Microsc. Microanal. Res. 2023 36(1) 17-22

Microscopy and Microanalysis Research
- The Journal of The Microscopy Society of Thailand

Journal Home Page: https://www.tci-thaijo.org/index.php/mmres/

Microsc. Microanal. Res.

Morphological Observation of Lime (*Citrus aurantifolia* (Christm.) Swingle) Fruit with Disease Symptoms by Microscopic Techniques

Umroong P.[1], Sangsawang J.[2*] and Chaemsai S.[1]

[1] Scientific Equipment and Research Division, Kasetsart University Research and Development Institute, Kasetsart University, Bangkok, 10900, Thailand
[2] Agricultural Research and Technology Transfer Center, Faculty of Agriculture, Kasetsart University, Bangkok, 10900, Thailand.
*Corresponding author's e-mail address: : rdipru@ku.ac.th, pangjung@yahoo.com

ARTICLE INFO

Article history
Submitted: 21 May 2023
Revised: 28 December 2023
Accepted: 29 December 2023
Available online: 31 December 2023

Keywords:
Microscopy; Transmission Electron Microscopy; Citrus canker; Lime *(Citrus aurantifolia* (Christm.) Swingle) fruit; Ultrastructure

ABSTRACT

This research aimed to morphological and ultrastructure studies of Lime peels (*Citrus aurantifolia* (Christm.) Swingle) in citrus diseases. Therefore, the application of microscopic techniques to examine the infestation of pathogens in plants in order to gain further insights into the deterioration of cells at the cellular and subcellular level in plants showing disease symptoms are currently commonly used to characterize their morphology and ultrastructure. In this study the morphology and cellular structure were explored through cell cross section studied with compound microscopy (OM). Cellular ultrastructure was examined under transmission electron microscope (TEM). In lime showed canker symptoms cellular degeneration which has been clearly morphological demonstrated within the cell organelles of chloroplasts enlarges in area of thylakoid messy and mitochondria have vacuoles were found.

INTRODUCTION

Lime (*C. aurantifolia* (Christm.) Swingle) fruit or 'Ma-Now-Pan' in Thai, is one of the most popular citrus fruits and widely grown crop in the world. Lime is rich vitamin C, mineral and nutrients fruit. Lime juice which contain citric acid is commonly used as a food and beverage ingredient in both commercial and household. In traditional medicine, lime was a source of many chemical composition such as essential oils, flavonoids, alkaloids, coumarins, tannins, cardiac glycoside, phytosterols, phenols, caffeine, pectin and minerals. Phytochemicals in lime fruit have a large spectrum of benefits including antibacterial, antifungal, antidiabetic, anticancer, antiviral, weight loss, skin care, good digestion, relief from constipation, eye care and treatment of many diseases such as scurvy, piles, peptic ulcer, respiratory disorders, gout, gums, urinary disorders, etc. [2,12,15,20]

Lime tree is evergreen large shrub in genus *Citrus*, Rutaceae family is native to Asia. Lime flower is small, white or creamish-white that borne in axillary cymes. Leaves, sepals, and petals composed of numerous oil glands (monoterpenes with limonene) giving pleasant smell. Dark greenish leaves are arranged alternately on the stem. Lime fruit is oval shape with smooth porous skin, greenish yellow to bright yellow color. Fruit of lime is hesperidium type, exocarp or flavedo with color range from yellowish-orange to green was called 'flavedo', rich of oil glands along the surface. Mesocarp or albedo is spongy-white tissue cover the inner area which composed of small sac with in any segments. Most segment contain a few seeds fixed around the central placenta of the fruit (Figure 1). [16,18]

One type of an economic important disease in lime is canker. Citrus canker is an infection of citrus bacterial canker (CBC) including *Pseudomonas citri* or *Xanthomonas citri* which highly present in Asia, Africa and America. The symptoms of the disease occur in many parts of plant including leaves, old branches and fruits. In lime, it causes

