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Arbuscular mycorrhizal symbiosis improves tolerance of Carrizo citrange to excess boron supply by reducing leaf B concentration and toxicity in the leaves and roots

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ABSTRACT

This study explores the possibility of using mycorrhization as a novel technique for diminishing the negative effects of boron (B) in the nutrient solution on seedlings of Carrizo citrange rootstock plants. For this, an experiment was planned for studying the physiological (gas exchange and chlorophyll fluorescence parameters), morphological (vegetative growth parameters), nutritional (organic solutes, carbohydrates) and oxidative stress responses of seedlings that were either mycorrhized (+AM, *Rhizophagus irregularis*; previously known as *Glomus intraradices*) or not mycorrhized (-AM), and irrigated with water containing different concentrations of B (0.5, 5 and 10 mg L^{-1}). It was observed that an excess of B in the nutrient solution decreased the vegetative growth in both +AM and -AM plants, but this decrease was greater in -AM plants. Mycorrhized plants (+AM) under high B concentration accumulated less B in the leaves, and had a smaller reduction of net assimilation rate of CO_2 and lower MDA concentration than non-mycorrhized plants. Thus, it can be concluded that mycorrhization increased the tolerance to high boron concentration in the irrigation water of citrange Carrizo seedlings by reducing both the B concentration in the plant tissue and the B toxicity in the physiological processes. The study of organic solutes and carbohydrates also pointed to a different response model between +AM and -AM plants that could be related to the different tolerance observed between these plants.

1. Introduction

Boron (B) is an essential micronutrient for plants, although it may not be essential for fungi or bacteria (with the exception of cyanobacteria). In nature, it is found as boric acid (H_3BO_3), borate [$B(OH)_4$], or as the mineral borosilicate. In most soils, B is found in extremely small quantities, oscillating between 2 and 100 mg L^{-1}. Most of it is not usable for plants, as they can only assimilate it when it is found at a concentration that ranges from 0.4 to 5 mg L^{-1}, and as it is mostly provided by the organic fraction (Reid, 2014). However, in the last 10 years, the crops from arid and semi-arid climates, as in the case of citrus in eastern Spain, have started to experience toxicity problems due to B excess. This has come from the use of water from waste treatment and desalinating plants, which have a B concentration that is higher than 0.5 mg L^{-1} (Martínez-Álvarez and Martín-Górriz, 2014). In agriculture, B concentrations of 0.5 mg L^{-1} in the irrigation water is considered to be harmful to crops.

The most common symptom found in plants exposed to B is the presence of burns that appear as chlorotic and/or necrotic patches on the margins and tips of the most mature leaves (Papadakis et al., 2004; Gimeno et al., 2012; Simón-Grao et al., 2018). These toxicity symptoms have been generally correlated to the accumulation of high concentrations of B in the leaves, which is proportionally correlated to the concentration of B in the soil and exposure time (Simón-Grao et al., 2018). This high concentration of B in the leaves culminates in the alteration of a wide range of physiological processes, which include: i) inhibition of photosynthesis and decrease of stomatal conductance (Gimeno et al., 2012; Simón-Grao et al., 2018), ii) increase in lipid peroxidation and alterations of the antioxidant-pathway enzymes (Landi et al., 2014; Shah et al., 2017; Simón-Grao et al., 2018), iii) increase in membrane permeability (Piñero et al., 2017), iv) reduction of proton extrusion in the roots (Roldán et al., 1992) and v) depositions of suberin and lignin (Singh et al., 2013); with all of these damages negatively affecting the production and quality of fruit.

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In the specific case of citrus, one of the most important crops in the Mediterranean area of Spain, the optimal leaf B concentration oscillates between 36 and 100 mg L^{-1}, depending on the variety and age of the tree. This crop also has the peculiarity that it is very sensitive to B toxicity, shifting from deficiency to excess within a very narrow margin of concentration (Grattan et al., 2015). In this crop, the rootstock genotypes play a fundamental role in the tolerance of these trees to abiotic stresses, such as drought, cold, salinity, alkalinity, and B toxicity (Syvertsen and García-Sanchez, 2014; Nawaz et al., 2016). Among the most common commercial citrus rootstocks, sour orange (*Citrus aurantium* L.) is considered to be tolerant to salt, drought and B toxicity as compared to Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) which is considered sensitive (Syvertsen and García-Sanchez, 2014; Ribeiro et al., 2014; Simón-Grao et al., 2018). The greatest sensitivity of this rootstock to the high concentration of B in the irrigation water seems to be directly related to the high concentration of B that accumulates in the tissues. Diverse research studies have shown that this rootstock plant rapidly accumulates a high concentration of B in its leaves, as compared to other rootstocks such as sour orange or Foner-Alcaide n°5 (Gimeno et al., 2012; Simón-Grao, 2015). In normal growing conditions, however, the Carrizo citrange rootstock provides improved agronomic features to mandarin and orange trees related to growth, production and quality of fruit, as compared to other rootstocks. Thus, Carrizo citrange has become the most-utilized rootstock in Spain (Ribeiro et al., 2014).

As the Carrizo citrange rootstock confers good agronomic characteristics to citrus trees under normal conditions, the Spanish citrus sector has aimed to apply agronomic strategies that help mitigate the adverse effects produced by abiotic stresses including B excess, onto trees grafted onto this rootstock. For example, it has been shown that the use of this rootstock can improve the tolerance to cold (Oustric et al., 2017) and drought conditions (Oliveira et al., 2017), as well as B toxicity (Ruiz et al., 2016), by using its tetraploid form. Its tolerance to salinity can also be improved through the over-expression of its glyoxalase-system genes (Alvarez-Gerding et al., 2015) or through the use of mycorrhizal fungi (Navarro et al., 2014). Mycorrhizal inoculation not only affects root morphology but also the physiological status of host plants, becoming a potential and possible tool that can be used for increasing a plant's tolerance to environmental stress conditions (Shireen et al., 2018; Kumar et al., 2017).

This research study will explore the use of mycorrhizal fungi for mitigating the damages suffered by the Carrizo citrange rootstock when it is irrigated with water that contains high concentrations of B (Navarro et al., 2014). Thus, the objective of this research is to study the effect of mycorrhizae on the morphological, biochemical and nutritional responses of Carrizo citrange plants irrigated with a high concentration of B, in order to understand if the use of mycorrhizae could be a good agronomic strategy for increasing tolerance to excess B in irrigation water, and also to understand what mechanisms could be implicated in the different tolerances between plants with and without Arbuscular mycorrhizal fungi.

