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Density and distribution of calcium oxalate crystals in vegetative organs of "green" wild taro (*Colocasia esculenta* (L.) Schott.)

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Abstract
The study's aim was to investigate the distribution of calcium oxalate (CaOx) in vegetative organs of "green" morphotype wild taro Colocasia esculenta (L.) Schott. An anatomical study was conducted on the vegetative organs (root, corm, leaf blade, and petiole) of the "green" morphotype wild taro at different developing stages. The transverse sections were double-stained with carmine alum laque–iodin green dye to investigate the density and distribution of CaOx crystals. The four forms of CaOx crystals found in vegetative organs were druse, free needle-liked, raphide bundle, and prism crystals. Prismatic crystals were observed in the root only once. Biforine or biforine-liked cell, an unusual form of raphide bundle, was detected. These crystals were revealed in specialized cells such as epidermis, parenchyma, palisade mesophyll, and spongy mesophyll. The density, size, shape, and distribution of crystals in the vegetative organs varied depending on the type and developmental stage of the organs. The diameter of the druses and the width of the raphide bundles were both increased in the developing leaves compared to that of the young leaves. The width of the raphide bundles of the old leaves was reduced compared to that of the developing leaves. The minimum and maximum sizes of all CaOx crystals varied greatly. The density and distribution of CaOx in the vegetative organs made it facility to protect plants against herbivores. The presence of CaOx in the vegetative organs created a warning signal when used as a source of green vegetables or traditional medicine.

Keywords: "green" wild taro, *colocasia esculenta* (l.) schott, calcium oxalate crystals, vegetative organs

Introduction

Wild taro (Colocasia esculenta (L.) Schott) is wild grow and widely distributed in many territories of Asia, Western Pacific, New Guinea, Australia [1], Southern Europe [2], New Zealand [3], and U.S. [4]. However, it has been little studied and is poorly known about the wild taro in Southeast Asia and the western Pacific [5].

Wild taro is tolerant and grows well in diverse habitats such as canals, lagoons, black water ponds, and wetlands. Based on morphological characteristics, wild taro is separated into three distinct morphotypes: "purple", "green" and "green with purple vein junction on lamina" [6]. For ancient years, wild taro has been used as foodstuff for humans and fodder for animals in Southeast Asia and East Asia [7]. Leaves, which are high in vitamins A, B, and C, are cooked and eaten like spinach, and young shoots are prepared to taste like mushrooms [8]. Leaf and spathe are sold as vegetables in local markets in Assam, India [9]. Local people in Chiang Mai, Thailand are picked young leaves for vegetable cuisine [10]. Salting, sour soup, curry, sauce, packet are cuisines made from the leaf, peduncle, and spathe of inflorescences in Malaysia, Vietnam and Philippines [1, 11, 5]. Farmers are also used as green fodder for pigs [12, 13] and for broilers [14]. Besides, the leaf and flowers of wild taro are used as medicinal herbs in India [15]. Ethnic Communities in Borneo (Indonesia) is used wild taro as medicine such as high blood pressure lowering and for consumption of diabetics [7]. At Uttarakhand Himalayas, corm juice of wild taro is used to treat solastalgia, alopecia areata, hemorrhoids, and congestion of the portal system; leaf juice of wild taro is used to cure hemorrhages, adenitis, otorrhoea, otalgia, and buboes, and petiole juice is used to cure hemorrhages, earache, and rubefacient [16]. Wild taro is one of the medicinal plants sold at local markets in Johor, Malaysia [17]. Taro (*C. esculenta*) has been shown to have anti-tumor, antibacterial (antibacterial and antifungal), antidiabetic, antitoxic and anticancer biological activities [18]. Therefore, taro has various potential for investigating its medicinal and pharmaceutical properties. As of 2019, the COVID-19 pandemic is an ongoing global pandemic that causes acute respiratory syndrome. *C. esculenta* one of 15 medicinal plants that may be useful to treat the novel coronavirus [19].

