Occurence and variation of calcium oxalate crystals in ten different genera of family araceae

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Abstract: Calcium oxalate crystals are the most abundant insoluble mineral found in plants and can be observed in 215 different plant families including gymnosperms and angiosperms. This study was conducted to determine the types and to compare the densities and sizes of the different types of calcium oxalate crystals in the leaves comparing those in the leaf margins and midveins of the ten selected genera of family Araceae. Leaf discs were cleared by using modified clearing method of Coté and Gibernau (2012). Two types of calcium oxalate crystals, *i.e.*, raphides and druses were present in nine genera of aroids, except Typhonium which only had raphides. The densities of raphides and druses vary with genera from 4 - 431 per mm² and 730 - 5401 per mm², respectively. Dieffenbachia had the highest density of raphides both in the leaf margin and midvein while the Dieffenbachia and Philodendron had the highest densities of druses in the leaf margin and midvein, respectively. The sizes of raphides and druses also vary from each of the genera from 47.4 µm - 175.07 um and 14.26 µm - 45.67 µm, respectively. Dieffenbachia and Anthurium had the longest raphide crystals and largest druses, respectively, while Typhonium and Colocasia had the shortest raphides and smallest druses, respectively.

Keywords: calcium oxalate crystals, raphides, druses, leaf margin, midvein, Araceae

INTRODUCTION

Family Araceae referred to as the aroids, belong to subclass Arecidae and under order Arales. This family is characterized by monopetalous flowers with an inflorescence of a spike (spadix) surrounded by an often brightly colored bract called spathe (Mayo *et al*., 1997). Araceae is also known because of its characteristic possession of calcium oxalate crystals in which raphides are common (Olson, 2013).

A series of cladistic analyses recognize seven major subfamilies of aroids: Gymnostachydoideae, Orontioideae, Pothoideae, Monsteroideae, Lasioideae, Calloideae and Aroideae which are further subdivided into 32 tribes consisting of 105 genera (Mayo *et al*., 1998) and more than 3300 species (Thompson, n.d.). Aroids are extraordinarily diverse with its probably most appreciated foliage (Mayo*et al*., 1997).

Many of the aroids have economic importance. The group includes crops that can produce edible tubers (*Colocasia*), used in hybridizing for cut flowers

(*Anthurium*), as medicine (*Lasia*), and as ornamental water plant (*Typhonodorum*). However, some plants are poisonous if ingested like *Zamioculcas* or commonly known as Zanzibar Gem (Joudi, 2016).

The morphological characteristics of the family give significance to the classification of plant taxa. Much accentuation is given to its floral structure. Once their inflorescence is being noticed, it is clear that they belong to the same family, with their characteristic cowl-like spathe and central fleshy spike, known as the spadix (Mayo *et al*., 1997). However, several studies have shown that some features are consistent in the family. One of such features is the formation of calcium oxalate (CaOx) crystals in the different parts of the plant body. These CaOx crystals are often classified into five categories: raphide, druse, crystal sand, styloid, or prismatic. The formation of crystals have significant biological roles which includes high-capacity calcium regulation, protection against herbivory, and tolerance to heavy metals (Nakata, 2012). Hence, the study will be conducted to give insights for further classification and identification of natural relationships of aroids based on the formation of different types of CaOx crystals in ten selected genera.

Objectives of the Study

This study was conducted to:

1. determine the types CaOx crystals found in ten selected different genera of family Araceae.
2. compare the densities and sizes of the different types of CaOx crystals in the different regions of the leaves of the ten selected different genera of family Araceae.
3. determine the relationship between different genera based on the type, size and density of CaOx crystals.