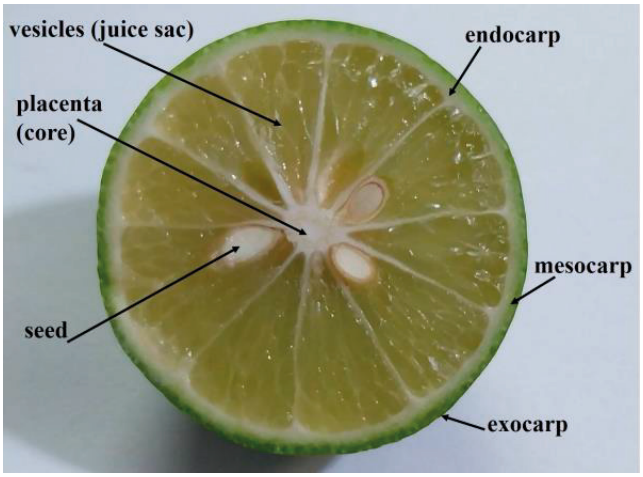

Figure 1. Transverse anatomy of lime fruit.

premature leaf and fruit fall. Citrus canker on fruit surface start with small spot and expand to be dark tan or black with the progression of disease, the margin of lesions is water soaked (Figure 2). After an infection Citrus canker start appearing in 15-20 days. The spread of citrus canker can occur through water, wind and human/bird/insect-assisted movement. [6,10, 11]

METHODOLOGY

Plant material

Lime (*Citrus aurantifolia* (Christm.) Swingle) fruit were collected from the private garden in Bangkok, Thailand (Figure 2). The pericarp was examined by a compound microscope and a transmission electron microscope.

Ultrastructure under compound microscopy

Lime peels normal and showing canker were cut into small pieces 1×3 mm^2. and primarily fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 for overnight at 4 ˚C in refrigerator. After that, the samples were washed 3 times (15 min per time) in the same buffer. After washing, the specimens were secondarily fixed in 2 % osmium tetroxide in distilled water for 2 hours at room temperature and then washed with distilled water 3 times (15 min per time). They were dehydrated in acetone series for about 20 min per series adapted from [16]. The samples were transferred to 2:1,1:1, and 1:2 of acetone and Spurr's resin repectively (4 hours per time). After that, were transferred to pure Spurr's low viscosity epoxy resin (6 hours per time). Then, the specimens were embedded in Spurr's low viscosity epoxy resin. To polymerize plastic, the samples were incubated using a vacuum oven at 80 °C for 7 hours, adapted from [1,3,4,18]. Then, the samples were cut semi-thin section (1,000 nm.) by using an ultramicrotome EM UC7 (Leica, Austria) and mounted onto a glass slide. Semi-thin section was stained in Toluidine blue 1% in borax at 85 ˚C for 2 minutes. After that, they were washed with distilled water then stained in Basic Fuchsin 1% in 50% alcohol at room temperature for 2 minutes, was washed with distilled water then dried at 85 ˚C for 30 minutes, and closed with a cover slide [17]. Measure the length and width of the exocarp and mesocarp cell and measure thickness cell wall mesocarp layer from 50 positions and ranges, the average is reported. The specimens were examined under compound microscope (AxioScope5, Carl Zeiss, Germany), which was equipped with a photographic camera under normal bright-field imaging.

Figure 2. Lime peel with sign of Citrus canker symptoms.

Ultrastructure under transmission electron microscopy

For transmission electron microscopy, the sample preparation steps to the polymerize plastic mold implantation process were identical with preparation of observed by compound microscope but differed at the cutting process. The samples were sectioned with an ultrathin section 70 nm., with a diamond knife, and mounted onto a copper grides, then were stained with lead citrate and 5% uranyl acetate [5,22]. Ultrathin section samples were observed on a transmission electron microscope (Hitachi; HT7700) with a high voltage of electron. at 80keV.

Statistical Analysis

The cell morphological data of normal and Citrus canker symptoms lime peels were also analyzed with One-way ANOVAs, when P-value ≤ 0.01. Mean values were compared with Tukey's Post Hoc multiple comparisons using SPSS vers. 26 (SPSS Inc, Chicago).

