Rodriguésia 75: e01352023. 2024
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202475012

Plant anatomy: history and future directions

Anatomy of stem lesions caused by Citrus leprosis

João Paulo Rodrigues Marques[1], *Elliot Watanabe Kitajima*[2] & *Beatriz Appezzato-da-Glória*[3,4]

Abstract

The citrus leprosis is responsible for causing major damage in fruit production. The disease, in the stems, is characterized by caused "scaly bark" lesions of reddish tone, depending on the stage they can be called blisters and make ruptures in the longitudinal direction. The aim of this study was to analyze the evolution of leprosis lesions of type CiLV-C and the anatomical alterations in the twigs of field-collected plants in order to compare to the evolution of the foliar and twigs lesions of inoculated plants already described in literature. For anatomical analysis, the samples were fixed in Karnovsky solution, dehydrated in a graded ethylic series, embedded in hidroxy-ethyl methacrylate resin (Leica Historesin), sectioned (5–7 μm thick), and stained with Toluidine blue for usual histological analysis. Histochemical tests were also carried out. The digital images were capture in a microscope with video camera. The analyzed lesion followed the same pattern as described to foliar lesions, *i.e.*, there was a necrotic center surrounded by a halo also necrotic. In the necrotic region there was the accumulation of lipid compounds. The cortical and, or phloematic parenchyma exhibited hyperplasia. In both lesions it was verified the decrease of starch amount and the increase of quantity of prismatic calcium oxalate crystals, and the presence of gummosis traumatic ducts.

Key words: *Citrus sinensis*, crystals, gummosis ducts, starch.

Resumo

A leprose dos citros é responsável por causar grandes danos à citricultura. Nos troncos e ramos a doença caracteriza-se por causar lesões corticosas de tonalidade avermelhada. Dependendo do estágio da doença, as lesões podem ser denominadas pústulas e apresentar fendas no sentido longitudinal. O objetivo desse estudo foi analisar a evolução das lesões da leprose do tipo CiLV – C e as alterações anatômicas nos ramos de plantas provenientes do campo a fim de compará-las com a evolução das lesões foliares e com as lesões de ramos de plantas inoculadas descritas na literatura. Para tal, amostras de lesões em diferentes estágios de desenvolvimento foram coletadas, fotografadas e fixadas em solução de Karnovsky. Posteriormente, as amostras foram submetidas à desidratação em série etílica e infiltradas em resina hidroxi-etil-metacrilato (Leica Historesin). As amostras foram seccionadas em micrótomo rotativo a 5–7 μm de espessura e coradas com azul de toluidina para as análises histológicas usuais. Também foram realizados testes histoquímicos. A captura de imagens digitais dos materiais preparados em lâminas foi realizada em microscópio equipado com câmera de vídeo. A evolução das lesões nos ramos seguiu o mesmo padrão descrito para as lesões foliares, ou seja, houve a formação de uma pontuação necrótica central e de um halo necrótico circundante. Na região da necrose havia o acúmulo de compostos lipídicos. O parênquima cortical e floemático apresentava hiperplasia. Em ambas as lesões houve a diminuição da quantidade de amido, aumento de cristais de oxalato de cálcio e a presença de ductos traumáticos gomosos.

Palavras-chave: *Citrus sinensis*, cristais, ductos gomosos, amido.

[1] University of São Paulo, Faculty of Animal Science and Food Engineering, Department of Basic Sciences, Pirassununga, SP, Brazil. ORCID: <https://orcid.org/0000-0001-5052-234X>.

[2] University of São Paulo, "Luiz de Queiroz" College of Agriculture, Department of Phytopathology and Nematology, Piracicaba, SP, Brazil. ORCID: <https://orcid.org/0000-0002-9138-2918>.

[3] University of São Paulo, "Luiz de Queiroz" College of Agriculture, Department of Biological Science, Piracicaba, SP, Brazil. ORCID: <https://orcid.org/0000-0003-2989-6931>.

[4] Author for correspondence: bagloria@usp.br

Introduction

Citrus leprosis is considered the most important virus-related disease in orchards in the state of São Paulo, Brazil (Bassanezi *et al*., 2019), causing damages to citrus plants, such as leaf and fruit drop, drying branch, and eventually plant death, reducing up to 100% of yield, depending on the citrus plant variety.

Citrus leprosis has a viral etiology with mite *Brevipalpus phoenicis* as the main disease vector in Brazil (Bastianel *et al*. 2006). Control of this mite requires prevention measures, which in turn increase production costs, otherwise the disease may cause annual losses of US$ 54 million in orchards in São Paulo state (Bassanezi *et al*., 2019).

