*Microsc. Microanal. Res. 2021 34(1) 19-22*

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.

# Double Staining Technique for Identifying Ultrastructure of *Citrus hystrix* DC Leaves

Dararat Changjan[1]* and Patcharee Umroong[1]

[1] *Scientific Equipment and Research Division, Kasetsart University Research and Development Institute, Kasetsart University, Bangkok, 10900, Thailand*
*Corresponding author's e-mail address: : rdidrc@ku.ac.th

**ARTICLE INFO**

*Article history*
Submitted: 18 April 2021
Revised: 22 June 2021
Accepted: 23 June 2021
Available online: 29 June 2021

*Keywords:*
Kaffir lime leaves; Double staining technique; Basic fuchsin; Toluidine blue O; Ultrastructure



**ABSTRACT**

Kaffir lime (*Citrus hystrix* DC) leaves is part of Rutaceae family. It is medicinal herb that is native to various parts of Southeast Asia. Kaffir lime leaves are widely used as a part of many food ingredients due to its unique fragrance. The aroma is derived from the oil glands on the leaf's surface. In addition, Kaffir lime leaf extract has been reported to contain bioactive properties such as antileukemic, antioxidant, antimicrobial and anti-inflammatory. In this report, we used a light microscope and a double staining technique using toluidine blue O and basic fuchsin to study the ultrastructure of Kaffir leaves. The semi-thin section of leaves were stained using 5 conditions. The double staining technique helped distinguish and enhance visualization of leaf tissue. The study showed that when compared, the 3 conditions of double staining, the optimum combination was obtained as 1 minute of 1% toluidine blue O in 2.5% sodium carbonate and 2 minute of 2.5% basic fuchsin in 50% ethanol. Toluidine blue O clearly showed chloroplast and xylem while basic fuchsin excellently stained idioblast cells and phloem. This study details the protocols of a variety of staining methodologies that could be applied to identify ultrastructure of other plants.

## INTRODUCTION

Staining techniques are excellent at revealing the many elements of the cell. Each stain is helpful in its own way for detection of the structure of tissues. Double staining will help to show the ultrastructure of cells because the first stain may have a weak reaction while the second stain may have stronger reaction in the same area. This is according to studies reported by Hernan and Tanya (2014) involving combination between Toluidine blue O (TBO) and ruthenium red for identifying secondary compound in Myrtaceae, lignified vessel and fiber were stained with TBO while nonlignified primary cell walls in the xylem, secondary phloem and non vascular tissues were stained with ruthenium red. TBO and ruthenium red have a different structure in the cell, so each secondary compound can be more clearly visible [1]. TBO and basic fuchsin are widely used to stain plant tissue. For example, maize leaf, vascular bundle cells are stained blue green, bulliform cells appear dark pink-purple and subsidiary cells of guard cells are stained pink by TBO. TBO is used to stain tissue that contains polysaccharide, lignin, polyphenol, tannin and suberin [2, 3]. The basic fuchsin stain showed an association of tissue that were lignified, suberized and cutinized such as cell wall and vascular bundle [4, 5, 6]. The excellent visual contrast of samples is associated with duration of staining, concentration of dyes and element of tissue [1].

*Citrus hystrix* DC commonly called kaffir lime, Thai bergamot, makrut lime or makroot, is part of Rutaceae family. It is native to various parts of Southeast Asia. Kaffir lime leaves are widely used as part of many food ingredients due to its unique fragrance that derives from the oil glands on leaf surface [7]. Kaffir lime leaf extracts have some bioactive properties such as antileukemic, antioxidant, antimicrobial and anti-inflammatory [8, 9, 10, 11]. Moreover, kaffir lime leaves extract were used as insecticides and pesticides. Previous studies show that leaf extracts from *C. hystrix* DC showed efficiency in killing the *Spodoptera litura* larvae [12] and were also used against *Lasioderma serricorne* [13]. Despite the benefits of kaffir lime leaves, the ultrastructure studies of such are rare and with limited reports. Thus, this report was done to investigate an optimum staining protocol using different duration of staining with TBO and basic fuchsin to identify ultrastructure of Kaffir leaves.