2. Materials and methods

2.1. Plant materials and experimental conditions

In this experiment, one and a half years old Carrizo citrange (*Poncirus trifoliata* L. × *Citrus sinensis* L.) rootstock plants acquired from a commercial nursery (Viveros Vivercitrus 2000 S.L.), were used. The seedlings were grown in a greenhouse under the same conditions as described by Simón-Grao et al. (2018). Half of the plants were inoculated in the soil at transplanting time with 0.5 g of a commercial preparation of the *Rhizophagus irregularis* strain (MYC 4000, ITHEC, Castelmaurou-France; previously known as *Glomus intraradices*). The plants were irrigated with a complete nutrient solution of macronutrients and micronutrients (Simón-Grao et al., 2018). A 0.25 mg L^{-1} B concentration was considered as the control, as this is an adequate B concentration for normal plant growth (Hoagland and Arnon, 1950). Each group of plants (with mycorrhizae, +AM; or without mycorrhizae, -AM) were divided into 3 subgroups. One of the groups was irrigated with 0.25 mg L^{-1} B, while the others, in order to induce B toxicity, were provided with H_3BO_3 until reaching concentrations of 5 mg L^{-1} B and 10 mg L^{-1} B, respectively. The B treatments were applied for 80 days (from June to September), after which the plants were harvested. For each mycorrhizae and B treatment combination, 8 plants were used, and these were randomly distributed throughout a surface area of 30 m^2.

2.2. Plant growth parameters

At the end of the experiments (plants watered with B for 80 days), the plants were harvested and processed as described by Simón-Grao et al. (2018). The dry masses of the leaves, stem and roots were used to calculate the total plant dry mass.

2.3. Mycorrhizal colonization and proliferation of the intraradical mycelium

The root samples taken at the end of the experiment were used to determine the mycorrhizal colonization, following the method described by Phillips and Hayman (1970), and this was quantified with the use of the formula described by Hayman et al. (1976): % colonization = (number of small roots infected/ number of total small roots observed) × 100. To determine the percent proliferation of the hyphae within the intraradical mycelium, different values were assigned according to the results (Simón-Grao, 2015): Null (presence of 0% of the hyphae in the root sample, Low (1–40%), Normal (41–70%), High (71–90%) and Very high (> 90%). For this, twenty-five root segments per plant were mounted onto slides, squashed by pressing on the coverslips, and examined for AM colonization.

2.4. Boron concentration in the different plant tissues

The B concentrations in the different plant tissues (leaves, stem and root) were determined in dry samples by inductively coupled plasma optical emission spectrometry (ICP-OES, Iris Intrepid II, Thermo Electron Corporation, Franklin, USA) following the same procedure described by Simón-Grao et al. (2018). Leaf samples were collected at 0, 25, 50 and 80 days from the start of the assay from 8 plants per treatment (three leaves per plant), and stem and root samples were harvested at the end of the experiment.

2.5. Leaf gas exchange and chlorophyll fluorescence parameters

The net assimilation rate of CO_2 (A_{CO2}), and leaf transpiration (E_{leaf}), were measured at 0, 25, 50 and 80 days using a portable photosynthesis system (model CIRAS-2, PP-System, Amesbury, MA, USA). During the measurements, the equipment was set to maintain the light (PAR: 1000 μmol m^{-2} s^{-1}) and the CO_2 concentration (400 ppm) constant in the measurement chamber. The measurements were conducted between the hours of 8:00–10:00 a.m. in one leaf per plant, for a total of eight plants per treatment. Leaves from the middle part of the plants were used, as these leaves were fully developed and healthy. New shoots and old leaves were not utilized.

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 (Simón-Grao et al., 2018).

2.6. H_2O_2 and MDA determination

At the end of the experiment, before completely harvesting the

plants, leaves were frozen in liquid nitrogen for the study of oxidative stress. The quantification of H_2O_2 was conducted following the method described by Yang et al. (2007) and modified by Simón-Grao et al. (2018). Lipid peroxidation was determined by measuring malondialdehyde (MDA) using the method by Hodges et al. (1999) and modified by Simón-Grao et al. (2018).

2.7. Proline and QAC 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) and modified by Simón-Grao et al. (2018). Quaternary ammonium compounds (QAC) were extracted from dry tissue with 1 M H_2SO_4 and quantified using a glycine-betaine standard according to the method described in Grieve and Grattan (1983) and modified by Simón-Grao et al. (2018).

2.8. Carbohydrate determination

The carbohydrate concentration was also analyzed at the end of the experimental period in the different tissues of the plant. The extraction of the carbohydrates was carried out using 80% ethanol with constant mixing for 30 min. A sulfuric acid assay with the anthrone reagent was performed to measure the total soluble carbohydrates. The procedure used for the measurement of the reducing sugars included an extraction with copper sulfate and arsenic-molybdate reagents (Hodge and Hofreiter, 1962; Simón-Grao et al., 2018). And lastly, the protocol for the measurement of starch included an extraction from the pellet with MES solution and gelatinization using a heat-stable alpha-amylase (Haissig and Dickson, 1979; Simón-Grao et al., 2018). Soluble sugars, reducing sugars and starch were quantified using glucose as a standard.

2.9. Statistical analysis

The statistical analysis included a two-way ANOVA, which was performed with the statistical package SPSS (Chicago, IL, USA), with two mycorrhizae strategies (Carrizo citrange without mycorrhizae, -AM; and Carrizo citrange with mycorrhizae, +AM) × three B treatments (control, 0.25 mg L^{-1}; moderate boron, 5 mg L^{-1}; and severe boron, 10 mg L^{-1}). There were 8 biological repetitions for each Mycorrhizae × Boron combination. When the variables were significant ($P < 0.05$), the treatment means were separated using Duncan's multiple range test.

3. Results

3.1. Percent mycorrhization and proliferation of the intraradical mycelium

The mycorrhized Carrizo citrange plants (+AM) did not show significant differences in the percentage of colonization after 80 days of being watered with 5 or 10 mg L^{-1} B with respect to the +AM plants from the control treatment (0.25 mg L^{-1}; Table 1). As for the proliferation of the intraradical mycelium however, a significant effect was observed when compared with the B treatments. While the +AM/control plants had a normal proliferation of mycelium (40–69%), the +AM plants subjected to B excess (5 and 10 mg L^{-1}) had a reduced proliferation, reaching values of less than 40%.

3.2. Growth parameters

At the end of the experiment, all the plants were harvested, and their growth parameters were measured. In the control B treatment, the mycorrhized plants (+AM) had 34% less total dry biomass as compared to the non-mycorrhized plants (-AM). The decreased growth of the +AM plants was due to the mycorrhization process reducing the growth of leaves, stems and roots down 43%, 37% and 23%, respectively (Fig. 1).