Lesions caused by citrus leprosis affect the citrus fruits, leaves, and branches. The lesions are localized, generally ring-shaped, with necrotic regions and punctures alternated with chlorotic regions. Branch lesions are cortical, salient, greyish, brown or reddish and can coalesce, causing branch drying and death (Rossetti 2001). In branches, inoculated via viruliferous mites, lesions begin as a chlorotic projection which can evolve into a necrotic pustule where cracks are perceptible. The lesions may coalesce causing the branch to gird (Marques *et al*. 2007) and leading to subsequent branch death. Lesions in branches in the field start as small spots, slightly raised, due to gum formation in sub-epidermal tissues, rounded with a pale green yellow color (Bitancourt 1955).

Stems inoculated with the cytoplasmic leprosis virus (CiLV - C) showed changes in vascular tissues, mainly in the phloem and in the production of traumatic gum ducts (Marques *et al*. 2007). Gum production by traumatic ducts can be referred to as gummosis (Fahn 1988). Two other diseases that lead to gummosis have already been identified in *Citrus* sp., both caused by fungi, namely gummosis caused by *Phytophtora citrophthora* (Gedalovich & Fahn 1985) and gummosis caused by *Dothiorella gregaria* (Moraes *et al*. 2007). Both diseases cause irreversible damage to crop yield and plant longevity.

This study assessed the evolution of lesions caused by CiLV - C and the anatomical changes in branches from field material and compared them to the evolution of foliar lesions and to lesions of plants inoculated with the same viruses described by Marques *et al*. (2007).

Materials and Methods

Plant material

Branches of *Citrus sinensis* (L.) Osbeck ‘Pêra’ showing different symptoms of cytoplasmic leprosis (CiLV - C) were collected from the Citrus Collection of the Vegetal Production Department of the Luiz de Queiroz College of Agriculture (ESALQ/USP) in the municipality of Piracicaba, São Paulo state, Brazil.

Morphoanatomical analyses

Samples of branches with lesions in different stages and with healthy branches (control) were collected and the morphology of the lesions were analysed under a Leica stereo microscope M205 C and the images were captured with aDFC 295 video camera coupled to the stereo microscope. The branches were fixed in Karnovsky’s solution (Karnovsky 1965) and taken to a vacuum pump to remove the air from the tissues. Then, the samples were dehydrated in ethylic series and infiltrated in hydroxy-ethyl-methacrylate resin (Leica Historesin). The blocks were sectioned at 5–7 µm thickness (transversal and/or longitudinal sections) using a rotary microtome (Leica RM 2045) with a C-type steel razor. Subsequently, the sections were stained with 0.05% toluidine blue (Sakai 1973) for the usual histological analyses. Dyes and reagents were also used in sections prepared on slides. Sudan black B (Pearse 1968) was used to detect substances of a lipidic nature, ferric chloride (Johansen 1940) to verify the formation of phenolic compounds, iodized zinc chloride (Jensen 1962) to detect starch and ruthenium red (Chamberlain 1932) to detect pectic compounds. After staining, histological slides were mounted in Entellan synthetic resin and the images were captured with a Leica DC 300F video camera coupled to the Leica DMLD microscope. Observation under polarized light was also used to visualize calcium oxalate crystals and fibers. The micrometric scales were obtained under the same optical conditions.

Results

Morphological analyses

The lesions present an intermediate lesion, that is, with a central necrotic pit and a surrounding necrotic halo (Fig. 1a-e) and the presence of successive necrotic halos (Fig. 1e). However, in the following stages, branch lesions differed from foliar lesions, as the lesion surface was circular and necrotic (Fig. 1b-c), and slightly depressed. In a subsequent stage, the lesion becomes cortical with the presence of scales or pustules that stand out in relation to the stem axis (Fig. 1f-g), some pustules may have a circular morphology (Fig. 1f) or contain slits. Green branches presented lesions at the bases (Fig. 1h). In dry branches, the presence of lesions at the bases (Fig. 1i-j) indicates that the lesions could be correlated to the withering of the branches.

Figure 1 – a-j. CiLV - Citrus leprosis lesions of branches of *Citrus sinensis* 'Pêra' from the field – a, d-e. intermediate lesions. Note the necrotic halos (arrows) ; b-c. depressed necrotic lesions. The arrow in b indicates the necrotic halo of the intermediate lesion; f-g. cortical lesions. Lesions may have halos (arrow in f) or cracks (arrow in g); h. lesions at the base of green branches (arrows); i. lesions at the base of a dry branch (arrows); j. set of dry branches where leprosy lesions were visible at their bases. Scale bar = 1 mm.