RESULTS AND DISCUSSION

observed under compound microscope and transmission electron microscope showed that the peels.

The lime peels anatomy of is composed of cuticle layer, exocarp layer, mesocarp layer, endocarp layer, juices sac and seed. The cuticle covered on exocarp layer (Figure 1,3). The fruit of lime is type hesperidium. The transverse results of normal and canker symptoms tissues examined by compound microscopy showed that morphology and cellular structure were explored through cell cross section (Figure 3A and B).

The measurement of exocarp cells showed that normal lime peels it was found that the size the mean length in the range of the exocarp cells was 2.89-17.32 µm, the mean width in the range of the exocarp cells was 4.40-12.60 µm. As for the results of studies of exocarp cells showed that canker symptoms lime peels it was found that the size the mean length in the range of the exocarp cells was 4.58-15.67 µm, the mean width in the range of the exocarp cells was 7.47-14.31 µm, (Table 1). The results of the measurements the size of cell exocarp of canker symptoms showed exocarp length more than normal and width less than normal due to cell degeneration. For exocarp layer, cell size was slightly changed. Cell width of the canker infected cell larger than the normal cell, while non significantly change in cell length. Typically, the exocarp layer consists of a cuticle and a layer of epidermal cells. Epidermal cells play an important role in interaction with the environment [21,13]. Citrus canker causes the epidermis disruption cuticle coating was destroyed by the bacteria, the area of spot thickened cuticle forms until the spot was degraded [23,18]. After the deterioration of tissues at the lesion area, water precipitation from atmosphere easily enter to intercellular space of epidermis. By hypotonic condition water was high potential to osmosis into the cell, cell size may be expanded by the turgid pressure [14].

The measurement of mesocarp cells showed that normal lime peels it was found that the size the mean length in the range of the mesocarp cells was 10.32-35.32 µm, the mean width in the range of the mesocarp cells was 8.46-31.18 µm. As for the results of studies of mesocarp cells showed that canker symptoms lime peels it was found that the size the mean length in the range of the exocarp cells was 10.42-46.83 µm, the mean width in the range of the mesocarp cells was 77.82-18.64 µm, (Table 1). The results of the mesocarp cells size

