Characterization of Calcium Oxalates Generated as Biominerals in Cacti[1]

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The chemical composition and morphology of solid material isolated from various Cactaceae species have been analyzed. All of the tested specimens deposited high-purity calcium oxalate crystals in their succulent modified stems. These deposits occurred most frequently as round-shaped druses that sometimes coexist with abundant crystal sand in the tissue. The biominerals were identified either as $CaC_2O_4.2H_2O$ (weddellite) or as $CaC_2O_4.H_2O$ (whewellite). Seven different species from the Opuntioideae subfamily showed the presence of whewellite, and an equal number of species from the Cereoideae subfamily showed the deposition of weddellite. The chemical nature of these deposits was assessed by infrared spectroscopy. The crystal morphology of the crystals was visualized by both conventional light and scanning electron microscopy. Weddellite druses were made up of tetragonal crystallites, whereas those from whewellite were most often recognized by their acute points and general star-like shape. These studies clearly demonstrated that members from the main traditional subfamilies of the Cactaceae family could synthesize different chemical forms of calcium oxalate, suggesting a definite but different genetic control. The direct relationship established between a given Cactaceae species and a definite calcium oxalate biomineral seems to be a useful tool for plant identification and chemotaxonomy.

Calcium oxalate is considered to be the most commonly occurring biomineral in higher plants (for example, see reviews of Arnott and Pautard, 1970; Franceschi and Horner, 1980; Arnott, 1982). It has been demonstrated that crystal growth is a highly controlled intracellular process (Mann, 1989; Fraústo de Silva and Williams, 1991; Baran, 1995). The cells in a plant tissue that produce the crystals are generally referred to as crystal idioblasts (Foster, 1956; Arnott, 1982).

Calcium oxalate occurs in two hydration states in plants, as the monohydrate (whewellite) or as the dihydrate (weddellite; Frey-Wyssling, 1981; Arnott, 1982). A number of crystal habits have been found for both hydration states: raphids, prisms, styloids, druses, and crystal sand (Franceschi and Horner, 1980; Arnott, 1982). Other less common shapes could be considered as variations of the mentioned forms. Both the chemical nature and the morphology of these crystals, as well as their localization within the plant body, could be specific for a given species.

Some higher plants may accumulate enormous quantities of inorganic material, and this is especially true for some members of the Cactaceae family (Franceschi and Horner, 1980). For example, as early as in 1938, a cactus species (*Cactus senilis*) was described as containing as much as 85% of its dry weight as calcium oxalate (Cheavin, 1938).

We described recently the isolation and characterization of biominerals from two different Cactaceae species. This bioinorganic material appears in the form of highly pure and well-crystallized calcium oxalates that typically grow in the form of druses, i.e. spherical aggregates of thousands of individual crystallites. These deposits were identified either as weddellite (Monje and Baran, 1996) or whewellite (Monje and Baran, 1997). We have also found that certain cactus species accumulate crystalline SiO_2 as α-quartz (Monje and Baran, 2000).

We have extended these studies by exploring the occurrence of solid biomineral deposits in other members of this plant family. In this context, we have now performed a systematic infrared spectroscopic study to characterize the chemical nature of these deposits and describe further the crystals in terms of their morphology. This survey among different species allowed us to verify the general occurrence of weddellite and whewellite as biominerals in cactus plants and their presence in very pure chemical forms. On the other hand, the crystals were usually very prominent, and most of them could be easily detected without the aid of a microscope.

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RESULTS

Identification of the Biominerals

The screening of the different Cactaceae species looking for the presence of mineral deposits allowed us to establish the occurrence of calcium oxalate crystals in all of the analyzed plant specimens. These biominerals were identified either as weddellite ($CaC_2O_4.2H_2O$) or whewellite ($CaC_2O_4.H_2O$), by comparison with literature data (Babic-Ivancic et al., 1985; Varetti and Volponi, 1995). A summary of the analyzed species and the type of biomineral found in each one are presented in Table I.

As seen in Table I, members of the Cereoideae subfamily always deposited weddellite, whereas the Opuntioideae subfamily mineralizes whewellite. Coexistence of the two types of oxalates could never be demonstrated in any of the investigated species.

