Morphological characterization of leaves at different crown positions of tangerine genotypes

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ABSTRACT: The leaf anatomy has been shown as an important tool to understand the interaction between this organ and its microclimate but this topic is still unclear for citrus plants. The objective of this study was to analyze the leaf structure of Ponkan, Fremont and Thomas tangerine and Murcott tangor. Fully expanded leaves were collected collected, from the 2nd and 3rd nodes of plagiotropic branches facing West, at three crown heights (lower, middle and upper). The leaves were fixed in 70% ethanol and embedded in historesin and sectioned in a semi-automatic microtome. The sections were stained with toluidine blue; images were captured under a light microscope coupled and analyzed to measure leaf tissue thickness. The Fremont tangerine leaves had greater epidermis thickness on the adaxial and abaxial surfaces and secretory cavity diameter as well as a shorter distance between secretory cavities, whereas the Murcott leaves had greater palisade and spongy parenchyma thickness. Thus, Fremont and Murcott are potential varieties for xeric conditions, as they are able to reduce transpiration and make optimal use of high incident radiation. The microclimates created by plant architecture may affect the leaf anatomy of the Ponkan, Fremont and Thomas and Murcott trees, allowing anatomical plasticity.

Key words: citrus; *Citrus reticulata*; ecological anatomy; leaf anatomy; plant morphology

Caracterização morfológica de folhas em diferentes posições da copa de genótipos de tangerineiras

RESUMO: A anatomia foliar vem se estabelecendo como uma importante ferramenta para se compreender a interação entre este órgão e o seu microclima, contudo, pouco se sabe sobre este assunto em citrus. Objetivou-se com este trabalho, estudar a estrutura foliar das tangerinas Ponkan, Fremont, Thomas e do tangor Murcott em diferentes alturas na copa. Folhas completamente expandidas foram coletadas, do segundo e terceiro nó de ramos plagiotrópicos, voltados para o oeste, em três alturas na copa (base, meio e topo). As folhas foram fixadas em etanol 70%, emblocadas em historesina e seccionadas em micrótomo; coradas com azul de toluidina e imagens foram capturadas em microscópio de luz e analisadas para mensuração das espessuras dos tecidos foliares. A tangerina Fremont apresentou maior espessura da epiderme nas faces adaxial e abaxial, maior diâmetro das cavidades secretoras e menor distância entre elas. Folhas de tangor Murcott apresentaram maior espessura do parênquima paliçádico e esponjoso. Fremont e Murcott se caracterizam como potenciais para condições xéricas, o que lhes permite redução na transpiração e melhor aproveitamento de uma grande quantidade de radiação incidente. Os microclimas criados pela arquitetura da planta influenciam a anatomia foliar da Ponkan, Fremont, Thomas e da Murcott, conferindo uma plasticidade anatômica.

Palavras-chave: citros; *Citrus reticulata*; anatomia ecológica; anatomia foliar; morfologia vegetal

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Introduction

Brazil is the third largest producer of tangerine in the world, with approximately 52.000 hectares of planted area and one million tons of production. The state of São Paulo ranks first in Brazil, followed by Paraná, Minas Gerais and Rio Grande do Sul, which combined account for 86% of Brazilian production. In São Paulo, which accounts for 35.5% of Brazilian production, two genotypes, i.e., Ponkan tangerine (*Citrus reticulata* Blanco) and Murcott tangor [*C. reticulata* Blanco x *C. sinensis* (L.) Osbeck], account for more than 80% of the planted area, demonstrating the low number of cultivated varieties (IBGE, 2016).

Cultivated citrus plants are constantly exposed to thousands of microorganisms and abiotic factors that may compromise their production, including drought periods resulting from irregular rainfall and dry spells during the rainy season (Pinto et al., 2008). Knowing the environmental contribution to phenotypic manifestation enables optimization of the selection of new cultivars and therefore, the selection of superior genotypes under any condition (Marchioro et al., 2005).

