the identification and quantification of metabolites, calibrated with TSP-d4, and the pH was adjusted to approximately 6, allowing for the detection of metabolites (see Alfosea-Simón et al., 2021). The metabolites analyzed were 4-Aminobutyrate (GABA, non-protein), Alanine (Ala), Aspartate (Asp), Glutamate (Glu), Glutamine (Gln), Proline (Pro), Threonine (Thr), Tyrosine (Tyr), Valine (Val), Acetate (Ace), Citrate (Cit), Formate (For), Fumarate (Fum), Malate (Mal), Succinate (Suc), Fructose (Fru), Glucose (Glu), Myo-Inositol (MI), and Sucrose (Suc).

2.3. Statistical analysis

For this study, a bi-factor design was used for each specie, with the use of the 'Cultivar' and 'Boron' factors (control and 5 mg L^{-1}). For each 'Cultivar x Boron' combination, a total of six plants were used that were randomly placed in the greenhouse ($n = 6$). For each specie (lemon, orange, and mandarin), an analysis of variance (ANOVA) was performed with the SPSS statistical package version 24, which included 'Cultivar', 'Boron' treatment, and 'Cultivar x Boron'. When the ANOVA was significant ($p \leq 0.05$), Tukey's multiple range test ($p \leq 0.05$) was applied to separate the means. A cluster analysis was performed with the standardized data for hierarchical associations, employing Ward's method for agglomeration, and the squared Euclidean distance as the dissimilarity measurement.

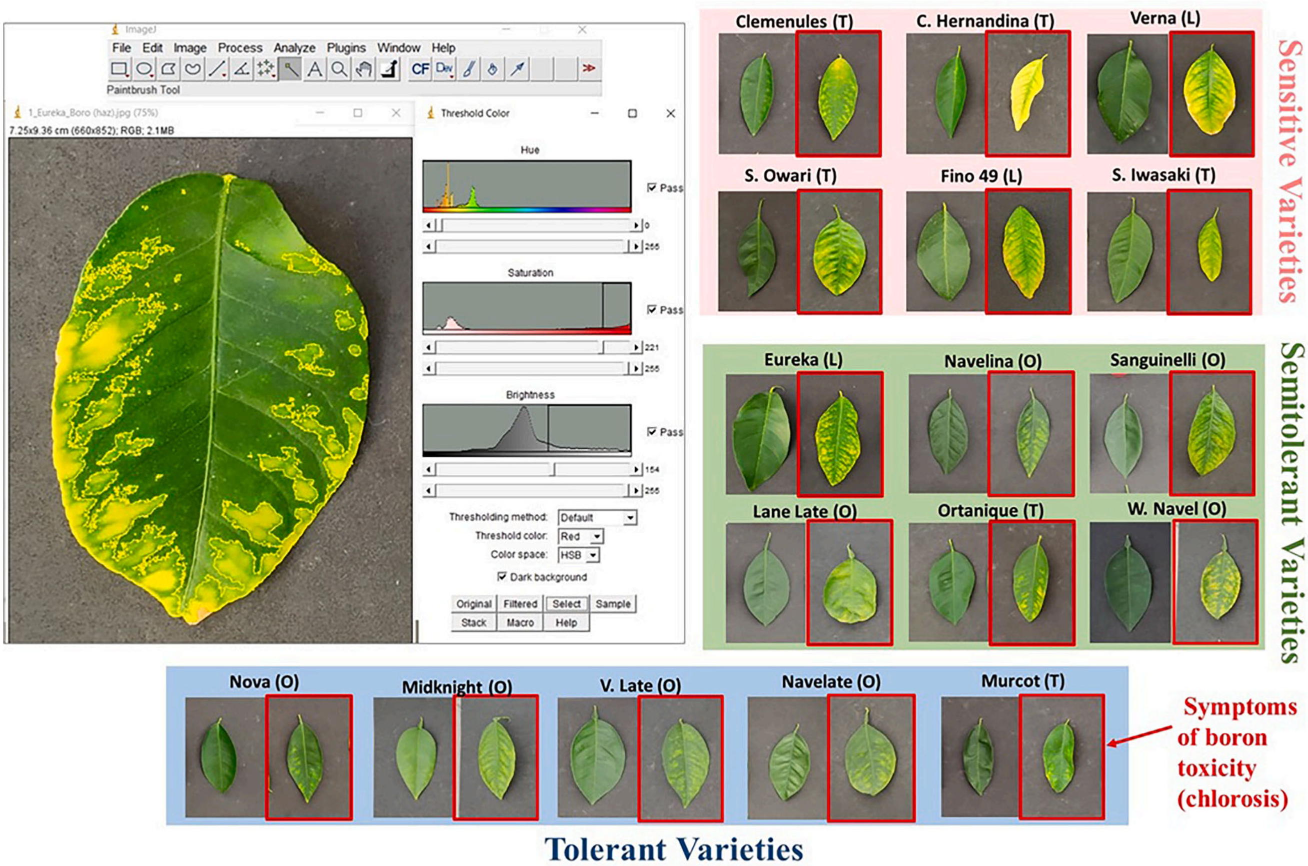

Fig. 1. Toxicity symptoms in leaves of citrus plants grown under greenhouse conditions that were irrigated with two types of water: i) control without B (0.25 mg L^{-1}) and ii) with excess B (5 mg L^{-1}; highlighted in red); and classified as: 'Sensitive' - Clemenules (tangerine), C. Hernandina (tangerine), Verna (lemon), S. Owari (tangerine), Fino 49 (lemon), and S. Iwasaki (tangerine); 'Semi-tolerant' - Eureka (lemon), Navelina (orange), Sanguinelli (orange), Lane Late (orange), Ortanique (tangerine), and W. Navel (orange); and 'Tolerant' - Nova (tangerine), Midknight (orange), Valencia Late, (orange), Navelate (orange) and Murcot (tangerine). Quantification of the percentage of chlorosis as a result of B toxicity using ImageJ® software.