For humans and animals, however, leaves, stolons, and inflorescences of *C. esculenta* can cause pain, itching, and swelling of the mouth, throat, stomach and even suffocation if not properly prepared before eating, due to the occurrence of CaOx crystals. In large doses, CaOx is a toxic effect and causes salivation, swollen lips, difficulty in swallowing, ulcers of the oral cavity, abdominal pain,, thoracodynia, and chest tightness [20]. In the case of CaOx poisoning, recovery is still possible but can cause permanent damage to the liver and kidneys [21]. In Canada, two cases of poisoning by raw taro leaf were reported to the British Columbia Drug and Poison Information Centre [22]. CaOx crystals in some wild plants that are used in traditional medicine could be caused harmful effects to patients [23]. Calcium oxalate crystals are part of the toxicity mechanism, so research is needed to show little (or no) harm to children, adults, and pets [24].

The aim of this study was to investigate the density and distribution of CaOx crystals in the vegetative organs of wild taro with "green" morphology (Colocasia esculenta (L.) Schott.) to provide data calcium oxalate to identify their species and warn about toxicity.

Materials and Methods

Collection of plant materials

"Green" morphotype Colocasia esculenta (L.) Schott was collected at Cantho province, Mekong Delta, VietNam. The fresh vegetatives of wild taro consisting of the leaf (included petiole and blade leaf) and tuber (including root and corm) were washed to remove dust and dried at room temperature (Figure 1).

- Leaf 1 (young leaf): consists of leaf blade (emerging, curled up, thin, soft, easy to tear, light green color) and petiole (soft, easy to break down, small, slightly green)
- Leaf 2 (developing leaf, counting from the young leaf): consist of leaf blade (opened, soft, easy to tear, green color) and petiole (harder, large, green)
- Leaf 3 (old leaf, counting from the young leaf): consist of leaf blade (thick, dark green) and petiole (hard, larger size, dark green)

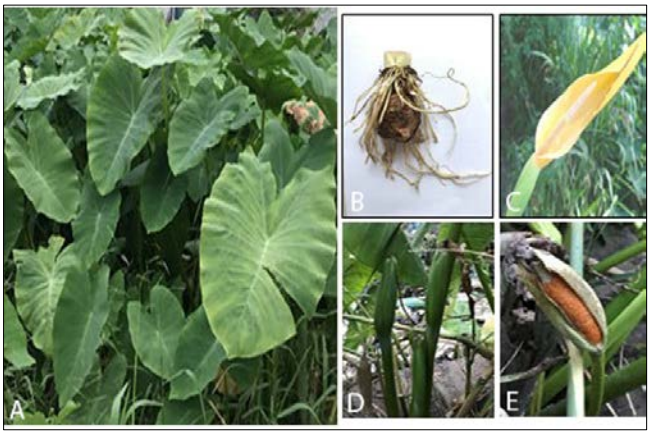

Fig 1: "Green" morphotype wild taro *Colocasia esculenta* (L.) Schott. A. Leaf, B. Tuber, C. Flower, D-E. Fruit

Anatomical study

Leaf blade was cut into thin slices 8 mm long (including leaf lamina and vein). Petiole was divided into 3 parts: near the leaf blade, the middle part, and near the tuber. Split longitudinally each part of petiole, and cut horizontally into a circular arc (radius 5-8 mm). For corm, peel off shaggy dry bark and shell, and cut thin slices horizontally (edges 5-8 mm) through starchy flesh. At root, cut thin slices horizontally of root hair (maturation) and lateral zone. All sections were double-stained with carmine alum laque-iodin green dye (carmine stained pink cellulose cell wall, and green iodine stained green carpentry cell wall). The staining protocol was following: soaked slices in sodium hypochlorite (bleach solution) for 15 minutes; rinse with distilled water to clean sodium hypochlorite (at least 5 times); soak in 5% acetic acid for 5 minutes; rinse with distilled water (at least 5 times) until there is no more smell of acetic acid; stain with carmine alum laque–iodin green dye for 3 minutes; wash samples with distilled water to remove the dye and keep specimens in water. An Olympus light microscope was used to view the slices in drops of glycerin.