Leaf anatomy is directly affected by environmental factors, and plant water status is one of the most important factors for leaf growth; thus, leaf morphology is a key issue for crop production (Castro et al., 2009). Other factors, such as radiation condition and nutrition may change the citrus leaf anatomy (Reed, 1931; Bondada & Syvertsen 2003; Zambrosi et al., 2017). Furthermore, height differences in canopy position affect leaf anatomy and may change leaf physiology because leaves respond differently to variations in light intensity, water availability, air temperature, vapor pressure, and wind speed as a function of plant height (Yoshimura 2011; Gebauer et al. 2015). Upper crown leaves are subjected to greater light availability and lower hydraulic conductivity and nutrient availability and therefore, these leaves will be thicker, with a higher mesophyll ratio and epicuticular wax deposition, among other specificities (Pires et al., 2015).

The effect of light intensity on leaf growth may affect leaf structure, as species with high acclimation potential commonly show changes in leaf anatomy resulting from differences in light intensity (Bjorkman, 1981). Thus, plant adaptation to light environments depends on the adjustment of the photosynthetic system towards using radiation as efficiently as possible (Boardman, 1977), i.e., a plant's response to the environment will affect its growth, development and production. Thus, growth efficiency is related to the adaptability of plants to different environmental radiation conditions.

More studies on citrus leaf morphological and anatomical characteristics are needed, as plant adaptation to the cultivation environment largely depends on the surface chemical properties and/or morphological characteristics of the leaves, which determine the amount of absorbed or reflected light, degree of leaf hydrophobicity, leaf-air vapor pressure differences, efficiency of leaf defense against parasites and pathogens, amount of pollutants or pesticides absorbed and the magnitude of cuticular transpiration (Santiago et al., 2001).

Given the above, the objective of this study was to assess possible changes in the leaf structure of Ponkan, Fremont and Thomas tangerine and Murcott tangor trees resulting from different crown positions (lower, middle and upper) and their possible effects on plant physiology and production.

Material and Methods

Ponkan - IAC 172, Fremont – IAC 543 and Thomas – IAC 519 tangerine and Murcott – IAC 221 tangor leaves were collected from parent plants at the Sylvio Moreira Citrus Center (Centro de Citricultura Sylvio Moreira – CCSM) of the São Paulo Agribusiness Technology Agency (Agência Paulista de Tecnologia dos Agronegócios – APTA) located in Cordeirópolis, São Paulo, and sent to the Plant Anatomy Laboratory, Department of Biology, Federal University of Lavras (Universidade Federal de Lavras - UFLA) for histological evaluation.

Four fully expanded 7.5-cm-long leaves were collected from plagiotropic branches facing West, at three crown heights, namely the lower (lower third), middle (mid-third) and upper (upper-third) crown, from ten plants of each of the four study genotypes (Ponkan, Fremont, Thomas and Murcott). The plants were 12 years old and 4.5 meters high. The leaves were fixed in $FAA_{70\%}$ (formaldehyde, glacial acetic acid pro analysis (p.a.) and 70% ethanol, at a 0.5:0.5:9 ratio) for a 72-hour period and subsequently preserved in 70% ethanol (v v^{-1}) (Kraus & Arduin, 1997).

To prepare the permanent slide collection, mid-leaf fragments were dehydrated in an increasing ethanol series, infiltrated and embedded in hydroxyethyl methacrylate (Leica®, Heidelberger, Germany), according to the manufacturer's instructions. The samples were sectioned in a semi-automatic microtome (KD-3368, Ciencor Scientific, São Paulo, Brazil), stained with 1% toluidine blue and mounted in Canada balsam.

The slides were analyzed under an Olympus CX41 trinocular microscope (Olympus, Tokyo, Japan) coupled to an image acquisition system. The image analysis was performed using the UTHSCSA ImageTool software version 3.0. The following anatomical characteristics were assessed: the epidermis thickness on the abaxial and adaxial surfaces, palisade parenchyma thickness, spongy parenchyma thickness, secretory cavity diameter and distance between secretory cavities on the adaxial side of leaves. One slide was prepared per leaf, and four histological sections were evaluated per slide for each study characteristic.