## METHODOLOGY

### Plant Materials

*Citrus hystrix* DC leaves were collected from the garden of Dararat's house, Nonthaburi, Thailand (Figure 1).

### Sample preparation

The leaves were cut into 1x2 $mm^2$. Then the specimens were pre-fixed with 2.5% glutaraldehyde in 0.1M sodium phosphate phosphate buffer (pH 7.2) overnight at 4°C, rinsed thrice in the same buffer. After that they were post-fixed with 2% osmium tetroxide in distilled water for 2 h. at room temperature and rinsed three times with distilled water. Next, they were dehydrated by acetone series (30%, 50%, 70%, 90% and 100%) for about 15 min for each. The samples were then transferred to 2:1, 1:1 and 1:2 of 100% acetone and Spurr's resin respectively (3-4 h. for each) and then to pure Spurr's resin overnight. For the purpose of the polymerization of resin, they were placed in flat embedding

**Figure 1.** *Citrus hystrix* DC Leaves.

molds containing Spurr's resin and heated at 80°C for 7 h [14]. Semi-thin sections of 1 m thickness were cut by an ultramicrotome (Leica; UC7) with a glass knife and put on a glass slide.

Staining procedure and light microscope (LM) observation

The sections were stained with one or both of 1% TBO in 2.5% sodium carbonate and 2.5% basic fuchsin in 50% ethanol for different periods of time according to 5 conditions (Table 1). For double staining,

**Table 1.** Staining conditions applied in this study using different combinations duration of staining.

| Condition | Duration of staining (min) | |
|---|---|---|
| | 1% toluidine blue O in 2.5% sodium carbonate | 2.5% basic fuchsin in 50% ethanol |
| C1 | 1 | 0 |
| C2 | 0 | 2 |
| C3 | 0.5 | 1 |
| C4 | 1 | 2 |
| C5 | 2 | 3 |

