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Comparative of the Morphology and Ultrastructure of Kaffir Lime (*Citrus hystrix* DC.) Leaves Attacked by Citrus Canker Symptoms with Microscopic Techniques

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ABSTRACT

The application of microscopic techniques to examine the infestation of pathogens in plants in order to gain further insights into the microbiological processes at the cellular and subcellular level in plants showing disease symptoms are currently commonly used to characterize their morphology and ultrastructure. Therefore, this research aimed to morphological and ultrastructure studies of kaffir lime leaves in citrus diseases. Citrus canker symptoms in *Citrus hystrix* DC. with transmission electron microscopy and microscopy to visualize how Citrus canker activity in kaffir lime leaves destroyed the ultrastructures. For the study of morphology and cell structure biology the samples must be prepared and infiltrated with resin, embedded, cut and stained. The results showed that morphology and cellular structure of Citrus canker symptoms in kaffir lime leaves were studied with light microscopy and transmission electron microscope. The cuticle of the leaf surface was damaged and the infection found in the mesophyll layer.

INTRODUCTION

Kaffir lime (*C. hystrix* DC.) is a plant member of family Rutaceae, Kaffir lime leaves are assembled into single leaves. The leaves are spread out into fins resembling leaf plates, the leaves are thick, smooth, oily, green, and dark green with the age of the leaves. The leaves have a coddle in the middle of the leaves, causing the leaves to be divided into 2 parts or 2 leaves in a row. The leaves are very fragrant because they contain oil glands. The leaves are often used in folk medicine. *C. hystrix* DC extract has been reported to protect against diabetes, high blood pressure. and hyperlipidemia It reduces the viability of cervical cells [3,8] and nerve cells and also testing by BioAssay confirmed the anti-epidemic activity of natural phytol and lupole products. The results showed that there was an anticancer activity of isolated phyto and lupine to reduce the proliferation of white blood cells. as well as being used in the cosmetic industry as well [6].

Citrus canker symptoms

In kaffir lime leaves with Citrus canker symptoms, bulging spots are shown to have a small brown blotch surrounded by yellow circles found on both sides of the leaves (Figure 1). It is a disease caused by the bacterium *Xanthomonas axonopodis* pv. *citri*, a gram-negative bacterium that destroys plant wounds, especially citrus plants such as tangerines, grapefruits, lemons, bergamot, etc. It can spread through wind and water and destroys plants when the environment is suitable. If there is a large infestation, the tree will deteriorate and the leaves drop, poor fruit skin quality with low productivity that will affects agricultural production.

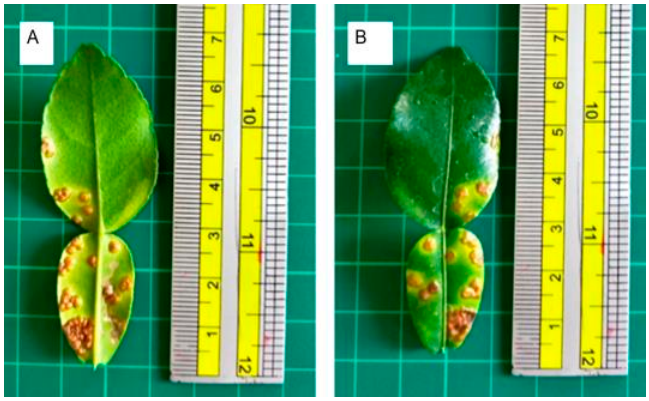

Figure 1. Shown of *Citrus hystrix* DC. leaves Citrus canker Symptoms A: upper leaves B: lower leaves.

Application of microscopic techniques to study the infestation of the bacterium *X. axonopodis* pv. *citri* in kaffir lime leaves. In nature, biological samples are composed of more than 70 percent water in cells. In sample preparation techniques, the cell is stabilized, and the intracellular elements are replaced by resins that are most similar to natural ones. The expertise and expertise of the sample preparer and tooling skills

are required in the sample cutting process. The intracellular structure was observed under transmission electron microscopy and light microscopy to accurate analysis results.

METHDOLOGY

Plant material

C. hystrix DC. were collected from the private garden in Bangkok, Thailand [Figure 1]. The leaves were examined by a compound microscope and a transmission electron microscope.