The density of crystals was investigated by counting the number of each type of crystals per 1 mm^2 unit of specimen area. The density of CaOx crystals is conventionally expressed as follows: 0 crystal (-), 1 - 10 crystals/ 1mm^2 (+, rarely/scatteredly), 10 - 30 crystals/ 1mm^2 (++, occationally), 30 - 60 crystals/ 1mm^2 (+++, frequently), > 60 crystals / 1mm^2 (++++, very frequently).

Results

The density of CaOx crystals in vegetative organs of "green" morphotype wild taro was showed in Table 1.

Table 1: Distribution of calcium oxalate crystals in the root, corm, petiole, and leaf blade

	Root	Corm	Petiole			Leaf blade		
			1	2	3	1	2	3
Druse	+	++++	+	++	++++	++	+++	++++
Raphide bundle	++	++++	+++	++++	+++	++	++	++
Free needle-liked	++	++++	+++	++	++	+	+	+
Prism	*							

Druses were found sparely in root and petiole 1; occasionally in petiole 2 and leaf blade 2; frequently in leaf blade 2; very frequently in leaf blade 3. Raphide bundles were present occasionally in the root, leaf blade 1, leaf blade 2, and leaf blade 3; frequently in petiole 1 and petiole 3; very frequently in the corm, and petiole 2. Free needle-liked crystals were scattered in leaf blade 1, leaf blade 2, and leaf blade 3; occasional in the root, petiole 2, and petiole 3; very frequently in the corm. Prism was very rare. It was observed only once.

The density and distribution of calcium oxalate crystals in root

Four forms of CaOx crystals were detected in the root of "green" wild taro as free needle-liked crystals, raphide bundles, druses, and prisms (Figure 2A-D). The cross-sections of maturation (root hair) and lateral zone of root revealed differences in the densities and forms of these crystals.

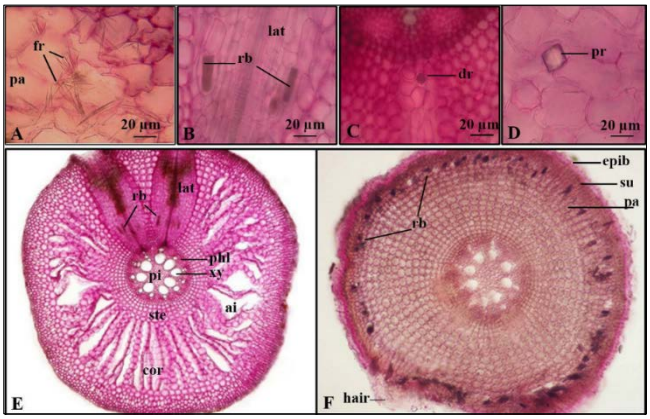

Fig 2: Calcium oxalate crystals and distribution of calcium oxalate crystals in root

A-D: Calcium oxalate crystals, E: Lateral zone (4X), F: Maturation (Root hair) zone (4X)

Abbreviations: fr – free needle-liked, rb – raphide bundle; dr – druse, pr – prism, lat – lateral zone, ai – air space, phl – phloem, xy – xylem, pi – pith, ste – stele, cor – cortex, epib – epiblema,

su – suberin, pa – parenchymal cell, hair – root hair At the lateral zone, druses and prisms were rarely observed in the parenchyma tissue of the cortex. Specially, prism was observed only once. Free needle-liked crystals (but not seen at low magnification) were scattered distributed in the stele. In the phloem region of the emerging lateral root, several raphide bundles and free needle-liked crystals were found (Figure 2E).

At the maturation (root hair) zone, free needle-liked crystals were occasionally distributed from cortex to stele. The raphide bundles were scattered circularly in the parenchymal tissue of the cortex. No druse and prism crystals were detected at the mature zone (Figure 2F).

The size of free needle-liked crystals, raphide bundles, druses, and prisms was indicated on Table 2. The minimum and maximum sizes of all forms of CaOx crystals varied greatly, specially at the length of raphide bundle and free needle-liked crystals.

Table 2: Size of CaOx crystals in root and corm (Mean±SD µm).

