THE DISTRIBUTION OF CALCIUM OXALATE CRYSTALS IN GENUS
DIEFFENBACHIA SCHOTT. AND THE RELATIONSHIP BETWEEN
ENVIRONMENTAL FACTORS AND CRYSTAL QUANTITY AND QUALITY

By

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4.4. Numbers of CaOx crystals within 1 cm from leaf margin in leaf blade cross sections of *Dieffenbachia maculata* ‘Rebecca’ which were grown in potting media......................................................................................................................................61
A popular ornamental foliage plant in commercial nurseries, *Dieffenbachia*, is also famous for its irritative nature that is caused by the combination of calcium oxalate (CaOx) crystals and a proteolytic enzyme. Although there were numerous reports of *Dieffenbachia* toxicity, the distribution of CaOx crystals in the whole plant and the relationship between these crystals and substrate nutrition levels, irrigation systems, and light intensities have not been previously investigated.

*D. maculata* ‘Carina’, *D. maculata* ‘Rebecca’, and *D. x ‘Star Bright’* were used to investigate the distribution of CaOx crystals in roots, stems, leaves, and inflorescences of *Dieffenbachia*. Many druse and raphide idioblasts were observed in stems, leaves, and male, female, and neutral flowers, but their distribution varied. In stems, raphide idioblasts formed a ring under 1 or 2 layers of epidermal cells, while druses were scattered in the cross section. In leaves, an increased density of druse idioblasts at close
proximity to the leaf margin was observed. In inflorescences, CaOx idioblasts were mainly distributed in anthers of male flowers, in styles and staminodes of female flowers, and in individual neutral flowers. Moreover, this study revealed that CaOx idioblast formation is closely related to differentiation activities, i.e., cell metabolic rate affects CaOx idioblast quantity. In addition, cultivars differed significantly in CaOx idioblast quantity. ‘Star Bright’ had a lower density of druses in the stem than the other two cultivars, and ‘Carina’ had a lower density of druse and raphide idioblasts in the stem than ‘Rebecca’.

Substrate nutrition levels proved to affect CaOx idioblast quantity in *Dieffenbachia*. When plants were subjected to different nitrogen (N) levels, the highest numbers of CaOx crystals were found in plants grown under 200 ppm N level, which is also the optimum level of N for *Dieffenbachia* growth. Thus, it appears that CaOx crystal quantity is closely related to the optimum growth of *Dieffenbachia*. Evaluation of the effect of calcium (Ca) and magnesium (Mg) levels on CaOx crystal formation in *D. maculata* ‘Rebecca’ indicates that CaOx crystal quantity increased as substrate Ca level increased when Mg level remained constant. Additionally, druse and raphide crystals responded differentially to Ca levels. Druses were more sensitive to changes in substrate Ca and Mg concentrations than raphides. Light intensity also influenced CaOx crystal formation as *Dieffenbachia* grown in production light level (285 μmol·m⁻²·s⁻¹) produced more CaOx crystals than those grown under interior low light level (8 μmol·m⁻²·s⁻¹). A general conclusion could be reached that plants with higher cell metabolic rates synthesized more oxalate from its precursors, ascorbic acid and galactose, and CaOx idioblast density increased as Ca uptake also increased.
CHAPTER 1  
LITERATURE REVIEW  

Dieffenbachia Genus

Dieffenbachia, a member of the family Araceae, has about 30 species of erect herbs. They are native to tropical America, with stout, unbranched stems bearing leaves toward the top. Leaves are entire, and often variegated with sheathed petioles (Bailey and Bailey, 1976). The inflorescence of Dieffenbachia consists of a spadix and a spathe. The spadix is an upright central axis covered with numerous small petalless flowers (Henny, 1977). Male flowers are located at the apex of the spadix, female flowers are at the basal end, and sterile flowers are located between the male and female flowers. The spathe is a specialized leaf attached at the base of the spadix which covers the spadix until anthesis (Henny, 1977). Dieffenbachia is dichogamous: the female flowers are receptive earlier than when male flowers shed their pollen. This helps to prevent self-pollination (Henny, 1977).

In its native habitat Dieffenbachia grows in shady, moist lowlands of tropical America, extending as far south as the Amazon drainage basin in Brazil and north to the islands of the West Indies (Barnes and Fox, 1955). The name of this genus comes from a famous German physician and botanist - J. F. Dieffenbach (1794-1847). These plants were first placed in the genus Arum but were later transferred to the genus Caladium. In 1829, Schott described the genus Dieffenbachia to compliment Dr. Dieffenbach (Barnes and Fox, 1955).
Dieffenbachia is one of the most important groups of ornamental tropical foliage plants (Fig. 1.1). Because of their attractive foliar variegation and capability of adapting to interior low light conditions, Dieffenbachia has been an important ornamental foliage plant used as a living specimen for interior decoration (Chen et al., 2002) since its introduction in 1759 (Sweet, 1839). Several of the most popular varieties are mutations or “sports” from Dieffenbachia maculata (Lodd.) (formerly D. picta Schott).

The beautiful outward appearance of these plants masks their uniform genetic background and, coupled with the commercial practice of asexual propagation, large populations of identical plants had been produced (Conover et al., 1973). The lack of genetic diversity in Dieffenbachia can lead to high susceptibility to disease or pests. This is the reason why numerous breeding experiments were carried out in 1970s and 1980s.

**Calcium Oxalate**

**Biomineralization**

A required element for plant growth and development, calcium (Ca) plays many important roles, e.g., as a structure component of cell walls, a signal in various physiological and developmental pathways, and as an osmoticum (Webb, 1999).

Ca deposition in plants may be amorphous or crystalline. Because of the wide distribution, ease of observation, and aesthetic qualities, crystalline deposits in plant cells have attracted substantial interest (Arnott, 1976). Cells containing many needle-shaped crystals of calcium oxalate (CaOx) are extremely abundant and can be found in many common plants, like grape, kiwi, and taro. These crystals are birefrigerant, and under polarized light microscope, they become brilliant, sometimes multicolored objects.

Crystal-containing cells with needle-shaped CaOx crystals are termed raphide idioblasts. Three other types of CaOx crystals can be defined by their characteristic shape
and developmental patterns (Arnott, 1976). Styloids are large elongated crystals usually found singly in a cell; the crystals may or may not exhibit characteristic facets and sometimes have curved faces. Prismatic crystals represent another crystal type in which the crystals are prism-shaped and show characteristic facets. The final type, druses, are spherical conglomerates of many CaOx crystals (Arnott, 1976).

Plants make CaOx crystals in an intriguing variety of defined shapes. In higher plants, the distribution of crystals, like their morphology, follows species-specific patterns, indicating regulation over the sites and modes of CaOx accumulation (Webb, 1999). Two general types of CaOx crystals, druses and raphides, have been identified in Dieffenbachia so far (Genua and Hillson, 1985).

Al-Rais et al. studied oxalate crystals in 10 species of flowering plants in 1971. They revealed that under normal conditions, the oxalate crystals consist almost entirely of CaOx, but when calcium was replaced by magnesium (Mg), barium (Ba), or strontium (Sr) these elements substituted for Ca in the crystals.

**Functions**

Why do plants sequester CaOx? Ca is very abundant in the natural environment. Nonetheless, cytosolic free Ca must be restricted to levels of $\sim 10^{-7}$ M or less, because higher concentrations interfere with a variety of crucial cell processes, including Ca-dependent signaling, phosphate-based energy metabolism, and microskeletal dynamics (Webb, 1999). Ca uptake by many plants does not appear to be tightly regulated by the roots, and the amount of Ca taken up may be more closely related to levels of available Ca in the soil rather than the plant Ca requirement (Franceschi, 1989). Higher plants appear to modulate differences between the natural abundance of environmental Ca and the very low levels required for cytosolic free Ca by controlling the distribution of Ca and
its compartmentation within the cell. The cell wall and the vacuole provide major sinks for Ca in plants (Webb, 1999).

A detailed description of CaOx precipitation process was made by Franceschi (1989) in his study of crystallization and solubilization of CaOx crystals in *Lemna minor* root:

Ca ions will naturally follow their electrochemical potential gradient into cells where cytoplasmic free Ca is kept low. The plant cell maintains a low cytoplasmic Ca level by actively pumping Ca back out to the apoplast and/or compartmentalizing it in mitochondria, vacuoles or endoplasmic reticulum. The activity of apoplastic Ca may also be lowered by binding of Ca to carboxylic acid groups of some wall components such as pectic acids. If apoplastic Ca reaches some critical level where wall binding sites are saturated and passive influx exceeds active efflux plus compartmentation, cytoplasmic free Ca concentration increases and some cells are induced to begin the differentiation necessary for crystal formation. (p.135)

Franceschi’s study (1989) indicated calmodulin was involved in the signaling of induction or change in direction and magnitude of Ca transport. The machinery necessary for rapid Ca transport and CaOx precipitation was quickly assembled. This included the membranes within which crystals were formed and, presumably, Ca transport proteins. The induced cell had then become a high capacity system for active removal of Ca from the apoplast and chelation of large amounts of Ca as a physiologically and osmotically inactive, highly insoluble precipitate.