Table 1
Mycorrhization and proliferation of the intraradical mycelium. Percent root colonization and proliferation of the intraradical mycelium in roots of the citrange Carrizo rootstock with mycorrhizae (+AM) under boron excess conditions (0.25, 5 y 10 mg L^{-1}).

	Boron treatment (mg L^{-1})			*ANOVA*
Into the root	**0.25**	**5**	**10**	**Boron**
Mycorrhizal colonization (%)	65.1	62.5	65.8	**ns**
Mycelial proliferation (%)	40–69 (normal)	< 40 (low)	< 40 (low)	*

*In the ANOVA: "ns" means non-significant differences at 95%, and * indicates significant differences at $P < 0.05$ between boron treatments ($n = 8$).*

The total dry biomass results showed that the -AM and the +AM plants had a reduced biomass that was dependent on the concentration of B applied, with the reduction being 19% and 47% for the 5 and 10 mg L^{-1} treatments, respectively, in -AM plants, and 22% and 37% in +AM plants. The dry weight of the leaves decreased due to the excess boron treatments (5 and 10 mg L^{-1}) in +AM plants as well as the -AM plants. However, this decrease was smaller in +AM plants. Thus, the +AM/5 mg L^{-1} and +AM/10 mg L^{-1} treatments had a reduction of leaf dry weight of 13% and 57%, respectively, as compared to the control treatment, while the -AM/5 mg L^{-1} and -AM/ 10 mg L^{-1} plants had a reduction of 30% and 77%, respectively (Fig. 1).

The results of the stem and the root with an excess of boron was similar for +AM and -AM plants, as shown by the lack of significant differences in the interaction M × B. Thus, independently of the plants having mycorrhizae or not, the increase in the concentration of B in the nutrient solution tended to decrease the dry weight of these plant parts (Fig. 1).

3.3. Concentration and distribution of B in the plant tissues

The excess of B in the nutrient solution resulted in a gradual increase of B in the leaves (B_{leaf}) with time, with a greater concentration of B always being observed in the treatment with 10 mg L^{-1} B as compared to the 5 mg L^{-1} B one (Fig. 2). The significant differences between the treatments with excess B (5 and 10 mg L^{-1}) and the control treatment were observed from the second sampling date (after 25 days), reaching the highest values at the end of the experiment (80 days). Thus, the accumulation of B_{leaf} was progressive, without reaching a stationary state during the entire experimental period.

The mycorrhizae also played a fundamental role in the accumulation of B in the leaves. For each excess B treatment, the +AM plants always had the lowest concentration of B_{leaf} as compared to the -AM plants. At the end of the experiment, the concentration of B increased in the following order: +AM/Control = -AM/Control < +AM/5 mg L^{-1} < -AM/5 mg L^{-1} < +AM/10 mg L^{-1} < -AM/10 mg L^{-1}. However, at 25 and 50 days, the leaf B concentration of -AM/5 mg L^{-1} plants was higher than plants from the +AM/5 mg L^{-1} and +AM/10 mg L^{-1} treatments.

The concentration of B in the stem and the root also increased, as its concentration increased in the nutrient solution (Fig. 2), although there were no significant differences between the 5 and 10 mg L^{-1} B treatments. The general effect of the mycorrhizae in these plant tissues was also to decrease the concentration of B with respect to -AM plants. Also, when comparing the concentration of B according to tissue, the following order was observed (from greater to lower concentration): leaves > root > stem, with this order maintained in -AM and +AM plants.

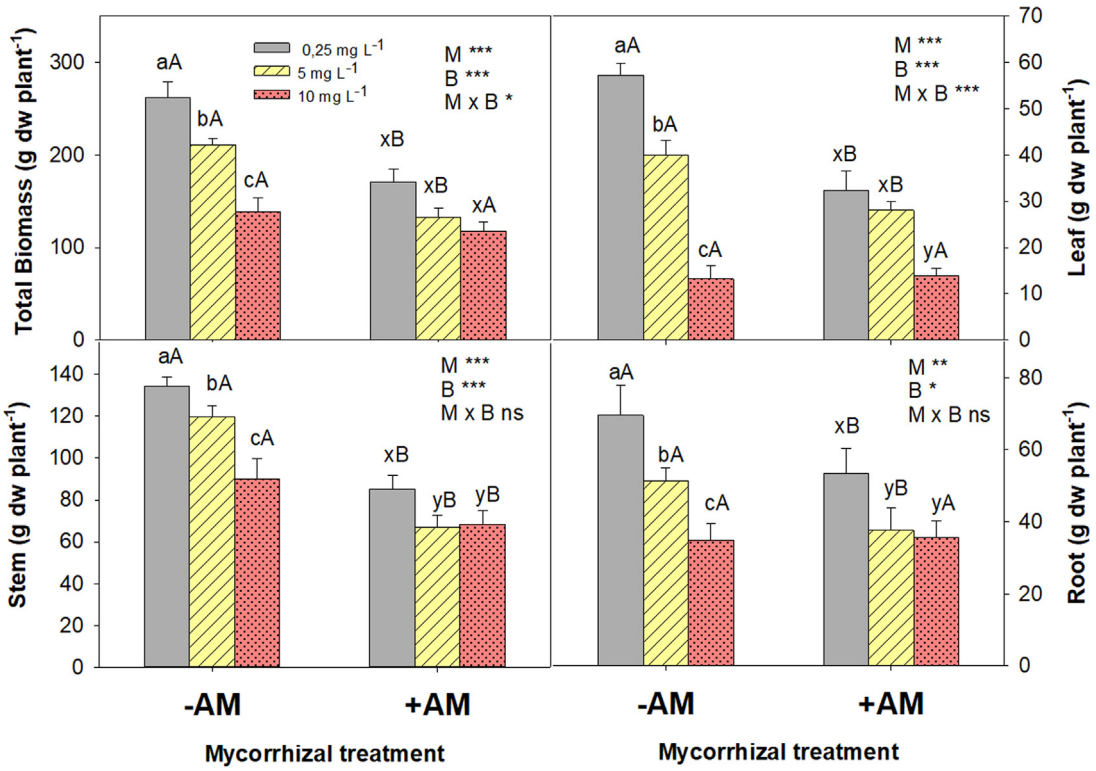

Fig. 1. Effects of B excess in the nutrient solution on the total, leaf, stem and root dry biomass in Carrizo citrange plants without (-AM) and with (+AM) mycorrhizae. In the ANOVA, "*ns*" means non-significant differences at 95% confidence level; on their part, *, ** and *** indicate significant differences for $P < 0.05$, 0.01 and 0.001, respectively. For each mycorrhizae treatment, the different *lowercase letters* indicate significant differences between the boron treatments at $P < 0.05$ as established by Duncan's multiple range test (*a, b,c for -AM; x, y,z for +AM*). The different *uppercase letters*, for each boron treatment, indicate significant differences between -AM and +AM. The vertical bar indicates the standard error of the mean ($n = 8$).