		Druse	Raphide bundle		Free needle-liked	Prism
		Diameter	Length	Width	Length	
Root	Mean± SD	20.40±8.61	71.81±20.64	16.70±4.61	39.51±22.9	38x25
	Min - Max	4.8 - 33.60	26.40 - 148.8	2.4 - 26.4	9.60 - 100.80	-
Corm	Mean± SD	36.83±6.96	130.05±61.51	29.75±10.51	84.20±22.28	-
	Min - Max	24.0 - 50.4	57.60 - 340,8	12.24 - 69.6	26.40 - 122.4	-

Prism: n =1; druses: n_{root}=48, n_{corm}=46; raphide bundles: n_{root}=74, n_{corm}=151; free needle-liked: n_{root}=110, n_{corm}=73 (n: number of crystals were measured size)

The density and distribution of calcium oxalate crystals in corm

Three forms of CaOx crystals detected in corm of "green" wild taro were free needle-liked crystals, raphide bundle, and druses. These crystals were densely distributed throughout the starchy flesh of corm. Free needle-liked crystals are scattered individually or clustered in groups. Raphide bundles were found in large idioblasts. An unusual raphide bundle to be known as biforine or biforine-liked cells was revealed. A few bundles were observed to be expelling crystals into air spaces. Druses located in specialized cellsknown as idioblasts (Figure 3).

The size of free needle-liked crystals, raphide bundles, and druses was recorded. The minimum and maximum sizes of all forms of crystals differed greatly, specially at the length of raphide bundle and free needle-liked crystals (Table 2).

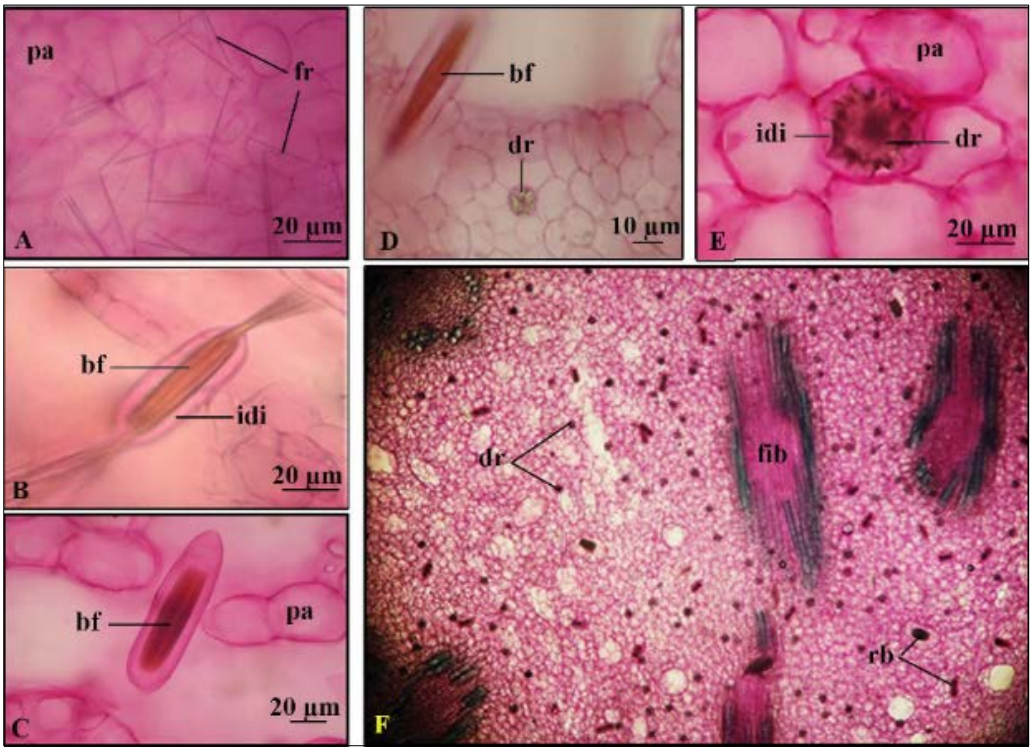

Fig 3: Calcium oxalate crystals and distribution of calcium oxalate crystals in corm