Apart from the druses, other crystal habits were observed in samples obtained from several species. Thousands of tiny individual crystallites similar to crystal sand were usually seen among fresh isolated preparations, as well as inside living parenchymal cells from the modified stems. Quite often, individual prisms were observed among the most abundant druses. Radiograph elemental analyses on single isolated prismatic crystals showed a predominance of calcium and oxygen. Raphides and styloids were not observed in samples from any of the studied species, although their existence in cacti plants has been reported for other species (Rivera and Smith, 1979). Figure 1 shows scanning electron microscopic (SEM) images from samples of *Gymnocallycium platense* (Cereoideae) and *Opuntia penicilligera* (Opuntioideae) selected as representative examples of each plant subfamily. Note the dramatic change in crystallite morphology related to the different chemical composition.

In general, infrared (IR) spectra of the oxalate crystals appeared very well defined and totally free from spurious bands, showing the high purity of the isolated biominerals as can be seen in the spectra presented as typical examples in Figure 2. A brief analysis of the spectra of both hydrated forms of calcium oxalate has been given in our earlier reports (Monje and Baran, 1996, 1997). On the other hand, the comparison of the two spectra clearly shows that both forms are easily distinguishable from each other. Whereas in the high-frequency range weddellite only presents a very broad and poorly structured band with a shoulder on its lower frequency side, whewellite shows a well-structured band multiplet with five defined components. The spectra are similar in the region of the stretching vibrations of the carboxylate groups (1,620–1,350 cm^{-1}), although the whewellite bands are slightly displaced to lower energies in comparison with those of weddellite. Finally, important differences in the spectral pattern are also evident in the lower frequency range in which whewellite presents again a greater number of IR absorptions.

Crystal Morphology

Biomineralized calcium oxalate crystals from cactus species were mainly found as more or less round-shaped transparent druses. Figures 3 through 7 show the general aspect of these druses after they were removed from the plant soft stem parenchyma. The druses were made up of hundreds of microcrystals tightly packed together in a single macrostructure. Some of them, particularly those of weddellite, were more than 300 to 400 μm in diameter, facilitating their handling by direct visual inspection. This fact, together with its high abundance in the tissues, facil-

Table I. *Occurrence of biomineral deposits in Cactaceae species*

The chemical nature of the calcium oxalate crystals has been determined by IR spectroscopy.

Species	Main Biomineral	Crystal Habits
Opuntioideae subfamily		
Opuntia longispina	Whewellite	Druses
Opuntia microdasys	Whewellite	Druses
Opuntia penicilligera	Whewellite	Druses
O. auranthiaca	Whewellite	Druses
Puna clavarioides	Whewellite	Druses
Tephrocactus articulatus	Whewellite	Druses
Maihueniopsis glomerata	Whewellite	Druses
Cereoideae subfamily		
Chamacereus silvestrii	Weddellite	Druses
Cleistocactus baumanii	Weddellite	Druses/crystal sand
Gymnocallycium cytianum	Weddellite	Druses
G. platense	Weddellite	Druses
Rebutia margarethae	Weddellite	Druses
Pyrrhocactus strausianus	Weddellite	Druses/prisms/crystal sand
Wigginsia tephracantha	Weddellite + α-quartz	Druses/bipyramids(few)/Crystal sand (abundant)

