

## Scanning electron microscopy of leaf and petal cuts of citrus trees fertigated with two nitrogen sources

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### SUMMARY

Calcium exerts a structural role on cell walls and medium lamellae in plants, which might have implications for tolerance to abiotic and biotic stresses in the field. Based on this later, our work was taken from a long-term field study on fertigation of a citrus orchard that compared the use of ammonium nitrate (AN) and calcium nitrate (CN) fertilizers via fertigation in Valencia sweet orange trees. Sample of leaves and flower petals of trees were collected and observed under scanning electron microscopy, from selected treatments receiving either 80 (N80) or 320 (N320) kg ha<sup>-1</sup>year<sup>-1</sup> of N, which correspond to 100 or 400 kg ha<sup>-1</sup>year<sup>-1</sup> of soluble Ca via CN. Trees fertigated with N320 as CN developed leaves and petals with higher mechanical resistance than those with AN. Results also demonstrated that structured cell walls were associated with higher calcium contents and greater mechanical resistance of the tissue during sample preparation for microscopy. From a more practical point of view, such improved structure of leaves and flower petals of CN-treated trees could contribute to an enhanced plant tolerance to diseases commonly affecting the citrus orchards. Additionally, such finding is important for a more sustainable citrus production, because it raises the possibility of reducing pesticides inputs by adjusting nutritional management of citrus orchards.

**Index terms:** fertigation, calcium, anatomy, disease resistance.

### Microscopia eletrônica de varredura de folhas e de pétalas de plantas cítricas fertirrigadas com duas fontes de nitrogênio

### RESUMO

O cálcio exerce papel estrutural nas paredes celulares e lamelas médias das plantas, o que pode ter implicações para a tolerância destas a estresses bióticos e abióticos no campo. Nosso trabalho fez parte de um estudo de campo de longo prazo sobre fertirrigação dos citros, o qual comparou o uso das fontes fertilizantes nitrato de amônio (NA) e de nitrato de cálcio (NC) em laranjeiras Valencia. Amostras de folhas e de pétalas de flores de tratamentos que receberam 80 (N80) ou 320 (N320) kg ha<sup>-1</sup>ano<sup>-1</sup> de N (os quais corresponderam a 100 ou 400 kg ha<sup>-1</sup>ano<sup>-1</sup> de Ca solúvel para NC) foram coletadas e observadas em microscópio eletrônico de varredura. As árvores fertirrigadas

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com N320 como NC desenvolveram folhas e pétalas com maior resistência mecânica do que aquelas fertirrigadas com NA. Os resultados também demonstraram correlação entre a melhor estruturação de paredes celulares com maiores concentrações de Ca e com a maior resistência mecânica dos tecidos durante processamento das amostras para microscopia. Na prática, tal melhora na estruturação de folhas e de pétalas de flores quando o NC foi utilizado pode contribuir para o aumento da tolerância a doenças que comumente afetam os pomares cítricos. Adicionalmente, essa constatação é importante para uma produção de citros mais sustentável, porque aumenta a possibilidade de reduzir o uso de pesticidas ao ajustar o manejo nutricional dos pomares.

**Termos de indexação:** fertirrigação, cálcio, anatomia, resistência a doenças.

## INTRODUCTION

Nitrogen (N) and calcium (Ca) are nutrients with high interdependence on citrus mineral nutrition, by impacting tree growth, fruit yield and quality. The need for improved N use efficiency (NUE) is remarkable for the environmental and economic sustainability of citrus production (Alva et al., 2006, 2008). Therefore, the best management of nitrogen (N) fertilization in fertigated orchards is required because the application of nutrients via fertigation affects the dynamic of N-fertilizers applied to the soil compared to the conventional fertilization practice in non-fertigated groves. For instance, the conversion of ammonium ( $\text{N-NH}_4^+$ ) to nitrate ( $\text{N-NO}_3^-$ ) in the soil does not occur at the same extent as observed in the traditional production systems (Cantarella et al., 2003; Souza et al., 2006).

Herein, ammoniacal sources cause acidification of soil, which in turn, limits nitrification process and increases  $\text{N-NH}_4^+$  availability in the soil solution (Alva et al., 2006; Souza et al., 2012). Thus, greater absorption of  $\text{N-NH}_4^+$  by the citrus trees occurs after the fertigation with ammoniacal sources what decreases the absorption of others positive charged cations, specially Ca (Hinsinger et al., 2003), which nutrient is one of the most accumulated by this species (Mattos Junior et al., 2003).

