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Freeze Drying *versus* Chemical Fixation Technique for Scanning Electron Microscope of Succulent and Aquatic Plant Leaves

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ABSTRACT

The maintenance of perfect leaf surface structure is important for morphological study by scanning electron microscope (SEM). Researchers normally use a chemical fixation technique to prepare the leaf samples because it obtains a good result to preserve leaf surface structures. Although chemical fixation is a popular technique, it is harmful to users and environment. Freeze drying is another good technique to prepare the samples for observation by SEM because it is safer than chemical use. The aim of this study is to investigate the optimum time duration of freeze drying for leaf samples of a succulent (*Pepperomia pellucida* (L.) Korth) and an aquatic (*Limnophila heterophylla* (Roxb) Benth) plant. When samples of *P. pellucida* and *L. heterophylla* were subjected to both techniques, the complete structure of the leaf surface was preserved. The leaves of both species took different periods of time to dry. *P. pellucida* took 36 hrs while *L. heterophylla* needed only 24 hrs. However, the compounds used in the chemical fixation are harmful and may contaminate the environment. Therefore, the freeze drying technique is a good way to prepare leaf samples for SEM analysis.

INTRODUCTION

The study of leaf surface by scanning electron microscope normally uses chemical fixation technique for sample preparation. This technique consists of fixation by glutaraldehyde and osmium tetroxide, dehydration using ethanol or acetone and drying by critical point dryer (CPD). It is a good technique to preserve leaf surface structures such as epidermal cells and trichomes. Freeze drying is another technique to prepare a fresh sample into a dry sample. This is a physical fixation technique in which fresh samples are rapidly frozen in liquid nitrogen under low vacuum (Gabriel, 1982). Thus moisture is removed from tissues under very low temperature and low pressure conditions. However, this technique requires a good vacuum system and effective cold trap to remove moisture in samples (Pathan and et.al, 2009). Small pieces of samples are completely dried in 3 days (Hayat, 2000). Several researchers have mentioned that freeze dried samples show less shrinkage than samples dried with the critical point drying method (Robinson and et.al., 1987). The advantage of the freeze drying method is to preserve the cuticle layer of plants because the samples are prepared without chemical solvents. However, this technique is more difficult than critical point drying because it has no proper conditions (such as duration to prepare the sample, size of sample and variation of sample properties) for all samples. The critical point drying method is popular because it has an obvious process and takes a short time to perform (Flegler and et.al., 1993)

Peperomia pellucida(L.) Korth. is an herb. Its common name is shiny bush and Thai name is pak kra sang. The stem and branch are translucent. The leaves are succulent. It is found in wasteland, marshy areas, slightly shaded areas, and is widespread throughout the country. The young shoot and stem are eaten raw or used in vegetable side dishes with chilli paste or minced meat in Thai cooking (Paisooksantivatana and Nakahara, 2009).It is used for antibacterial activity against *Esherichia coli*, *Bacillus subtilis*, *Pseudomonas areuginosa* and *Staphylococcus aureus* (Humboldt et.al., 1816).

Limnophila heterophylla (Roxb) Benth is an aquatic and medicinal plant. Its common name is ambulia and Thai name is sarai chat. The stem is erect and creeping with submerged leaves. It is found in rice fields and irrigation systems. It is used as medicine (Cook, 1996) and for antimicrobial and anti- inflammatory purposes (Goraiet.al. 2014).

METHODOLOGY

Plant Materials

Two plant species are *Peperomia pellucida*(L.) Korth and *Limnophila heterophylla* (Roxb) Benth representing succulent and aquatic plants, respectively. The samples were collected from Kasetsart University, and then prepared by chemical fixation and freeze drying techniques.

