CALCIUM OXALATE CRYSTALS IN THE LEAVES OF *NELUMBO NUCIFERA* AND *NYMPHAEA TETRAGONA*[1]

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Abstract: Both *Nelumbo nucifera* Gaertn. and *Nymphaea tetragona* Georgi contain crystals in their leaves, but the crystals differ in shape, size and distribution. In *Nelumbo* the crystals, with a druses form, occur in the central vacuole of crystal idioblast. Generally, there is only a single druse per cell. As the crystal idioblasts mature, the cell wall partly collapses around the crystal and the sharp points or facets of the crystal protrude into the adjacent air space. In *Nymphaea*, the crystal has a prismatic form. It was observed in the extraplasmic space between the primary and secondary cell wall of the sclereids. As the sclereids grow, the sharp facets of the crystal become protruded but remain covered by primary wall material. The crystal idioblasts in these two aquatic plants are found associated with aerenchyma. Their formation is suggested to linked with evaporation of water. The crystals in both *Nelumbo* and *Nymphaea* were identified as calcium oxalate by means of X-ray micro-analysis and acid-etching tests.

INTRODUCTION

Crystal formation in animals is usually a pathological phenomenon and occurs extracellularly, e. g. urinary stones. However, in plants crystals typically accumulate in specialized cells, called crystal idioblasts, during the ordinary physiological processess (Cody and Horner, 1985; Franceschi and Horner, 1980). Crystals are secondary products of plant tissues and they may be calcium or silica salts in various chemical and structural modification (Mauseth, 1988). The appearance and number of crystals inside a crystal idioblast vary considerably, and the location of the crystal idioblasts in a plant body also varies in different taxa. However, within a given taxon, the shape and distribution of crystals may be specific and some investigators have used it in classification (Heintzelman and Howard 1948; Metcalfe, 1983).

Crystals can often be observed in different organs and tissues of a single plant. In the stem and root they occur in the pith, cortex, rays and vertical parenchyma of wood and bark (Rao and Dave, 1984). Besides, they increase in number in the tissues which soon cease to function, e. g. abscission zones (Chiang and Chiu, 1989). Crystals have also been found in the leaves of various plant species. Various types of crystal occur in leaves, e. g. crystal sand in *Beta vulgaris*; raphides in *Lemna minor*; prismatic in *Glycine canescens* and druse in *Arthrostema ciliatum* (Franceschi and Shueren, 1986).

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The occurrence of biogenic crystals in vascular plants have been most studied in land plants, but rare in aquatic plants. *Nelumbo* and *Nymphaea*, two aquatic perennial herbs, are phyllogenetically closely related to each other (Li, 1976). Both contain crystals in the leaves, but the crystals are quite different in their shape and distribution. In this paper, the crystals of these two species were investigated by means of scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray microanalysis and acid-etching in order to understand their ultrastructure and chemical composition.

MATERIALS AND METHODS

The leaves of *Nelumbo nucifera* Gaertn. and *Nymphaea tetragona* Goergi were obtained from aquaria in the greenhouse of the Department of Botany, National Taiwan University.

Material for SEM were fixed for 2 h in 2.5% glutaraldehyde followed by 1% OsO_4 for 2 h, dehydrated in an ethanol-acetone series, dried with a Hitachi Critical Point Dryer (HCP-1), coated with IB-2 ion coater (Dawes, 1979), and examined with the Hitachi S-550 SEM.

For the acid-etching test, material was first treated with acetic, hydrochloric or sulfuric acid for 1 or 20 min, followed by preparation for SEM as above.

For TEM, the material was fixed in glutaraldehyde and OsO_4 followed by dehydration in a acetone series and embedded in Spurr's resin (Spurr, 1969). Sections were stained with methanolic uranyl acetate and lead citrate and viewed with a Hitachi H-600 TEM at 75 kV.

The samples for X-ray microanalysis were quenched in freon 22 (cooled by Liq. N_2) for 20 sec, transferred into Liq. N_2 for 1 h, transferred into a freeze-dryer with Liq. N_2 for two days and then coated with carbon (Yang, 1986). A Jeol JXA-733 EPMA at 10-100 μm, 25 kV was used for examining the preparations.

RESULTS

Both *Nelumbo nucifera* and *Nymphaea tetragona* have solitary, petiolated leaves. Like most aquatic angiosperms, the vascular tissue in the leaves of these species is much reduced while the aerenchyma is well developed. In the transection of petioles 6-10 air channels can be observed in the ground tissue with diaphragms sepatating the channels transversly. The crystal idioblasts of these leaves are often associated with the air channel.

Crystal idioblast in *Nelumbo*

In the petiole of *Nelumbo* crystals are formed mainly in peripheral cells around the air channels (Figs. 1b, 3) and occasionally in some cells of the diaphragm (Fig. 1c). In the mesophyll they are distributed mostly in the cells of the spongy tissue (Figs. 1a, 2d).

Constantly, there is only one crystal per idioblast. The crystal is in a druse form, about 15-20 μm in diameter. The crystal exhibits sharp points or facets and often becomes so large that the cell becomes deformed and ultimately takes on the same shape as the crystal (Figs. 1d, 2a-2c). In later stage of development, some crystal cells protrude into the adjacent air channel, then the cell wall

becomes partly ruptured. Consequently the protruding portion of crystal would be exposed to the air channel. During the sectioning the crystals would be lost with the remain of excavated cavities (Figs. 4a, 4b). In a well developed crystal idioblast there is usually no cytoplasm.