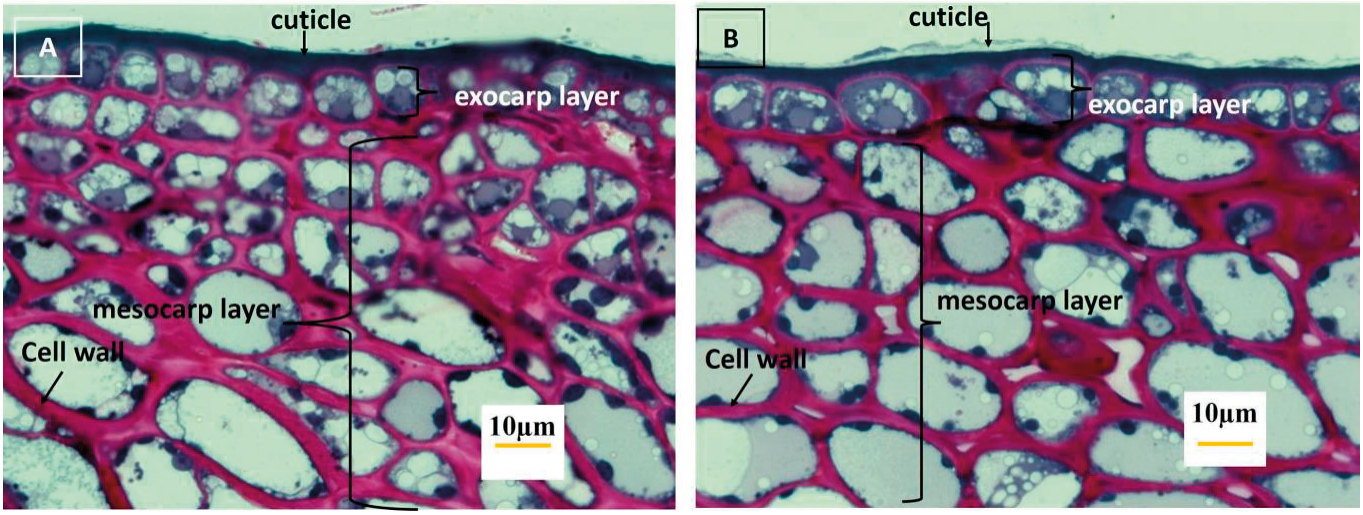

Figure 3. Anatomical characters observed under light microscope showed the exocarp and mesocarp layers of A. normal lime peel and B. canker infected lime peel. A: The anatomical structure of the normal lime peel (100X). B: The anatomical structure of the canker infected lime peel (100X).

Table 1. Comparison of exocarp and mesocarp cells size between normal and canker symptoms lime peels.

		Comparison of exocarp and mesocarp cells			
		Normal		Canker infected	
		Range	Average	Range	Average
Exocarp	length (µm)	2.89-17.32	9.79±3.22	4.58-15.67	10.21±2.31
	Wide (µm)	4.40-12.60	8.92±1.82a	7.47-14.31	10.79±1.39b
Mesocarp	length (µm)	10.32-35.32	19.73±6.23	10.42-46.83	22.79±6.42
	Wide (µm)	8.46-31.18	16.95±5.55a	7.82-18.64	13.59±2.49b

Remark: N=50, P ≥ 0.01

measurement showed that the canker symptoms lime peels had length more than normal lime peels but had width less than normal lime peels. This clearly shows that when canker symptoms are present, the cell enlarges due to cell degeneration. Which, show the deterioration of cell in the cytoplasm degeneration too (Table 1) (Figure 1,4-6) [17,25]. Non-infected mesocarp cell was long-flat shape, while canker bacterial infected cell was elliptic shape. After canker infected, mesocarp cell changed by vertical expansion and horizontal contraction (Table 1 and Figure 3). Water in apoplast way resulting in the mechanical properties of the cell walls and middle lamella [19,24]. By the water entering pass through the canker lesion into mesocarp layer, middle lamella expanded (Figure 6). The expansion of middle lamella in canker infected tissue resulting cell size changed.

The results of the cell wall of mesocarp cells thickness measurement showed that the normal lime peels had thickness in the range of 0.9-3.69 µm and the thickness of canker symptoms lime peels in the range of 1.7-14.6 µm. This clearly shows that when canker symptoms are present, the cell wall enlarges due to cell wall degeneration in the middle lamella and cell in the cytoplasm too (Table 2) (Figure 1,4-6) [17, 20, 23].

CONCLUSION

In conclusion, microscopy and electron microscopy techniques were able to study the microstructure changes of lime peels as a result of cell degeneration. The results of the ultrastructure showed that canker infected were the cell enlarges due to cell degeneration. The cellular degeneration which has been clearly morphological demonstrated that within the cell organelles of chloroplasts enlarges in area of thylakoid messy and mitochondria had vacuoles (Figure 6). However, response of tissues to determine the relative magnitude of the contributions of turgor pressure, cell wall, and middle lamella.

ACKNOWLEDGEMENT

The authors wish to thank the Kasetsart University Research and Development Institute, Bangkok, Thailand, for the financial support of this project.

