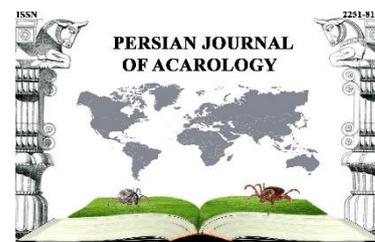




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Article

Histopathological aspects in ripe fruits of Tahiti lime *Citrus citrus x latifolia* (Rutaceae) affected by phytophagous mites

Edgar Javier Rincón-Barón^{1*} , Gerardo Andrés Torres-Rodríguez²  and Beatriz Elena Guerra-Sierra¹ 

1. Universidad de Santander, Facultad de Ciencias Médicas y de la Salud y Facultad de Ciencias Naturales, Grupo de Investigación Agroambiente y Salud-MICROBIOTA, calle 70 No 55-210, Campus Universitario Lagos del Cacique, Bucaramanga, Colombia; E-mails: ed.rincon@mail.udes.edu.co, bguerra@udes.edu.co
2. Unidad de Microscopía Electrónica, Universidad del Cauca, carrera 2 # 1A-25 Museo de Historia Natural, Popayán, Cauca, Colombia; E-mail: gator@unicauca.edu.co

* Corresponding author

ABSTRACT

This study describes in detail the anatomical and histochemical aspects of the phytophagous mites-Tahiti lime pathosystem. More than 300 healthy pieces of fruit affected by these mites were collected from agricultural crops in the province of Santander-Colombia. Identification of the mites associated with the lesions observed was conducted via specialized literature and consultations with expert taxonomists of this group. The pericarps were processed according to standard protocols for embedding and sectioning in paraffin, and the sections obtained were stained with Safranin-Alcian Blue, Fast Blue B, Floroglucinol, Lacmoid, and PAS-Amido Black. Additional sections were processed in resin and stained with Toluidine Blue. For observation under scanning electron microscopy (SEM), the material was fixed and dehydrated in 2,2-dimethoxypropane, then in critical point dried and coated with gold. The surface of healthy fruit was smooth, with a bright, intense green color and stomata. The exocarp is composed of various cell layers; the outermost being the epidermal tissue, which is formed of a single layer of cells covered by a thick cuticle. The remaining layers consist of photosynthetic and reserve parenchyma with abundant starch grains. The fruit affected by the three mites showed deterioration of the exocarpal layers varying according to the severity of the attack and the species of mite responsible. In general, there were fissures and/or cracks on the surface of the fruit in addition to structures such as circular whitish spots in the case of *Schizotetranychus hindustanicus*. The affected tissues tested positive for polyphenols and lignin, but not for callose. The primary walls were positive for PAS-Amidoblack but no proteins or starch granules were detected. Likewise, staining with Toluidine Blue indicated the presence of primary wall components, lignin, and polyphenols. Phytophagous mite attack affects mainly the exocarpal layers. The histopathological response of the plant to the attack of these mites is similar among the three, and results in the formation of periderm, lignin deposits, and polyphenols in the affected tissues. Greater severity of tissue damage was observed in the case of attack by *Polyphagotarsonemus latus*.

KEY WORDS: Histochemistry; histopathology; lime fruit; pericarp; plant mite.

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INTRODUCTION

Worldwide production of citrus fruits has increased in response to high consumer demand, with some

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146,429,018 tons registered in 2016 (Food and Agriculture Organization [FAO] 2016), with an expected further 4% increase between 2020 and 2021 (United States Department of Agriculture 2021). Within the citrus family, the most commonly cultivated species are oranges, followed by tangerines, limes, lemons, and grapefruits; with China being the largest producer, followed by Brazil and the European Union (United States Department of Agriculture 2021).

Tahiti limes belong to the Rutaceae family and to the genus *Citrus*, and although the species has long been referred to as *Citrus latifolia* Tanaka, this name is without nomenclatural validity. In reality, the species refers to a triploid hybrid with the precise scientific name of *Citrus citrus* x *latifolia* Tanaka ex Q. Jiménez (Grayum *et al.* 2012). This fruit is widely commercialized and consumed worldwide, being seedless, bright green, varying in shape from round to oval, and exhibiting a respectable weight (Aguilar *et al.* 2012; Grayum *et al.* 2012; Arévalo *et al.* 2016); the latter of which makes it suitable for the extraction of juice used in drinks and cocktails, and is consumed in many North American and European nations (Botina *et al.* 2019). According to the United Nations Food and Agricultural Organization (Food and Agriculture Organization 2016), the 10 nations with the greatest production of Tahiti limes are Brazil, China, the United States, Mexico, India, Spain, Iran, Italy, Nigeria, and Turkey. In Latin America, Mexico and Brazil are the largest producers of this fruit; however, other developing nations such as Colombia, Guatemala, and Peru are emerging as future exporters (Arias and Suárez 2017; Centre for the Promotion of Imports from Developing Countries 2018).

In Colombia, Tahiti limes are grown from sea level to an elevation of 2100 m, with a maximum of 1600 m being recommended for commercial production (Hernández *et al.* 2014; Murcia *et al.* 2020). Colombian agricultural land dedicated to Tahiti lime production was estimated at 467,558 hectares as of 2018, with a total production of 8,401,463 tons (Ministerio de Agricultura y Desarrollo Rural-Agronet 2019). Within Colombia, the provinces of Santander, Cauca, Antioquia, Tolima, Quindío, Atlántico, and Nariño represent the principal centers of cultivation (Murcia *et al.* 2020). In these areas, however, the production of Tahiti limes - as well as that of other tropical cultivars - is subject to attack by various pests and phytophagous mites. These latter cause damage to the exocarp of the fruit, which negatively affects its external appearance (Departamento Administrativo Nacional de Estadística 2015). The damage caused by these mites makes the fruit undesirable for sale, as consumers generally rate the quality of fruits and vegetables based upon external factors such as appearance, color, size, and an imperfection-free surface (Peña 1990; Peña *et al.* 2000; Navia and Marsaro 2010; Opara and Pathare 2014; León and Kondo 2017; Sarada *et al.* 2018; Botina *et al.* 2019).