Ultrastructure of leaf under compound microscopy

The normal kaffir lime leaves and kaffir lime leaves showing canker symptoms were cut into small pieces 1×2 mm^2. and primarily fixed in a solution containing 2.5 % glutaraldehyde in 0.1 M sodium with phosphate buffer pH 7.2 for overnight at 4 ˚C. in refrigerator. 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). The specimens were dehydrated in a graded series of acetone (30% ,50%,70%,90% and 100 %) for about 15 min per series modified from [4]. 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, using a formulation of Spurr's resin (1969) [7]. Then, the samples were cut semi-thin section at 1 µm by using an ultramicrotome EM UC7 (Leica, Austria) with a glass knife, and mounted onto a glass slide. The specimens were stained in Toluidine blue 1% in borax and stained in Basic Fuchsin 1% in 50% alcohol at room temperature for 2 minutes, then dried at 85 ˚C for 20 minutes, and closed with a cover slide [10]. Measure the length and width of the palisade cell and measure thickness cuticle layer and epidermis cell layer from 100 positions and ranges, the average is reported. The specimens were examined under compound microscope (Carl Zeiss; Axiostar Plus), which was equipped with a photographic camera under normal bright-field imaging.

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 [6]. The ultrathin samples were observed under a transmission electron microscope (Hitachi; HT7700) with a high voltage of electron. at 80keV.

RESULTS AND DISCUSSION

Observation by compound microscopy and transmission electron microscopy.

The transverse results of healthy kaffir lime leaf tissues examined by compound microscopy revealed that the anatomy of *C. hystrix* DC. leaves consisted of a cuticle layer, mesophyll layer, vascular bundle and oil glands (circle). The abaxial and adaxial epidermis was the outer layer of the leaf on the abaxial and adaxial epidermis. The cuticle layer covered on the outside (Figure. 2). Crystals were distributed between different cell types in kaffir lime leaves. Mostly found in palisade and spongy mesophyll cells, and crystals were found in some idioblast cells (Figure. 3). The palisade mesophyll layer duty to absorb light efficiently, cells are packed with many chloroplasts. For observation by transmission electron microscopy found that transverse section of healthy *C. hystrix* DC. leaves composed of cell wall, chloroplasts and organelles within the cell very complete cells (Figure. 4).

The transverse results of kaffir lime leaves showed canker symptoms, observing compound microscopy revealed that the anatomy of *C. hystrix* DC. leaves consisted of a cuticle layer, mesophyll layer

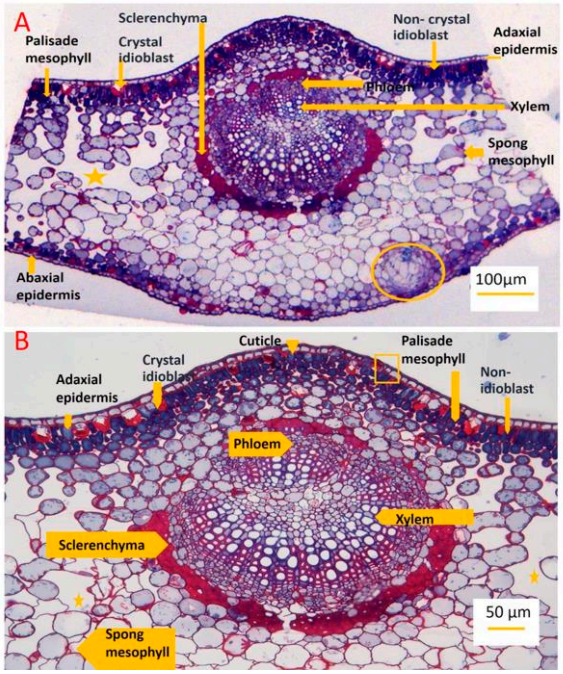

Figure 2. Compound microscope micrograph show transverse section of healthy *C. hystrix* DC. leaves showing characteristics. A: The anatomical structure of the characteristics oil glands (circle), air space (star) (10X). B: The anatomical structure of the characteristics air space (star), stomata (square) (20X).