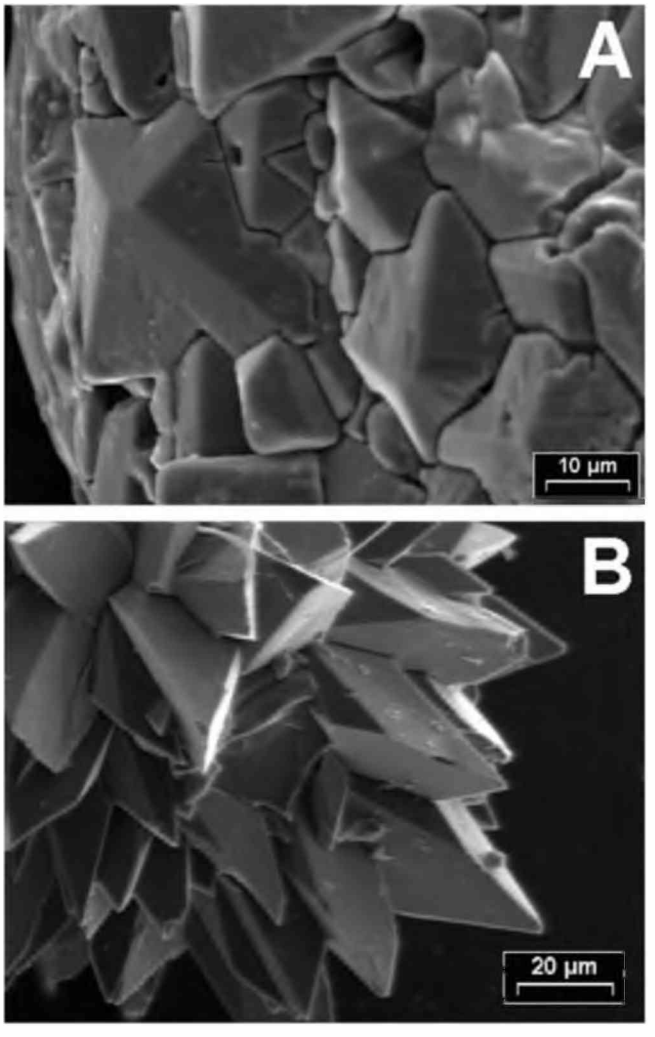

Figure 1. Portion of calcium oxalate druse biominerals from two representative Cactaceae spp. that emphasize microcrystal appearance and arrangement at the surface of single druses. A, Weddellite from *G. platense* (Cereoideae). B, Whewellite from *O. penicilligera* (Opuntioideae).

itated both isolation and purification procedures before the IR studies.

Druses composed of weddellite were usually made up of individual tetragonal crystals (Figs. 1A, 3, and 4), clearly reflecting the tetragonal crystal system of the biomineral. The general aspect of single, isolated druses varies among species, especially with respect to size and angles between individual crystal faces, as can be seen in Figures 3 and 4. Weddellite bodies from *Rebutia margarethae* represented an exception to this pattern (Fig. 5). In this case, single, solid structures acquired curious final shapes conformed by fine crystal layers deposited tightly one above another.

Whewellite druses were distinguished from weddellite druses mainly by their stellate shapes, with individual crystallites having acute sharp points emerging from the center of the druse (Figs. 1B, 6, and 7). Crystals from the *Opuntia* genera were found visually indistinguishable among the species analyzed by us (Fig. 6). They were also similar to those of other *Opuntia* species mentioned in the literature (Rivera and Smith, 1979). However, druses from *Opuntia* spp. were remarkably different from the ones isolated from other members of the Opuntioideae subfamily, namely *Puna*, *Tephrocactus*, and *Maihueniopsis*, based on size, individual crystal shape, and packing (Figs. 7 and 8). We speculate that biophysicochemical parameters within the crystal chamber, as well as mechanical forces, could strongly affect crystal development. Thus, open-airy or compact druses, big or small aggregates, might be developed from identical chemical oxalate species. The cellular environment controlling crystal formation and development should be species specific in nature.

The simultaneous presence of SiO_2 deposits, generally termed as phytoliths (Arnott, 1982; Volcani, 1983), could be detected in the crystal sand of some samples, and we confirmed their characteristics in the case of *W. tephracantha* (Monje and Baran, 2000).

DISCUSSION

Most vascular plants deposit some form of mineralized material (Lowenstam, 1981; Arnott, 1982; Fraústo da Silva and Williams, 1991; Baran, 1995). The most widely distributed biomineralization system among different plant taxa is that of crystalline calcium oxalate, which is absent from only a few angiosperm families such as Juncaceae and Cyperaceae (McNair, 1932; Arnott, 1982; Smith, 1982).

The presence of large amounts of crystalline calcium oxalate distributed throughout the tissues of various cacti plants has been also reported (Rivera and Smith, 1979; Franceschi and Horner, 1980). The presence of such biomineral deposits has been reported for a number of species belonging to the Cereoideae (Cheavin, 1938; Rivera and Smith, 1979; Monje and Baran, 1996, 2000), Opuntioideae (Rivera

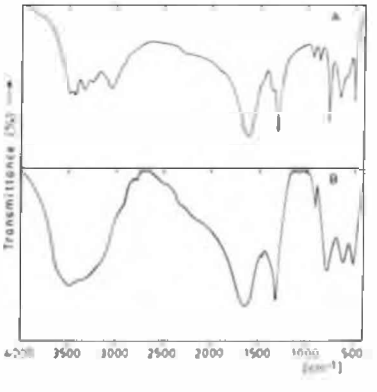

Figure 2. Infrared spectra, in the range 4,000 to 400 cm^{-1}, of whewellite from *Puna clavaroides* (A) and weddellite from *Pyrrhocactus strausianus* (B).

