Taiwania, **47**(1): 1-10, 2002

Formation of Calcium Oxalate and Calcium Carbonate Depositions in the Leaves of *Ficus pumila* L. var. *awkeotsang* (Makino) Corner

Ling-Long Kuo-Huang [1, 2], Tsair-Bor Yen [1], Shian-Cheun Chen [1]

(Manuscript received 25 January, 2002; accepted 27 February, 2002)

ABSTRACT: By using light microscopy and scanning electron microscopy, the composition, shape and localization of calcium depositions in the leaves of *Ficus pumila* L. var. *awkeotsang* (Makino) Corner were studied. In the mature leaves, many calcium oxalate and calcium carbonate were accumulated. All calcium carbonate depositions (cystoliths) were found in the lithocysts of the lower epidermis. The calcium oxalate crystals were in forms of druses or prismatic crystals. Most of the druse crystals were located sporadically in the cells of palisade mesophyll, while the prismatic crystals were found in the crystal cells of the bundle sheath. The formation of these two kinds of calcium deposits was related to the levels of calcium supply. The size of calcium carbonate depositions increased with the calcium concentration, but the density of lithocysts was not affected by different concentrations of calcium supply. Nevertheless, the density of calcium oxalate crystals was higher in high calcium solutions.

KEY WORDS: Calcium carbonate, Calcium oxalate, Calcium supply, *Ficus pumila* var. *awkeotsang*.

INTRODUCTION

Calcium crystals are structural elements in the leaves of many higher plants (Mauseth, 1988). When crystals occur in a given species, they are consistently found in specific compositions, locations and have predetermined shapes (Franceschi and Horner, 1980; Genua and Hillson, 1985; Kuo-Huang *et al.*, 1994; Wu and Kuo-Huang, 1997). Calcium oxalate is the substance that occurs most commonly, however, calcium carbonate is only found in the Moraceae, Urticaceae, Acanthaceae and some other families (Pentecost, 1980; Demarty *et al.*, 1984; Yu and Li, 1991; Kuo-Huang and Yen, 1993). The presence or abscence of crystals is one of the important characters for understanding the evolutionary relationships of plant species (Franceschi and Horner, 1980).

The accumulation of calcium oxalate crystals in the plant bodies has been studied for many years (Franceschi and Horner, 1980; Kuo-Huang, 1990). Functions of calcium oxalate formation in plants during ordinary growth and development are still unclear. They may represent the storage forms of calcium and oxalate acid, and/or act as the depositories for regulation of cytosolic calcium concentration (Franceschi and Horner, 1980; Webb, 1999). By changing the availability of calcium ions, the formation of calcium oxalate crystals in the crystal idioblasts is affected (Frank, 1972; Borchert, 1985; Kuo-Huang and Zindler-Frank, 1998). In times of calcium depletion, calcium redissolved from the crystals was observed (Franceschi, 1989; Wu and Kuo-Huang, 1995).

1. Department of Botany, National Taiwan University, Taipei 106, Taiwan.
2. Corresponding author. E-mail: linglong@ccms.ntu.edu.tw

Several of the earlier investigations had been done on the influence of calcium nutrition, and some other factors on the precipitation of cystoliths (Freisleben, 1933; Ajello, 1941; Rabiger, 1951). However, no attention was paid to the comparison of the formation of calcium carbonate and calcium oxalate crystals. Virtually only a few reports were concerned about both calcium oxalate and calcium carbonate in a given species (Fahn, 1990, Wu and Kuo-Huang, 1997).

The jelly fig (*Ficus pumila* var. *awkeotsang*) is an endemic woody vine growing at an elevation of 800-1800 m in Taiwan (Lin *et al*., 1989). In the previous study, it was interesting to note that both calcium carbonate and oxalate calcium depositions were found in the leaf of *F. pumila* var. *awkeotsang* (Kuo-Huang and Yan, 1993). Therefore, *F. pumila* var. *awkeotsang* is suitable for the study of the functional anatomy of calcium idioblasts. In this paper, the composition, shape, and location of these two kinds of calcium deposition were investigated by means of light microscopy, scanning electron microscopy and x-ray microanalysis. The influence of calcium supply on the formation of the calcium depositions in the hydroponically grown leaves was also studied.

MATERIALS AND METHODS

The branches and mature leaves of *Ficus pumila* var. *awkeotsang* were collected from the greenhouse of the Department of Botany, National Taiwan University. Some mature leaves were decolorized in 95% ethanol, cleared in 50% lactic acid (Sporne, 1948) and then stored in 95% ethanol. Each segment of leaf was mounted on a slide in 50% lactic acid solution. Observation and photographs were prepared with a Leica Diaplan Microscope, under bright field or polarized light. Materials for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4, dehydrated in an ethanol - acetone series, dried with a Hitachi Critical Point Dryer (HCP-1), coated with IB-2 ion coater, and then examined with the Hitachi S-2400 SEM. The Kevex Level IV at 25kV was set for x-ray microanalysis (Dawes, 1979). For the identification of calcium crystals chemical compositions, the acid-etching test was used (Horner and Wagner, 1992).