About 60% of the total cellular Ca is located in the middle lamella like Ca-pectates (Iwahori & Oohata, 1980; Iwahori & Van Stevenick, 1994; Tobias et al., 1993; McLaughlin & Wimmer, 1999). The adequate Ca supply to plants provides superior fruit yield (Conway & Sams, 1983; Hanson et al., 1993; Xisto et al., 2002; Taylor et al., 2004; Brackmann et al., 2010). For instance, guava fruits harvested from limed orchards exhibited greater total leaf-Ca concentration and cell walls and middle lamellae more defined compared to those harvested from unlimed orchards (Natale et al., 2005). In accordance, Tahiti acid limes are less prone to water loss and present longer shelf-life storage due to higher Ca contents in the peel (Mattos Junior et al., 2010).

Furthermore, the application of Ca also decreases the occurrence or severity of plant diseases (Bangerth, 1979), since the resistance of plants can be improved, among other factors, by changes in tissues anatomy that offer a physical barrier to pathogenic agents (Marschner, 1995).

A long-term field study evidenced higher NUE and 40% higher fruit yield for fertigated orange trees with CN compared to those fertigated with AN (Quaggio et al., 2014). Such differences resulted from the maintenance of higher pH of the soil solution with the CN treatment, as well improved absorption of N by plants in the form of  $\text{NO}_3^-$  in relation to  $\text{NH}_4^+$ , which also favored the availability and uptake of Ca by the trees.

In view of the above, we consider that citrus trees fertigated with ammonium nitrate or calcium nitrate present differences in the structure of leaves and flower petals in response to the improved Ca uptake. To evaluate this hypothesis, leaves and flowers samples collected from long-term field experiment reported by Quaggio et al. (2014) were analyzed in a scanning electron microscope.

## MATERIAL AND METHODS

### Plant material and field conditions

The present work is part of the study on citrus fertigation (Quaggio et al., 2014), whose experiment was installed in 2003 in a commercial orchard with Valencia sweet orange trees [*Citrus sinensis* (L.) Osb.] grafted on citrumelo Swingle [*C. paradise* Macfad. x *Poncirus trifoliata* (L.) Raf.], in a medium texture Entisol ( $\text{CEC} = 37 \text{ mmol}_c \text{ dm}^{-3}$ ). The soil received dolomitic limestone to increase the base saturation to  $\text{V}\% = 70$ , in the 0-20 cm depth layer every two years). The experimental area was implanted with density of 444 trees per hectare, South-central region of the state of São Paulo, Brazil ( $21^\circ 88'S$  and  $49^\circ 14'O$ ).

The experiment was designed in a completely randomized blocks with four replicates. Each plot consisted of a

line of 16 uniform trees, with only the 10 central plants being used for sampling and evaluating the effects of the treatments. The trees were fertigated with doses of N applied as ammonium nitrate (AN) or calcium nitrate (CN) at 80, 160, 240 and 320 kg ha<sup>-1</sup> year<sup>-1</sup> of N from 2009 to 2014.

The fertigation system had two drip lines per planting line (one on each side) placed 60 cm away from the trees' trunk. The distance between drippers (3.5 L h<sup>-1</sup>) was 85 cm. Irrigation management was based on the daily evaporation measured by the class A tank and monitored by tensiometry to 30 and 60 cm soil depth. Nitrogen and potassium (K) were supplied twice a week by fertigation, summing up 70 applications per season. The annual rate of K corresponded to 183 kg ha<sup>-1</sup> as potassium chloride. Phosphorous (P) was supplied during the winter by a single application of MAP (26 kg ha<sup>-1</sup> year<sup>-1</sup> of P). Micronutrients were supplied annually with three foliar applications at optimum rates (Quaggio et al., 2005).

## Leaf and petals sampling

Only those plots that received the lowest and the highest N dose via AN or CN (N80 and N320) were selected for sampling, justified by the *a priori* objective of the study. When CN was used, it was provided 100 or 400 kg ha<sup>-1</sup> year<sup>-1</sup> of soluble Ca for N80 and N320 respectively.