In Colombia, the main phytophagous mites associated with citrus cultivation belong to the Tetranychidae, Tarsonemidae, Eriophyidae, and Tenuipalpidae families. Within these groups, several species stand out as being especially damaging to Tahiti lime production; for example, the broad mite, *Polyphagotarsonemus latus* Banks (Tarsonemidae), the rust mite *Phyllocoptruta oleivora* Ashmead (Eriophyidae), and several species of the genus *Brevipalpus* (Tenuipalpidae), with the latter being furthermore key vectors for the transmission of viruses such as Citrus Leprosis. The Hindu mite *Schizotetranychus hindustanicus* Hirst (Tetranychidae) has also been discovered in Colombia, a pest which exhibits a preference for oranges, tangerines, and Tahiti lime (Mesa 1999; Instituto Colombiano Agropecuario 2012; Mesa 2015; Álvarez 2017; León and Kondo 2017; Ministerio de Agricultura y Desarrollo Rural-Agronet 2017; Murcia *et al.* 2020; Pérez-Artiles *et al.* 2021). The chelicerae or mouthparts of the mites penetrate individual tissue cells, causing damage with symptoms that vary according to the type of mite responsible. Rust mites, for example, cause browning of leaves and fruit due to the production of melanin and the formation of lignified tissue (McCoy and Albrigo 1975; McCoy 1996; Moraes and Flechtmann 2008; Álvarez 2017; León and Kondo 2017; Murcia *et al.* 2020). Broad mites attack lime fruit from early on in its ontogeny, causing alterations to its physiology and development, as well as giving mature fruits the unpleasing appearance of scalding or erosion of the exocarp via the formation of peridermal tissue (Childers

1994; Childers *et al.* 2003; McCoy 1996; Moraes and Flechtmann 2008; Navia and Marsaro 2010; Bensoussan *et al.* 2016; Álvarez 2017; Rodríguez *et al.* 2017; Murcia *et al.* 2020). The symptoms of attack by species from the Tenuipalpidae family are varied and depend on the exact species responsible. In general, the damage involves brownish or reddish spots on the fruit which, in cases of severe infestation, may also cause the leaves to discolor and fall (Álvarez 2017; León and Kondo 2017). In the case of attack by the Hindu mite, the leaves and fruits exhibit round, whitish spots like freckles, with these patterns mirroring the spider webs with which these mites construct their nests. These structures are thought to affect the photosynthetic capacity of the plant and, thus, the filling of the fruit (León and Kondo 2017; Ministerio de Agricultura y Desarrollo Rural-Agronet 2017).

Although advances in knowledge have been made with respect to how herbivorous arthropods in this patho-system evade plant defense systems (Blaazer *et al.* 2018; Stahl *et al.* 2018), little is known about the histopathological aspects of the response to the damage caused by this type of mite in plants (McCoy and Albrigo 1975; Peña 1990; Achor *et al.* 1991; McCoy 1996; Childers and Achor 1999; Peña *et al.* 2000; Petanović and Kielkiewicz 2010a, b; Álvarez 2017; de Lillo *et al.* 2018; Sarada *et al.* 2018; Murcia *et al.* 2020). A thorough understanding of the type and extent of citrus lesions caused by mites, as well of the plants' response from a histological and histochemical perspective is of critical importance, as this provides a greater understanding of the biology of these sorts of interactions for the taking of measures for prevention and control. Proper control of these parasites is a key in maintaining the external quality of fruit. Loss of this quality not only limits the ability for the producing nation to participate in the global economy, but the pericarp of citrus fruit, itself, represents a valuable subproduct used in the food, pharmaceutical, and cosmetics industries (Mahato *et al.* 2018).

The present research study involves a detailed investigation of the histopathological and histochemical aspects related to the damage caused by the Hindu mite *S. hindustanicus*, rust mite *Ph. oleivora*, and broad mite *Po. latus*, which are considered to be the species which most greatly affect the quality of Tahiti lime fruit in Santander, Colombia. The information obtained by the present study contributes to the scientific literature regarding citrus defense and response mechanisms against the herbivores in question.

MATERIAL AND METHODS

More than 300 pieces of Tahiti lime fruit were collected from both healthy plants, as well as others affected by *S. hindustanicus* (Hindu mites), *Ph. oleivora* (rust mites), and *Po. latus* (broad mites). These specimens were collected in the Llano Grande region of the municipality of Giron in Colombia's Santander province; specifically, from the El Diamante and Los Matarratones farms, during the months of July and August 2020. These sources are geographically located at 7° 00' 39.3" N, 73° 09' 43.7" W and 7° 02' 44.0" N, 73° 09' 09.0" W and are characterized by average temperatures of 29.3 °C and 28 °C and elevations of 800 and 760 m a.s.l. respectively. More than 30 samples were taken for each condition (that is, healthy or affected). The mites responsible for the damage were identified using specialized literature, and with the support of experts in this taxonomic group (Ochoa *et al.* 1991; Mesa 1999; Moraes and Flechtmann 2008; Vacante 2010a, b; Mesa and Rodríguez 2012; León and Kondo 2017).

The pericarps of the collected fruits were fixed in a mixture of formaldehyde, ethanol, and acetic acid (FAA) for 24 to 48 hours at 6 °C. These were then cut into pieces of 1 cm length, dehydrated via a series of alcohols and two clarification steps in Xylol (Ruzin 1999), before being embedded in Paraplast Plus (Mc Cormick®) for 12 hours at 55 °C. Transversal and longitudinal sections were obtained using a LEICA RM 2125 rotary microtome set to a thickness of 4–5 µm. The samples were then stained as indicated in Table 1 (Ruzin 1999; Demarco 2017; Rincón *et al.* 2020a, b).

Table 1. Histochemical tests applied to pericarpal sections of Tahiti lime fruit *Citrus citrus x latifolia*, both healthy and affected by *Schizotetranychus hindustanicus*, *Phyllocoptruta oleivora*, and *Polyphagotarsonemus latus*, in Santander, Colombia.

stain	highlighted composition/structure
Toluidine Blue	Lignified secondary walls (xylem and sclerenchyma), stained green or blue-green.
Safranin-Alcian Blue (FASGA)	Primary walls stained blue.
Lacmoid	Highlights callose deposits in light blue shade. This is specific, with no cross reactions.
Wiesner Technique (Phloroglucinol-HCl)	Specific for lignin (coniferyl aldehyde and sinapil aldehyde groups), highlighted in magenta. No cross reactions are observed with other compounds.
Fast Blue B	Polyphenols can be observed highlighted from red to dark brown to black. This is specific, with no cross reactions.
PAS-Amidoblack	Polysaccharides (reserve and structural) are highlighted in fuchsia. Proteins and nuclei are highlighted in bright blue. This process cross reacts with polyphenols (brown).