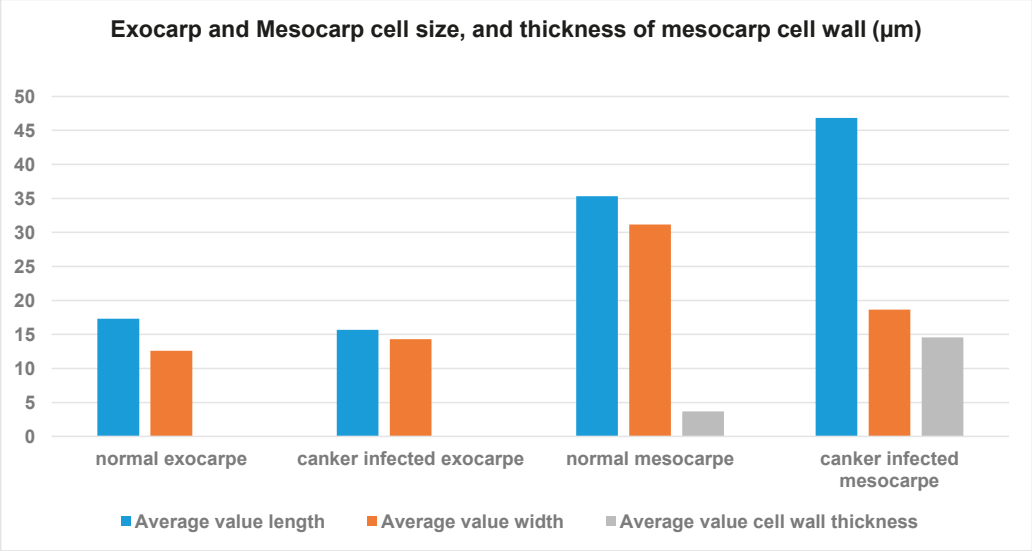

Figure 4. Comparison of exocarp and mesocarp cells size, mesocarp cell wall thickness between normal and canker infected lime peels.