Anatomical aspects

Changes occur in the epidermis, in the cortex, and in the vascular tissue (Figs. 2-3). In the vascular tissue, changes are possibly correlated to branch withering, as pectic compounds accumulate in intercellular spaces (Figs. 2f-g; 3g-h), and to the collapse of phloem cells (Fig. 3g-h).

Depressed necrotic lesions show similarities in terms of the anatomical structure when compared to intermediate lesions therefore only the anatomical characteristics of intermediate lesions are described here. Intermediate lesions presented a central necrotic region and two adjacent necrotic regions, which corresponded to the halo that surrounds the lesions (Figs. 1a; 2a). In the necrotic region, epidermal and cortical cells accumulated compounds of a lipid nature (Fig. 2e). The cortical cells divided into a periclinal direction (Fig. 2b) both in the region of central necrosis and in the region of halo necrosis (Fig. 2a). Hyperplasia promoted by the cortical cells adjacent to the necrosis caused the rupture of pressured necrotic tissues and formation of cracks (Fig. 2d).

As for cortical lesions, the regions still covered by the epidermis began to accumulate compounds of a lipid nature. The cortical and phloem parenchyma showed intense cell division in the anti- and periclinal direction (Fig. 3a,d) in the entire stem length, since all stem circumference is injured (Fig. 3e-f), pressuring necrotic tissues above with the formation of elevations (Fig. 3f) and, posteriorly, of cracks (Fig. 2d).

Traumatic gum ducts were observed in lesions in the field (Fig. 4a-e) is in fact induced by the leprosy virus and not by its interaction with other viruses, as initially questioned. The mother cells of the xylem parenchyma originated traumatic gum ducts, which could be observed between the cells of the secondary xylem (Fig. 4a-b) as growth progressed. The longitudinal section showed that the axial parenchyma cells surrounded the already formed ducts (Fig. 4b) with a pectic content.

Discussion

Stages in lesion evolution of the branches were similar to those in the leaves, as observed by Appezzato-da-Glória *et al*. (2006), regarding the presence of the intermediate lesion, that is, with a central necrotic pit and a surrounding necrotic halo. This type of lesion was frequently observed in older branches and this stage of the lesion is called "scaly bark", according to Knorr & Price (1958). Sometimes distinguishing lesions on severely infected branches becomes impossible to due to coalescence. In dry branches, the presence of lesions at the bases indicates that the lesions could be correlated to the withering of the branches (Bitancourt 1955).

Lesions of plant branches collected in the field follow the same pattern described by Marques *et al*. (2007) for branches inoculated with CiLV - C. Symptoms already found in inoculated branches by Marques *et al*. (2007). Virus translocation from lesioned regions to non-lesioned regions is also compromised, as discussed by Marques *et al*. (2007), virus movements over long distances occur according to the translocation of photoassimilates (Roberts *et al*. 1997).

The formation of cracks in different lesions of branches from the field, as well as in inoculated branches (Marques *et al*. 2007), resulted from the lysis and separation of necrotic cells due to the pressure exerted by the increase in the number of cell layers in the underlying cortex. This increase resulted from hypertrophy and hyperplasia of cortical cells in the lesion area and not from gum accumulation in the subepidermal tissues, as described by Bitancourt (1955) in materials collected in the field. Necrotic cells in the cortex showed only compounds of a lipid nature, as tests for phenols and pectic substances were negative.

However, a healing meristem formed from cortical or phloem cells was observed under necrotic cortical cells and following the entire crack length (Figs. 2h; 3f). In cortical lesions (Fig. 3f), the replaced tissues (epidermis and cortex) detached from the branches constitute the 'scales' mentioned by Knorr & Price (1958). These 'scales' were composed of cells that accumulated compounds of a lipid nature (data not shown). Compounds of a phenolic nature accumulated in the layer of cells of the healing meristem in contact with the environment (Fig. 3e). The healing meristem, also observed in branches inoculated by Marques *et al*. (2007), is normally formed in lesioned areas and may play a protective role, regardless of the cause of the lesion, as discussed by Melo-de-Pinna *et al*. (2002). The authors also described the formation of the healing meristem and the increase in the content of phenolic substances in leaf-mining cells of *Richterago riparia* caused by a lepidopteran larva. The deposition of phenolic compounds in cell walls are common responses in different species and pathosystems and may restrain the colonization process or prevent eventual abiotic damages (Pratyusha 2022; Marques *et al.* 2018, 2020).

