

Formation of Calcium Carbonate Deposition in the Cotyledons during the Germination of *Justicia procumbens* L. (Acanthaceae) Seeds

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ABSTRACT: During seed germination of *Justicia procumbens*, the formation of lithocysts, trichomes and diacytic stomata in the epidermis of cotyledons was following a specific distribution pattern. During the first 1-3 days, many young stomata and trichome initial cells were formed sporadically in the adaxial and abaxial epidermis, but no lithocyst was found. Three to five days after seed sowing, two cotyledons were exposed to light and then opened. In the meantime, some lithocysts were recognized on both adaxial and abaxial epidermises. The lithocysts on the adaxial epidermis occurred in the radially arranged cells located between the central area and the margin. However on the abaxial epidermis, they were found only in the marginal cell layer and their axes were along the margin of cotyledons. The total number of lithocysts in a cotyledon at this stage was 32.2 ± 4.3 and the cystolith inside the lithocyst was spindle in shape and $48.2 \pm 21.1 \mu\text{m}$ in length. Three weeks after seed sowing, the cotyledons were mature and the total number of lithocysts in a cotyledon was 112.2 ± 10.1 and the cystolith in the lithocyst was enlarged to be $119.8 \pm 27.8 \mu\text{m}$ in length. The cystolith was extracellularly formed in the cell wall of lithocyst. Its surface was with many protuberances and surrounded by a cystolith sheath connecting to cytoplasmic strands. The core of cystolith was surrounded by concentrically stratified fibrils and the calcium carbonate was concentrically accumulated. The waved stratified fibrils were also deposited in the protuberances. The EDX spectra showed that the main mineral elemental compositions of cystoliths were Ca and P. Ca was deposited more in the central part of cystolith than in the marginal area.

KEY WORDS: Calcium carbonate deposition, Lithocyst, Cystolith, Cotyledon, Seed germination, EDX, *Justicia procumbens*.

INTRODUCTION

Under the ordinary conditions, the biological calcium deposition is a common phenomenon. It has long been known that insoluble calcium salts in form of the carbonate, sulfate, and oxalate occur in a wide variety of plants (Solereider, 1908). In the plant bodies, calcium oxalate and calcium carbonate are the predominantly deposited calcium salts (Arnott and Pautard, 1970). Many studies have been made on the formation of calcium oxalate crystals, however only a few reports are concerning the deposition of calcium carbonate (Franceschi and Horner, 1980; Smith, 1982).

The calcium carbonate is frequently formed in algae or aquatic plants. They are generally

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deposited on the plant outer surface or in the intercellular spaces (Borowitzka, 1984). In land plants, the most encountered calcium carbonate deposition is cystolith formation (Arnott, 1980). Cystoliths are formed in the specialized cells called lithocysts. They are mostly located in the epidermis and generally consist of a stalk and cystolith body. In contrast to the formation of calcium oxalate crystals in many families of angiosperms, cystoliths have been found only in the species of Moraceae, Urticaceae, Acanthaceae and some other families. They may occur in various organs and tissues. The morphology and distribution of lithocysts in the plant bodies vary among different taxa; therefore, they have been used for the plant systematic study on the familial, generic, or species levels (Werner, 1931; Pireyre, 1961; Hsieh and Huang, 1974; Smith, 1982; Okazaki *et al.*, 1986; Yu and Li, 1991).

The occurrence of cystolith consisting of calcium carbonate in the lithocyst is one of the most prominent characters of Acanthaceae and is valuable for the recognition of the genera (Hsieh and Huang, 1974). In the leaves and sepals of *Justicia*, the lithocysts occurred frequently in the epidermis (Kuo-Huang and Yen, 1996). Their shapes and distribution patterns are specific between organs and may be correlated with the developmental pattern of these organs. In this study, the distribution and morphology of lithocysts in the developing and mature cotyledons were investigated. In addition, the ultrastructures and mineral elemental compositions of the cystoliths were studied by TEM and EDX microanalysis.

MATERIALS AND METHODS

The seeds of *Justicia procumbens* L. were collected from the campus or the greenhouse of the Department of Life Science, National Taiwan University. The seeds were soaked and germinated in petridishes with wetted filter papers in the growth chamber (26-28 °C, 3750 Lux 12 h light / 25-26 °C night). The lengths of cotyledons were measured every 3 days. For counting the number and length of lithocysts, cotyledons in different developing stages were bleached in 95% ethanol kept at 100 °C in a boiling waterbath for 20 min. After cooled, the samples were cleared in pure lactic acid for at least 30 min and then mounted in 50% aquatic glycerin (Spore, 1948). The cleared samples were investigated and photographed with Leica Diaplan light-microscope under polarized light. Quantitative results of the number of lithocysts in a cotyledon and the lengths of cystoliths in lithocysts were based on the counts and measurements from 10 cleared cotyledons in each size category.

For light and transmission electromicroscopy, cotyledons in various developing stages were collected and fixed for 2 h in 2.5% glutaraldehyde followed by 1% OsO₄ for 2 h, dehydrated in ethanol-acetone series, infiltrated and embedded in Spurr resin. Thin sections (70-90 nm) were stained with uranyl acetate and lead citrate, and then investigated under Hitachi H-600 TEM.

The materials for SEM were fixed and dehydrated as for TEM. The samples were then dried with Hitachi Critical Point Dryer (HCP-1), Au coated with an IB-2 ion coater, and examined with the Hitachi S-520 or S-2400 SEM (Fu *et al.*, 2003). For energy dispersive X-ray microanalysis (EDX), some resin embedded cystoliths were grounded to the core or to the edge of cystoliths with fine mesh polishing papers, and polished with alumina paste. The samples were then etched with EDTA, coated with thin layer of Au, and examined with the Kevex Level 4 EDS and Hitachi S-2400 SEM.