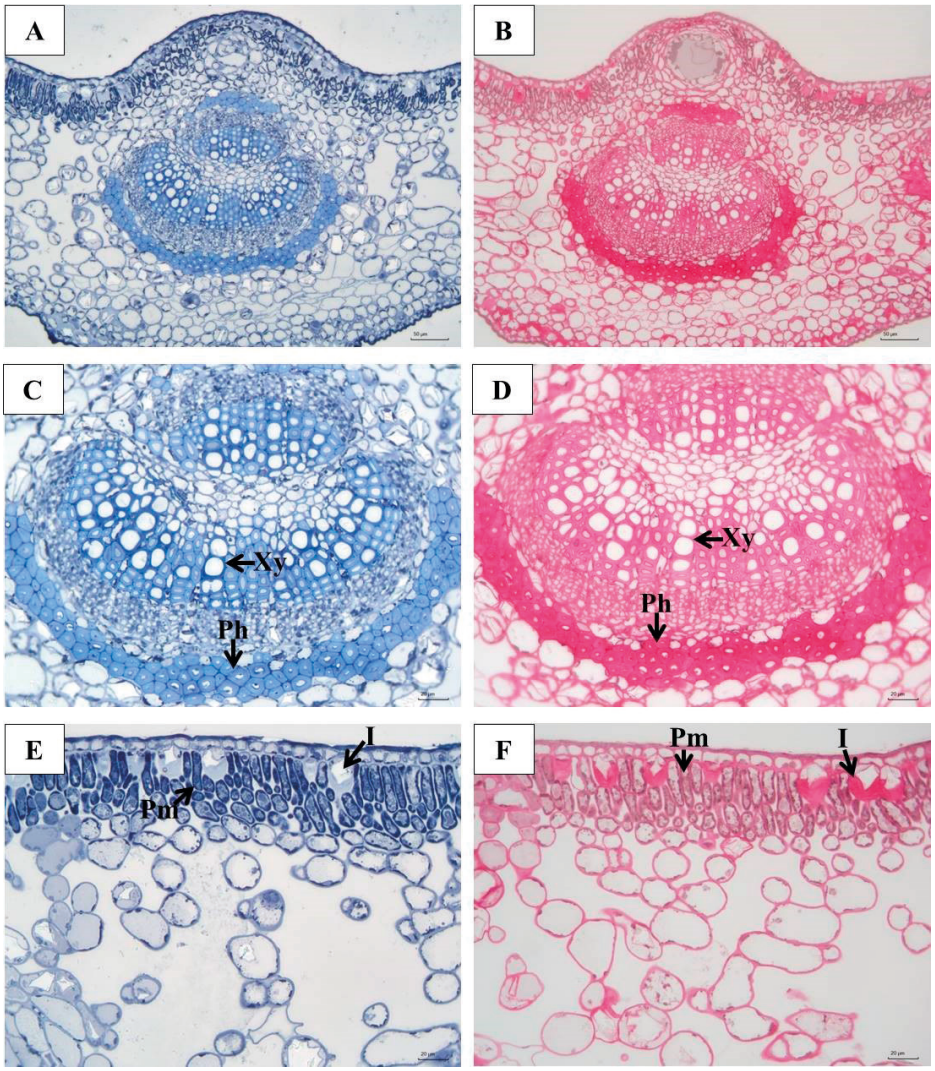


**Figure 2.**Transverse light micrograph of *C. hystrix* DC leaf, showing comparisons between condition C1 (A, C, E) and C2 (B, D, F). Images were analyzed under 20X and 40X magnification for A-B and C-F respectively. (I = Idioblast cell, Ph = Phloem, Pm = Palisade mesophyll cell, Xy = Xylem).

samples were stained with TBO first at 85°C and then they were stained with basic fuchsin at room temperature. Finally, they were observed using a light microscope (Carl Zeiss; Axiostar plus) with Motic image plus 3.0 software.

## RESULTS AND DISCUSSION

Above, Figure 2 shows that when staining with TBO, the areas of kaffir lime leaf that have a darker colour than other areas of chloroplast, xylem and cuticle. Xylems are stained blue, chloroplast and cuticle were stained dark blue (Figure 2 A, C and E), while condition C2, xylem and chloroplast showed a light colour by basic fuchsin (Figure 2 B, D and F). Moreover, TBO has a weak reaction with phloem and idioblast cells which were stained light blue, but basic fuchsin have a strong reaction with them and they are stained dark pink. TBO is a polychromatic dye which has the ability to stain diverse components and produce in different colours depending on chemical components of the tissue [3, 4].

The double staining, with TBO for 0.5 min and basic fuchsin for 1 min (condition C3) did not give clearly contrast between palisade mesophyll cells (PM) and idioblast cells (I) due to weak reaction of TBO (Figure 3 A-C).

Condition C4 produces contrast better than C3 due to TBO having a strong reaction with tissue. Moreover, C4 proved to be an effective condition for differentiating kaffir lime leaves structure based on colour when compared with other conditions. Under this condition, idioblast cells were stained dark pink and chloroplasts were stained dark blue (Figure 3 D-F).

C5 (Figure 3 G-I) showed similar results to C4 in terms of contrast between palisade mesophyll cells and idioblast cells, but the pink colour of the idioblast cells were lighter compared to those of the C4. Similarly, stained xylem and phloem were easily distinguishable under the conditions C4 and C5 while vascular bundles were not clearly contrasted when stained with condition C3 (Figure 3).

