First Evidence for the Presence of Weddellite Crystallites in *Opuntia ficus indica* Parenchyma

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Calcium oxalate crystallites occur very often in the plants tissues and their role is still poorly known. We report here the experimental protocol leading to the isolation of two forms of calcium oxalate crystallites differing in their hydration level in the parenchymal tissues of *Opuntia ficus indica* (Miller). Whereas the whewellite crystallites are habitual in all *Opuntia* species, the weddellite form has never been isolated from these species before, which is probably due to their small size (about 1 μm). We have identified these forms using X-ray diffraction and scanning electron microscopy.

Key words: Opuntia ficus indica, Weddellite, Whewellite

Introduction

Opuntia ficus indica (prickly pear tree) is a highly productive crassulacean acid metabolism (CAM) plant (Garcia De Cortázar and Nobel, 1991). Originating from the American continent, it is widely cultivated in the world for its exotic fruits and for the flattened stem segments called cladodes (Nobel *et al.*, 1992). The young stems are used as vegetable in Mexico and the southern states of the USA, where it is called ‘Nopalitos’. These cladodes contain a fairly large amount of minerals (ash = 20 % of dry weight), and exhibit a high Ca/P ratio (Malainine *et al.*, 2003).

Calcium oxalate crystallites are present in many species of plants. They are generally formed inside cells (called crystal idioblasts) and may have various functions. They could be related to the plant’s need to keep up an ionic equilibrium, and to regulate its osmotic pressure. It had been demonstrated, on one hand, that oxalic acid is a by-product of various cellular metabolic processes, on the other hand, that the plant could hardly survive with elevated amounts of calcium. The formation of crystals could be a means to solve both of these problems: trapping oxalates and calcium as insoluble salt, and controlling their concentration to avoid having a high level of toxic entities inside the cell (Franceschi and Horner, 1980). From another point of view, one could consider the calcium oxalate crystallites as storage substances, consisting of essential ions for the plant’s growth and development (Lane, 1994). The presence of calcium oxalate crystallites in the plant tissues appears not to be directly related to the soil content of calcium carbonate (Scharrer and Jung, 1954). In contrast, for *Beta vulgaris*, it seems that nitrogen-rich fertilisation increases the amount of calcium oxalate accumulation in the plant (Scharrer and Jung, 1954).

Calcium oxalate crystallites in *Opuntia imbricata* and *Opuntia englemannii* were identified as the whewellite form ($CaC_2O_4 \cdot H_2O$), while crystallites in Cereoideae species were found to be of the weddellite form ($CaC_2O_4 \cdot 2H_2O$) (Rivera and Smith, 1979). Monje and Baran (1996, 1997) have studied two other Cactaceae species from Argentina. While *Opuntia microdasys* give a high purity whewellite, *Chamacereus silvestrii* deposit only the weddellite form (Monje and Baran, 1996, 1997). Further evidences for this selective biomineralisation inside the Cactaceae subfamilies were gathered by the same authors in recent studies. They have found that all species of Cactaceae (14 species verified) deposit calcium oxalate crystallites. Upon those species, seven produce only the whewellite form and all were from the *Opuntioideae*

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subfamily. The others deposit only weddellite and were all from the *Cereoideae* subfamily. The authors conclude that the hydration level of oxalate crystallites seems to be a functional criterion in chemotaxonomy of Cactaceae subfamilies (Monje and Baran, 2002).

For *Opuntia* species, studies on the presence and distribution of calcium in crystal idioblasts had begun very early. Besides crystal idioblasts, for example *Opuntia ficus indica* has shown a massive presence of calcium in mucilage cells and cell walls. Trachtenberg and Mayer (1981) found that the growth regime of oxalate crystals resembles that of parenchyma cells. They suggest that calcium is actively transferred from oxalate crystals to the mucilage cells.

Elsewhere of the plant kingdom few observations of the coexistence of two forms of calcium oxalate have been reported. Scurfield *et al.* (1973) and Gibson (1973) have shown that crystals of different morphologies may appear in adjacent cells. Calmès *et al.* (1970) have found two forms of morphologically different oxalate crystals, in different parts of the plant: raphides in buds and druses in leaves. Calmès and Carles (1970) have reported that raphides (weddellite) disappeared while druses (whewellite) are not degraded.

To the best of our knowledge, there is no previous report about the coexistence of the dihydrate and the monohydrate forms of calcium oxalate inside the same plant cell.

The aim of this work is to provide a protocol allowing to isolate and identify weddellite in *Opuntia ficus indica* tissues. This kind of calcium oxalate has never been identified in *Opuntioideae* tissues before.

Materials and Methods

Plant material

Three months old cladodes of *Opuntia ficus indica* were collected from an experimental plantation in Amezmiz, 30 km far from Marrakech, Morocco.