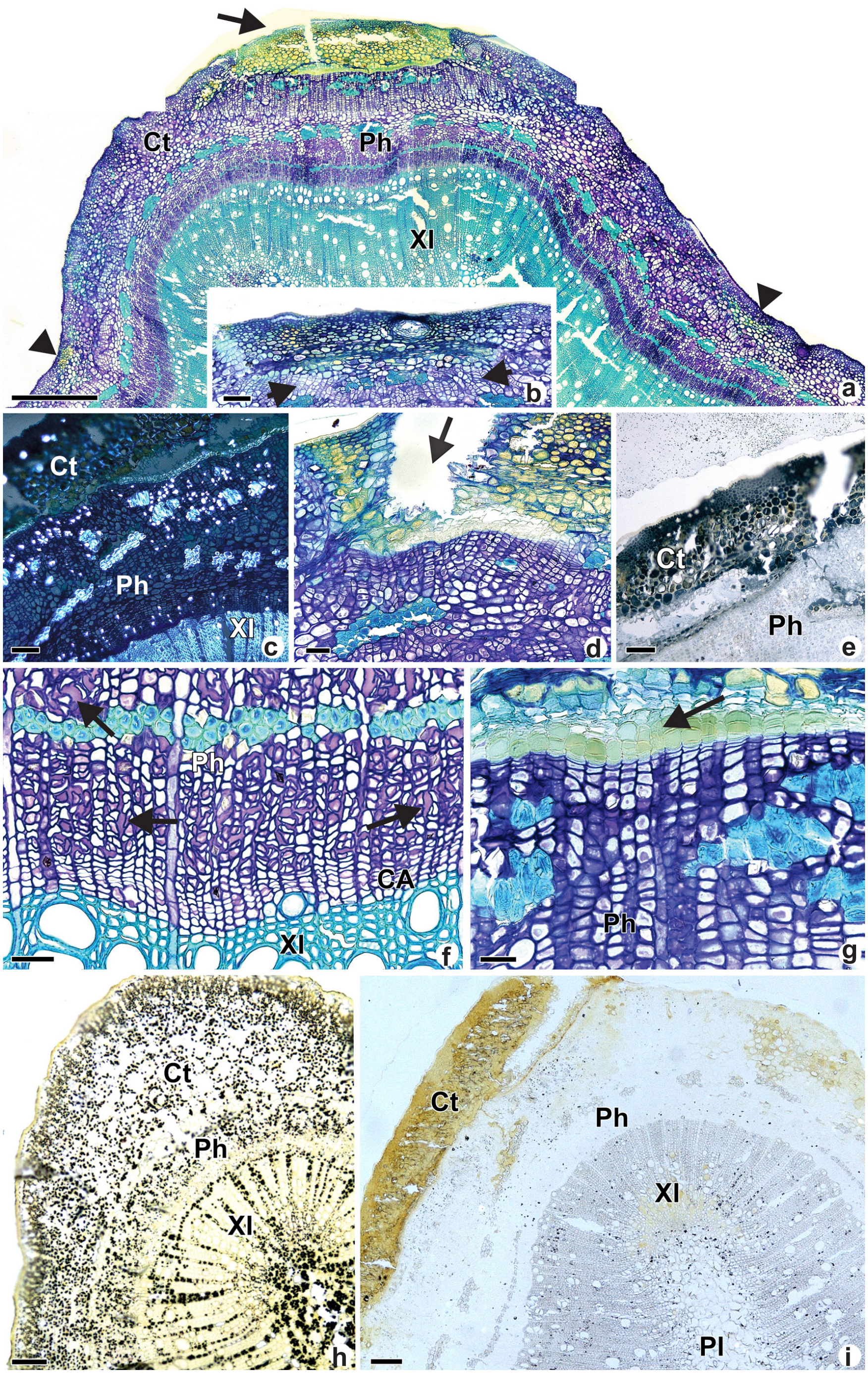

Figure 2 – a-i. Cross-sections of intermediate lesions caused by CiLV-Citrus leprosis in branches of *Citrus sinensis* 'Pêra' – a. central region of the lesion (arrow) and the necrotic halo (arrowheads) (bar = 200 μm); b. detail of the necrotic halo (bar = 50 μm); c. calcium oxalate crystals in the cortex and phloem observed under polarized light. (bar = 30 μm); d. crack on stem surface resulting from cortical cell hyperplasia (bar = 30 μm); e. necrotic region stained with Sudan Black B (bar = 30 μm); f. secondary phloem showing accumulation of pectic compounds in intercellular spaces (arrows) (bar = 30 μm); g. region of phloem hyperplasia and healing meristem formation (arrow) (bar = 30 μm); h-i. test with iodized zinc chloride. Note the large amount of starch (blackened dots) in healthy tissues (h) in relation to injured tissues (i) (bars = 100 μm). (a-b, d, f-g. toluidine blue staining; c. polarized light; e. Sudan Black B test; h-i. iodized zinc chloride test. Ct = cortex; Ca = vascular cambium; Ep = epidermis; PI = pith; Ph = phloem; Xl = xylem).