RESULTS

The cotyledons of mature seeds of *Justicia procumbens* were oval form and 0.8-0.9 mm in length (Figs. 1 and 2A). During the first 1-3 days of seed germination, they were still enclosed in the mature seed coat. On the adaxial and abaxial surfaces, many young stomata with guard mother cells or young guard cells and trichome initial cells were distributed randomly (Figs. 2B, 3A, and 3B). The diacytic type of stoma was distinguishable. However, no lithocyst was found on both adaxial and abaxial epidermises. The arrangement of the axis of ordinary epidermal cells in the cotyledons was different among areas. In the abaxial surface, all the epidermal cells were arranged almost parallel to the axis and the margin of cotyledons (Fig. 3A). In the central area of the adaxial surface, the epidermal cells lined almost parallel to the axis of cotyledon, while the peripheral 2-3 rows of epidermal cells were along the margin of cotyledon. Between these two areas, the epidermal cells, particularly only on the adaxial surface were radially arranged (Fig. 2B). The axes of cells in this area were perpendicular to the margin.

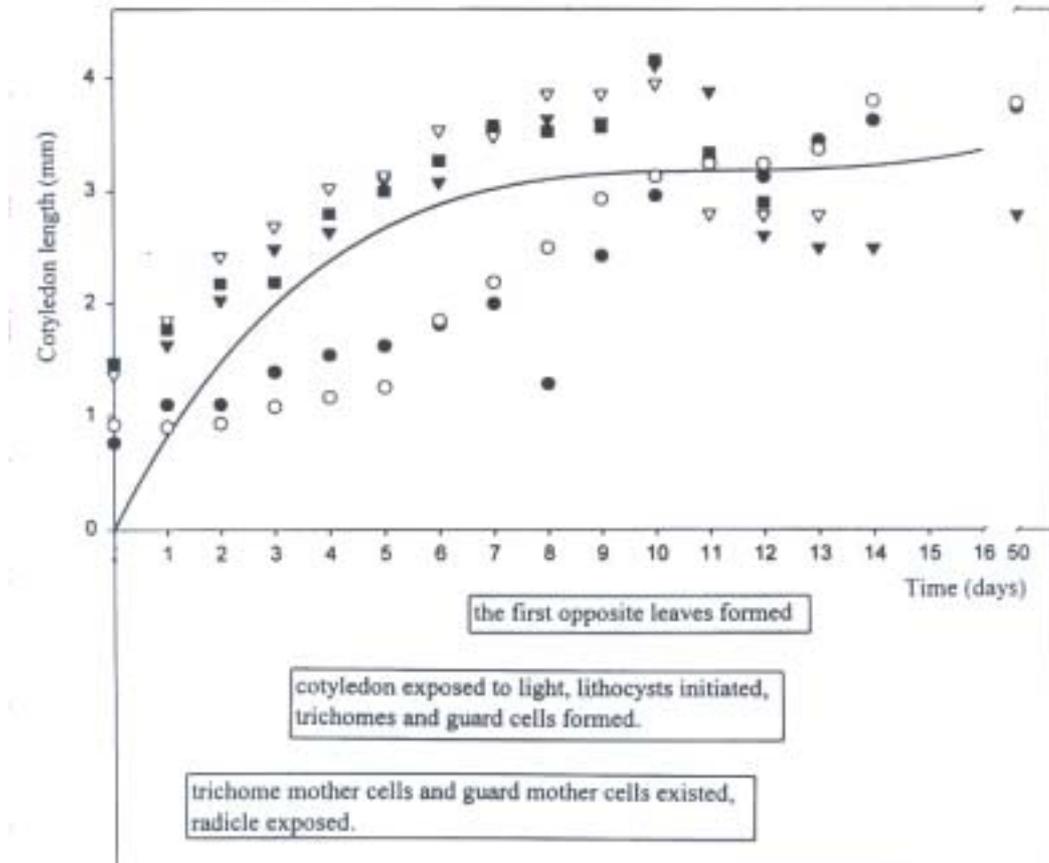


Fig. 1. The regressive lines of length of cotyledons and morphogenous changes of seedlings during germination. The different symbols represent different individuals.

During the first 3-5 days of germination process, the two cotyledons were exposed to light and opened. The young green cotyledons at this developmental stage were 1-1.5 mm long and the midrib and lateral veins were found (Figs. 1 and 2C). The stomata pores and the head cell

