



Techniques for Preparing Fragile Pollen of *Curcuma supraneeana* W.J. Kress & K.Larsen Škornick. for Morphological Observation

Yupadee Paopun*, Piyanan Thanomchat, Dararat Changjan and Rommanee Charoensub

Scientific Equipment and Research Division, Kasetsart University Research and Development Institute, Kasetsart University, Bangkok, 10900, Thailand

*Corresponding author's e-mail address: rdydp@ku.ac.th

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ABSTRACT

Scanning electron microscopy (SEM) has been used to study detail of pollen morphology such as structure of the wall pattern, shape and size of pollen grains. Sample preparation to preserve fragile pollens is critical to maintain pollen structure. This research aims to determine an optimum preparation technique of *Curcuma supraneeana* W.J. Kress & K.Larsen Škornick. fragile pollens for scanning electron microscopy observation. The acetolysis method is an approved technique to prepare pollen for light microscope and SEM. We investigated three conditions including drying, double fixation and boiling in 10% potassium hydroxide. The results showed that drying fresh pollens in a desiccator caused distortion and shrinkage of the pollen grain. The double fixation in chemical preparation technique is excellent to preserve structure of fragile pollen while boiling in 10% potassium hydroxide to remove pollenkitt on the pollen wall breaks and distorts the pollen grain. Therefore, the double fixation in chemical preparation technique is an optimum of way to condition fragile pollens for scanning electron microscope analysis. It is also found that growth stage of the pollen is also an important factor. The pollens from dehiscent anther have a little of pollenkitt on the pollen wall and should be chosen to study the exine pattern of the pollen wall. The fragile pollens are difficult to prepare. Although there are several techniques for pollen morphological study, the suitable methods have not been reported. Also, the pollen growth stage effects on the quality of pollens after accessed the preparation procedures have been providing this study.

INTRODUCTION

Pollen is a part of male gametophyte of flowering plants. Morphologies of pollen including size, shape and especially, exine sculpturing on the pollen wall can be used to classify and identify of seed plant. However, it is challenging to study the exine because it is usually covered by a lot of pollenkitt, which is mainly lipid and sticky substances. Normally, we can use acetolysis followed Erdman's method to clear the pollen wall. The procedure in acetolysis method is to boil pollens in 10% potassium hydroxide to remove pollenkitt on the pollen surface [1]. Hanks and Fairbrothers (1970) used acetolysis method to prepare pollens for morphologies observation of *Fagus* and *Nothofagus* pollens by scanning electron microscope [2]. Kajornjit et. al (2018) studied *Globba* pollen in family Zingiberaceae using ethanol treatment and air-drying the pollens on the aluminum stubs. The pollen grains and wall showed shrinkage [3]. Halbritter (1998) found that a quick method to prepare fresh pollens by decanting them in 2,2-dimethoxypropane and drying them in a critical point dryer [4]. This method helps conserve the details of pollen grains such as size, shape and wall surface, however, the chemical is highly flammable and irritant to people. Dušmann and Von der Ohe (1993) removed pollenkitt on the pollen wall of *Sapium sebiferum* and *Euphobia* spp. by ultrasonication, dehydrating with

acetone and drying in a critical point dryer [5]. Lastly, a chemical fixation technique is a good one to use for biological samples for scanning electron microscopy analysis. The fixatives are glutaraldehyde and osmium tetroxide, followed by dehydration using acetone and drying in a critical point dryer [6].

The genus of *Curcuma* belongs to a family Zingiberaceae. *Curcuma supraneeana* W.J. Kress & K.Larsen Škornick. is an endemic species of Thailand. The flower of this species [Figure1,2] is elegant, made them are valued cultivate for export to Europe. It can grow in deciduous forests on limestone, derived soil and in the grassland on limestone slopes. The flowers bloom during rainy season at the same time as Buddhist Lent days. Therefore, Thai people use this flower for religious ceremony [7]. The pollens of family Zingiberaceae are a nonaperturate type. The pollen grains are 40-150 µm. The exine is very thin and not resistant to acetolysis method. In this study, we investigated an optimal condition for the best result on remove pollenkitt on the fragile wall of the pollens while preserve the detailed morphology of the pollens. This study is to determine the appropriate preparation method of fragile pollen for scanning electron microscope observation. In addition, the effect of pollen growth stages are also important to observe.



Figure 1. *Curcuma supraneeana* inflorescences.



Figure 2. A flower of *Curcuma supraneeana*.

