Microsc. Microanal. Res. 2022 35(1) 26-32

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.

Calcium Oxalate Crystals in Thai *Peperomia pellucida* (L.) Kunth Stems and Leaves

Yupadee Paopun[1*], Piyanan Thanomchat[1], Patcharee Umrung[1] and Supapan Seraphin[2]

[1] *Scientific Equipment and Research Division, Kasetsart University Research and Development Institute, 50 Ngamwongwan Road, Ladyao, Chatujak, Bangkok 10900, Thailand*
[2] *Department of Materials Science and Engineering, University of Arizona, Tucson, Arizona 85721, U.S.A.*
*Corresponding author's e-mail address: rdiydp@ku.ac.th

ARTICLE INFO

Article history
Submitted: 28 April 2022
Revised: 16 June 2022
Accepted: 20 June 2022
Available online:30 June 2022

Keywords:
Peperomia pellucida, pepper elder, calcium oxalate, microcrystals, biomineralization

ABSTRACT

Peperomia pellucida (L.) Kunth is one of the most popular local Thai vegetables, especially in the northeast of Thailand where a relatively high rate of kidney stone illness was also found. It has been shown that there is a direct link between consuming high-oxalate vegetables and kidney stone incident. On a contrary, recent studies report the ancient traditional uses of *P. pellucida* in treating fever, high blood pressure, diabetes, skin disorder, among many others. Further studies are needed to investigate the controversial effects of *P. pellucida*. In this study, we used light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) to examine *P. pellucida* stems and leaves. Various crystal shapes of calcium oxalate were found including druses, prismatic, hexagonal and octahedron in both stems and leaves with about the same frequencies. One hundred of each crystal form were examined. The crystal sizes were varied drastically ranging from 0.11 to 27.64 µm. The raphide crystal form was not found, unlike some of the previously studied *Peperomia* species. EDS spectra and X-ray maps show that the crystals were composed primarily of calcium and oxygen. This study was a basic research with potentials to be used for the medicinal field and for anyone who wants to prevent themselves from getting too much calcium oxalate from plants.

INTRODUCTION

P. pellucida (L.) Kunth is a common, fleshy annual herb and a local vegetable of Thailand that belongs to the family *Piperaceae*. The common name is pepper elder and the Thai name is Phak krasang. The plant is found in many South American and Asian countries. The specie develops during rainy periods and thrives in loose, humid soils under the shade of trees. It grows in moist habitat and found throughout wet areas of forests and cultivated land. The plants can grow to about 20-40 cm high with tiny flowers on a spike [17]. The stem is erect, branched, and glabrous as shown in Figure 1. The leaf is shiny and has a shape of ovate or ovate-triangular (heart shaped). The leaf surface is glabrous and translucent. The base of the leaf is cordate and the apex is acute or obtuse [20]. The leaves and stalk of the plant are edible and can be eaten as fresh salad. Several studies reported that *P. pellucida* can be used as a medicinal plant for antihypertensive remedy, for treatment of headache, fever, eczema, abdominal pains, and convulsions [3,11]. Hamzah *et al*. (2012) shows that *P. pellucida* in diet can reduce hyperglycemia in diabetic rats. Akinnibosun *et al*. (2008) shows an antibacterial activity of aqueous and ethanol leaf extracts. Khan *et al*. (2008) and Magdale *et al*. (2014) reported that *P. pellucida* leaf-extract could be used to reduce fever.

On the contrary, several herbaceous plants generate calcium oxalate through biomineralization process to serve several functions. It is found that calcium oxalate is poisonous and harmful to human. It has been identified as one of the causes for kidney stone and urinary

Figure 1. A photograph of *P. pellucida* plant. It is about 10 cm tall. The stem is translucent and the leaves are heart-shaped and shiny. There are two spikes of tiny flowers on the left side of the plant.