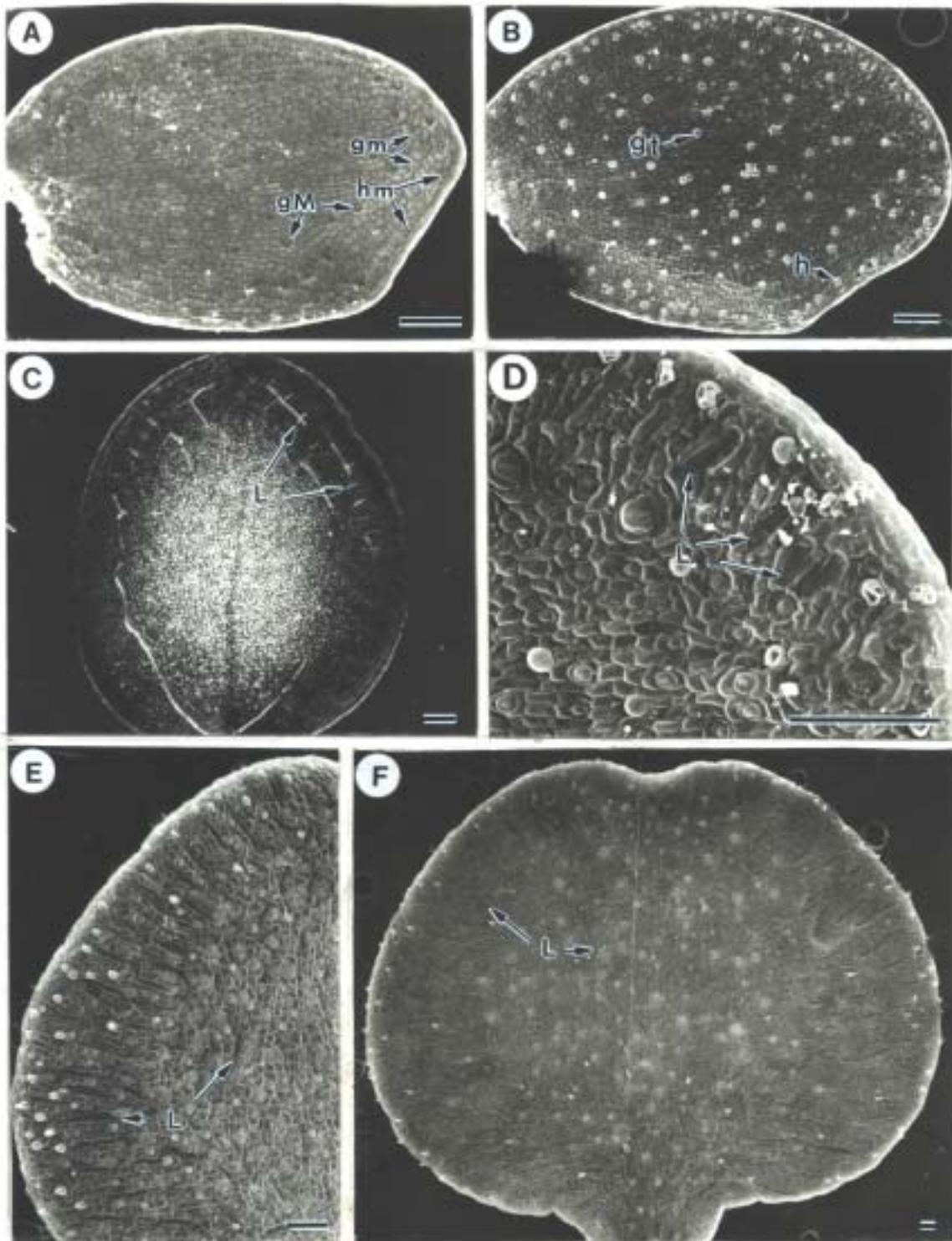


Fig. 2. SEM photographs of the adaxial surfaces of cotyledons in different developmental stages. A and B: Cotyledons before exposed to light (1-3 days after sowing) showing guard mother cells (gm) and mother cells of glandular trichomes (gM). C and D: Cotyledons after exposed to light (3-5 days after sowing) showing young lithocysts (L) anticlinally arranged near margin of cotyledons. E and F: Mature cotyledons (3 weeks after sowing) showing 1-2 rows of lithocysts (L) anticlinally arranged near margin of cotyledons and the others parallel to midrib in central areas of cotyledons. All bars = 100 μ m.