MATERIAL AND METHOD

Pollens of *Curcuma supraneeana* were collected from a field in Pukae Botanical Garden, Saraburi Province, Thailand during July-August 2018. Pollen grains of *Curcuma supraneeana* were studied through a scanning electron microscope, a compound light microscope and a transmission electron microscope at Scientific Equipment and Research Division, Kasetsart University Research and Development Institute.

Sample Preparation for scanning electron microscopy Observation

There are three preparation methods used in this study: 1) Natural drying. 2) Chemical preparation method by fixation by glutaraldehyde and osmium tetroxide and 3) Boiling samples in potassium hydroxide followed with chemical preparation method.

All samples were coated with platinum in sputter coater (Quorum: Q150RES) and examined in Field Emission Scanning Electron Microscope (Hitachi; SU 8020).

Natural dried sample

Fresh pollen grains were pasted on carbon tape onto a stub and kept in a desiccator for 1 week. Then the samples were coated with platinum using a sputter coater machine (Quorum; Q150 RES).

Sample preparation by fixative chemical method

Pollen grains were prefixed with 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, they were dehydrated in 20-100% acetone

series and dried in a critical point dryer (Quorum: K850). The dried samples were coated with platinum for SEM analysis.

Pre-treat sample in potassium hydroxide method

Pollen grains were boiled in 10% potassium hydroxide for 2 mins. Then, the samples were washed in distilled water 3 times and followed the above fixative chemical preparation procedure.

Anatomical investigation by compound microscope and transmission electron microscope

Pollen grains which derived from an anther were pre-fixed with 2.5% glutaraldehyde in sodium phosphate buffer pH 7.2 at 4 °C for 12 hours and were rinsed with sodium phosphate buffer pH 7.2 (3 times). Then, the samples were post-fixed with 1% Osmium tetroxide (OsO_4) for 1 hour and also were rinsed with distilled water for 3 times and also were rinsed with distilled water for 3 times. The samples were dehydrated with Acetone series (20%, 40%, 60, 80% and 100% acetone). The samples were infiltrated and embedded into Spurr's resin. To polymerize plastic, the samples were incubated using a vacuum oven at 80 °C for 7 hours. Then, the samples were cut with a thickness 1000 μm by using an ultramicrotome (Leica; UCT). The sectioned samples were stained with 1% Toluidine Blue for observing under a compound microscope. For transmission electron microscope observation, the samples were sectioned with a thickness 70 nm (as so called ultrathin section), then were stained with lead citrate and 5% uranyl acetate. The ultrathin samples were observed under a transmission electron microscope (Hitachi; HT7700) with a high voltage of electron at 80keV.

RESULTS AND DISCUSSION

The fresh natural dried pollen sample shows several areas of distortion and its shape is no longer round [Figure3]. The pollen grain after treated with chemicals is round and complete [Figure4]. However, the wall surface is covered with pollenkitt, which obscures detailed morphology of the pollen wall. The pollen grains after being boiled in 10% potassium hydroxide [Figure 5, 6, 7] to clean the pollen surface. It turns out that the pollens are damaged from the effect of temperature and potassium hydroxide. The grain appears broken, distorted and shrunk [Figure 8, 9]. The level of pollen damage occurs from slightly damage to highly damage. Therefore, it is not possible to report the size, shape and pattern of the pollen wall. We further investigated the pollens with light microscope and transmission electron microscope why pollens were damaged after being boiled in 10% potassium hydroxide. The light micrograph of a cross-section view of a pollen grain [Figure9] and the transmission electron micrograph of a cross-section view in more details [Figure10] shows the structure of pollen wall of *Curcuma supraneeana* consisting of an outer exine and an inner intine. On the outer exine, pollenkitt covers on the surface. From TEM images, we found that the exine layer is 1.380-2.390 μm thick and the pollenkitt layer thickness is varied from 22.30 nm to 1,290 nm. The intine layer is 54.60-243 nm thick. Note that, the intine layer is thinner than the exine layer. The pollenkitt thickness is varied widely.

Table 1. Summary data of *S. supraneeanae* layer thickness in the pollen wall.