The experimental design was completely randomized in a 3x4 factorial scheme (three plant crown positions and four *Citrus* genotypes) with nine replicates, considering one leaf per plant as the experimental unit. Statistical analysis was performed using the statistical software SISVAR 5.0.

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Before performing parametric analysis, the data were tested for normality by the tests of Shapiro-Wilk and Kolmogorov-Smirnov and the homocedasticity was tested by the Lavene test. Data without normal distribution were transformed using the following formula: $(X+1)^{\frac{1}{2}}$ where "X" represents the original data. The data were subjected to analysis of variance, and the means were compared using the Scott-Knott test (p<0.05).

Results and Discussion

The Fremont leaves had the thickest epidermis on the adaxial surface (ade) (Figure 1 D-F), which was 19.52% thicker than that of the Ponkan (Figure 1 J-L) and Thomas leaves (Figure 1 G-I). The ade of the Murcott leaves (Figure 1 A-C) was 17.76% thicker than that of the Ponkan and Thomas varieties (Table 1). Plants with a thicker epidermis presumably tend to withstand a greater amount of radiation, drought

Table 1. Adaxial and abaxial epidermis thickness (µm) measured in cross sections of leaves collected from different crown positions (lower, middle and upper) of four citrus varieties (Fremont, Ponkan and Thomas tangerine and Murcott tangor).

Varieties	Epidermis thickness on the adaxial surface (µm)		
Ponkan	7.99 c*		
Thomas	8.11 c		
Murcott	8.89 b		
Fremont	9.55 a		
CV (%)	20.45		
	Epidermis thickness on the abaxial surface (µm)		
	Lower	Middle	Upper
Ponkan	5.63 Ab	6.10 Ab	5.70 Ab
Thomas	6.28 Aa	5.48 Bb	5.28 Bb
Murcott	5.48 Bb*	5.64 Bb	6.56 Aa
Fremont	6.65 Aa	6.80 Aa	6.45 Aa
CV (%)	21.37		