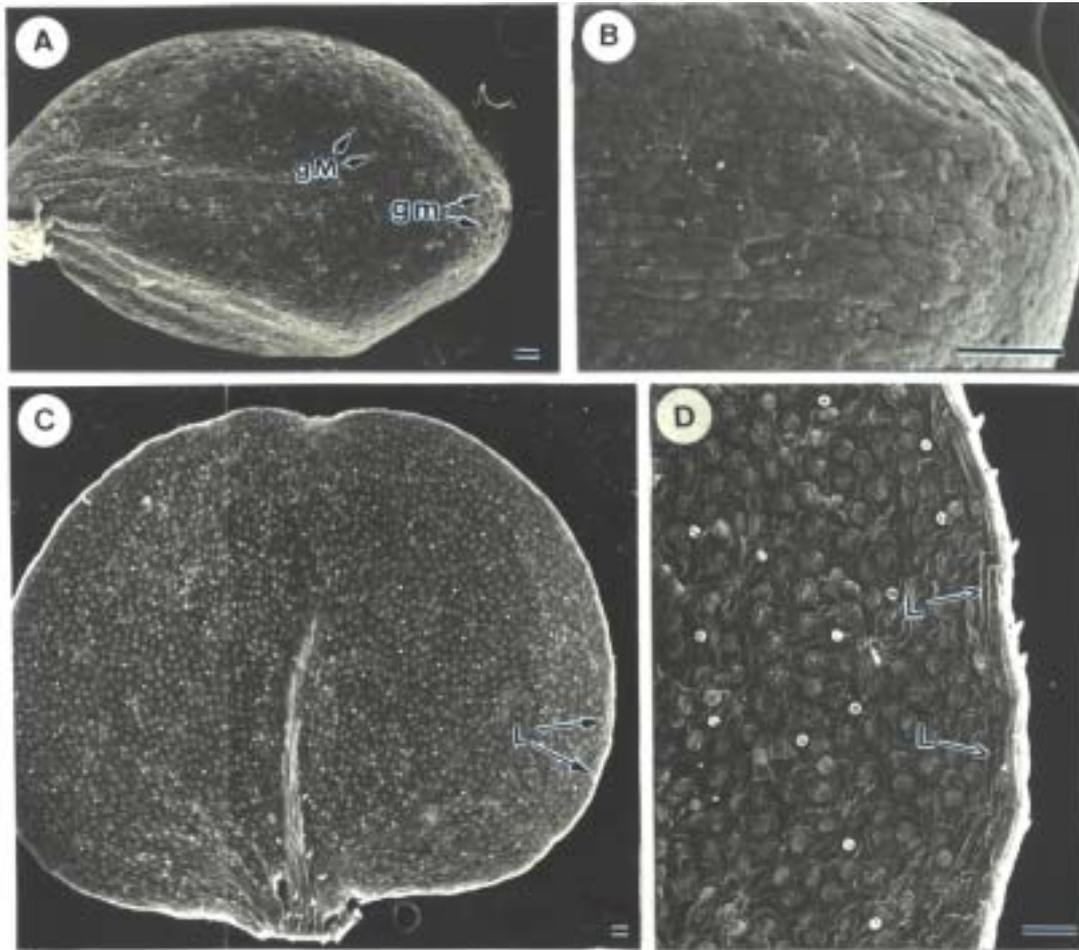


Fig. 3. SEM photographs of the abaxial surfaces of cotyledons in different developmental stages. A and B: Cotyledons before exposed to light (1-3 days after sowing) showing guard mother cells (gm) and mother cells of glandular trichomes (gM). C and D: Mature cotyledons (3 weeks after sowing) showing a row of lithocysts (L) parallel arranged to the margin of cotyledons and many stomata (g) sporadically distributed. All bars = 100 μ m.

of glandular trichomes were distinct. Both on the adaxial and abaxial epidermises some lithocysts were recognizable (Fig. 2D). The cystoliths inside the lithocysts were spindle in shape and $48.23 \pm 21.1 \mu\text{m}$ in length. The lithocysts on the adaxial epidermis occurred only in the radially arranged cells located between the central area and the margin. The axes of lithocysts were orientated almost perpendicularly to the margin of cotyledon. The total number of lithocysts in a cotyledon at this stage was 32.2 ± 4.3 .

Three weeks after seed sowing, the mature cotyledons were 2-2.5 mm long and they were in a round form (Figs. 1, 2F, and 3C). The cystoliths in the lithocysts were enlarged to a length of $119.8 \pm 27.8 \mu\text{m}$. Besides, many lithocysts occurred in the central area of the adaxial epidermis (Fig. 2E). The cystoliths in these cells were $53.2 \pm 26.2 \mu\text{m}$ in length. On the abaxial epidermis, the lithocysts were found only in the cells of marginal layers and their axes were along the margin of cotyledons (Fig. 3D). In the central area of the abaxial epidermis, many guard cells and glandular trichomes were sporadically differentiated from the epidermal cells, however no lithocyst was found. The total number of lithocysts in a cotyledon at this stage was 112.2 ± 10.1 .