3. Results

3.1. Index of tolerance to excess B in the nutrient solution and concentration of chlorophylls

Fig. 2 shows the index of tolerance to excess B for each of the cultivars in the different citrus groups studied, and the index of tolerance between the citrus groups. In the case of the lemon species, in which the cultivars Eureka, Fino 29, and Verna were compared, it can be observed that the 5 mg L^{-1} B treatment reduced the leaf biomass with respect to the control, with this response being similar in all three cultivars (Fig. 2) The index of relative tolerance, calculated as the percentage of biomass of the plants in the B treatment with respect to the control, oscillated between 73 and 57 %, with Eureka being the most tolerant (73 %), followed by Fino 49, and Verna (57 %). Significant differences were found between Eureka and Verna.

In the mandarin group, seven cultivars were compared. The irrigation with the nutrient solution containing 5 mg L^{-1} B did not reduce the growth of Murcot, although it did affect the rest of the varieties, but without significant differences between Murcot and Nova. The index of relative tolerance oscillated between 100 (Murcot) to 52 % (Clemenules), in the following order, from more to less tolerance: Murcot $\geq$ Nova $\approx$ Ortanique $>$ Satsuma Iwasaki $\geq$ Satsuma Owari $\geq$ Hernandina $\approx$ Clemenules.

In the group of oranges, seven cultivars were compared. The irrigation with 5 mg L^{-1} B slightly reduced the shoot biomass. The index of tolerance of the plants irrigated with B oscillated between 100 (Navelate) to 81 % (Navelina), in the following order, from more to less tolerance: Navelate $\approx$ Valencia Late $\approx$ Midknight $\geq$ Washington Navel $\approx$ Lane Late $\geq$ Sanguinelli $\approx$ Navelina.

When comparing the three groups studied, independently of the cultivar, it can be affirmed that the group of orange trees were more tolerant (mean index of tolerance of 90), while the mandarin and lemon groups obtained a similar index of tolerance between them (64 and 73 %, respectively). When studying the global data, and using the indices of tolerance, all the varieties can be classified into the following groups: 1) Sensitive: Clemenules, Hernandina, Verna, Owari, Fino and Iwasaki, with an index of tolerance below 69 %; 2) Semi-tolerant: Eureka, Navelina, Sanguinelli, Lane Late, Ortanique and Washington, with indices of tolerance between 70 and 89 %; and lastly, 3) Tolerant: Midknight, Valencia Late, Navelate and Murcot, with indices of tolerance higher than 90 %.

3.2. Symptoms of phytotoxicity in the leaves

In the present study, the 'chlorophyll concentration' and the 'chlorosis index' were also measured as indicators of B phytotoxicity symptoms (Fig. 3). Generally, the symptoms observed in the leaf tissues of different citrus varieties irrigated with high concentrations of B in the present study were burn on the edges or tips of older leaves (yellow chlorosis and necrosis); this leaf chlorosis was first observed on the leaf tips, and later expanded towards the edges, to finally cover practically the entire leaf in some varieties (Fig. 1, 4). All the varieties had external symptoms of B toxicity on their leaves. Nevertheless, the degree of severity of these symptoms was dependent on the variety. In the case of the group of lemon cultivars, the high B concentration in the irrigation water decreased the amount of chlorophylls by 62 %, independently of the cultivar, from 51.6 to 19.5 (dimensionless) for the control and B-