3.4. Leaf gas exchange and chlorophyll fluorescence parameters

Fig. 3 shows the gas exchange, A_{CO2}, and the E_{leaf} parameters of the +AM and -AM plants according to the B treatments (5 and 10 mg L^{-1}), as a relative percentage of the values from their respective control treatments (0.25 mg L^{-1} B). The change of A_{CO2} and E_{leaf} with time of the -AM plants shows that these parameters were affected by the excess of B starting at day 50. On this day, the A_{CO2} and the E_{leaf} decreased in plants watered with 10 mg L^{-1} B with respect to the control treatment; however, the 5 mg L^{-1} treatment did not have an effect on these parameters. In the measurement from day 80, the A_{CO2} continued to decrease in the 10 mg L^{-1} B treatment (not the E_{leaf}), and the A_{CO2} and E_{leaf} in the -AM/ 5 mg L^{-1} treatment were also reduced. The results of the changes from the +AM plants showed a tendency similar to the -AM plants. At day 50, the +AM plants had a significantly reduced A_{CO2} under the 10 mg L^{-1} treatment, although the E_{leaf} was not affected by any of the B treatments. In the measurement at 80 days, the two B treatments decreased both A_{CO2} and E_{leaf} parameters.

Although the changes in A_{CO2} and E_{leaf} were similar for both +AM and -AM plants, the A_{CO2} results showed that the +AM plants suffered less damage with the excess B than the -AM plants. For example, in the -AM plants under the 10 mg L^{-1} treatment at day 50, and in the 5 and 10 mg L^{-1} treatment at day 80, the A_{CO2} was reduced by 70%, 65% and 90%, respectively; while in the +AM plants these reductions were 44%, 23% and 78%, respectively (Fig. 3). The chlorophyll fluorescence parameters were not affected neither by the mycorrhization nor the B treatments (Table 2).

3.5. Biochemical determinations

The leaf concentration of proline of the +AM plants did not change significantly with the excess B treatments (Table 2); however, in the -AM plants, there was an observed significant decrease of 20% and 33% for the 5 and 10 mg L^{-1} treatments, respectively. The concentration of the QAC and starch were significantly affected by the B treatments, but only in the +AM plants. With respect to the QAC, the +AM/10 mg L^{-1} plants had a higher concentration (7.51 mg g^{-1} dw) with respect to the other two treatments and the three B treatments with -AM plants (Table 2). The starch concentration in +AM plants decreased with the B excess treatments, although there were no significant differences between +AM/5 mg L^{-1} and +AM/10 mg L^{-1}.

The MDA concentration of +AM plants from the control treatment was half the concentration of the -AM plants (Fig. 4). However, the mycorrhization did not affect the concentration of H_2O_2 (Fig. 4). Nevertheless, the effects of the B treatments on the concentration of MDA and H_2O_2 were dependent on the presence or not of mycorrhizae. Thus, in -AM plants, the concentration of these compounds progressively increased as the B concentration increased in the nutrient solution. However, in +AM plants, only an increase in MDA and H_2O_2 was observed when these plants were watered with 10 mg L^{-1}, and this increase was smaller than the one observed for -AM plants.

In leaves, there were small changes in the total soluble (SS_{leaf}) and reduced (RS_{leaf}) sugars due to the different factors studied in this experiment. Thus, independently of the mycorrhization, the concentration of SS_{leaf} decreased with the increase of the concentration of B in the nutrient solution. While for RS_{leaf}, the mycorrhization altered their concentration, so that the +AM plants had a greater concentration than the -AM ones (Fig. 5).

In the stems, there was a mycorrhization × boron treatment interaction in the total soluble sugars (SS_{stem}) as well as the reducing sugars (RS_{stem}). For the SS_{stem}, this interaction was evident. Thus, while in the -AM plants the concentration decreased 13% and 28% with the 5 and 10 mg L^{-1} B treatments, respectively, in +AM plants no significant differences were observed among the treatments (Fig. 5). The opposite