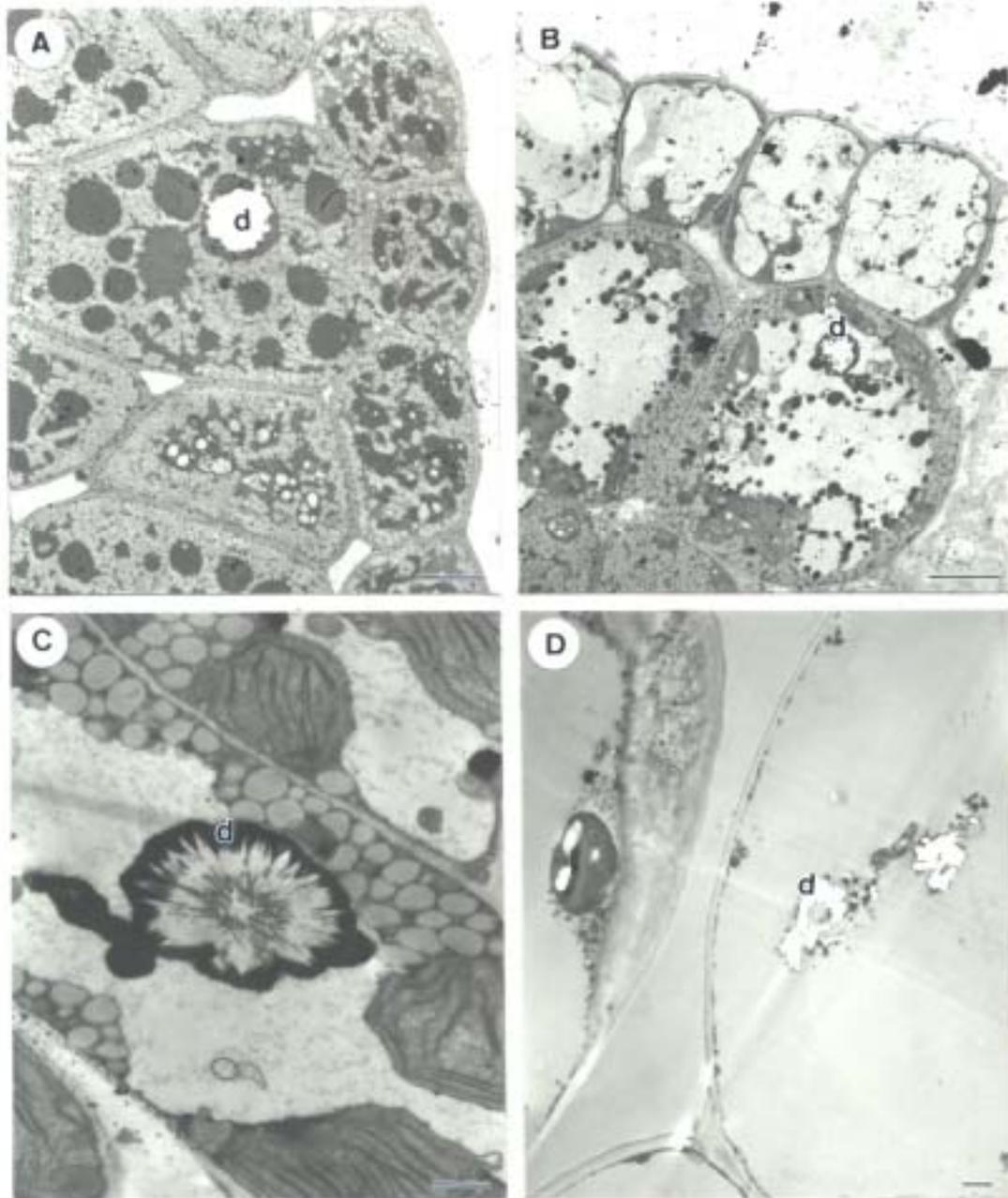


Fig. 4. TEM photographs of the cross sections of cotyledons in different developmental stages. A: Young cotyledon before exposed to light (1-3 days after sowing) showing dense cytoplasm and druse calcium oxalate crystal (d) in mesophyllous cell. B-D: Mature cotyledons (3 weeks after sowing) showing chloroplasts and druse calcium crystals in vacuoles. Bars = 5 μm (A and B); 8 μm (C); 4 μm (D).

The cytoplasm of epidermal cells and mesophyllous cells of the cotyledons, which were still enclosed in the seed coat, was dense with oil droplets. In the vacuoles there were storage proteins. In addition, some druses calcium oxalate crystals were found in the vacuoles of mesophyllous cells (Fig. 4A). In the mature cotyledons, the cytoplasm of epidermal cells and mesophyllous cells was full of large vacuoles (Fig. 4B). In the mesophyllous cells there were still many oil droplets. Besides some chloroplasts with thylakoide membrane, grana, and small starch grains were easily observed and occasionally small druses crystal were found in

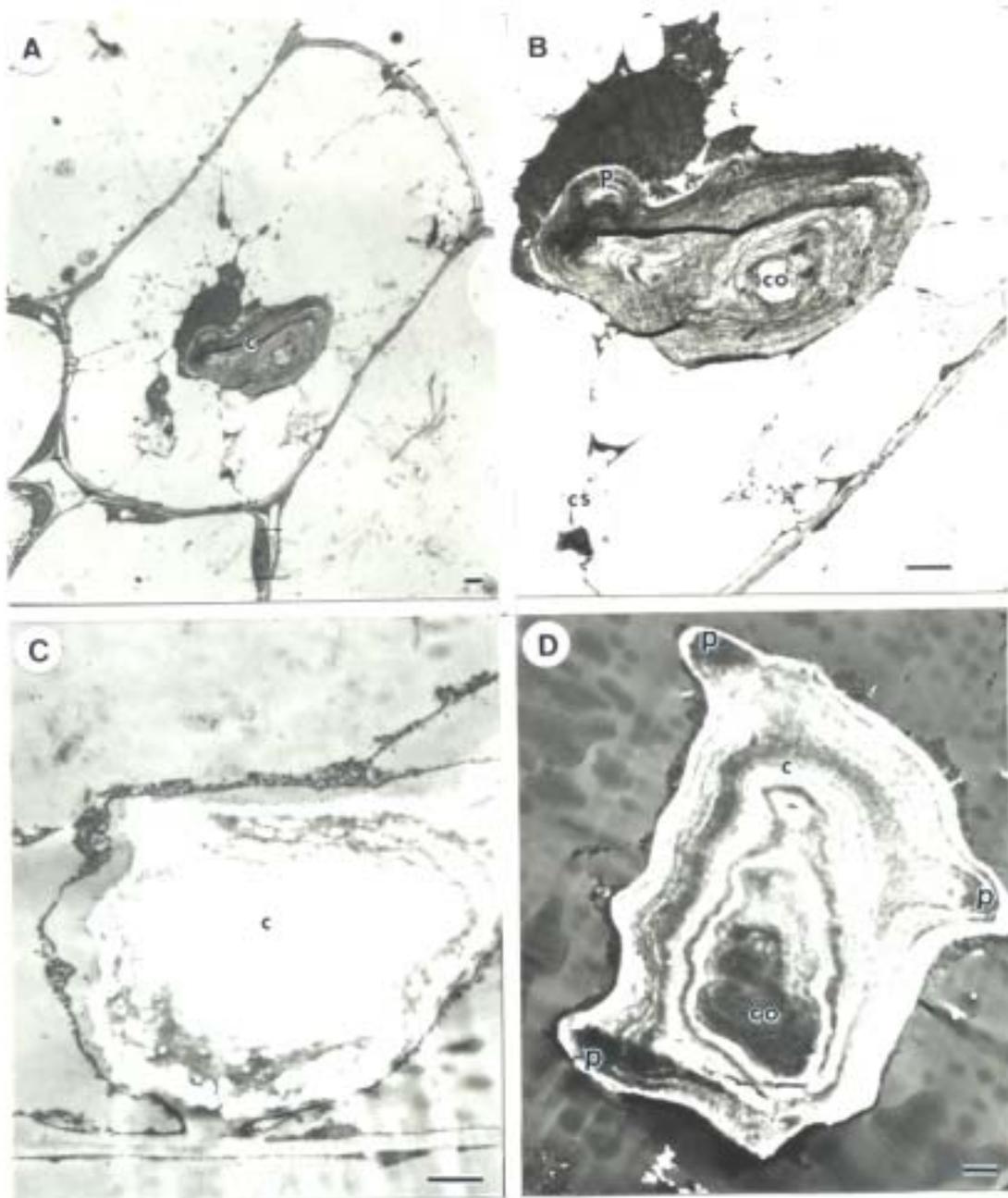


Fig. 5. TEM photographs of the cystoliths in the lithocysts of mature cotyledons. A: Cross section of cystolith (c) in a lithocyst. B: Enlargement of A, showing core (co) and protuberances (p) of cystolith surrounded by concentrically stratified fibrils. C: Cross section of cystolith (c) showing the cytoplasmic strands connected with crystal sheath of cystolith. D: Cross section of cystolith (c) showing core (co) of cystolith surrounded by concentrically stratified fibrils with many protuberances (p). All bars = 100 μ m.