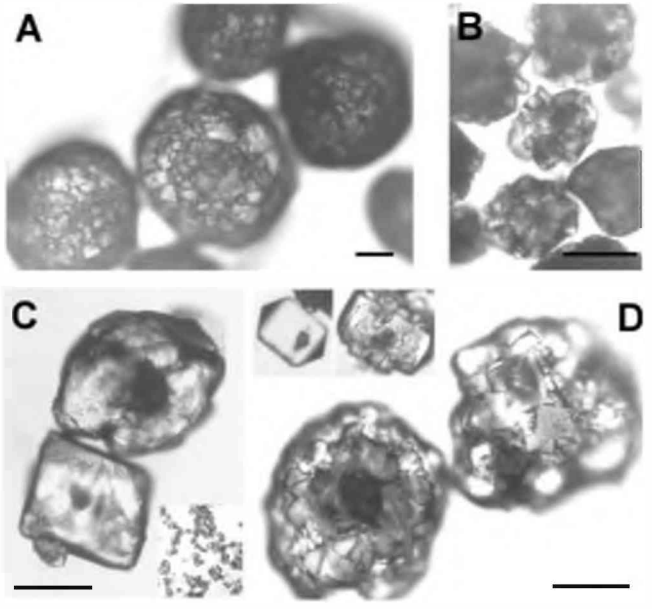

Figure 3. Weddellite druses from Cereoideae: light microscopy. Crystalline aggregates from *Chamaecereus silvestrii* (A), *Cleistocactus baumanii* (B), *Wigginsia tephracantha* (C), and *P. strausianus* (D) (scale bars = 200 μm). C, Inset, a purified sample of crystal sand isolated from *W. tephracantha*. The inset in D tries to lay stress on developmental stages toward the formation of a spherical crystalline cluster in *P. strausianus*. Small mineral fragments seem to be deposited from a starting point at the center of prismatic solid skeletons. Scale bars = 200 μm.

and Smith, 1979; Braun and Pereira, 1991; Monje and Baran, 1997), and the ancient Pereskioideae (Bailey, 1961; Leuenberger, 1986) subfamilies. It is known that the deposits occur prevalently in crystal idioblasts and usually have a defined arrangement in some tissues (Arnott, 1982; Braun and Pereira, 1991). However, limited information about the exact chemical composition of the isolated crystals found in cactus plants is now available. To our knowledge, the crystallographic studies performed by Rivera and Smith (1979) on five different cactus species from the United States are, up to now, the most compelling studies ever published on this matter. Our survey among different species from Argentina confirmed that these members of the Cactaceae family have also strongly mineralized stem tissues. These minerals apparently derive from at least two different and independent biomineralization processes.

The number and location of crystal idioblasts within the plant body vary among taxa, and some investigators have used them in classification. Most plant families tend to contain one crystal type or a range of morphologically related types. The size and shape of the crystals within a given group could be a very specific feature.

Our results clearly show that Cereoideae and Opuntioedeae subfamily members can deposit different hydration states of calcium oxalate. The less commonly found dihydrate weddellite (Arnott and Pautard, 1970; Franceschi and Horner, 1980) was observed in large quantities in all the species coming from the first subfamily. This fact is especially interesting because weddellite is the metastable form of calcium oxalate and, thus, less widely distributed than the stable form whewellite. This behavior is not uncommon for biominerals, as shown, for example, in the case of calcium carbonate. Calcite is the most thermodynamically stable polymorph at normal atmospheric temperatures. Aragonite is less stable than calcite, and the most unstable polymorph is vaterite (Addadi and Weiner, 1992). Notwithstanding, all three modifications are found as biominerals in plants and other forms of life (Lowenstam, 1981).