Crystal idioblast in *Nymphaea*

In *Nymphaea* crystalline bodies are on the outer surface of sclereids (idioblasts) (Figs. 5a-5c). All the sclerereids found bear crystals. As described previously (Chiang and Huang, 1983), the sclereids are well branched and quite different in their size and shape. Most of them occur sporadically in the parenchymatous tissue around the air channel (Fig. 5a).

The crystals distribute densely but are in irregular arrangement on the wall (Fig. 5c). They deposite on whole surface of a sclereid. On the other words, the crystals are found on the wall surfaces in contact with the other types of cells (mainly parenchyma) as well as the surfaces exposed to the air channel. The crystals have a prismatic form and their diameter varies from 3-5 μm. The relationship between the crystal and the cell wall can be fairly showed on the complementary fracture faces under SEM (Figs. 5d, 5e). At maturity, the crystals are apparently embedded firmly in the cell wall. This observation is supported by the fact that it is not possible to remove the crystals from the cell wall by sonic oscillations.

TEM photographs reveal that the primary wall is densely stained while the secondary wall is lightly stained (Figs. 4c, 4d). The crystals are located in the extraplasmic space between the primary and secondary walls. As the sclereids grow, the crystals remain covered by wall materials. The wall beneath the crystal is thinner than the other part of the same cell wall (Fig. 4c).

Chemical compositions of the crystals

By using the X-ray microanalysis technique it is possible to determine the elemental compositions of a selected sample area (Smith and Cameron, 1987). Using the wavelength for calcium to scan the samples of *Nelumbo* and *Nymphaea* (Figs. 3a-3d, 7a-7d), it could be observed that the crystals of both these species contain substantial amounts of calcium.

All crystals observed in *Nelumbo* and *Nymphaea* are insoluble in acetic acid (Figs. 2c, 5f), but they dissolved in hydrochloric and sulfuric acid without forming bubbles (Figs. 2d-2g, 6a-6h). These crystals are thus calcium oxalate, like the crystals in most other plant tissues.

DISCUSSION

Calcium oxalate crystals are widely distributed in plants. The presence or abscence of crystals is one of the important characters for understanding the evolutionary relationships of plant species (Franceschi & Horner, 1980). In a preliminary observation by the present work in *Nymphoides*, it is interesting to note that *Nymphoides coreana* (Gentianaceae, aquatic herbs) can grow together with *Nymphaea tetragona* in the same pond. As in *Nymphaea*, there are many sclereids in the plant bodies of *Nymphoides*, yet the outer surfaces of the sclereids of *Nymphoides* are free of crystals.

If crystal shape is regarded as a valuable taxonomic criterium then one must assume that it is under genetic control (Chartschenko, 1932; Heinzelman and Howard, 1948). In *Nymphaea* calcium oxalate crystals are formed in the cell wall by a multitude of small crystals rather than by a large druse crystal in the central vacuole as in *Nelumbo* (Figs. 1b, 5c). Each of these two plants contains only one type of crystal irrespective the tissue in which it occurs. However, in some other plants two or more different shapes can be found (Scott, 1941). In general, the different types of crystals are separately located in cells of the definite tissues. Arnott and Pautard (1970) have described that the crystal shape may be determined by the shape of the vacuole chamber in the crystal idioblast. However, various factors, such as temperature, pressure or pH gradient, can affect crystal growth (Franceschi and Horner, 1980). Presently the mechanism controlling crystal type is not yet clear.

Crystal cells are alwas distributed in a specific region of a tissue (Metcalfe, 1983). Scott (1941) suggested that the fate of a cell in a apical meristem, whether destined to become a crystal idioblast, or a typical parenchyma cell, is already determined. In mesophyll, the crystals mostly occur in the cells adjacent to large intercellular spaces or air channels. Hence, their formation may be linked with the evaporation of water (Franceschi and Horner, 1980). In both *Nelumbo* and *Nymphaea* the aerenchyma is well developed and the crystal idioblasts are associated with the well organized air channels. But in the wall between the sclereid and the adjacent parenchymatous cell of *Nymphaea* the crystals can also be found.

In plants the calcium oxalate crystals are usually found to develop intracellulary, within the central vacuole. Deposition of crystals in the wall rather than in the vacuoles is apparently not common amongst flowering plants (Franceschi and Shueren, 1986). In *Nymphaea*, it is possible that the crystals are produced

Key to labeling (For Figs. 1-7)

A —air channel
Co —collenchyma
Cr —crystal
D —diaphragm
E —epidermis
El —lower epidermis
Eu —upper epidermis
L —lacuna
P —palisade tissue
S —sclereid
V —vascular tissue
W —cell wall
Wp—primary cell wall
Ws—secondary cell wall

Fig. 1. SEM photographs of *Nelumbo*
(a) Transection of a blade bearing many crystal idioblasts in peripheral cells around the air channels.
(b) Longisection of an air channel of a petiole, bearing densely distributed crystal idioblasts.
(c) Crystal idioblasts forming in some cells of the diaphragm across an air channel.
(d) Enlargement of (c), showing the details of the crystal idioblast.