MATERIALS AND METHODS

Collection of Leaf Material

Plants (Figure 1) representing ten local genera of family Araceae were collected within the vicinity of VSU Main campus. These are *Aglaonema sp*., *Alocasia macrorhiza*, *Anthurium sp*., *Colocasia esculenta*, *Dieffenbachia sp*., *Homalomena philippinensis*, *Philodendron sp*., *Spathiphyllum sp*. and *Syngonium sp*., *Typhonium trilobatum.*

Leaf sampling was based on the method of Barsalote (2004). Two mature, fully expanded and healthy leaves per plant per genus were randomly collected from sample plants with three replicates. The collected samples were placed in labelled plastic bags, into an ice bucket and were immediately brought to the laboratory for microscopic examination.

Slide Preparation and Microscopic Examination

Ten circular disks were obtained from the margins and the areas near the midvein of the leaf blades of each of the three leaf samples using a paper puncher.

The leaf disks were put in test tubes containing 30 mL 5% sodium hydroxide and were stored overnight.

The leaf disks were subjected to clearing procedure for ease in counting the calcium oxalate crystals. The method of leaf clearing was based on Coté and Gibernau (2012) with slight modifications. From 5% NaOH, the samples were treated with commercial household bleach (Sodium Hypochlorite) until the samples turned white, then were washed three times in distilled water for at least 10 min per washing. Generally, about 30 mL of liquid was used to allow free movement of the samples. For confirmation of the chemical components of the crystals, cleared samples were treated with 5% acetic acid for 24 hours. The samples were washed three times with distilled water at least 15 min per washing. Cleared samples were passed through a dehydration process using 10%, 30%, 50%, 75%, 85% and 95% reagent grade ethanol, and were rinsed finally with Ter- Butyl Alcohol for at least 50 min with shaking. Then, the samples were placed in xylene for 10 min with occasional swirling.

The cleared samples were mounted on glass slides with Canada Balsam, and were examined under the microscope to identify and count the number of crystals using a tally counter.

Documentation

Photographs of the plant species studied were taken using a camera. Likewise, photographs of the CaOx crystals were taken using a photomicroscope.

Aglaonema sp.

Alocasia macrorrhiza

Anthurium sp.

Colocasia esculenta

Figure 1. Ten genera of family Araceae used in this study.

Data Analysis

Data were analyzed using descriptive statistics such as means. To determine possible relationship between genera based on crystal characteristics, data on crystal type, size and density of crystals were analyzed using cluster analysis through Ward's Minimum Variance Method and Euclidian Distance to measure distances. This was run using R Statistical Software.

Data Gathered

1. Type of CaOx crystal - This was assessed based on the shapes of the crystals.

a. Raphides

b. Druses

c. Prismatic

d. Styloids

2. Density of CaOx crystals - This was determined by counting the number of CaOx crystals per microscopic field of vision and was extrapolated into number of crystals per mm^2. For raphide crystals, density of CaOx idioblasts rather than individual crystals was determined. This was done because raphide crystals are enclosed in the vacuole of specialized cells called idioblasts which made it difficult to count individual crystals.

3. Size of CaOx crystals - This was determined by measuring the length of the crystals using the stage and ocular micrometer.

RESULTS AND DISCUSSION

Type and morphology of Calcium Oxalate Crystals

Morphology of the calcium oxalate crystals observed in the ten genera studied are presented in Figures 2 and 3. Results show that only two types of CaOx crystals were present in the species studied. All the ten genera studied have both raphides and druses except *Typhonium*, which produced raphide crystals only. Gulliver (1877) noted that raphide crystals can not only be observed in the family Araceae but also in thirteen other families including Balsaminaceae, Amaryllidaceae, Liliaceae, Trilliaceae, Lemnaceae (except *Wolffia*), Orchidaceae and Typhaceae.

Other species from other families possess other types of crystals. Anitha and Sandhiya (2014) observed prismatic crystals in the leaf section of *Murraya koenigii*, large number of small druse in *Moringa pterygesperma*, abundant raphides and druse in *Cissus quadrangularis*, and large and small druses in *Amaranthus gangeticus* and *Mentha arvensis*, respectively. It was stated that the occurrence of these calcium oxalate crystals in these medicinal plants have definite roles in their medicinal activity. Moreover, each type of crystal has different morphology. In the study of Chairiyah *et. al.* (2013), calcium oxalate crystals observed, *i.e.* raphides and druses, in *Amorphophallus muelleri* were differentiated and classified according to their morphology.