Chemical Fixation Technique

The leaf samples were cut into small pieces (3x3 mm), pre-fixed in 2.5% glutaraldehyde in sodium phosphate buffer pH 7.2 at 4 ˚C

for 12 hrs and post-fixed in 1% osmium tetroxide for 1 hr. Then the samples were dehydrated in 20-100% acetone series and dried in critical point dryer (Quorum: K850) (Gabriel, 1982)

Freeze Drying Technique

The leaf samples were cut into small pieces (3x3 mm). These small pieces were immersed in liquid nitrogen and dried in freeze dryer (Quorum: K750X) for 6, 12, 18, 24, 30 and 36 hrs. The dried leaf samples were coated with gold particles using ion coater (IKO Engineering: IB-2) and then examined by scanning electron microscope (Hitachi: SU8020) operated at 4 kV.

RESULTS AND DISCUSSION

In this experiment, two techniques for leaf sample preparation, chemical fixation and freeze drying, were compared for scanning electron microscope. The aim of this study was to find the appropriate technique to preserve the samples and to reduce the use of osmium tetroxide and other chemicals. These chemicals are verified as heavy metals which are toxic to users. We also studied the appropriate duration to completely dry leaves in a freeze dryer. Six periods of time for freeze drying 6, 12, 18, 24, 30 and 36 hrs were compared (Fig. 1-3).

Leaves of *P.pellucida*

When *P. pellucida* was subjected to the chemical fixation

Table 1 The percentages of complete cells on leaf surfaces of *P. pellucida* and *L. heterophylla* obtained from chemical fixation and freeze drying techniques.

No.	Plant species		Percentages of complete cells on leaf surface (%)						
			Chemical fixation technique	Freeze drying technique (hrs)					
				6 hrs	12 hrs	18 hrs	24 hrs	30 hrs	36 hrs
1	*P. pellucida*	Lower epidermis	46	0	0	1	4	10	67
		Upper epidermis	62	0	0	1	0	8	61
2	*L. heterophylla*	Lower epidermis	66	1	4	7	87	49	60
		Upper epidermis	72	3	2	17	78	42	89