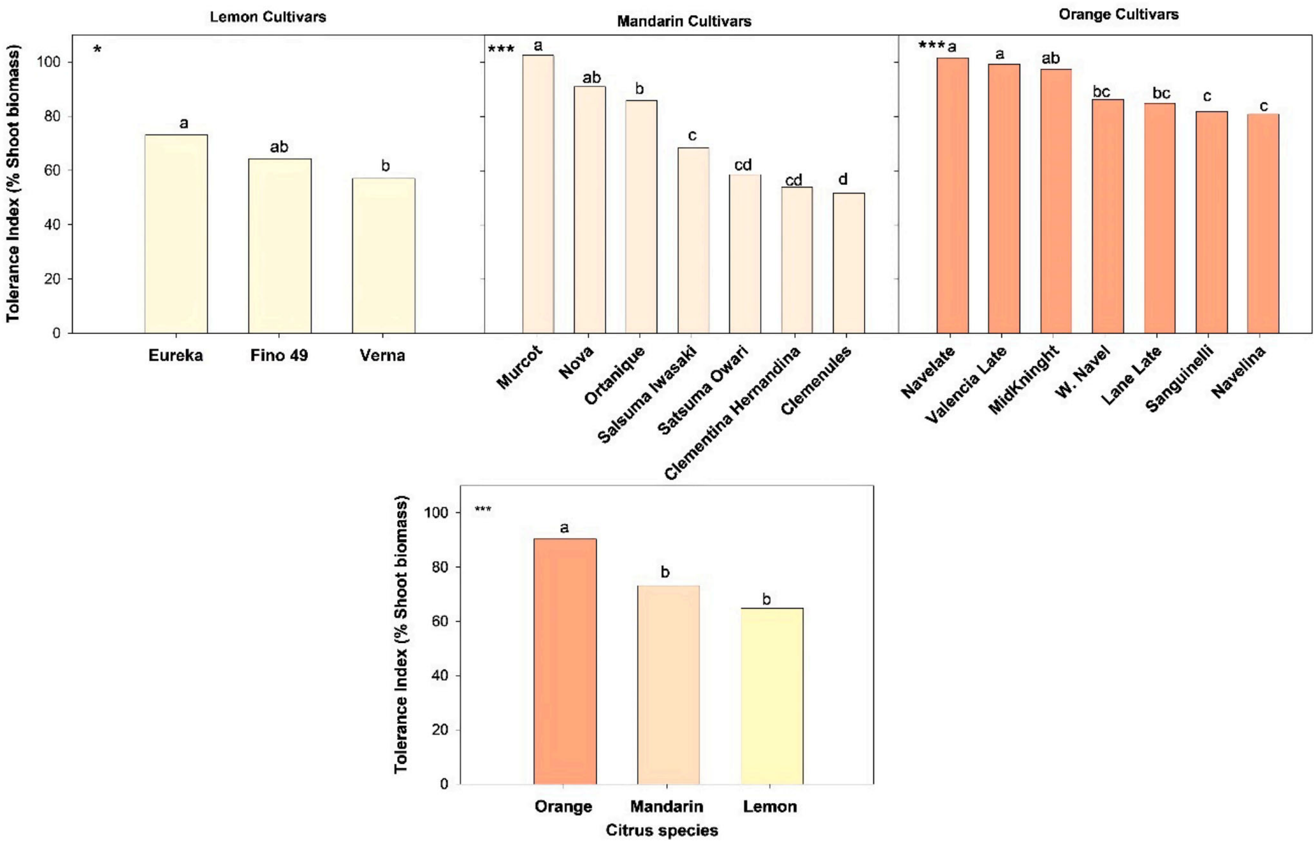

Fig. 2. Tolerance index of the varieties tested in the study (a) and tolerance index grouped by species (b). The Biomass of the control plants without excess B was (g DW): Eureka 154, Fino 156, Verna 169, Murcot 125, Nova 132, Ortanique 126, Satsuma Iwasaki 159, Satsuma Owari 127, Clementina Hernandina 117, Clemenules 119, Navelate 124, Valencia Late 124, MidKnight 131, W. Navel 151, Lane Late 132, Sanguinelli 141, Navelina 143. In the ANOVA (upper left area in each panel): "ns" denotes non-significant differences within a 95 % confidence interval; conversely, * and *** denote significant differences at $p \leq 0.05$ and $p \leq 0.001$, respectively. Different lowercase letters represent significant differences between treatments at $p \leq 0.05$, as determined by Tukey's multiple range test ($n = 6$).

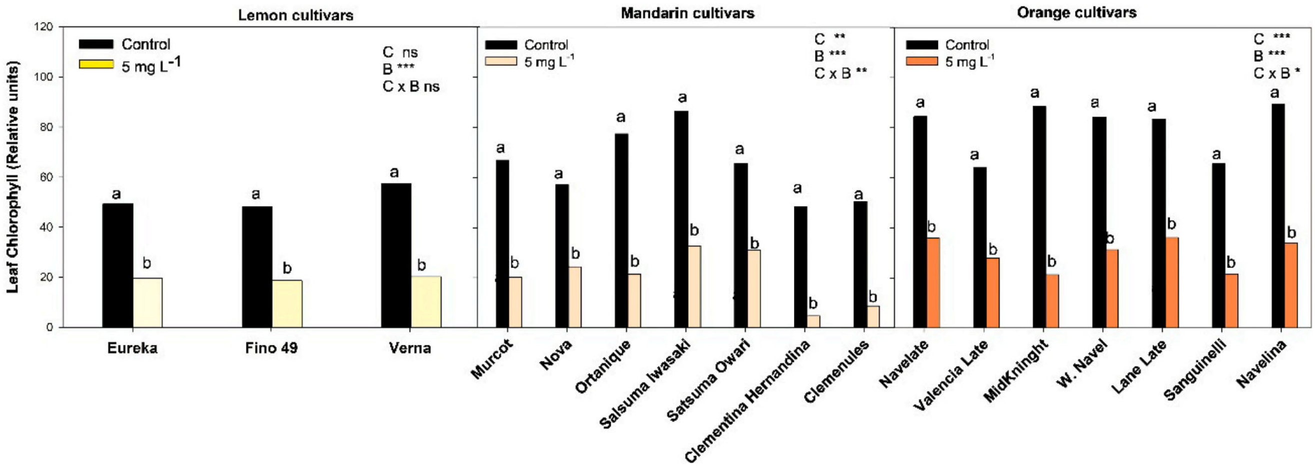

Fig. 3. Chlorophyll level (dimensionless, Hansatech) for each of the varieties tested. The trees were watered with control nutrient solution (B: 0.25 mg L^{-1}) or with a high concentration of B (5 mg L^{-1}). In the ANOVA (upper left area in each panel, V = variety, B = Boron treatment; V x B = interaction variety x Boron): "ns" denotes non-significant differences within a 95 % confidence interval; meanwhile, * and *** signify significant differences at $p \leq 0.05$ and $p \leq 0.001$, respectively. Different lowercase letters indicate significant differences between treatments at $p \leq 0.05$, as established by Tukey's multiple range test (n = 6).

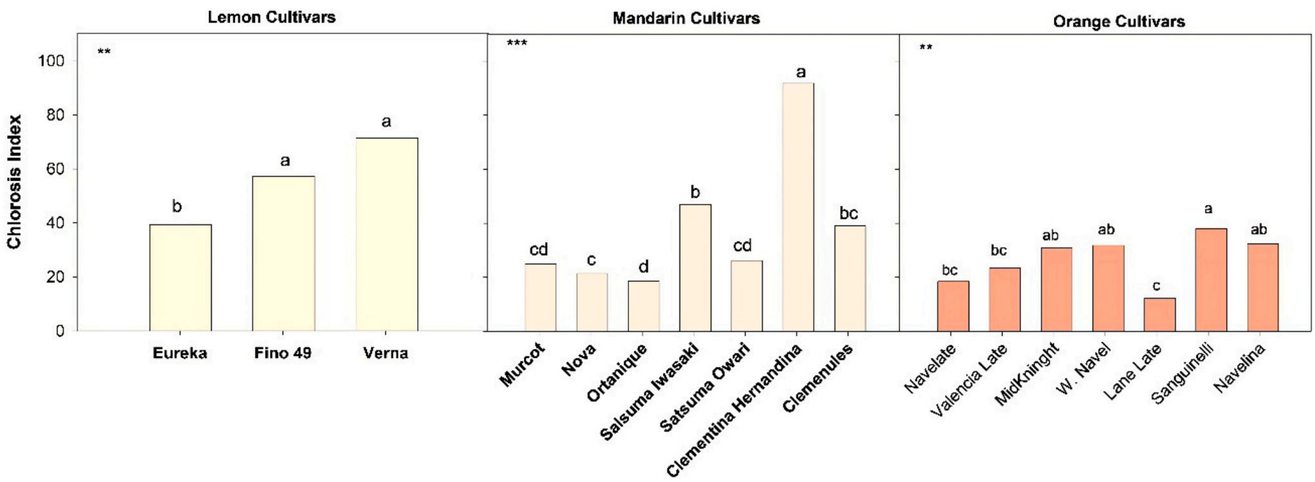

Fig. 4. 'Chlorosis index' (%) for each of the tested varieties irrigated with a high concentration of B. ** and *** denote significant differences at $p \leq 0.01$ and $p \leq 0.001$, respectively, within a 95 % confidence interval. Different lowercase letters signify significant differences between treatments at $p \leq 0.05$, as determined by Tukey's multiple range test (n = 6).