In some instances, crystals have been classified as whewellite or weddellite solely on the basis of their shape. Certain evidence indicated that crystal shape might be independent from the hydration state of calcium oxalate. Although the mechanism controlling shape has not been clearly elucidated, it is assumed that the final crystal is molded inside the crystal chamber. The formation of crystals with different habits seems to be associated with complex membranous systems within idioblast vacuoles that restrict environmental parameters where crystalogenesis takes place (Arnott and Pautard, 1970; Arnott, 1982). Therefore, crystal formation is not a random or haphazard process. The coordinated operation of proton pumps and ion channels to mobilize calcium and oxalate through the vacuole membrane is considered to underlie the whole process.

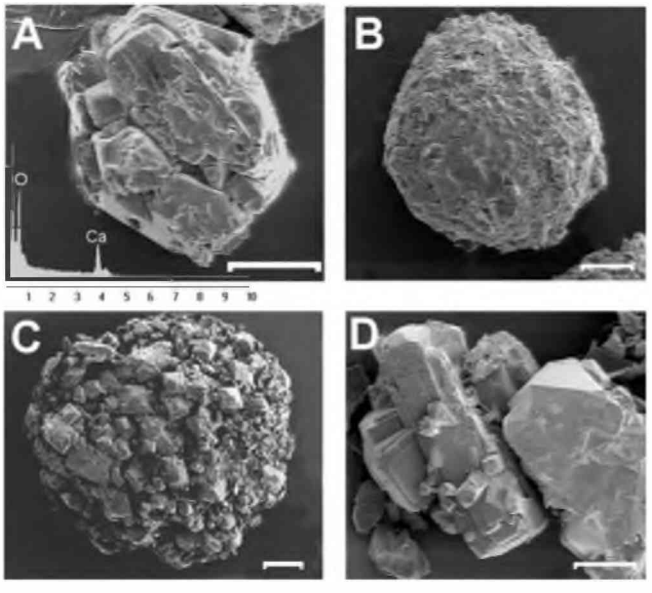

Figure 4. Weddellite druses from Cereoideae: scanning electron microscopy. Fine structure of typical freshly isolated deposits from *W. tephracantha* (A), *G. platense* (B), *C. silvestrii* (C), and *P. strausianus* (D). Individual tetragonal crystallites are usually tightly clustered to form a more or less spherical dense mineral body. A, Inset, area-restricted EDAX spectrum from the above druse. The expected peaks for calcium and oxygen in the calcium oxalate are denoted. Scale bars = 60 μm.

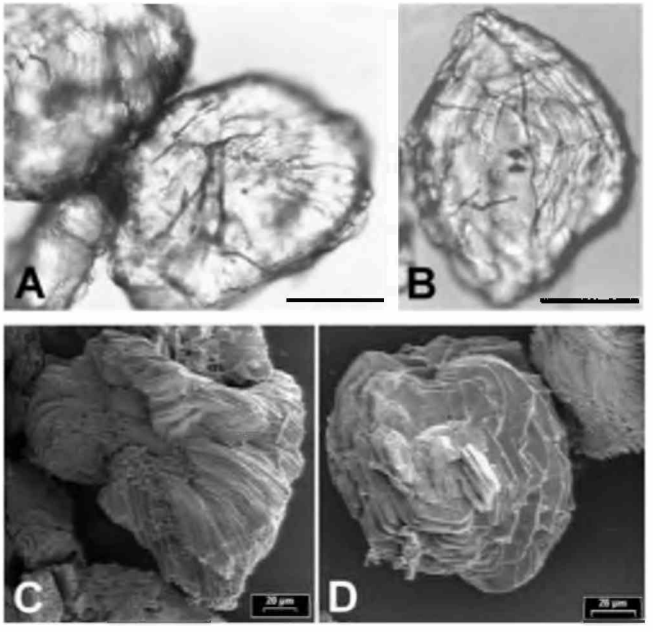

Figure 5. Gill-like weddellite druses from the Cereoideae *R. margarethae*. A and B, Light microscopy (scale bars = 80 μm). C and D, Scanning electron microscopy. Most druses were made up of very thin tightly packaged flat crystalline lamella. The general appearance of these agglomerates is noteworthy.