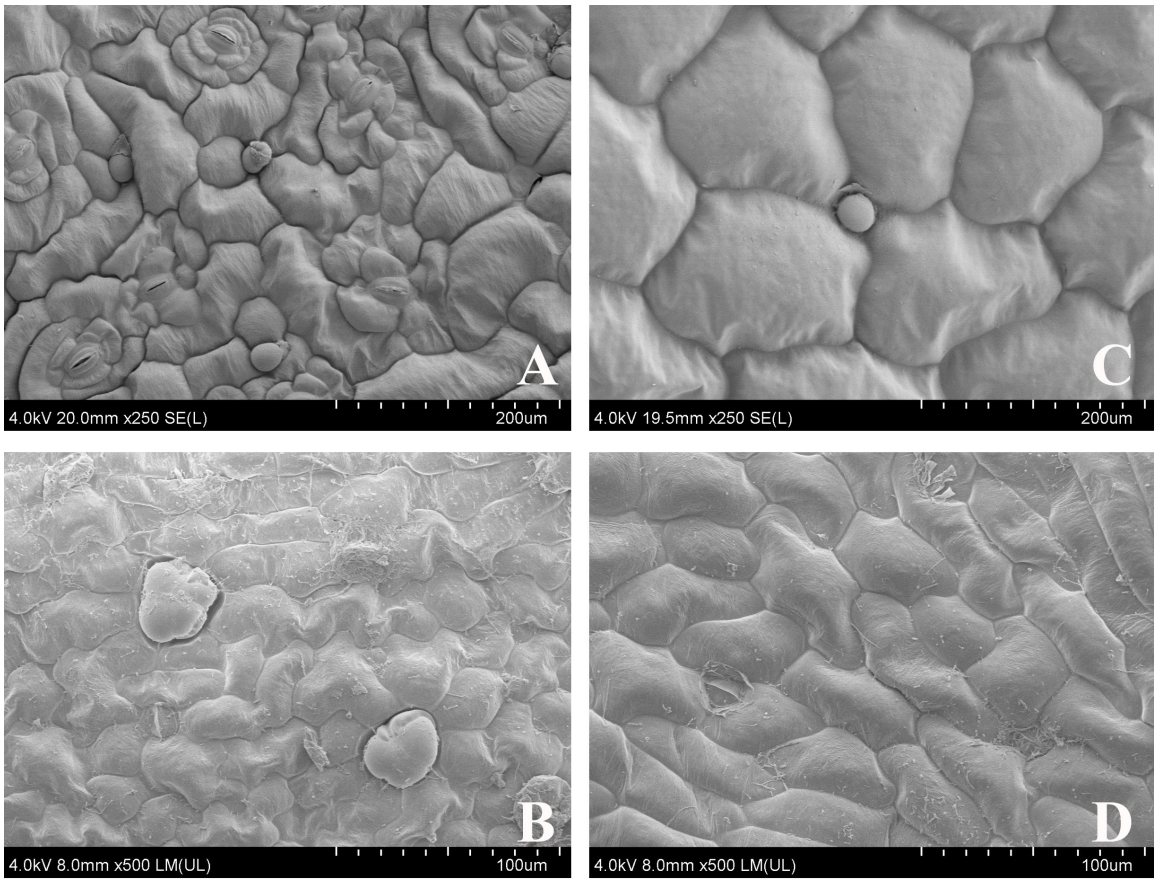

Figure 1 Scanning electron micrographs of leaf epidermal cells of *P. pellucida* and *L. limnophylla* leaf surfaces when prepared by chemical fixation technique. (A) lower epidermis of *P. pellucida* (B) lower epidermis of *L. heterophylla* (C) upper epidermis of *P. Pellucida* (D) upper epidermis of *L. heterophylla*