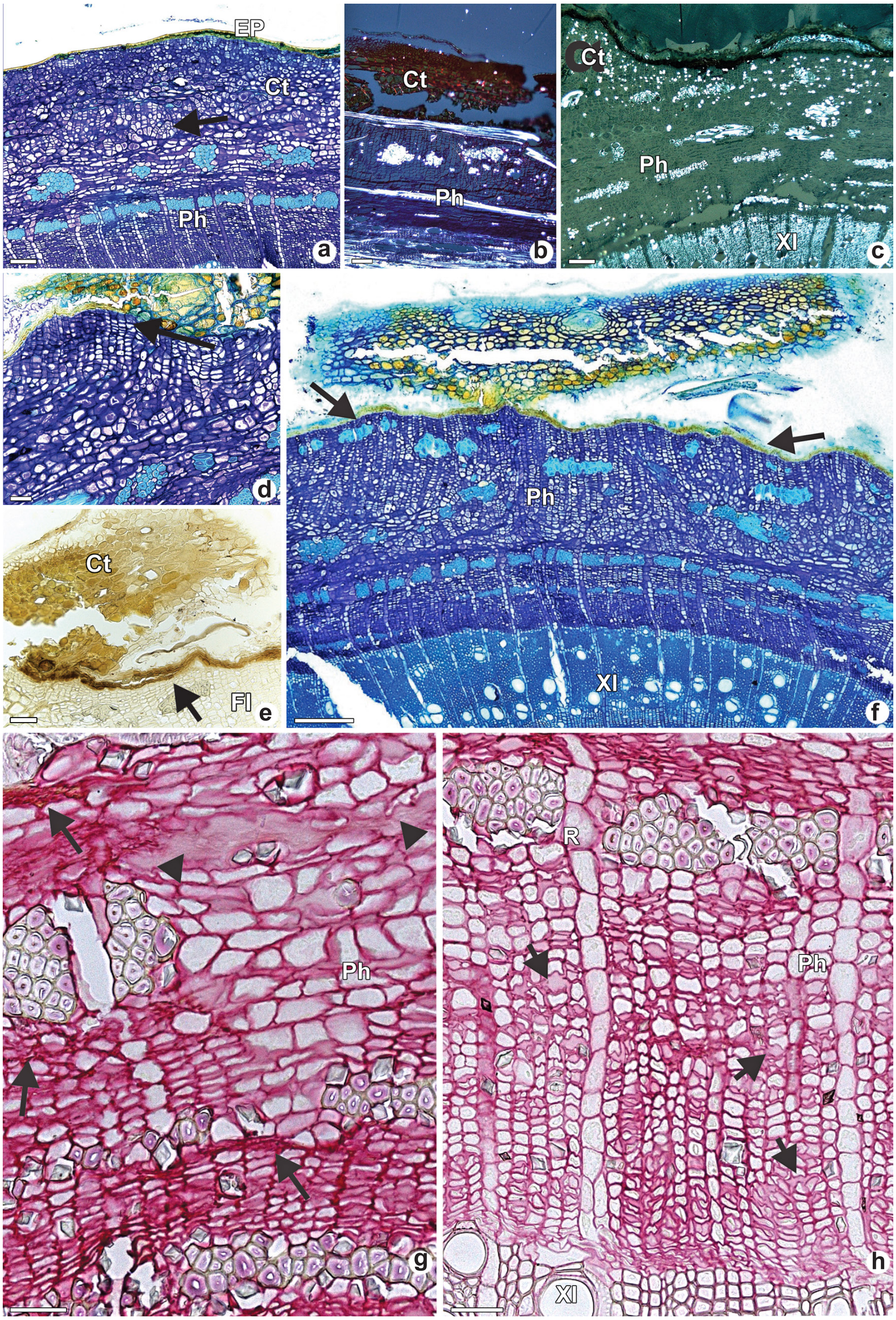

Figure 3 – a-h. Cross-sections of cortical lesions caused by CiLV-C leprosis in branches of *Citrus sinensis* 'Pêra' – a. hyperplasia in the cortical tissue (arrows) (bar = 50 µm); b-c. calcium oxalate crystals observed under polarized light (bars = 50 µm); d. cell divisions in the cortex below the necrotic cells (arrow) (bar = 30 µm); e-f. detachment of the cortex under which the healing meristem is located (arrows) (bars = 30 µm); g-h. pectic compounds in the intercellular spaces of the phloem (arrowheads in g and arrows in h) and presence of collapsed cells (arrows in g) (bars = 10 µm). (a,d,f. toluidine blue staining; b-c. polarized light; e. ferric chloride test; g-h. ruthenium red test. Ct = cortex; Ca = vascular cambium; Ep = epidermis; Ph = phloem; R = vascular radius; Xl = xylem).

Traumatic gum ducts in branches inoculated by CiLV - C (Marques *et al*. 2007) indicate that their formation in lesions in the field. However, it was not verified the lysis of hypertrophied cells in the initial stages of formation of ducts in inoculated branches, which characterizes their lysigenous origin (Marques *et al*. 2007). The gum ducts caused by *Phytophora critricarpa* in *Citrus* sp. (Gedalovich & Fanh 1985) and the ducts formed in Rosaceae (Saniewski *et al*. 2006) result from the dissolution of the middle lamella and consequent formation of a schizogenous cavity. Pectic responses has been reported as a important component of plant resistance response in different