irrigated plants, respectively. In the case of the mandarin cultivars, the chlorophyll content was reduced by the excess B, with this reduction dependent on the cultivar. The percentage of reduction oscillated between 52 and 90 %, with the percentage of reduction being lower for Satsuma Owari, and the highest for Clementina Hernandina. Just as with the group of mandarins, all the orange cultivars experienced a reduction in the concentration of chlorophylls with the B treatment, with this reduction being dependent on the cultivar. The reduction oscillated between 57 and 76 %; the lowest reduction was observed in Valencia late and Lane Late, while the highest was observed in Sanguinelli. The values from the 'chlorosis index' were used to verify that the Verna cultivar had the most severe phytotoxic damage in the lemon group, while in the mandarin and orange groups, these were Clementina Hernandina and Sanguinelli, respectively. On the contrary, the mandarin cultivars Nova and Murcot, and the orange cultivars Midknight, Valencia Late and Nave Late barely had any signs of phytotoxicity.

3.3. B concentration in the leaves

Fig. 5 shows the leaf concentration of B (B_{leaf}; mg kg^{-1} DW) for each of the cultivars studied within each citrus group. The general response was similar in the three groups. All the cultivars within each citrus group showed the same accumulation of B except for some exceptions. In the lemon group, all of them accumulated a similar quantity, with a mean of 611 mg kg^{-1}. In the mandarin group, all of them accumulated a mean concentration of 821 mg kg^{-1}, except for Ortanique, which accumulated the highest concentration, with a value of 1123 mg kg^{-1}. The orange group followed the same pattern, with all of the cultivars accumulating a mean value of 701 mg kg^{-1}, except for Navelina, which accumulated the lowest concentration, with a value of 473 mg kg^{-1}.

When the data were analyzed according to citrus group and not according to the different cultivars, it was observed that lemons and oranges accumulated a mean of 640 mg kg^{-1}, and this value was significantly lower that the value of 864 mg kg^{-1} in the mandarins. These B_{leaf} values were also used for a cluster analysis to establish homogeneous groups within the citrus varieties, as a response to B accumulation in their leaves. In the dendogram obtained (Fig. 5), a line at a distance of 10 was traced. The analysis grouped the different citrus varieties into two clearly-differentiated groups that were far from each other: i) Group 1, with a mean value of B_{leaf} of around 664 mg kg^{-1}, which included the varieties Navelate, Midknight, Valencia Late, Eureka, Fino, Iwasaki, Lane Late, Sanguinelli, Washington, Verna and

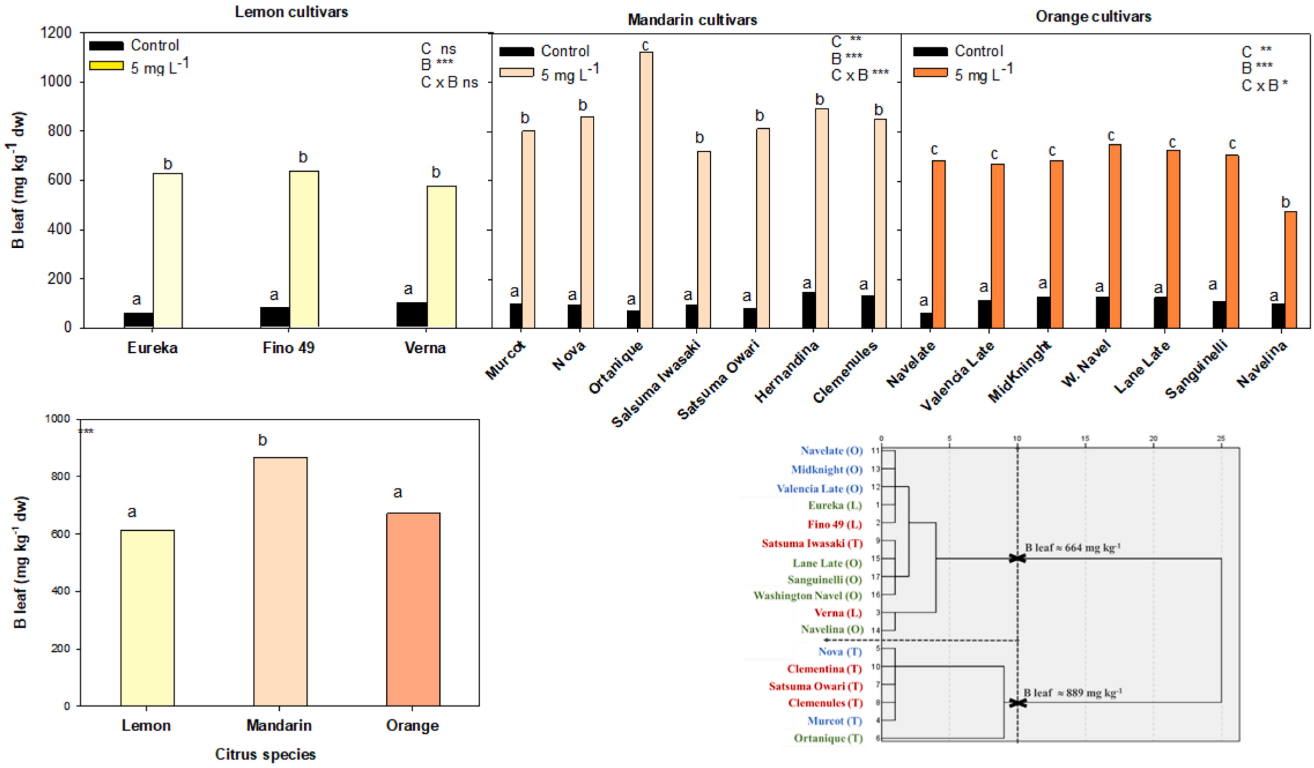

Fig. 5. B_{leaf} concentration for each of the varieties tested. The trees were watered with control nutrient solution (B: 0.25 mg L^{-1}) or with a high concentration of B (5 mg L^{-1}). In the ANOVA (V = variety, B = Boron treatment; $V \times B$ = interaction variety × Boron): "ns" denotes non-significant differences within a 95 % confidence interval; whereas *, **, and *** represent significant differences at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Different lowercase letters indicate significant differences between treatments at $p \leq 0.05$, as determined by Tukey's multiple range test ($n = 6$). The bottom right panel displays the dendrogram generated using Ward's agglomeration method and the squared Euclidean distance as the measure of dissimilarity.