From our study, double staining improves visibility for identifying cell structures better than single staining due to both stains binding differently to each structure of the cell. Such as TBO having a weak reaction with idioblast cells (light blue). However, when stained with basic fuchsin, idioblast cells are a darker colour (dark pink), so basic

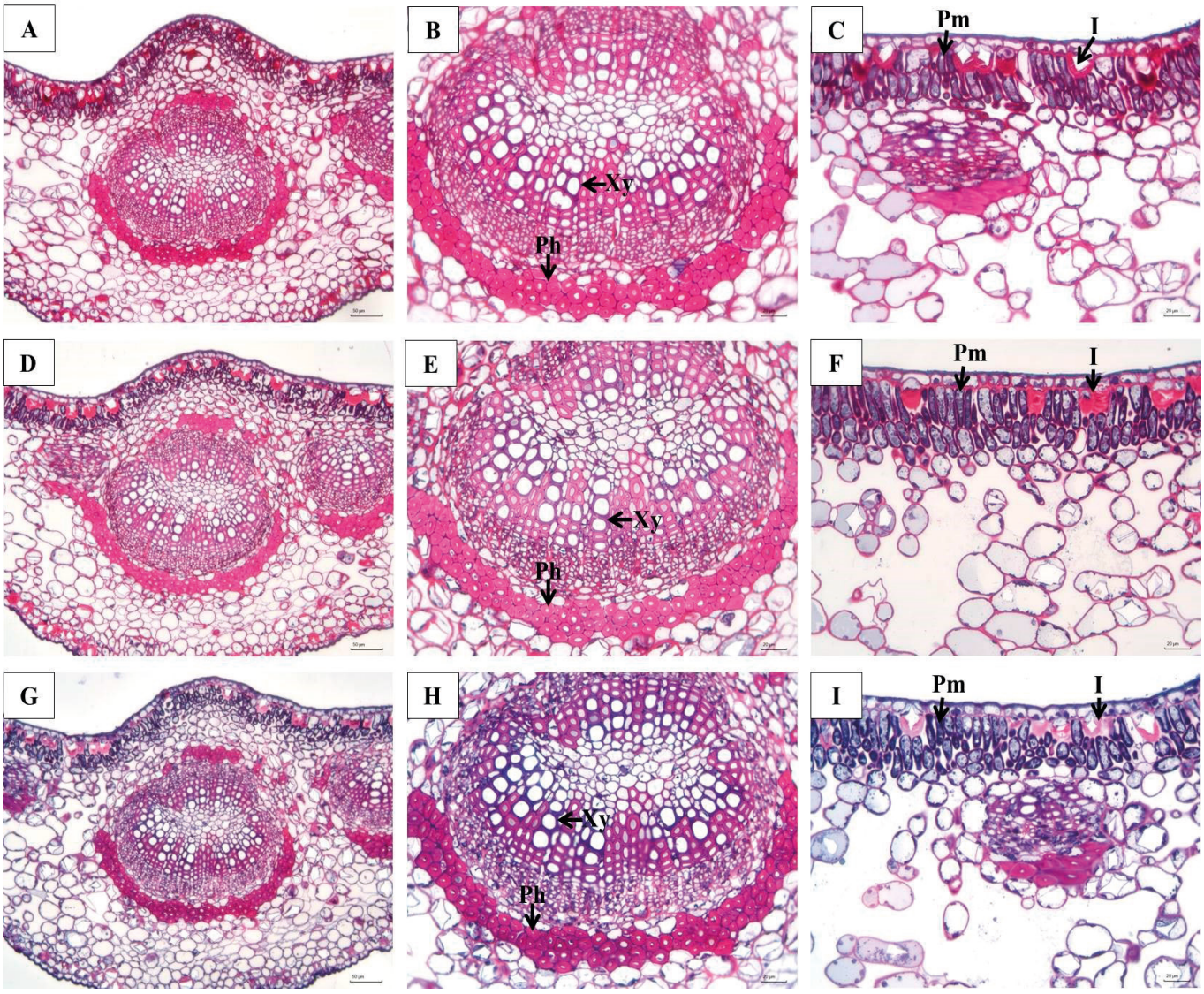


**Figure 3.** Transverse light micrograph of *C. hystrix* DC leaf, showing comparisons between condition C3 (A-C), C4 (D-F) and C5 (G-I). Images were taken with magnification of 20X for A, D, G and 40X for B, C, E, H, I. (I = Idioblast cell, Ph = Phloem, Pm = Palisade mesophyll cell, Xy = Xylem).