This viewpoint reinforces the idea that a differential genetic control directs the particular biomineralization of calcium oxalate to weddellite or whewellite as the final forms. Information on crystal composition consequently might be a useful criterion to discriminate among members from each of the main cactaceae subgroups. As it happened to be for other plants at lower taxonomic levels, related taxa tend to have similar crystallization patterns. Despite this, we believe that a careful study of the differences/similarities of calcium oxalate druses from different plant sources (size, development and arrangement of individual crystallites, localization, etc.) might be developed as a useful tool to discriminate among certain

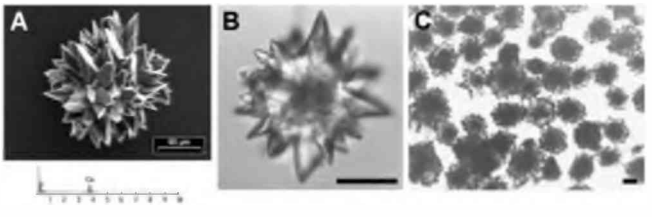

Figure 6. Whewellite from the Opuntioideae subfamily: stellate druses from the genera *Opuntia*. Crystals from these genera were found visually indistinguishable among the examined species. A, SEM micrograph of a representative whewellite druse from *Opuntia auranthiaca* (above) and area-restricted EDAX-spectrum from the same crystal (below) confirming the sole presence of calcium and oxygen in the aggregate. B, Detail of a typical stellate druse from *Opuntia longispina* (light microscopy, 500× magnification). C, Population of purified stellate druses from *O. penicilligera* (light microscopy, 100× magnification). Scale bars from light microscopy = 80 μm.

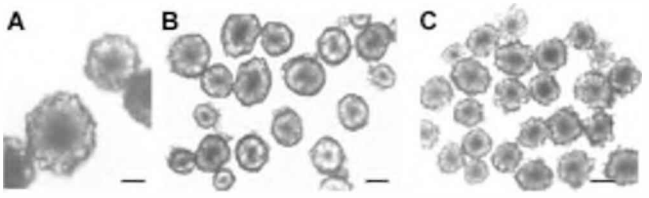

Figure 7. Whewellite druses from other Opuntioideae members: conventional light photographs of purified crystal preparations of druses from *Tephrocactus articulatus* (A), *Maihueniopsis glomerata* (B), and *Puna clavarioides* (C). These whewellite deposits were much smaller in diameter than that of the *Opuntia* spp. Morphological differences were also evident. Scale bars = 100 μm.

cactus genera or perhaps even species. The oxalates have the particular advantage to be very resistant water-insoluble plant products and, thus, they could be found where other plant residues are no longer evident. Perhaps in a large enough sampling of different Cactaceae species, one might find common crystal patterns for systematic phylogenetics. Because crystal biomineralization involves a highly controlled and complex process that needs the coordination of many physiologically independent events, one might speculate that Opuntioideae and Cereoideae ancestors could have turned different pathways at an early stage in evolution.

An intriguing issue is the reason why most cactus plants develop an extraordinary abundance of calcium oxalate crystals in some of their tissues. Although their presence is shared by a variety of plant taxa, the abundance of minerals found in Cactaceae makes them unique in the plant kingdom. There are results suggesting that the development of crystal idioblasts may be related to the amount of calcium available in the soil (Frank, 1972). However, calcium availability is not always a limiting factor. The function of calcium oxalate deposits in plants is still controversial. They have been implicated in many different functions that range from intracellular regulation of pH and calcium ions, to gravity perception, mechanical support, and even plant defense. We speculate that precipitation of calcium oxalate in stem tissues may be related to a particular physiological aspect of this succulent plant family, specialists in the matter of preserving water. Ruiz and Mansfield (1994) presented evidence that deposition of calcium oxalate in cells of the leaf could be neces-

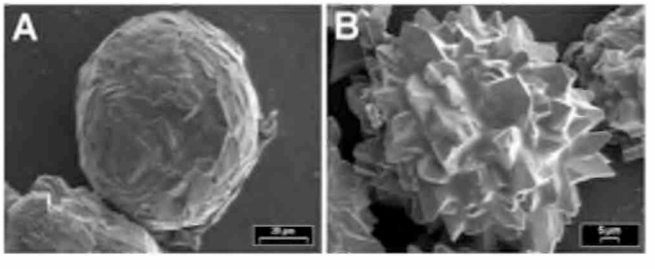

Figure 8. SEM photographs of whewellite druses from other Opuntioideae members: *M. glomerata* (A) and *P. clavarioides* (B).

sary to maintain a low calcium concentration in the vicinity of stomatal guard cells, as the sap traverses the apoplast from the xylem toward the epidermis. Calcium ions are implicated in many signal transduction events that control the stomatal aperture. Apoplastic calcium concentration must be maintained at a low level if stomatal opening is to be avoided. In cactus plants having crassulacean acid metabolism, a special requirement for stomata is that they should be kept tightly closed during the day to avoid gas exchange and subsequent water loss.

