Characterization of Calcium Oxalate Biominerals in *Pereskia* Species (Cactaceae)

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Z. Naturforsch. **64c**, 763–766 (2009); received June 29, 2009

Calcium oxalate druses were isolated from the stems and leaves of six Pereskioideae family members and investigated by infrared spectroscopy, showing that in all samples the biomineral was present in the form of whewellite, $CaC_2O_4 \cdot H_2O$. As *Pereskia* is thought to represent the "ancestral" condition of the leafless stem-succulent cacti, these results suggest that the biomineralization of calcium oxalate in Cactaceae represents a primitive characteristic of the group and also support a close genetic relationship between *Pereskia* and *Opuntia*.

Key words: Pereskia, Biominerals, Whewellite, Infrared Spectroscopy

Introduction

Crystalline oxalates are widely distributed in nature and have been observed in rocks, soil, and among a variety of living organisms, including plants and animals (Baran and Monje, 2008). The most widely distributed biomineral of this type is calcium oxalate, which is especially common in the plant kingdom (Arnott, 1982; Khan, 1995; Monje and Baran, 2004a; Baran and Monje, 2008). The presence of other metal oxalates is extremely rare in biological systems, although the presence of crystalline magnesium, manganese, copper, and ammonium oxalates in different forms of life has been reported, whereas soluble forms of sodium and potassium oxalates are widely distributed in plants and fungi (Baran and Monje, 2008).

Crystalline calcium oxalates have been found in two different hydration states in plants, either as the monoclinic monohydrate (whewellite, $CaC_2O_4 \cdot H_2O$) or as the orthorhombic dihydrate (weddellite, $CaC_2O_4 \cdot 2H_2O$). Whewellite is the most stable form from the thermodynamic point of view (Monje and Baran, 2004a; Baran and Monje, 2008). However, the morphology (druses, raphids, solitary crystals, crystal sand) and the hydration state of calcium oxalates in plants differ significantly among different groups.

Because higher plants incorporate calcium in excess to the cellular requirements and most of them, unlike animals, do not have well-developed excretory systems to eliminate such excess, they modulate the difference between the natural abundance of calcium and the very low intracellular levels required by tightly controlling the distribution of calcium and its compartmentation within cells. In this context, with a solubility product of $1.3 \cdot 10^{-9}$ mol^2 l^{-2} in water, calcium oxalate effectively sequesters calcium and renders itself metabolically and osmotically inactive for plant cells. Therefore, many plants accumulate crystalline calcium oxalate in response to the presence of an excess of calcium (Webb, 1999; Baran and Monje, 2008).

On the other hand, a number of recent studies indicate that these calcium oxalate crystals do not form an inert, non-retrievable deposit of calcium, but can act as a sort of reservoir to which plants can recur in cases of calcium deficiency. Apart from this primary function as a calcium regulator, the great variety of crystal shapes and sizes, as well as their localization in different plant tissues, suggests various other functions for calcium oxalates which might be evolved secondarily. Some of these functions include mechanical support, gravity perception, intracellular pH regulation and ion balance, detoxification and even light gathering and reflection (Nakata, 2003; Baran and Monje, 2008).

The Cactaceae family is a well-known lineage of stem-succulent plants that are widely recog-

0939–5075/2009/1100–0763 $ 06.00

nized for their specialized adaptation to drought. These include an extensive and shallow root system with dynamic hydraulic properties that allow for maximum water uptake following rain, a succulent pith and cortex with high capacitance for water storage, and a long-lived stem cortical tissue system that has replaced leaves as the primary photosynthetic organ. These anatomical and histological adaptations come along with the acquisition of crassulacean acid metabolism (CAM photosynthesis), which minimizes the transpirational water loss by allowing the stomata to open during the night (Edwards and Diaz, 2006; Edwards and Donoghue, 2006). Cactaceae species are also unique in their ability to biomineralize calcium oxalate (Rivera and Smith, 1979; Monje and Baran, 2002; Hartl *et al*., 2007), and the content of the biomineral can reach up to 80–90% of the plant's dry weight (Monje and Baran, 2004a). However, the connection between calcium oxalate biomineralization and water stress adaptation remains obscure.

