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Boron modulates the plasma membrane H^+-ATPase activity affecting nutrient uptake of *Citrus* trees

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Abstract

Boron (B) affects plasma membrane (PM) integrity and consequently modulates the P-type PM H^+-ATPase activity creating a driving force for nutrient influx at the root level. Because citrus rootstocks respond differently to B supply, we hypothesised that PM H^+-ATPase activity of varieties contrasting in horticultural traits would affect nutrient uptake by trees. Sweet orange (*Citrus sinensis*) trees grafted onto Rangpur lime (RL; *Citrus limonia*) or Swingle citrumelo (SW; *Citrus paradisi* × *Poncirus trifoliata*) were grown in nutrition solution with four B concentrations (0 [control concentration], 46, 230 and 460 μM B, as H_3BO_3) up to 7 days of treatment imposition after plant adaptation into the hydroponic condition. SW exhibited higher B absorption, leaf B and enzyme activity than RL. The highest enzyme activity was achieved with 230 μM of B 1 day after treatment imposition (ATI), whereas B excess impaired the PM H^+-ATPase in all periods evaluated. Absorption of mineral nutrients correlated with PM H^+-ATPase activity, with greater nutrient uptake per root unit in SW compared to RL. Leaf and root nutrient concentrations were equivalent to amounts absorbed and enzyme activity, with greater increments exhibited by trees grafted onto SW compared to RL. Effects of B supply on PM H^+-ATPase activity explain distinct nutrient uptake patterns by trees, what supports fine-tuning fertilisation guidelines of citrus taking into account rootstock varieties.

KEYWORDS

enzyme activity, membrane hyperpolarisation, nutrient absorption, proton extrusion, root medium, rootstocks

1 | INTRODUCTION

Boron (B) is essential for vascular plants, exerting key metabolic and physiological functions, and therefore regulating yield and quality of crops (Landi, Margaritopoulou, Papadakis, & Araniti, 2019; Reid, 2014; Shireen et al., 2018). In citrus, B deficiency frequently affects orchards worldwide (Liu, Wang, Liu, Wu, & Jiang, 2013; Wang, Yang, Pan, Liu, & Peng, 2015; Zhou et al., 2014), usually associated with low soil availability and environmental stresses, such as drought or excess rain (Wu, Lu, Riaz, Yan, & Jiang, 2018). Citrus rootstocks exhibit distinct absorption and demand for B, which results from different physiological and anatomical traits of trees (Boaretto, Quaggio, Mourão Filho, Giné, & Boaretto, 2008; Mattos Jr., Hippler, Boaretto, Stuchi, & Quaggio, 2017; Mesquita et al., 2016) and which also affects water uptake and transport (Mesquita et al., 2016). The most common rootstock genotypes used in the Brazilian citrus industry have been replaced in the last few decades by new ones exhibiting improved resistance/tolerance to diseases or highest adaptation to adverse

environmental conditions (Hippler et al., 2018; Mattos Jr. et al., 2017; Zambrosi, Mesquita, Tanaka, Quaggio, & Mattos Jr., 2013).

Boron maintains the integrity, elasticity and function of the cell wall and plasma membrane (PM), auxin and phenol metabolism as well as affects the transcription of genes related to nitrogen metabolism and oxidative stress (Camacho-Cristóbal & Gonzáles-Fontes, 2007; Landi et al., 2019; Macho-Rivero et al., 2018; Turan, Taban, Kayin, & Taban, 2018). Moreover, investigations reported that B acts as a modulator agent of the root PM H^+-ATPase in selected plant species (Camacho-Cristóbal & Gonzáles-Fontes, 2007; Martinez-Ballesta et al., 2008). Indeed, the role of B on the cell wall, PM and mainly on the PM H^+-ATPase likely provide a rationale for differences observed on plant nutrient uptake varying B supply (Carvalho, Castro, Kozak, & Azevedo, 2020; Macho-Rivero et al., 2018; Mesquita et al., 2016; Riaz et al., 2019).

Ion pump ATPases are transmembrane enzymes with the ability to discriminate two compartments by pumping specific ions through cell membranes (Falhof, Pedersen, Fugslang, & Palmgren, 2016; Palmgren & Morsomme, 2019). The P3-type PM H^+-ATPase is considered a master enzyme, only found in a plant's PM (Palmgren & Morsomme, 2019; Zhang, Bu, & Liang, 2016). This enzyme uses the energy of ATP hydrolysis to pump H^+ from the cytoplasm to the apoplast (Falhof et al., 2016), which promotes a driving force for nutrient uptake through the PM after hyperpolarisation and generation of a transmembrane pH gradient (Dalir, Khoshgoftarmanesh, Massah, & Shariatmadari, 2017; Palmgren & Morsomme, 2019; Zhang et al., 2016). In fact, many physiological processes are mediated by this enzyme and each one requires specific regulators for modulation of proton pump activity (Dalir et al., 2017; Haruta, Tan, Bushey, Swanson, & Sussman, 2018; Palmgren & Morsomme, 2019). However, the relevance of each factor in the modulation of PM H^+-ATPase activity is unclear.

Absorption dynamics of B is not affected by PM H^+-ATPase activity in situ, because this nutrient is mostly absorbed by diffusion and its distribution is driven by the plant transpiration process (Macho-Rivero et al., 2018; Wimmer & Eichert, 2013). However, previous work has demonstrated that potassium (K) concentration in citrus trees increased with the increment of B applied to the soil (Mattos Jr. et al.,-2017), likely related to the role of K in the maintenance of charge equilibrium in root cells (Shabala, 2017). The PM H^+-ATPase activity has recently been correlated with K retention (Shabala, 2017), expanding its known role in the plant nutrition, also reported for absorption of nitrogen (N) (Luo et al., 2013; Zhang et al., 2018), phosphorus (P) (Yan, Zhu, Müller, Zörb, & Schubert, 2002) and iron (Fe) (Santi & Schmidt, 2009). Previously, it was found that B deficiency can decrease the vanadate-sensitive ATPase activities and ATP-dependent H^+ pumping in sunflower cells (Ferrol, Belver, Roldán, Rodriguez-Rosales, & Donaire, 1993) and the expression of H^+-ATPase in the PM of tobacco roots (Camacho-Cristóbal & Gonzáles-Fontes, 2007). Thus, it is tempting to speculate that B supply in the root medium could improve nutrient uptake by modulating the PM H^+-ATPase activity of root cells.