Navelina, and Group 2, with a mean B_{leaf} value of 889 mg kg^{-1}, and with the varieties Nova, Clementina, Owari, Clemenules, Murcot and Ortanique. This indicates that the mandarin varieties accumulated the most B in the leaves, with all of them found in group 2, except for Satsuma Iwasaki.

3.4. *Metabolic study in the citrus leaves*

The metabolites detected and quantified with the H—NMR technique in citrus leaves were: 1) Amino Acid Group – 4-Aminobutyrate (GABA, non-protein), Alanine (Ala), Aspartate (Asp), Glutamate (Glu), Glutamine (Gln), Proline (Pro), Threonine (Thr), Tyrosine (Tyr), and Valine (Val); 2) Organic Acid Group – Acetate (Ace), Citrate (Cit), Formate (For), Fumarate (Fum), Malate (Mal), and Succinate (Suc); 3) Sugar Group – Fructose (Fru), Glucose (Glu), Myo-Inositol (MI), and Sucrose (Suc); these metabolites are involved in primary metabolism, and have an influence on diverse physiological processes (Teixeira et al., 2017). Next, the most important results from each of the metabolite groups are described.

3.4.1. *Metabolic profile of the control group without excess B (0.25 mg L^{-1})*

Fig. 6 shows the metabolic profile of each citrus specie assayed (lemon, orange, and mandarin) corresponding to the control treatment of 0.25 mg L^{-1} of B. In this case, the means of all the varieties found in the same groups are provided. In the case of the lemon group, within each chemical compound group, the highest concentration was found in proline and glutamate, citrate and malate, and sucrose and glucose. In the mandarins, the highest concentration was found in proline and glutamine, citrate, and malate, and sucrose and fructose. And in the oranges, the highest concentrations found within each group were in proline and glutamate, malate and citrate, and sucrose and fructose. The statistical analysis revealed that the variety also had an influence on the concentration of these metabolites. To define a generalized behavior of citrus cultivation in control conditions without B excess (0.25 mg L^{-1}), the mean values for the crop in general were (mg g^{-1} DW): i) for the amino acid group, Pro (7.46) > Glu (2.60) ≈ Gln (2.30) > Tyr (1.52) > GABA (0.72) > Asp (0.48) > Thr (0.28) ≈ Ala (0.25) > Val (0.09); ii) for the organic acids group, Citrate (6.57) > Malate (5.72) > Acetate (0.37) > Fumarate (0.14) ≈ Succinate (0.11) > Formate (0.02); and iii) for the sugar group, Sucrose (19.4) > Fructose (5.16) > Glucose (2.36) > Myo-Inositol (1.95).

3.4.2. *Metabolic profile of trees irrigated with an excess of B (5 mg L^{-1})*

To show how the metabolic profile changed in the leaves of trees irrigated with a high concentration of B, the leaf concentration of the metabolites of these trees is provided. The color map technique was used, labeling the cells with yellow or orange colors to indicate if the metabolite significantly decreased or increased, respectively, with respect to the control treatment. Using these data for each variety, the percentage of the metabolites that changed with B was calculated, as well as the number of metabolites that increased or decreased (Table 2). For example, in the case of the Fino 49 lemon, 60 % of the metabolites changed with the irrigation with B, with 6 increasing in concentration, while 6 decreasing. In the case of the Nova variety of mandarin, its metabolic profile changed by 40 %, while for the oranges Navelate and Navelina, their metabolic profile changed by 60 and 45 %, respectively. A correlation study was conducted with this data, in absolute terms: i) Pearson's linear regression matrix (index of tolerance *vs* metabolites of trees irrigated with B, *data not shown*), ii) index of tolerance *vs* metabolites of trees irrigated without an excess of B; and iii) statistical study of principal components, and linear regression of PC1 and PC2 with the