The *Pereskia* genus (subfamily: Pereskioideae, family: Cactaceae) consists of species of leafy shrubs and trees and is the only cactus group that has persistent non-succulent stems and leaves. It has long been considered as the best living representation of the "ancestral cactus" (Edwards *et al.*, 2005; Edwards and Donoghue, 2006; Metzing and Kiesling, 2008). As opposed to succulent cacti, most species of Pereskioideae grow in tropical, humid environments.

We have extensively studied the biominerals present in the two main succulent subfamilies of cacti, Cereoideae and Opuntioideae, and have found the presence of calcium oxalate (Monje and Baran, 1996, 1997, 2002, 2004b), magnesium oxalate (Monje and Baran, 2005), and silicon dioxide (Monje and Baran, 2000, 2004b). We have now investigated the presence of calcium oxalates in different species of *Pereskia*, the non-succulent "relictual cacti", in order to investigate the possible relationship between calcium oxalate biomineralization and adaptation of cacti drought.

Material and Methods

Plant material

The plant material was provided by the South Florida Cactus and Succulent Society (Miami, Florida). The stems and leaves of six representative species of *Pereskia* were harvested from healthy and well-hydrated specimens growing under normal greenhouse conditions, regardless of the season and other environmental conditions.

Crystal isolation and purification

Crystal druses were isolated from fresh plant tissues. Plant stems and leaves were carefully washed with abundant distilled water to remove any possible external contamination, cut with a razor blade into thin tissue sections and immersed in a 6% sodium hypochlorite solution for 48 h. The tissue was then mechanically dissociated by passing it repeatedly through a plastic transfer pipette, filtered through gauze, and allowed to decant for 30 min. The pellet containing crystalline products was suspended in absolute ethanol, and the crystals were manually collected by inspection through a dissecting light microscope. The isolated crystalline material was repeatedly washed with ethanol until the tissue debris was no longer evident. This procedure renders crystalline samples consisting mainly of intact druses. The purified crystal samples were finally dried under a nitrogen flow before spectroscopic analysis.

Synthesis of $CaC_2O_4 \cdot H_2O$ samples

For comparative purposes we have prepared high-purity crystalline $CaC_2O_4 \cdot H_2O$ samples. They were prepared by simultaneous dropping of highly diluted sodium oxalate and calcium chloride solutions in a great volume of distilled water, maintained at 70 °C, as described by Grases *et al.* (1990).

Infrared spectroscopy

The IR spectra were obtained by means of a Bruker IFS 66 spectrophotometer in the spectral range between 4000 and 400 cm^{-1} using the KBr pellet technique (4 mg of the powdered sample dispersed in 100 mg of KBr).

Results and Discussion

Infrared spectroscopy is a powerful tool for the investigation of the chemical composition of plant material (Baran, 2005); we have applied this methodology successfully in the investigation of plant biominerals (Monje and Baran, 1996, 1997, 2000, 2002, 2004b, 2005; Baran and Rolleri, 2009). In the present study, we have used this sensitive spectroscopic technique for the first time to analyze the

composition of crystalline material isolated from the stems and leaves of six different members of the Pereskiodeae subfamily (*Pereskia aculeata*, *P. bleo*, *P. portulacifolia*, *P. nicoyana*, *P. quisqueyana*, and *Pereskiopsis* sp.); all species showed the presence of highly abundant druses in tissues of the stems and leaves.

The infrared spectra of biominerals present in the purified druses of *Pereskia* species showed clearly that the crystals consisted of highly pure whewellite ($CaC_2O_4 \cdot H_2O$). This was a consistent finding regardless of the species analyzed and their localization in the plant body. Fig. 1 shows a comparison of the infrared spectra of a pure synthetic sample of $CaC_2O_4 \cdot H_2O$ a representative crystalline sample of *Pereskia* (*P. bleo*), and a crystalline sample isolated from an Opuntioideae family member (*Tephrocactus articulatus*), that was identified as whewellite in a previous study (Monje and Baran, 2002). As it can be seen the three spectra are identical, indicating that whewellite is the main biomineral present in the druses of Pereskioideae members. Four other species of this family (*P. grandifolia*, *P. sacharosa*, *P. stenantha*, *P. zinniflora*) recently, investigated by X-ray diffractometry, also show the presence of whewellite (Hartl *et al*., 2007).