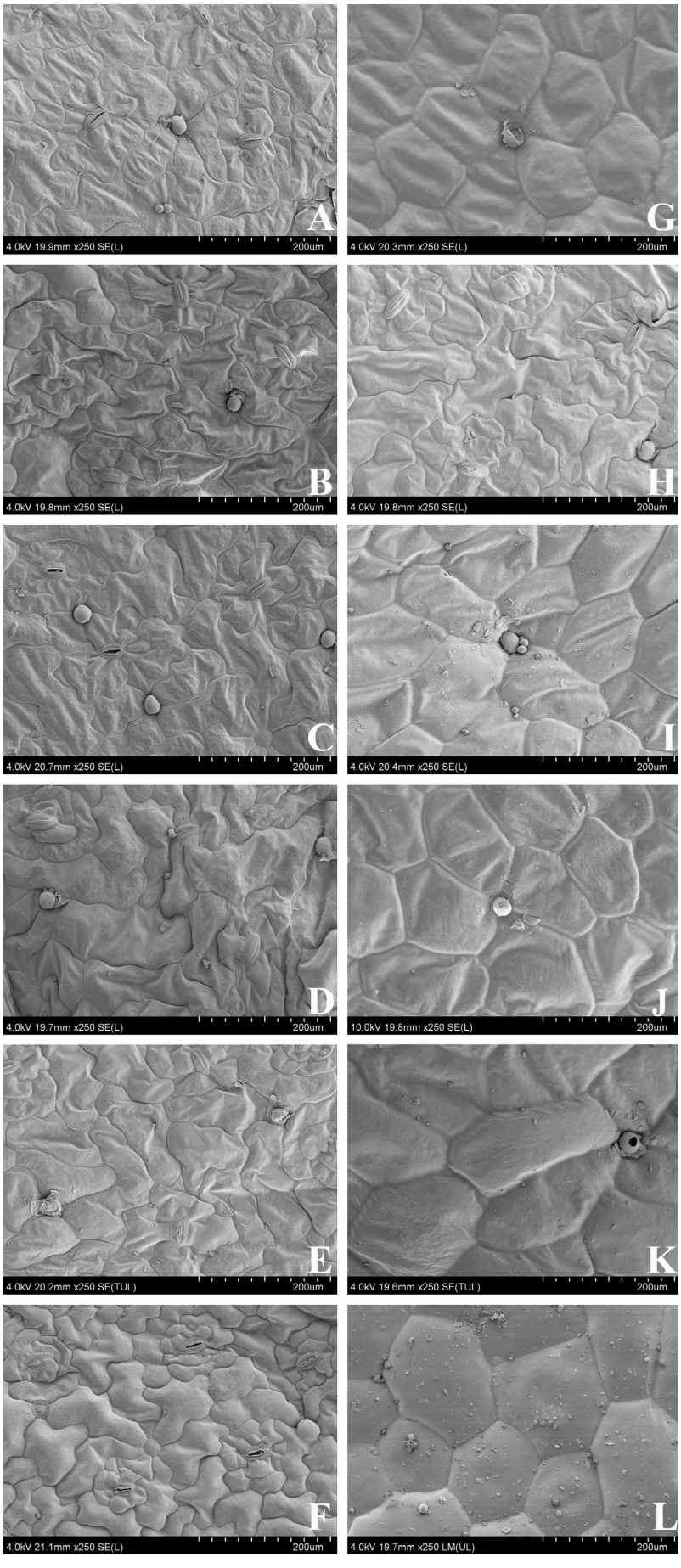

Figure 2 Scanning electron micrographs of epidermal cells of *P. pellucida* leaf surface when dried by freeze drying technique. A-F lower epidermis subjected for 6, 12, 18, 24, 30 and 36 hrs, respectively. G-L upper epidermis subjected for 6, 12, 18, 24, 30 and 36 hrs, respectively.