Some branches were transferred into modified Hoagland solutions in a growth chamber under conditions as described by Kuo-Huang and Zindler-Frank (1998). Three concentrations of calcium nutient solutions (as $Ca(NO_3)_2$): 3750 μM Ca^{2+}/L, 750 μM Ca^{2+}/L, and 94 μM Ca^{2+}/L were used. Replacements of calcium were made with equal concentrations of $NaNO_3$. After two months, small leaf squares were sliced from the mature leaves of the plants growing in the three levels of calcium nutrient solutions. For light or SEM microscopy, these square specimens were prepared as above.

RESULTS AND DISCUSSION

The mature leaves of *Ficus pumila* var. *awkeotsang* are coriaceous. The upper leaf surface was glabrous and shiny, but the lower surface was hirsute and prominently hairy brown (Liao, 1996). By scanning electron microscopy, we found two kinds of trichomes (including glandular and non-glandular trichomes) occurring on the upper leaf surface (Fig 1A), but no stoma was found. However, there were some hydathodes and within them many stomata were observed (Fig. 1B). In general, some glandular trichomes were located near each hydathode. Such type of hydathode was also found on the upper leaf surface of *F. formosana* (Chen and Kuo-Huang, 1991).

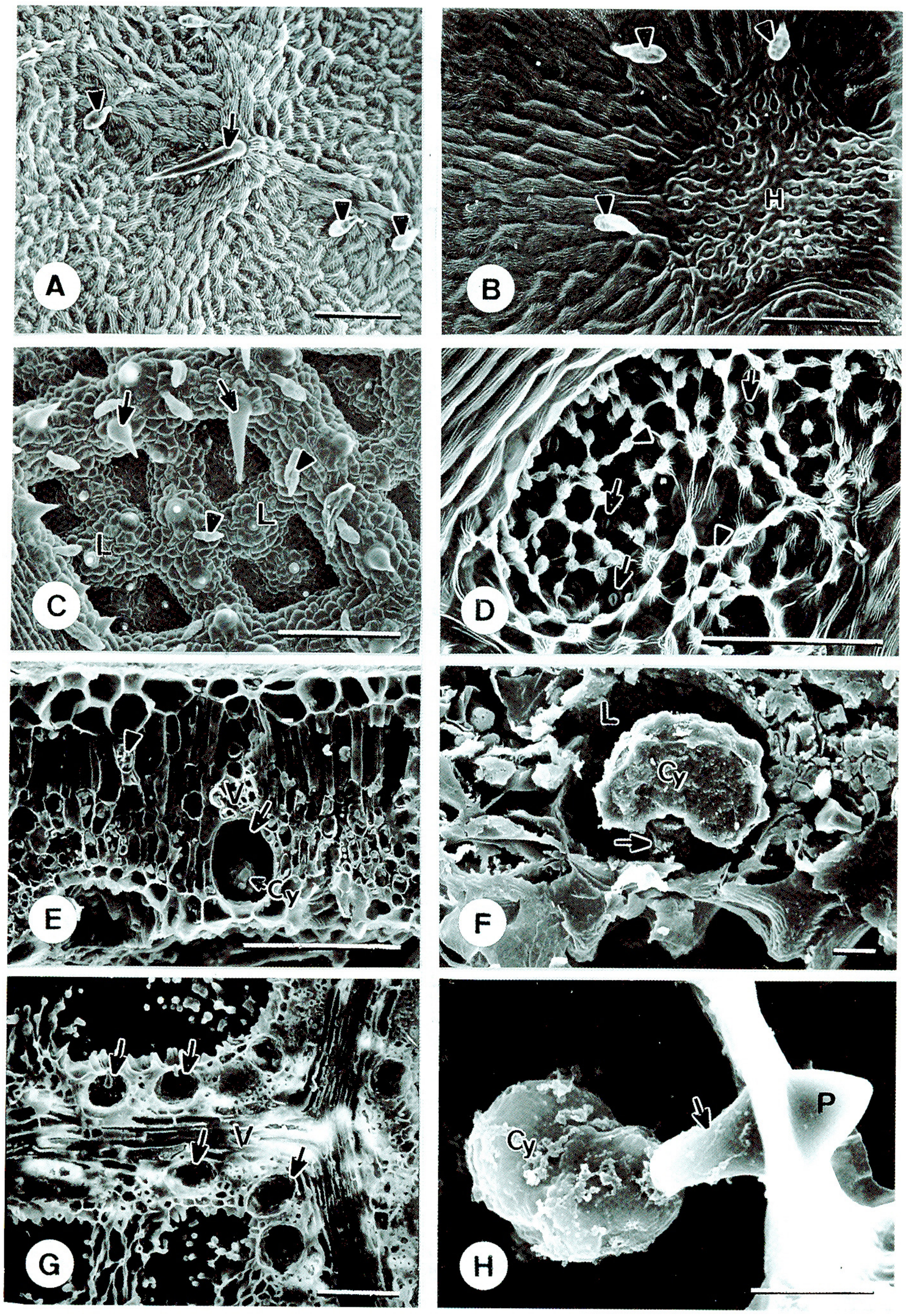

Fig. 1. SEM photographs of mature leaves of *Ficus awkeotsang*. A. Upper leaf surface showing the non-glandular (arrow) and glandular trichomes (arrowheads). No stoma was found. Bar=100 μm. B. Upper leaf surface showing the hydathode (H) with many stomata. Some glandular trichomes (arrowheads) located near the hydathode. Bar=100 μm. C. Lower leaf surface showing the uneven structure. Many non-glandular (arrows) and glandular trichomes (arrowheads) and the lithocysts (L) located on the leaf veins. Crypts were orderly arranged between the leaf veins. Bar=100 μm. D. In the stomata crypt many stomata (arrows) were observed. The ordinary epidermal cells had prominent tuberculate (arrowheads). Bar=100 μm. E. Transverse section of the leaf showing the 1-2 layers of upper and lower epidermis. Lithocyst (arrow) with cystolith (Cy) located on the lower epidermis beneath the leaf veins (V), while the druse crystal (arrowhead) was found in the most upper layer of palisade mesophyll. Bar=100 μm. F. Transverse section of the lithocyst (L) showing the stalk (arrow) and the body of cystolith (Cy). Bar=10 μm. G. Paradermal section of leaf showing the distribution of lithocysts (arrows) along the leaf veins (V). Bar=100 μm. H. Transverse section of the lithocyst showing the papilla (P) connected with the stalk (arrow) and the body of the cystolith (Cy). Bar=10 μm.

The lower leaf surface of *F. pumila* var. *awkeotsang* was obviously uneven (Fig. 1C). Crypts were orderly arranged and many stomata were found (Fig. 1D). The outer surface of ordinary epidermal cells had prominent tuberculate. This kind of organization minimizes air movement near the stomata (Mauseth, 1989). Both the upper and lower epidermis were 1-2 layered (Fig. 1E). In the lower epidermis there were many lithocysts which could be recognized as papillae while viewing from the leaf surface (Fig. 1C). The lithocysts occurred mostly along the leaf veins (Fig. 1G). Each lithocyst had a cystolith. The cystolith contained a stalk which was connected to the papilla of the lithocyst outer cell wall (Fig. 1H). Beneath the stalk, calcium carbonate accumulated and formed the body of the cystolith (Fig. 1F). By using the EDX microanalysis, the papilla contained silica, while calcium was detected mostly in the cystolith body (Figs. 2A-C). The cystoliths were soluble in acetic acid and hydrochloric acid with bubbles formation, suggesting that they were composed of calcium carbonate (Franceschi and Horner, 1980).