On the other hand, it was often considered that calcium oxalate was formed only to maintain low soluble levels of the potentially toxic oxalic acid (Franceschi and Horner, 1980; Franceschi and Loewus, 1995). Notwithstanding, most recent studies suggest that the biosynthetic pathway can be induced as a response to increased calcium levels in calcium oxalate accumulating plants (Keates et al., 2000; Kostman et al., 2001). In addition, the biosynthesis of L-ascorbic acid (Smirnoff et al., 2001) and its conversion to oxalic acid in plants (Kostman et al., 2001) is another interesting point to explore in relation to calcium oxalate biomineralization.

Many open questions remain to be investigated in relation to the genesis and development of crystal idioblasts, and Cactaceae seem to be highly suited plant models to continue with such studies.

MATERIALS AND METHODS

Plant Material

Samples of *Wigginsia tephracantha*, *Gymnocalycium platense*, *Opuntia penicilligera*, and *Opuntia auranthiaca* were collected from their natural habitats in the Provincial Park "Ernesto Tornquist" (Sierra de la Ventana, Buenos Aires). Opuntioedeae subfamily members (*Puna clavarioides*, *Tephrocactus articulatus*, *Opuntia longispina*, and *Maihueniopsis glomerata*) and Cereoideae specimens (*Cleistocactus baumanii*, *Rebutia margarethae*, *Phyrrocactus strausianus*, and *Gymnocalycium cytianum*) were provided by Dr. Roberto Kiesling (Instituto de Botánica Darwinion, Buenos Aires). *Chamacereus silvestrii* and *Opuntia microdasys* were grown by the authors. All of the mentioned cactaceae species are native from different regions of Argentina (Kiesling, 1975, 1984), with the exception of *O. microdasys*, which is from Mexico.

Crystal Isolation and Purification

Crystal druses were isolated from both fresh and dry plant specimens. However, dry material was preferred to increase crystal recovery. With the purpose of avoiding potential contamination of crystalline samples by soil particles, plant stems were carefully washed with abundant distilled water. After removal of needles and epidermis, thin sections from the succulent stems were excised and washed several times. The druses could be easily separated manually. Tissue sections were macerated in water and crystals were mechanically freed with the help of dissection knives, as first reported by Rivera and Smith (1979). Alternative isolation methods using cell wall digestive enzymes or acetic acid to degrade the fresh stem tissue were also employed (Monje and Baran, 1996, 1997). Crystalline products were first separated by direct visual inspection. The final separation of solid material was performed by manual collection under a dissecting light microscope. Isolated druses were washed several times until plant debris were no longer evident. A similar procedure was employed to isolate and purify samples of crystal sand. The samples were finally dried under a nitrogen flow and submitted to microscopic or spectroscopic analyses.

Physicochemical Studies

IR Spectroscopy

The IR spectra were obtained by means of a Bruker IFS 66 spectrophotometer in the spectral range between 4,000 and 400 cm^{-1} using the KBr pellet technique (4 mg of the powdered sample dispersed in 100 mg of KBr).

Scanning Electron Microscopy and EDAX Analysis

SEM and area-restricted x-ray analysis by energy dispersive spectrometry (EDAX) were carried out with a Jeol 35 CF instrument (Jeol Co. Inc., Tokyo) with attached energy dispersive x-ray analytical system, containing a germanium window and a lithium-drifted silicon detector. Druses were mounted on glass coverslips and coated with gold in the usual way. SEM analysis was carried out using an acceleration voltage of 5 kV.

Light Microscopy

Druses were visualized using a Zeiss Axiolab light microscope (Carl Zeiss, Thornwood, NY) in transmission mode. Samples were mounted on glass slides and inspected using 10× or 20× dry objectives. Photographs were taken using Kodak Plus X-Pan 125 white and black film (Eastman-Kodak, Rochester, NY). After digitalization, images were exported to Adobe Photoshop (Adobe Systems, Mountain View, CA) for digital processing.

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