Completed open flowers present during the major citrus blooming period (50 flowers per treatment; Spring 2012) as well fully expanded mature leaves, about six-month-old (50 per treatment; early Summer 2013), from branches with a terminal fruit of approximately 4-cm in diameter were collected from treatments representing both doses and N sources of interest.

Mesophilic tissue pieces of approximately 3 mm × 5 mm were cut in random regions of leaf blades, but always with a secondary vein in its center. The petals were cut into six equal pieces and the two middle ones were selected. These leaf and petal samples were fixed in Karnovsky's solution (Karnovsky, 1965) yet in the field. Once in the laboratory, those samples were submitted to a vacuum for 5 min and stored in the refrigerator for at least 24 h.

## Scanning electron microscopy imaging

The fixed samples were infiltrated in 30% glycerol for 1 h, immersed in liquid nitrogen and transversely fractured with scalpel. These were dehydrated in series of acetone

concentrations [30, 50, 70, 90 and 100% (3 times)], remaining 20 min at each stage. Soon after, they were dried to the critical point using CO<sub>2</sub>, glued to stubs and metalized with gold. The samples were then analyzed by scanning electron microscopy (SEM, model LEO 435VP, Zeiss, Germany) to produce images saved in TIFF format.

The same leaf or petal's tissue regions were photographed for every sample. Mesophyll regions presenting tertiary veins were captured, which were close to a secondary vein. The middle region of petal samples were also captured, which presented secondary and tertiary veins (note that petal veins are smaller than leaf veins). Even we didn't use veins anatomic characteristics for results or discussion, we used them to standardize the regions of tissues which were targets of analysis. Then, images that best illustrated anatomical characteristics repeated in observations of six samples per treatment were selected in the laboratory.