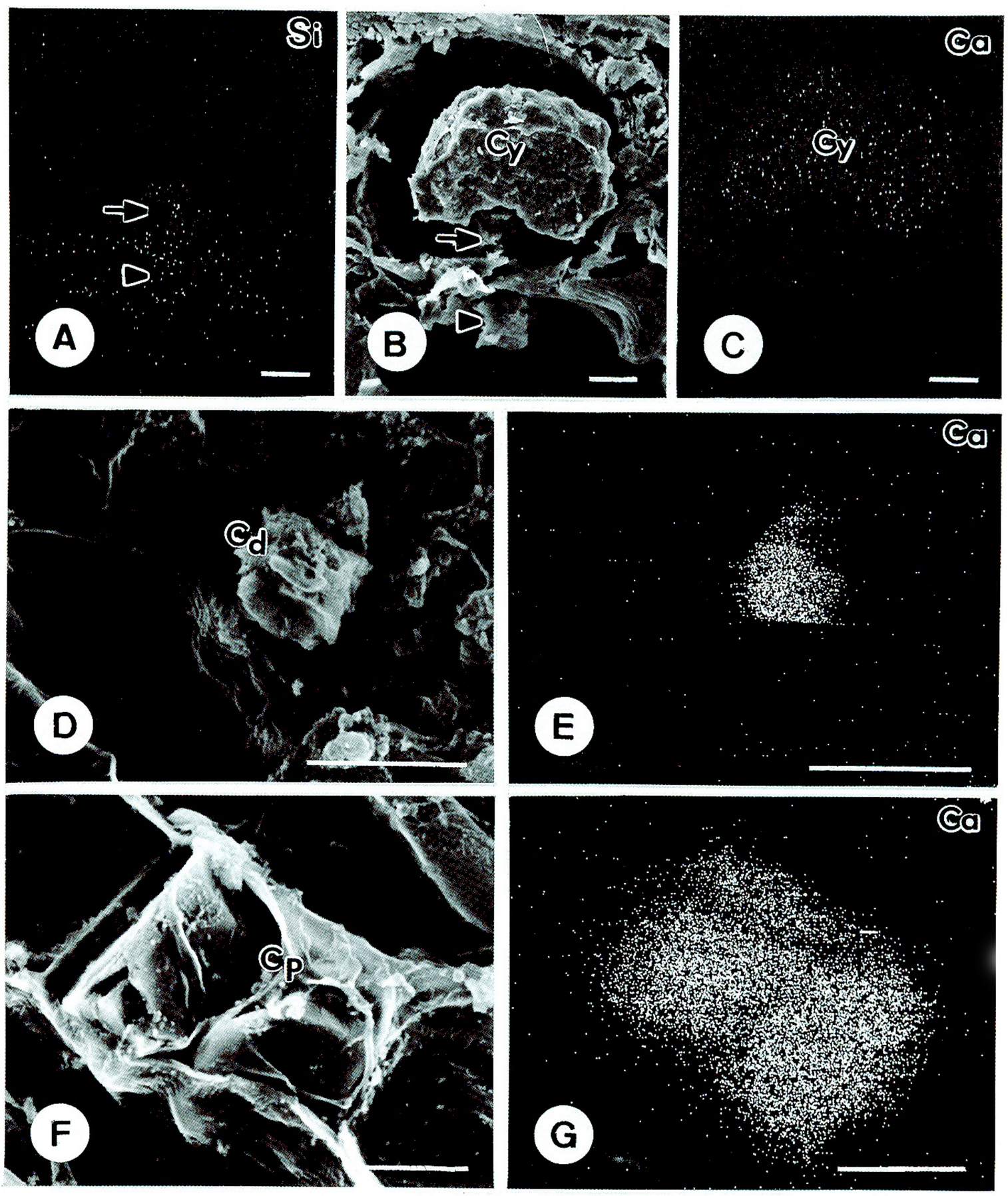

Fig. 2. SEM photographs and X-ray maps for localizing the electron transition energy of calcium and/or silica. A-C. Transverse section of lithocyst indicating the high calcium content of cystolith (Cy) and high silica content in the stalk (arrow) and papilla (arrowhead). D and E. Druse crystal (Cd) showing the high calcium content in the crystal area. F-G. Prismatic crystal (Cp) showing the high calcium content in the crystal area. All bars = 10 μm.