There is a lack of information about B supply and modulation of the PM H^+-ATPase activity in roots, especially in woody plants such as citrus, in which plant nutritional and water demand varies according to the combination of scion and rootstock genotypes. In this work, we tested the hypothesis that modulation of the PM H^+-ATPase enzyme activity by B supply in the root medium could differentially affect the absorption of nutrients by sweet orange trees grafted onto two rootstocks contrasting in B demand and horticultural characteristics.

2 | MATERIAL AND METHODS

2.1 | Plant material and growth conditions

Young sweet orange (*Citrus sinensis* (L.) Osbeck cv. Valencia) trees grafted onto Rangpur lime (RL; *Citrus limonia* (L.) Osbeck) or Swingle citrumelo (SW; *Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.) were used as biological models. Rootstocks were produced in nursery pots with organic substrate (80% pine bark, and 20% vermiculite), fertilised with macro and micronutrients for 6 months, then grafted to continue to grow under similar management conditions for another 6 months before starting treatment imposition. The experiment was set up in a completely randomised, 2 × 4 factorial design, with two citrus rootstocks (SW and RL) and four B concentrations in the nutrient solution (NS; 0 [control], 46, 230 and 460 μM B, as H_3BO_3), with three replicates per evaluation period ([before B addition], 1 day, 3 days and 7 days). The B concentration of 46 μM in the NS was considered adequate for growth of young citrus trees (Boaretto et al., 2008). Before transferring trees to the hydroponic condition, conducted into pots with 6 L of NS, roots were washed with distilled water to remove particles of substrate from the root tissue. Thereafter, plants were let to adapt at 25% of the final NS concentration for 1 week and at 50% of the final NS for 3 weeks. Plants were then maintained at full NS, in mM: 12.85 N (80% N-NO_3), 0.64 P, 4 K, 5 Ca, 1.2 Mg and 1.2 S, and in μM: 89 Fe, 9.1 Mn, 3.06 Zn, 0.95 Cu, 0.52 Mo (modified from Zambrosi et al., 2013) for other 4 weeks that concluded the adaptation period without B supply to plants. During adaptation of plants to the hydroponic condition, new roots grew and no visual symptoms of B deficiency were observed. Indeed, B acquired by trees in the nursery sustained adequate growth until B treatment imposition.

After 7 weeks of tree transplanting to the NS, treatments were started by adding B at various concentrations to the NS. Furthermore, volume of solution in the pots was kept constant by addition of deionised water when necessary and the pH of the NS (~7.0) was adjusted to 5.0–5.5 with 1.0N H_2SO_4.

Three plants of each treatment were destructively harvested in four periods (before B addition, 1 day, 3 days and 7 days ATI). In each evaluation period, root PM H^+-ATPase activity was assayed as described in the following section. Then, plants were destructively harvested and separated into leaves and roots. Total leaf area was measured using the Leaf Area Integrator LI-3100 (LI-COR; Lincoln, NE). Dry mass (DM) was determined by oven drying at 60–63°C till constant weight, and the plant material was ground to pass through a 200-mesh sieve, B was extracted in 0.1N HCl and quantified with azomethine-H colorimetric method (Gaines & Mitchell, 1979), while

the other nutrients were determined by nitro-perchloric digestion (Bataglia, Furlani, Teixeira, Furlani, & Gallo, 1983) in plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC; Norwalk, CT).

At 0-day and 7-day ATI, 50 ml of the NS of each pot was sampled to estimate amounts of nutrients taken up by trees based on nutrient depletion in the NS and determined by ICP-OES. Nutrient absorbed per root (NtAR) unit for each nutrient was estimated as follows:

$$\text{NtAR} = (\text{NC}_{\text{initial}} - \text{NC}_{\text{final}})/\text{root DM}, \quad (1)$$

where $\text{NC}_{\text{initial}}$ (mmol/L) is the initial concentration of the nutrient in the NS before provided to tree (at 0 day), NC_{final} (mmol/L) is the final concentration of the nutrient in the NS after 7-day ATI and root DM (g/plant) is the total root dry biomass of each tree.

2.2 | Determination of H^+-ATPase activity in PM of root cells

The PM H^+-ATPase activity was assayed in enriched microsomal vesicles isolated from citrus roots. Ten grams of fresh fine roots (<3.0 mm Ø) per tree were homogenised in 20 ml of 250 mM sucrose, 10% glycerol (wt/vol), 0.5% polyvinylpyrrolidone-40 KDa (PVP-40) (wt/vol), 2 mM EDTA, 0.2% bovine serum albumin (wt/vol), 100 mM tris-(hydroxymethyl) aminomethane-HCl buffer (TRIS; pH 7.5), 150 mM KCl, 2 mM dithiothreitol (DTT), 1 mM benzamidine hydrochloride and 1 mM methylphenylsulfonyl fluoride (PMSF). The obtained homogenate was filtered and subjected to three subsequent centrifugations (1,500*g* for 15 min; 10,000*g* for 15 min; 100,000*g* for 40 min) to obtain the microsomal fraction according to De Michelis and Spanswich (1986) with some modifications (Façanha & De Meis, 1995). The microsomal fraction was solubilised in buffer solution containing 15% glycerol (vol/vol), 1 mM DTT, 1 mM PMSF and 10 mM Tris–HCl (pH 7.5). The total protein concentration was obtained by the method described by Bradford (1976).

The ATP hydrolysis activity by H^+-ATPase was determined by measuring colorimetrically by measuring the release of inorganic phosphate (Lebel, Poirier, & Beaudoin, 1978). The composition of the reaction medium contained: 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS; pH 6.5), 3 mM $MgCl_2$, 100 mM KCl, 1 mM ATP and 30 μg of protein, which was obtained from the microsomal fraction. The reaction started after the addition of protein extract and was interrupted by the addition of trichloroacetic acid (TCA) at final concentration of 10% (vol/vol). The value of hydrolysed inorganic phosphate was obtained by the addition of solution with 2% $(NH_4)_6Mo_7O_{24}$ (wt/vol), 2% H_2SO_4 (vol/vol) and 1% ascorbic acid (wt/vol). After 10 min in ambient temperature the inorganic phosphate was determined by spectrophotometry at 790 nm. The specific activity of PM H^+-ATPase was revealed using P-ATPase inhibitor, 0.2 mM Na_3VO_4 (Façanha & De Meis, 1998). The difference between activity measured without P-ATPase inhibitor and activity measured with the P-ATPase inhibitor represented the PM H^+-ATPase activity.