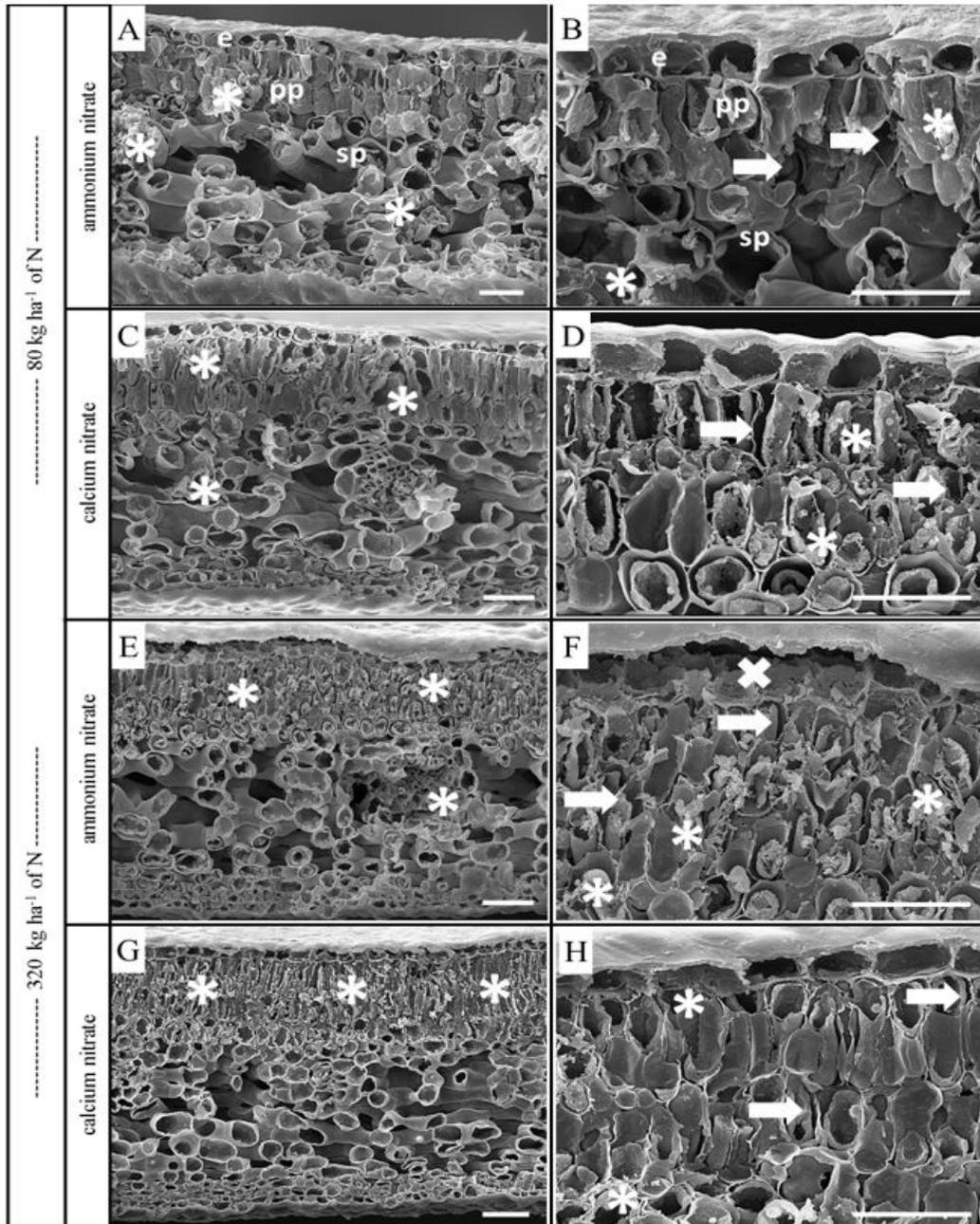
## RESULTS

### Leaf samples

Leaf tissue of trees fertigated with N80 as AN exhibited poor adhesion between the parenchyma palisade cells, associated to repeated rupture of the middle lamellae (Figure 1A, B) what likely resulted from laceration after sample cut processing in the laboratory for microscopy observation. When CN was applied at the same dose, parenchyma maintained a better organized structure between cells (Figure 1C, D), likely resulted from the increased Ca concentration of the leaves (30% greater than that observed with AN; Petená, 2016).

With N320, leaf mesophyll presented greater cell elongation irrespectively of the N source used in the fertigation (Figure 1E-H). However, it was observed differences in tissue organization when comparing N320 as AN or CN.

Leaf samples from trees fertigated with AN at N320, after laboratory processing, showed significant proportion of rupture of epidermal cell walls as well fractures of the parenchyma palisade cell walls (Figure 1F). On the contrary, better cellular structure of leaf tissues was observed in trees fertigated with N320 as CN, suggesting that adhesion between cells exerted by the middle lamellae was less



**Figure 1.** Scanning electron microscopy of cross section of leaves of Valencia oranges trees fertigated with: 80 kg ha<sup>-1</sup>year<sup>-1</sup> of N as ammonium nitrate (A and B) or calcium nitrate (C and D), and 320 kg ha<sup>-1</sup>year<sup>-1</sup> of N as ammonium nitrate (E and F) or calcium nitrate (G and H). Legend: e = epidermis; pp = palisade parenchyma; sp = spongy parenchyma; \* = breakage of cell walls; arrows = loss of contact surface between cells of the palisade parenchyma; x = rupture of cell walls of epidermal cells. Horizontal bar = 15 μm.

impaired during processing (Figure 1H). Difference in Ca concentration of leaves receiving CN and AN was similar to that observed between treatments at N80 (about 30% greater with CN), that is 44 g kg<sup>-1</sup> of Ca with CN and 33 g kg<sup>-1</sup> of Ca with AN (Petená, 2016).