tract infections [16]. Siener (2006) reported impacts of dietary habits on kidney stone formation in human. In Thailand, kidney stone patients were found at the highest prevalence (16.9 %) in the northeast provinces [9]. Tosukhowong *et al*. (2007) reported that kidney stone patients in that region consumed mainly local high-oxalate vegetables including *P. pellucida*, which is found abundantly in Thailand. Several groups of Thai researchers reported observations using light microscopes to catalog a list of indigenous plants in Thailand that contain calcium oxalate, for example, a study by Khoomgratok *et al*. (2006) showing *P. pellucida* contains calcium oxalate in druse form only. To the best of our knowledge, there is no study on the distribution of calcium oxalate crystals in the stems and leaves of *P. pellucida*. Therefore, in this study, we used LM, SEM, EDS and TEM to identify size, shape, and elemental composition of calcium oxalate crystals in the stems and leaves of *P. pellucida*. We studied crystal types and sizes in both plant organs because Thai people eat the whole plant as a vegetable. As stated, *P. pellucida* could cause kidney stone and yet could cure several illnesses as well. Obviously, further studies in various aspects of this species of common Thai plant are needed. Therefore, the aims of this study were to investigate the diversify and frequency of calcium oxalate crystals present in the stem and leaf cells of *P. pellucida* grown in Thailand. This study is a basic research of one of the local vegetables and the medicinal herbs in Thailand.

MATERIALS AND METHODS

Sampling and Preparation

The samples of *P. pellucida* leaves and stems were collected from plants that grew in a nursery at Kasetsart University. Fresh samples were washed with distilled water. To prepare specimens for observing under LM, a clean razor blade was used to cut a fresh clean stem into thin cross sections. The samples were stained with 1% safranin in distilled water. Those sections were transferred onto a glass slide and covered it with a cover slid. For a leaf sample, a clean leaf was submerged in 10% aqueous KOH for three days in order to make the sample transparent. Rinsed with distilled water for 5 minutes three times. Put it in 95% ethanol for one day to dehydrate the leaf. The samples were stained with 1% safranin in distilled water. Placed the sample on a glass slide. Put a drop of mounting medium and covered it with a cover slid. Waited one day for the slide to dry.

The preparation TEM and LM specimens, the leaf samples were cut into 1x2 mm^2 and pre-fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 for overnight at 4 °C in refrigerator. The samples were post-fixed in 1% osmium tetroxide for 1 hr. They were dehydrated in acetone series, infiltrated and embedded in Spurr's resin. They were cut into 2000 nm thin with a glass knife on an ultramicrotome (Leica: EM UC7), stained with 1% toluidine blue solution and examined under the LM. The 100 nm of thin sections were double stained with 5% uranyl acetate in distilled water and lead citrate for 15 minutes. Then, they were examined under the TEM.

To prepare SEM specimens, cut fresh clean stems and leaves into 3x5 mm^2 and dried in a freeze dryer (Quorum: K750X) for 15 hrs. The samples were coated with a thin layer of carbon to increase conductivity and reducing charging in SEM. We used a Quorum: Q150R sputter coater to deposit a carbon layer for 120 seconds.

Structural Analysis

The specimens were examined and observed under LM (Zeiss model Axiostar plus) and TEM, (Hitachi model HT7700), operated at 80 kV. For SEM analysis, we used Field-emission SEM (Hitachi SU8020 and Hitachi SU4800), operated at 10 kV for imaging and 20 kV for Energy Dispersive X-ray analysis (EDAX: Apollo X).

Crystal Size Measurement

SEM images were analyzed using Image J software to determine crystal shapes and sizes. Image J software is an image processing program developed at the National Institutes of Health (http://imagej.nih.gov/ij/). The measurement scale is set by entering known distance between two points in an image before the dimensions of crystals were measured.

RESULTS AND DISCUSSION

The location of calcium oxalate crystals in the cells is shown in Figure 2 (A) for a stem specimen and (B) for a leaf specimen. Arrows on the images indicate examples of crystals in the cells. In stems, the crystals were found mostly in the cortex. In leaves, the crystals located along the veins to form macropatterns as showed in Figure 2 (B). The palisade parenchyma cell in the mesophyll layer of the leaf contained calcium oxalate crystals as showed in Figure 3. The calcium oxalate crystals are very clear in a palisade cell when viewed under TEM as showed in Figure 4. The parenchyma cells in the cortex of stem contain calcium oxalate crystal as showed in Figure 5. Horner *et al*. (2009) illustrated systematic investigation of macropatterns in *Peperomia* spp. They studied leaves from 45 species to identify their macropatterns and to compare macropatterns with molecular data. They found that