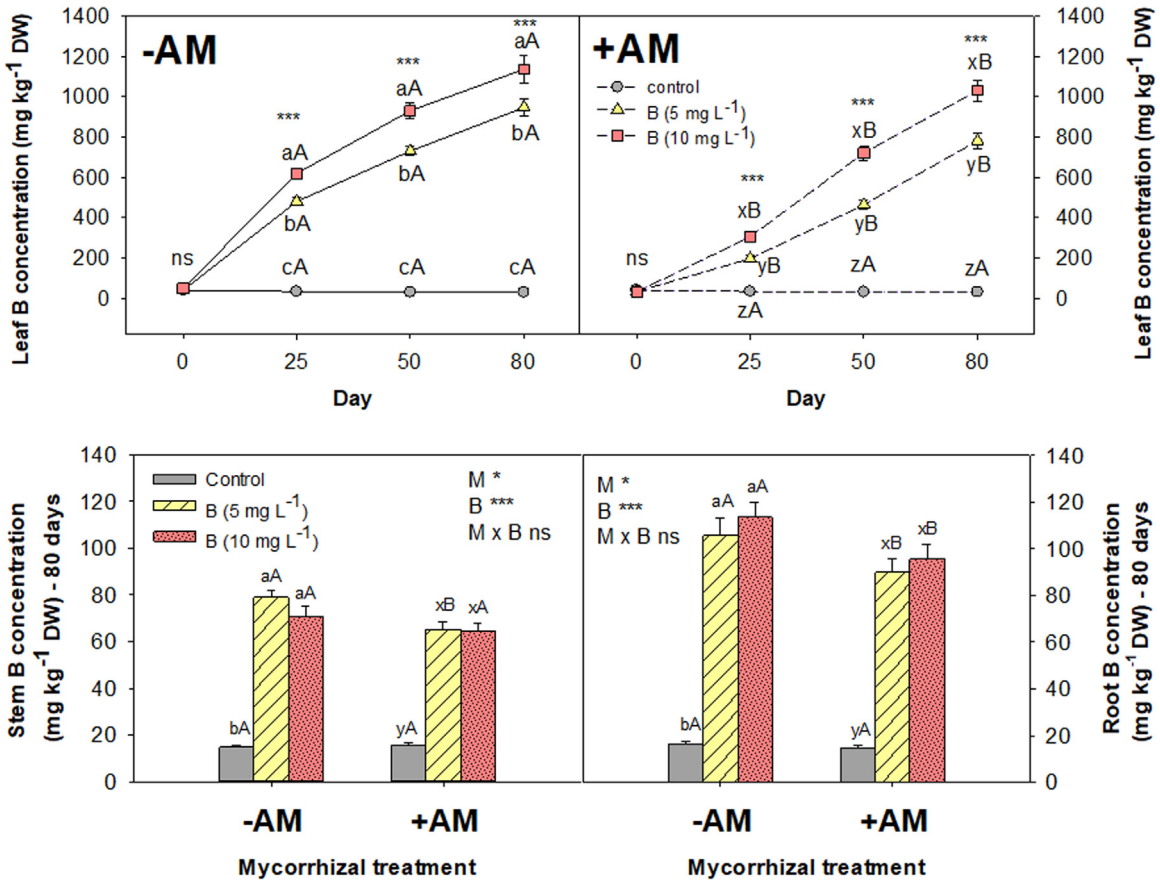

Fig. 2. Changes in B_{leaf} with time, and B concentration in the stem and root at the end of the experiment as a response to an excess of boron (0.25, 5 and 10 mg L^{-1}) in Carrizo citrange plants without (-AM) and with (+AM) mycorrhizae during the 80 days of experiment. In the ANOVA, "*ns*" means non-significant differences at 95% confidence level; on their part, * and *** indicate significant differences for $P < 0.05$ and 0.001, respectively. For each mycorrhizae treatment strategy, the different *lowercase letters* indicate significant differences between the boron treatments at $P < 0.05$ (*a, b,c for -AM; x, y,z for +AM*); for each B treatment, the different *uppercase letters* indicate significant differences between -AM and +AM at $P < 0.05$. In both cases, as established by Duncan's multiple range test. The vertical bar indicates the standard error of the mean ($n = 8$).

was observed in the RS_{stem}, where no changes were observed in the -AM plants under the B treatments, but these decreased with B excess in +AM plants.

In the root, there was also a mycorrhization × boron treatment interaction, in the SS_{root} and the RS_{root} results. The interaction with SS_{root} was made evident in that in -AM plants, the 5 mg L^{-1} treatment reduced their concentration with respect to the other B treatments; while in the +AM, differences were only found between the 5 mg L^{-1} and 10 mg L^{-1} treatments, with the latter having the least concentration (Fig. 5). As for the RS_{root} results from the -AM plants, the same tendency was found as the concentration of SS_{root}, but in the +AM plants, the concentration progressively decreased when the B concentration increased in the nutrient solution.

4. Discussion

The positive effect of the fertilization with B in the formation of mycorrhizae was first observed in citrus plants with *Citrus jambhiri* in the year 1989 (Dixon et al., 1989). However, little is known about the effect that an excess of B could have on the fungi-plant symbiotic relationship. In this study, it was observed that the percentage of colonization of the mycorrhizae was not altered. However, an effect was observed in the proliferation of the mycelium within the root. Our results showed that in conditions of excess of this micronutrient, mycelium growth was less than 40%, while in control plants, these values were normal (40–60%). In normal conditions, when the fungus finds a root that is susceptible to colonization, there is a notable stimulation that is manifested as an abundant proliferation and ramification of the mycelium. This allows the hyphae to contact the root, adhering to its surface through appressoriums (pre-colonization structures), through which it will initiate the penetration of the plant's root. Once the fungus-plant association is established, the host plant cells provides sucrose to the interfacial matrix through passive transport, which will be later hydrolyzed into glucose and fructose (the main reducing sugars), and lastly, the glucose is absorbed by the fungus through a process of active transport in the arbuscules, where it will transformed into more useable forms (mainly poly-alcohols, trehaloses and glycogen; Dixon et al., 1989). In this sense, the lower growth rate of the +AM plants with respect to –AM plants observed at the end of our experiment could also be due to +AM plants going through a period of adjustment in which their growth rate stopped or decreased. During the establishment of the plant/AM symbiosis, the plant's resources are diverted towards the defense against the fungus and/or the establishment of the symbiosis with the fungus, instead of investing these resources for its own growth. Nevertheless, taking into account the above, the negative effect of excess B on the proliferation of the intraradical mycelium could be due to two factors: I) the decrease of A_{CO2} due to the B toxicity in the leaves could have decreased the amount of sugars the fungus normally received for its optimum growth; and/or II) the formation of *B-polyol* complexes in the extraradical mycelium, which impedes the proper use of the sugars from the plant by the mycorrhizae. These organisms allocate most of the sugars from the plant to the extraradical mycelium, where they are transformed to *polyols*. These fungal carbohydrates have a high affinity for forming complexes with B (immobile