The proton gradient generated by the PM H^+-ATPase was evaluated according to De Michelis and Spanswich (1986), with some modifications (Façanha & De Meis, 1998), by monitoring the decrease in fluorescence (Δfmax%min^{-1}) of the metachromatic fluorescence probe, 9-amino-6-chloro-2-methoxyacridine (ACMA), excited at wavelength of 415 nm and emission captured at 485 nm using a spectrofluorometer (RF-5301PC, Shimadzu; Tokyo, Japan). The ACMA has the ability to transit through the lipid bilayer of the membranes when not protonated, and loses this capacity when protonated. Thus, the pumping of H^+ ions promotes a quenching of probe fluorescence by accumulation in the acidified vesicles. The reaction solution has 10 mM Mops-Tris (pH 6.5), 100 mM KCl, 5 mM $MgSO_4$, 1 μM ACMA and 30 μg of protein. The reaction started with the addition of 1 mM ATP and the electrochemical gradient is disrupted by 20 mM NH_4Cl.

2.3 | Statistical analyses

Descriptive statistics were applied, and the data belonging to each time point (not between different time points), as a complete 2×4 factorial design with two rootstocks and four B in the NS were submitted to the analysis of variance (ANOVA) at 5% level of significance. When significant, data were fitted to regression models to describe relationships between B concentration in plants and NS, PM H-ATPase activity assays, NtAR, both leaf and root nutrient concentration.

3 | RESULTS

3.1 | Plant biomass

Trees were grown with four concentrations of B in the NS for 7 days after treatment imposition (ATI), and no differences were observed on the DM of leaves and roots for B treatments (Table 1). Moreover, trees grafted onto SW exhibited 10% less roots DM than those grafted onto RL, regardless of the B in the NS (Table 1 and Supplementary Figure 1), which difference was already characterised before treatment imposition.

3.2 | Uptake and accumulation of boron by citrus trees on different rootstocks

Boron concentration in roots increased along with the increment of B in the NS up to 82 mg/kg, whereas the same in leaves increased up to 67 mg/kg (Figure 1a,b). Trees grafted onto SW exhibited 15% higher concentration of B in roots than those grafted onto RL (Figure 1a). Furthermore, mean B depletion in the NS was up to 30% higher for plants grafted onto SW compared to RL, whereas B absorbed per root DM unit (NtAR) was 1.6-fold higher in plants grafted onto SW than those onto RL (Figure 1c,d). Moreover, the B concentration in roots and leaves of trees, in spite of the rootstock genotypes, without B

TABLE 1 Dry mass (DM) of leaves and roots, and leaf area of young sweet orange trees (*Citrus sinensis* (L.) Osbeck cv. Valencia) grafted onto Rangpur lime (RL) or Swingle citrumelo (SW), after 7 days grown under different boron (B) concentrations in the nutrient solution

	Rootstock genotype (RG)		*F* test		
B concentration	RL	SW	B	RS	B × RS
Leaf DM					
μM	g/plant				
0	15.5[a]	15.8	ns[b]	ns	ns
46	15.9	15.6			
230	15.6	15.5			
460	16.0	15.6			
Leaf area					
μM	cm^2/plant				
0	1,541	1,524	ns	ns	ns
46	1,559	1,547			
230	1,558	1,537			
460	1,500	1,519			
Root DM					
μM	g/plant				
0	22.7	20.3	ns	*	ns
46	23.2	21.1			
230	22.2	20.8			
460	21.7	19.4			

[a]n = 12.
[b]Not significant (p > .05).
*Significant (p < .05) by F test.

supply exhibited normal B levels (Boaretto, Quaggio, Mattos Jr, Muraoka, & Boaretto, 2011; Mesquita et al., 2016; Figure 1a,b), which was an important indicator that plants did not suffer B deficiency along the short conduction period of the experiment.

3.3 | PM H^+-ATPase activity in citrus rootstocks

Maximum ATP hydrolysis activities were verified for trees with approximately 207 μM B as estimated with data pooled for rootstock and periods of evaluation (Figure 2a,c,e). The highest ATP hydrolysis was ~600 μmol Pi mg^{-1} protein hr^{-1} for trees grafted onto SW at 1-day, 3-day or 7-day ATI, whereas for RL, a similar ATP hydrolysis stimulation was observed only at 1-day ATI (Figure 2a). Thus, SW rootstock exhibited up to 20% higher ATP hydrolysis over time than RL (Figure 2a,c,e). Furthermore, no difference was observed in PM H^+-ATPase activity in roots of RL in any of the B, 7-day ATI (Figure 2e).

The activity of H^+-pumping exhibited a differential pattern of activation in each rootstock genotype. The most remarkable change was recorded at 230 μM B and after 1-day ATI, as the H^+-pumping in SW roots was twofold higher than that of RL (Figure 2b), while in the same condition, there was no meaningful change in the pattern of the ATP hydrolysis driven by the PM H^+-ATPase between the two rootstocks (Figure 2a). Moreover, the overall modulation of the PM H^+-ATPase by B in SW was greater and indicated a much higher coupling between ATP hydrolysis and the H^+-pumping activities than in RL at 1-day and 3-day ATI (Figure 2a–d). However, the H^+-pumping in roots of SW did not vary with B at 7-day ATI, while in roots of RL, the highest activity was observed when grown in 230 μM B for the period (Figure 2f).

3.4 | Absorption of nutrients by citrus rootstocks after B supply

Trees exhibited increased NtAR when B was added in the NS up to 230 μM B, for both rootstocks (Figure 3a–e, g–k), with the exception of Mg^{+2} NtAR in trees grafted onto SW (Figure 3f). However, the increments of nutrient absorption in trees grafted onto SW were higher than those grafted onto RL, mainly for N-NO_3^-, K^+, Ca^{+2}, SO_4^{-2}, Fe^{+2} and Cu^{+2} (NtAR; Figure 3). The increment of the amounts of nutrients taken up followed a polynomial-type response, increasing when 46 μM was added in the NS and decreasing with the highest B in the NS (460 μM). Response curves for K^+, N-NO_3^-, Ca^{+2}, P-$H_2PO_4^-$, S-SO_4^{-2}, Zn^{+2} and Mn^{+2} were similar and in accordance with the PM H^+-ATPase activity (ATP hydrolyses and H^+-pumping), with highest NtAR for these nutrients verified in trees with 230 μM B in the NS. As observed, the different B in the NS also affected the concentration of nutrients in roots and leaves of citrus at 7-day ATI, which also differed between the rootstocks.