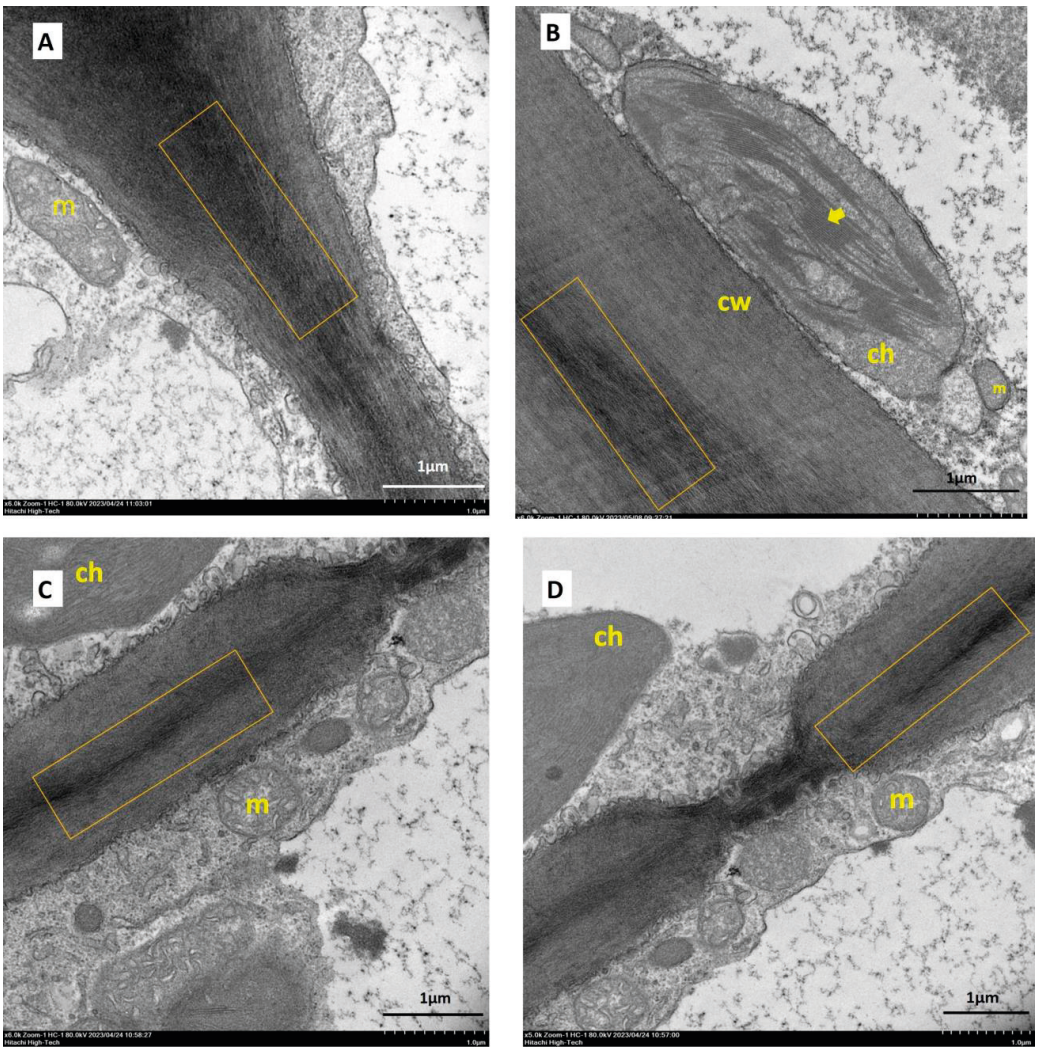

Figure 5. Transmission electron microscopy micrograph of the normal lime peels showing the cell wall structure and organelle inside the cells. (m = mitochondria, ch = chloroplasts,) A. Middle lamella (rectangle), mitochondria (magnification 6,000x) Scale bar, 1 µm. B. Middle lamella (rectangle), mitochondria, chloroplasts, cell wall and thylakoid (yellow arrow) (magnification 6,000x) Scale bar, 1 µm. C. Middle lamella (rectangle), mitochondria and chloroplasts (magnification 6,000x) Scale bar, 1 µm. D. Middle lamella (rectangle), mitochondria and chloroplasts (magnification 5,000x) Scale bar, 1 µm