Additional samples were fixed in 2.5% Glutaraldehyde in a 0.2 M phosphate buffer with pH 7.2 for 24 to 48 hours at 6 °C. After fixing, the samples were washed in the same buffer solution, and thereafter with distilled water before postfixing with 2% Osmium Tetroxide for 4 hours at 6 °C in darkness and using constant agitation. They were then dehydrated for one hour in a graduated ethanol series, and for 12 hours in 100% ethanol at 6 °C. The samples were then embedded using progressive mixtures of Spurr propylene oxide for one week at ambient temperature. The pure resin was refreshed at several points during the six day process, and was kept under constant agitation. The resin was polymerized at 60 °C for 48 hours. Sections of 0.4–0.5 µm thickness were then obtained using glass knives in a Leica® ultramicrotome. These sections were then stained with Toluidine Blue (TBO) in 1% borax, pH 3.6, for 30 to 60 seconds. Both the sections in paraffin, as well as those in resin were examined with a Nikon Eclipse Ni photonic microscope with a Differential Interference Contrast (DIC) using the Nikon NIS-Elements program, version 4.30.02.

For morphological descriptions undertaken via scanning electron microscopy (SEM), the pericarps of the healthy and mite-affected samples were fixed in 3% Glutaraldehyde 0.2M phosphate buffer at pH 7.2 for 24 to 48 hours at 6 °C and dehydrated in acidified 2,2 Dimethoxypropane for 2 to three days, with the solution being refreshed once during this period (Lin *et al.* 1977; Dykstra and Reuss 2003). Lastly, two 30-minute passes were carried out in pure ethanol, and the samples were subjected to critical point drying using a SAMDRI®-795 desiccator. They were then mounted on double-sided conductive carbon tape and coated with gold in a DENTON VACUUM DESK IV ionizer for 10 minutes. Final observations and photographic records were then made using a JEOL JSM-6490LV scanning electron microscope.

RESULTS

Morphoanatomy of Tahiti lime fruit, both healthy and affected by phytophagous mites

Healthy Tahiti lime fruit appears as a hesperidium berry with a smooth surface and bright green color (Fig. 1A). Under SEM, the surface can be seen to be homogeneous, with stomata distributed randomly over the exocarp (Fig. 1B). In the transversal section, the exocarpal outer layer can be observed to be composed of a unicellular epidermal stratum of rectangular to square-shaped cells, with a thick cuticle on its periclinal walls, while the rest of exocarpal tissue is made up of various layers of parenchymal tissue, mainly photosynthetic and reserve (Fig. 1C).

Fruit affected by *Po. latus* exhibited silvery grey lesions of scalded appearance which covered the full or partial surface of the exocarp; and the fruit generally lost its luster and bright green color (Fig. 1D). The SEM detail of the affected exocarp reveals deep fissures and grooves which expose the underlying periderm, which has formed in response to the mite attack (Fig. 1E).