Fig. 2. SEM photographs of *Nelumbo*
(a) Crystals protruding into the adjacent air space.
(b) Cell wall of a crystal idioblasts partly collapsed.
(c) Leaf pre-etched with 50% acetic acid for 20 min, showing that the crystals did not dissolve and sharp points or facets are intact.
(d) Transection of a blade, pre-etched with 50% hydrochloric acid for 1 min.
(e) Enlargement of (d), showing that the crystal dissolved.
(f) Longisection of petiole, pre-etched with conc. hydrochloric acid for 1 min.
(g) Enlargement of (f), showing that the crystals dissolved, that the protruded wall of the crystal idioblast became shrunken and distorted.

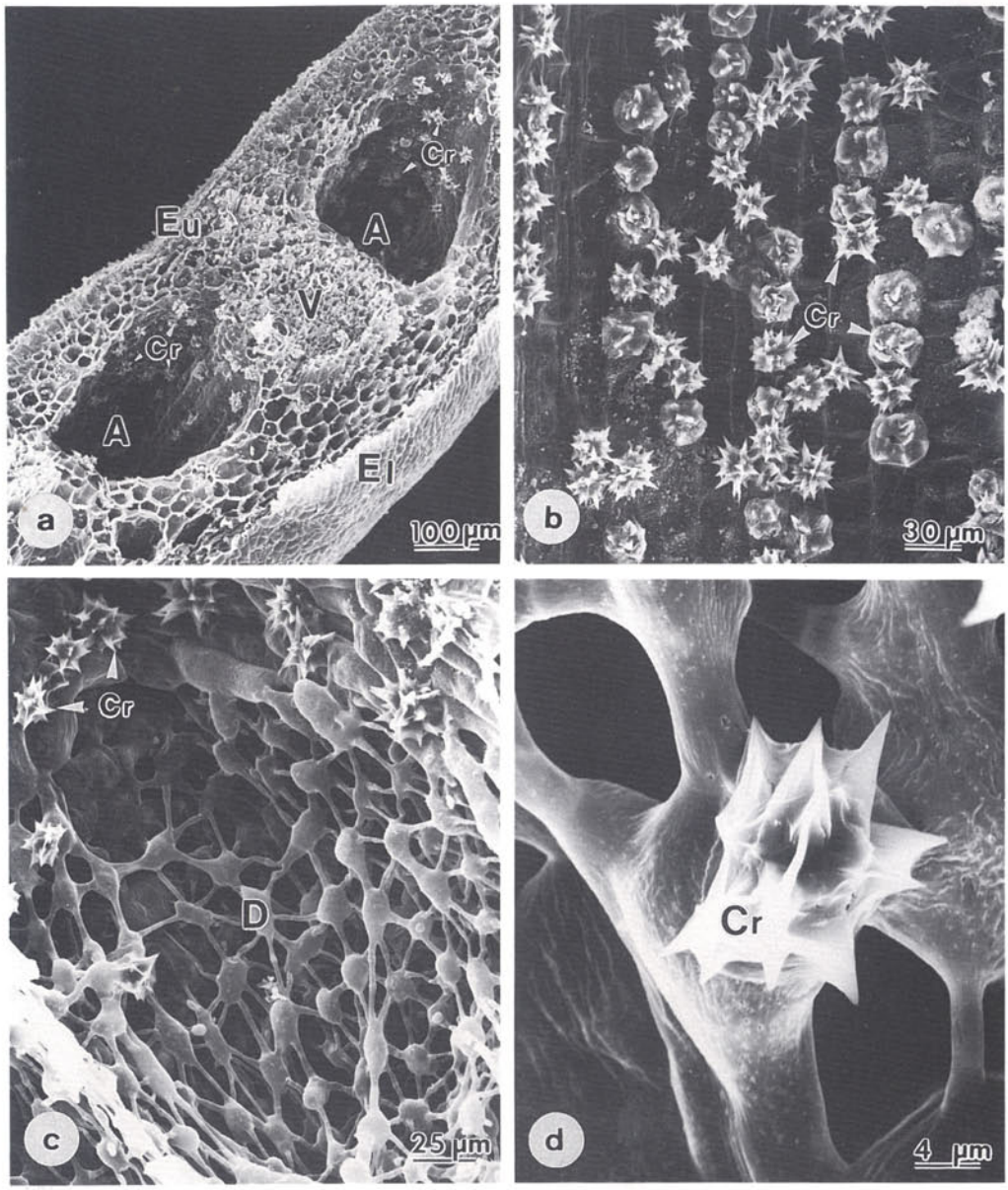

Fig. 1.