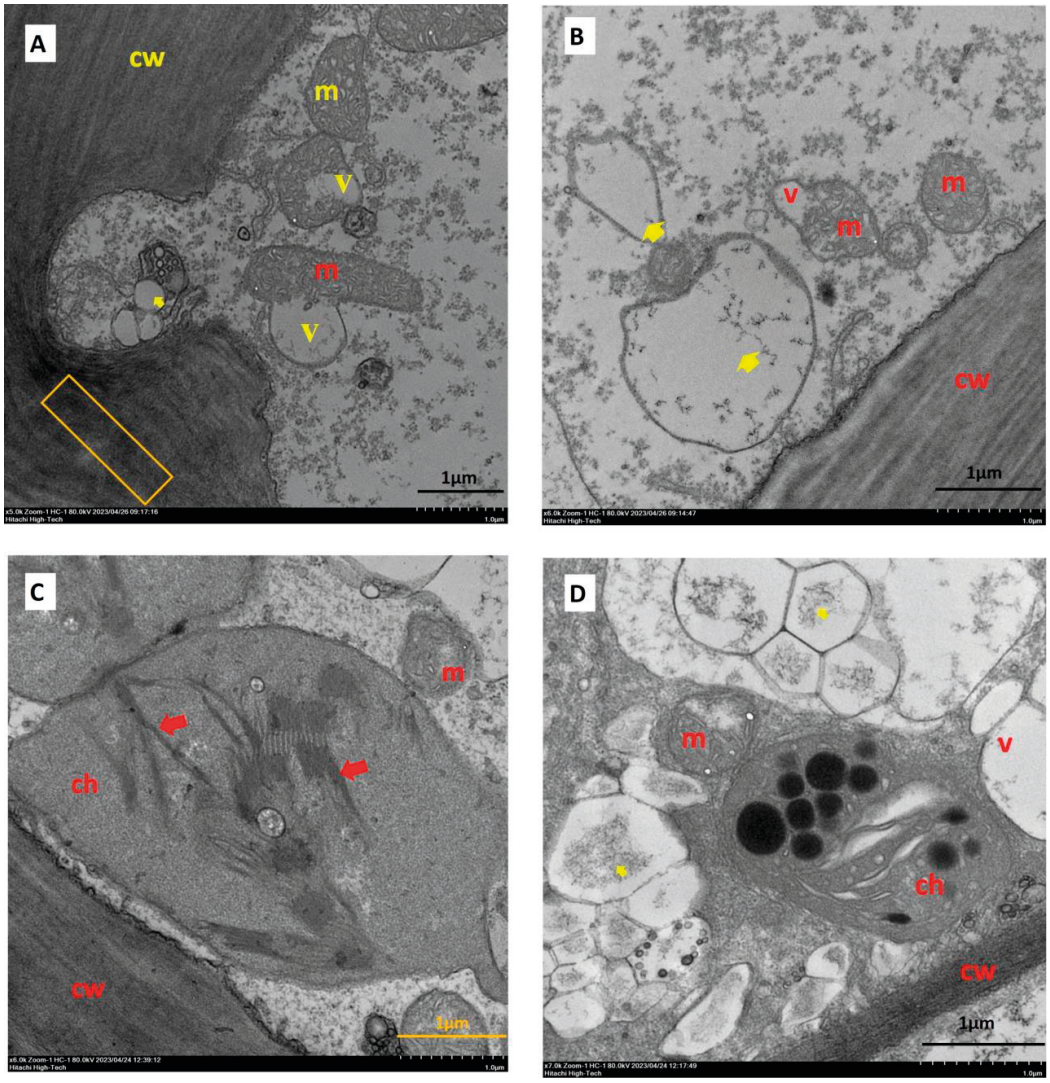

Figure 6. Transmission electron microscopy micrograph of the canker infected lime peels showing the characteristics cell wall organelles deterioration inside the cells in cytoplasm. (m = mitochondria, ch = chloroplasts, v = vacuoles) A. The cells wall deteriorate middle lamella (rectangle), mitochondria had enlarged vacuoles, (yellow arrow) autophagosome-like vesicles (magnification 5,000x) Scale bar, 1 µm. B. The cells wall deteriorate had space inserted in cell, mitochondria, autophagosome-like vesicles (yellow arrow) (magnification 6,000x) Scale bar, 1 µm. C. The cells wall deteriorate has space inserted in cell, mitochondria, chloroplasts and large in area of collapsed thylakoids messy (red arrow) (magnification 6,000x) Scale bar, 1 µm. D. The cells wall deteriorate, autophagosome-like vesicles (yellow arrows), vacuoles, chloroplasts enlarge (magnification 7,000x) Scale bar, 1 µm.

Table 2. Comparison of mesocarp layer cell wall thickness between normal and canker infected lime peels.

Lime peels	Thickness of mesocarp cell wall (µm)	
	Range	Average
Cells wall of normal	0.9-3.69	1.86±0.62a
Cells wall of canker infected	1.7-14.6	4.49±2.5b

Remark: N=50, P ≥ 0.01

REFERENCES

[1] A.R. Spurr, A low viscosity epoxy resin embedding medium for electron microscopy, *Journal of Ultrastruct Research.*, **26**, 31-43 (1969).

[2] B.B. Mathew, S. Jatawa, A. Tawari, Phytochemical analysis of *Citrus limonum* pulp and peel, *International Journal of Pharmacy and Pharmaceutical Sciences*, 4, 369-371 (2014).

[3] B.L. Gabriel, *Biological Electron Microscopy*. Van Nostrand Reinhold Company Inc., New York. 1982.

[4] D. Changchan, P. Umroong, Double staining technique for identifying ultrastructure of *Citrus hystrix* DC Leaves, *Microsc. Microanal Res*, **34**, 19-22 (2021).

[5] E.S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy, *J. Cell Biol.*, **17**, 208-212 (1963).

[6] H. Houng, 2022, Citrus canker: reproduction and control measures, *Journal of Plant Pathology and Microbiology,* **13**, 615 (2022).

[7] H.N. Siti, S. Mohamed, Y. Kamisah, Potential therapeutic effects of *Citrus hystrix* DC and its bioactive compounds on metabolic disorders. *Pharmaceuticals*, **15**, 1-21 (2022).