Druses were classified solid, semisolid, loose and small while the raphide crystals were classified according to color, length and neatness of edges. In the study of Meric (2009a), different plant parts of different species under family Asteraceae varied in the type of CaOx crystals present. *Inula graveolens* possessed druses in the stem, style and leaf, styloid in the anther and prismatic crystals in the ovary. *Pulicaria dysenterica* possessed druses in the filament and style, styloid in the anther and prismatic crystals in the ovary. *Filago eriocephala*, *Logfia arvensis and Logfia gallica* only produced prismatic crystals in the ovary.

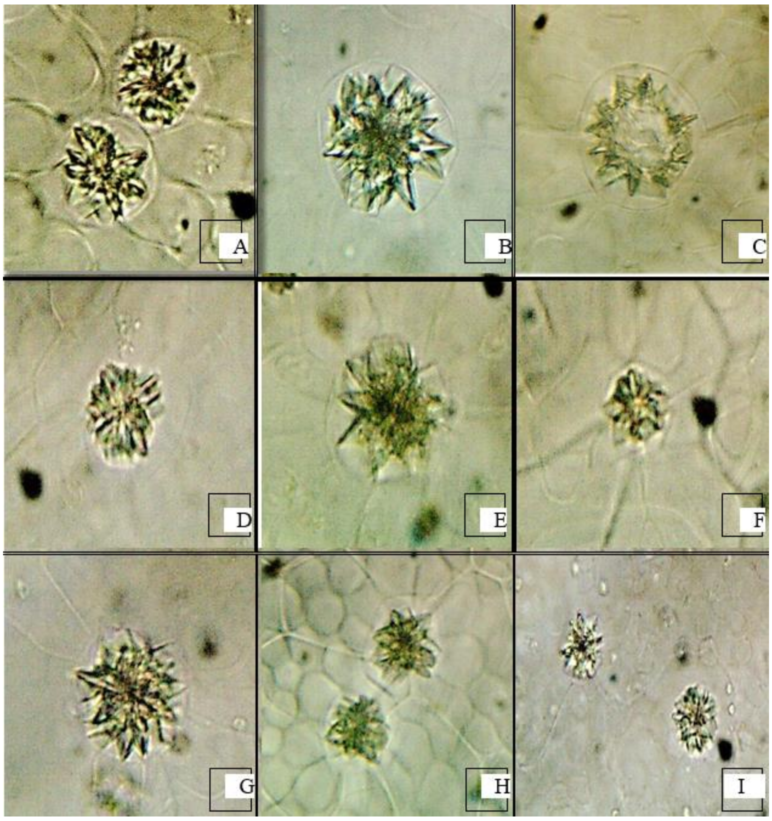

Figure 2. Druses observed in: A. *Aglaonema sp.*, B. *Alocasia macrorrhiza*, C. *Anthurium sp.*, D. *Colocasia esculenta*, E. *Dieffenbachia seguine*, F. *Homalomena philippinensis*, G. *Philodendron sp.*, H. *Spathiphyllum commutatum* and I. *Syngonium podophyllum*. Magnification: 400x