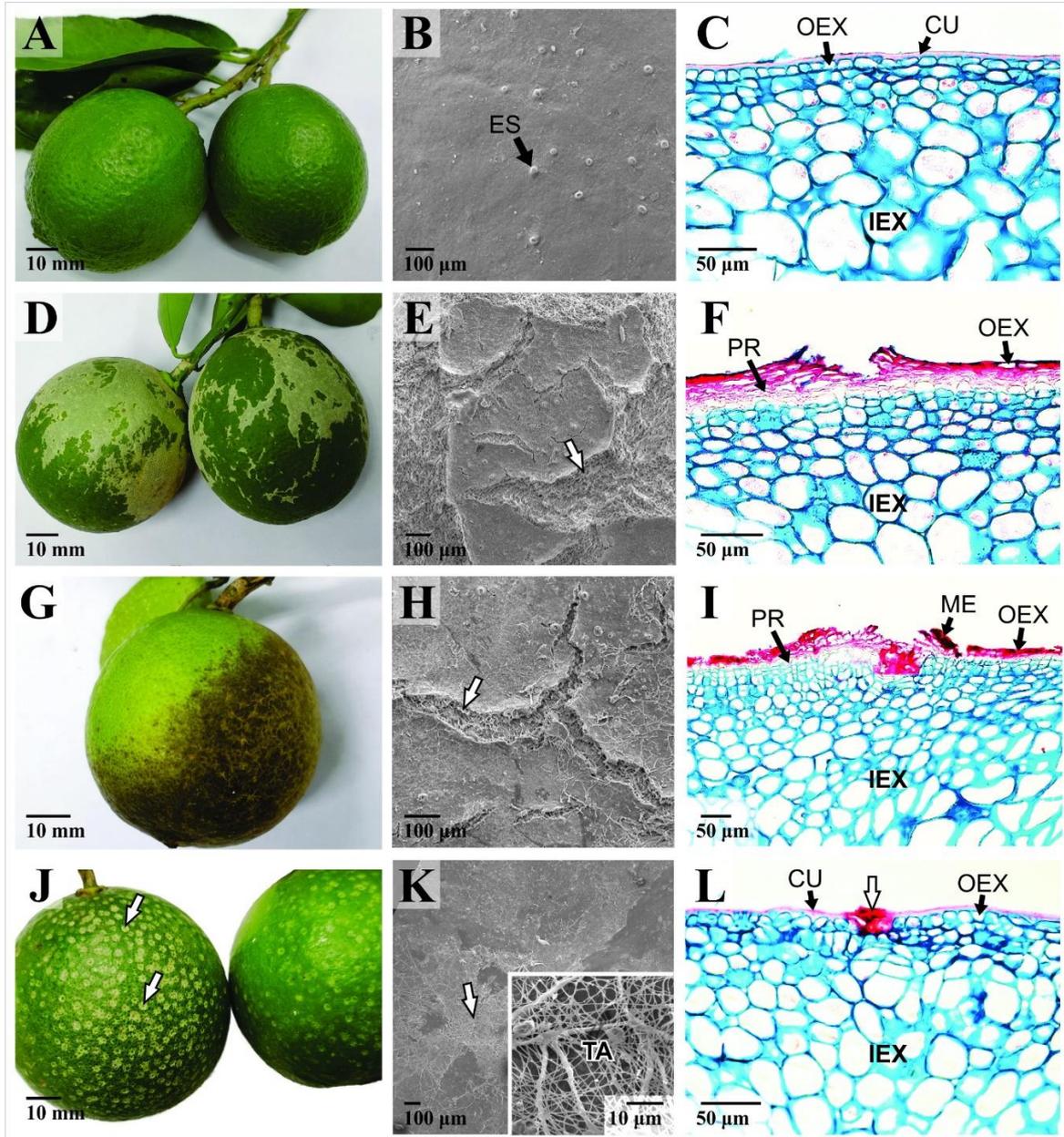


Figure 1. Macroscopic and microscopic lesions on the pericarps of healthy Tahiti lime fruits affected by phytophagous mites. **A–C.** Healthy fruit and tissue. **A.** Pericarp surface; **B.** Pericarp surface viewed under scanning electron microscopy (SEM). Stomata can be observed; **C.** Cross section of the pericarp, showing the exocarp (Safranin-Alcian blue). **D–F.** Fruit and tissues affected by *Polyphagotarsonemus latus*. **D.** Lesions on the pericarp; **E.** Detail of the lesions on the pericarp surface (white arrow) (SEM); **F.** Cross section of the pericarp, showing the lesion affecting the exocarp and the formation of the peridermis (Safranin-Alcian blue). **G–I.** Fruit and tissues affected by *Phyllocoptruta oleivora*. **G.** Lesions on the pericarp; **H.** Detail of the lesions on the pericarp surface (white arrow) (SEM); **I.** Cross section of the pericarp, showing the lesion affecting the exocarp and the formation of peridermis and melanin deposits (Safranin-Alcian blue); **J–L.** Fruit and tissues affected by *Schizotetranychus hindustanicus*. **J.** Lesions on the pericarp. Mites nests (white arrows) can be observed; **K.** Mites nests in SEM (white arrow). Spider web of nest formation can be clearly seen; **L.** Cross section of the pericarp, showing the lesion affecting the exocarpal layers (white arrow) (Safranin-Alcian blue). CU: cuticle; EN: stomata; IEX: inner exocarp; ME: melanin; OEX: outer exocarp; PR: Peridermis; TA: spider web.

The transversal sections reveal deterioration and collapse of the outer exocarp, with this tissue being heavily stained in fuchsia by Safranin; while the innermost exocarp does not exhibit any histopathological alterations (Fig. 1F). Fruits affected by *Ph. oleivora* display a dark hue referred to as “bronzing” which ranges from brown to black, with the exact shade depending upon the severity of the attack, and which can cover the entire surface of the fruit (Fig. 1G). Observations of the transversal sections made using the SEM reveal very similar histopathological effects to those described for *Po. latus*, although in this case the grooves and fissures caused by the mites' damage to the exocarp are neither as extensive nor as deep. The presence of melanin was noted in the remaining exocarp cells surrounding the lesion (Fig. 1H & 1I). Fruit affected by *S. hindustanicus* exhibited whitish spot-like structures across the surface of the exocarp that covered the fruit either partially or completely, with the effect of giving the fruit a silvery sheen (Fig. 1J). Observations under the SEM showed these structures to correspond with the mites' nests, which are formed from spider web material that they produce (Fig. 1K). Observations of the transversal sections indicates that the lesions caused by this species are limited in location to the feeding zones under the mites' nests, and demonstrate a similar histopathological response to that described for the other two species in question (Fig. 1L). In some sections, the formation of periderm can also be confirmed.

Histochemical tests applied to healthy Tahiti lime fruit

Healthy Tahiti lime fruit did not exhibit accumulation of polyphenols in the exocarp or mesocarp (Fast Blue B); however, a cross-reaction was evident with the cuticle and chloroplasts, which appears as a light brown color (Fig. 2A). Likewise, the presence of lignin was not detected in the epidermal tissue that forms the outer exocarp, nor in the photosynthetic parenchymal tissue of the inner exocarp; nevertheless, tissues that are normally lignified with xylem showed a positive reaction (magenta color) with phloroglucinol acid (Fig. 2B). Callose was not found in the tissues that comprise the exocarp and mesocarp, although it is commonly found in tissue such as phloem, where it can be observed with a blue tint in the presence of Lacmoid stain (Fig. 2C). With PAS-Amidoblack stain, structural and reserve carbohydrates appear fuchsia, as can be observed in the parenchymal and epidermal tissues that form the mesocarp and exocarp, respectively. With this stain, it was not possible to observe the presence of proteins, and the cuticle did not color. In contrast, a large number of starch granules can be seen, mainly in the photosynthetic parenchyma of the exocarp (Fig. 2D). The Toluidine Blue stain colored the primary walls of the outer and inner exocarp and mesocarp in purple, as well as the cuticle in dark blue. The starch grains were not colored by this stain (Fig. 2E).

Histochemical tests applied to Tahiti lime fruit affected by phytophagous mites

The exocarpal layers of the fruit exhibited an abundant accumulation of polyphenols in the areas damaged by the three mite species; the plants' response was illustrated by a brown or black color under the Fast Blue B test (Fig. 3 A–C). Additionally, in the damaged zones, lignin was found in the exocarpal layers, appearing as a magenta shade under the phloroglucinol acid test (Fig. 3D–F). Callose was not found to be present in the mite-damaged zone when using Lacmoid stain (Fig. 3G–I). Additionally, heavier staining was always observed for polyphenols and lignin in the case of tissues attacked by *Ph. oleivora*, and melanin deposits were noted in the exocarp in the areas damaged by this species (Fig. 3H). The PAS-Amidoblack technique did not result in different shades of staining between the lesions caused by the three mite species. The primary walls exhibited the fuchsia color typical of this test, and it was not possible to determine the presence of proteins, nor were starch grains found (Fig. 3J–L). Using Toluidine Blue stain, the primary walls of the parenchymal tissues of the exocarpal layers and the epidermal tissue display the purple coloration characteristic of this test for primary wall components. This technique also indicates the presence of lignin with a blue-green color which can be observed on various sections of the exocarpal layers in damaged areas. The same test also detected the presence of polyphenols, seen in a brown or black coloration (Fig. 3M–O). Nevertheless, these stains can overlap, which complicates the visualization and analysis of tissues

with this coloration. In all cases, peridermal tissue was found to be present to a greater or lesser extent depending on the severity of the lesions (Fig. 3A–O).