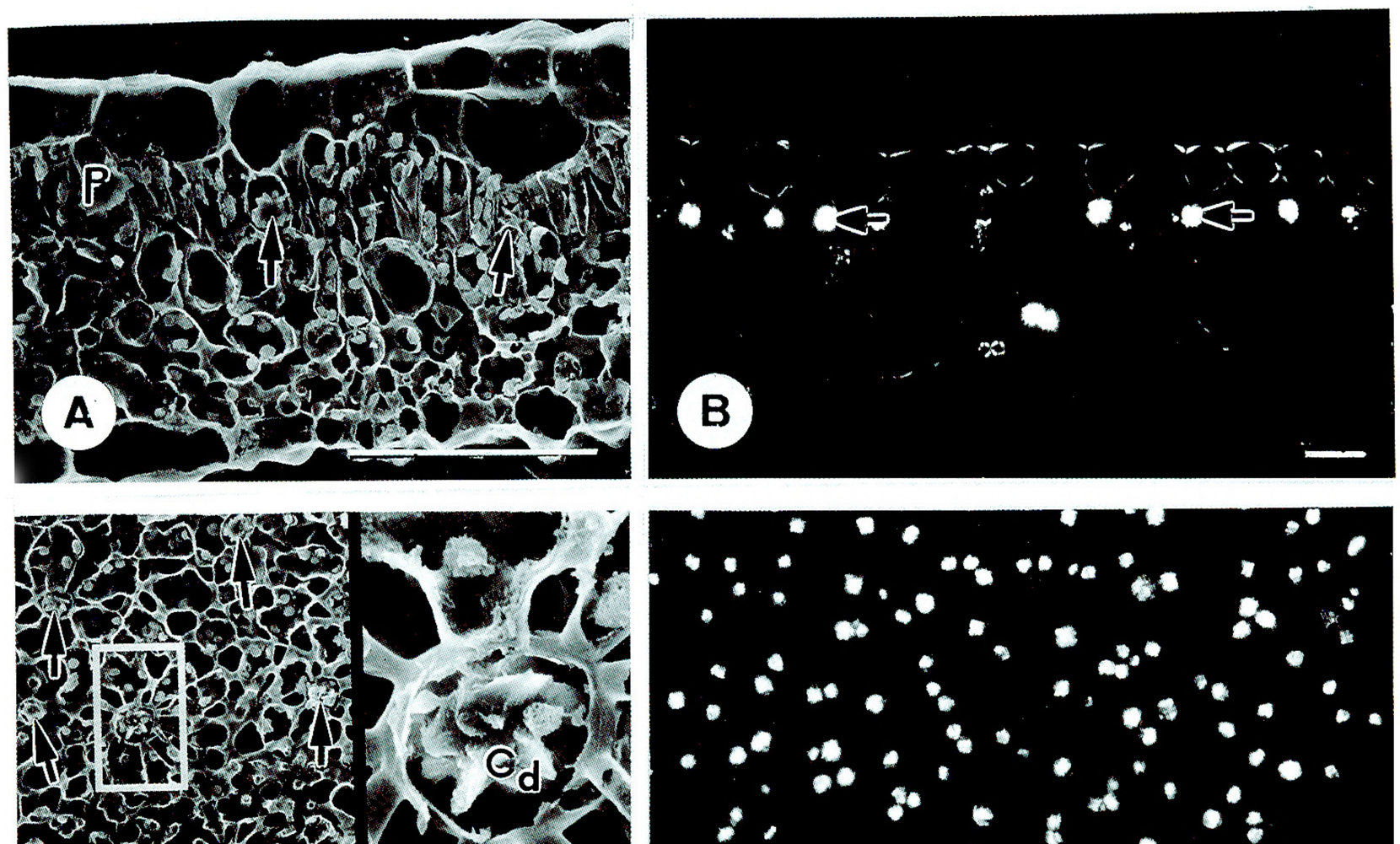

Fig. 3. Sections of leaves. A and B. SEM photograph and LM photograph with partial polarized light on the transverse sections, showing the druse crystals (arrows) located in the first layer of palisade mesophyll (P). Bars=100 μm. C. SEM photograph of the paradermal section on the palisade tissue showing the druse crystals (Cd, arrows). Bar=10 μm. D. Enlargement of C. Bar=100 μm. E. LM photograph with polarized light showing the distribution of druse crystals (bright areas). Bar=100 μm. F. SEM photograph of prismatic crystals (Cp). Bar=10 μm. G. LM photograph with polarized light of paradermal section on the bundle sheath showing the distribution of prismatic crystals (arrows, bright areas). Bar=100 μm.

The morphology and distribution of cystolith and lithocyst are genus- and species-specific in the Acanthaceae (Hsieh and Huang, 1974; Kuo-Huang and Yen, 1996) and Moraceae (Wu and Kuo-Huang, 1997). Besides, in Moraceae, the types of lithocysts were found to be related to the number of leaf epidermal layers, i.e., hair-like lithocyst in uniseriate epidermis, and papillate lithocysts of multiseriate epidermis (Wu and Kuo-Huang, 1997). In *F. pumila* var. *awkeotsang*, the papillate lithocysts were located in the multiseriate lower epidermis of the mature leaves.

Both prismatic crystals and druses were found in the mesophyll. By the X-ray microanalysis technique, calcium was detected in these crystals (Figs. 2D-G). These calcium oxalate crystals were insoluble in acetic acid and soluble in hydrochloric acid, without forming bubbles. Besides, they showed prominent bright areas when viewed with the light

microscope under polarized light (Figs. 3B, E, and G). The idioblasts with druse crystals were distributed sporadically in the palisade parenchyma, however, they were generally found in the most upper layer of the palisade tissue (Figs. 3A-E). The prismatic crystals (Fig. 3F) occurred in the bundle sheath along the leaf veins (Fig. 3G). In the leaves, this kind of crystal distribution supports the suggestion that the formation of calcium oxalate crystals in plants may be linked to evaporation of water (Franceschi and Horner, 1980; Kuo-Huang, 1990).