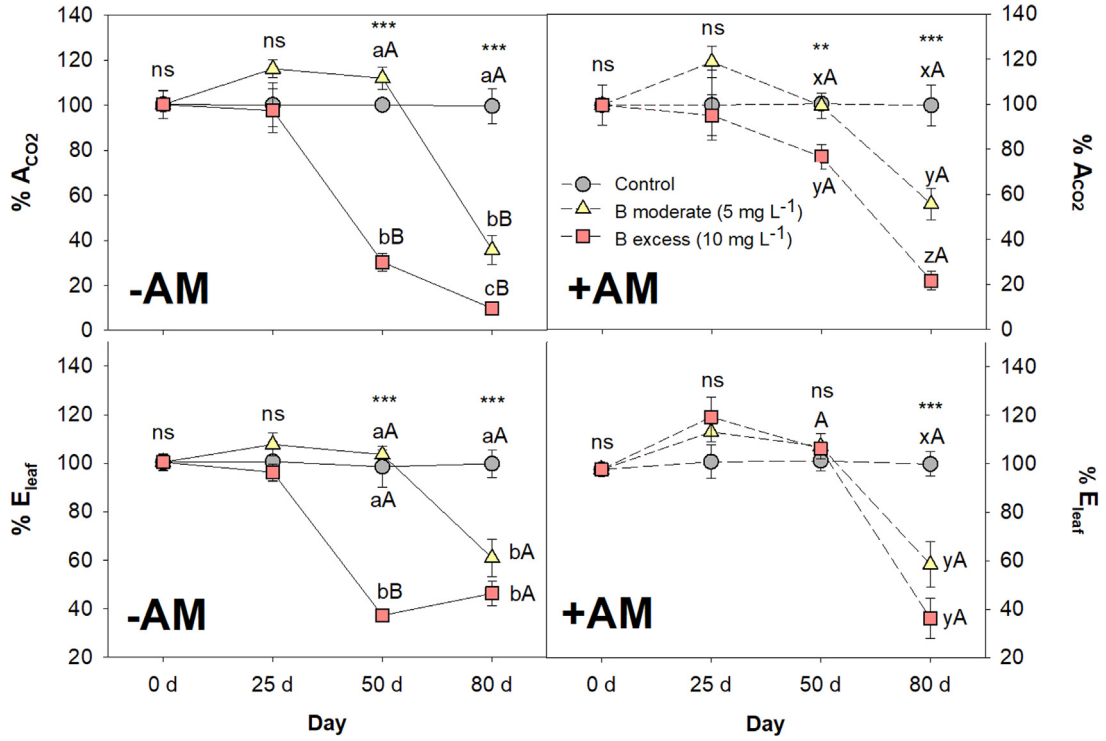

Fig. 3. Changes in the net CO_2 assimilation rate (A_{CO2}) and leaf transpiration (E_{leaf}) in response to an excess of boron (0.25, 5 and 10 mg L^{-1}) in Carrizo citrange plants without (-AM) and with (+AM) mycorrhizae during the 80 days of experiment. The data are shown as a relative percentage of the data from the control treatment. In control plants, 0.25 mg L^{-1} B, the average values were the following ACO_2, μmol m^{-2} s^{-1}, -AM = 10.9, +AM = 10.4; E_{leaf}, mmol m^{-2} s^{-1}, -AM = 2.72; +AM = 2.57). In the ANOVA, "*ns*" means non-significant differences at 95% confidence level; on their part, ** and *** indicate significant differences for $P < 0.01$ and 0.001, respectively. For each mycorrhizae treatment strategy, the different *lowercase letters* indicate significant differences between the boron treatments at $P < 0.05$ (*a, b,c for -AM; x, y,z for +AM*) as established by Duncan's multiple range test. The vertical bar indicates the standard error of the mean ($n = 8$).

Table 2
Effect of an excess of boron 0.25, 5 mg L^{-1} and 10 mg L^{-1} B) on the following leaf parameters: quantum efficiency of photosystem II (ΦP_{SII}), concentration of proline, quarternary ammonium compounds (QAC) and starch.

	Boron treatments	Φ_{PSII}	Proline (mg g^{-1} dw)	QAC (mg g^{-1} dw)	Starch (mg g^{-1} dw)
-AM	0.25 mg L^{-1} B	0.453	19.17 aA	6.33 aA	18.43 aB
	5 mg L^{-1} B	0.404	15.35 bA	6.77 aA	19.47 aA
	10 mg L^{-1} B	0.409	12.94 cB	6.39 aB	21.43 aA
+AM	0.25 mg L^{-1} B	0.461	17.96 xA	6.22 yA	21.65 xA
	5 mg L^{-1} B	0.411	16.45 xA	5.78 yB	15.97 yB
	10 mg L^{-1} B	0.471	17.52 xA	7.51 xA	17.14 yB
	ANOVA (M × B)	ns	***	**	***

In the ANOVA, "ns" means non-significant differences at 95% confidence level; on their part, *, ** and *** indicate significant differences for $P < 0.05$, 0.01 and 0.001, respectively. For each mycorrhizae treatment, the different lowercase letters indicate significant differences between the boron treatments at $P < 0.05$ (a,b,c for -AM; x,y,z for +AM); for each B treatment, the different uppercase letters indicate significant differences between -AM and +AM for $P < 0.05$. In both cases as established by Duncan's multiple range test (n = 8).

form) and thus could not be used as a source of energy by the growth of the fungus.

The Carrizo citrange plants are very sensitive to B, as they accumulate great concentrations of this micronutrient in their leaves. However, despite the proliferation of the fungus being limited by the excess of B, the vegetative growth data showed that the mycorrhization improved the tolerance of the leaves of Carrizo citrange plants watered with excess B. The 5 and 10 mg L^{-1} B treatments reduced the growth of +AM plants by 13% and 57% with respect to the control treatment, respectively, while for -AM plants it was reduced by 30% and 77%. The greater tolerance of the mycorrhized plants could be due to these plants accumulating a lesser concentration of B in the leaves as compared to the non-mycorrhized plants. These data are in agreement with results described by Sonmez et al. (2014), who observed that the concentration of B in mycorrhized wheat plants was lower than in plants that had not been infected. In plants, the total B that accumulates in their tissues depends on various factors such as leaf transpiration, water use efficiency, the shoot: root ratio, net absorption of B by the roots, etc.

In this experiment, taking into account all the variables analyzed, the differences observed between plants with mycorrhizae and plants without could be due to the different capacity of net absorption of B by the roots, as there were hardly any differences in the physiological parameters between +AM and -AM plants. Nevertheless, the present study did not make clear if the decrease in the net absorption of B by the mycorrhizae could be due to another mechanism that restricts the uptake or favors the efflux of B from the interior of the root towards the outside, the place where the sugars that are typical of the fungi could play a fundamental role.

Besides the negative effects produced by B toxicity in the leaves, the decrease in the root growth is another typical response found in crops that are sensitive to B excess, and it is believed that this is due to the high sensitivity shown by the meristematic areas of the root (Reid et al., 2004). Our results showed that the roots from the Carrizo citrange plants were very sensitive to the excess of B in the nutrient solution. But our results also showed two important aspects, such as: i) the concentration of B in the nutrient solution, rather than the concentration of B that accumulated in the root, could be inhibiting the root's growth, and on the other hand, ii) that in the highest B treatment (10 mg L^{-1}), the inoculation with *R. irregularis* could be protecting the root, thereby decreasing B toxicity. These two aspects are backed by the fact that a