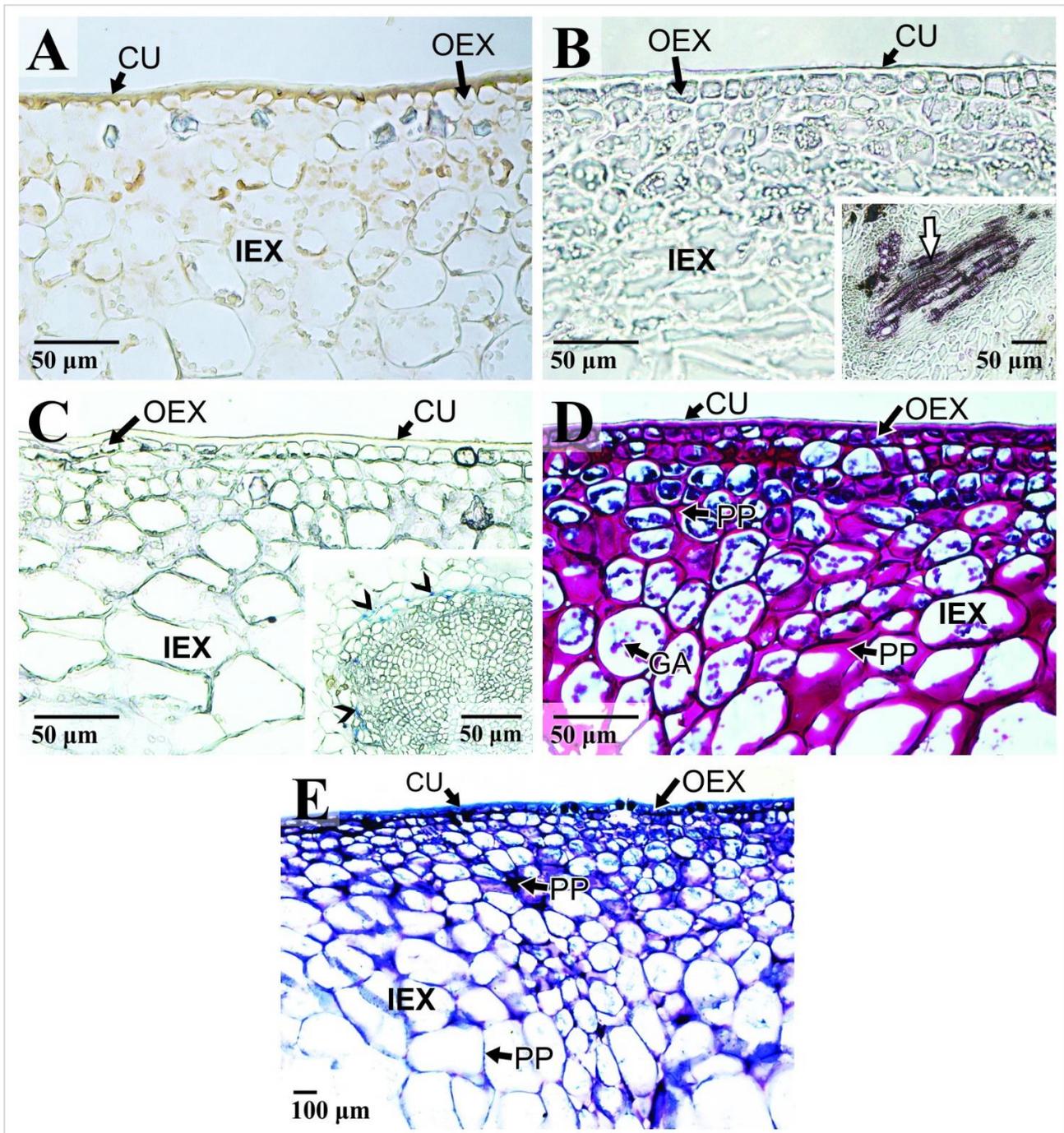


Figure 2. A–E. Histochemical tests applied to healthy Tahiti lime pericarps (cross sections). **A.** Polyphenols Test (Fast Blue B). No positive reaction for polyphenols is observed in the exocarpal layers; **B.** The reaction for lignin detection (phloroglucinol acid) is negative in the exocarpal layers, but it is positive in lignified tissues (reddish staining) such as the fruit xylem (detail, white arrow); **C.** Callose reaction (Lacmoid) is negative in the exocarpal layers, but positive (blue staining) in fruit phloem (detail, arrow heads); **D.** PAS-Amidoblack test, the primary walls of the exocarpal layers and starch granules are stained magenta; **E.** Toluidine Blue stain, the primary walls of the exocarpal layers stain violet and cuticle dark blue. CU: cuticle; GA: starch granules; IEX: inner exocarp; OEX: outer exocarp; PP: primary walls.