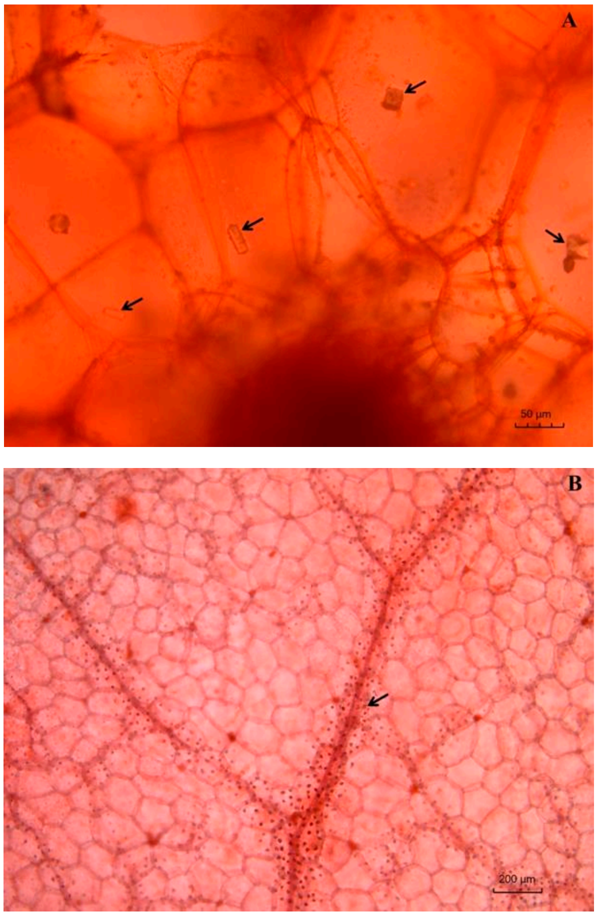


Figure 2. Optical micrographs of *P. pellucida.* (A) a cross-section view of a stem; (B) an image of a leaf showing crystal macropatterns. Arrows show examples of crystals in the specimen.

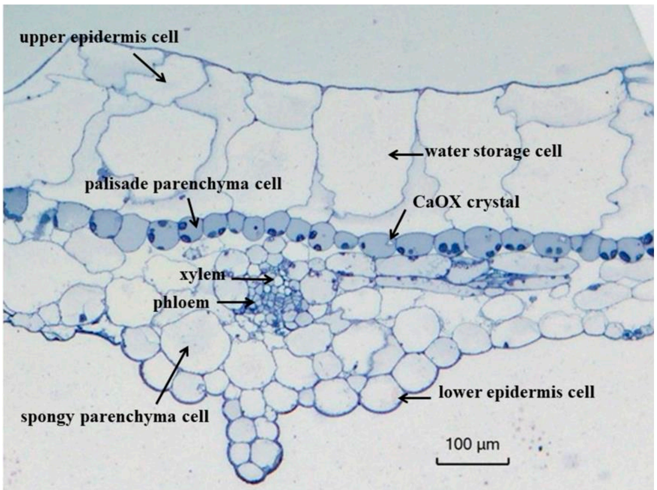


Figure 3. LM image of calcium oxalate crystal in palisade parenchyma cells of *P. pellucida leaf.*

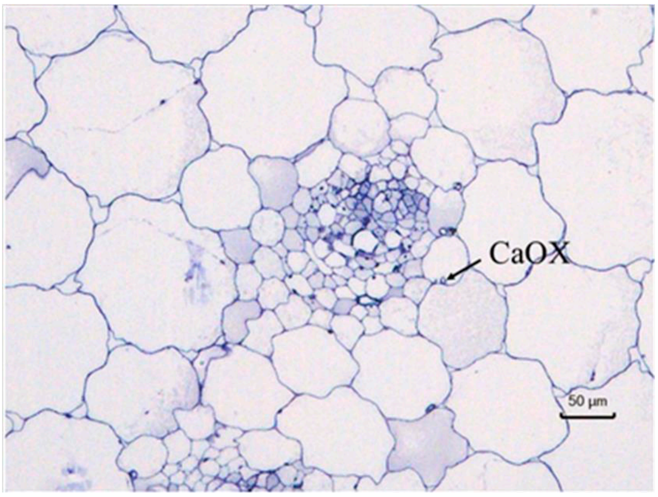


Figure 5. LM image of calcium oxalate crystal in cortex parenchyma cells of *P. pellucida stem.*