* Means followed by the same lowercase letter within columns and uppercase letter within rows are not significantly different (Scott-Knott test at 5% probability).
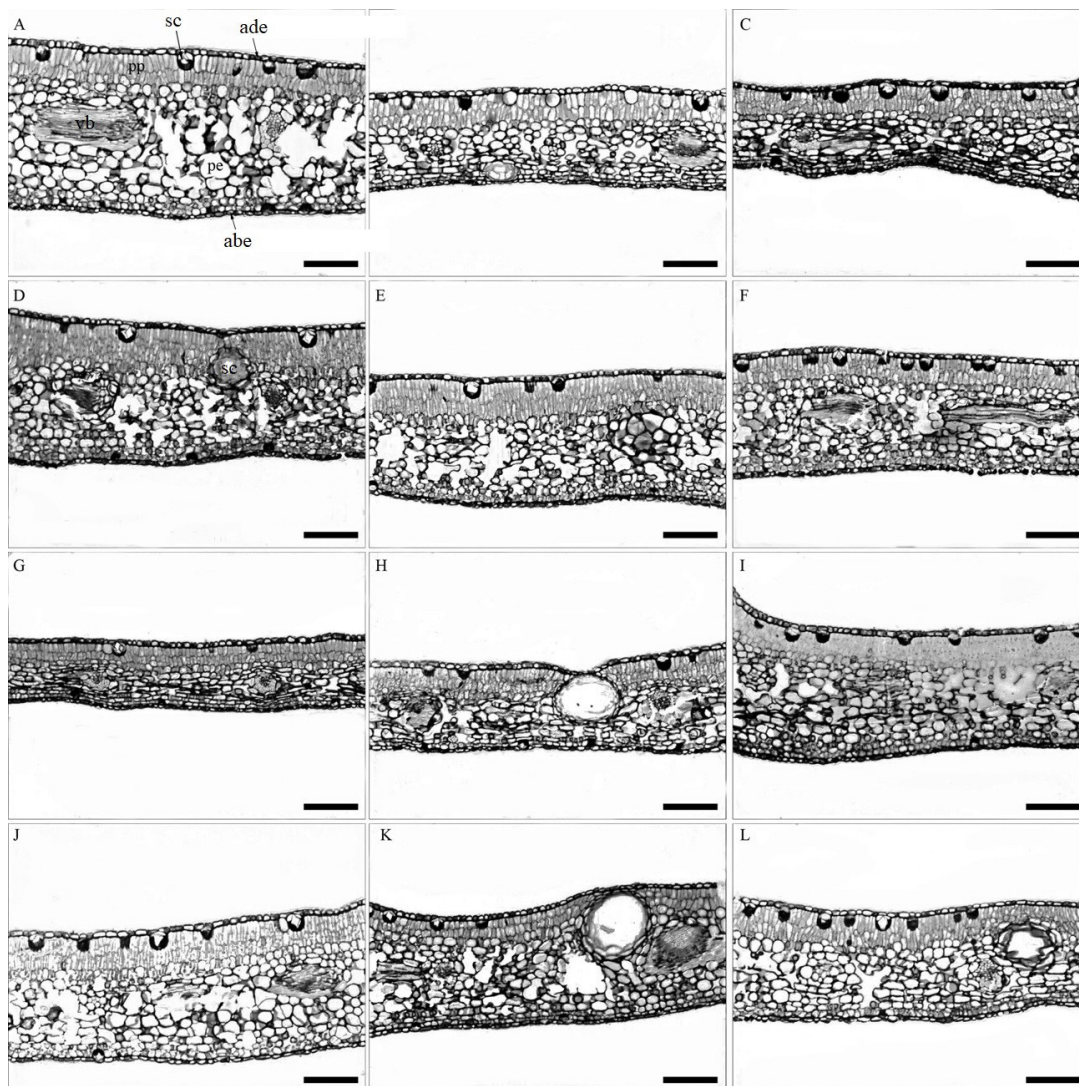
Figure 1. Cross sections of leaves collected at various crown heights (A,D,G,J = upper crown; B,E,H,K = middle crown; C,F,I,L = lower crown) of different *Citrus* genotypes (A-C: Murcott; D-F: Fremont; G-I: Thomas; J-L: Ponkan). pp = palisade parenchyma, sp = spongy parenchyma, ade = adaxial epidermis, abe = abaxial epidermis, sc = secretory cavity, vb = vascular bundle. Bars = 100 µm.

and other environmental pressures (Nunes et al., 2012). The two genotypes with a thicker epidermis, Fremont and Murcott, might transpire less and be more protected against drought and excess radiation. Ribeiro et al. (2012) reported that cassava leaves with an increased adaxial epidermis thickness showed increased tolerance to drought and high light intensity. Similarly, Medri & Lleras (1980) stated that epidermal cell wall thickening is an adaptation to prevent excessive water loss in most xerophytic trees. Conversely, Chazdon & Kaufamann (1993) reported that *Piper arieianum* leaves under high solar radiation had increased epidermal thickness, suggesting that light intensity is directly related to an increase in leaf blade thickness.

The epidermis thickness on the adaxial surface did not differ throughout the crown regardless of the study variety, which indicates some homogeneity regarding this leaf characteristic. Thus, the protection capacity provided by the epidermis was independent of the location at which this characteristic was measured. However, this characteristic allows a comparison of the four study varieties, with Fremont tangerine and Murcott tangor standing out as the varieties that are generally the most tolerant to environments with increased incident radiation.

Combined studies of different radiation intensities and hormone dosages show that cell expansion may be stimulated by the presence of light, drought and several hormones, including auxins, cytokinins, gibberellins and brassinosteroids. However, the mechanisms are not yet fully understood, requiring further studies involving endogenous (phytohormones) and exogenous (environmental factors) characteristics (Lee et al., 2000). Hectors et al. (2010) reported that leaf morphological changes resulting from UV radiation are spatiotemporally dependent processes, that is, the effect of UV radiation on adaxial epidermis cell size is apparently permanent, whereas leaf shape is transiently changed, with a decrease in length compared with width in young leaves.