### Flower petals

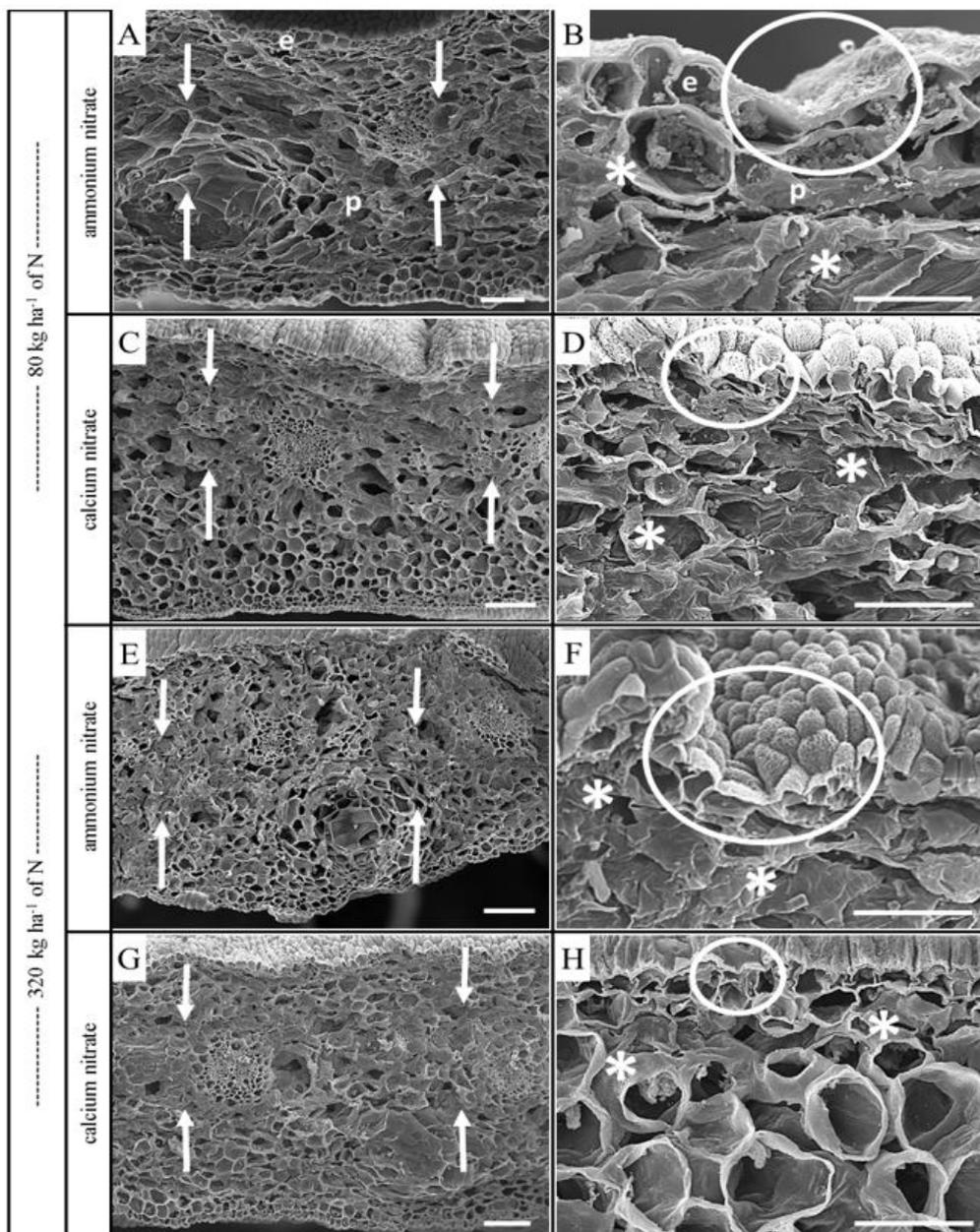
As for the leaves, the mechanical force exerted by the laboratory processing damaged the tissue structure of the petal samples irrespectively of the studied treatments

(Figure 2A-H). However, plants that received AN, most of the petal tissue cells was more affected by the cutting procedures required for microscopy observation (Figure 2B, F).

When AN was the N source in the fertigation system, the adaxial epidermis of petals was rippled (Figure 2B, F) as a consequence of exacerbated cell elongation, what was more evident with N320 (Figure 2F) and likely

associated with lower Ca concentration in the petal tissue ( $3.4 \text{ g kg}^{-1}$  of Ca; Petená, 2016). With CN, epidermal roughness was softened, mostly at N320 (Figure 2D, H), in which condition Ca concentration in the petal was  $4.4 \text{ g kg}^{-1}$  of Ca (Petená, 2016).

Moreover, the laboratory processing was less severe to the cellular petal structure of trees fertigated with CN (Figure 2C, G). These treatments improved adhesion



**Figure 2.** Scanning electron microscopy of cross section of flower petals of Valencia oranges trees fertigated with:  $80 \text{ kg ha}^{-1}\text{year}^{-1}$  of N as ammonium nitrate (A and B) or calcium nitrate (C and D), and  $320 \text{ kg ha}^{-1}\text{year}^{-1}$  of N as ammonium nitrate (E and F) or calcium nitrate (G and H). Legend: e = epidermis; p = parenchyma; \* = breakage of cell walls; arrows = crushed tissue; circles = epidermal roughness. Horizontal bar =  $15 \mu\text{m}$ .

between cells by middle lamellae (Figure 2D, H). This fact was more evident with N320, observing the integrity of epidermal and parenchymal cell walls of petal tissue (Figure 2H).