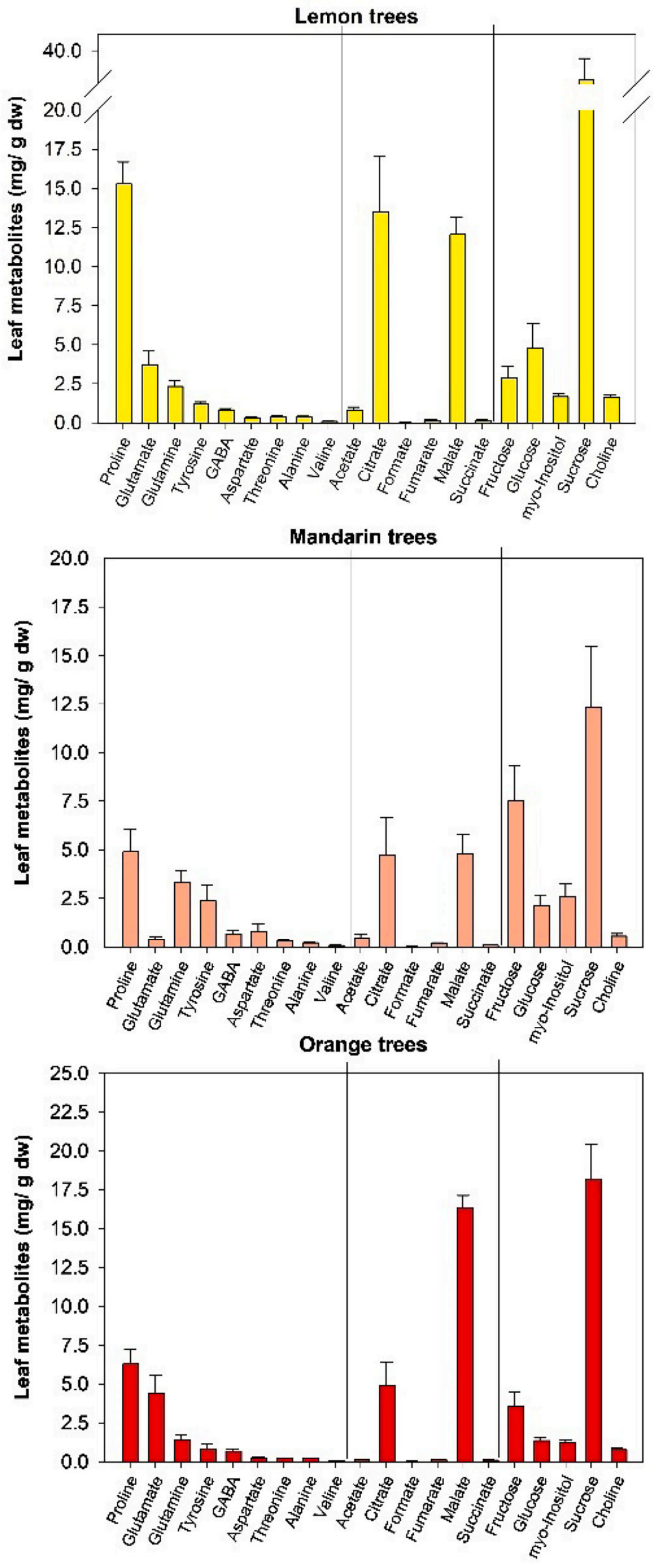

Fig. 6. Concentration of amino acids, organic acids and sugars identified in the H—NMR study. Each value is the average of the varieties belonging to each group ($n = 3$–7) irrigated with the control B treatment of 0.25 mg L^{-1}.

Table 2
Concentration of metabolites (mg g^{-1} DW) in leaves of trees irrigated with excess B (5 mg L^{-1}). Cells in 'yellow' indicate a reduction of the metabolite and cells in 'orange' indicate an increase in the metabolite in relation to the values of their respective control treatment without B (0.25 mg L^{-1}). "Decrease", "Increase", "%Total" refers to the number of metabolites that increased or decreased and the percentage of the total metabolites affected for each cultivar.

	Eureka	Fino	Verna	Murcot	Nova	Ortanique	Iwasaki	Owari	Hernandina	Clemenules	Navelate	Valencia Late	MidKnight	Washington	Lane Late	Sanguinelli	Navelina
Proline	8.98	5.41	7.47	1.86	0.99	4.95	3.63	5.86	2.41	4.58	4.40	3.62	2.90	4.25	4.08	1.48	4.24
Glutamate	4.10	2.37	4.13	0.11	0.06	0.55	0.60	0.17	0.44	0.49	2.75	0.93	0.69	6.44	5.87	0.91	3.36
Glutamine	2.93	3.57	3.32	2.53	1.88	3.76	4.06	3.46	2.43	2.99	0.86	0.71	0.84	1.35	0.53	1.12	0.59
Tyrosine	1.47	0.98	1.07	0.47	0.48	3.03	2.29	3.59	2.87	6.51	1.50	0.15	0.09	3.11	1.33	0.17	1.53
GABA	0.49	0.46	0.65	0.15	0.12	0.81	0.84	0.73	0.69	0.39	0.18	0.18	0.11	0.59	0.47	0.12	0.49
Aspartate	2.41	2.01	1.09	1.12	1.00	0.06	0.62	0.15	0.62	0.42	1.76	1.07	0.54	0.26	0.59	0.34	0.18
Threonine	0.45	0.41	0.40	0.11	0.09	0.30	0.23	0.28	0.26	0.52	0.24	0.15	0.13	0.31	0.20	0.12	0.22
Alanine	0.34	0.54	0.45	0.07	0.05	0.18	0.21	0.16	0.21	0.16	0.13	0.06	0.05	0.20	0.16	0.06	0.20
Valine	0.11	0.24	0.13	0.05	0.03	0.11	0.11	0.19	0.09	0.07	0.05	0.03	0.04	0.08	0.07	0.05	0.08
Citrate	16.64	10.77	14.42	0.81	0.98	0.74	3.79	2.11	1.39	2.78	1.19	0.18	0.25	0.74	2.20	0.17	0.69
Malate	12.28	12.86	12.56	1.45	1.24	0.64	1.23	0.79	1.11	0.92	0.38	0.22	0.27	0.51	0.69	0.35	0.49
Acetate	0.69	0.06	0.32	0.06	0.05	0.22	0.36	0.13	0.39	0.23	0.05	0.02	0.03	0.10	0.22	0.08	0.07
Fumarate	0.16	0.11	0.11	0.03	0.07	0.12	0.23	0.16	0.24	0.26	0.15	0.13	0.02	0.14	0.19	0.02	0.19
Succinate	0.09	0.05	0.12	0.07	0.05	0.06	0.10	0.19	0.09	0.06	0.08	0.02	0.01	0.04	0.01	0.02	0.02
Formate	0.02	0.04	0.04	0.01	0.05	0.04	0.05	0.01	0.05	0.04	0.01	0.02	0.01	0.01	0.03	0.01	0.04
Fructose	3.34	5.48	6.40	1.17	2.69	10.54	11.35	11.77	17.70	8.48	5.47	0.87	1.45	7.67	3.73	2.11	3.96
Glucose	1.97	5.85	2.32	0.34	1.50	7.22	7.88	3.83	3.02	2.70	1.55	1.00	1.02	2.52	1.36	0.82	1.75
myo-Inositol	1.98	3.96	1.88	1.19	0.66	2.81	1.98	2.18	3.14	1.58	1.17	0.94	1.25	2.24	1.00	1.01	1.21
Sucrose	31.68	26.00	39.22	3.37	0.09	15.66	12.25	21.19	2.32	29.36	25.23	21.12	19.34	28.07	30.47	11.43	30.43
Choline	1.63	1.61	1.38	0.10	0.13	0.65	0.57	0.41	0.65	0.52	0.54	0.58	0.84	1.02	1.46	0.41	0.53
Decrease	4	6	4	1	2	7	6	6	5	9	11	8	7	3	6	8	8
Increase	7	6	5	1	6	5	3	2	2	2	1	1	2	4	2	0	1
%Total	55	60	45	10	40	60	45	40	35	55	60	45	45	35	40	40	45

index of tolerance (*data not shown*). However, no significant relationships were found between these parameters. The generalized response among all of the varieties was a decrease in proline, GABA, citrate, malate, and succinate, which was found in 65–70 % of the varieties studied.