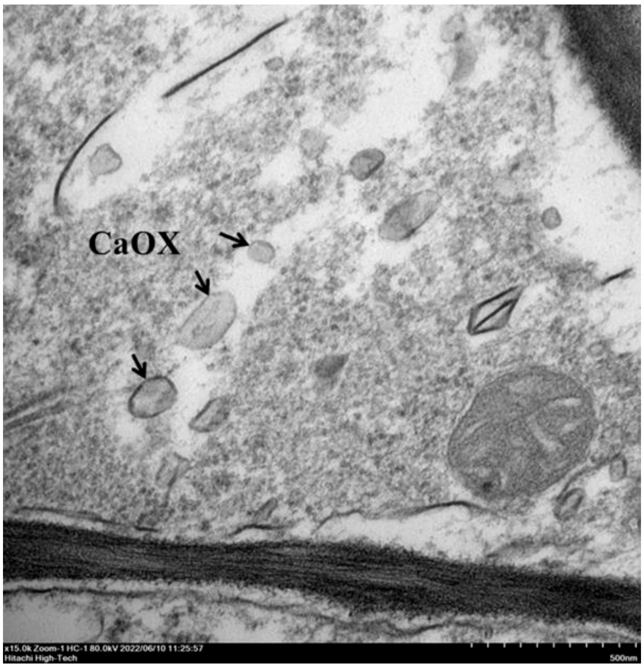


Figure 4. TEM image of a cluster of calcium oxalate crystals in a palisade parenchyma cell of a *P. pellucida* leaf. the idioblast. Bars¯10 μm.

all but one species (81.8%) had a uniform layer of crystals. The rest (18.2%) had a single druse per cell above veins forming a reticular pattern. Crystal macropattern evolution in *Peperomia* spp. is generally characterized by an increasing complexity of the distribution of various forms of calcium oxalate crystals. Kuo-Huang *et al*. (2007) reported a role of calcium oxalate crystals in the photosynthesis process. They found that the position of crystals within the cells changed in response to growth under different light intensity. Horner *et al*. (2012) studied crystal macropatterns in leaves of *Piper* spp. and *Peperomia* spp. and suggested that plants might use crystals for light gathering and reflection for efficient photosynthesis under low-intensity light environments. Some researchers suggested that plants generated calcium oxalate as part of a defense mechanism when there is too much calcium in their environment [16]. This surviving mechanism of plants could be harmful to human because calcium oxalate is poisonous and can cause kidney stone disease.

Calcium oxalate crystals in plants could have variety of morphologies including crystal sands, prisms, styloids (block-like rhombohedral or prismatic), druse (multifaceted conglomerate spherical cluster of crystals having many facets radiating from a central core), and raphides (needle-like crystals in bundles of tens to hundreds of crystals). The shape, size, and distribution of calcium oxalate crystals are specific to plant species [10]. We found that there were patterns of CaOX crystals inside the *P. pellucida* stem as shown in Figure6 (A); an overview of cells containing relatively dense calcium oxalate crystals, Figure 6 (B); including a group of druse crystals, Figure 6 (C); a big prism crystal among much smaller crystals of other forms, Figure 6 (D); numerous crystals in octahedral forms, Figure6 (E); a mixture of druses and octahedral crystals, Figure 6 (F); a chain of about a dozen octahedral crystals fused together. We found the same various types of CaOX crystals in the stems as in the leaves. Figure7 shows various crystal shapes and sizes in *P. pellucida* leaves. Figure 7 (A); is a single druse in a cell, Figure 7 (B); numerous crystals in octahedral form, Figure 7 (C); a pair of hexagonal crystals, Figure 7 (D); a druse on a prism, which appears like a "gift box" in a micrometer size, Figure 7 (E); side view of a druse on a prism, Figure 7 (F); a cluster of large octahedral crystals. The average sizes of these crystals were listed in Table 1. Overall, crystal sizes were ranging from 0.11 to 27.64 μm. The crystals had the same range (8-10 μm) as those found in purple allamanda (Thai name -rang cheut). However, in purple allamanda, the calcium oxalate had needle-like structure [18]. On the other hand, calcium oxalate crystals in sweet basil leaves were of crystal-sand type with cluster size about 2 to 4 μm in diameter and the size of individual crystal in the cluster was about 200-500 nm [19].