A-E: Calcium oxalate crystals; F: Cross section of starchy flesh of corm (4X)

Abbreviations: bf – biforine or biforine-liked cell, idi – idioblast, fib – fibre, others as in Figure 2

Density and distribution of calcium oxalate crystals in leaf blade

Free needle-liked crystals, raphide bundles, and druses were present in the leaf blade. Free needle-liked crystals were found

rarely in leaf blades 1, 2, and 3 (but not seen at low magnification). Raphide bundles occasionally occurred in the central spongy mesophyll cells of leaf blades 1, 2, and 3. Raphide bundles were detected in large idioblasts. The biforine or biforine-liked cell crystals were suspended in the intercellular airspaces**.** Druses were distributed occasionally in leaf blade 1, frequently in leaf blade 2, and very frequently in leaf blade 3. The palisade mesophyll cells were revealed the abundance of druses with variable sizes. The spongy mesophyll cells near the lower epidermis contained a small number of the larger size druses. The spongy mesophyll cells located inside of leaf were occasionally detected druses.

At leaf vein, druses were detected at the upper side of the vein, but not present on the underside. At the center of the veination, free needle-liked crystals, druses and raphide bundles were scatteredly detected (Figure 4).

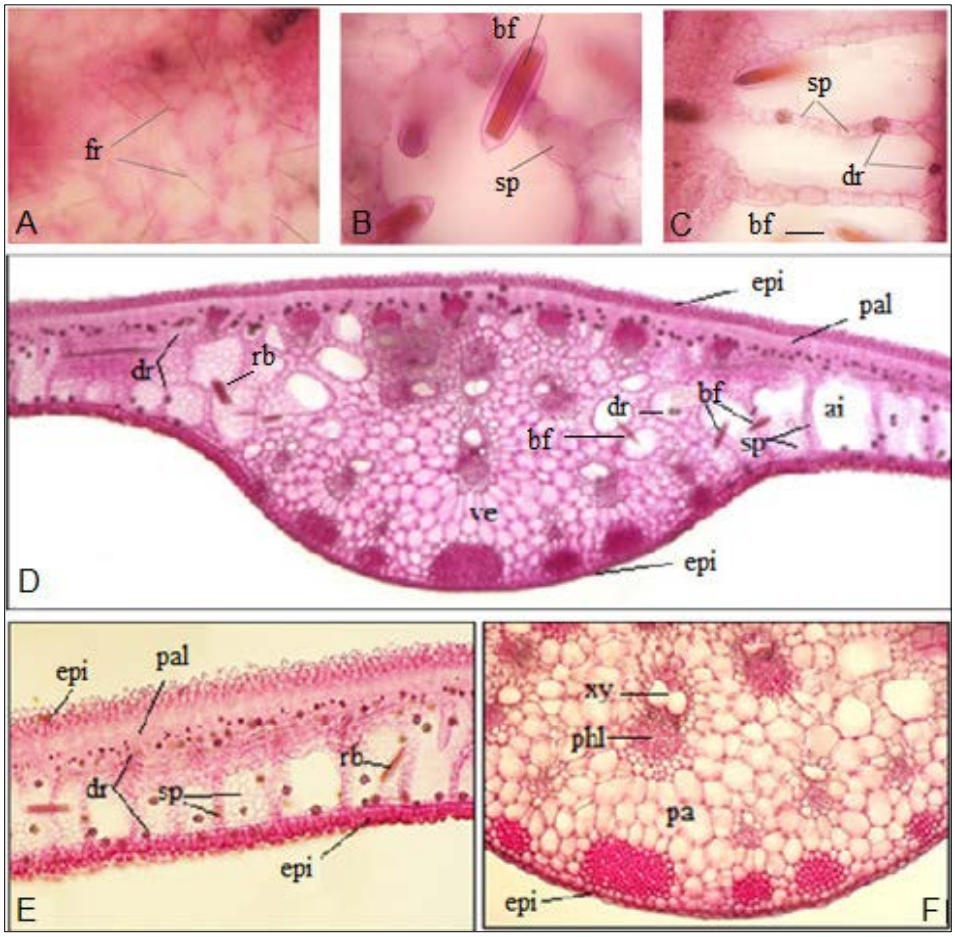

Fig 4: Calcium oxalate crystals and distribution of calcium oxalate crystals in leaf blade 2; A-C: Calcium oxalate crystals; D: Cross section of leaf blade (4X); E: Lamina (10X); D: Lower part of vein (10X)