4. Discussion

4.1. Index of tolerance and classification with respect to an excess of B in citrus varieties and species

In citrus, it is known that the element B can cause toxicity in leaves when they are irrigated with water with a B concentration higher than 0.25 mg L^{-1}. In general, the high B concentrations in the nutrient solution inhibit the growth of these plants (shoots, roots, or both), affecting productivity, and resulting in toxicity symptoms, especially in older leaves (Gimeno et al., 2012; Navarro et al., 2022; Papadakis et al., 2003; Sheng et al., 2008; Simón et al., 2018, 2019). In most of the citrus B tolerance studies, the level of tolerance provided by the rootstocks to a specific variety were compared. In these studies, it was observed that the Carrizo citrange rootstock was very sensitive to abiotic stress. However, this rootstock is still being used due to its excellent agronomic behavior, production, and harvest quality, in optimal conditions. Thus, it is necessary to understand which cultivar within each variety would be the most ideal for dealing with conditions of high B toxicity. This study presents, as a novel objective, a never before carried out study on the tolerance to excess B of the three most important citrus groups in the Mediterranean area of Eastern Spain, such as lemons, mandarins, and oranges. For the first group, we selected the C*itrus macrophylla* rootstock, as it is the predominant one used in lemons (Eureka, Fino 49 and Verna); for the oranges (Murcot, Nova, Ortanique, Satsuma Iwasaki, Satsuma Owari, Clementina Hernandina, and Clemenules) and mandarins (Navelate, Valencia Late, Midknight, Washington Navel, Lane Late, Sanguinelli, Navelina), the Carrizo citrange rootstock was used, a rootstock that is sensitive to excess B in the irrigation water, although it has an excellent behavior when irrigated with good quality water.

The results obtained through the index of tolerance, established with the use of the percentage of the shoots of the plants irrigated with excess B as related to those irrigated in control conditions, allowed us to classify the varieties of each of the three species into the tolerant, semi-tolerant, and sensitive groups (Table 1, Fig. 1). This resulting classification is the following: 1) Lemons – *Semi-tolerant*: Eureka; and *Sensitive*: Verna and Fino. 2) Oranges – *Tolerant*: Murcot and Nova; *Semi-tolerant*: Ortanique; and *Sensitive*: Clemenules, Hernandina, Iwasaki and Owari. 3) Mandarins: *Tolerant:* Navelate, Valencia Late and Midknight; *Semi-tolerant*: Washington, Lane Late, Sanguinelli and Navelina. Also, after studying the data in a global manner, according to species, we can state that in general, the oranges were more tolerant to B toxicity as compared to mandarins or lemons. These results are especially relevant for selecting species and varieties that will be planted in areas that are irrigated with waters that have a high B concentration, such as water coming from desalinating plants.

4.2. The index of tolerance to B stress is not directly related with the B concentration in leaves

In citrus, in conditions of high B concentration in the irrigation water, B is accumulated in plants via the transpiration stream (Brdar-Jokanovic, 2020; Brown and Shelp, 1997; Takano et al., 2008). In addition, its mobility is restricted to the plant's interior, where the transpiration flow determines the B concentration that is accumulated in the apical part and the edges of older leaves (Garcia-Sanchez et al., 2020). In our previous experiments, we observed that citrus trees accumulate a high concentration of B in their leaves when they are irrigated with 5 mg L^{-1} of B. Thus, Verna lemon trees (Gimeno et al., 2012) irrigated with this concentration accumulated up to 7.4 more B than the trees irrigated with a B concentration of 0.25 mg L^{-1} (leaf concentration of 100 mg kg^{-1} DW). With respect to the rootstock, in the previous experiment, it was also observed that it had a great influence on B accumulation in the leaves, with Carrizo citrange being considered as a B includer, while the Cleopatra mandarin considered an excluder. In the present assay, however, it was observed that the variety had a very little influence on the concentration of the B in the leaves in the combinations scion/rootstock that have the same rootstock for each specie studied. Thus, the varieties of lemon, mandarin, and orange obtained means of 600, 850, and 700 mg kg^{-1} de B, respectively, independent of the cultivar. The only exceptions were found in the Ortanique mandarin, which obtained a greater B concentration than the rest of the mandarin varieties (approx. 1100 mg kg^{-1}), and the Navelina orange, which obtained the least concentration, with respect to the other orange varieties (approx. 400 mg kg^{-1}). Thus, it can concluded that in citrus, the variety has a very little influence on the accumulation of B in leaves, with the rootstock being the main factor that regulates the concentration of B.

To discover if the index of tolerance of each citrus specie was related with the accumulation of B in the leaves, a statistical study based on the linear regression model between these two parameters (index of tolerance *vs* B_{leaf} concentration) was performed, as well as a cluster conglomeration study (Fig. 5, Table 1). The results showed that there was no direct relationship between the index of tolerance within each specie and the accumulation of B in their leaves. Thus, the second most important conclusion that can be extracted from this study (aside from the classification of the cultivars according to the tolerance of each specie), is that the tolerance to B toxicity between cultivars of the same species does not exclusively depend on the concentration of B accumulated in the leaves. Thus, the reactivity of B within plants depends on other factors, such as the physiological and metabolic responses of each of the cultivars; this hypothesis is supported by the fact that although the mandarins accumulated a high concentration of B (except for Iwasaki), they had a broad range of sensitivities and tolerances.

As for the B phytotoxicity study performed according to the measurement of chlorophyll concentration, it was observed that within a concentration range of 50–150 mg kg^{-1}, no negative visual symptoms were observed (Fig. 1– *control plants*). However, concentrations higher than 150 mg kg^{-1} DW resulted in symptoms and damage to the leaves, although the degree of severity of these symptoms was dependent on the variety itself, and not the B concentration in the leaves. The clementine varieties of Hernandina and Clemenules had the most severe symptoms of chlorosis, with chlorophyll values lower than 10 (Fig. 1, 3 and 4). These symptoms appeared as a result of B in crops such as citrus having a limited mobility in the phloem, which leads to the development of burns in the tips and edges of the older leaves due to the high concentration of B in these tissues (Brown et al., 1998). These data support our hypothesis that the level of incidence for the same concentration of B in leaves depends on the reactivity of B for each variety, and the responses and mechanisms of said variety for limiting or palliating its toxicity. A possible biochemical mechanism for B tolerance could include the internal compartmentation of B in vacuoles, an antioxidant defense response to oxidative stress, or by counteracting the B-induced upregulation of the cell wall-associated proteins pectin methylesterase (PME) and expansins (Chatzissavvidis and Antonopoulou, 2020).