the vacuole (Figs. 4B-D). The cystolith was formed extracellularly in the cell wall of lithocyst. Its surface was with many protuberances and surrounded by a cystolith sheath (Figs. 5A-D). The calcium carbonate in the cystolith was concentrically accumulated. In the cross sections of cystoliths, the core of cystolith was surrounded by concentrically stratified fibrils and concentrically waved stratified fibrils were also deposited in the protuberances. There were cytoplasmic strands connected with cystolith sheath (Figs. 5B, D, and Figs. 6A-D).

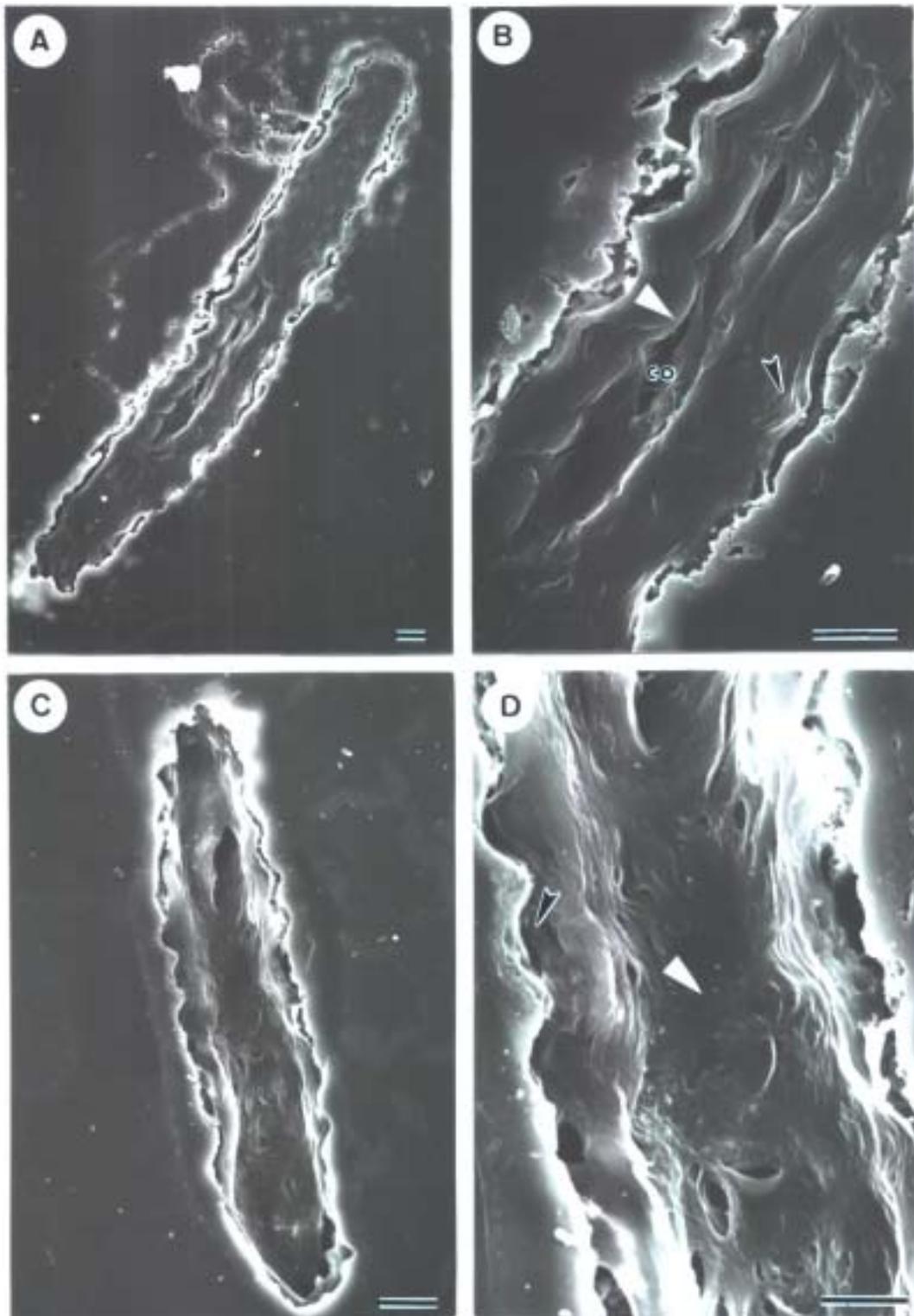


Fig. 6. SEM photographs of the grounded and polished faces of cystoliths in the mature cotyledon. A: Central longitudinal face through the crystal core (co). B: Enlargement of A, near core (co) showing EDX spot locations at center (white arrow head) and margin (black arrow head). C: Tangential face through the crystal edge. D: Enlargement of C, near edge showing EDX spot locations at center (white arrow head) and margin (black arrow head). Bars = 10 μm (A, B, and C); 5 μm (D).