Abbreviations: epi – epidermis, pal – palisade mesophyll cell, sp – spongy mesophyll cell, ve – vein, others as in Figure 2.

The average size of druses, raphide bundles, and free needle-liked crystals in leaf blades was recorded in Table 3. The minimum and maximum sizes of all forms of CaOx crystals differed greatly. The diameter of druse crystals in leaf blade 1 was smaller than that of druses in leaf blades 2 and 3 ($p < 0.05$). The length of raphide bundles and free needle-liked crystals at leaf blade 1, leaf 2, and leaf blade 3 were no statistically significant differences ($p > 0.05$). The width of raphide bundles in leaf blade 2 was larger than that of raphide bundles in leaf blade 1, but smaller than that of leaf blade 3 ($p < 0.05$).

Table 3 Size of calcium oxalate crystals in leaf blade (Mean ± SD µm).

	Druse diameter	Raphide bundle		Free needle-liked length
		Length	Width	
Leaf blade 1	15.01±5.93[b]	101.07±39.56[a]	20.42±8.83[b]	54.20±20.9[a]
Leaf blade 2	18.00±6.15[a]	107.35±47.90[a]	23.02±10.19[a]	50.99±23.26[a]
Leaf blade 3	18.08±5.50[a]	105.45±51.60[a]	17.63±5.83[c]	55.62±22.22[a]
Min - Max	2.4 - 28.8	28.8 - 456	4.8 - 79.2	7.2 - 158.4

Druses: n_1=167, n_2=190, n_3=180; Raphide bundles: n_1=237, n_2=266, n_3=194; Free needle-liked: n_1=89, n_2 =73, n_3 =86; $n_{1,2,3}$: number of crystals were measured at leaf blade 1, leaf blade 2, or leaf blade 3 Values are expressed as Mean ± SD and analyzed by ANOVA ($p < 0.05$). a, b: $p < 0,05$ when compared size of calcium oxalate crystals among leaf blade 1, leaf blade 2, and leaf blade 3

Density and distribution of calcium oxalate crystals in petiole

Three forms of CaOx crystals were detected in the petiole of "green" wild taro as free needle-liked crystals, druses, and raphide bundles. Druses were rarely distributed in petiole 1, occasionally in petiole 2, and very frequently in petiole 3. Raphide bundles were very frequently in petiole 2 and frequently in petiole 1, and petiole 2. Raphide bundles were investigated with variable shapes. Free needle-liked crystals were frequently distributed in petiole 1, and occasionally in petiole 2 and petiole 3. The distribution of the CaOx crystals in petiole was different depending on the type of crystals.

Free needle-liked crystals were distributed at epidermis, parenchymal cells, and secretory sinuses (but not seen at low magnification). Raphide bundles were mainly distributed in the parenchymal cells. The closer to the center of petiole, the greater the number of raphide bundles. Druse crystals were mainly observed in parenchyma cells and secretory cavity (Figure 5 A-I).