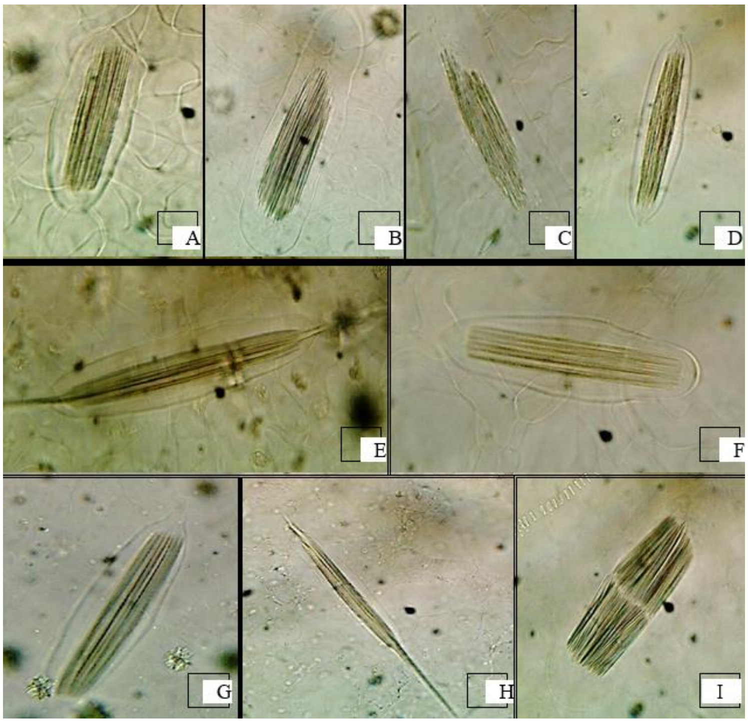

Figure 3. Idioblasts containing raphide crystals observed in: A. *Aglaonema sp.*, B. *Alocasia macrorrhiza*, C. *Anthurium sp.*, D. *Colocasia esculenta*, E. *Dieffenbachia seguine*, F. *Homalomena philippinensis*, G. *Philodendron sp.*, H. *Syngonium* and I. *Typhonium sp.* Magnification: 400x

Raphides can be found as a single crystal or as bundles in a cell of a plant (Chairiyah, 2013). In the ten genera, raphide crystals occured as bundles of spindle-shaped. Druses are combinations of multifacet crystals. It could be an aggregation of prism crystals or styloid crystals (Chairiyah, 2013). According to Franceschi and Nakata (2005), CaOx formation is regulated genetically and can be indicated by constancy of crystal morphology within species, cell specialization, and the noticeable coordination of crystal growth and cell expansion.

Size and Density of Calcium Oxalate Crystals

Size of the crystals, measured in terms of length for raphides and diameter for druses, did not vary greatly between the leaf margin and the midvein. However, large variation between genera was evident (Figure 4). Among the ten genera studied, *Dieffenbachia* had the largest raphides measuring 173.22 µm and 175.07 µm, respectively. The biggest druses were recorded in *Anthurium* with average diameters of 43.77 µm for those found in the leaf margins and 45.67 µm in those found in the midvein. *Typhonium*, on the other hand, had the shortest raphide crystals both in the leaf margin and midvein with 47.4 µm and 50.46 µm, respectively. *Colocasia* and *Homalomena*, which had the lowest druse diameter both in the margin and the midvein, have comparable diameters of 14.26 µm and 16.80 µm, and 14.91 µm and 17.32 µm, respectively.

Density of crystals also did not differ much in the margin and the midvein in all genera but varied greatly with genera (Figure 5). Raphide crystal idioblasts ranged from 4 to 400 per mm^2 and druses ranged from 700 per mm^2 to 5400 per mm^2. Among the ten genera, *Dieffenbachia* had the highest mean raphide crystal count both in the margin and midvein with 431 and 386 crystals per mm^2, respectively. Likewise, *Dieffenbachia* and *Philodendron* had the most number of druse crystals, both in margin and midvein with 4066 per mm^2 and 4493 per mm^2, and 4013 per mm^2 and 5401 per mm^2, respectively. On the other hand, *Spathiphyllum* had the least number of raphide crystals both in margin and midvein with 9 per mm^2 and 4 per mm^2, respectively. Meanwhile, *Homalomena* varied greatly in the density of druses per mm^2 in its margin and midvein wherein it had 730 and 2861 druse crystals per mm^2, respectively.