4.3. Metabolomics as a tool for discerning the metabolic routes and/or metabolites that are affected due to B toxicity in citrus crops

This metabolic study aimed to describe how the metabolic profile of leaves from the different citrus crops changed when irrigated with a high concentration of B in the irrigation water, using the –omic technique of H—NMR for this, as it allows us to analyze a large number of primary metabolites in a fast and simple manner. This study reveals that the metabolites that were most affected, as a generalized response to B toxicity in citrus, were proline, GABA, citrate, malate, and succinate, whose concentration decreased, with respect to the control treatment, in 65–70 % of the varieties studied. This indicates that B damages a large variety of metabolic processes and routes pathway in citrus leaves (Table 2).

The decrease in the concentration of amino acids such as proline, and GABA indicates that B toxicity negatively affects the following physiological and metabolic functions of citrus trees: i) proline – the capacity for osmotic adjustment, protection of cell membranes, and deactivation of reactive oxygen species (ROS) and nitrogen reserve compounds (Dar et al., 2016); ii) GABA – pH regulation, nitrogen reserves, and regulation of nitrogen metabolism, etc., (Bown and Shelp, 1997; Satya Narayan and Nair, 1990). In the specific case of proline, it is broadly described that in citrus cultivation, B excess produces a reduction in the levels of leaf proline (Papadakis et al., 2004; Simón-Grao et al., 2018); however, its role in the decrease of GABA concentration in a generalized manner was not known until now. This response could be related with the fact that; i) an increase in the synthesis of protein was produced, which have amino acids in their chemical composition, ii) that the synthesis routes of these amino acids are directly interrupted by the high concentration of B and/or iii) that the citrus plants use these amino acids as antioxidant molecules or sources of nitrogen, etc., as with proline (Simón-Grao et al., 2018).

The decrease in the concentration of the organic acids acetate, citrate, and succinate clearly indicates that B toxicity could affect the Krebs cycle (tricarboxylic acid cycle), with the associated negative effects, such as the loss of energy in the form of ATP, the loss of reducing power, unbalancing of organic acids, and the loss of amino acid precursors and phenolic compounds (Alasalvar et al., 2001; Balasundram et al., 2006; Camacho and Salinas, 2005; Lattanzio, 2013; Naiko et al., 2019).

On the other hand, the differential responses of the tolerant varieties with respect to the semi-tolerant or sensitive ones are difficult to identify if the metabolites are studied individually, although it was observed that in tolerant varieties, some responses could be related with this tolerance. In the case of lemons, Eureka was the most tolerant to B toxicity, and in this variety, a decrease in acetate, citrate, and succinate was not observed; but on the contrary, an increase in concentration was observed, together with an increase in thyroxine and glutamic acid. Thyroxine has been identified as a compound with a great antioxidant activity, while aspartic acid is closely related with the acclimation to stress. It also acts as a biomarker, has roles in signaling, and is associated with phytohormones (Khalid et al., 2022). In the case of mandarins, the cultivars tolerant to an excess of B in the irrigation water were Murcot and Nova, and their metabolites were the least affected by B toxicity. In Murcot, only formate decreased, and in Nova, proline and sucrose. Thus, in these cultivars, the reactivity of B could have decreased, with respect to the rest of the varieties, due to any of the following causes: i) internal compartmentalization of B in vacuoles, ii) an antioxidant defense response against oxidative stress, or iii) counteraction of the positive regulation induced by B of the proteins pectin methylesterase (PME) and expansins associated with the cell wall (Chatzissavvidis and Antonopoulou, 2020). In the case of the oranges, the most tolerant were the varieties Navelate, Valencia Late, and MidKnight. In this case, a clear relationship was not observed between the metabolites that were affected or not by the index of tolerance, although an increase in aspartate was observed in these varieties, which is related with acclimation to stress (Han et al., 2009).

5. Conclusions

In the present study, the relative tolerance of different varieties of lemon, mandarin, and oranges to B toxicity, produced by an excess of this nutrient (5 mg L^{-1}) in the solution used for irrigation, was studied. The index of tolerance for each group of plants, calculated as the ratio between the biomass of the plants irrigated with a high concentration of B and the plants irrigated under low concentrations of B, classified the different varieties studied in the following manner: 1) Lemons – *Semi-tolerant*: Eureka; and *Sensitive*: Verna and Fino. 2) Oranges – *Tolerant*: Murcot and Nova; *Semi-tolerant*: Ortanique; and *Sensitive*: Clemenules, Hernandina, Iwasaki, and Owari. 3) Mandarins: *Tolerant:* Navelate, Valencia Late, and Midknight; *Semi-tolerant*: Washington, Lane Late, Sanguinelli, and Navelina. This index of tolerance was not related with the concentration of B accumulated in the leaves, so that the degree of B reactivity or toxicity could be different in each variety. The metabolic profile indicates that as a general response, B toxicity alters proline, GABA, and the tricarboxylic acid route (mainly the acetate, citrate, and succinate acids), reducing their concentration. As a general response, it was also observed that aspartate plays an essential role in the tolerance to this stress, as the more tolerant varieties in each citrus group experienced a significant increase in its concentration. In future studies, it would be necessary to delve into this metabolite, and its relationship with the tolerance observed in each of the varieties studied.

CRediT authorship contribution statement

V. Navarro-Pérez: Visualization, Software, Resources, Investigation, Formal analysis, Data curation. **I. Simón:** Writing – review & editing, Methodology, Formal analysis. **José M. Cámara-Zapata:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **J. Muñoz-Acero:** Writing – original draft, Visualization, Software, Data curation. **M. Alfosea-Simón:** Writing – original draft, Validation. **F. Garcia-Sánchez:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **S. Simón-Grao:** Writing – original draft, Validation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research has been carried out thanks to a grant from the Valencian Agency for Agrarian Development and Guarantee (AGCOOP_A/2019–008). We thank the Rural Development Program of the Valencian Community for cooperation projects related to innovative and sustainable experiences between producers and research centers with crops adapted to climate change and produced with agroecological models, and the following public administrations: THE European Union (EAFRD), THE Ministry of Agriculture, Fisheries and Food (MAPA), and THE Department of Agriculture, Rural Development, Climate Emergency and Ecological Transition.

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