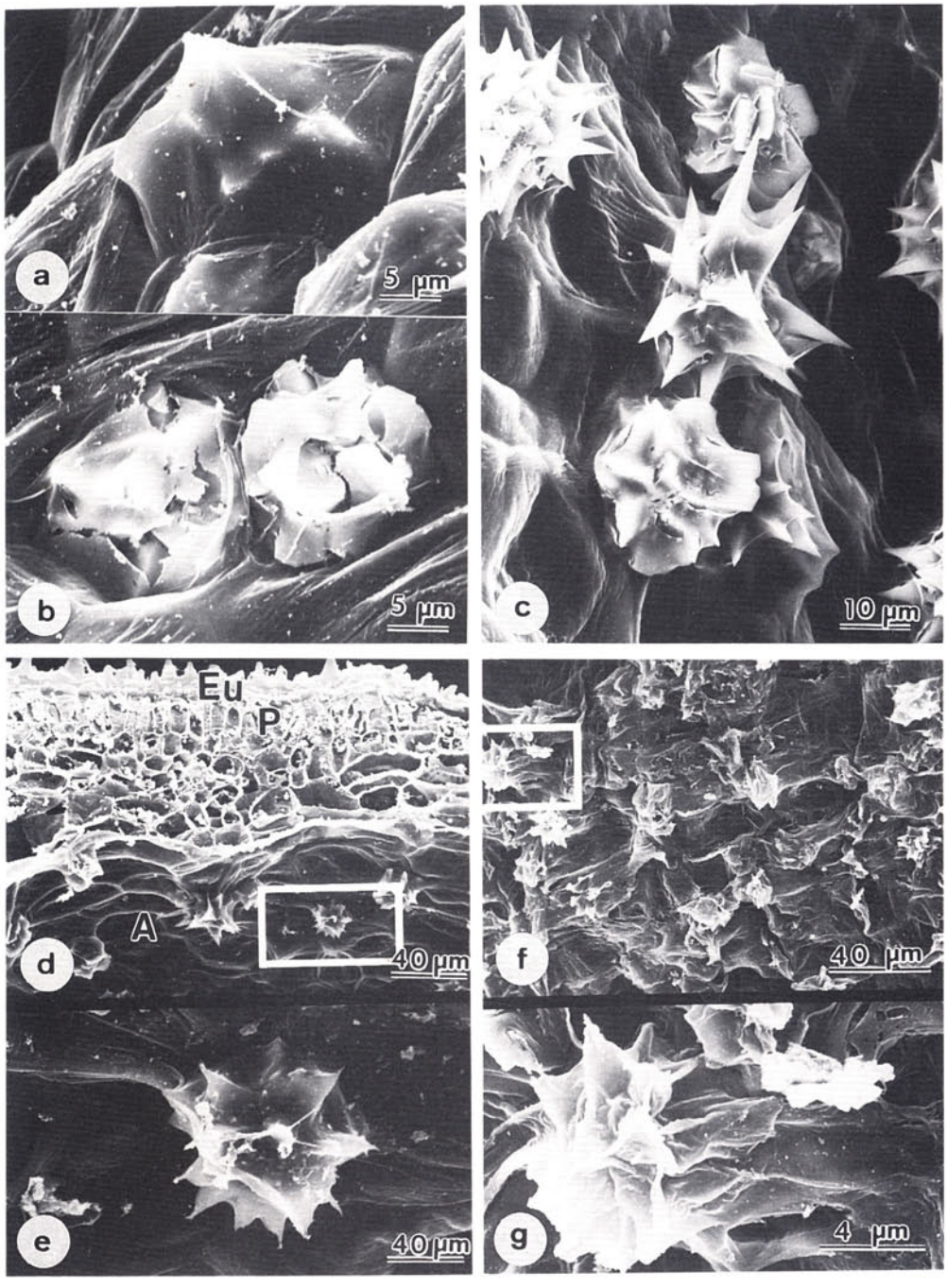

Fig. 2.