## DISCUSSION

The use of CN in citrus fertigation in tropical soils promotes fruit yield and NUE, given the improved ion balance in plants (optimizes the  $\text{NO}_3^-:\text{Ca}^{+2}$  absorption ratio), as well as the increased pH in the soil ( $\text{pH} \geq 6.3$ ) when compared to the management of fertilization using AN (Quaggio et al., 2014). On the other hand, the application of AN limits the nitrification of N in the soil, results in a high  $\text{NH}_4^+$  concentration in the soil solution and, consequently, in the reduction in the net absorption of cations by the trees, mainly of Ca (Hinsinger et al., 2003).

These results suggest that, with the same N dose, it is possible to obtain higher citrus yields changing the  $\text{NO}_3^-:\text{NH}_4^+$  ratio in the fertilization management. It is also interesting to note that, in part, this increase in fruit yield may be related to the better anatomical structuring of plant tissues, given greater wall thickening, number of cells, number and diameter of vascular vessels occurring in leaf and flowers of plants (Philip et al., 1991; Prabhpreet et al., 2000; Drazeta et al., 2004; Castro et al., 2005). All the later together are likely to improve nutrient and water transport, overall photosynthesis and carbohydrate allocation in plants (Taiz & Zeiger, 2006).

These aspects of tissue integrity and quality might also contribute to the reduction of fruit losses caused by pathogens that commonly affect citrus crop in Brazil (Feichtenberger, 1991, 1994) and in Florida (USA) (Peres et al., 2000). For instance, the Brazilian harvest in the 2009/2010 crop season, when climatic conditions during the winter were very favorable to the occurrence of postbloom fruit drop, resulted from the infection of citrus flowers by the fungus *Colletotrichum acutatum*, caused a reduction of up to 85% in the production of oranges in the Southwest of the state of São Paulo (Souza et al., 2012).

The establishment of diseases in plants is characterized by the occurrence of successive and ordered events. During infection, the phytopathogen (or its vector) first needs to fix to the surface of the tissue (Amorim et al., 2011). Epidermal roughness in petals of plants that received AN, mainly at N320 (Figure 2B, F), favors a microenvironment for such fixation (protecting the pathogens or their vectors of the wind, rain and predators). Moreover, the roughness

favors the accumulation of water on petal's surface, which would also facilitate the direct penetration of fungi (Amorim et al., 2011). The easier fixation, vector feeding and direct penetration for treatments that received NA, especially at N320, likely favors the higher incidence of diseases.

During penetration, the phytopathogen (or its vector) is forced to break cell walls of the epidermis. The pathogen is then required also to break the cell walls of the parenchyma to obtain nutrients of the cellular content necessary for its development and movement from cell to cell (Amorim et al., 2011). Therefore, epidermal cells provided with greater resistance to mechanical force would probably hinder direct penetration and transmission (by vector feeding) of pathogens in citrus orchards. Also, parenchyma cells with the same characteristic would decelerate pathogenic colonization and, in consequence, severity of diseases. Indeed, samples of trees fertigated with CN, mainly at N320, showed greater resistance to the mechanical force exerted during laboratory sample processing, presenting lower proportion of rupture of cell walls and greater contact surface between cells in leaves (Figure 1G, H) and petals with improved cell integrity (Figure 2G, H).

## FINAL CONSIDERATIONS AND PERSPECTIVE

Considering the structural role of Ca, the anatomical changes observed in leaf and petals of trees fertigated in tropical condition are supported by the increase in 30% of leaf-Ca and petal-Ca when orchard was managed with 320N as CN compared to the same dose as AN. Thus, we could also argue that a greater resistance of citrus trees to disease infection and progress by the development of thicker cell walls and medium lamellae of leaves and flower petals would be attained with higher concentrations of Ca in plants. Then, further studies need to be performed in order to validated such relationship and contribute for a more sustainable management of citrus diseases

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