Analysis of the abaxial epidermis (abe) thickness revealed an interaction between the variables variety and leaf position in the crown (Table 1). The Thomas and Fremont varieties exhibited an increased abe in the lower crown (Figure 1 C; I), corresponding to a 11.55% thicker abe than the other study varieties. Fremont tangerine also showed a 11.48% thicker abe in the mid-crown leaves (Figure 1 A). Conversely, the Murcott variety, followed by the Fremont variety, exhibited a thicker abe in the upper crown leaves compared with the other varieties, an increase of 13.16% (Figure 1 D). This result may be explained by the increased epidermal cell wall thickness of the upper crown leaves of these plants because they were subjected to water deficit, which was also observed by Zhan et al. (2015), who reported an increased water deficit in the upper crown layer of cotton plants.

Savé et al. (1995) studied some drought-resistant morphological and physiological characteristics of citrus plants and also observed that orange and tangor plants subjected to: water stress showed elastic adjustment (increased tissue elasticity). In addition, the tangor plants showed a more efficient water conservation strategy than the orange plants based on crown architecture characteristics (smaller crown areas and a more closed canopy with leaves almost vertically oriented). This helps to explain the presence of thicker leaves at the ends of tree branches, in the upper third of the crown, and the decreased susceptibility to drought of plants with larger crown basal areas, among the study varieties.

Behavior analysis of each variety separately showed that the Murcott tangor leaves in the upper crown had a thicker abe (Table 1; Figure 1 A). This response is common among plants with globose crowns because this characteristic is related to higher incident radiation in this canopy location. Conversely, the abaxial epidermis thickness of the Ponkan leaves was homogeneous throughout the crown (Table 1; Figure 1 J-L), whereas the Thomas tangerine trees showed an unexpected behavior, as their leaves exhibited a thicker abe in the lower crown (Table 1; Figure 1 I), which usually has more shading. However, this finding may be explained by the more triangular architecture of this variety, which increases the incidence of solar radiation in the lower crown. Crown height had no effect on abaxial epidermis thickness in the Fremont tangerine, as it was mostly homogeneous (Figure 1 D-F). However, it is important to mention that the Fremont leaves were consistently thicker than the leaves of the other study varieties (Table 1).

These results highlight the importance of identifying an optimal citrus plant architecture for local conditions, that is, one that provides ease of cultivation, good productivity and quality fruits for farmers. Citrus plant architecture is determined by several factors, including variety and rootstock, soil, and phytosanitary and climate conditions, which, combined, determine plant vigor and growth habit (Stuchi, 2012).

Cohen et al. (1987) evaluated the effect of radiation distribution within the crown of adult orange trees and found that the location of the leaves throughout the crown affected the distribution of photosynthetically active radiation, revealing that crowns whose leaves received more solar radiation were thicker and, therefore, had a higher photosynthetic potential.

It is important to note that the plants in this study were located in planting rows running North-South, i.e., arranged in the orchard to prevent them from shading each other. According to Sganzerla (1991), this is the correct planting alignment because plants receive approximately the same amount of energy.

An interaction was observed between the variables variety and leaf position in the crown when analyzing the palisade and spongy parenchymas. Murcott tangor had thicker palisade parenchyma in the three crown locations than the other genotypes (Table 2), with 19.44% thicker palisade parenchyma in the lower crown than the other study varieties (Figure 1 C; F; I; L), 46.03% thicker palisade

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parenchyma in the middle crown than the Thomas and Ponkan tangerines (Figure 1 B; H; K), and 61.52% thicker palisade parenchyma in the upper crown compared with the Thomas tangerine (Figure 1 A; G; J). Thus, the Murcott tangor variety exhibited a plant architecture characterized by a wider crown in the upper part, with a shaded lower crown. The Ponkan tangerine trees were more homogeneous, with the same palisade parenchyma thickness throughout the crown. The Thomas variety had a lower palisade parenchyma thickness in the upper than in the middle and lower crown, which explains its triangular crown architecture. The Fremont tangerine trees had a more globular crown pattern, with the thickest leaves in the upper crown.