In the study of Meric (2009a), the ovaries of the different plant species under family Asteraceae possessed prismatic crystals with lengths varying from

8.70 µm to 25.96 µm depending on the species. Anitha and Sandhiya (2014) also reported variation in crystal sizes of different crystal types in the leaves of medicinal plants. It was recorded that *Amaranthus* had large druses measuring 22.1 µm, *Murraya* produced rosettes which measured 16.8 µm; while *Cissus quadrangularis* had rosette, druse and raphides that measured 25 µm, 15.4 µm and 69.7 µm, respectively. *Mentha arvensis* had acicular crystals and hexagonal crystals that measured 30-50 µm.

According to Franceschi and Nakata (2005), crystals may greatly vary in their sizes and distribution. But even with large range in size and distribution among species, these characteristics are constant within a species. Meric (2009a) agreed to this statement. Moreover, Ruiz (2002) discussed that there was no significant difference among plants from different populations with or without herbivory in calcium oxalate crystal production regardless of soil characteristics and other treatments. Additionally, it was also stated that plant populations have a fixed defensive genotype that is always expressed and changing through evolution but functioning independently of current herbivory. Thus these constancy, shape and distribution, are used as taxonomic characters that can indicate genetic regulation of crystal deposition.

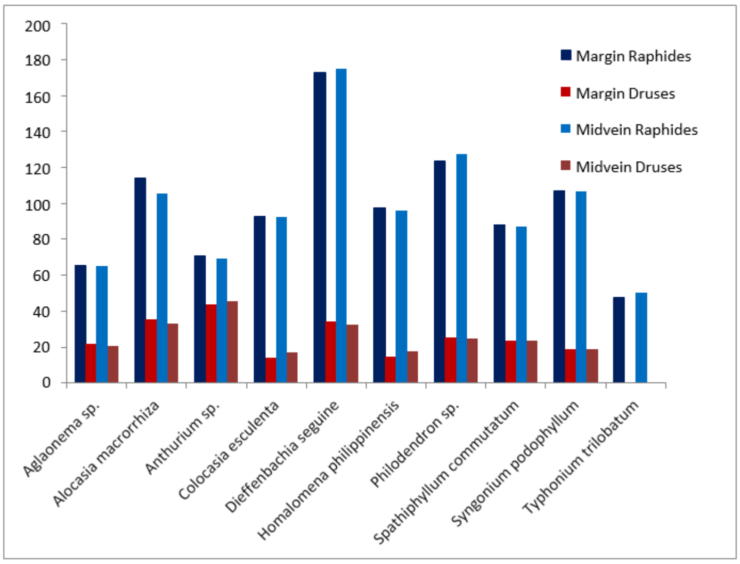

Figure 4. Comparison on the mean crystal size (µm) in different regions of the leaves of the ten genera of family Araceae.

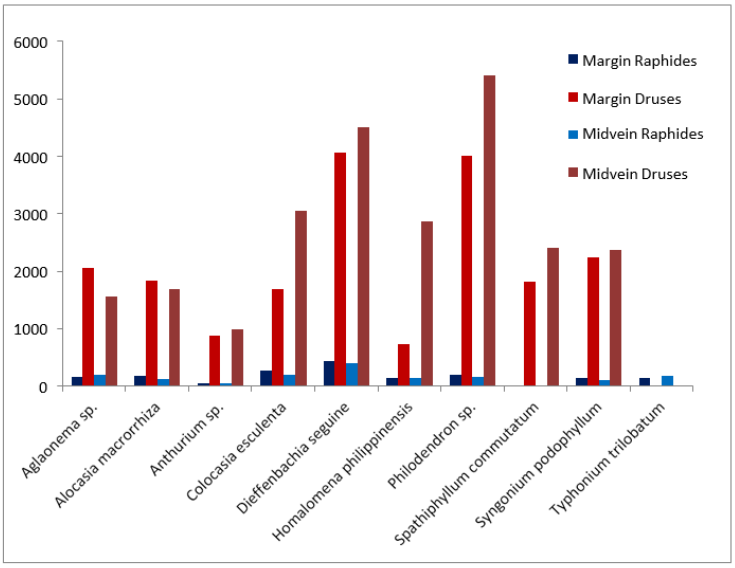

Figure 5. Comparison on the mean densities of crystals per mm^2 leaf disc in different regions of the leaves of the ten genera of family Araceae.