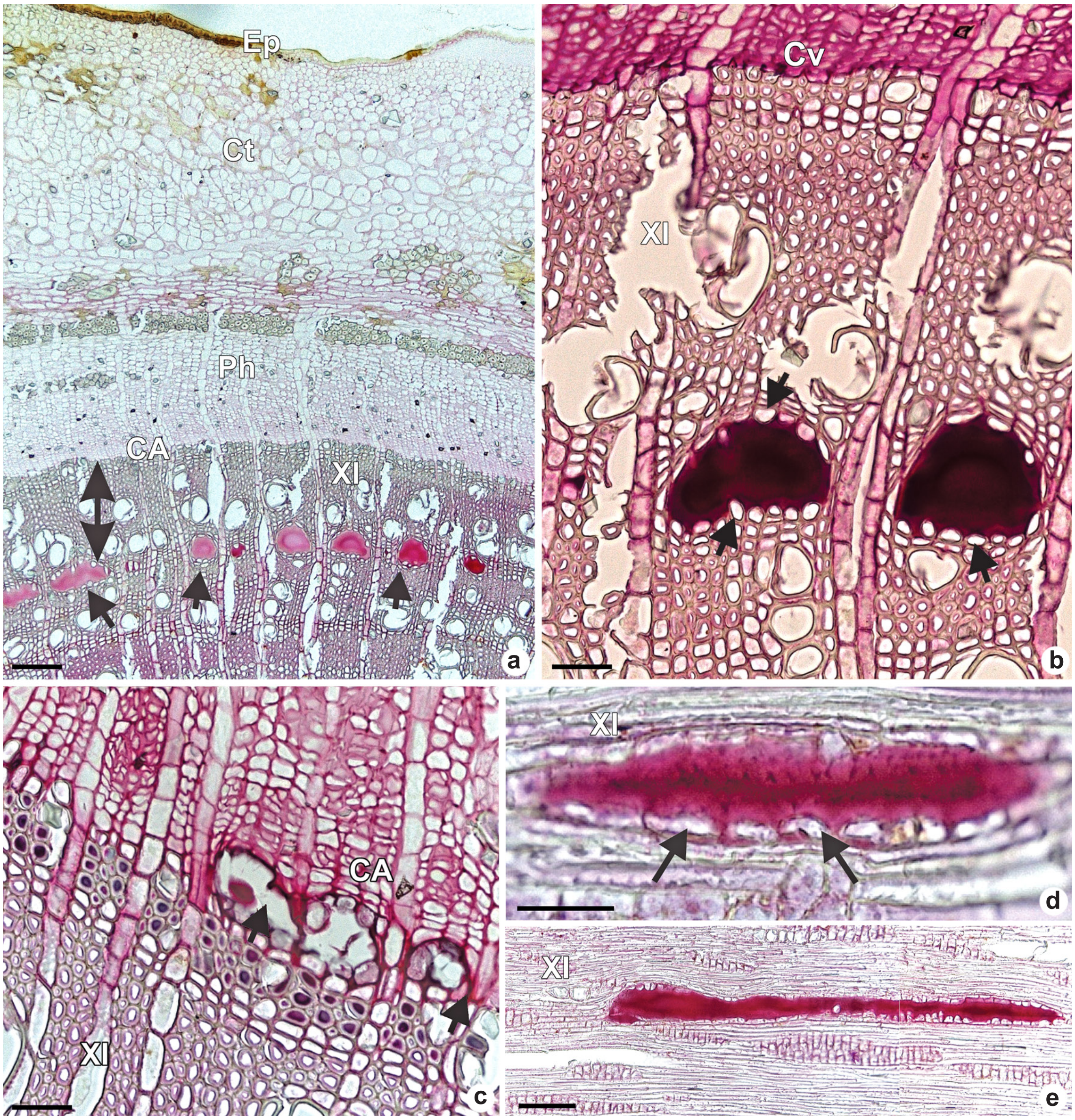

Figure 4 – a-e. Cross-sectional (a-c) and longitudinal (d-e) sections of traumatic gum ducts in lesions caused by CiLV-C leprosis in branches of *Citrus sinensis* 'Pêra' stained with Ruthenium Red – a. ducts in the xylem (arrows), note the distance from the vascular cambium (key) (bar = 50 µm); b. detail of ducts shown in a (bar = 30 µm); c. ducts near the vascular cambium (arrow) (bar = 30 µm); d. epithelium (arrows) of the duct in formation with a pectic content (bar = 10 µm); e. duct inside the xylem (bar = 50 µm). (Ct = cortex; Ca = cambium; Ep = epidermis; Ph = phloem; Xl = Xylem).

pathossystems (Wang *et al.* 2022, 2023; Marques & Nuevo 2022), demonstrating that the cells wall-mediated responses are important in order to restrain possible systemic infection of CiLV - C.

The stem analyzed under polarized light revealed a large accumulation of prismatic crystals in the tissues in the region of the lesion compared to healthy tissues (Figs. 2c; 3b-c). Marques *et al*. (2007b) reported this accumulation in branches inoculated with CiLV - C. Ceita *et al*. (2007) verified an increase of intracellular prismatic calcium oxalate crystals in tissues of *Theobroma cacao* infected by *Moniliophthora perniciosa* fungus that causes the witches' broom disease. The authors suggest that calcium oxalate degradation may be correlated to cell apoptosis, as hydrogen peroxide is produced in the oxalate degradation reaction, a beneficial molecule for plants by neutralizing the strategy of some pathogens.