The leaves of the Murcott tangor variety had thicker palisade parenchyma compared with the other study genotypes (Table 2; Figure 1 A-C), with no relationship with resistance to the fungus that causes Alternaria brown spot (ABS), *Alternaria alternata*, to which this variety is considered susceptible, corroborating the findings of Sanine & Rodella (2012) and in contrast to the results reported by Veiga et al. (1994), who observed increased leaf parenchyma development in resistant plants. Thus, palisade parenchyma cell growth strictly depends on sunlight and is proportional to light intensity, according to the analysis by Rizzini & Mors (1995).

Changes in leaf blade thickness may be related to factors other than plant resistance mechanisms, including water stress, high temperatures, excess solar radiation, and photosynthetic mechanisms (Castro et al., 2009). The palisade parenchyma is closely related to photosynthesis, and increased palisade parenchyma thickness may enable greater CO_2 fixation (Santos et al., 2015). Thus, the increased palisade parenchyma thickness of the Murcott tangor plants may be more closely related to the increased adaptability to high solar radiation intensities and drought than to varietal resistance, as the greater the solar radiation incident on

leaves, the thicker the palisade parenchyma and therefore, the higher the photosynthetic potential.

The Ponkan tangerine leaves had thicker spongy parenchyma in the middle crown (Table 2; Figure 1 K), reflecting a more homogeneous crown, whose center may have received an optimal amount of solar radiation, water and nutrients, leading to improved leaf growth in this location. Conversely, the Murcott tangor leaves followed the palisade parenchyma pattern (Table 2), that is, thicker in the upper crown (Figure 1 A) and were therefore considered sun leaves, with increased photosynthetic potential in this location. In contrast, the Fremont tangerine leaves had thinner spongy parenchyma in the lower crown (Figure 1 F), similar to the palisade parenchyma (as shown in Table 2). This shows that the upper crown of this plant variety receives more solar radiation, forming thicker leaves, which is very common in most plants and shows the greater photosynthetic potential of leaves in the upper and middle crown (Pires et al., 2015). However, the Thomas variety showed a different pattern, that is, its lower crown leaves had a thicker spongy parenchyma than the other study varieties (Table 2 Figure 1 I). This may result from the triangular crown shape, which resulted in more radiation incident on the lower crown leaves, without upper crown shading.

Batista et al. (2010) studied leaf anatomy and water potential in coffee varieties and found increased spongy parenchyma thickness in varieties with decreased tolerance to water stress and increased palisade parenchyma thickness in high-performing varieties under water stress conditions. This helps to understand the results found in this study (Table 2), wherein the plants may have developed more efficient photosynthesis because CO_2 is potentially more easily diffused in the spongy parenchyma. The main functions of the spongy parenchyma are CO_2 accumulation for photosynthesis and radiation reflection inside leaves. Studies report the direct correlation between CO_2 diffusion in the leaf spongy parenchyma and an increase in the photosynthetic rate (Pereira et al., 2016).

Analysis of the interaction between the variables variety (Fremont, Murcott, Ponkan and Thomas) and leaf position in the crown (lower, middle and upper) with respect to the secretory cavity diameter (scd; Table 3) shows increased scd in leaves located in the lower and middle crown of the Murcott tangor trees (Figure 1 B-C) and increased scd in leaves located in the middle and upper crown of the Fremont tangerine trees (Figure 1 D-E). Secretory cavities are related to plant defense against pathogen invasion, microbial growth and herbivory (Castro et al., 2009). Thus, the greater the foliar secretory cavity diameter and number, the higher the potential to produce and store essential oils in the structural lumen, which serve as plant defense substances.