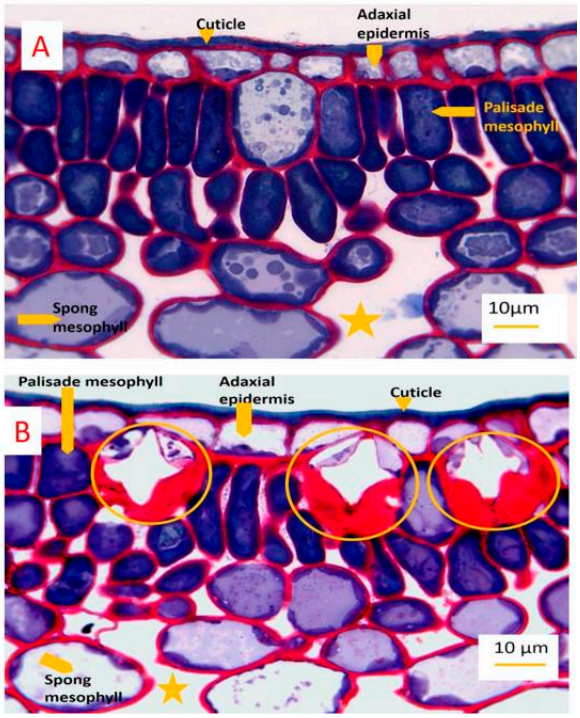

Figure 3. Compound microscope micrograph show transverse section of *C. hystrix* DC. leaves healthy showing anatomical characteristics. A: The anatomical structure of the characteristics air space (star) (100X). B: The anatomical structure of the characteristics air space (star), crystal idioblast (circle) (100X).

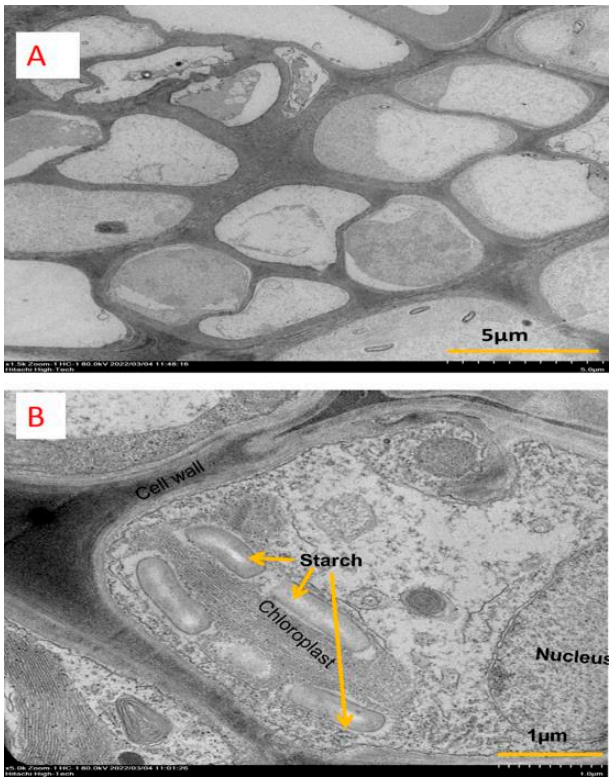

Figure 4. Transmission electron microscopy micrograph of *C. hystrix* DC. leaves healthy showing organelles within the cells in mesophyll layer very complete cells. A. (magnification 1,500x) B. (magnification 5,000x).

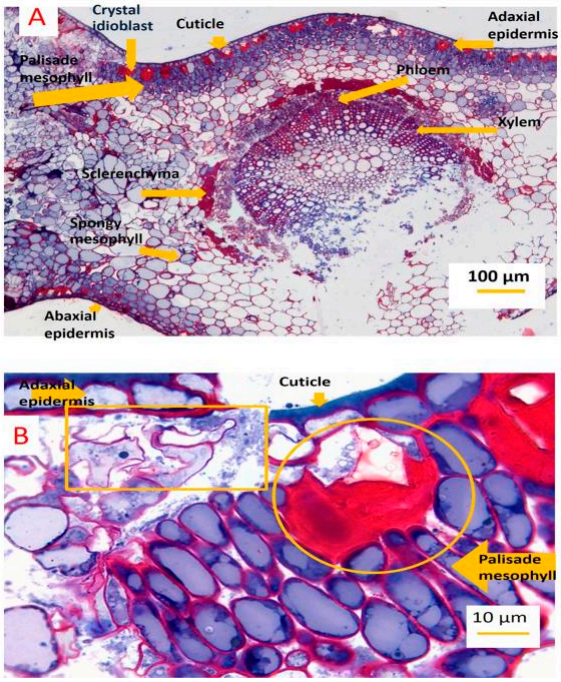

Figure 5. Compound microscope micrograph show transverse section of *C. hystrix* DC. leaves canker symptoms showing anatomical characteristics and non- complete. A: The anatomical structure of the characteristics (10X). B: The anatomical structure of the characteristics within the cells deteriorate (square), crystal idioblast (circle) (100X).