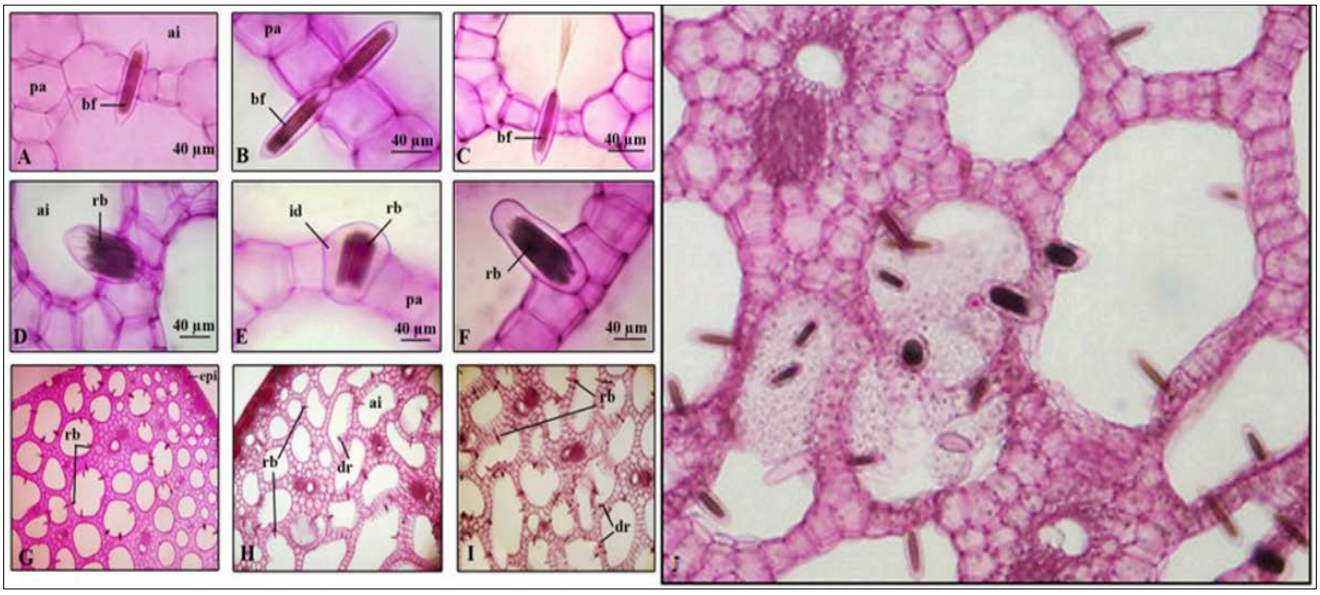

Fig 5: Calcium oxalate crystals and distribution of calcium oxalate crystals in petiole; A-C. Biforine or biforine-liked cells; D-F. Raphide bundles; G-I. Cross section of petiole 1, petiole 2, and petiole 3 (4X); J. Different size of raphide bundles in petiole Abbreviations: as in Figure 2.

The size of free needle-liked crystals, raphide bundles, and druses was recorded on Table 4. The minimum and maximum sizes of CaOx crystals differed greatly. The length of free needle-liked crystals for petiole 1, 2, and 3 was no statistically significant difference ($p > 0.05$). The diameter of druse crystals in petiole 1 was smaller than that of druses in petiole 2 and 3. The length of raphide bundles in petiole 3 was slightly larger than that of raphide bundles in petiole 1 and 2. The width of raphide bundles in petiole 2 was larger than that of raphide bundles in petiole 1 but smaller than that of petiole 3 ($p < 0.05$).

Table 4: Size of calcium oxalate crystals in petiole (Mean±SD µm).

	Druse diameter	Raphide bundle		Free needle-liked length
		Length	Width	
Petiole 1	$17.90±7.68^b$	$95.63±30.18^b$	$24.84±7.90^b$	$64.51±22.0^a$
Petiole 2	$24.40±7.20^a$	$95.41±26.4^b$	$26.92±10.19^a$	$61.98±27.16^a$
Petiole 3	$24.84±6.16^a$	$106.56±46.40^a$	$24.50±10.02^b$	$64.86±30.84^a$
Min - Max	2.04 - 46.6	33.60 - 465.6	4.8 - 63.84	12 - 122.8

Druses: $n_1=83$, $n_2=181$, $n_3=187$; Raphide bundles: $n_1=289$, $n_2=197$, $n_3=220$; Free needle-liked: $n_1=56$, $n_2=98$, $n_3=75$; $n_{1,2,3}$: number of crystals were measured at petiole 1,2, or 3

Values are expressed as Mean ± SD and analyzed by ANOVA ($p < 0.05$).

a, b: $p < 0,05$ when compared size of calcium oxalate crystals among petiole 1, petiole 2, and petiole 3

The various size of raphide bundles was clearly shown in Figure 6. Idioblasts containing raphide bundles were showed the differences in size. A number of raphide crystals that were accumulated within idioblasts varied from vacant to bundles.