Our observation was in contrast to the study by Khoomgratok *et al*. (2006) who reported only druse crystal form in *P. pellucida* and the study by Horner *et al*. (2009) who reported that *P. pellucida* had crystals in the forms of druse, raphides, and prism. We did not observe any raphides in neither the stems nor leaves in this present study.

The chemical composition of crystals was determined using Energy-Dispersive X-ray Spectroscopy (EDS) equipped on the SEM. The EDS spectrum from the central region of the druse in Figure 7 (A) was shown in Figure 8. The spectrum indicated the presence of carbon,

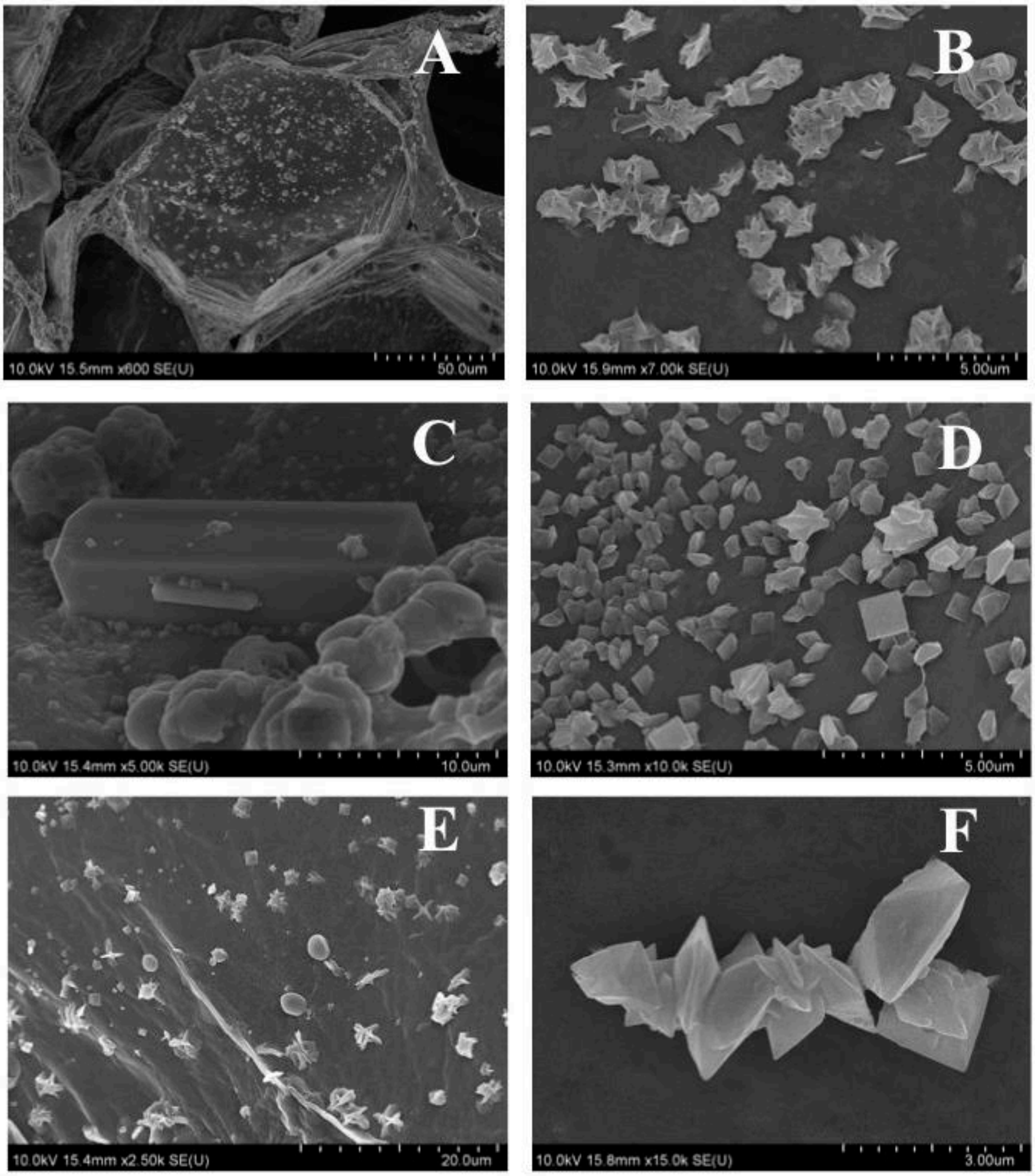


Figure 6. SEM images of various forms of crystals in a stem. (A) an overview of cells containing relatively dense calcium oxalate crystals; (B) a group of druse crystals; (C) a big prism crystal among much smaller crystals of other forms; (D) numerous crystals in hexagonal and octahedral forms; (E) a mixture of druses and octahedron crystals; (F) a chain of about a dozen octahedron crystals fused together.