fuchsin showed idioblast cells more clearly than TBO. Moreover, we have demonstrated that the time for staining is important, if the time of the staining is not optimal for the sample, it might be difficult to clearly vision and identify components of the tissue.

## CONCLUSION

The optimum combination for double staining was 1 minute of 1% toluidine blue O in 2.5% sodium carbonate and 2 minute of 2.5% basic fuchsin in 50% ethanol (condition C4). Under this condition, the ultrastructure of kaffir lime leaves was clearly visible and each tissue type was distinguishable .

## REFERENCES

[1] H.A. Retamales, T. Scharaschkin, A staining for identifying secondary compounds in Myrtaceae, *Appl Plant Sci*., **2014**, 2, 1-8.

[2] M.A. Bellinger, S.K. Sidhu, C.G. Rasmussen, Staining Maize epidermal leaf peels with toluidine blue O, *BioProtoc*, **2019**, 9, 1-11.

[3] P.P. Mitra, D. Loque, Histochemical of *Arabidopsis thaliana* secondary cell wall elements, *J Vis Exp*., **2014**, 87, 1-11.

[4] J. Elizabeth, H.C. de Sousa, M.H. Rezende, N.M. Castro, C. Vecchi, R. Luque, Astra blue and basic fuchsin double staining of plant materials, *Biotech Histochem*., **1998**, 73, 235-243.

[5] N. Kapp, W. J. Barnes, T. L. Richard, C. T. Anderson, Imaging with the fluorogenic dye Basic Fuchsin reveals subcellular patterning and ecotype variation of lignification in *Brachypodium distachyon*, *JXB*, **2015**, 66, 4295–4304.

[6] J.H. Gourle, Basic fuchsin for staining vascular bundle, *Stain tech*, **1930**, 5, 99-100.

[7] R. Budiarto, R. Poerwanto, E. Santosa, D. Efendi, A. Agusta, Production, post-harvest and marketing of kaffir lime (*Citrus hystrix* DC) in Tulungagung, Indonesia, *J Tropical Crop*., **2019**, 6, 138-143.

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

[9] N. Hutadilok-Towatana, P. Chaiyamutti, K. Panthong, W. Mahabusarakam, V. Rukachaisirikul, Antioxidative and free radical scavenging activities of some plants used in Thai folk medicine, *Phamaceutical Biology*, **2006**, 44, 221-228.

[10] S. Siripongvutikorna, P. Thummaratwasikb, Y. W. Huang, Antimicrobial and antioxidant effects of Thai seasoning, Tom-Yum, *LWT*, **2005**, 38, 347-352.

[11] P. Lertsatitthanakorn, S. Taweechaisupapong, C. Aromdee, W. Khunkitti, In vitro bioactivities of essential oils used for acne control, *IJA*, **2006**, 16, 43-49.

[12] F.S. Loh, R.M. Awang, D. Omar, M. Rahmani, Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura* fabricius, *JMPR*, **2011**, 5, 3739-3744.

[13] S. Ikawati, M.S. Dhuha, T. Himawan, Bioactivity of *Citrus hystrix* D.C. leaf extract against Cigarette Beetle *Lasioderma serricorne* (F.), *JTLS*, **2017**, 7, 189-196.

[14] P. Umroong, Leaf anatomy and minimal structure in leaves of *Hydrocotyle umbellata L*., obtained from water stress, were examined under electron microscope and light microscope, *Microsc. Microanal. Res*., **2018**, 31, 29-33.