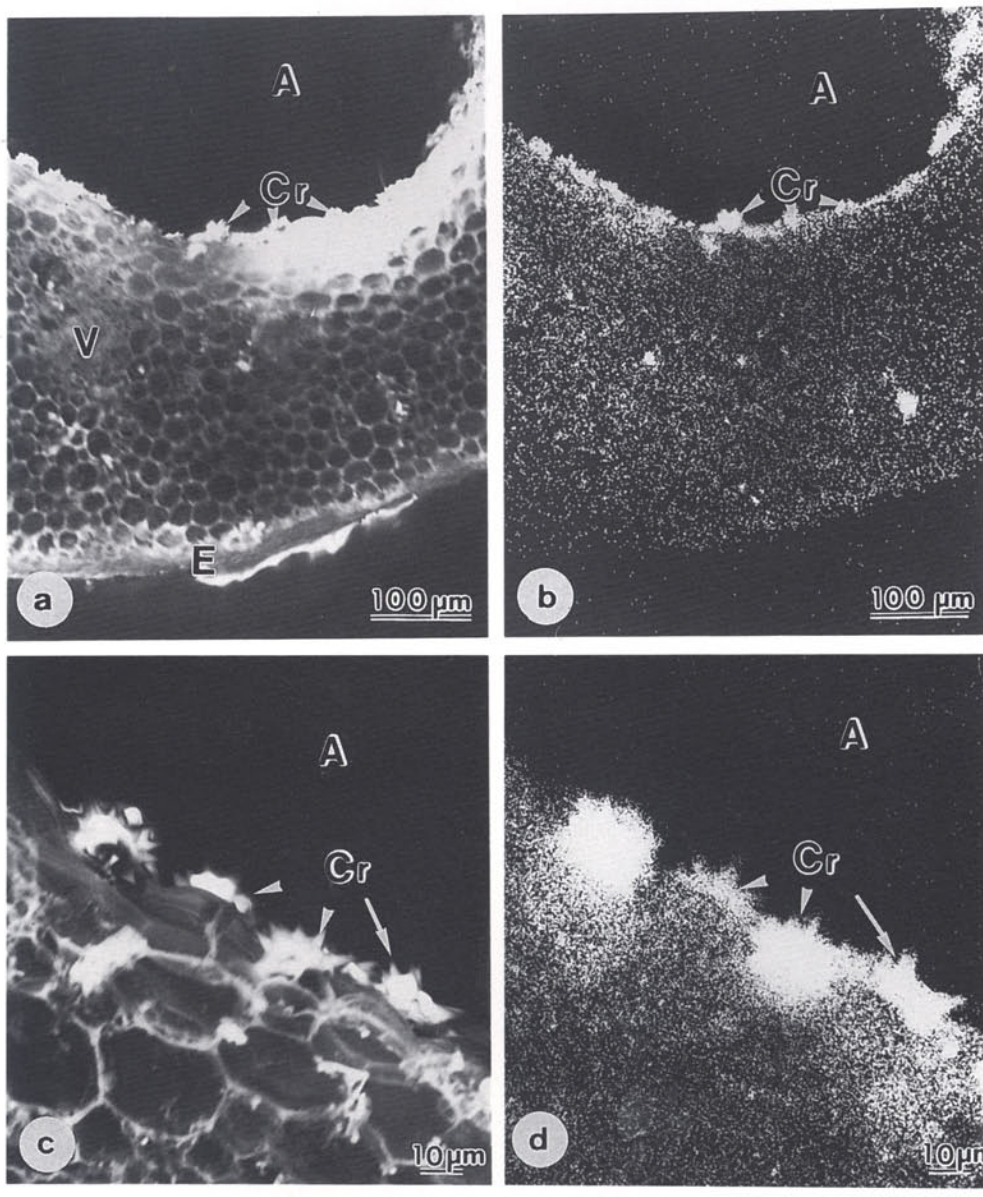

Fig. 3. *Nelumbo*

(a) SEM photograph of a transection of petiole, showing the distribution of crystals along the periphery of air channel.

(b) An X-ray map localizing the calcium electron transition energy, the map corresponding with the secondary electron image in (a), indicating the high calcium content of the crystals.

(c) Enlargement of (a).

(d) Enlargement of (b).

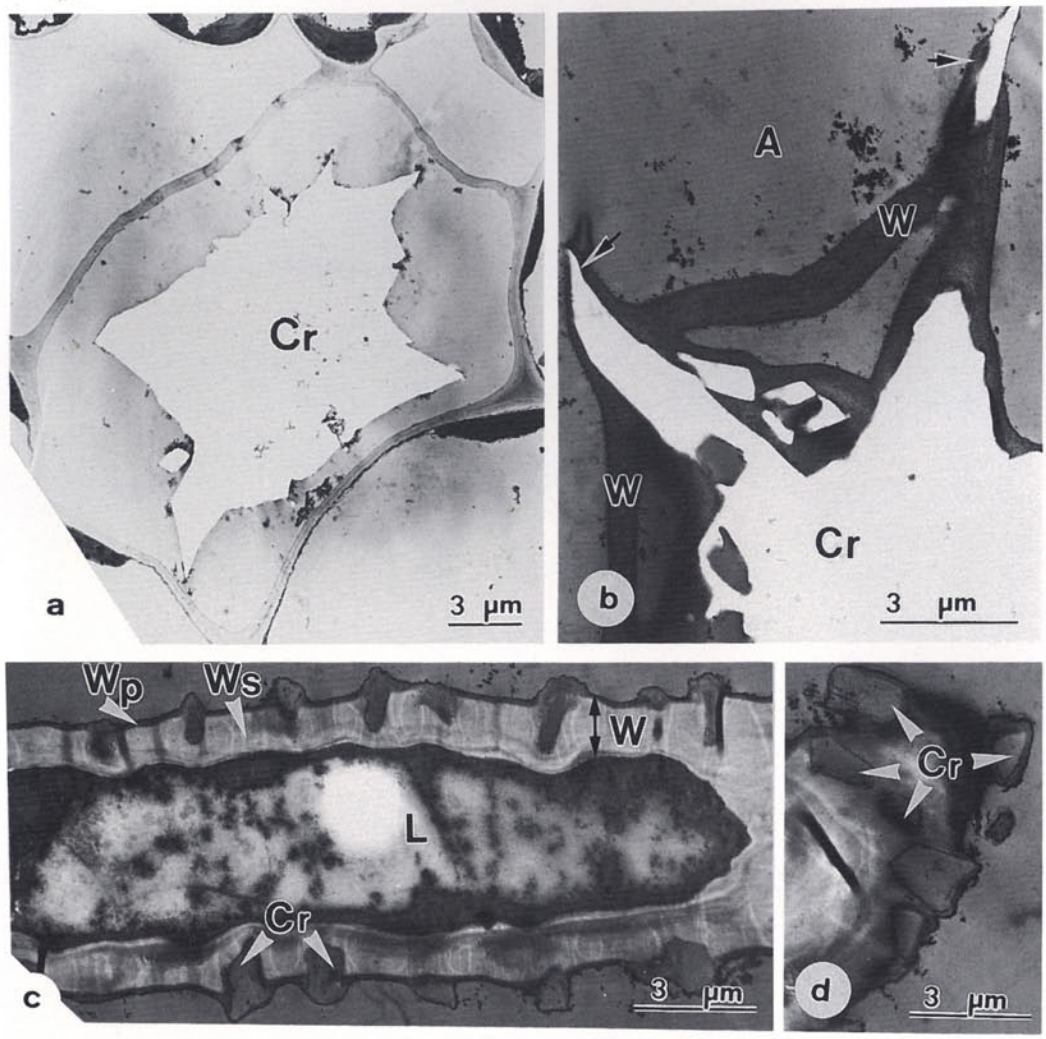

Fig. 4. (a, b) TEM photographs of *Nelumbo*
(c, d) TEM photographs of *Nymphaea*
(a) A crystal idioblast from the mesophyll, containing a druse crystal, showing no cytoplasm in the cell.
(b) The sharp points of crystals protruding into the air channel (→).
(c) Sectional view of an arm of sclereid, showing the crystals forming between the primary and secondary cell wall, densely stained primary wall and lightly stained secondary wall.
(d) Crystals protruding from the wall of a sclereid.