Another characteristic observed in the field material and in inoculated branches (Marques *et al*. 2007b) was the change in the position of xylem fibers in the region where the ducts are present. Tests for starch presence were performed in all types of lesions, with a negative result observed in the presence of this polysaccharide in lesioned tissues, differing from observations in non-injured tissues. According to Fahn (1988), starch is the raw material for gum synthesis (polysaccharides) by the traumatic ducts. However, the hypothesis that degraded starch provides energy and carbonic skeleton to intense hyperplasia in lesioned stem tissues cannot be disregard.

Acknowledgements

This research had financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2006/5492-6; 2019/25078-9).

Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

References

Appezzato-da-Gloria B, Marques JPR, Astua JF & Kitajima EW (2006) Análise morfológica da evolução das lesões lepróticas foliares de *Citrus sinensis* (L.) Osbeck 'Pêra'. *In*: Congresso Nacional de Botânica 57, 2006. Gramado. Anais... Sociedade Brasileira de Botânica, São Paulo. 1 CD-ROM .

Bassanezi RB, Czermainski ABC, Laranjeira FF, Moreira AS, Ribeiro Júnior PJ, Krainski ET & Amorim L (2019) Spatial patterns of the citrus leprosis virus and its associated mite vector in systems without intervention. Plant Pathology 68: 85-93.

Bastianel M, Freitas-Astua J, Kitajima EW & Machado MA (2006) The citrus leprosis pathosystem. Vol. 32, n. 3. Summa Phytopathologica, Botucatu. Pp. 211-220.

Bitancourt AA (1955) Estudos sobre a leprose dos *Citrus*. I. Distribuição geográfica e sintomatologia. Vol. 22. Arquivos do Instituto Biológico, São Paulo. Pp. 161-184.

Ceita GO, Macedo JNA, Santos TB, Alemanno L, Silva Gesteira A, Micheli F, Mariano AC, Gramacho KP, Costa Silva D, Meinhardt LW, Mazzafera P, Pereira GAG & Cascardo JCM (2007) Involvement of calcium oxalate degradation during programmed cell death in *Theobroma cacao* tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. Vol. 173, n. 2. Plant Science, Shannon. Pp. 106-117.