Relationship Between Genera Based on CaOx Crystal Characteristics

Based on the type of CaOx crystals found as to size and density, the ten aroid genera studied can be categorized into four clusters. As shown in the dendrogram in Figure 6, each cluster was represented by the genera that were closely related in terms of CaOx crystal characteristics. The first cluster (Cluster I) comprises *Dieffenbachia* and *Philodendron*. The second cluster (Cluster II) consists of *Anthurium* and *Typhonium*. The third cluster is divided into two clusters; Cluster IV comprises *Aglaonema*, *Alocasia* and *Homalomena* while Cluster V comprises *Spathiphyllum*, *Colocasia* and *Syngonium*. Clusters IV and V are the most closely related while Cluster I is the most distant to the other clusters.

Mayo *et. al.* (1997) classified family Araceae into two major groups: the Proto-Araceae and the True Araceae. The former comprises subfamilies Gymnostachydoideae and Orontioideae while the True Araceae includes Pothoideae, Monsteroideae, Lasioideae, Calloideae and Aroideae. Based on the classification of Mayo *et. al.* (1997), the ten genera in this present study belong to the True Araceae. *Anthurium* and *Spathiphyllum* belong to subfamily Pothoideae and Monsteroideae, respectively while the other eight genera belong to subfamily Aroideae. In the dendrogram (Figure 6), *Spathiphyllum*, which belong to subfamily Monsteroideae, was grouped with *Colocasia* and *Syngonium* that belong to subfamily Aroideae. *Anthurium* and *Typhonium*, which belong to subfamily Pothoideae and Aroideae; respectively, were grouped in one cluster.

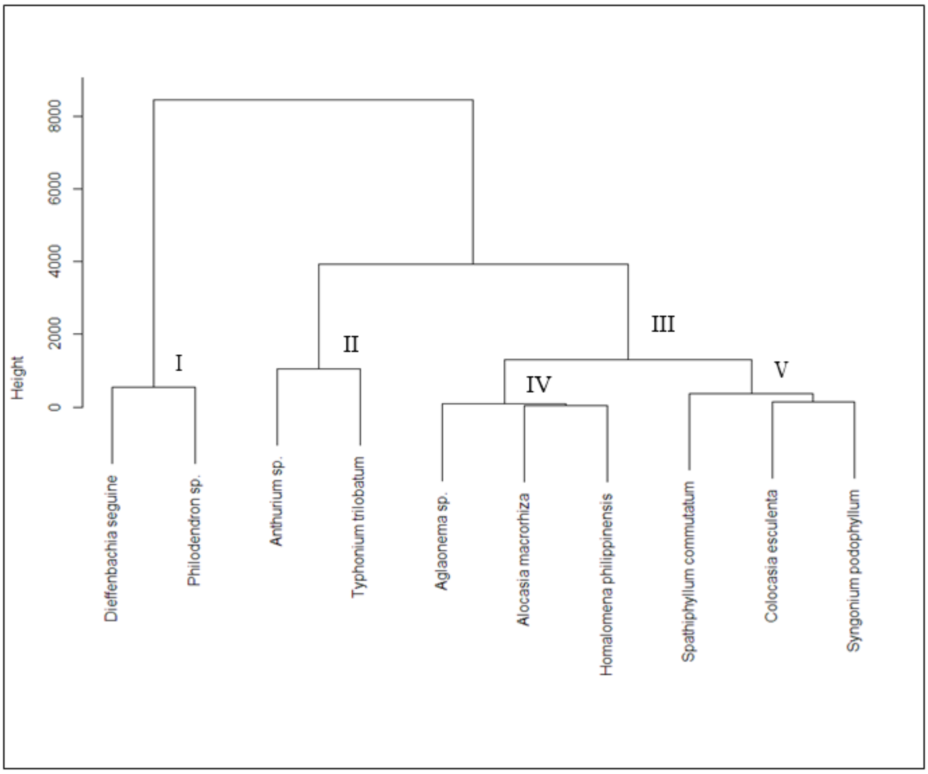

Figure 6. Dendrogram showing the relationship between the ten genera based on the type, density and size of CaOx crystals.