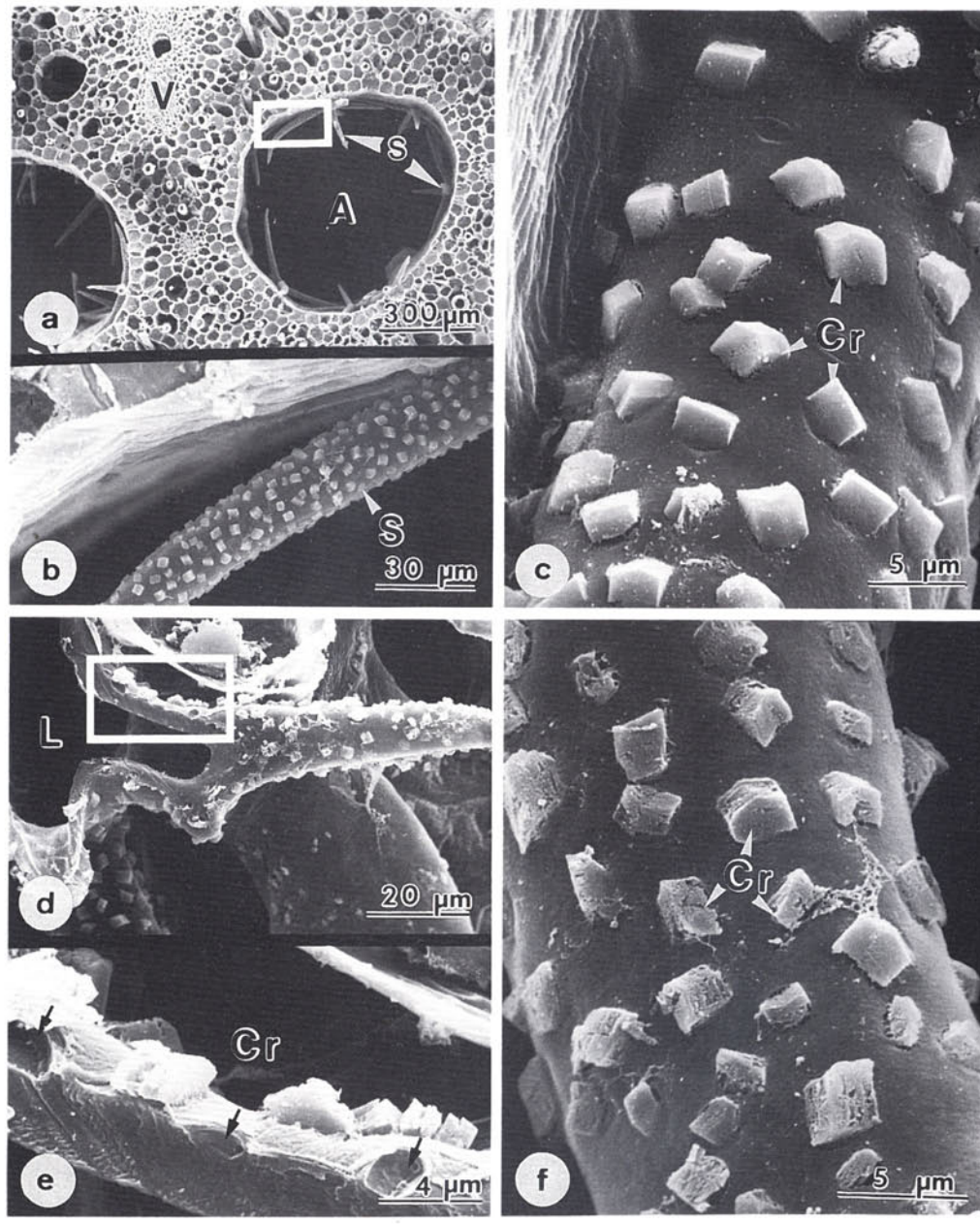

Fig. 5. SEM photographs of *Nymphaea*
(a) Transection of a petiole, showing the distribution of sclereids.
(b) Enlargement of (a).
(c) Crystalline bodies on the outer surface of the sclereids.
(d) Leaf pre-etched with 50% acetic acid for 20 min. showing that the crystals did not dissolve.
(e) Enlargement of (d), showing the complementary fracture faces of the crystals on the wall of a sclereid (→).
(f) Leaf pre-etched with 50% acetic acid for 20 min, showing that the crystals did not dissolve, but that the cell wall covering the crystals partly collapsed.