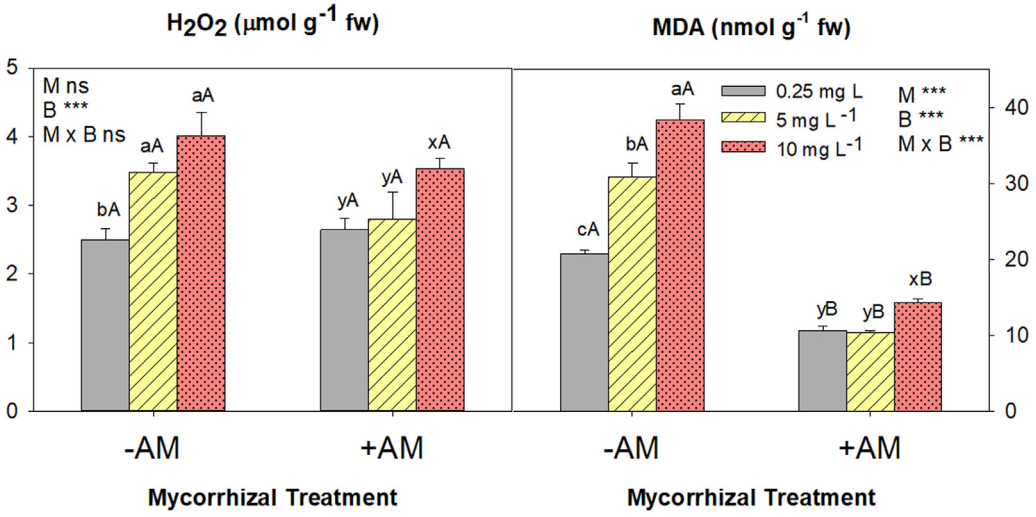

Fig. 4. Effect of an excess of boron (0.25, 5 and 10 mg L^{-1}) on the concentration of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in Carrizo citrange plants without (-AM) and with (+AM) mycorrhizae during the 80 days of experiment. In the ANOVA, "*ns*" means non-significant differences at 95% confidence level; *** indicates significant differences for $P < 0.001$. For each mycorrhizae treatment strategy, the different *lowercase letters* indicate significant differences between the boron treatments at $P < 0.05$ (*a, b,c for -AM; x, y,z for +AM*); for each B treatment, the different *uppercase letters* indicate significant differences between -AM and +AM at $P < 0.05$. In both cases, as established by Duncan's multiple range test. The vertical bar indicates the standard error of the mean ($n = 8$).

direct correlation between the concentrations of B that accumulated in the root and the decrease in growth was not observed. For example, in -AM plants, the concentration of B in the root was similar (110 mg kg^{-1} dw) for both excess B treatments. However, the reduction of the root biomass was 26% and 50% for the 5 and 10 mg L^{-1} treatments, respectively. As for the second aspect mentioned previously, this was backed by the fact that the reduction of the root growth from the +AM plants was similar for both B treatments (5 and 10 mg L^{-1}), despite the different concentrations in the nutrient solution.

Besides the different accumulation of B in the leaves of +AM and -AM plants, it seems that in the +AM plant leaves, B was less toxic than in the -AM plants. This could be deduced from the response observed for the gas exchange parameters (A_{CO2} and E_{leaf}). In both +AM and -AM plants, there was a linear and significant relationship between the B concentration in the leaves versus the net CO_2 assimilation rate. In this correlation, it was observed (*data not shown*) that in +AM plants, A_{CO2} decreased 14% for every 100 mg kg^{-1} dw of B accumulated in the leaves; while that in -AM plants, this descended to 22% for each 100 mg of B kg^{-1} dw.

Thus, this indicates that B toxicity was more severe in -AM plants than in +AM plants, and therefore the mycorrhizae were, in some way, protecting the photosynthetic machinery of the plants from the toxic effects of B. In other plant species such as wheat, it has been described that mycorrhization increases their tolerance to salinity, because in these plants a series of mechanisms are activated that protect the plants from Na^+ toxicity. Among these mechanisms, the osmotic adjustment, the improvement of antioxidant systems, optimization of carbon and nitrogen metabolism, are notable (Talaat and Shawky, 2014).

Our chlorophyll fluorescence results excluded the idea that the photochemical machinery of the leaves (ability to capture light and convert it to chemical energy) was damaged, as the Φ_{PSII} parameter was not altered due to an excess of B, neither in the mycorrhized nor non-mycorrhized plants. However, although this parameter was not altered by B, there was a decrease in the A_{CO2}/Φ_{PSII} ratio when A_{CO2} decreased (*data not shown*), which meant that in the photosynthetic process there was an excess of electrons that did not participate in the electron transport chain, and thus, were directed to an alternative acceptor such as O_2. As a result, there was an over-production of reactive oxygen species that caused oxidative stress at the cellular level (Cakmak and Römheld, 1997; Cakmak, 1994). Nevertheless, the reduction of the A_{CO2}/Φ_{PSII} ratio was less in -AM plants, which could have had important consequences for the different responses of the mycorrhized and non-mycorrhized plants to excess B. Also, the fact that in the leaves from +AM plants the concentration of MDA was not affected by the 5 mg L^{-1} B treatment, and little affected by the 10 mg L^{-1} B treatment, despite the decrease in the A_{CO2}/Φ_{PSII} ratio, suggests that the plants inoculated with *R. irregularis* must have had a very powerful and efficient antioxidant system, thus being able to manage the reactive oxygen species (ROS) that were been created as result of the excess B.

The synthesis of organic solutes, mainly proline and quaternary ammonium compounds, is one of the most common responses of plants to stress (Parvaiz and Satyawati, 2008; Ashraf and Foolad, 2007). The role of proline in the process of osmotic adjustment, the detoxification of ROS and the protection of membranes against lipid peroxidation has been studied before (Hong et al., 2000; Martínez-Cuenca et al., 2015). Under conditions of excess B in citrus, a reduction in the levels of proline has been observed. Along with Papadakis et al. (2004), we suggest that leaf proline could be used as a negative biomarker of the degree of tolerance to B stress in citrus. Thus, a greater concentration of proline in the leaves signifies a lesser B toxicity. The results obtained in this study on the organic solutes back the idea that +AM plants are more tolerant to excess B as compared to -AM plants, as the concentration of proline hardly changed in the former, but significantly decreased in the latter.

The study on the carbohydrates suggests that the reducing sugars could play a decisive role on the decreased incorporation of B in the +AM plants. The root's cells depend on the supply of sugars from leaf tissues for their growth. These sugars are synthesized by the process of photosynthesis, and are transported through the phloem in the form of sucrose towards the root. In B toxicity conditions this sugar supply gains more importance, especially its reducing form (glucose and fructose), as shown in tolerant species (Choi et al., 2007). On its part, when plants are mycorrhized, the demand for these sugars by the root increases even more (Tinker et al., 1994; Graham, 2000). However, not too many studies have shed light on what occurs with the distribution of carbohydrates in mycorrhized plants that suffer conditions of excess B. In this experiment, it was observed that the effects of the B excess treatments on the concentration of soluble and reducing sugars and their relationship, were dependent on the mycorrhization of the plants. What was observed in mycorrhized plants was the following: the total soluble sugars were not affected by the B excess treatments, neither in leaves, stems nor roots. Thus, sugars are being produced, and these compounds are transported to the mycorrhized root. However, the concentration of reducing sugars in the root diminishes with the excess B treatments of 5 and 10 mg L^{-1}. Having in mind the metabolism of carbon in fungi, these reducing sugars from the plant could have been transformed to sugar *–diols*. As previously mentioned, B has a high affinity for these types of sugars, which lead to the formation of *B-polyol*