The impurities present in crystal cells may have been a factor in the formation of different crystal shapes (Scurfield *et al.*, 1973). In the leaves of a given species, two or more shapes of calcium oxalate crystals were commonly found. Doaigey (1991) observed various shaped calcium oxalate crystals in individual plants of *Datura*, *Nerium* and *Rumex*. In this study we also found two different shapes of oxalate crystals in the mesophyll of a leaf.

Calcium ions are essential nutrient for the plants and known to influence many of their developmental processes (Arnott and Pautard, 1970). The presence of calcium crystals is certainly not detrimental to the plant. Physical and chemical conditions may affect crystal formation and properties (Franceschi and Horner, 1980; Webb, 1999), but information concerning the function of these crystals remains scarce.

The formation of calcium carbonate and oxalate crystals in the cells of *F. pumila* var. *awkeotsang* leaves was prominently affected by the different concentrations of calcium supply. In plants grown in low calcium (94 μM Ca^{2+}/L) solution, most lithocysts contained only the stalk of cystolith (Fig. 4A). But in plants grown in normal calcium (750 μM Ca^{2+}/L) solutions, the cystoliths formed both the stalks and the crystal bodies (Fig. 4B). Although cystoliths without prominent crystals existed, they were rare and never seen in plants grown with high calcium solutions. In the plants grown in the high calcium (3750 μM Ca^{2+}/L) solution, the lithocyst was almost filled with the cystolith (Fig. 4C). Nevertheless, the density of lithocysts was not affected by the different concentrations of calcium supply.

In *F. pumila* var. *awkeotsang*, the formation of calcium oxalate crystals was also influenced by different concentrations of calcium supply. Many druses and prismatic crystals were observed in leaves of both the plants grown in normal (750 μM Ca^{2+}/L) and high calcium (3750 μM Ca^{2+}/L) solutions (Figs. 4E and G), and fewer oxalate crystals were found in the leaf of plants grown in low calcium solutions (Figs. 4D and F).