Separation of oxalate crystals

Spines and peels of the cladode were removed, and small cubes (1–2 cm^3, 10 g) were cut out, mixed with distilled water and disrupted in a Waring Blender. The resulting suspension was filtered through a 50 μm mesh nylon sieve. The residue was resuspended in distilled water and filtered. The liquid and the pulp residue were both investigated for crystals. The pulp residue was suspended in 2 % NaOH at 80 °C for 2 h, under slow mechanical stirring. Crystals released during this step have gradually deposited as a white sediment, while other cell components remained in suspension. The liquid was carefully removed and the residue (sediment A) washed and suspended twice in distilled water. 200 mg of white crystals were obtained and allowed to dry in air for X-ray analysis.

The filtrate (liquid passing through 50 μm nylon sieve) was centrifuged and the resulting sediment suspended in 2 % NaOH at 80 °C for 2 h. Organic cell components were solubilized while crystals stay without change. After centrifugation 300 mg of white crystals (sediment B) were obtained and allowed to dry in air for X-ray analysis.

Scanning electron microscopy

Small cubes (0.5 cm × 0.5 cm) were cut out from fresh *Opuntia* cladodes. These specimens were subjected to solvent exchange (water-alcohol-liquid CO_2), then dried under critical point conditions in a E3000 critical point drying apparatus (Polaron Equipment Limited, Watford, UK). The samples were vacuum-coated with a gold-palladium alloy before observation with a scanning electron microscope (SEM) JEOL-JMS-6100 operated at acceleration voltages ranging from 5 to 8 kV.

X-ray analysis

Powdered samples were investigated by a Siemens D500 X-ray Diffractometer $\theta/2\theta$, operated at 45 kV and 40 mA. Fully automated data collection and analysis with use of the library of the Joint Committee for Powder Diffraction Standards (JCPDS) on CD-ROM, was performed with an associated IBM/PS2 station.

Results

Morphology of oxalate crystals

Crystals in *Opuntia ficus indica* observed in this study were ordered into two discernible classes,

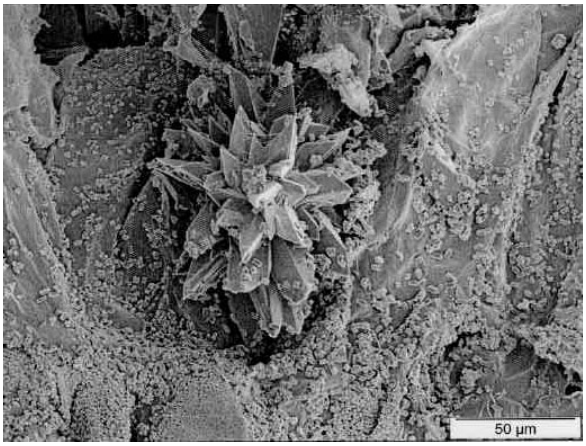

Fig. 1. SEM micrograph of crystals in *Opuntia ficus indica* parenchyma cells.

differing in shape and form. Fig. 1 shows both of families in the same cell. Starlike crystallite conglomerates appear as large druses of 20–500 times larger than the quadratic individual crystals. The size of those conglomerates ranged from 100 to 250 μm. Fig. 2 shows quadratic crystals spread all over the cell wall. Their average size is about 1 μm.

Table I. Comparison of whewellite and sediment A X-ray diffraction patterns.

Whewellite			Sediment A	
d_{hkl} [Å][a]	I/I_0 (%)[b]	≥ 10 %	d_{hkl} [Å]	I/I_0 (%)
5.93	100	*	5.92	100
5.79	30	*	5.78	28.3
4.52	4		4.52	9.7
3.78	6		3.78	13.1
3.65	70	*	3.65	85.3
3.01	10		3.00	12.9
2.98	45	*	2.97	64.3
2.91	10	*	2.91	12
2.84	10	*	2.84	14.8
2.49	18	*	2.49	29.7
2.35	30	*	2.35	35.8
2.26	8		2.26	16.6
2.21	6		2.21	9.4
2.13	2			
2.07	14	*	2.08	17
1.98	10	*	1.98	16.2
1.95	10	*	1.95	11.8
1.93	8		1.93	13.7
1.89	6		1.89	11.5
1.82	6			
1.74	6			

[a] Interatomic spacing (d-spacing); peaks of low intensity were skipped.
[b] relative intensity.

X-ray diffraction analysis

X-ray diffraction analysis, shows that sediment A – starlike druses – is constituted of high purity whewellite (Table I). All the reflections in the diffractogram fit perfectly the American Society for Testing and Materials (ASTM) file 20-231 (de Wolff, Delft, The Netherlands) specific to a class of calcium oxalate ($CaC_2O_4 \cdot H_2O$) set with a single water molecule in the crystal.