The increase in NtAR with B supply through the modulation of the PM H^+-ATPase activity was reflected in nutrient concentration in leaves (Figure 4) and roots (Figure 5) 7-day ATI. Trees grafted onto SW were more responsive to B treatments in the NS, exhibiting higher N, K, P, Fe, Ca and Mg concentrations in leaves when compared to RL (Figure 4). Similarly, trees grafted onto SW exhibited higher root nutrient concentrations compared to RL (Figure 5). Interestingly, the response type observed for nutrient concentrations for both leaves and roots were comparable with those observed for NtAR and PM H^+-ATPase activity, with the highest concentration of nutrients verified when 230 μM of B was added to the NS (Figures 4 and 5). Furthermore, excess of B (460 μM) decreased nutrient concentrations in leaves (Figure 4) and roots (Figure 5), such as observed for enzyme activity (Figure 2) and NtAR (Figure 3).

4 | DISCUSSION

Boron is a key nutrient for citrus, because B status of trees (deficient, sufficient or excessive) explains distinct physiological and nutritional responses affecting plant growth and fruit yield, in part, because of induced structural impairment of root cells and distinct sensitivity of citrus rootstocks to B supply (Boaretto et al., 2008; Mesquita et al., 2016; Wu et al., 2018; Zhou et al., 2014). Under an

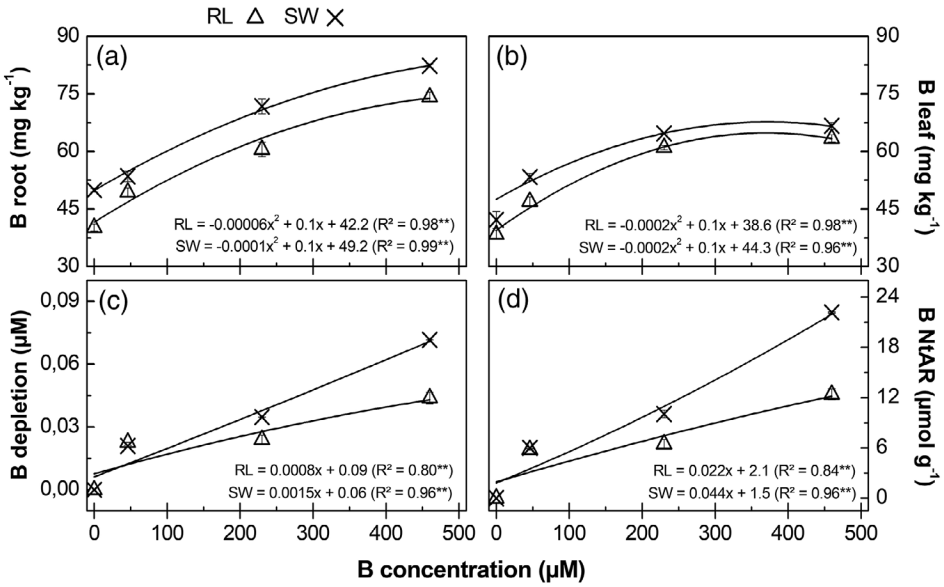

FIGURE 1 Boron (B) concentration in roots (a) and leaves (b), B depletion of nutrient solution (c) and B nutrient absorption per root unit (NtAR) (d) of young sweet orange trees grafted onto Rangpur lime (RL) or Swingle citrumelo (SW), after 7 days grown under different B concentrations in the nutrient solution. Vertical lines represent standard error of the mean (n = 3); ns, not significant (p > .05), *p < .05 and **p < .01

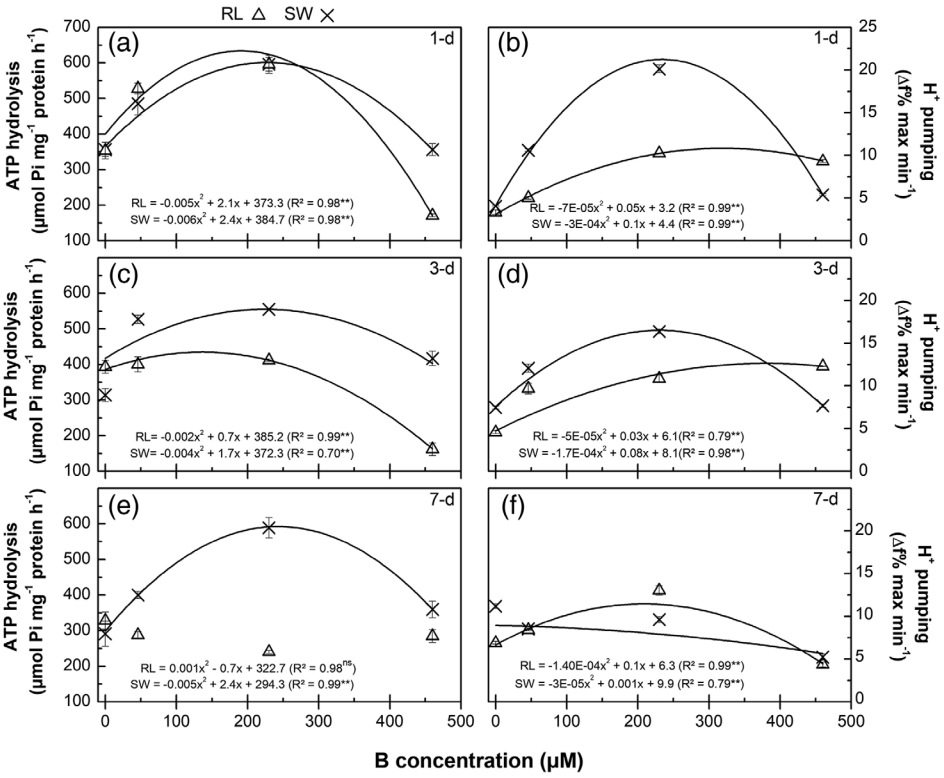

FIGURE 2 ATP hydrolysis (a, c, e) and H^+ pumping activities (b, d, f) by the plasma membrane H^+-ATPase from root microsomes of young sweet orange trees grafted onto Rangpur lime (RL) or Swingle citrumelo (SW) rootstocks, after 1-day, 3-day and 7-day (days) of supplementation with increasing boron (B) concentrations in the nutrient solution. Vertical lines represent standard error of the mean (n = 3); ns, not significant (p > .05), *p < .05 and **p < .01