The calcium crystal cells may act as Ca-sinks, and enable plant tissues to deposit surplus Ca absorbed under experimental and, implicitly, natural conditions as Ca-carbonate or –oxalate, and to maintain relatively low concentrations of soluble calcium in the cells and tissues (Webb, 1999). The results of this study demonstrated that in the leaves of *F. pumila* var. *awkeotsang*, both calcium oxalate and calcium carbonate serve as storage forms for calcium. If the capacity of existing Ca-sinks is insufficient to precipitate most apoplastic Ca, the tissue content in soluble Ca will rise, and new calcium oxalate crystal cells may be induced. The literature shows that the number and size of calcium oxalate crystals may be influenced by calcium nutrition to a degree varying from subject to subject (Zindler-Frank, 1980; Franceschi and Horner, 1980; Franceschi, 1989). It seems probable that the origin of these specialized cells is correlated with the properties of the calcium ion. Factors which control oxalate synthesis and cellular calcium uptake and mobility may affect calcium oxalate crystal induction and formation (Scott, 1941; Frank, 1972; Borchert, 1984, 1986; Franceschi, 1987).

The density of lithocysts in the mature leaves of *F. pumila* var. *awkeotsang* was not significantly affected by the different concentrations of calcium supply which suggests that

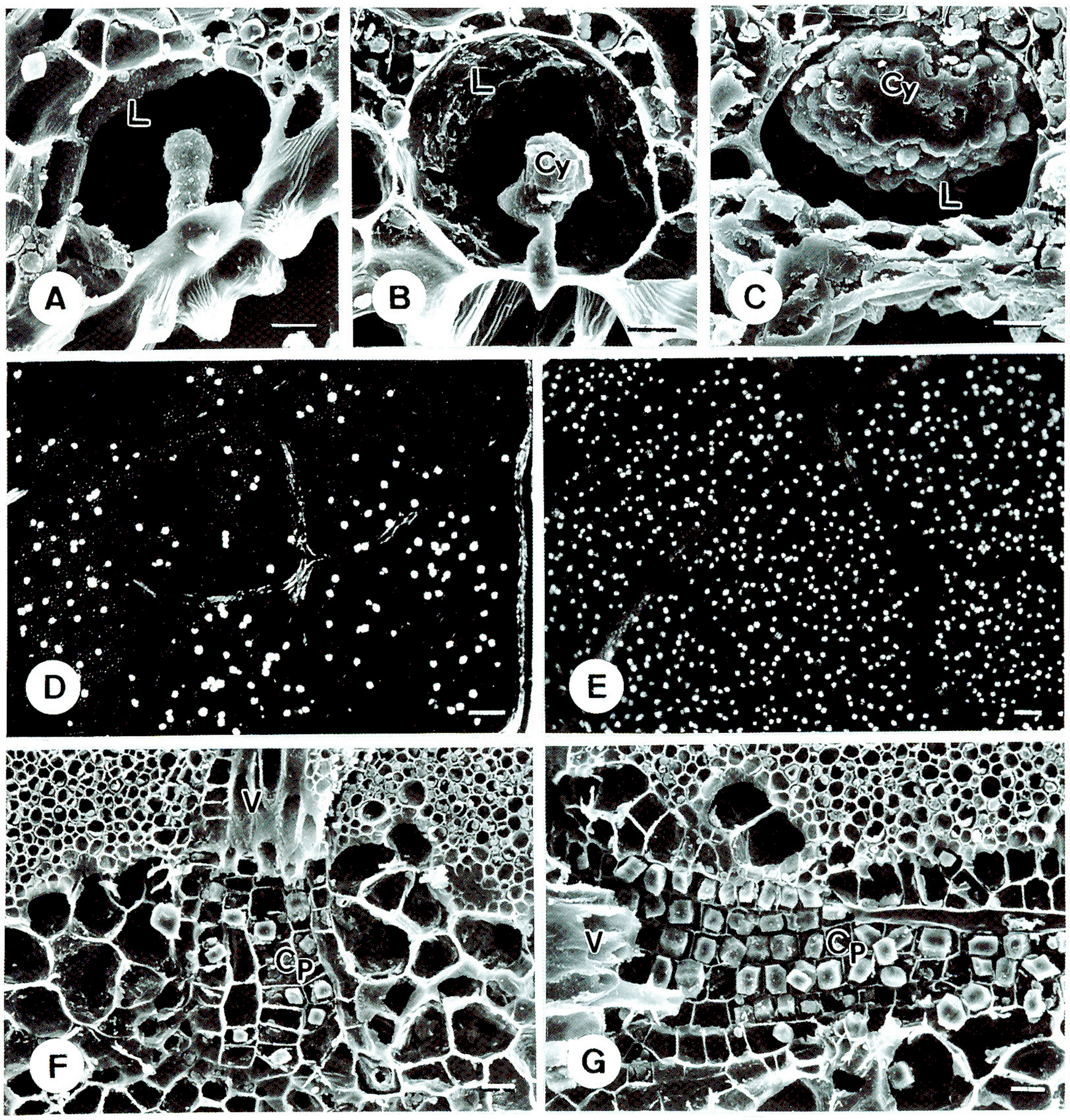

Fig. 4. Calcium depositions in the leaves with different calcium supplies. A, B, D, and F: 1/8 Ca supply; C, E, and G: 5 Ca supply. A and B. The lithocyst with none or little cystolith accumulation. Bars=10 μm.C. The lithocyst almost filled with cystolith. Bar=10 μm. D. LM photograph with polarized light showing fewer calcium oxalate crystals occurring in the mesophyll. Bar=100 μm. E. LM photograph with polarized light showing more druse crystals occurring in the mesophyll. Bar=100 μm. F. SEM photograph showing fewer prismatic crystals (Cp) occurring in the bundle sheath cells around the leaf vein (V). Bar=100 μm. G. SEM photograph showing more prismatic crystals (Cp) occurring in the bundle sheath cells around the leaf vein (V). Bar=100 μm.

neither extra lithocysts are induced under the high calcium supply, nor fewer lithocysts are formed under low calcium supply. Only the sizes of calcium carbonate depositions increased from the low calcium (94 μM Ca^{2+}/L) to the high calcium (3750 mm μM Ca^{2+}/L) solutions. The formation of the number of lithocyst initials in the epidermal tissue is determined, but their differentiation is affected by the amount of Ca^{2+} supply. During the leaf development, a continuous surplus of Ca^{2+} influx from the high calcium concentration solution enables the lithocyst initial to form a large calcium carbonate deposition inside. Otherwise, when under calcium deficiency, most of the calcium carbonate depositions contain only the stalk and no obvious body structure was found. Further experiments are needed to understand the regulation mechanisms of the formation and redistribution of the calcium depositions.