The cystoliths in the mature cotyledons were eyebrow- or spindle-shaped. Figure 6A showed the grounded and polished central longitudinal face through the core of a cystolith and figure 6 C showed the tangential face through the edge of a cystolith. The EDX spot tests were done on the spots at the center (Fig. 6B) and the margin (Fig. 6D). The EDX spectra (Figs. 7A-D) showed that the main elements of cystoliths were Ca and P. The spot tests on the tangential faces (Figs. 7C and D), a small peak of Cl was also found. When taking the mass of Au as the standard, Ca was deposited more in the central part of cystolith than in the margin area.

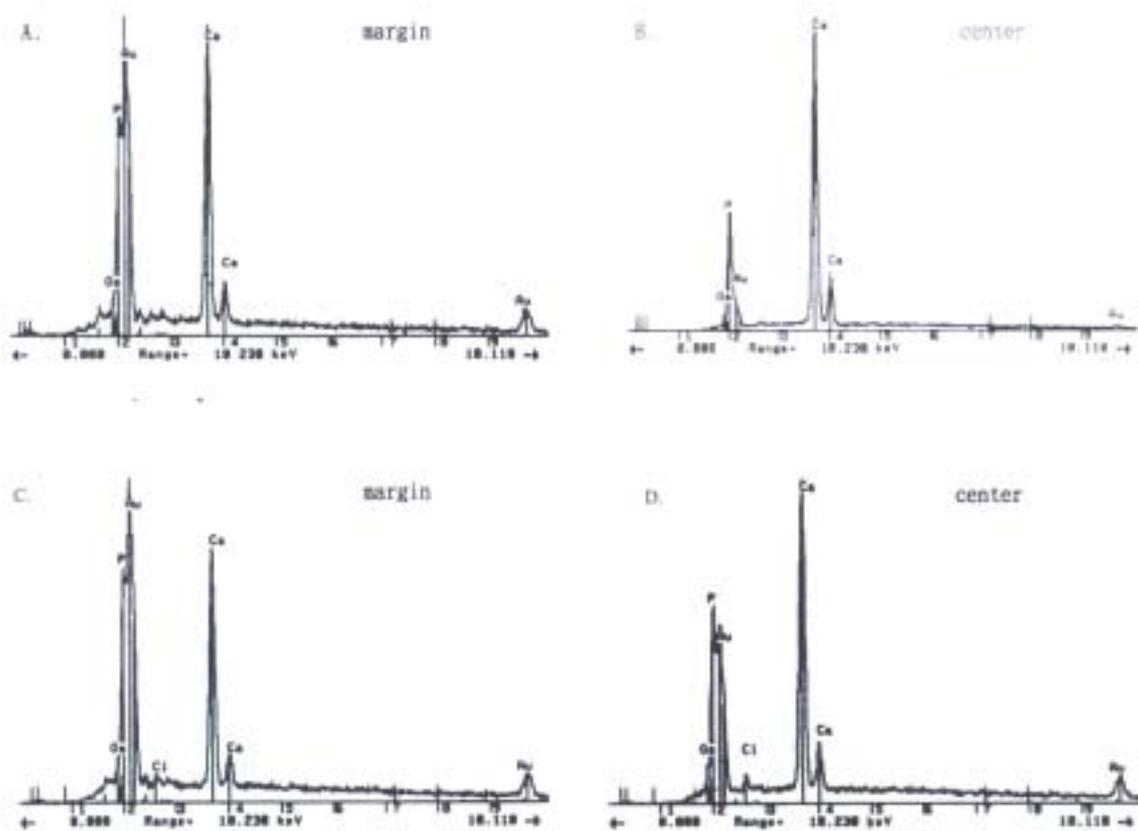


Fig. 7. Representative EDX spectra. EDX spectrum of the spot located at the margin (A) and near the center (B) of the central longitudinal face through the core (spots location showed in figures 6 B). EDX spectrum of the spot located at the margin (C) and near the center (D) of the tangential face through the edge (spots location showed in figures 6 D).

DISCUSSION

In angiosperms, the formation of calcium carbonate depositions in form of cystoliths occurred mostly in the epidermal lithocysts. Appearance and location of the lithocysts may be specific and useful in taxonomic classification (Arnott, 1980). Smith and Watt (1986) studied the occurrence and distribution of various epidermal cell types in the leaves of *Pilea cadierei*, and found that the development of lithocysts was an orderly and regulated process. It may be controlled by the spacing patterns caused by the mutual incompatibility between the initial cells of the same or different types (Buenning, 1965). As in the developing leaves and sepals of *Justicia procumbens* (Kuo-Huang and Yen, 1996), the formations of lithocysts, trichomes,

and diacytic stomata in the adaxial and abaxial epidermises of cotyledons exhibit a specific distribution pattern. In accordance with the developmental pattern of the located organ types, there is a gradient of origin and maturation of the lithocysts from the tip to base and from the margin to midrib. Besides, during the development of cotyledons, the orientation of lithocysts showed a correlation between the development of the neighbour cells and the time of lithocysts elongation.

The lithocysts form in the early stages of organ development. In the cotyledons of *Justicia procumbens*, they were originated and differentiated very early in the germinating process, however, the lithocysts and the cystoliths inside were formed only when the located tissue was exposed to light. Franceschi and Horner (1980) reported that various physical and chemical parameters such as light, pressure, pH, and ion concentration affect the growth and habit of the calcium depositions. In plants, calcification is mainly the result of photosynthetic CO₂ or HCO₃⁻ assimilation. This raises the local pH and CO⁻ concentration resulting from shifts in the dissolved inorganic carbon equilibrium.