Recently, the role of CaOx formation in regulating excess tissue Ca was brought into question by genetic research (Nakata and McConn, 2000). Leaves from a chemically mutagenized *Medicago truncatula* were visually screened for alterations in CaOx crystal formation. Seven different classes of CaOx defective mutants were identified that exhibited alterations in crystal nucleation, morphology, distribution, and/or amount. Genetic analysis suggested that crystal formation is a complex process involving more
than 7 loci. Phenotypic analysis of a mutant that lacks crystals, cod 5, did not reveal any difference in plant growth and development compared with controls. Thus the hypothesized roles of CaOx formation in supporting tissue structure and in regulating excess tissue Ca were questioned (Nakata and McConn, 2000).

Another function of CaOx crystals was described as a defence mechanism against herbivores (Ward et al., 1997; Molano-Flores, 2001). In the study by Ward et al. on desert lily, 3 species of herbivores ate only those parts of the leaves where CaOx raphides were absent. In Molano-Flores’ study, Sida leaves from seedlings subjected to increased herbivory were induced to produce greater amount of crystals than those seedlings not subjected to herbivory.

*B. Dieffenbachia Toxcity*

*History*

Barnes and Fox (1955) compiled an extensive literature review on reports of *Dieffenbachia* toxicity. According to their review, one of the best known members of genus *Dieffenbachia*, *D. seguine* Schott, was commonly called “dumb cane”. In 1707 Hans Sloane explained the origin of this common name:

> If one cut this Cane with a Knife, and put the tip of the Tongue to it, it makes a very painful sensation, and Occasions such a very great irritation on the salivary Ducts, that they presently swell, so that the person cannot speak, and do nothing for some time but void Spittle in a great degree, or Salivate, which in some time goes off, in this doing in a greater degree, what European *Arum* does in a lesser, and from this its quality, and being jointed, this *Arum* is called Dumb-Cane…. Strangers must be warned of these Canes, they looking like those of Sugar. (p.168)

It was cited by Barnes and Fox (1955) that Bigelow wrote in his *American Medical Botany* in 1818:

The acrid property, which resides in this species of *Arum*, appears to depend upon a distinct vegetable principle in chemistry, at present little understood. It is extremely volatile and disappears almost entirely by heating, drying or simple exposure to air. (p.55)

Barnes and Fox (1955) also reported that Sloane was among the first to warn us of *Dieffenbachia* acridity (1696). Yet he also mentioned that dumb cane is eaten by the Indians for want of better meat. Early legends and accounts concerning the toxicity of *Dieffenbachia* report many varied and unique ways in which dumb cane was employed. The Tucuna Indians of the upper Amazon, according to Tschirch (1925) in his *Handbuch der Pharmacognosie*, used the stems of *D. seguinum* in the formulation of curare as an arrow poison (Barnes and Fox, 1955).

According to Barnes and Fox (1955), Martyn described the use of dumb cane as a means of punishing slaves in 1807:

… in Jamaica, where it is said they sometimes rub the mouths of their negroes with it, by way of punishment. (p.1)

Hooker in 1823 related a similar experience, cited by Barnes and Fox (1955):

…When Mr. MacNab, the excellent Superintendent of the Edinburgh Botanic Garden, was at Kew, a box of these plants arrived there from Cayenne. One of the men employed to remove the individuals to the stove, incautiously bit a piece of one of them, when his tongue swelled to such a degree that he could not move it; he became utterly incapable of speaking and was confined to the house for some days, in the most excruciating torments. (p.1)

And a similar warning by Heal (1925) was also chronicled by Barnes and Fox (1955):

In taking cuttings or potting the plants be careful not to allow the knife or tying material to go near the mouth, as a poisonous, acid juice produced by the plant gives intense pain and causes the tongue to swell so as to render speech impossible; I knew a gardener who was ill for a long time by accidentally putting the tying material in his mouth. (p.77, p.353)
Barnes and Fox (1955) also chronicled an interesting story (Kremmens, 1952) of *Dieffenbachia* being rubbed into the mouth of an eye witness to a crime by the culprit, and when the witness was called into court in the Bahamas, he could not testify and the criminal was acquitted.

The reports that *Dieffenbachia seguine* produces sterility is one of the most interesting and unusual aspects of its action. Madaus (1938) stated that this plant has been used by the Brazilian Indians as a means of sterilizing their enemies, the juice being administered either in food or as an arrow poison (Barnes and Fox, 1955). In 1950’s, it was still a common belief among the inhabitants of many islands of the Caribbean that a temporary male sterility of some 24 to 48 hours’ duration is effected by chewing *Dieffenbachia seguine*, and it was thus employed by them as a contraceptive (Barnes and Fox, 1955).

According to *Trials of War Criminals before the Nuernberg Military Tribunals*, certain members of the Third Reich in Germany attempted to produce mass sterilization of undesirable races, and employed *Dieffenbachia* juice in sterilization experiments on concentration camp inmates. These experiments were limited by the unavailability of *D. seguine* (Arditti and Rodriguez, 1982).

Preparations containing *Dieffenbachia* were once used to treat gout, dropsy, sexual impotence, and frigidity (Fochtman et al., 1969). Sloane reported that *Dieffenbachia* stalk can effectively cure hydropic legs, according to Arditti and Rodriguez (1982). In addition, *D. seguine* was used to open obstructions and against inflammations. Sliced root was employed against gout (Arditti and Rodriguez, 1982).
Symptoms

Dieffenbachia is second only to Philodendron in the number of cases reported to United States poison control centers through 1988 (Pamies et al., 1992). During 1976, 485 cases of Dieffenbachia poisoning were reported. However, in 1990 the number increased to 4,124. Nursery workers are particularly susceptible to the ocular effects of Dieffenbachia. As the plant’s stalk is cut, droplets may be expressed into the eyes resulting in eyelid edema and inability to open the eyes (Pamies et al., 1992). Contact with Dieffenbachia may cause following symptoms:

Digestive. Ingestion of the leaves or stems of Dieffenbachia species results in rapid development of local mouth and throat irritation (Spoerke and Smolinske, 1990). Redness, swelling, and burning pain of the tongue and mucous membranes are noted initially. Swelling may rarely become severe enough to produce obstruction and respiratory compromise. Profuse salivation and dysphagia are often present. In severe cases, impairment or loss of speech may occur, lasting for several days. Painful tongue and buccal bullae and necrosis may persist for a week or longer (Spoerke and Smolinske, 1990). It is uncommon for plant parts to be swallowed because of immediate pain upon ingestion. If swallowed, laryngeal, esophageal, or gastric edema may occur, also respiratory obstruction due to edema and constriction of the glottis. Inflammation peaks in an hour and may continue from a few days to two weeks (Pamies et al., 1992).

Ocular. Exposure to the juice has resulted in immediate intense pain, photophobia, followed by eyelid edema, blepharospasm, watery eyes, conjunctival chemosis, and corneal abrasions. Needle-like CaOx crystals are frequently visible on the corneal epithelium (Spoerke and Smolinske, 1990).
Dermal. Exposure to cut stems or juice may produce local inflammation (Spoerke and Smolinske, 1990).

Through an intensive literature review, Pedaci et al. (1999) ranked the types of toxicity caused by Dieffenbachia. The most common were oral irritation, dermal pain, vomiting, erythema, throat irritation, and dermal edema.

Related Studies

The Causes of the Toxicity

The nature of the irritant effects of Dieffenbachia has long been a source of controversy in the literature. That CaOx-containing raphides play a part is undisputed (Spoerke and Smolinske, 1990). A bundle of 100 to 200 needle-like CaOx monohydrate raphide crystals are located within each idioblast. They are fired in a projectile fashion in response to mechanical force (Spoerke and Smolinske, 1990).