and vascular bundle. The abaxial and adaxial epidermis was the outer layer of the leaf on the abaxial and adaxial epidermis. The cuticle layer covered on the outside. Anatomy shows the epidermis, adaxial epidermis, palisade mesophyll and non- complete epidermis from the micrographs (Figure 5-7). For observation by transmission electron microscopy found that transverse section of *C. hystrix* DC. leaves canker symptoms found cell wall, chloroplasts and organelles within and organelles within the cells deteriorate (Figure 8). Due to adjacent host mesophyll cells were hypertrophied and collapsing between cells. Concurrently, the cell infectivity of bacteria placed in the mesophyll developed in leaves of Kaffir lime (*Citrus hystrix* DC.) leaves attacked by canker symptoms (Figure 5 -8). [8,10].

The results of the cuticle thickness measurement showed that the healthy kaffir lime leaves had cuticle thickness in the range of 4.61-12.62 µm and the thickness of the epidermis layer in the range of 1.00-4.68 µm. The thickness of the canker symptoms of kaffir lime leaves was lower. The thickness of the cuticle layer was in the range of 1.89-7.19 µm. However, it had higher thickness of the epidermis layer which was in the range of 3.96-15.23 µm. It can be concluded that the thickness of the healthy kaffir lime leaves and the epidermis was less than that of the canker symptoms. Because at this stage, the leaves continue to expand, the cuticle quickly thickens (Table1) [7].

The measurement of palisade cells showed that healthy kaffir lime leaves with crystals inserted the average length of the palisade cells was in the range 7.01-26.09 µm, the average width of the palisade cells was in the range 3.85-14.23 µm, and the average area of the palisade cells was 45.21-273.54 µm. In healthy kaffir lime leaves without crystals the mean length in the range of the palisade cells was 3.73-

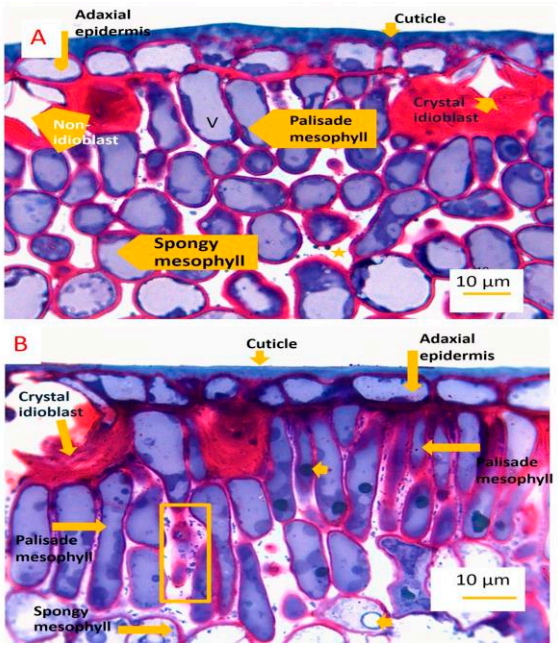

Figure 6. Compound microscope micrograph show transverse section of *C. hystrix* DC. leaves canker symptoms showing anatomical characteristics and non- complete. A: The anatomical structure of the characteristics air space (star) (100X). B: The anatomical structure of the characteristics within the cells deteriorate (square), oil glands (arrow) (100X).

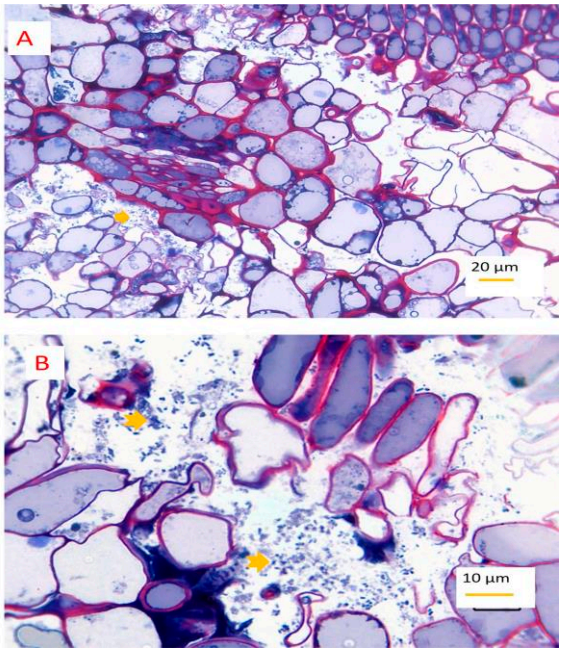

Figure 7. Compound microscope micrograph show transverse section of *C. hystrix* DC. leaves canker symptoms showing anatomical characteristics and non- complete. A and B. The anatomical structure of the characteristics within the cells deteriorate infectivity of bacteria(arrow) (100X).