oxygen, sodium, chlorine, and calcium. Figure 9 (A) was a SEM image of a crystal chain from a stem. Figure 9 (B) and (C) were the corresponding X-ray maps of calcium and oxygen, respectively. Both X-ray maps were acquired using K_{α}-line of each element. It could be seen that the X-ray intensity in the map corresponded to the region of the crystals in the image (A), suggesting that the crystals contain calcium and oxygen. Figure 10 (A) was a SEM image of druse on a prism crystal from a leaf. Figure10 (B) and (C) were the corresponding X-ray maps of calcium and oxygen, respectively. This result confirmed that the crystals composed of calcium and oxygen.

We planned to extract these crystals from the plant tissued in our further study in order to compare crystal density in stem versus in leaves. Additional analysis using X-ray diffraction will be used to identify the specific phase of calcium oxalate and Raman spectroscopy will provide information about types of bonding in the crystals. Further information is needed in order to elucidate possible formation mechanisms of calcium oxalate crystals in *P. pellucida*.

The presence of calcium oxalate crystals in the stems and leaves cells promotes the growth of *P. pellucida* since these crystals help to reflect light to the chloroplasts, allowing them to photosynthesize more. The light is good so the plants are growing well. Horner (2012) found that the druse of calcium oxalate crystal was surrounded by chloroplast in palisade parenchyma cells of *P. obtusifolia* that the palisade parenchyma cells were involved in photosynthesis of plants.The light waves pass