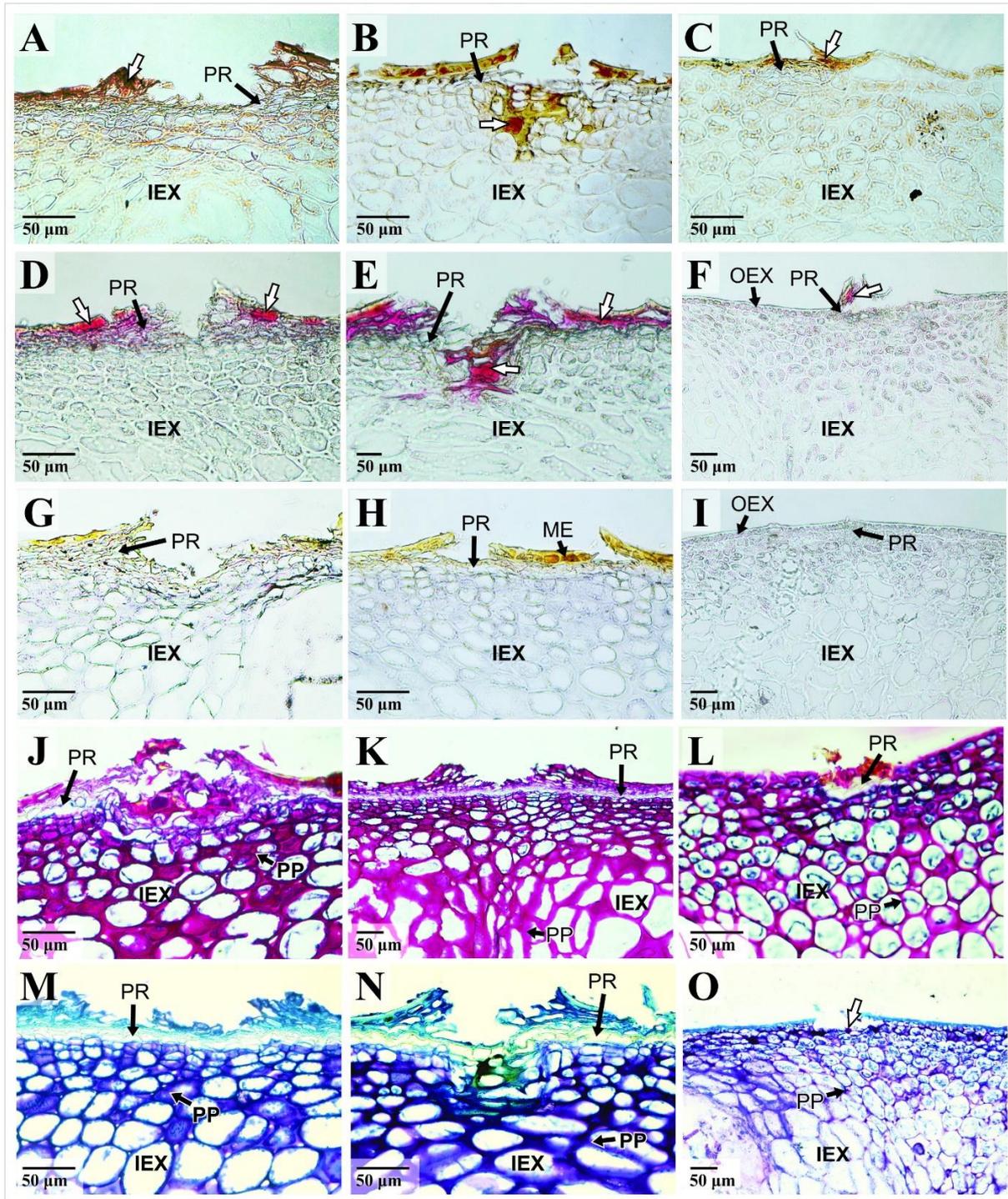


Figure 3. Histochemical tests applied to Tahiti lime pericarps affected by phytophagous mites (Cross sections). **A–C.** Pericarps affected by *Polyphagotarsonemus latus*, *Phyllocoptruta oleivora* and *Schizotetranychus hindustanicus* respectively. A dark brown stain can be observed due to the accumulation of polyphenols in the OEX: exocarpal layers (white arrows, Fast Blue B); **D–F.** Pericarps affected by *Po. latus*, *Ph. Oleivora* and *S. hindustanicus* respectively. A magenta stain can be observed due to the accumulation of lignin in the exocarpal layers (white arrows, Phloroglucinol acid); **G–I.** Pericarps affected by *Po. latus*, *Ph. Oleivora* and *S. hindustanicus* respectively. Callose was not detected in the exocarpal layers (Lacmoid). Melanin deposits were observed; **J–L.** Pericarps affected by *Po. latus*, *Ph. Oleivora* and *S. hindustanicus* respectively. Primary walls stain magenta and no protein or starch granules were detected (PAS-Amidoblack); **M–O.** Pericarps affected by *Po. latus*, *Ph. Oleivora* and *S. hindustanicus* respectively. Primary walls are stained purple, lignified tissues blue-green, and polyphenols brown or black (Toluidine Blue). *Schizotetranychus hindustanicus* lesions are restricted to feeding zones below nests (Fig. 3O) (White arrows). Peridermis formation was observed in all cases (Fig. 3A–O). CU: cuticle; IEX: inner exocarp; ME: Melanin; OEX: outer exocarp; PP: primary walls; PR: Peridermis.

DISCUSSION

Several authors have indicated that citrus fruits in general correspond morphoanatomically with a modified type of berry denominated “hesperidium” (Sadka *et al.* 2019; Tadeo *et al.* 2020), an argument with which the findings of the present study concur with respect to the Tahiti lime in particular. These same authors indicate that the ripe citrus fruit consists of an external multilayered layer called the exocarp that corresponds to the epidermis and several photosynthetic and reserve parenchyma layers; as well as an innermost layer of several cellular strata called the mesocarp, made up of parenchymal tissue that is usually reserve; as well as an endocarp made up of juicy hairs, this being the edible portion of the fruit. The preceding description agrees with the observations of the present study with respect to the Tahiti lime, specifically. Nevertheless, it should be mentioned that although the aforementioned authors also use the terms “flavedo” and “albedo” to refer to the exocarp and mesocarp, respectively; we consider that these terms are histologically imprecise, due to the fact that the flavedo would be composed by various histological entities such as the epidermis of the fruit (the exocarp), together with parenchymal components of the mesocarp. Thus, we prefer to use the terms exocarp and mesocarp (Evert 2006; Crang *et al.* 2018). Various studies in the literature report that some species of the *Citrus* genus exhibit stomata distributed randomly across the exocarp, as well as epicuticular wax deposits which form crystal-like structures and large platelets distributed irregularly over the surface of the fruit, and giving it a rough appearance under SEM observation (Liu *et al.* 2012; Wang *et al.* 2014; Tadeo *et al.* 2020). Although we concur with these authors in relation to the presence and distribution of stomata on the surface of Tahiti lime fruit, the present study did not discover the presence of epicuticular patterns such as those described in the literature, with the surface of this fruit being smooth under SEM observation.