The Murcott variety, susceptible to the fungus that causes ABS, had longer distances between secretory cavities, that is, a lower density of these structures (Table 3 and Figure 1 A-C). In contrast, the Fremont tangerine, a resistant variety, had shorter distances between these structures, that is, higher

Table 2. Palisade and spongy parenchyma thickness (μm) measured in cross sections of leaves collected from different crown positions (lower, middle and upper) of four citrus varieties (Fremont, Ponkan and Thomas tangerine and Murcott tangor).

Varieties	Lower	Middle	Upper
Palisade parenchyma thickness (μm)			
Murcott	51.00 Ba*	58.88 Aa	57.89 Aa
Ponkan	43.73 Ab	41.75 Ac	45.70 Ab
Thomas	42.70 Ab	40.32 Ac	35.84 Bc
Fremont	46.88 Bb	51.23 Bb	60.83 Aa
CV (%)	19.82		
Spongy parenchyma thickness (μm)			
Murcott	155.10 Ba*	167.69 Ba	185.45 Aa
Ponkan	135.31 Bb	175.06 Aa	143.92 Bb
Thomas	167.10 Aa	97.45 Cb	135.90 Bb
Fremont	142.55 Bb	175.06 Aa	171.15 Aa
CV (%)	25.15		

* Means followed by the same lowercase letter within columns and uppercase letter within rows are not significantly different (Scott-Knott test at 5% probability).

Table 3. Diameter of secretory cavities (μm) and distance between secretory cavities (μm) measured in cross sections of leaves collected from different crown positions (lower, middle and upper) of four citrus varieties (Fremont, Ponkan and Thomas tangerine and Murcott tangor).

Varieties	Diameter of secretory cavities (μm)		
	Lower	Middle	Upper
Murcott	32.47 Aa*	30.56 Aa	27.86 Bb
Ponkan	24.70 Ab	24.30 Ab	26.02 Ac
Thomas	26.40 Ab	24.05 Bb	22.80 Bd
Fremont	26.24 Cb	28.89 Ba	31.70 Aa
CV (%)	15.97		
Distance between secretory cavities (μm)			
Ponkan	83.14 b*		
Thomas	97.97 a		
Murcott	110.51 a		
Fremont	79.78 b		
CV (%)	84.72		

* Means followed by the same lowercase letter are not significantly different (Scott-Knott test at 5% probability).

secretory cavity density (Figure 1 D-F). A similar result was found by Torres & Martínez (1992) and Veiga et al. (1994), who related resistant plants to greater numbers of idioblasts (internal secretory structures), supporting the hypothesis that a greater number and size of secretory cavities in citrus are correlated with resistance to pathogens.

The Fremont tangerine leaves mostly had secretory cavities with larger diameters that were present in higher numbers, promoting increased leaf protection and therefore, further protecting its subsequently formed fruits because its crown is homogeneous with respect to this study attribute. Specifically, the following characteristics are important for the production of this genotype: thicker adaxial and abaxial epidermis in the upper third of the crown, which is directly related to increased radiation and drought tolerance, and thicker palisade and spongy parenchyma in the upper third of the crown, which enables the optimization of radiation use in this area, i.e., the thickening of these structures compensates for shading, maintaining the photosynthetic potential of the leaves, ensuring refraction and reflection of the light absorbed by the leaves and promoting greater similarity in fruit quality, a characteristic of extreme interest for tangerine farmers. These results corroborate the findings of Sanine & Rodella (2012), who successfully differentiated peanut cultivars, i.e., susceptible and resistant to cercosporiosis, through leaf structure analysis based on abaxial epidermis thickness and the numbers of idioblasts, stomata and calcium oxalate crystals, among other attributes.

Conclusions

The leaf anatomy varied among the Ponkan, Fremont and Thomas tangerine and Murcott tangor trees.

These trees may be differentiated using quantitative structural characteristics of the leaf blade related to the adaxial and abaxial epidermis, palisade and spongy parenchyma and secretory cavities.

The canopy height the leaf anatomy of citrus plants.

Fremont tangerine and Murcott tangor exhibit xerophytic characteristics.

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