overall perspective, B deficiency symptoms are commonly observed in citrus orchards of major growing areas in the American continent and Asia (Boaretto et al., 2011; Liu et al., 2013; Mesquita et al., 2016; Wu et al., 2018). The present study reports the modulation of PM H^+-ATPase activity influenced by B in NS, in two citrus rootstocks with contrasting nutrient demand for B (Mattos Jr. et al.,-2017; Mesquita et al., 2016). The enhancement of PM H^+-ATPase activity by B addition in the NS increased NtAR by citrus trees and consequently root and leaf nutrient concentrations in a rootstock dependent manner.

4.1 | Supply of boron modulates PM H^+-ATPase

Differential modulation of PM H^+-ATPase activity in citrus rootstocks is reported in this study. The addition of 230 µM B in the NS enhanced PM H^+-ATPase activity of root cells (Figure 2), which was higher for trees grafted onto the SW rootstock compared to RL. Despite B absorption not being directly related to the H^+-ATPase function in roots, this enzyme activity was sensitive to B availability in the growing medium (Figure 2). Activity of PM H^+-ATPase is regulated by several plant physiological factors, indicating multiple regulatory

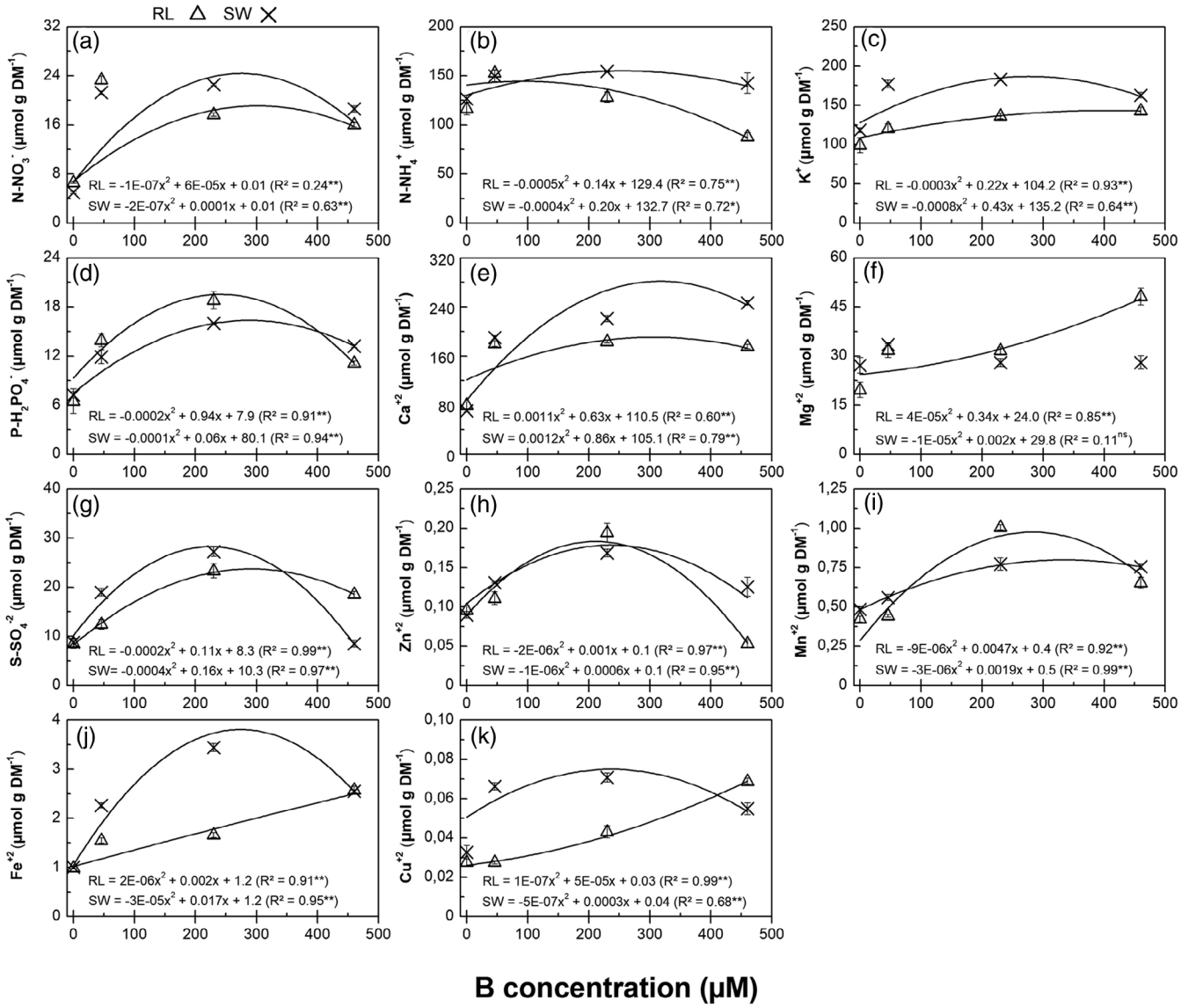

FIGURE 3 Amount of nutrients absorbed per root dry mass (DM) unit (NtAR) of young sweet orange trees grafted onto Rangpur lime (RL) or Swingle citrumelo (SW) (a–k), after 7 days grown under different boron (B) concentrations in the nutrient solution. Vertical bars represent standard error of the mean (n = 3); ns, not significant ($p > .05$), $^{*}p < .05$ and $^{**}p < .01$