The unique raphide structure in Dieffenbachia maculata was investigated by Sakai and Nagao in 1980. SEM work revealed there were 2 types of raphides and 2 types of raphide idioblasts in D. maculata. Small raphides are about 25 µm long and 0.4 µm wide. They have 2 grooves on opposite sides of the crystal, which run the entire length. Barbs occur on ridges on either side of the grooves along the length of the raphide. Tips of the barbs are oriented in one direction. These small raphides were found in idioblast cells similar in shape to adjacent ground parenchyma cells. Large raphides, about 120 µm long and 4 µm wide, with wide grooves were found in spindle shaped, obtuse ended idioblasts, about 165 µm long and 40 µm in diameter. One end of these raphide crystals is narrow, acute, and tapering, the other is broad and more abruptly acute. Two wide grooves on opposite sides of the crystal extend along 1/4 the length from the broad end.
A similar pair was found extending along 3/8 the length from the narrow end (Sakai and Nagao, 1980).

Sakai and Nagao (1980) suggested the barbs made dislodging difficult once the raphide penetrated mouth tissues, and the grooves might prevent mouth and throat tissue from sealing around the crystal or served as a means for moving a chemical irritant toxin into mouth and throat tissue.

Using light microscopy, Middendorf (1983) reported that it was easy to observe raphide idioblasts shoot out needle after needle like some kind of automatic microscopic blowgun. The ejection of the raphides is apparently caused by the swelling of the polysaccharide material associated with the raphides, which breaks the end walls and causes the ejection of the raphides (Sakai and Nagao, 1980).

The theory that CaOx crystals are the only cause of *Dieffenbachia* toxicity was questioned by many investigations. Madaus was among the earliest to discuss the cause of *Dieffenbachia* toxicity. He listed a poisonous alkaloid, glycoside, or bitter substance in 1938 (Barnes and Fox, 1955).

Fochtman *et al.* (1969) tested the juice of *D. picta* and *D. exotica* on rats and rabbits. Their work supported the view that the toxicity of *Dieffenbachia* juice is attributed to a proteolytic enzyme similar to trypsin, not to the oxalate content as previously believed. The mechanism of toxicity seemed to be associated with histamine release because the blood histamine levels increased significantly after the topical application of *Dieffenbachia* juice to the rat tongue.

In the investigation of chemicals in *Dieffenbachia* by Walter and Khanna (1972), it was found that *D. seguine, D. amoena,* and *D. picta* all contained a proteolytic enzyme.
Presence of this enzyme tends to explain most of the poisonous properties attributed to *Dieffenbachia*. The authors believed the proteolytic enzyme acts on sensitive tissues after the tissues have been injured by the long sharp CaOx raphides. The combination of the enzyme and the raphides were responsible for the immediate caustic nature of these plants.

In 1983, Saha and Hussain studied 4 aroids and found that the least irritating variety had more Ca and oxalate than the moderately irritating varieties, and the most irritating variety had the highest content. Thus, they concluded that the raphides of CaOx crystals might not be the only irritating principle. Through a series of biochemical tests they suggested the irritating factor might be a glycoside. Their assumption was corroborated by the fact that the irritant (glycoside) was reduced by 47% when samples were dried, which was resulted from the breakdown of the glycoside into its non-acrid components. Another supportive fact was that the most irritating variety had the highest content of glycoside and the least irritating variety had the minimum amount (Saha and Hussain, 1983).

Bradbury and Nixon (1998) studied 5 species in the Araceae family which are the major staple foods of people in several tropical countries. They listed 3 facts to support their statement that the acridity of aroids was not due to raphides acting alone: (1) acridity of plant tissue could be eliminated by cooking and raphides were undamaged by heat; (2) the aridity was not related to the amount of raphides extracted from the aroid as the yield of raphides from weakly acrid species was much greater than from strongly acrid species; (3) the acridity of raphides was removed by immersion in methanol, ethanol, distilled water, water containing a non-ionic detergent, or CCl₄, however,
immersion in PE (petroleum ether) caused no loss of acidity. The irritant was identified as a protease in their earlier work (Bradbury and Nixon, 1998).

It appears after an extensive review of literature that *Dieffenbachia* toxicity is caused by a raphide/irritant complex.

**Toxicity Comparison**

The first study to report and compare the toxicity of several *Dieffenbachia* varieties was by Fox and Barnes (1955) who placed the expressed juice of several varieties into the conjunctival sacs of rabbit’s eyes and scored the amount of irritation. The varieties tested in decreasing order of their power to produce irritation were: *D. seguine*, *D. picta*, *D. Oestedii*, *D. Pitterii*, and *D. Amoena* (Barnes and Fox, 1955). In 1968, Walter and Muni compared the toxicity of 3 species of *Dieffenbachia* and found *D. memoria-corsii* the most toxic, followed by *D. amoena* and *D. seguina-nobilis*. Fox and Barnes (1954) found the expressed juice of the stems of several varieties more irritating to rabbit’s eyes than the juice from leaves. The expressed juice from old stems was more irritating than that from young stems (Barnes and Fox, 1955).

**Crystal Formation Regulation Mechanisms**

The process of CaOx raphide crystal growth and how crystal growth is coordinated with cell growth was studied using microautoradiographic, immunological, and ultrastructural techniques (Kostman and Franceschi, 2000). Incorporation of $^{45}\text{Ca}^{2+}$ directly demonstrated that Ca transport was strictly regulated at the plasma membrane; and, relative to surrounding mesophyll cells, crystal idioblasts acted as high-capacity $\text{Ca}^{2+}$ sinks. In very young idioblasts, Ca is incorporated along the entire length of the needle-shaped raphide crystals but as they mature incorporation only occurs at crystal tips.
in a bidirectional mode. This indicated a morphological polarity and an apparent nucleation point from which crystals grow bidirectionally.

CaOx crystals isolated from callus cultures and intact plants were compared in 8 species known to produce CaOx crystals of different habits (Kausch and Horner, 1982). Three species produced callus crystals which were abnormal with respect to their intact-plant counterparts. For example, *Cissus* callus produced styloids in culture when its explants contained raphides and druses. They suggested CaOx crystal production and habit generally were under genetic control, but were influenced by the metabolic status of the cells.

**CaOx Deposition in Relation to Nutrient Levels**

The formation of CaOx crystal idioblasts at different levels of Ca was studied as early as 1972 (Frank). *Canavalia ensiformis* were grown in nutrient solution at 3 different levels of Ca supply: normal, minimum, and excess Ca. Plants grown with minimum Ca showed obvious signs of Ca deficiency yet still formed crystals. This indicated that crystal development was genetically programmed, and Ca allocation for crystal development had a precedence over other plant needs. With excess Ca, the number of crystals rose above the “normal” level. Therefore, Ca was suggested to be a deciding factor in idioblast cell differentiation. The results from her further study in this area (1975) which showed a causal relationship between Ca level, intermediate stages of oxalate biosynthesis, and the differentiation of idioblasts containing CaOx crystals. Her conclusions were supported by Borchert (1985) who showed that exposure of isolated leaflets to inductive Ca\(^{2+}\) (calcium acetate) triggered the differentiation of mature parenchymatous leaf cells into crystal idioblast cells.
The first use of callus culture to study CaOx crystal idioblast formation was reported in 1979 (Franceschi and Horner). The number of crystal idioblasts formed per unit volume of callus varied directly with Ca concentration of the medium. Adding oxalate to the medium decreased the number of idioblasts probably because of Ca precipitation. During induced Ca deficiency, the callus lost crystals that had formed prior to the deficiency. L-ascorbic acid, an oxalate precursor in some plants, stimulated idioblast formation when added to the medium at low concentrations. At higher concentrations, it inhibited idioblast formation. Inhibitors of enzyme-catalyzed reactions capable of converting glycolate or glyoxylate to oxalate, caused a large decrease in idioblast formation. Their results showed that the availability of Ca and oxalate and idioblast initiation are definitely related.

Although CaOx crystal formation had been considered a time-consuming and dead-end process, an in vivo test aimed at examining the raphide crystal bundle formation in roots of intact Lamna minor (Franceschi, 1989) indicated that the CaOx formation was a rapid and reversible process. When plants growing on culture medium with 5 mM Ca were exposed to 7 mM Ca, new raphide crystal bundles were formed within 1 hour. When plants were pretreated on Ca-free medium, crystal bundle formation occurred even more quickly (within 30 minutes). The process was reversible with recently formed crystal bundles being dissolved over a period of about 3 hours, but older bundles were more resistant to dissolution. This study supported the function of CaOx crystals as localized sites for “locking” excess Ca.