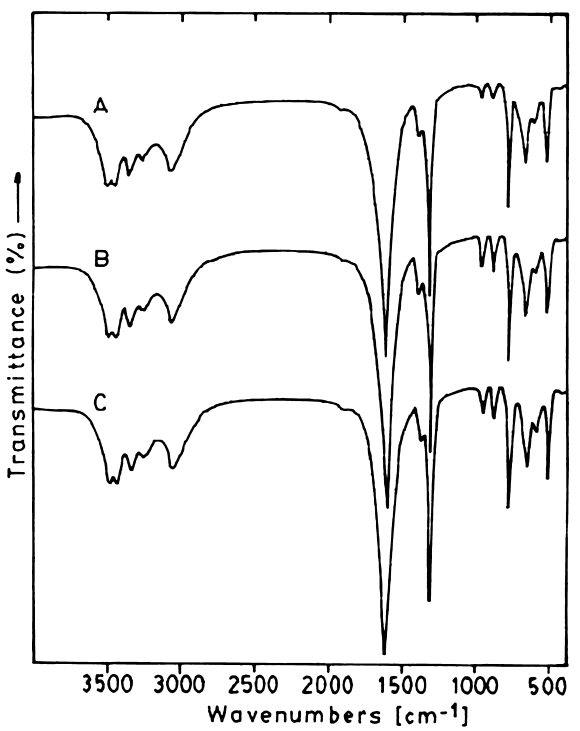

Fig. 1. FTIR-spectra of (A) a pure sample of synthetic $CaC_2O_4 \cdot H_2O$, (B) druses of *Pereskia bleo* and (C) druses of *Tephrocactus articulatus*.

Our previous investigations on biominerals present in the Cactaceae family have shown that Opuntioideae family members biomineralize calcium oxalate as whewellite, $CaC_2O_4 \cdot H_2O$, whereas Cereoideae members biomineralize calcium oxalate as weddellite, $CaC_2O_4 \cdot 2H_2O$, suggesting a definite but different genetic control over the biomineralization process in the two most representative groups of the succulent Cactaceae (Monje and Baran 2002, 2004a). This conclusion was confirmed by a recent X-ray diffraction study of the total crystalline material present in a large number of Cactaceae species (Hartl *et al*., 2007). Even though the simultaneous presence of the two hydration states of calcium oxalate in a single taxon seems to be rare in angiosperms (Arnott, 1982), Hartl *et al*. (2007) showed that certain Cactaceae species display the concurrent presence of weddellite and whewellite. One of the few known examples in which the presence of both forms of calcium oxalate has been unambiguously demonstrated is *Opuntia ficus indica* (Malainine *et al*., 2003).

Overall, the results of our study confirmed a close genetic relationship between *Pereskia* and *Opuntia* (Metzing and Kiesling, 2008), as both groups biomineralize calcium oxalate as whewellite druses, which were also highly similar in size and morphology. Therefore, we conclude that whewellite must have been the ancestral biomineral in the Cactaceae lineage and that weddellite must have evolved after branching of the Cereus as a separate group. Besides, the observation of whewellite druses in all species of *Pereskia* suggests that the biomineralization of calcium oxalate in Cactaceae represents a primitive characteristic of the family, most likely acquired before adaptation to water stress conditions. Whereas primitive cacti deposit calcium oxalate in the form of the thermodynamically most stable monohydrate, in the course of evolution certain lines of Cactaceae perhaps conserved calcium oxalate mainly in the form of the metastable and convertible dihydrate.

Acknowledgements

This work was supported by Universidad Nacional de La Plata (UNLP) and Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). E. J. B. is a member of the Research Career of this organism.

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