features that integrate signals for enzyme activity modulation (Haruta et al., 2018; Janicka-Russak, Katarzyna, & Wdowikowska, 2013). In the post-translational regulation of PM H^+-ATPase, B likely regulates the membrane environment and modulates the enzyme activity (Camacho-Cristóbal & Gonzáles-Fontes, 2007; Landi et al., 2019; Wimmer, Lochnit, Bassil, Mühling, & Goldbach, 2009). Furthermore, because of the high number of diverse B-binding proteins in roots, many of which are usually enriched in membrane micro domains, it was reported that B contributed to stabilise the PM of citrus where B cross-linked glycoproteins are found (Carvalho et al., 2020; O'Neill, Ishii, Albersheim, & Darvill, 2004; Wimmer et al., 2009). Therefore, the B function in PM is similar to that at cell wall level where B cross-linked cell wall pectin is present as a borate-diol ester that cross-links the chains of rhamnogalacturonan (O'Neill et al., 2004). Genes encoding H^+-ATPases in other plant species have been shown to be modulated by B availability in the root medium. In tobacco roots, B deprivation reduced the expression of genes that encode H^+-ATPase (Camacho-Cristóbal & Gonzáles-Fontes, 2007). Increased expression and H^+-ATPase activity in response to B supply has also been reported for the enzyme in the PM of maize root cells (Martinez-Ballesta et al., 2008).

In citrus roots, we found that 1-day ATI, ATP hydrolysis was increased to the same extent for both rootstocks; however, at the same time, the H^+-pumping in SW roots was twofold higher than in RL at the most effective B supply (i.e., 230 µM; Figure 2a,b). This result suggests that B regulatory effects on the SW H^+-ATPase underlies increased efficiency of the coupling between H^+ transport and ATP hydrolysis activities. The control of the tightness of coupling of H^+ transport and ATP hydrolysis has previously been related to auxin-like hormonal activities and other proton pumps (Zandonadi

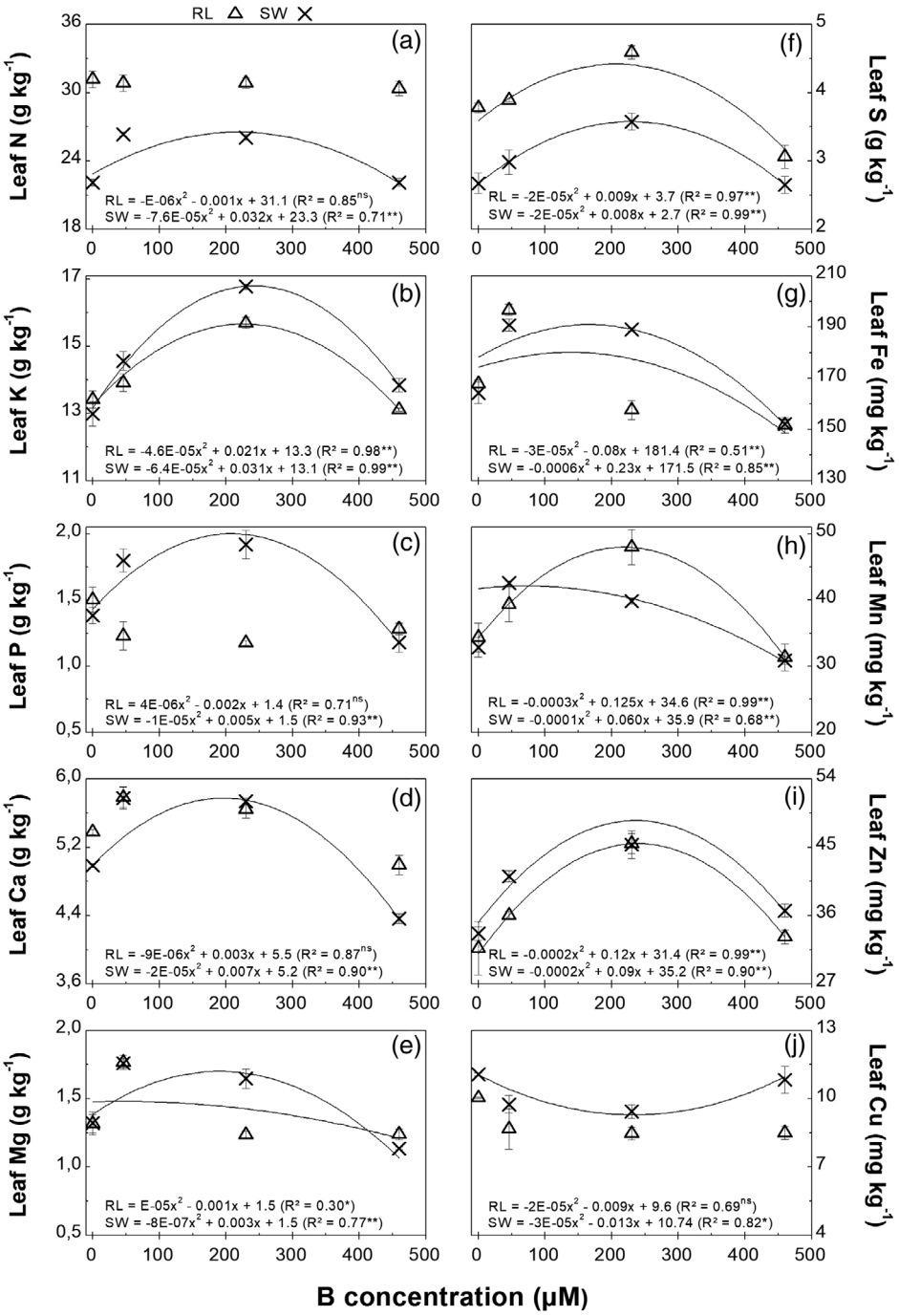

FIGURE 4 Nutrient concentrations in leaves of young sweet orange trees grafted onto Rangpur lime (RL) or Swingle citrumelo (SW) (a–j), after 7 days grown under different boron (B) concentrations in the nutrient solution. Vertical bars represent standard error of the mean (n = 3); ns, not significant (p > .05), *p < .05 and **p < .01

et al., 2010; Zandonadi, Canellas, & Façanha, 2007). In accordance with this later finding, a relationship between B nutrition and auxin metabolism has already been described for several other plant genera including citrus (Ghanem et al., 2011; Li, Liu, Pan, Xie, & Peng, 2016). Therefore, it is possible to speculate that such B-regulated phytohormonal activity could account for the higher coupling efficiency exhibited by the proton pumps from SW rootstock compared to RL. Additionally, the PM H^+-ATPase exists on basal or activated states (Falhof et al., 2016), which differ in the coupling ratio between ATP hydrolysis and proton pumping, with ATP/H^+ stoichiometry of one proton per ATP hydrolysed in the activated state and less than one proton translocated per ATP split in the basal state (Falhof et al., 2016; Haruta et al., 2018; Palmgren & Morsomme, 2019). This activated state is dependent on environmental factors as well the overall physiological status of the plant, including nutritional status, hence would explain differences in H^+-pumping curves in response to B in both rootstocks (Figure 3).