In the leaves of *Justicia procumbens*, most lithocysts occur in the adaxial epidermis, however the lithocysts in the sepals are located only in the abaxial surface where the light intensity is higher (Kuo-Huang and Yen, 1996). In the present study the results showed that no lithocyst was found as the cotyledons still enclosed in the seed coats. However during the germinating process the two cotyledons were exposed to light and opened. For the ordinary leaves, the lithocysts form mostly in the adaxial epidermis. Okazaki *et al.* (1986) supposed that the deposition of calcium carbonate in the lithocysts of the leaf epidermis of *Morus* can be related with the photosynthesis. In *Justicia procumbens*, the different ratios of light intensities between the adaxial and abaxial surfaces of the leaves, sepals, and cotyledons may play a role in the formation of lithocysts and the cystolith inside.

In *Pilea cadierei* the lithocysts initials are firstly recognizable in the abaxial epidermis of leaves in 4 mm long (Smith and Watt, 1986). However, in these lithocysts no cystolith is formed. In the later developing or mature leaves lithocysts are easily found in the adaxial and abaxial epidermises. They contain cystoliths and all of them are approximate at the same stage of development. In the cotyledons of *Justicia procumbens*, the formations of lithocysts on both abaxial and adaxial surfaces were alike, but the newly originated lithocysts on the abaxial surface were smaller and fewer. It is probably that the origin and maturation of the lithocysts are genetically controled, but the accumulations of the calcium carbonate in the cells are mostly depended on the calcium ion supply (Zindler-Frank, 1980; Wu, 1995).

The cystoliths are extracellularly formed in the cell wall of lithocysts. The plant cell wall may be important in CaCO₃ nucleation by acting as an epitaxial substratum or template (Okazaki *et al.*, 1984). Cystoliths have the stalks and the amorphous calcium carbonate deposited with the regular cellulosic fibrillils in the cystolith body. Two types are known in ways of cystolith formation. In *Ficus elastica* (Moraceae), apical cell wall (peg) of lithocysts grows down into the lumen of the cell to form stalk and calcified body (Ajello, 1941). In *Beloperone californica* (Acanthaceae), however, incipient cystolith originates in a vacuole containing CaCO₃ depositions (Scott, 1946).

In *Justicia procumbens*, the cystoliths were surrounded by sheath and some cytoplasmic strands were found to be connected with cystolith sheath. It suggests an essential role of cytoplasm in the cystolith formation. The results of EDX spectra showed that the main mineral elemental compositions of cystoliths in the cotyledons of *Justicia* were Ca and P. Ca was deposited more in the central part of cystolith than in the margin area. These statements may suggest that P could be originated from the cytoplasm, but the P/Ca ratio in the cystoliths

both in the margin (Figs. 7A and C) and center (Figs. 7B and D) parts are greater than 0.5. From the thermodynamical point of view, the solubility products of Ca-P (such as apatite) is much smaller than that of calcium carbonate (such as calcite). Therefore, an unknown coprecipitation formed by Ca-P and CaCO₃ may be deposited in the cystoliths inside the cotyledons of *Justicia*

Histochemical studies on organic matrix of cystoliths show that cellulose is found in both stalk and body, but other neutral polysaccharides such as callose and acid polysaccharides are detectable only in the body (Pireyre, 1961). Little is known about Ca⁺²-binding substances for serving as an epitaxial template of CaCO₃ depositions. It is probable that Ca⁺² is an important mediating agent in the control of metabolism of plant cells, as it is clearly demonstrated in animal cells (Okazaki *et al.*, 1984). Through its cellular regulation and involvement in cell walls, Ca⁺² may have a specific, direct effect on plant growth, including in plant organ senescence and fruit ripening. The physiological significance of cystoliths is obscure. However, a possible role of cystolith is proposed to serve as CO₂ or Ca⁺² reservoirs for photosynthesis.

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爵床（爵床科）種子發芽過程子葉內碳酸鈣沉積物的形成

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摘 要

爵床 (*Justicia procumbens*) 的種子發芽過程中，子葉表皮之石胞、毛茸、與氣孔的形成均遵循著特有的分布型式。在種子發芽的第 1 至 3 天，子葉仍包裹於種皮內，許多氣孔與毛茸始源細胞在子葉之向軸與背軸的表皮分散的形成，但並無觀察到石胞。當種子發芽 3 至 5 天後，兩片子葉突破種皮並打開，同時在子葉之向軸與背軸的表皮上都可區分出石胞。在子葉的向軸面，石胞僅形成於中央部位與周邊之間的放射狀排列細胞，而在背軸面，石胞則僅形成於子葉邊緣，其長軸並與子葉邊緣平行。此時期每片子葉平均有 32.2 ± 4.3 個石胞，而其內的鐘乳體呈紡錘形，長 $48.2 \pm 21.1 \mu\text{m}$ 。當種子發芽 3 星期後，子葉已發育成熟，在子葉向軸面的中央區域，可觀察到許多石胞的形成，但在背軸面的中央區域則僅密佈氣孔，此時期每片子葉平均有 112.2 ± 10.1 個石胞，而其內鐘乳體的長度則增加至 $119.8 \pm 27.8 \mu\text{m}$ 。鐘乳體沉積於石胞的細胞壁，外緣具有許多瘤狀突起並為鞘狀物所包圍，其外與細胞質絲所相連。鐘乳體之碳酸鈣沉積物呈向心的推積，其核心與邊緣部位為纖維層向心的層層堆積。X 光能微量元素分析顯示鐘乳體主要的無機元素為鈣與磷，鈣元素在鐘乳體之核心部位較其在邊緣部位堆積為多。

關鍵詞：碳酸鈣沉積物、石胞、鐘乳體、子葉、種子發芽、X 光能微量元素分析、爵床。

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