The effect of 0, 3, and 7 mM Ca\(^{2+}\) on the allocation and deposition of Ca\(^{2+}\) into intracellular and sub-cuticular periplasmic CaOx crystals was examined in leaf primordia
of rooted cuttings of *Dracaena sanderiana* by Pennisi and McConnell (2001). They observed the number of sub-cuticular crystals in leaf primordia of cuttings rooted in deionized water grown in solutions supplemented with either 0, 3, or 7 mM Ca\(^{2+}\) was similar, but crystals were considerably smaller in plants grown in 0 mM Ca\(^{2+}\). Sub-cuticular crystals appeared developmentally earlier in leaf primordia of all cuttings grown in either 3 mM or 7 mM Ca\(^{2+}\) than in cuttings rooted in deionized water grown in 0 mM Ca\(^{2+}\). They suggested that deposition of sub-cuticular crystals is modulated by Ca\(^{2+}\) levels and could be induced at an earlier ontogenetical stage by rhizospheric Ca\(^{2+}\) levels or delayed by lowering rhizospheric Ca\(^{2+}\) levels.
Figure 1.1. *Dieffenbachia* production in a commercial foliage nursery.
CHAPTER 2
INVESTIGATIONS ON THE RELATIONSHIP OF CALCIUM OXALATE CRYSTAL WITH NITROGEN LEVELS, IRRIGATION SYSTEMS, AND LIGHT INTENSITIES

Comparison of CaOx Raphide Idioblast Numbers in Dieffenbachia Grown under Different Nitrogen Levels and Irrigation Systems

The objective of this experiment was to evaluate the effects of N levels and irrigation systems on CaOx raphide idioblast formation in three Dieffenbachia cultivars.

Materials and Methods

Tissue cultured explants of Dieffenbachia maculata ‘Exotic Perfection’, Dieffenbachia x ‘Snow Flake’, and Dieffenbachia x ‘Tropic Breeze’ 10 weeks out of culture and rooted in 2.5-cm plastic cell trays were obtained from Twyford International Inc., Apopka, Florida on June 7, 2001. After removal from the trays, 60 uniform single plants of each cultivar were planted into 15-cm diameter pots filled with Vergo Container Mix A (Verlite Co. Tampa, FL): Canadian peat, vermiculite, and perlite (3:1:1 by volume). Each cultivar was divided into 2 equal groups, and each group was either irrigated by ebb-and-flow or overhead system. All plants were grown in a shaded and evaporated pad cooled greenhouse under a maximum photosynthetically active radiation (PAR) of 285 µmol•m⁻²•s⁻¹ (normal greenhouse condition). Temperatures ranged from 18.3 to 32.2°C and relative humidity from 50 to 100%.

One group of 54 plants (18 plants of each cultivar) were placed on grooved 2.4 m² ebb-and-flow trays leveled on benches in a shaded greenhouse and sub-irrigated with nutrient solutions stored in closed polybutylene 75-L receptacles (Rubbermaid,
Winchester, VA) under the benches. The solutions were made from a water-soluble fertilizer 17N-2.1P-15.7K (Peter’s 24N-8P2O5-16K2O, Grace-Sierra Horticultural Products, Milpitas, CA) to provide N concentrations of 50, 200, or 800 mg L⁻¹. Each ebb-and-flow tray was equipped with a receptacle and a submersible pump. An automatic timer controlled all pumps, and solutions were pumped into corresponding ebb-and-flow trays to a depth of 2.5 cm for 10 minutes 2-3 times a week so that all plants were fertigated through the bottom of the pots by capillary action. Solutions were then drained back into the storage receptacle for continuous use. Ammonium- and nitrate-N concentrations in each receptacle were analyzed weekly using the methods described by Diatloff and Rengel (2001). Appropriate amounts of N were added to maintain total N in each receptacle at designated concentrations.

The other group of 54 plants grown under overhead irrigation were placed on a greenhouse bench, and fertilized with an 18.0N-2.6P-10.0K controlled-released fertilizer with micronutrients (Osmocote 18-6-12, 8-9 month duration, The Scotts Co., Marysville, Ohio) by applying 1, 5, or 10 g per pot, respectively, to the surface of substrate. The N contained in these three fertilizer levels are equivalent to 50, 200, and 800 mg L⁻¹ used in the ebb-and-flow irrigation system. The experiment was arranged in a completely randomized design with 6 replications.

Leaf samples were taken on February 8, 2002 to determine if the number of CaOx raphide idioblasts of Dieffenbachia cultivars were affected by different N rates and irrigation systems. Samples were taken from all 3 cultivars under ebb-and-flow irrigation at 3 N levels, and leaf samples were taken from ‘Snow Flake’ grown under overhead irrigation at the 1 g, 5 g, and 10 g N levels. Within each treatment, we randomly chose 3
plants out of the 6 replicates and cut the first unfurled leaf from each plant and stored them in a sealed bag with a piece of wet paper towel to maintain relative humidity. Leaf samples were dissected and observed in the lab during the next several days.

Leaf lengths were measured, leaf blades removed and the basal half of the midrib was divided into 10 equal units and cut. The basal half of the midrib was used since preliminary observations showed that upper half of the midrib contained few CaOx raphide idioblasts. Five to ten continuous free-hand sections were made from each unit and CaOx idioblasts in cross sections were counted using a polarized light microscope (Nikon Optiphot-Pol). Leaves were used in this experiment because removal and examination was compatible with other data collected from the nutrition and irrigation experiment.

Data were analyzed using Statistical Analysis System (SAS Institute, Inc., 1992, Cary, NC). Analysis of variance (ANOVA) was followed by Tukey tests at 5% level.

**Results and Discussion**

Only raphide idioblasts were observed in the leaf midrib of all 3 cultivars, and they were distributed predominantly in the central area of the abaxial side (Fig. 2.1).

From leaf base to midpoint, the number of raphide idioblasts decreased in all the cultivars. Quadratic equations fit the data very well with a high coefficient determination ($R^2$) (Figs 2.2 to 2.5).

Comparing the 3 cultivars, ‘Snow Flake’ had the greatest number of CaOx raphide idioblasts, while idioblast numbers in ‘Exotic Perfection’ and ‘Tropic Breeze’ were similar. Under 285 $\mu$mol m$^{-2}$ s$^{-1}$, plants grown under 200 ppm or 5 g nitrogen level always contained the greatest amount of CaOx raphide idioblast in their leaf midrib.
Through visual evaluation and measurement of the leaf length, all 3 cultivars exhibited optimum growth under 200 ppm or 5 g nitrogen level (Fig. 2.6). Plants grown under 50 ppm and 800 ppm did not have obvious visual difference, and in both ‘Exotic Perfection’ and ‘Tropic Breeze’, Tukey’s test showed no significant differences in the number of CaOx raphide idioblasts between these two N levels.

In previous studies on the CaOx crystal in relation to N, two N sources – ammonium and nitrate-N were compared (Franceschi, 1987). He concluded significantly more crystal idioblasts were formed by plants grown on ammonium-N than by plants grown on nitrate-N. But his research did not compare different N levels. The results of this experiment showed that CaOx raphide idioblasts in *Dieffenbachia* were closely related to the growth rate of the plant. Under 200 ppm N, plants have larger leaves and more CaOx raphide idioblasts per unit length of leaf midrib, therefore, these plants contained greater amount of CaOx raphide idioblasts than plants grown under other two N rates.

When CaOx raphide idioblast numbers in ‘Snow Flake’ grown with the same N level but different irrigation systems were compared, ANOVA tests showed no significant differences. Therefore, the quantity of CaOx idioblasts in *Dieffenbachia x ‘Snow Flake’* were not affected by the irrigation system.

**Comparison of CaOx Raphide Idioblast Number in Leaf Midribs of *Dieffenbachia* Grown under Different Light Intensities**

The objective of this experiment was to determine the effects of light intensity on CaOx raphide idioblast numbers in *Dieffenbachia*. 
**Materials and Methods**

The same plants grown for the N level experiment were used to evaluate light intensity effects on raphide idioblast numbers. Fertilizer programs were terminated at January 15, 2002, and 6 of the 7 replicates were moved to interior evaluation rooms with 8 or 16 µmol•m$^{-2}$•s$^{-1}$ PAR (3 plants each light intensity) on February 15, 2002 to determine how CaOx crystals in *Dieffenbachia* cultivars produced using different N rates and irrigation systems were affected by interior light levels.

The experimental rooms were maintained on a 12 hr light / 12 hr dark cycle with a temperature range of 20 to 24$^\circ$C and relative humidity 50 to 60%. Leaf samples were taken on October 15, 2002 from the same treatments which were selected for the N level experiment, and CaOx raphide idioblast numbers in leaf midribs were also determined by the same method.

Comparison of leaf samples from plants grown under 8 and 16 µmol•m$^{-2}$•s$^{-1}$ revealed no significant differences, therefore, samples from plants grown under 8 µmol•m$^{-2}$•s$^{-1}$ were chosen to compare with samples from plants grown under greenhouse light level (285 µmol•m$^{-2}$•s$^{-1}$).