ACKNOWLEDGEMENTS

The financial support of the National Science Council of Taiwan (NSC-82-0211-B002-266) is highly appreciated. We are also grateful to Professor Tsan-Piao Lin for the supply of mature branches of *Ficus pumila* var. *awkeotsang* and the useful discussion.

LITERATURE CITED

Ajello, L. 1941. Cytology and cellular interrelations of cystolith formation in *Ficus elastica*. Am. J. Bot. **28**: 589-594.

Arnott, H. J. and F. G. E. Pautard. 1970. Calcification in plants. In: Schraer H. (ed.). Biological calcification, cellular and molecular aspects. Appleton-Century-Crofts, New York.

Borchert, R. 1984. Functional anatomy of the calcium-excreting system of *Gleditsia triacanthos*. Bot. Gaz. **145**: 474-482.

Borchert, R. 1985. Calcium-induced patterns of calcium oxalate crystals in isolated leaflets of *Gleditsia triacanthos* L.and *Albizia julibrissin* Durazz. Planta **165**: 301-310.

Borchert, R. 1986. Calcium acetate induces calcium uptake and formation of calcium oxalate crystals in isolated leaflets of *Gleditsia triacanthos* L. Planta **168**: 571-578.

Chen, C.-C. and L.-L. Kuo-Huang. 1991. The study on hydathodes in the leaves of *Ficus formosana*. Annual meeting of Chinese Botanical Society. **17**: 6.

Dawes, C. J. 1979. Biological Techniques for Transmission and Scanning Electron Microscopy, pp. 231-251. The Offset Hause Co., Burlington.

Demarty, M., C. Morvan and M. Thellier. 1984. Calcium and the cell wall. Plant, Cell and Environ. **7**: 441-448.

Doaigey, A. R. 1991. Occurrence, type, and location of calcium oxalate crystals in leaves and stem of 16 species of poisonous plants. Am. J. Bot. **78**: 1608-1616.

Fahn, A. 1990. Plant Anatomy. 4th ed. pp. 153, 230-231.Pergamon Press, Oxford.

Franceschi, V. R. 1987. Oxalic acid metabolism and calcium oxalate formation in *Lemna minor* L. Plant, Cell and Environ. **10**: 397-406.

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

Franceschi, V. R., H. T. Horner. 1980. Calcium oxalate crystals in plants. Bot. Rev. **46**: 361-427.

Frank, E. 1972. The formation of crystal idioblasts in *Canavalia ensiformis* DC. at different levels of calcium supply. Z. Pflanzenphysiol. **67**: 350-358.

Freisleben, R. 1933. Untersuchungen ueber Bildung und aufloesung von Cystolithen bei den Urticales. Flora N. F. **27**: 1-45.

Genua, J. M. and C. J. Hillson. 1985. The occurrence, type and location of calcium oxalate crystals in the leaves of fourteen species of Araceae. Ann. Bot. **56**: 351-361.