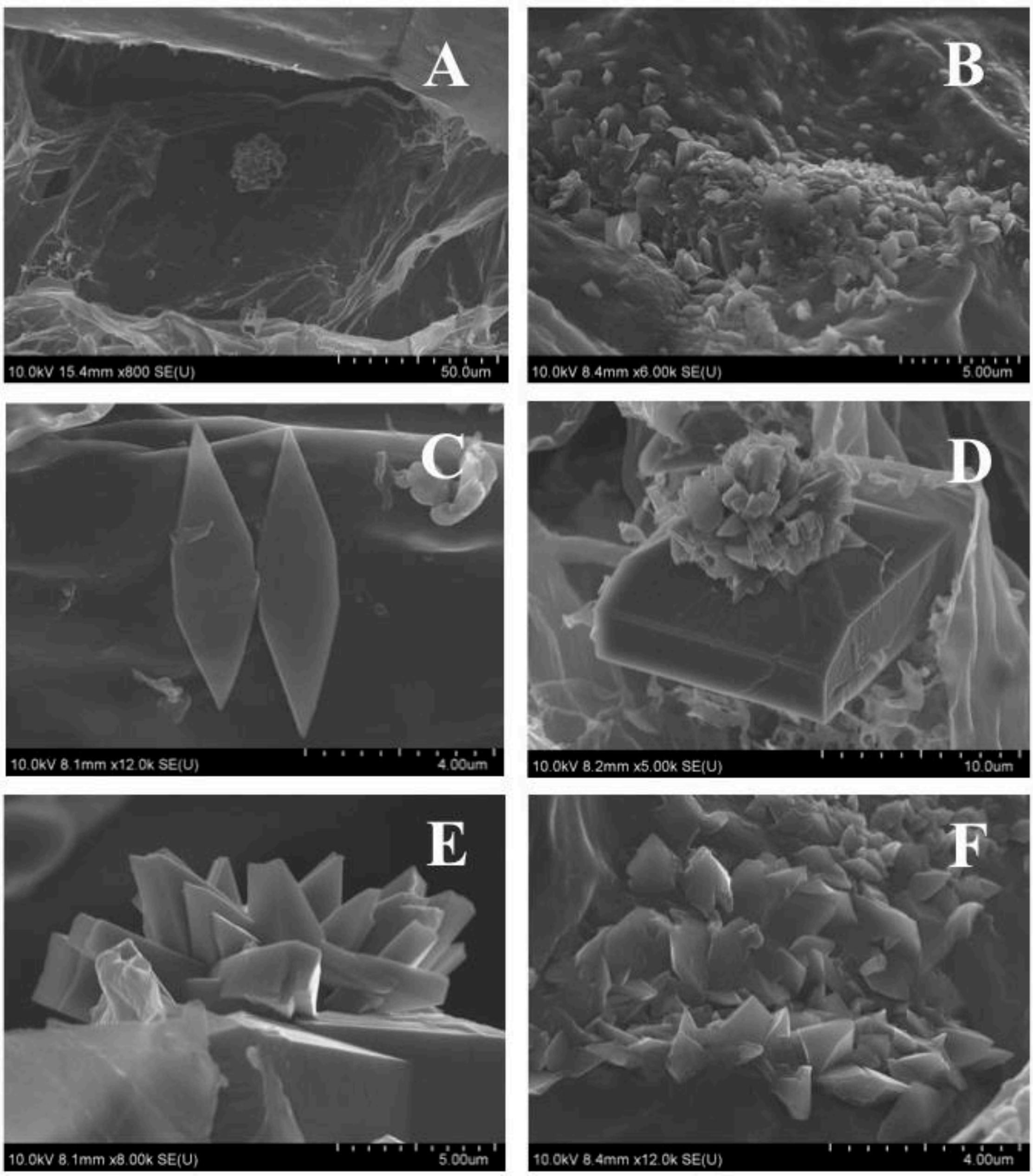


Figure 7. SEM images of various crystal forms in leaves. (A) A single druse in a cell; (B) numerous crystals in a octahedral form; (C) a pair of hexagonal crystals; (D) a druse on a prism, which appears like a “gift box” in a micrometer size; (E) side view of a druse on a prism; (F) a cluster of large octahedron crystals.

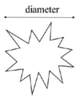
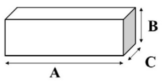
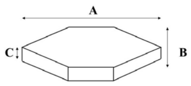
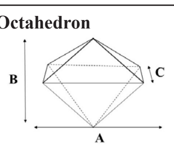
into the mesophyll cells and are collected and dispersed by the druses to surrounding chloroplasts in the mesophyll cells.

Generating calcium oxalate crystals in plant cells is a protective mechanism of plants from receiving too much calcium. However, it is harmful to human, especially when people consume too much of plant organs containing high calcium oxalate. It can cause kidney stones. The study on leaf surface, leaf anatomy could be used to classify plants. The knowledge of calcium oxalate in plant cell can be used for the medicinal field and for anyone who wants to prevent themselves from getting too much calcium oxalate. The obtained information can be used as a guideline for consumption or adjusting the amount and frequency of consuming this local vegetable.

CONCLUSIONS

P. pellucida stems and leaves contained numerous calcium oxalate crystals having sizes ranging from 0.11 to 27.64 **μ**m. The crystal shapes and forms were varied slightly in stems versus in leaves. We observed druse, prisms, hexagonal, and octahedral shapes. None of them were needle-like raphides. EDS X-ray analysis confirmed that the crystals were calcium oxalate.

Table 1. Size analysis of various crystal forms in *P. pellucida* leaves and Stem.

Types of crystal	**Crystal Size**			
Crystal form	**Diameter of druse crystal** (μm)	**Length (Side A) (μm)**	**High (Side B) (μm)**	**Width (Side C) (μm)**
Druse 	1.06-4.37 (1.91±0.59)	-	-	-
Prism 	-	0.48-2.40 (0.98±0.42)	0.35-1.98 (0.88±0.35)	0.33-1.51 (0.76±0.31)
Hexagonal 	-	1.40-9.22 (4.73±1.85)	0.51-7.58 (1.76±1.18)	0.11-2.18 (0.57±0.35)
Octahedron 	-	2.02-27.64 (9.85±5.94)	0.48-7.38 (2.79±1.58)	0.42-7.53 (2.87±1.67)