Layer of pollen wall	Thickness of layer (nm)	
	Range	Average
Pollenkitt layer	22.30-1,290.00	277.85±375.24
Exine layer	1,380.00-2,390.00	1,797.00±284.27
Intine layer	54.60-243.00	124.17±39.78

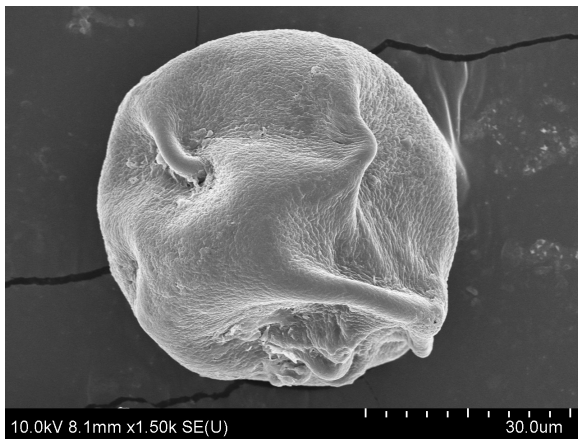


Figure 3. Scanning electron micrograph of a fresh pollen grain of *Curcuma supraneeana* after kept in a desiccator showing distorted pollen grain and withered surface.

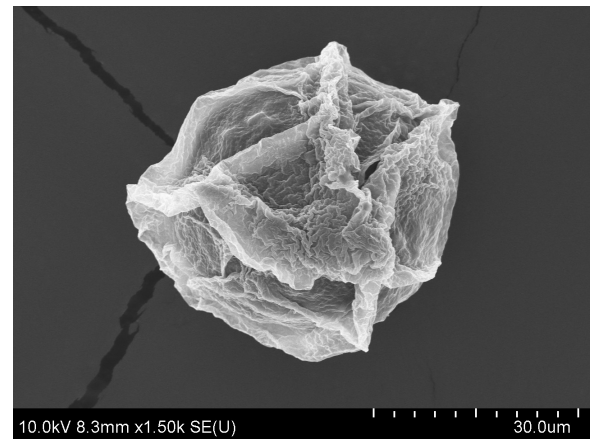


Figure 6. Scanning electron micrograph of a pollen grain of *Curcuma supraneeana* after being boiled in 10% potassium hydroxide and treated with the chemical preparation showing distorted pollen grain.

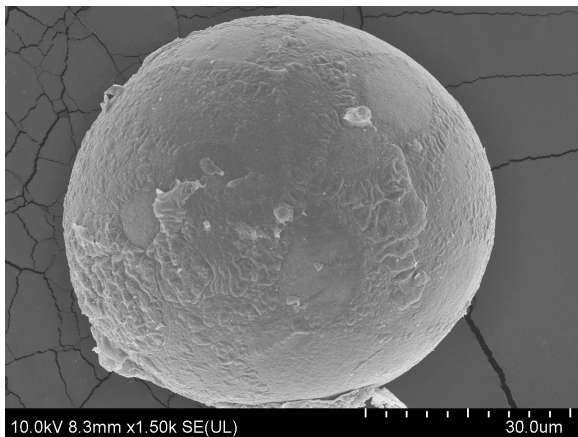


Figure 4. Scanning electron micrograph of a pollen grain of *Curcuma supraneeana* after prepared by the chemical preparation showing a round shape and a fuzzy surface.

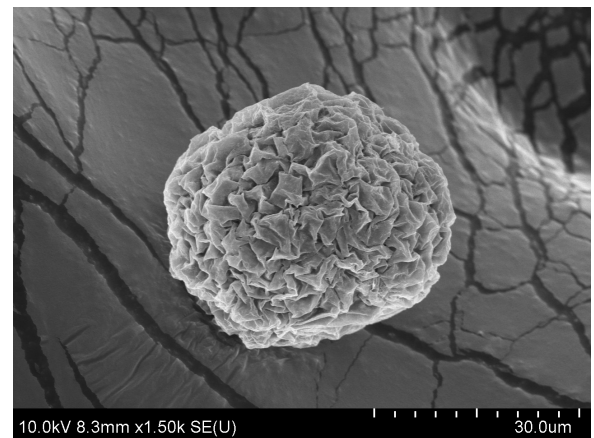


Figure 7. Scanning electron micrograph of a pollen grain of *Curcuma supraneeana* after being boiled in 10% potassium hydroxide and treated with the chemical preparation showing distorted and shrunk pollen grain.

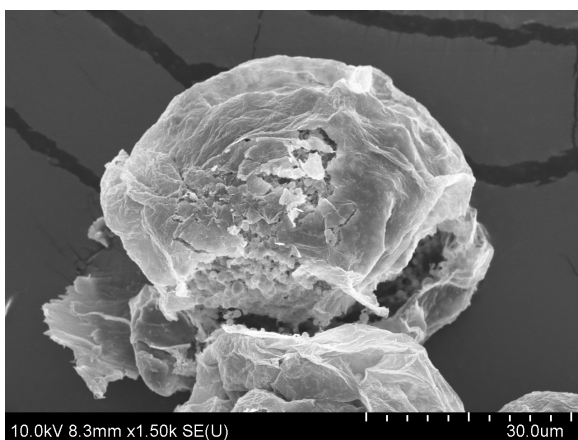


Figure 5. Scanning electron micrograph of a pollen grain of *Curcuma supraneeana* after being boiled in 10% potassium hydroxide and treated with the chemical preparation showing broken pollen grain.