The macroscopic lesions associated with the *Po. latus*, *Ph. oleivora*, and *S. hindustanicus* mites have been widely reported for an array on the mature fruits of several citrus species, including oranges, tangerines, lemons, and limes; the description of which aligns with the findings of the present study with respect to Tahiti limes. Mature fruit attacked by *P. latus* was observed to exhibit a silvery grey coloration, along with cracks or fissures across the exocarp which may extend across the entire surface (Peña 1990; Gerson 1992; Peña *et al.* 2000; Umeh *et al.* 2007; Mesa and Rodríguez 2012; León and Kondo 2017; Rodríguez *et al.* 2017; Cabedo-López *et al.* 2021). In the case of citrus fruit attacked by *Ph. oleivora*, in addition to the same cracks and fissures of the exocarp, the fruit was noted to take on a coloration from brown to black, giving a bronzed appearance; hence the reference to “bronzing” of fruit being an important characteristic in identifying the damage caused by this species of mite (McCoy and Albrigo 1975; Achor *et al.* 1991; McCoy 1996; Childers and Achor 1999; Petanović and Kielkiewicz 2010a, b; Álvarez 2017; de Lillo *et al.* 2018; Sarada *et al.* 2018; Murcia *et al.* 2020). The melanin deposits discovered in the exocarp of Tahiti limes attacked by this mite appear to be created by the accumulation of polyphenolic compounds that react with quinones; with these, in turn, reacting with proteins to form melanin, as described with reference to the “bronzing” of *Citrus limona* L. shoots attacked by *Aceria sheldoni* mites (Ishaaya and Sternlicht 1971; Petanović and Kielkiewicz 2010a). Nevertheless, this hypothesis would not explain why melanin was not formed in lesions caused by the attack of *Po. latus* and *S. hindustanicus* in Tahiti limes, wherein the formation of abundant quantities of polyphenolic compounds was observed, these being an input for the formation of melanin.

Lesions caused by *S. hindustanicus* in citrus fruits were not macroscopically visible, as they formed beneath the nests constructed by spider web materials produced by the mites. Thus, damage can only be observed as structures of whitish spots on the fruit exocarp. This condition is a good indicator to detect the attacks of this species of mite (Navia and Marsaro 2010; Instituto Colombiano Agropecuario-ICA 2012; León and Kondo 2017; Pérez-Artiles *et al.* 2021). It is important to reiterate that the formation of spider webs is a common behavior of the Tetranychidae family (Vacante 2010a), by which the organism protects itself from predators and from adverse climatic conditions (Aponte and McMurtry 1997; Lemos *et al.* 2010; Shimoda *et al.* 2010). The webs, themselves, were not visible

in the histological samples prepared with resin or paraffin; likely being eliminated by the solvents utilized for the preparation of the tissues.

Some recent studies take the mite species *Tetranychus urticae* C.L. Koch, as a model of plant-mite herbivory, and report that surface lesions on the leaves of the plant are not highly visible at the macroscopic level, since the mite does not damage the epidermis and generally inserts its long chelicera into the stoma or the spaces between epidermal cells, with its preferred targets being the deepest cells within the leaf mesophyll (Bensoussan *et al.* 2016, 2018; Rioja *et al.* 2017). This general model successfully explains the herbivory mechanism in the leaves of an array of plant groups affected by *T. urticae*, but does not fully elucidate the effects of *S. hindustanicus* on mature Tahiti lime fruit, given that the lesions, although difficult to observe, retain a histopathological and histochemical similarity to the observations of lesions caused by *Po. latus* and *Ph. oleivora*; a finding which indicates that the *S. hindustanicus* mite exhibits a preference both for epidermal cells as well as the parenchymal tissues of the exocarp. It is also important to mention that the morphology of lesions caused by phytophagous mites can vary depending on whether the observations are made under real-world conditions in the field or experimental conditions in a greenhouse; and also depending on the developmental state of the vegetal structure that is affected, given that the mites can cause damage to immature fruit, leaves at various phenological stages of their development, and even the stem of the plant (McCoy and Albrigo 1975; Peña 1990; McCoy 1996; Achor *et al.* 1991; Childers and Achor 1999; Peña *et al.* 2000; Rodríguez *et al.* 2017). Finally, some observable lesions may be similar among various species of mite, and can even be confused with other conditions such as mineral deficiencies, phytotoxicity due to pesticides, and physiological disorders (Gerson 1992; Grafton-Cardwell *et al.* 2017; Cabedo-López *et al.* 2021). It is thus recommended that the characteristics of lesions observed should be cross-checked with the taxonomic identification of the mite that caused them.

The histochemical response to the lesions caused by *Po. latus*, *Ph. oleivora*, and *S. hindustanicus* are very similar in terms of the production of lignin and polyphenols in both the exocarpal layers. Plants produce an array of defensive molecules in response to attack by various pathogens, with studies reporting the generation of lignin, polyphenols, callose, flavonoids, and various types of terpenoids (Lattanzio *et al.* 2006; Aoun 2017; Rincón *et al.* 2020a, b). As such, both recent and older studies have reported the formation of these same compounds in the patho-system of phytophagous mites and citrus plants; with detailed descriptions of the formation of lignin, polyphenols, and volatile terpenoids (Petanović *et al.* 2010a, b; Agut *et al.* 2014, 2015, 2018; Rioja *et al.* 2017; de Lillo *et al.* 2018; Cabedo-López *et al.* 2021), which is congruent with the findings of the present study with respect to the production of lignin in Tahiti lime. Lignin reinforces cell walls against the mechanical attack of mite chelicerae, while polyphenols may be generated both as an important component for the formation of lignin, as well as to trigger molecular signaling mechanisms for the plant's immune response (Petanović *et al.* 2010a, b; Rioja *et al.* 2017; de Lillo *et al.* 2018). The intense shade of fuchsia staining from Safranin in areas damaged by the three mite species also indicates a strong response of lignin and polyphenol production, as this stain will non-specifically color both these chemical compounds (Ruzin 1999; Rincón *et al.* 2020 a, b). Furthermore, some studies have reported the presence of callose as a defensive mechanism surrounding the lesions caused by some phytophagous mites, primarily those that form galls (Petanović and Kielkiewicz 2010 a, b), although this was not observed in the present study for the three species of mite in question on Tahiti lime. One constant histopathological marker which can be observed due to attack by these mites is the development of peridermal tissue; a finding which has been previously documented in this type of patho-system (McCoy and Albrigo 1975; Peña 1990; McCoy 1996; Achor *et al.* 1991; Childers and Achor 1999; Peña *et al.* 2000). This formation of periderm has been reported not only as a mechanical response to mite attack, but also as a response to other phytopathogens such as fungi and bacteria (Biggs *et al.* 1984; Biggs and Stobbs 1986; Agrios 2005). Furthermore, McCoy and Albrigo (1975) observe that the cracks and fissures which form on the surface of fruit affected by these mites are

caused by the mass death of epidermal cells in the exocarp and the fruit's growth cycle. In this respect, our findings in the present study align with these authors; and we furthermore add the suggestion that the formation and expansion from the inner exocarp of the fruit as a protective tissue against mite attack leads to the fracturing and formation of cracks on the fruit's surface.