Table 1 Comparison of thickness of cuticle layer and epidermis between normal and canker symptoms (*C. hystrix* DC.) leave.

Kaffir lime (*C. Hystrix DC.*)	**Thickness layer parameters (µm)**
Cuticle layer of normal	4.61-12.62 (2.69 ± 0.686[a])
Cuticle layer of Canker symptoms	1.89-7.19 (3.81 ± 0958[b])
Epidermis cell layer of normal	1.00-4.68 (8.34 ± 1.466[a])
Epidermis cell layer of Canker symptoms	3.96-15.23 (9.06 ± 2.137[b])

Remark: N = 100, P ≥ 0.01

25.33 µm, the mean width in the range of the palisade cells was 3.29-14.46 µm, and the mean area of the palisade cells was 12.25-318.21 µm^2. As for the results of studies on kaffir lime leaves showed canker symptoms, it was found that the size of palisade cells with crystals inserted the mean palisade cell length was 4.39-34.27 µm, the palisade cell width was 2.73-16.35 µm, and the palisade cell area was 13.41-253.20 µm^2. In conclusion, the study found that in healthy kaffir lime leaves with crystal idioblast, the cell width and fence area were greater compared to the non- crystal idioblast, but in the kaffir lime leaves showing signs of rotting by crystal in idioblast the width and area of the fence cells were less, compared to non- crystal idioblast. In addition,