Data were analyzed through analysis of variance (ANOVA) tests using SAS software.

**Results and Discussion**

Examination of leaf midrib sections from plants grown under 8 µmol•m$^{-2}$•s$^{-1}$ revealed that CaOx raphide idioblast numbers decreased from the midrib base to the midrib midpoint, as previously observed, and quadratic equations continued to fit the data better than linear equations in all the cases (Figs 2.2, 2.5, 2.7, and 2.8). For each N level, each cultivar, comparison of the amount of CaOx raphide idioblasts before and after
plants were moved into interior condition showed that the number of CaOx idioblasts decreased significantly after placement in light levels typical of building interiors. ‘Tropic Breeze’ grown under ebb-and-flow irrigation and 800 ppm N level was the only exception.

It is known that oxalate synthesis, and the presence of Ca ions is essential to the induction of crystal idioblasts (Franceschi and Horner, 1979). As cell metabolic rates increase, the synthesis of ascorbic acid and galactose, precursors to oxalate formation (Keates et al., 2000) also increase. Thus the increase of CaOx crystal formation under higher light level is understandable, since the metabolic rates were elevated under the higher light intensity, meanwhile, Ca uptake was also elevated.

**Conclusions**

(1) CaOx idioblast numbers were influenced by fertilizer levels and light intensities but not by irrigation system.

(2) *Dieffenbachia* grown under standard greenhouse fertilizer and light levels had the greatest number of raphide idioblasts.

(3) Total number of CaOx raphide idioblasts in *Dieffenbachia* was closely related to growth rate. Optimum growth conditions produced the greatest number of CaOx raphide idioblasts.

(4) *Dieffenbachia* transferred to light levels typical of building interiors had a lower number of raphide idioblasts in the midrib than plants grown under greenhouse production light levels.

(5) Measures other than manipulation of fertilizer rates would be required to reduce the number of raphide idioblasts.
Figure 2.1. Distribution of CaOx raphide idioblasts in *Dieffenbachia* x ‘Tropic Breeze’ leaf midrib free hand cross sections. Raphide idioblasts mainly distribute in the central area of abaxial side. A. Bar = 1 mm. B. Bar = 200 µm.
Figure 2.2. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. maculata 'Exotic Perfection'* at different N levels under ebb-and-flow irrigation system. Raphide idioblast number decreased from leaf base to leaf midpoint. Quadratic equations fit the data well. Plants grown under 285 µmol•m⁻²•s⁻¹ contained less raphide idioblasts than those grown under 8 µmol•m⁻²•s⁻¹.
Figure 2.3. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. x Tropic Breeze* at different N levels under ebb-and-flow irrigation system and 285 µmol·m⁻²·s⁻¹ light level.
Figure 2.4. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. x 'Snow Flake'* at different N levels under ebb-and-flow irrigation system and 285 μmol•m⁻²•s⁻¹ light level.
Figure 2.5. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. x 'Snow Flake'* at different N levels under overhead irrigation system. Raphide idioblast numbers in plants grown under 285 µmol·m⁻²·s⁻¹ are significantly higher than plants grown under 8 µmol·m⁻²·s⁻¹ light level.
Figure 2.6. Performance of *D. x ‘Snow Flake’* under 3 N levels. A. 50 ppm N level. B. 200 ppm N level. C. 800 ppm N level. Plants grown under 200 ppm N have optimum growth.
Figure 2.7. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. x 'Tropic Breeze'* at different N levels under ebb-and-flow irrigation system and 8 µmol m⁻² s⁻¹ light level.
Figure 2.8. Number of CaOx raphide idioblasts in basal half of leaf midrib of *D. x 'Snow Flake'* at different N levels under ebb-and-flow irrigation system and 8 µmol·m⁻²·s⁻¹ light level.
CHAPTER 3
INVESTIGATION ON THE DISTRIBUTION OF CALCIUM OXALATE CRYSTALS
IN DIEFFENBACHIA

The objective of this experiment was to investigate the distribution and quantity of CaOx druse and raphide crystals in the stems, roots, inflorescences and leaves of three Dieffenbachia cultivars.

Materials and Methods

Three Dieffenbachia cultivars, D. maculata ‘Carina’ and ‘Rebecca’ and D. x ‘Star Bright’ were used in this investigation. Both ‘Carina’ and ‘Rebecca’ are sports selected from D. maculata ‘Camille’ and have white, cream, and yellow variegated, ovate leaves that are 30 cm long and 12 cm wide. ‘Rebecca’ and ‘Carina’ differ visually as they have distinctive variegation patterns. ‘Star Bright’ was developed via traditional breeding involving several D. maculata cultivars including D. maculata var. Angustior Angustifolia, which is native to Guyana (Henny, 1995). 'Star Bright' has a foliar variegation pattern highlighted by whitish petioles and narrow leaves half the width of most Dieffenbachia leaves.

Three uniform tissue cultured plantlets of each cultivar were transplanted into a 15-cm diameter pot filled with Vergro Container Mix A (60% Canadian peat, 20% perlite, and 20% vermiculite by volume; Verlite Co., Tampa, FL). Ten containers of each cultivar were potted. Plants were grown in a shaded and evaporated pad cooled greenhouse with a maximum photosynthetic active radiation of 380 µmol•m⁻²•s⁻¹ under
natural photoperiod. Greenhouse temperatures ranged from 20 to 32°C and relative humidity from 50 to 100%. Plants were fertilized once or twice a week, depending on temperature, with a nutrient solution made from a 20N-8.7P-16.6K water-soluble fertilizer (Peter’s 20-20-20, The Scotts Co. Marysville, OH) at a nitrogen concentration of 200 mg•L\(^{-1}\). Ten weeks later, four pots of each cultivar were dissected to obtain root, stem and leaf samples, and six pots of each cultivar were foliar sprayed with 500 mg•L\(^{-1}\) of gibberellic acid (GibGro 4LS, 4% GA\(_3\) and 96% isopropyl alcohol; Agtrol Chemical Products, Houston, TX) until runoff. Ten weeks later, all GA-treated plants flowered; inflorescences were removed at anther dehiscence and dissected to determine CaOx crystal distribution and density in the spadix of male, female, and sterile zones.

**Stem, root, and spadix samples.** CaOx crystal counts in stems, roots, and spadix were determined using both fresh and preserved tissues. Sections of the first, second, and third macroscopically visible internodes from the apex of each cultivar were killed in FAA (formalin-acetic acid), soaked in 5% NaOH for 1 hr, placed on glass slides, and gently squashed for observation. Three additional segments of the first, second, and third internodes and nodal regions of each cultivar were killed in FAA, dehydrated in tertiary butyl alcohol (TBA), and embedded in Paraplast. Twelve µm cross and longitudinal sections were cut using a rotary hand microtome (Spencer Lens Co., Buffalo, NY). The sections were mounted using Haupt’s adhesive. All slides were observed under a Nikon Optiphot-Pol research microscope (Nikon Nippon Kogaku K.K., Tokyo, Japan) equipped with polarizing optics. Crystal counts were taken from micrographs, and photographs were taken with an automatic Nikon UFX-II camera attachment (Nippon
Kogaku K.K., Tokyo, Japan). The described stem preparation procedures were also used for preparing root and spadix (male, female, and sterile zones) samples of each cultivar.

**Leaf samples.** The furled leaf (leaf blade tightly wrapped), the unfurling leaf, and the first unfurled leaf (laminae on both sides of the midvein were flat) were excised from ‘Carina’, ‘Rebecca’, and ‘Star Bright’. The procedures described by Sunell and Healey (1985) were used to clear leaves and count CaOx crystals. Briefly, leaves were placed in 70% ethanol at 60 ºC for one day, transferred to 95% ethanol at room temperature for 1 hr, washed in distilled water, transferred to 5% NaOH for 1 hr at room temperature, and rinsed three times in distilled water for transection examination. Transections 5 mm wide were cut from the leaf base, mid-section, and apex. Total counts of raphide and druse idioblasts on several small interveinal areas about 0.4 – 0.6 mm² on each transection were determined using a polarized light microscope (Nikon Optiphot-Pol) and multiplied by the specific area ratio. After counts were made on each transection, a narrow piece about 2 mm wide was removed, dehydrated in TBA, embedded in Paraplast, and sectioned at 12 µm using a rotary hand microtome (Spencer Lens Co.). Idioblast counts derived from these two different procedures were consistent.

Numbers or densities of CaOx raphide and druse idioblasts in the observed area of leaf blades, internodes, and different zones of spadix of three cultivars were analyzed using the Statistical Analysis System (SAS Institute, Inc., 1992, Cary, N.C.). When significant ($P < 0.05$) differences occurred within a measured parameter, means were separated using Tukey tests at the 5% level.