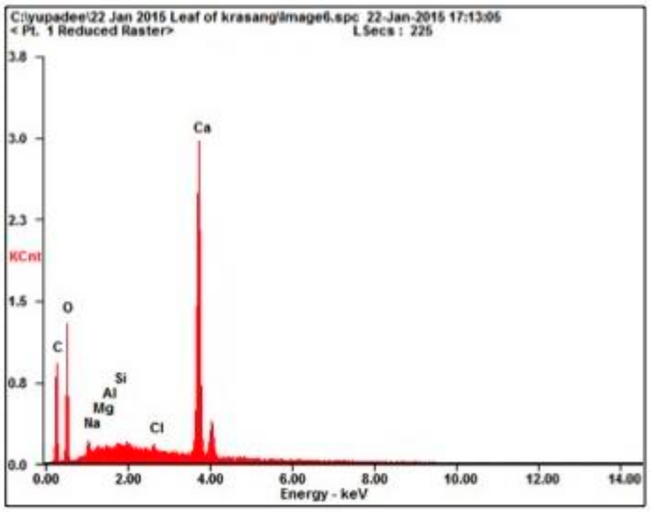


Figure 8. EDS of the druse crystal showed in Figure 4 (A).

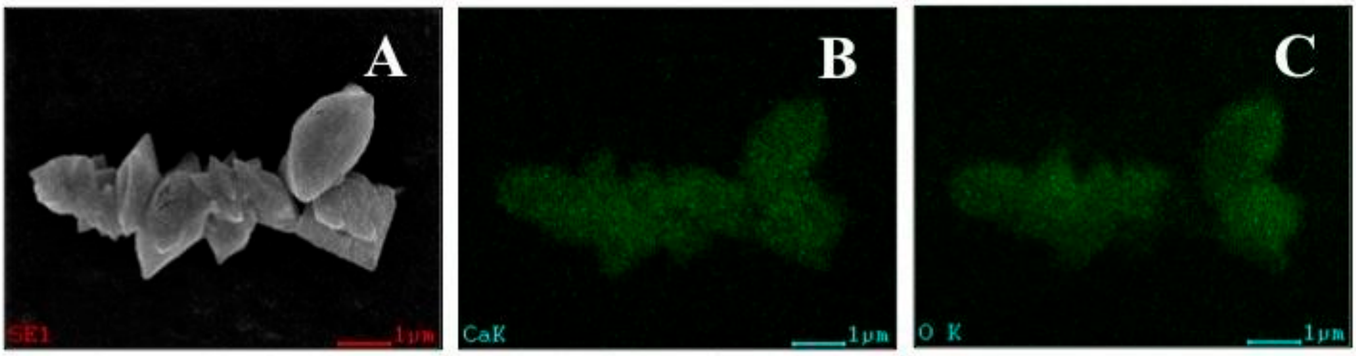


Figure 9. (A) SEM image of crystals in a stem and the corresponding X-ray maps of (B) Ca K_{α} and (C) O K_{α}.

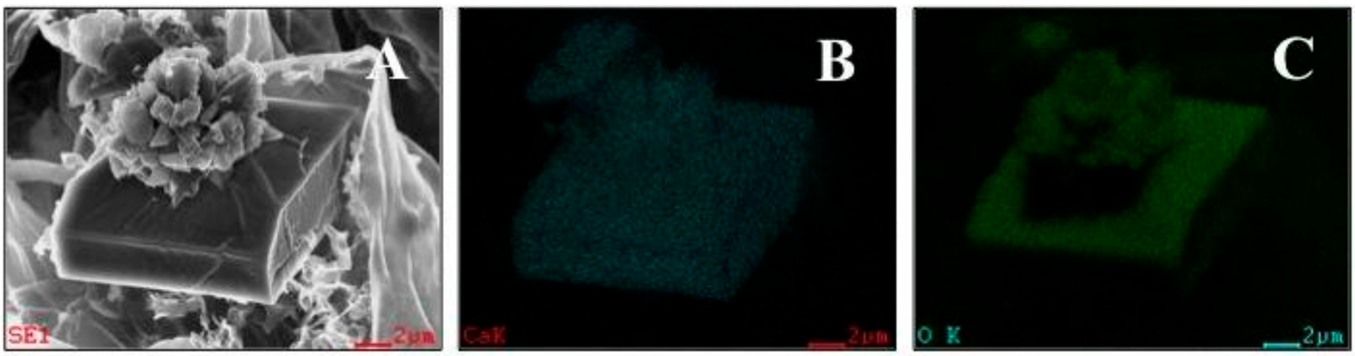


Figure 10. (A) SEM image of "gift box" druse on a prism crystal in a leaf and the corresponding X-ray maps of (B) Ca K_{α} and (C) O K_{α}.

ACKNOWLEDGEMENT

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

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