Based on the results, CaOx characteristics cannot be considered as a stable character in determining affinities between aroid subfamilies and genera although their presence seems to be a stable character of the family. According to Franceschi and Nakata (2005), even though crystals may have large ranges in size and distribution among species, these characteristics are reported to be constant within a species. It is thus highly possible that CaOx characteristics can be useful in defining different species within the genera of aroids.

SUMMARY, CONCLUSION AND RECOMMENDATION

Summary

The study was conducted to determine and compare the variation in terms of type, density and size of calcium oxalate crystals formed in the leaves of the ten different genera of family Araceae.

Two types of calcium oxalate crystals, *i.e.*, raphides and druses were present in ten genera of aroids, except *Typhonium* which only had raphides. The density of raphides and druses vary with genera from 4 - 431 per mm^2 and 730 - 5401 per mm^2, respectively. *Dieffenbachia* had the highest density of raphides both in the leaf margin and midvein while the *Dieffenbachia* and *Philodendron* had the highest densities of druses in the leaf margin and midvein, respectively. The sizes of raphides and druses also vary from each of the genera from 47.4 µm - 175.07 µm and 14.26 µm - 45.67 µm, respectively. *Dieffenbachia* and *Anthurium* had the longest raphide crystals and largest druses, respectively. In contrast, *Typhonium* and *Colocasia* had the shortest raphides and smallest druses, respectively.

In terms of CaOx crystal characteristics, the ten genera can be grouped into four clusters. *Philodendron sp*. and *Dieffenbachia seguine*, which belong to Cluster I, are more similar to each other but are the most distant to the other clusters. The second cluster (Cluster II) consists of *Anthurium* and *Typhonium*. The third cluster is divided into two smaller clusters; Cluster IV comprises *Aglaonema*, *Alocasia* and *Homalomena* while Cluster V comprises *Spathiphyllum*, *Colocasia* and *Syngonium*. Clusters IV and V are the most closely related clusters.

Conclusion

Raphide crystals were the most common type of CaOx crystals produced in the ten studied genera. Nine out of the ten genera possessed druses. The crystals were formed in both leaf margin and midvein. *Dieffenbachia* had the highest density of raphides while the same genus, together with *Philodendron*, had the highest densities of druses. *Dieffenbachia* and *Anthurium* had the longest raphide crystals and largest druses, respectively. In contrast, *Typhonium* and *Colocasia* had the shortest raphides and smallest druses, respectively.

Based on CaOx characteristics, it can be inferred that it cannot be considered as a stable character in determining affinities between aroid subfamilies and genera although their presence seems to be a stable character of the family. Other characters including molecular data must be considered in defining the relationships between different plant taxa.

Recommendations

This study has been focused only on the leaf blades of the ten genera of family Araceae being studied and the determination of its calcium oxalate crystal density. With these considerations, possible areas of study to further understand the relationship of the genera under family Araceae are suggested:

1. Inclusion of petiole, stem and organ for storage for quantification of crystal density in order to establish wider observations in the variation of calcium oxalate crystal formation.
2. Inclusion of the environmental factors to assess their effects in CaOx crystal formation.
3. Comparative study using more genera of family Araceae or other species or varieties of the genera to document possible similarities or differences on CaOx crystal formation.
4. Comparative study using other plant families in order to study their relationship based on their variation in CaOx formation.
5. Differentiation of crystal morphology to establish possible function of the crystals according to its morphology.

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