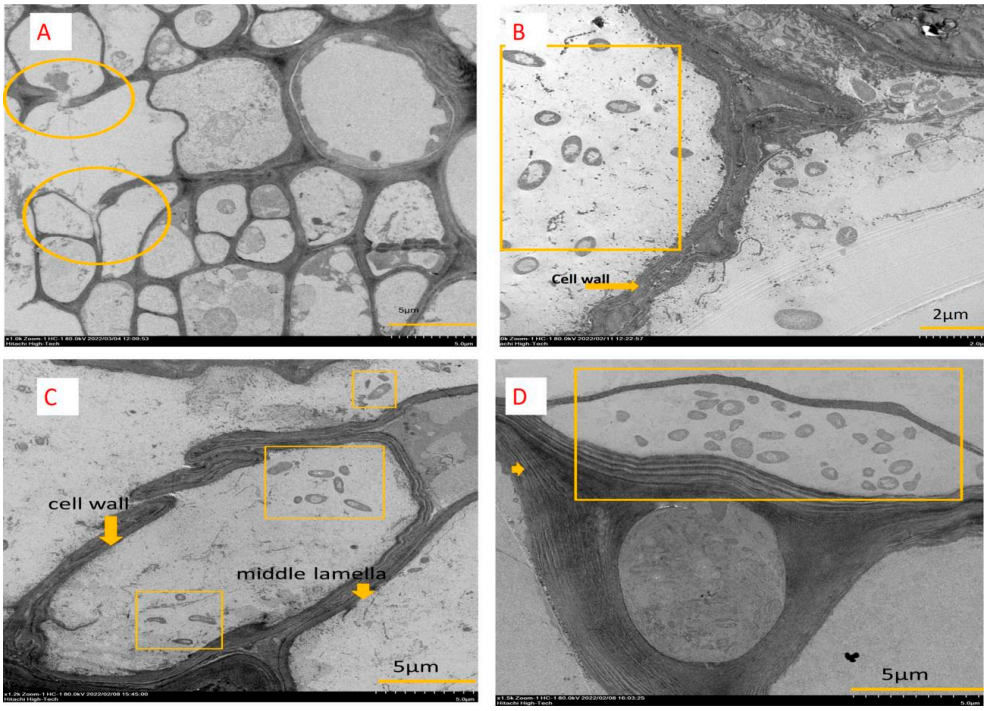

Figure 8. Transmission electron microscopy micrograph of *C. hystrix* DC. leaves canker symptoms showing anatomical characteristics and non- complete. showing organelles within the cells in mesophyll layer. A. Showing organelles within the cells in mesophyll layer non- complete organelles within the cells wall deteriorate (circle) (magnification 1,000x) B. Showing organelles within the cells in mesophyll layer non- complete organelles within the cells wall deteriorate and found infectivity of bacteria placed in the mesophyll (square) (magnification 2,000x) C. Showing organelles within the cells in mesophyll layer non- complete organelles within the cells wall deteriorate and found infectivity of bacteria placed in the mesophyll (square) (magnification 1,200x) D. Showing organelles within the cells in mesophyll layer non- complete organelles within the cells wall deteriorate and found infectivity of bacteria placed in the mesophyll (square) (magnification 1,500x).

Table 2. Comparison of palisade cell size between healthy and canker symptoms (*Citrus hystrix* DC.) leave (n=100).

Kaffir lime (Citrus Hystrix DC.)	**Palisade cell size parameters**	
	with crystal	**without crystal**
Length of palisade cell of healthy (µm)	7.01-26.09 (15.90 ± 4.988)	3.73-25.33 (15.08 ± 4.841)
Width of palisade cell of healthy (µm)	3.85-14.23 (8.13 ± 2.184[b])	3.29-14.46 (8.66 ± 2.571[b])
Area of healthy (µm2)	45.21-273.54 (129.40 ± 53.482)	12.25-318.21 (132.40 ± 59.594)
Length of palisade cell of canker symptoms (µm)	3.90-40.45 (16.80 ± 7.124)	4.39-34.27(16.05 ± 6.780)
Width of palisade cell of canker symptoms (µm)	2.19-13.15 (6.85 ± 2.208[a])	2.73-16.35 (6.74 ± 2.212[a])
Area of canker symptoms (µm2)	12.09-384.96 (121.16 ± 70.899)	13.41-253.20 (108.75 ± 59.914)

Remark: N=100, P ≥ 0.01

when compared to kaffir lime leaves showing canker symptoms, palisade cells without crystal inserts in healthy kaffir lime leaves had more cell length, width and area. less palisade However, the width of palisade cells was greater compared to kaffir lime leaves showing canker symptoms. (Table2) [7].

CONCLUSION

In conclusion, microscopy and electron microscopy techniques were able to study the microstructure of *C. hystrix* DC. cells within leaves and can focus on the study of infection and colonization processes independently and with specific mechanisms. This varies with the interaction between pathogens and pathogens of canker symptoms independently and with specific mechanisms. In addition, the result showed that in palisade cells with crystal idioblast, the length, width and area of cells of healthy leaves were greater than kaffir lime leaves showing canker symptoms. Moreover, in palisade cells with non-crystal idioblast in kaffir lime leaves showing canker symptoms showed that the length and area of cells higher than healthy kaffir lime leaves, but they have smaller cell width.

REFERENCES

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

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

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

[4] 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).

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

[6] T.R. Gottwald, J.H. Graham, T.S. Schubert, Citrus canker: The pathogen and its impact, *Plant Health Prog.*, **3**, (2002).

[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] S. Anuchapreeda, F. Chueahongthong, N. Viriyaadhammaa, P. Panyajai, R Anzawa, S. Tima, C. Ampasavate, A. Saiai, M. Rungrojsakul, T. Usuki, S. Okonogiet, Antileukemic cell proliferation of active compounds from kaffir lime (*Citrus hystrix*) leaves, *Molecules*, **25**, 1-16 (2020).

[10] R.E. Stall, G. M. Marco, B.I. Canteros de Echenique, Importance of mesophyll in mature-leaf resistance to cancrosis of citrus. *Phytopathology*, ***72***, 1097-1100 (1982).

[11] R. Storeyand, R.A. Leigh, Processes modulating calcium distribution in citrus leaves. An investigation using x-ray microanalysis with strontium as a tracer, *Plant Physiol.*, **136**, 3838–3848 (2004).

[12] W.A. Sri Tunjung, J. Cinatl, M. Michaelis, C.M. Smales, Anti-cancer effect of kaffir lime (*Citrus hystrix* DC) leaf extract in cervical cancer and neuroblastoma cell lines, *Procedia Chem.*, **14**, 465–468 (2015).

[13] P. Umroong, P. Kotepong, Ultrastructure studies of fungus *Colletotrichum gloeosporioides* cause of anthracnose disease of mango by microscope technique, *Microsc. Microanal* Res, **32**, 1-5 (2019).