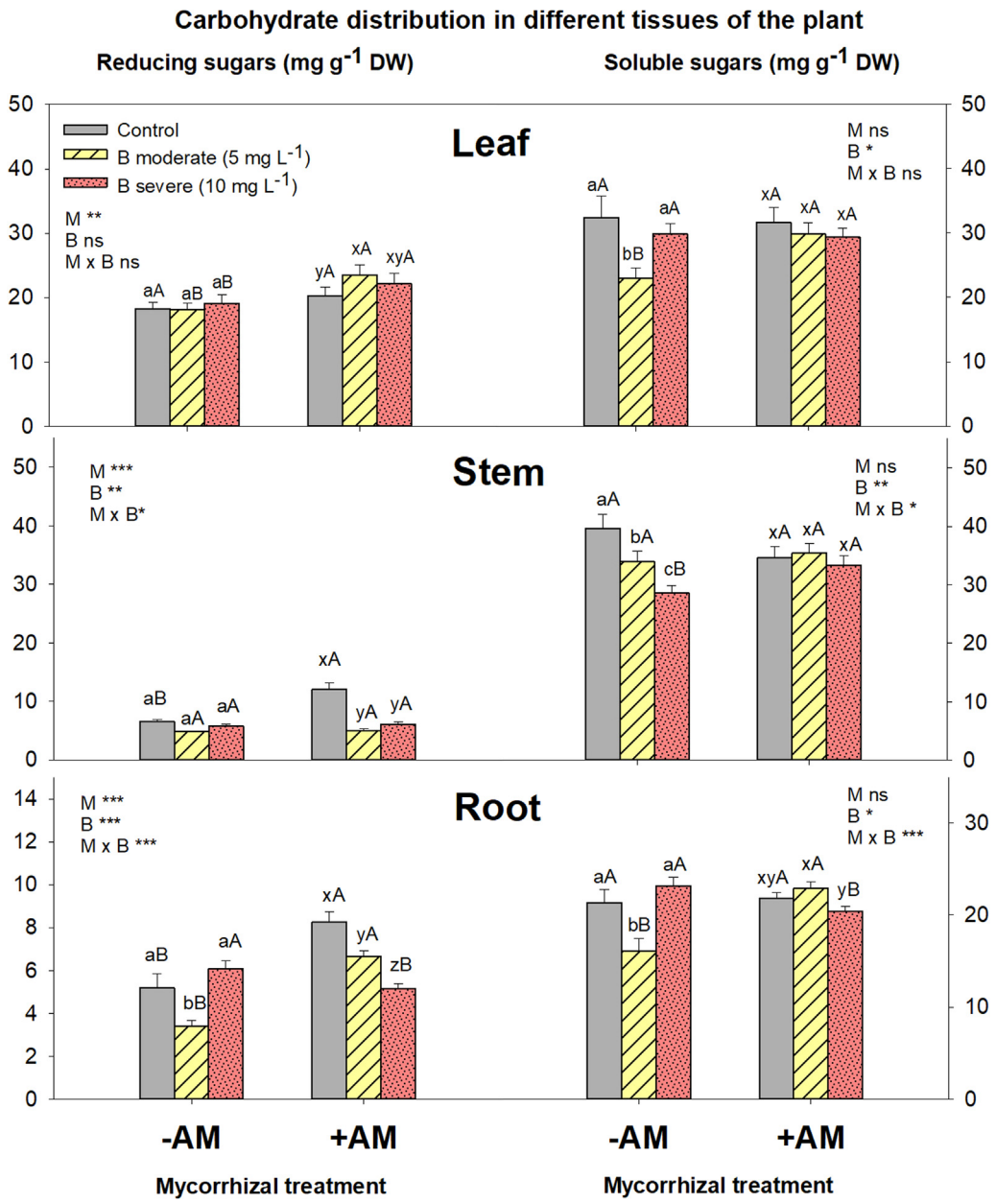

Fig. 5. Effect of boron excess (0.25, 5 and 10 mg L^{-1}) on the concentration of reducing (RS) and soluble (SS) sugars present in the different plant tissues of the Carrizo citrange rootstocks without (-AM) and with (+AM) mycorrhizae during the 80 days of experiment. In the ANOVA, "*ns*" means non-significant differences at 95% confidence level; on their part, *, ** and *** indicate significant differences for $P < 0.05$, 0.01 and 0.001, respectively. For each mycorrhizae treatment, the different *lowercase letters* indicate significant differences between the boron treatments at $P < 0.05$ (*a, b,c for -AM; x, y,z for +AM*); for each B treatment, the different *uppercase letters* indicate significant differences between -AM and +AM for $P < 0.05$. In both cases as established by Duncan's multiple range test. The vertical bar indicates the standard error of the mean ($n = 8$).

complexes. Thus, on the one hand, this implies that mycorrhizae cannot use part of the sugars from the plant, as these are complexed with *B-polyols*, and therefore their proliferation in the interior of the root is reduced; and on the other hand, when B is complexed with the *diols*, it could be immobilized within the mycorrhizae's structures and/or expelled towards the exterior of the plant. Thus, this hypothesis could explain: i) the lesser proliferation of the mycelium within the root, ii) the lesser incorporation of B to the root tissue of the Carrizo citrange rootstock and, therefore, iii) the lesser concentration of B accumulated in leaves as compared to -AM plants in every case.

5. Conclusions

It was observed that tolerance to B excess supply can be improved in Carrizo citrange, a citrus rootstock that is considered sensitive to this stress. This was shown by the mycorrhizae/plant symbiosis avoiding a high B accumulation in the leaves and decreased B toxicity in leaves and roots as reported by the physiological and oxidative stress study. However, more studies are needed in order to understand in detail why and how mycorrhizae limit the accumulation and toxicity of boron in plant tissue. The results of the carbohydrate study could suggest that the fungal *–diol* sugars, and the high complexation affinity with B to form *B-polyol* compounds could play a decisive role in the observed response of the Carrizo citrange plants to an excess of B. These *B-polyol* complexes could limit the toxicity and the entry of B in the plants, although this interesting hypothesis has yet to be explored. In addition, another important aspect observed in this experiment was that although high B concentration in the soil medium did not affect the percentage of mycorrhization, it did affect the proliferation of mycelium within the roots. Thus, it seems logical to suggest that the search for mycorrhizae strains that are more tolerant than *R. intraradices* to an excess of B could greatly improve the results of mycorrhization.

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