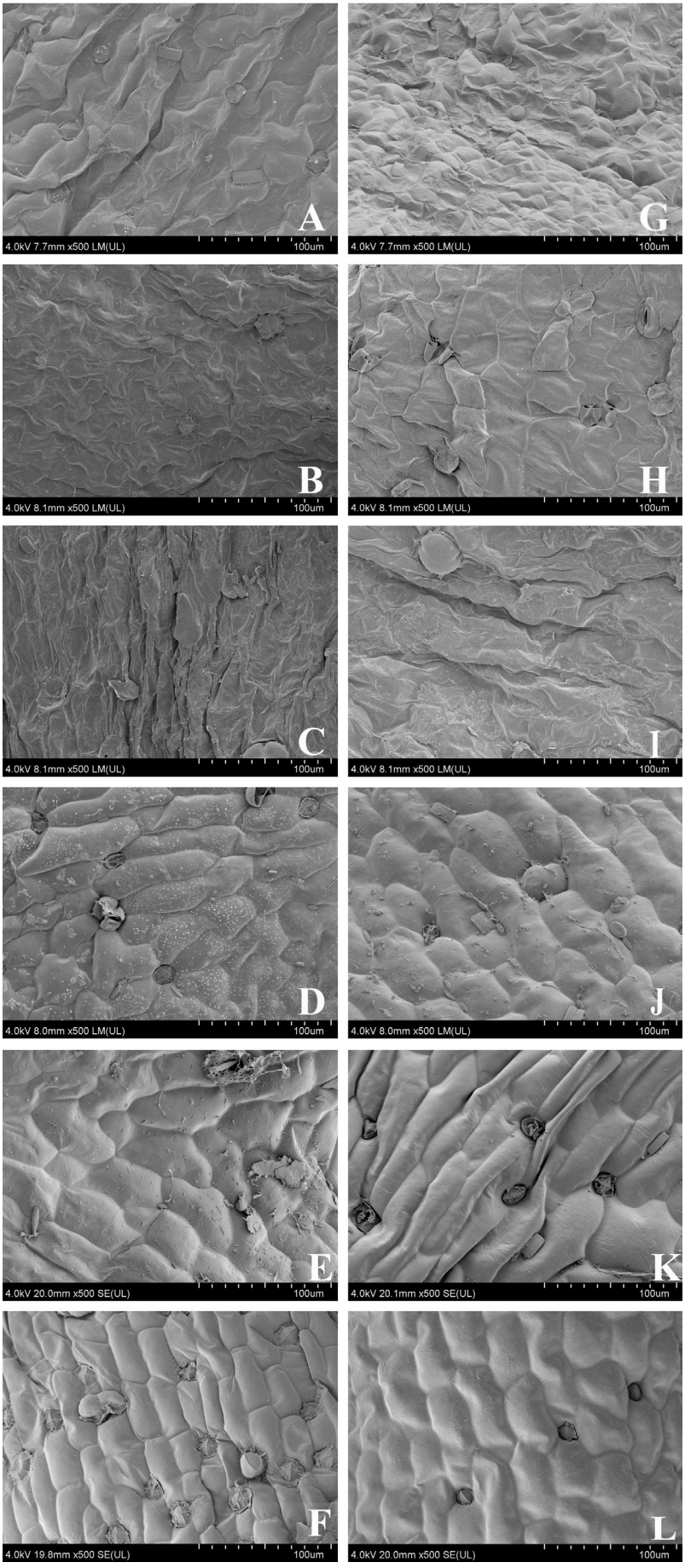

Figure 3 Scanning electron micrographs of epidermal cells of *L. heterophylla* leaf surface when dried by freeze drying technique. A-F lower epidermis subjected for 6, 12, 18, 24, 30 and 36 hrs, respectively. G-L upper epidermis subjected for 6, 12, 18, 24, 30 and 36 hrs, respectively.

technique, 46% of the cells in the lower epidermal layer were complete, and 62% in the upper layer were complete (Fig 1). After 6 and 12 hrs of freeze drying, all the cells showed shrinkage on both sides of the leaf surface. The freeze dried leaves at18 hrs had only 1% of complete cells on both sides of leaf surface. At 24 and 30 hrs, 4% and 10 % of the cells on lower epidermis were complete, and 0 % and 8 % of the cells on upper epidermis were complete, respectively. At 36 hrs, 67 % of the cells on lower epidermal layer were complete, with 61 % complete on upper epidermal layer (Fig.2).

Leaves of *L.heterophylla*

When *L .heterophylla* was subjected to the chemical fixation technique, 66 % of the cells in the lower epidermal layer and 72 % in the upper epidermal layer were complete (Fig.1).After freeze drying for 6 hrs,1% of the cells on the lower epidermal layer and 3 % of the cells on the upper epidermal layer were complete. At 12 hrs, 4% of the cells on the lower epidermal layer and 2 % of the cells on the upper epidermal layer were complete. At 18 hrs, 7% the cells on lower epidermal layer and 17 % of the cells on upper epidermal layer were complete. Finally at 24 hrs, 87 % of the cells on the lower epidermal layer and 78 % of the cells on the upper epidermal layer were complete. At 30 hrs, 49 % of the cells on the lower epidermal layer and 42 % of the cells on upper epidermal layer were complete. At 36 hrs, 60 % of the cells on the lower epidermal layer and 89 % on the upper epidermal layer were complete (Fig.3).Therefore the freeze drying technique can preserve the structure of the leaf surface including epidermis cells and trichomes when using the appropriate period of time to prepare dry leaf samples. The appropriate time for *P. pellucida* leaves is 36 hrs and is 24 hrs for *L. heterophylla* leaves. The results are shown in table 1.

CONCLUSION

Both chemical fixation and freeze drying techniques are good for leaf samples of succulent and aquatic plants. From this study, the freeze drying method is recommended because it can eliminate harmful chemicals. The optimum duration of drying is 36 hrs for *P. pellucida* and 24 hrs for *L. heterophylla*.

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