**Results and Discussion**

**Stems.** Raphide idioblasts were distributed in a ring-like fashion in the first or second cortical cell layer under the epidermis (Fig. 3.1). They were also observed in the
inner ground parenchyma. Druse idioblasts were distributed sporadically throughout the cortex (Fig. 3.1). ‘Carina’ had the highest number of druses while ‘Star Bright’ had the lowest in all three internodes examined. Druse idioblast densities increased as tissue matured in all three cultivars, and the druse idioblast densities in the third internode was up to 10 times greater than the first internode (Fig. 3.2). No significant differences existed in raphide densities between the second and third internodes of all 3 cultivars, while significant differences of druse densities were detected in each developmental stage in all cultivars.

Additionally, raphide densities increased dramatically at axillary bud initiation sites from stem cross sections of nodal regions (Fig. 3.3). Furthermore, crystal sand was also frequently observed in the cortex and inner ground parenchyma in the second and third internodes (Fig. 3.4). No previous reports have documented CaOx crystal sand formation in Dieffenbachia. Genua and Hillson (1985) reported negative results using the Pizzolato and Rubeanic acid-silver nitrate tests in Dieffenbachia maculata leaves. This crystal type may only occur in stems of Dieffenbachia.

The increase of CaOx crystal density as stem tissue matured was observed in another aroid, taro. Sunell and Healey (1979) reported that both number and density of CaOx crystal idioblasts increased as taro corms (a special form of stem) enlarged. However, the increased raphide densities in axillary bud initiation regions and occurrence of crystal sands have not been reported in Dieffenbachia before.

**Roots.** Only raphide idioblasts were observed in cross sections of roots, including the root cap and root meristematic region (data not shown). The raphide idioblasts were arranged in one or two rings, under the epidermis and one or two layers of
cortical cells. In the maturation region of the root, raphide idioblasts were also formed in the cortex and vascular system. Similar to the axillary bud initiation sites on the stem, numbers of raphide idioblasts increased dramatically at sites where lateral roots initiated. Druse idioblasts were first detected in the maturation region of the root outside the vascular tissues in close proximity to the endodermis. All cultivars showed the same patterns with no detectable differences in CaOx densities. An association of druse idioblasts with vascular tissues was also observed in taro corms by Sunell and Healey (1979). They found numerous druses concentrated at root trace junctions along a ring of vascular tissue about 3 mm from the surface of the corm.

Male flower zone. Druse and raphide idioblasts of ‘Rebecca’ were observed primarily in the anthers, with few in the filaments, and none in the spadix where male flowers attached. In contrast, spadices of ‘Carina’ and ‘Star Bright’ contained a low density of druses and raphides (Fig. 3.5). At new flower initiation sites in male flower zone cross sections, a raphide distribution pattern similar to that found in lateral roots and axillary buds was observed. The CaOx crystal density increased dramatically where new flowers initiated (Fig. 3.6).

Female flower zone. Cross sections of female flowers revealed the greatest number of raphides at the ovarian end of style, and the greatest number of druses at the stigmatic end of the style. No crystals were found in the spadix where female flowers developed in ‘Carina’ and ‘Rebecca’, however, ‘Star Bright’ had a crystal density in the spadix of 67.43 cm$^2$ for druses and 5.34 cm$^2$ for raphides. Interestingly, the staminodes associated with the female flowers contained numerous raphide crystals as well as starch grains (Fig. 3.7).
**Sterile flower zone.** No crystals were detected in cross sections of the spadix sterile flower zone of ‘Rebecca’. However, the spadix of ‘Carina’ and ‘Star Bright’ were characterized by a low density of both druses and raphides. The density of CaOx druse and raphide idioblasts was much greater in sterile flowers than female or male flowers (Figs 3.7 and 3.8).

**Leaves.** Leaves contained more druse than raphide idioblasts regardless of developmental stages (Fig. 3.9), but both idioblasts were located primarily in interveinal areas of the lamina. Genua and Hillson (1985) observed druses in both palisade and spongy mesophyll and raphides in each tissue layer of *Dieffenbachia maculata* leaf blades, i.e., adaxial epidermis, palisade mesophyll, spongy mesophyll, and abaxial epidermis. Our study also found druse idioblasts in the leaf epidermis, but no raphide idioblasts were detected in the abaxial epidermis.

The raphide density in unfurled and unfurling leaves of the three cultivars was similar. However, druse density of unfurling leaves of ‘Rebecca’ and ‘Star Bright’ was less than unfurled leaves, while druse density of unfurled and unfurling leaves were similar in ‘Carina’. The highest druse densities were observed in furled leaves. As leaves unfurled and expanded, both druse and raphide densities decreased. The greatest density of druse idioblasts was observed near leaf margins (Fig. 3.10A, B). Parallel to the leaf margins, five to seven rows of druse idioblasts were observed in all three cultivars. This concentration of druse idioblasts has not been previously reported in *Dieffenbachia* or other aroids.

**CaOx Crystal Quantity in Relation to Differentiation**

In this study, we observed dramatically increased numbers of CaOx raphide idioblasts at lateral root, axillary bud, and new flower initiation sites. Franceschi and
Horner (1980) investigated CaOx crystals in callus cultures of *Psychotria punctata* Vatke and reported increased idioblast formation in regions of vascular nodule formation and root initiation during callus cultures. They suggested that the high density of CaOx crystals in differentiation sites of callus cultures was a consequence of the high metabolic rate in those sites. Our investigation of greenhouse-grown *Dieffenbachia* revealed that raphide formation was closely associated with regions of elevated cellular division and differentiation that lead to the development of secondary meristems.

Oxalate synthesis and the presence of Ca ion are two major prerequisites for the formation of crystal idioblasts (Franceschi and Horner, 1979; Webb, 1999). At differentiation sites, these two components are present. Cells with a high metabolic rate have an increased synthesis of oxalate from ascorbic acid and galactose, precursors to oxalate formation (Keates *et al*., 2000). Cellular regions undergoing differentiation during the initiation process can function as a physiological sink where ionic concentrations significantly increase. Calcium, as a ubiquitous signal in plants, could reach micromolar levels at the growing apex (Sanders *et al*., 1999), and Ca gradients in pollen tubes correlates closely with growth (Miller *et al*., 1992). Thus, elevated levels of the two constituents for CaOx crystal formation at differentiation sites could lead the formation of CaOx. However, the roles of formed raphides at these sites to plant growth remain to be determined.

Another region with increased CaOx is the leaf margin (Fig. 3.10). This increase is mainly associated with the formation of druse idioblasts. Sunell and Healey (1985) proposed that the deposition of druses was more sensitive to factors such as growth rate or physiological variables. Volk *et al.* (2002) believed that druse idioblast formation in
*Pistia stratiotes* L. is strictly involved in Ca regulation, as druses are more sensitive to Ca levels with respect to size and number. Druses are induced to form in differentiating chlorenchyma under high Ca levels, and druses can be dissolved when Ca is limited. They also claimed that this proposition is supported by the fact that druse deposition in the leaf primarily around or close to the xylem of the vascular bundles. Our observation of high levels of druses in leaves may support the proposition that druses act as reversible storage sites for Ca, but function and formation factors of the high densities of druses in the leaf margins require further investigation.

CaOx raphide density increased where the new flowers initiated in male flower zone, but this was not observed in the female flower zone of the spadix. This is possibly because the female flowers on the collected samples had already matured, but the male flowers were still undergoing the final stages of maturation. In *Dieffenbachia*, female flowers matured earlier than male flowers, thus reducing the possibility of self-pollination (Henny, 1977).

**The Relationship of R/D Ratio with Developmental Stage**

Only raphide idioblasts, not druses, were detected in cross sections of the root cap and region of cell multiplication and cell elongation, while in the region of root cell maturation both druse and raphide idioblasts were present. Our results support the conclusion by Sunell and Healey (1985) that raphide idioblasts are formed earlier than druse idioblasts.

Raphides were the dominant idioblasts in the first internodes of all three cultivars. As stem tissues matured, the densities of druse idioblasts increased more rapidly than the densities of raphide idioblasts (Fig. 3.2). However, it was druses that were dominant in the furled leaves, and as leaves unfurled, the density of both druses and
raphides decreased. To describe the dynamic changes of idioblast quantities with the developmental stages, we developed a raphide to druse ratio (R/D). The R/D ratios decreased in both stems and leaves as tissues matured (Table 3.1). Additionally, the ratios in stems were larger than 1 but less than 0.2 in leaves, revealing that the dominant type of crystal idioblast was organ specific. Although numerous studies have attributed the skin irritation to raphides in *Dieffenbachia*, the large number of druse idioblasts in leaf margin suggests that druses as well as raphides exacerbate the toxicity of proteolytic enzymes of *Dieffenbachia*.