[8] J.H. Graham, T.R. Gottwald, J. Cubero, D.S. Achor, *Xanthomonas axonopodis* pv. citri: factors affecting successful eradication of citrus canker, *Mol. Plant Pathol.*, **5**, 1–15 (2004).

[9] J.J. Bozzola, *Electron Microscopy Principles and Techniques for Biologists*, second ed., Jones and Bartlett publishers, Massachusetts, 1992.

[10] K. Kirasa, T. Sansayawichai, W. Polrakdee, Programmed cell death in the process of cells sugarcane white leaves disease, KHON KAEN AGR.*J.*,**40**,222-230 (2012).

[11] L.A. Rigano, M.R. Marano, A.P. Castagnaro, A.M.D. Amaral, A.A. Voinov, Rapid and sensitive detection of Citrus Bacterial Canker by loop-mediated isothermal amplification combined with simple visual evolution methods, *BMC Microbiology*, **10**, 1-8 (2010).

[12] M. Jadav, R.R. Charan, H. Solanki, Citrus canker diseases-a review, *International Journal of Creative Research Thoughts,* **10**, e764-e778 (2022).

[13] M. Javelle, V. Vernoud, P.M. Rogowsky, G.C. Ingram, Epidermis: the formation and functions of a fundamental plant tissue, New Phytol, **189**, 17-39 (2011).

[14] M. Majda, N. Trozzi, G. Mosca, R.S. Smith, How cell geometry and cellular patterning influence tissue stiffness, International Journal of Molecular Sciences, **23**, 1-16 (2022).

[15] M. Mohanapriya, L. Ramaswamy, R. Rajendran, Health and medicinal properties of lemon (*Citrus limonum*), *Journal of Ayurvedic and Herbal Medicine*, **3**, 1095-1100 (2013).

[16] M.R. Adams, Acids and fermentation, pp. 467-479, *In* Y. Motarjemi, H. Lelieveld, eds, *Food safety management 2014: a practical guide for the food industry*, Academic Press, Massachusetts, 2014.

[17] P. Karmakar, Pollination biology of *Citrus aurantiifolia* (Christm.) Swingle: a medicinally important fruit plant, *International Journal of Innovative Research and Development***, 2**, 138-142 (2013).

[18] P. Umroong, D. Changchan, J. Sangawang, Comparative of the morphology and ultrastructure of Kaffir lime (*Citrus hystrix* DC.) leaves Attacked by Citrus canker symptoms with microscopic techniques, *Microsc. Microanal* Res, **35**, 21-25 (2022).

[19] R.E. Pitt, Models for the rheology and statistical strength of uniformly stressed vegetative tissue, Transactions of the ASAE, **25**, 1776-1784 (1982).

[20] S. Nagy, P.E. Shaw, M.K. Veldhuis, *Citrus science and technology*, Vol. 2, AVI Publishing Company, Westport, 1977.

[21] S. Savaldi-Goldstein, C. Peto, J. Chory, The epidermis both drives and restricts plant shoot growth, Nature, **446**, 199-202 (2007).

[22] T. Qudah, U. Zahra, R. Rehman, M.I. Majeed, S. Sadique, S. Nisar, T.S. l-Qudah, R.W. Tahtamouni, Lemon as a source of functional and medicinal ingredient: A review, *International Journal of Chemical and Biochemical Science*, **14**, 55-61 (2018).

[23] T.R. Gottwald, J.H. Graham, T.S. Schubert, Citrus canker: The pathogen and its impact, *Plant Health Prog.*, **10**, 2002).

[24] V. De Smedta, P. Barreiro, B.E. Verlinden, E.A. Veraverbeke, J. De Baerdemaekera, B.M. Nicolai, A mathematical model for the development of mealiness in Apples, Postharvest Biology and Technology, **25**, 273-291 (2001).

[25] W. G. Van Doorn, E. J. Woltering, Physiology and molecular biology of petal senescence, *Exp. Bot. J.,* **59**, 453-480 (2008).