In general, the cracks and fissures which were observed on the exocarp of Tahiti lime fruit were more extensive and developed in the attacks from the *Po. latus* mites. This finding may suggest that the species is able to develop and feed more successfully on Tahiti lime, especially when we note the lesser quantity of defensive compounds such as lignin and polyphenols generated by the plant, as noted by the present study, which could indicate the presence of molecules in the saliva of many mite species which disrupt plant defensive systems (Bensoussan *et al.* 2016, 2018; Rioja *et al.* 2017; Rodríguez *et al.* 2017; Agut *et al.* 2018; Blaazer *et al.* 2018; de Lillo *et al.* 2018; Cabedo-López *et al.* 2021). In this sense, *Po. latus* appears to be more effective than its counterparts *Ph. oleivora* and *S. hindustanicus*. For the latter two species, histochemical stains specific to these compounds exhibited a weaker coloration when applied to the damaged area. Nevertheless, additional experiments would be required to support these observations and hypothesis.

One interesting observation of the present study involves the presence of a large number of starch granules in the inner exocarp of the healthy Tahiti lime fruit, which are absent in the same tissue of fruit attacked by *Po. latus*, *Ph. oleivora*, and *S. hindustanicus*. This finding may be related to other research which posits that one strategy plants use to avoid foraging by mites is to make their tissues less attractive for consumption (Shi and Tomczyk 2001; Petanović and Kielkiewicz 2010a, b; de Lillo *et al.* 2018). According to this theory, the absence of starch granules in the mesocarp of fruit attacked by the three mite species could correspond to an anti-predation strategy of the Tahiti lime plant. It is also possible that the starch granules are converted to small sugar molecules (glucose) by the enzymes secreted by the mites, preparing it for their consumption.

It is important to have a deep understanding of this type of patho-system from a morphoanatomical and histopathological point of view, as well as its relationship to plant defense mechanisms, as this knowledge makes possible the creation of effective strategies for prevention and control. This research is especially key considering the relatively scarcity in the literature of studies concerning resistance to phytophagous mites in citrus plants.

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جنبه‌های هیستوپاتولوژیک در میوه‌های رسیده لیموی تاهیتی *Citrus citrus x latifolia* (Rutaceae) تحت تأثیر قرار گرفته با کنه‌های گیاهخوار

ادگار خاویر رینکن-بارون^{۱*}، جراردو آندرس تورس-رودریگز^۲ و بناتریز النا گوئرا-سیرا^۱

۱. دانشگاه سانتاندر، دانشکده علوم پزشکی و بهداشت و دانشکده علوم طبیعی، کشاورزی، محیط‌زیست و بهداشت - گروه پژوهشی میکروبیوتانا، پلاک ۷۰ شماره ۵۵-۲۱۰، پردیس دانشگاه لاگوس دل کاسیک، بوکارامانگا، کلمبیا؛ رایانامه: ed.rincon@mail.udes.edu.co
 ۲. واحد میکروسکوپ الکترونی، دانشگاه کائوکا، کاررا # 1A-25 # 2 موزه تاریخ طبیعی، پوپایان، کائوکا، کلمبیا؛ رایانامه: gator@unicauca.edu.co
 * نویسنده مسئول

چکیده

این مطالعه به طور مفصل جنبه‌های تشریحی و هیستوشیمیایی کنه‌های گیاهخوار-لیموی تاهیتی را تشریح می‌کند. بیش از ۳۰۰ قطعه میوه سالم تحت تأثیر این کنه‌ها از محصولات کشاورزی استان سانتاندر-کلمبیا جمع‌آوری شد. شناسایی کنه‌های مرتبط با خسارات مشاهده شده از طریق منابع تخصصی و مشاوره با آرایه‌شناسان های خبره این گروه انجام شد. پریکارپ‌ها بنابر شیوه‌نامه‌های استاندارد برای جاسازی و برش در پارافین پردازش شدند و مقاطع به دست آمده با سافرانین-آسیان بلو، فست بلو B، فلوروگلو سینول، لاکموئید و PAS-Amido Black رنگ‌آمیزی شدند. بخش‌های اضافی در رزین پردازش و با تولوئیدین بلو رنگ‌آمیزی شدند. برای مشاهده زیر میکروسکوپ اسکن الکترونی (SEM)، مواد در ۲،۲-دی متوکسی پروپان ثابت و خشک شد، سپس در نقطه بحرانی خشک و با طلا پوشانده شد. سطح میوه سالم صاف، به رنگ یکدست سبز روشن بود و روزنه داشت. اگرزکارپ از لایه‌های سلولی مختلفی تشکیل شده است؛ بیرونی‌ترین آن بافت اپیدرمی است که از یک لایه سلولی پوشانده شده با کوتیکولی ضخیم تشکیل شده است. لایه‌های باقیمانده از پارانیشیم فتوستتزی و ذخیره‌ای با دانه‌های نشاسته فراوان تشکیل شده است. میوه آسیب دیده توسط سه کنه زوال لایه‌های اگرزکارپی را نشان داد که با توجه به شدت حمله و گونه کنه آسیب‌زننده متفاوت است. به طور کلی، افزون بر ساختارهایی مانند لکه‌های سفید دایره‌ای در اثر *Schizotetranychus hindustanicus*، شکاف‌ها و یا ترک‌هایی روی سطح میوه وجود داشت. بافت‌های آسیب دیده از نظر پلی فنل و لیگنین مثبت بودند، اما برای کالوز منفی بودند. دیواره‌های اولیه برای PAS-Amidoblack مثبت بودند اما هیچ پروتئین یا گرانول نشاسته شناسایی نشد. به همین ترتیب، رنگ‌آمیزی با تولوئیدین بلو نشان‌دهنده وجود اجزای اصلی دیواره، لیگنین و پلی فنول‌ها بود. حمله کنه‌های گیاهخوار بیشتر لایه‌های اگرزکارپی را تحت تأثیر قرار می‌دهد. پاسخ هیستوپاتولوژیک گیاه به حمله این کنه‌ها در بین سه مورد مشابه است و منجر به تشکیل پریدرم، رسوبات لیگنین و پلی فنول‌ها در بافت‌های آسیب دیده می‌شود. شدت بیشتر آسیب بافتی در مورد حمله *Polyphagotarsonemus latus* مشاهده شد.

واژگان کلیدی: هیستوشیمی؛ هیستوپاتولوژی؛ میوه‌های لیمو؛ پریکارپ؛ کنه گیاهی.

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