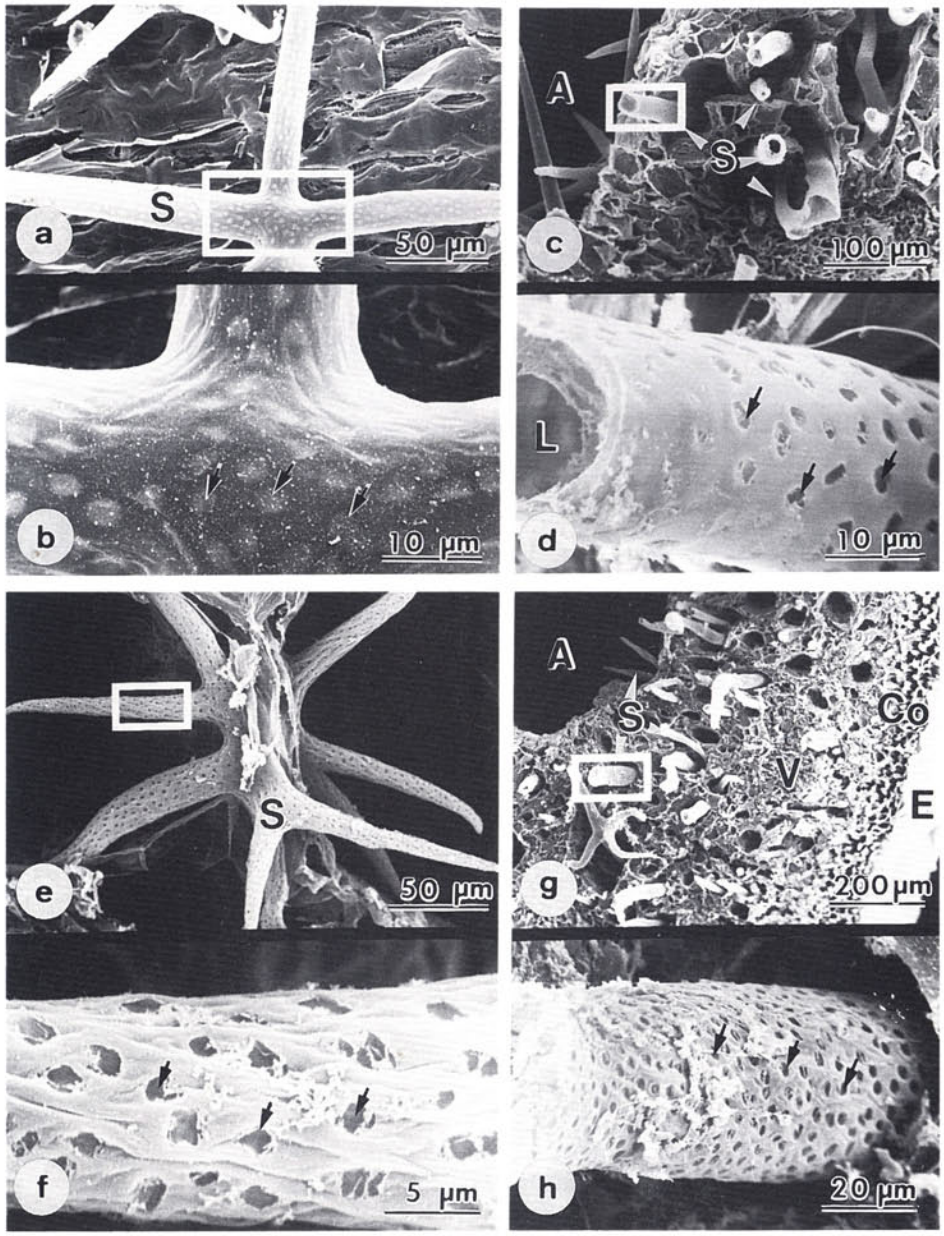

Fig. 6. SEM photographs of *Nymphaea*

(a) Leaf pre-etched with 50% hydrochloric acid for 1 min.
(b) Enlargement of (a), showing the partly dissolved crystals (→).
(c) Leaf pre-etched with 50% hydrochloride acid for 1 min.
(d) Enlargement of (c), showing the excavation cavities of dissolved crystals (→).
(e) Leaf pre-etched with conc. hydrochloric acid for 1 min.
(f) Enlargement of (e), showing the disappearance of crystals and the collapsed sclereid wall (→).
(g) Leaf pre-etched with 50% sulfuric acid for 1 min.
(h) Enlargement of (g), showing the absence of crystals and the collapsed sclereid wall (→).