**Cultivar Differences in CaOx Crystal Densities**

It is generally agreed that regardless of the underlying mechanisms involving the toxic effects of *Dieffenbachia*, raphides, druses, or both, are the vehicles of irritating substances. A solution to further strengthen the status of *Dieffenbachia* as an important indoor foliage plant is to reduce the quantities of CaOx through breeding. Screening of mutated populations of *Medicago truncatula* L., McConn and Nakata (2002) showed that calcium crystal formation and growth is a tightly regulated genetic process. For the first time, our study demonstrated the differences in CaOx densities exist among *Dieffenbachia* cultivars. ‘Star Bright’, a hybrid developed from multiple crosses, has significantly lower density of druses in stems than ‘Carina’ and ‘Rebecca’, which are genetically similar as both are sports of ‘Camille’. ‘Carina’ had consistently lower densities of both raphides and druses in stems and lower densities of druses in leaves than those of ‘Rebecca’. These results suggest that genetic reduction of CaOx crystal density in *Dieffenbachia* is possible.
Table 3.1. CaOx raphide and druse idioblast ratios of 3 different stem and leaf developmental stages of 3 *Dieffenbachia* cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>R/D Ratio</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stem</td>
<td>Leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1st Internode</td>
<td>2nd Internode</td>
<td>3rd Internode</td>
<td>Furled</td>
<td>Unfurling</td>
</tr>
<tr>
<td>‘Carina’</td>
<td>8.85</td>
<td>1.58</td>
<td>1.03</td>
<td>0.107</td>
<td>0.090</td>
<td>0.087</td>
</tr>
<tr>
<td>‘Rebecca’</td>
<td>7.47</td>
<td>6.61</td>
<td>1.35</td>
<td>0.134</td>
<td>0.118</td>
<td>0.068</td>
</tr>
<tr>
<td>‘Star Bright’</td>
<td>18.23</td>
<td>16.86</td>
<td>7.70</td>
<td>0.197</td>
<td>0.098</td>
<td>0.096</td>
</tr>
</tbody>
</table>
Table 3.2. Total CaOx crystal density in stems and leaf blades of 3 *Dieffenbachia* cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Stem 1st Internode</th>
<th>Stem 2nd Internode</th>
<th>Stem 3rd Internode</th>
<th>Leaf Furled</th>
<th>Leaf Unfurling</th>
<th>Leaf Unfurled</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Carina’</td>
<td>279</td>
<td>467</td>
<td>575</td>
<td>14919</td>
<td>9340</td>
<td>9470</td>
</tr>
<tr>
<td>‘Rebecca’</td>
<td>143</td>
<td>383</td>
<td>373</td>
<td>10977</td>
<td>6468</td>
<td>4391</td>
</tr>
<tr>
<td>‘Star Bright’</td>
<td>75</td>
<td>281</td>
<td>379</td>
<td>14321</td>
<td>9226</td>
<td>5942</td>
</tr>
</tbody>
</table>
Figure 3.1. Cross section of the third internode of D. x ‘Star Bright’, 12 µm thick. Bar = 0.5 mm. Note a ring of CaOx raphide idioblasts (R) under the epidermal cells. In cortex and ground parenchyma, druses (D), raphide idioblasts, and a huge amount of starch grains (SG) were observed.
Figure 3.2. Density of CaOx druse and raphide idioblasts in stems of 3 *Dieffenbachia* cultivars. R = raphides and D = druses.
Figure 3.3. Cross section of the first internode of D. x ‘Star Bright’. In the axillary bud, the density of CaOx crystals is much higher than other parts of the stem. Bar = 0.5 mm.
Figure 3.4. Crystal sand observed in cross section of the second internode of *D. maculata* ‘Rebecca’. Bar = 50 µm.
Figure 3.5. Cross section of *D. maculata* ‘Carina’ spadix male flower zone. Note increased CaOx crystal density in anthers. Bar = 0.5 mm.
Figure 3.6. Cross section of D. x ‘Star Bright’ spadix male flower zone. Density of CaOx crystal increases remarkably at where the new flower initiates. Bar = 0.1 mm.
Figure 3.7. Density of CaOx druse and raphide idioblast in the flowers of 3 Dieffenbachia cultivars.

*Spadix where male flowers developed.

**Spadix where sterile flowers developed.
Figure 3.8. Cross section of D. maculata ‘Rebecca’ spadix sterile flower zone. CaOx crystals located within the sterile flowers (SF). Bar = 0.1 mm.
Figure 3.9. Number of CaOx druse and raphide idioblast in 50 mm² area of leaf blades of 3 Dieffenbachia cultivars. R = raphides and D = druses.
Figure 3.10. CaOx crystal distribution in leaf blade of D. x ‘Star Bright’. A, leaf margin, has higher density of druses compared to other part of the leaf blade. B, leaf blade, druse idioblasts (D) and two forms of raphide idioblasts, defensive (DR) and nondefensive raphide idioblast (NR), were all observed. Bar = 0.1 mm.
CHAPTER 4
INVESTIGATION ON THE RELATIONSHIP BETWEEN CALCIUM OXALATE CRYSTAL AND RHIZOSPHERIC CALCIUM AND/OR MAGNESIUM LEVELS

The objective of this experiment was to determine the effect of different concentration of Ca, Mg or both Ca and Mg on the density, distribution and size of CaOx druse and raphide idioblasts in *Dieffenbachia maculata* ‘Rebecca’.

**Materials and Methods**

Tissue cultured liners of *Dieffenbachia maculata* ‘Rebecca’ (week 39) were transplanted on January 5, 2003 to a hydroponic system as described by Chen and Gabelman (1999) and into container media as described in Chapter 3. Plants were grown in a shaded green house under 285 µmol•m⁻²•s⁻¹, temperature of 20-32°C, and relative humidity of 50-100%.

Thirty hydroponic containers were divided into 10 groups with 3 containers for each group, and 2 tissue cultured liners per container. Each group was filled with nutrient solutions labeled as 1 to 10 (Table 4.1). For the plants grown in potting media, no fertilizer was added after potting. Fifty uniform plants were divided into 10 groups, 5 plants each group. Plants in each group were respectively watered with nutrient solution with Ca and Mg concentration as listed in Table 4.1.

One leaf transection, 2 cm by 5 mm, from the leaf margin in the middle of lamina was removed from the first unfurled leaf, 3 replicates were randomly selected from the 6 replicates in each group on May 23, 2003. Two weeks later (June 6, 2003),
another leaf transection from the symmetrical position of the same leaf was taken from plants previously sampled. Two weeks later (June 20, 2003), the third sample was taken from the first unfurled leaf at the same position.

Leaf transections were killed, dehydrated, microtomed at 12 µm, and observed by the same methods used on stems and inflorescences in the CaOx crystal distribution investigation (chapter 3). In each cross section, CaOx druse and raphide crystal numbers within 1 cm from leaf margin were counted.

Results and Discussion

Plant growth in hydroponic solutions varied with Ca and Mg concentration in the solution. A preliminary rating of the plants showed treatment 5 had the best growth, followed by treatments 4 and 6. Plants in treatments 2, 3, 7, and 8 were ranked next, while treatments 1, 9 and 10 had the least growth and were characterized by small and twisted new leaves. Plants grown in potting medium all had acceptable quality. This suggests that potting medium served as a nutrient buffer and limited amounts of nutrients were available.

Cross sections of the leaf blades revealed that both druse idioblasts and raphide idioblasts were mainly distributed within palisade mesophyll. Druse idioblasts were found in all the leaf layers: adaxial epidermis, palisade mesophyll, spongy mesophyll, and abaxial epidermis. Raphide idioblasts were found in all layers except the abaxial epidermis.

Sakai and Nagao (1980) stated in their investigation of CaOx raphide idioblasts that *Dieffenbachia maculata* contained two kinds of raphide in the stems: defensive (small) and nondefensive (large). We observed that nondefensive raphide idioblasts in *D. maculata* ‘Rebecca’ leaf blade occurred with greater frequency in the spongy mesophyll,
parallel to the epidermis; while most defensive raphide idioblasts were observed in the palisade mesophyll, and were oriented vertical to the epidermis. The size of nondefensive raphide idioblast was usually twice that of defensive raphide idioblasts (Fig. 4.1).