Indeed, sweet oranges grafted onto SW rootstock exhibited greater maintenance of B absorption than other rootstocks when subjected to similar conditions of B supply (Liu et al., 2013). Moreover, greater vessel diameter and optimised vascular system structure were noted in SW (Mesquita et al., 2016). Hence, SW is likely to exhibit a

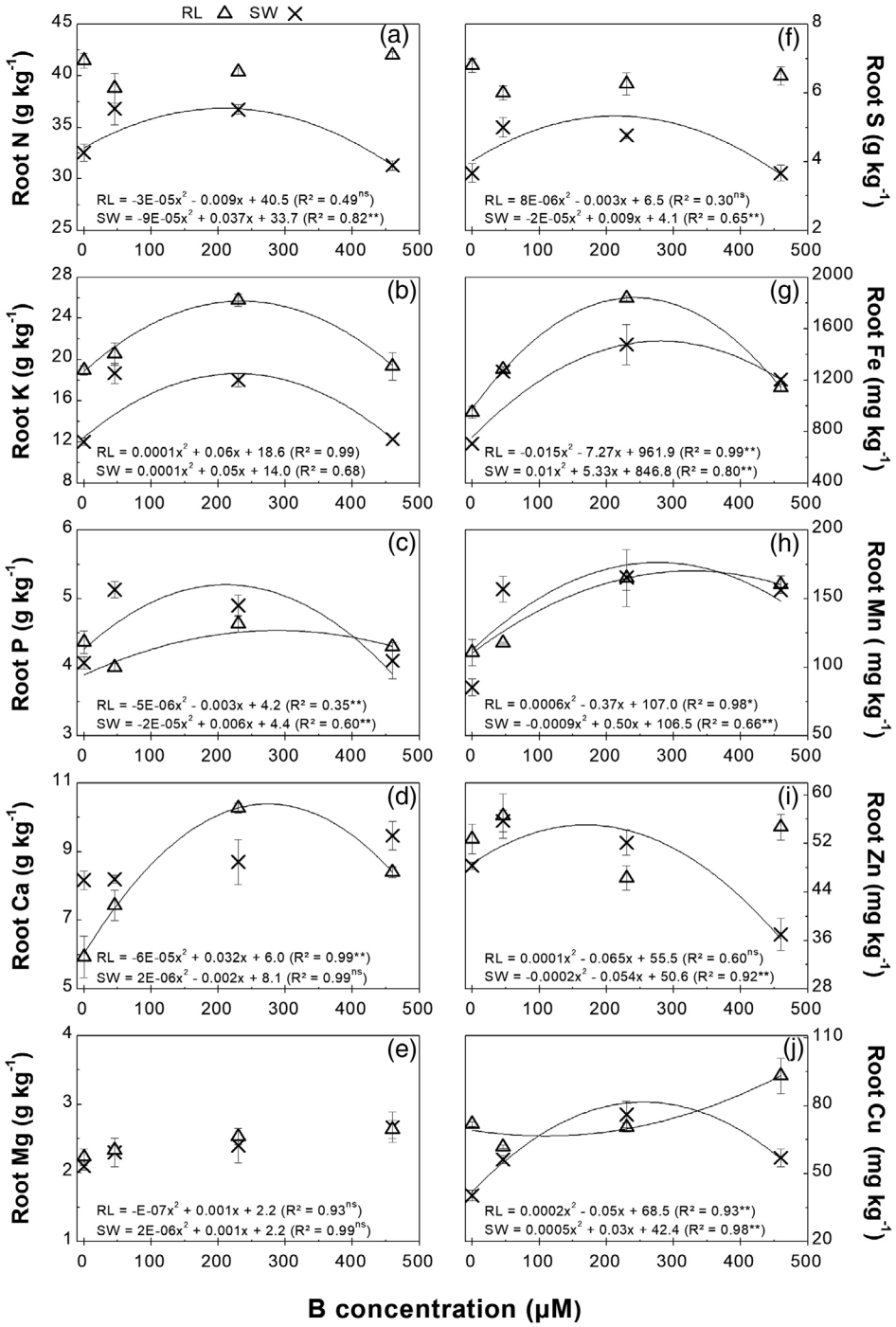

FIGURE 5 Nutrient concentrations in roots of young sweet orange trees grafted onto Rangpur lime (RL) or Swingle citrumelo (SW) (a–j), after 7 days grown under different boron (B) concentrations in nutrient solution. Vertical bars represent standard error of the mean (n = 3); ns, not significant (p > .05), $^{*}p$ < .05 and $^{**}p$ < .01

more efficient mechanism to absorb B, which positively improves cell wall structure through B cross-linked pectin, as well PM stabilisation by induced B cross-linked glycoproteins (O'Neill et al., 2004; Wimmer et al., 2009). Thus, we assume that well established cell structure by B in the SW is one of the factors for superior modulation of PM H^+-ATPase activity, which triggered the results of ATP hydrolysis activities and H^+ pumping observed in the present study. In addition, such benefits of B on SW responses support the positive correlation between plant nutrient demand, B fertilisation and fruit yield recorded in field studies (Mattos Jr. et al., 2017; Quaggio, Mattos Jr., & Boaretto, 2011).

4.2 | Nutrient absorption by citrus rootstocks modulated by PM H^+-ATPase activity

The PM H^+-ATPase activity controls secondary active transport processes through membranes in plant cells, such as root nutrient acquisition and xylem or phloem loading (Sondergaard, Schulz, & Palmgren, 2004). The increment of the PM H^+-ATPase activity when B was added in the growing medium modified the nutrient absorption by roots. That is, after B absorption by citrus roots, ATPase activity increased, which was higher in roots of SW compared to RL. The PM H^+-ATPase generated enhanced H^+ extrusion and a transmembrane

electrochemical gradient (negative inside the cell) that provided the proton motive force to immediately increase absorption of nutrients through nutrient transporters. In this process, SW rootstock exhibited higher ATP hydrolysis and H^+-pumping compared to RL (Figure 3), and consequently the former rootstock also presented higher absorption of nutrients, such as for N-NH_4^+, K^+, Ca^{+2}, Fe^{+2} and Cu^{+2} (Figures 3 and 4).