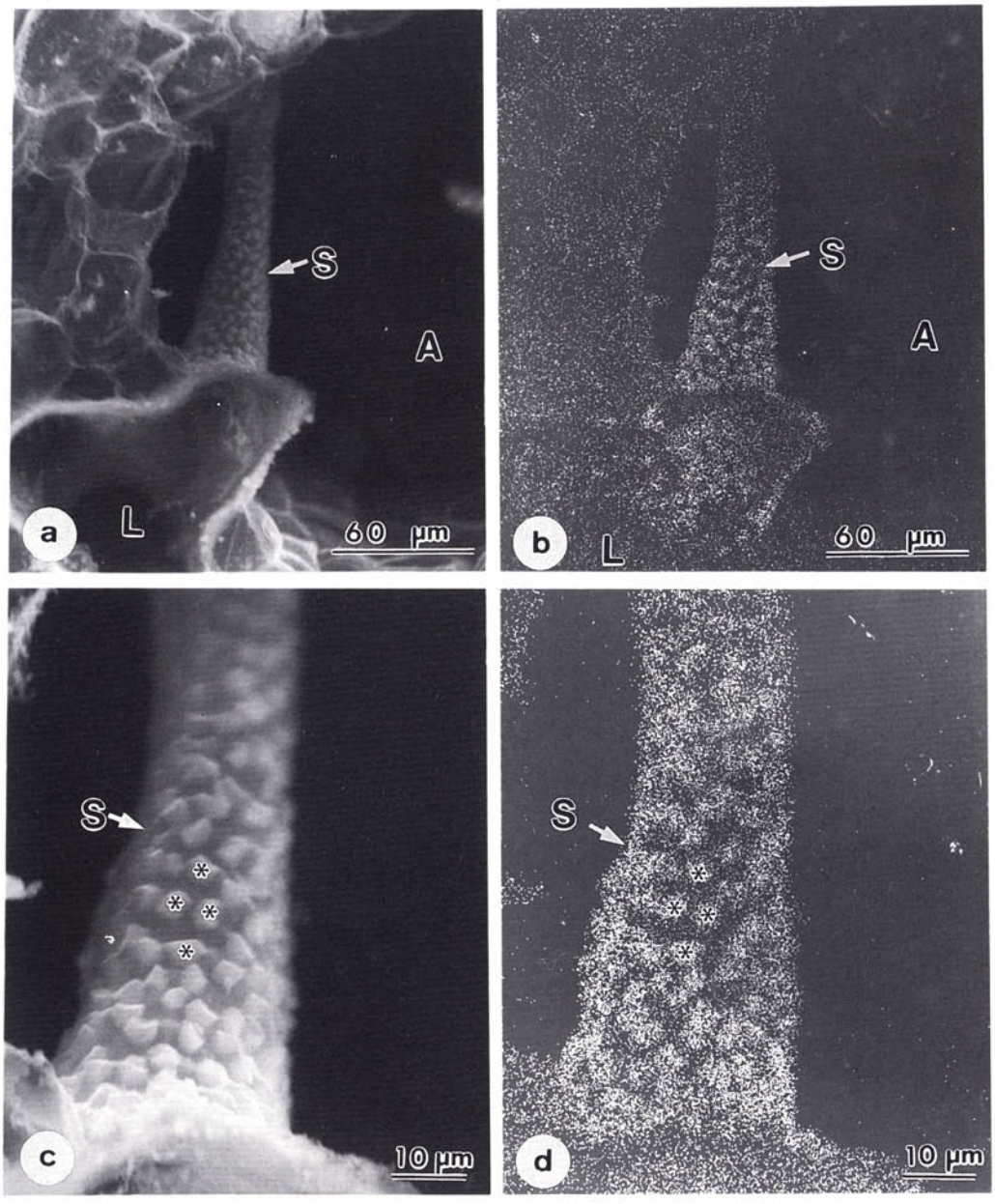

Fig. 7. *Nymphaea*

(a) SEM photograph of a transection of petiole, showing the distribution of crystals on the wall of partly cut sclereid.

(b) An X-ray map localizing the calcium electron transition energy, map corresponding to (a), indicating the high calcium content of the crystals.

(c) Enlargement of (a).

(d) Enlargement of (b).

intracellularly and then excreted from the protoplasm to be deposited on the surrounding cell wall. These crystals are covered by fibrous wall materials. Mature crystal idioblasts of *Nelumbo* are devoid of cytoplasm. Cheavin (1938) considered crystal idioblasts as being dead cells. Other workers have interpreted the disruption of cytoplasm the result of the preparation process of microscopical observation (Arnott and Pautard, 1970). In *Nymphaea*, a mature sclereid (with crystals on the wall) still contains some cytoplasm with a small nucleus (Chiang and Huang, 1984).

The presence of free calcium in the cytoplasm is considered an important regulator for many cellular functions in both animal and plant cells (Borchert, 1986). Thus, the tissues of stems and leaves are continuously exposed to an influx of calcium carried by the transpiration stream. Elimination of this excess calcium poses an important functional problem to plants. Borchert (1986) has demonstrated that in the leaflets of *Gleditsia triacanthos* the absorbed calcium has precipitated as calcium oxalate in the central vacuole of the crystal cell. In *Nymphaea* the cell wall of the sclereid initial has dynamic properties. It can undergo secondary changes and may act as a sink for metabolic end products, deposited as crystals. Lazzaro and Thomson (1989) studied the accumulation of waste materials in the extra-plasmic space of mature head cells of the trichome of *Cicer arietinum*. They found that, instead of the normal secreting of organic acids, the head cells accumulate excess calcium in the form of a calcium precipitate. This change in function of the trichomes may result from metabolic changes in the cells due to senescence.

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荷與睡蓮葉部之草酸鈣結晶體

黃 玲 瓏

摘 要

荷 (*Nelumbo nucifera* Gaertn.) 與睡蓮 (*Nymphaea tetragona* Georgi) 的葉部均含有結晶體，但其形狀、大小、與分佈均各有不同。荷葉中的結晶體呈晶簇狀，存在於結晶異形細胞的中央液胞內。一般而言，一細胞中只含有一晶簇狀晶體。當結晶異形細胞成熟時，其細胞壁局部瓦解，而晶體的尖銳面凸出於細胞空隙間。睡蓮中的結晶體呈角柱狀，存在於厚壁細胞之初生細胞壁與次生細胞壁之間，當厚壁細胞成熟時，其尖銳面突起仍覆蓋著初生細胞壁。此兩種水生植物的結晶異型細胞的位置均伴隨在通氣組織，因此結晶的形成可能與水份的蒸發有關。經由X光微量元素分析術及酸蝕測試，此兩種結晶體的成份爲草酸鈣。