As discussed earlier (chapter 3), an increased density of druse idioblasts in close proximity to leaf margins was found in all 3 cultivars. In this investigation these marginal druses were affected by substrate Ca and Mg levels. Hydroponic cultured plants grown without Ca, treatments 1, 8, 9, and 10 had no druse idioblasts observed near the leaf margin. Cross sections of other treatments usually had 4-8 druse idioblasts located within 0.5 mm from the margin (Fig. 4.2). This indicates that marginal druses did not form until Ca substrate levels attained a level of 1 mM. Thus, the proposition that CaOx druses serve as important sequestering sites to regulate Ca is supported (Volk et al., 2002).

Plants in hydroponic solutions without Ca (treatments 1, 8, 9, and 10) had significantly fewer crystals, and the density of druse idioblast decreased more than the density of raphide idioblasts (Fig. 4.3). Ca and Mg availability also affected the density of both crystal types. Two types of CaOx crystals showed difference in their sensitivity. Under 0 mM Mg, both druse and raphide idioblasts dramatically increased in plants cultured with 1.0 mM Ca. However, in solutions with Ca concentration of 2.5 mM and 4.0 mM, druse idioblast density decreased, but raphide idioblast numbers were not affected. These results suggest that druse density will not increase if hydroponic solutions do not contain an appropriate ratio of Ca to Mg. The density of druse and raphide idioblasts increased when plants were grown in hydroponic solutions that contained equal concentrations of Ca and Mg. The druse density of plants grown in solutions with 0 mM Ca was almost zero, but raphide density remained stable.
The observed response of druse crystal density to Ca and Mg levels in plants grown in potting media were not as dramatic as those in hydroponic plants (Fig. 4.4). The densities of raphide idioblast in leaves were similar under the different nutrient regimes. This is probably because container media contained residual Ca and Mg, and the effects of either low Ca or Mg were masked.

Studies by other researchers (Frank, 1972; Zindler-Frank, 1975; Borchert, 1986; Franceschi, 1989; Volk et al., 2002) indicated that CaOx crystal level was elevated when substrate Ca concentration increased, except when Ca concentrations were very high (Franceschi and Horner, 1979). Those studies did not examine the interaction of Ca and Mg. We believe that increases in CaOx idioblast quantity as Ca concentrations increase will only occur when substrate Mg is available. When Mg substrate concentration remains constant, increases in Ca levels increase CaOx idioblast quantities.

CaOx druse and raphide idioblasts are both common in all organs of Dieffenbachia. Why does Dieffenbachia have two types of CaOx crystals in the same organ? It appears that two types of CaOx crystals serve different functions. Compared to raphide idioblast formation, druse idioblast formation is more sensitive to rhizospheric Ca and Mg levels. When Ca and Mg substrate concentrations in hydroponic culture were changed, idioblast density was affected more than raphide idioblast density (Fig. 4.3). Additionally, the sizes of druse crystals were affected by Ca and Mg levels, as crystals were larger when the Ca/Mg ratio was 0.25 or higher. To use an analogy, if we view Ca as a currency, raphide idioblasts serve as a safety deposit box, while druse idioblasts are like checking account. Both may be necessary to regulate levels of cytosolic free Ca in Dieffenbachia.
One possible reason for the different response of druse and raphide idioblast formation to Ca concentrations was proposed by Volk et al. (2002). They suggested it is related to the relative complexity of the process of crystallization in the two systems, and possibly the hydration form of the two crystals. Raphide development in plants is known to be complex. It requires the coordination of growth of hundreds of individual crystals with each crystal exhibited bidirectional growth in an oriented manner that must be coordinated with cell elongation. Druse crystal formation, while controlled by cellular processes, is less complex since druse consist of a mass of single aggregated crystals and the idioblast does not show polar growth.

This investigation revealed that the CaOx druse idioblast formation is dependent on Ca and Mg concentrations. An appropriate Ca/Mg ratio is required before CaOx crystal densities follow the changes of substrate Ca concentrations. Marginal druse density increases as Ca concentrations increase. These facts lead to the conclusion that druse idioblast formation plays an important role in Ca regulation in Dieffenbachia.

In summary, this experiment demonstrates that the formation of CaOx crystals is a dynamic process. The level of either druse or raphide idioblasts can be elevated or reduced by substrate Ca and Mg levels.
Table 4.1. Ca and Mg concentrations used in groups 1-10.

<table>
<thead>
<tr>
<th>Treatment No.</th>
<th>Ca concentration (mM)</th>
<th>Mg concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
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<tr>
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<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Figure 4.1. CaOx crystals in *D. maculata* ‘Rebecca’ leaf blade cross section. Bar = 50 µm. A. Druse (D) and defensive raphide (DR) idioblasts. B. Nondefensive raphide (NR) idioblast. Most of the druse and defensive raphide crystals are located in palisade mesophyll, while nondefensive raphide crystals usually distribute in spongy mesophyll. Two kinds of raphide idioblasts have different orientation: the former are usually vertical to epidermis and the latter are parallel to epidermis. The size of nondefensive raphide idioblast is twice as big as defensive raphide idioblast.
Figure 4.2. Marginal druse idioblast densities are significantly different under different nutritional Ca levels. Bar = 250 µm. A. Hydroponic group 2 (1 mM Ca, 0 mM Mg). B. Hydroponic group 1 (0 mM Ca, 0 mM Mg).
Figure 4.3. Numbers of CaOx crystals within 1 cm from leaf margin in leaf blade cross sections of Dieffenbachia maculata ‘Rebecca’ which were grown in hydroponic solutions.
Figure 4.4. Numbers of CaOx crystals within 1 cm from leaf margin in leaf blade cross sections of *Dieffenbachia maculata* ‘Rebecca’ which were grown in potting media.
CaOx druse and raphide idioblasts were observed in all major organs of *Dieffenbachia*. Both druse and raphide idioblasts were detected in cross sections of stems, roots, inflorescences and leaves in all three cultivars examined, but the two types of CaOx crystals have different patterns of distribution. As tissue matured, druse idioblasts became more dominant. Raphide idioblasts formed earlier than druse idioblasts. An increase of CaOx crystal density was observed in secondary meristematic sites, such as axillary buds, new flowers and lateral roots, while in primary meristems, e.g., shoot meristem and root meristem, only a few crystal idioblasts were observed.

**CaOx crystal quantity and quality can be influenced by rhizospheric nutrition concentrations.** It was revealed that substrate N concentrations affected the number of CaOx raphide idioblasts in all three *Dieffenbachia* cultivars examined. Ca and Mg concentrations were proven to affect both the quantity and quality of CaOx crystals in *Dieffenbachia maculata* ‘Rebecca’. In addition, the formation of CaOx idioblasts was revealed to be a dynamic process that reflects cellular metabolic rates, absorption of Ca and Mg substrate concentrations.

**Low light intensity decreased the number of CaOx crystals in *Dieffenbachia* by reducing the cell metabolic rates.** When three *Dieffenbachia* cultivars were moved from high light levels typical of commercial production facilities to low light levels typical of building interiors, the number of CaOx raphide idioblasts decreased in all the
cultivars. Low light levels were believed to reduce CaOx crystal quantity by reducing cell metabolic rates in *Dieffenbachia*.

**Druses and raphides have different roles in regulating cytosolic Ca levels in Dieffenbachia.** When rhizospheric Ca and Mg levels fluctuated, the number of druse idioblasts in *Dieffenbachia* changed more than the number of raphide idioblasts. Additionally, the size of druse crystals was also influenced by substrate Ca and Mg nutrition levels.

**Significant differences of CaOx crystal quantity existed between cultivars.** Consequently, developing new *Dieffenbachia* cultivars with lower toxicity may be possible. Although the amount and type of CaOx crystals can be influenced by many environmental factors, such as light levels, and nutrient concentrations, the most practical means to develop *Dieffenbachia* cultivars with lower toxicity is through breeding programs and selection.
LITERATURE CITED


Franceschi VR. 1989. Calcium oxalate formation is a rapid and reversible process in *Lemna minor* L. *Protoplasma* 148: 130-137.


BIOGRAPHICAL SKETCH

Hui Cao was born on October 22\textsuperscript{nd}, 1978, in Anhui province, P.R. China. She obtained her Bachelor of Science degree in biology from Xiamen University in July 2001. At the same time, she was admitted to the master’s program in the Department of Environmental Horticulture, University of Florida. Her study was sponsored by Dr. Jianjun Chen with a research assistantship.

She worked as a research assistant in the plant ecology lab while she pursued her bachelor’s degree in Xiamen University and took part in several projects concerning ecological usage of mangrove. At the University of Florida, she was employed as research assistant in a plant physiology lab and helped with plant maintenance and microscopy.