Uptake of inorganic-N forms (nitrate and ammonium) by roots presented a close relationship with PM H^+-ATPase activity (Alvarez-Pizarro et al., 2014; Falhof et al., 2016; Hippler, Mattos Jr., Boaretto, & Williams, 2018; Zhang et al., 2018). Rice plants grown under N starvation exhibited an increment of root PM H^+-ATPase activity when N was resupplied in a hydroponic condition (Sperandio, Santos, Bucher, Fernandes, & Souza, 2011). In citrus trees, the highest root PM H^+-ATPase activity of the SW rootstock (Figure 2) resulted in greater N-NO_3^- NtAR and N-NH_4^+ NtAR compared to RL, with effect more evident for N-NO_3^- uptake (Figure 3a,b). In addition, only trees grafted onto SW exhibited an increment of N concentration in leaves, with polynomial-type curve response similar as observed for N-NO_3^- NtAR and N-NH_4^+ NtAR (Figures 3a,b and 4a). Gene expression of nitrate transporters (NRTs) is strictly related to PM ATPase isoforms (Hippler, Mattos Jr., et al., 2018; Tavares et al., 2016). Nitrate taken up by such specific NRTs is more dependent on energy provided by the membrane electric potential produced by the PM H^+-ATPase activity than N-NH_4^+ (Nikolic et al., 2012; Zhang et al., 2018). However, the quantification of the expression of genes encoding NRTs would be necessary to better characterise the induction of nitrate uptake in citrus rootstocks after B supply.

4.3 | H^+-ATPase activity and nutrient uptake in citrus rootstocks under high levels of boron

Growth of the root system is characterised to vary either under unfavourable environment or abiotic stresses (Landi et al., 2019; Riaz et al., 2019). Moreover, the main morpho-anatomical plant alterations induced by B excess encompass disorders at the root level, such as disarrangement of root meristems (Landi et al., 2019; Riaz et al., 2019). For instance, B excess increases reactive oxygen species (ROS) content and lipid peroxidation in root cells and consequently membrane leakage (Landi et al., 2019; Shah et al., 2017; White et al., 2013). Thereby, the highest concentration of B in the NS (460 μM) likely suppressed the PM H^+-ATPase activity further affecting the ATP hydrolysis and H^+-pumping of rootstocks (Figure 2f). The PM H^+-ATPase is a transmembrane enzyme that needs a resistant and stable membrane to reduce the differential electrical potential (Falhof et al., 2016; Riaz et al., 2019).

The B excess effects also suppress the PM H^+-ATPase activity by other pathways as photosynthesis (Landi et al., 2019; Riaz et al., 2019). Biochemical effects encompassed by excess B are reduced of CO_2 use efficiency, decline of electron transport rate and impairment of photosystem II (PSII) efficiency (Landi et al., 2019; Mesquita et al., 2016; Riaz et al., 2019). Thus, such effects reduce the availability of carbohydrates and, consequently ATP synthesis, which is the primordial and indispensable substrate for PM H^+-ATPase activity (Haruta et al., 2018; Palmgren & Morsomme, 2019; Riaz et al., 2019).

The reduction of PM H^+-ATPase activity by B excess also reflected in the NtAR, as well as in nutrient concentrations in roots and leaves (Figures 3–5). Except for Cu, concentrations of macronutrients, as well as Fe, Mn and Zn, in plant leaves decreased with increased B concentration in the NS, similarly to the ATP hydrolysis and H^+-pumping decreases (Figures 2, 4 and 5). Copper is mostly accumulated in roots compared to the leaves of citrus trees (Hippler, Dovis, et al., 2018). Moreover, despite that Cu concentration in the leaves did not change significantly with varying B rates, lower Cu concentration was detected in roots of the SW rootstock with the highest concentration of B (Figure 3k). Indeed, literature reported that B supply limits heavy metal accumulation in the root zone what correlates to the pH elevation in the root surface and PM H^+-ATPase activity stimulus (Carvalho et al., 2020; Li et al., 2018).

Despite SW rootstock being considered more tolerant to B excess when compared to other citrus rootstocks, such as RL and Sunki mandarin (Mattos Jr. et al., 2017; Mesquita et al., 2016), the PM H^+-ATPase activity was very sensitive in a short time period, in which the higher absorption capacity of B by SW rootstock exhibited faster inhibition of this enzyme activity when compared to RL (Falhof et al., 2016; Haruta et al., 2018; Wu et al., 2018).

In fact, roots are more susceptible to B excess than other plant parts (White et al., 2013), which causes unusual mitoses on meristems, compromising cellular division and root length growth (Simón-Grao et al., 2018). In this case, damage in the root system would disturb the absorption of water and nutrients and consequently reduce plant growth capacity (Huang et al., 2014; Simón-Grao et al., 2018). On the other hand, under B excess conditions in the root medium, sweet orange grafted onto SW exhibits less damage in anatomical and ultrastructural leaf cells, chlorophyll content, leaf gas exchange and carbohydrate concentrations compared to trees grafted onto other citrus rootstocks (Mesquita et al., 2016; Reid, 2007). The agreement observed in this study among B demand, B supply, PM H^+-ATPase activity and nutrient uptake supports the role of this enzyme affecting important processes and plant growth, as well presents the perspective to study responses of other fruit tree crops to B fertilisation (Camacho-Cristóbal & Gonzáles-Fontes, 2007; Falhof et al., 2016; Mattos Jr. et al., 2017; Palmgren & Morsomme, 2019; Shireen et al., 2018; Turan et al., 2018; Wu et al., 2018).

Therefore, our research demonstrates that different rootstocks can modulate the activity of PM H+-ATPase according to the demand of B. Moreover, the highest modulation of the PM H+-ATPase activity and increase of nutrient uptake by citrus trees grafted onto SW rootstock, varying B supply, allow us to outline a close relation with tree responses observed in previous investigations. The research also offers perspective for further studies of other fruit tree crops depending on rootstock use, because yield increase is mostly dependent on improved nutrient use efficiency by plants.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

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