Horner, H. T. and B. L. Wagner. 1992. Association of four different calcium crystals in the anther connective tissue and hypodermal stomium of *Capsicum annuum* (Solanaceae) during microsporogenesis. Am. J. Bot. **79**: 531-541.

Kuo-Huang, L.-L. 1990. Calcium oxalate crystals in the leaves of *Nelumbo nucifera* and

Nymphaea tetragona. Taiwania **35**: 178-190.
Kuo-Huang, L.-L., C.-R. Sheue, Y.-P. Yang, and S.-H. T. Chiang. 1994. Calcium oxalate crystals in some aquatic angiosperms of Taiwan. Bot. Bull. Acad. Sin. **34**: 179-188.
Kuo-Huang, L.-L. and T.-B. Yen. 1993. Calcium crystals in the leaves of *Ficus awkeotsang* (Moraceae) R.O.C. Symposium on electron microscopy, XIV: 9-10.
Kuo-Huang, L.-L. and T.-B. Yen. 1996. The development of lithocysts in the leaves and sepals of *Justicia procumbens* L. Taiwania **41**: 17-26.
Kuo-Huang, L.-L. and E. Zindler-Frank. 1998. Structure of crystal cells and influences of leaf development on crystal cell development and vice versa in *Phaseolus vulgaris* (Leguminosae). Bot. Acta. **111**: 337-345.
Liao, J.-C. 1996. Moraceae. In: Huang, T.-C. (ed). Flora of Taiwan. Editorial Committee of the Flora of Taiwan, 2nd edition. pp. 136-195 Taipei.
Lin, T.-P., C.-C. Liu, S.-W. Chen and W.-Y. Wang. 1989. Purification and characterization of *Ficus awkeotsang* Makino achenes. Plant Physio. **91**: 1445-1453.
Mauseth, J. D. 1988. Plant anatomy. pp. 32-34. The Benjamin, Cummings Publ. Company, Inc. Menlo Park, California.
Pentecost, A. 1980. Calcification in plants. International Review of Cytology **62**: 1-27.
Rabiger, F. H. 1951. Untersuchungen an einigen Acanthaceen und Utricaceen zur Funktion der Cystolithen. Planta **40**: 121-144.
Scott, F. M. 1941. Distribution of calcium oxalate crystals in *Ricinus communis* in relation to tissue differentiation and presence of other eragastic substances. Bot. Gaz. **103**: 225-246.
Scurfield, G., A. J. Michell and S. R. Silva. 1973. Crystals in woody stems. Bot. J. Linn. Soc. **66**: 277-289.
Sporne, K. R. 1948. A note on a rapid clearing technique of wide application. New Phyto. **47**: 290-291.
Webb. M. A. 1999. Cell-mediated crystallization of calcium oxalate in plants. Plant Cell **11**: 751-761.
Wu, C.-C. and L.-L. Kuo-Huang. 1995. The effect of calcium ion concentrations on the formation of calcium crystals in the leaves of *Morus australis*. R.O.C. Symposium on electron microscopy. XVI: 65-66.
Wu, C.-C. and L.-L. Kuo-Huang. 1997. Calcium crystals in the leaves of some species of Moraceae. Bot. Bull. Acad. Sin. **38**: 97-104.
Yu, F.-G., Z.-I. Li. 1991. Anatomy of the lithocyst in the epidermis of Leaf in *Boussonetia papyrifera*. Acta Botanica Sinica **33**: 249-255.
Zindler-Frank, E. 1980. Changes in leaf crystal idioblast differentiation in *Canavalia* caused by gibberellic acid through an influence on calcium availability. Z. Pflanzenphysiol. **98**: 43-52.

愛玉子葉部草酸鈣與碳酸鈣沉積物的形成

黃玲瓏[1, 2]、顏才博[1]、陳香君[1]

(收稿日期：2002 年 1 月 25 日；接受日期：2002 年 2 月 27 日)

摘　　要

應用光學與掃描電子顯微鏡技術來觀察愛玉子 (*Ficus pumila* var. *awkeotsang*)葉部鈣沉積物的成分、形態與分佈。愛玉子成熟葉片中，可觀察到草酸鈣與碳酸鈣的堆積。碳酸鈣鐘乳體分佈在葉片下表皮之石胞中，晶簇狀草酸鈣結晶分散於柵狀葉肉組織中，而多面體結晶則主要分佈於維管束鞘。此外，愛玉子葉片鈣結晶的形成受到所提供之不同鈣離子濃度的水耕培養液的影響，在較高濃度鈣離子培養液中，石胞內堆積多量碳酸鈣，而在低濃度鈣離子培養液中，石胞內則較少碳酸鈣堆積，然而，石胞的密度並不受不同濃度的鈣離子供應所影響。就葉肉組織中的草酸鈣結晶而言，隨著水耕培養液中鈣離子濃度之升高，其草酸鈣結晶之密度亦明顯增加。

關鍵詞：碳酸鈣、草酸鈣、鈣離子供應、愛玉子。

1. 國立台灣大學植物學系，台北市 106，台灣，中華民國。
2. 通信作者。