Chamberlain CJ (1932) Methods in plant histology. The University of Chicago Press, Chicago. 416p.

Fahn A (1988) Secretory tissues in vascular plants. Tansley review nº 14. Vol. 108. New Phytologist, Lancaster. Pp. 229-257.

Gedalovich E & Fahn A (1985) The development and ultrastructure of gum ducts in Citrus plants formed as a result of brown-rot gummosis. Vol. 127. Protoplasma, New York. Pp. 73-81.

Jensen WA (1962) Botanical histochemistry. WH Freeman, San Francisco. 408p.

Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York. 523p.

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Vol. 27. Journal of Cell Biology, New York. Pp.137-138.

Knorr LC & Price WC (1958) Leprosis. *In*: Pratt R (ed.) Citrus insects, diseases and nutritional disorders. Agricultural Experiment Station, Gainesville. 191p.

Marques JPR, Kitajima EW, Freitas-Astua J & Appezzato-da-Glória B (2007) Lesões foliares e de ramos de laranjeira-doce causadas pela leprose-dos-citros. Vol. 42, n. 11. Pesquisa Agropecuária Brasileira, Brasília. Pp. 1531-1536.

Marques JPR, Spósito MB, Amorim L, Montanha GS, Silva-Junior GJ, Carvalho HWP & Appezzato-da-Glória B (2020) Persistent calyxes in postbloom fruit drop: a microscopy and microanalysis perspective. Pathogens 9: 251. DOI: 10.3390/pathogens9040251

Marques JPR, Hoy JW, Appezzato-da-Glória B, Viveros AFG, Vieira MLC & Baisakh N (2018) Sugarcane cell wall-associated defense responses to infection by *Sporisorium scitamineum*. Frontiers in Plant Science 9: 698. DOI: 10.3389/fpls.2018.00698

Marques JPR & Nuevo LG (2022) Double-staining method to detect pectin in plant-fungus interaction.

Journal of Visualized Experiments 180: e63432. DOI: 10.3791/63432

Melo-De-Pinna GFA, Kraus JE & Menezes NL (2002) Morphology and anatomy of leaf mine in *Richterago riparia* Roque (Asteraceae) in the campos rupestres of Serra do Cipó, Brazil. Vol. 62, n. 1. Brazilian Journal of Biology, São Carlos. Pp. 179-185.

Moraes WS, Castro HA, Lima JD, Leite EAG & Souza M (2007) Susceptibilidade de três espécies cítricas à *Dothiorella gregaria* Sacc. em função do estado nutricional. Vol. 37, n. 1. Ciência Rural, Santa Maria. Pp. 7-12.

Pearse AGE (1968) Histochemistry, theoretical and applied. 3rd ed. Vol. 1. Churchill, London. 998p.

Pratyusha S (2022) Phenolic compounds in the plant development and defense: an overview. *In*: Hasanuzzaman M & Nahar K (eds.) Plant stress physiology - perspectives in agriculture. IntechOpen, Rijeka. Pp. 1-17. DOI: 10.5772/intechopen.102873

Roberts AG, Santa Cruz S, Roberts IM, Prior DAM, Turgeon R & Oparka KJ (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. Vol. 9. Plant Cell, Watesbury. Pp. 1381-1396.

Rossetti V (2001) Manual ilustrado de doenças dos citros: doenças de causas desconhecidas. FEALQ, FUNDECITRUS, Piracicaba. 270p.

Sakai WS (1973) Simple method for differential staining of paraffin embedded plant material using toluidine blue O. Vol. 48, n. 5. Stain Technology, Baltimore. Pp. 247-249.

Saniewski M, Ueda J, Miyamoto K, Hosbowing M & Puchalski J (2006) Hormonal control of gummosis in Rosaceae. Journal of Fruit and Ornamental Plant Research, Skierniewice 14: 137-144.

Wang D, Kanyuka K & Papp-Rupar M (2023) Pectin: a critical component in cell-wall-mediated immunity. Trends in Plant Science 28: 10-13. DOI: 10.1016/j.tplants.2022.09.003

Wang D, Yang L, Silva CJ, Sarwar R, Chen Y & Blanco-Ulate B (2022) Pectin-mediated plant immunity. Annual Plant Reviews 5: 55-80. DOI: 10.1002/9781119312994.apr0798

Area Editor: Dr. João Paulo Basso-Alves

Received on September 25, 2023. Accepted on February 02, 2024.