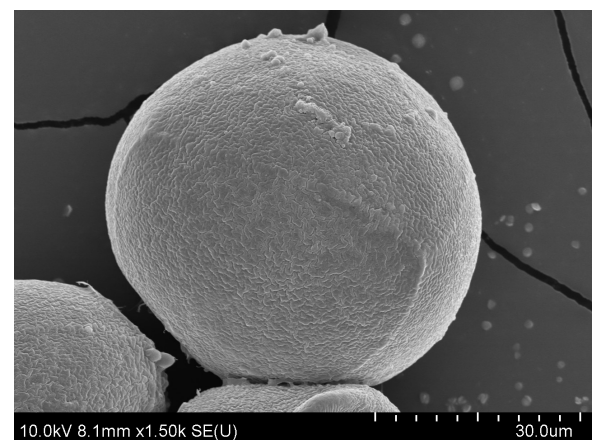


Figure 8. Scanning electron micrograph of a pollen grain of *Curcuma supraneeana* in dehiscient anther after treated with the chemical preparation showing a clear surface of the pollen grain.

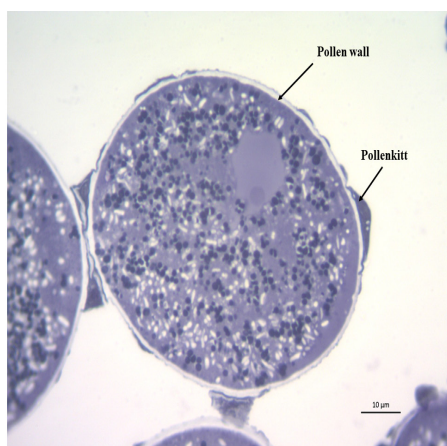


Figure 9. Photograph of a cross-section of *Curcuma supraneeana* pollen.

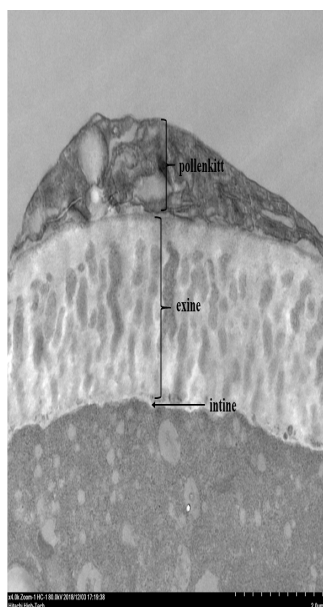


Figure 10. Transmission electron micrograph of a cross-section pollen of *Curcuma supraneeana* showing the wall, pollenkitt, exine, and intine.

It is noted that the pollen of *Curcuma supraneeana* cannot tolerate high temperature and alkaline solution in boiling process when compared with the pollens of other plants. Erdtman (1972) reported the exine thickness of *Aegialitis rotundifolia* pollen was about 8 μm and that of *Hakea bipinnatifida* pollen was 5 μm . Both are thicker than that of *Curcuma supraneeana* pollen.

On the observation of the effect of pollen stage on sample preparation, two growth stages were examined by scanning electron microscope. We found that the surfaces of pollen wall were different. At the stage of beginning to bloom, there is a lot of pollenkitt cover on the pollen wall of anther [Figure 4]. While the pollen wall of dehiscence anther from a full bloom flower are clear and shows better details on the exine pattern of the pollen wall as shown in Figure 8. We notice that the pollens from dehiscent anthers of a blooming stage showed a good result on preparation, may be because of they can withstand to chemical treatment better than pollens from the anthers at before blooming stage. The mature stage of pollen may reduce breaking, denting, and shrinking on sample preparation. Erdtman (1972) found that most of pollen grains of family Zingiberaceae are nonaperture and have a spheroidal shape. The pollen walls are thin and cannot resist acetolysis processes.

CONCLUSIONS

Among three sample preparation methods above. The chemical fixation technique is best method for fragile pollens because it can preserve morphology of the pollen for study under microscope. However, the pollen surface was not clear enough to study pollen wall pattern. Furthermore, growth stage of the pollen is also an important factor that should be kept in mind. We suggested that pollens from dehiscent anther should be selected to observe a clear detail of the exine pattern of the pollen wall because there are small amount of pollenkitt attach on the pollen wall.

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