Discussion

At the plant, five forms of CaOx crystal are described as raphide, druse, styloid, prism, and sand [25]. Raphide and druse are commonly present in Araceae [26]. In this research, four forms of calcium oxalate crystals were detected in vegetative organs of "green" morphotype wild taro (C. esculenta) as free needle-liked, raphide bundle, druse, and prism crystals. However, prism crystal was only observed one time at the root. Free needle-liked crystals were mostly distributed outside parenchymal cells throughout cross-sections. This could lead to the hypothesis that these crystals may originate from raphide bundles ruptured during anatomical manipulation or spontaneously ejected from biforine forms.

At leaf and tuber of "green" morphotype wild taro, CaOx crystals were also found in specialized cells (as epidermis, parenchymal cells, palisade mesophyll cell, and spongy mesophyll cells). CaOx occurred at phloem area, cortex, stele, lamina, venation, and starchy flesh with variable densities. CaOx crystals were distributed in the mesophyll and epidermal cells of the leaf [27]. Crystals in the cortex and pith cells of the stem [28]. Wild and cultivator *Colocasisa esculenta* existes many varieties. Forms of

CaOx differed within varieties of a species, therefore the presence or absence of different CaOx types in various parts of plants to be regarded as significant taxonomic characters [29]. The presence of CaOx in the leaves and tubers of "green" wild taro has raised alarm about the toxicity of CaOx to human and animal health.

This study also showed that the density and size of CaOx crystals in "green" wild taro were different depending on the developmental stage of the vegetative organs. Both density and size of the druse crystals increased from leaf 1 to leaf 3 (including leaf blade and petiole). The size of the raphide bundle on leaf 1 is smaller than that of leaf 2. However, the width of the raphide bundle on leaf 3 was reduced compared to leaf 2 that could be a result of ejecting of crystals from biforine. Besides, the minimum and maximum sizes of all forms of CaOx crystals differed greatly. CaOx crystals are formed by the combination of Ca^{2+} (obtained from root hairs by absorbing minerals of the environment) with oxalic acid produced by plants during metabolism. CaOx crystals in plants are related to biomineralization known as the formation and reformation process [30]. The density of CaOx crystals in leaf is an inducible mechanism that the decrease or increase of crystals depends on the impact levels of herbivory and concurrency of crystals also depend on the age of the leaf [31].

CaOx is one of the metabolic substances in plants, which are presumed to serve as defensive mechanisms that deter herbivory. Needle-liked crystals also produce toxins and facilitate the diffusion of toxins throughout the skin of herbivores [32]. Raphides are of two types: defensive and non-defensive raphides. Our research was revealed raphide bundles and an unusual form of raphide bundles (known as biforine or biforine-liked cells) [31]. The former crystals could relate to support and the later could relate to protective function. Our study also showed that the density of druse crystals on the upper surface of the leaf blade was greater than that on the underside of the leaf blade. This may lead to the hypothesis that the density and distribution of druses may associate with insect gnawing resistance. Infants and young larvae often scrape the leaf surface and leaf-eating insects generally avoid CaOx crystals [33]. Therefore, druse crystals could play a passive role in defense against herbivory damage [34].

Conclusion

Druse, free needle-liked, raphide bundle, and prism crystals were revealed in vegetative organs of “green” morphotype Colocasia esculenta (L.). The density, size, shape, and distribution of CaOx crystals were different depending on the kinds and developmental stages of the vegetative organs. CaOx crystals in “green” morphology wild taro accumulated during growth and were in a favorable position to protect plants against herbivores. Another suggestion that emerged from this study was the toxicity of CaOx in its use as a green vegetable and in traditional medicine.

Acknowledgements

Non

Conflict of Interests

All authors have no conflicts of interest in submitting this paper.

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