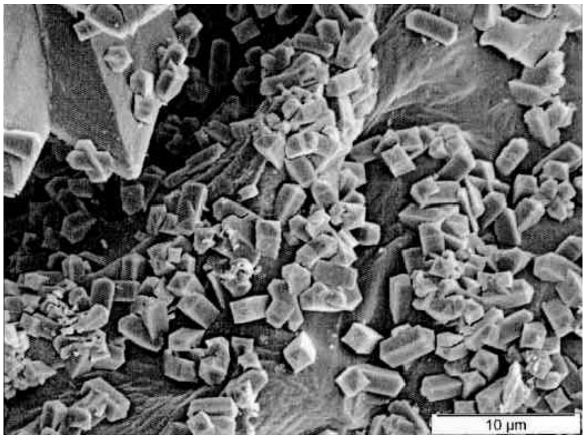

Fig. 2. SEM micrograph of quadratic crystals (weddellite form).

Sediment B showed the complicated X-ray diagram of a mixture. We found that it could be better explained on the basis of the coexistence of a small amount of cubic silicon (Si) (ASTM file 27-1402) and two forms of calcium oxalate: the whewellite and two classes of the calcium oxalate hydrate also called weddellite ($CaC_2O_4 \cdot 2\ H_2O$; ASTM file 17-451) and ($CaC_2O_4 \cdot 2.25\ H_2O$; ASTM file 20-233) (Table II). Comparing the diffraction lines to those from the ASTM files, we have assigned downright the major diffraction lines, except the reflection at $d_{hkl} = 5.21$ Å ($I/I_0 = 13\ \%$), which looks like the large diffraction bands of amorphous material.

Discussion

We succeeded to isolate the large starlike druses, which are almost ubiquitous in *Opuntia* species. Riviera and Smith (1979) have found crystals with the same morphological aspect in *Opuntia imbricata* and *Opuntia englemannii.* They

Table II. Comparaison of weddellite and sediment B X-ray diffraction patterns.

Whewellite			Sediment A		
d_{hkl} [Å][a]	I/I_0 (%)[b]	≥ 10 %	d_{hkl} [Å][a]	I/I_0 (%)[b]	Attribution
8.73	4		8.70	6.7	weddellite
6.18	100	*	6.18	41.8	weddellite
			5.93	100	whewellite
			5.21	13	amorphous
			4.64	12	whewellite
4.42	30	*	4.42	21.1	weddellite
3.91	8		3.90	13.2	weddellite
			3.78	10.7	whewellite
			3.64	47.9	whewellite
			3.13	20.9	Si
3.09	10	*	3.09	10.6	weddellite
			2.96	55.9	whewellite
			2.90	7.9	whewellite
			2.84	8.4	whewellite
2.81	12	*	2.81	10.4	weddellite
2.77	65	*	2.77	30.1	weddellite
			2.49	10.7	whewellite
2.41	16	*	2.42	9.6	weddellite
			2.35	11.1	weddellite
			2.26	7.3	whewellite
2.24	18	*	2.24	12.8	weddellite
			2.21	7.5	whewellite
2.13	8		2.12	7.6	weddellite
			2.09	7.1	whewellite
			2.07	6.6	whewellite
			1.98	14.9	whewellite
1.96	12	*	1.96	9.2	weddellite
			1.94	6.8	whewellite
			1.92	13	Si
1.90	12	*	1.90	9.5	weddellite
1.84	10	*	1.84	7.4	weddellite

[a] Interatomic spacing (d-spacing); peaks of low intensity were skipped.

[b] relative intensity.

have also attributed the X-ray diffraction to the whewellite form of calcium oxalate. Monje and Baran (1997, 2002) have identified the same shape in *Opuntia microdasys* and six other *Opuntioideae* as whewellite by means of IR spectroscopy.

In sediment B we have found a mixture of at least 3 compounds. The presence of small amount of silicon has been discussed by Monje and Baran (2000) in an explicit study. Disregarding amorphous material, we have clearly established the coexistence of two phases of calcium oxalate: the monohydrate and the dihydrate.

Previous studies have failed to discern those crystallites (in sediment B) as a result of the experimental protocol used to isolate the more obvious starlike druses (our sediment A). Because of their size (< 2.5 μm), they pass through the sieves used, and must be centrifuged to isolate them from the thick suspension of the mucilage filtrate.

What is the exact function of each of those forms of calcium oxalate, and how this plant has stocked them simultaneously?

Many authors have observed the disappearance of CaC_2O_4 during natural processes. Franceschi (1989) suggested that specific mechanisms in plant cause oxalate formation and dissolution. Moreover, germin-induced oxalate oxidation, and the subsequent disappearance of CaC_2O_4 during germination, has proven that the calcium oxalate crystals are storage of structurally important nutriment (Lane, 1994). This reaction releases Ca^{2+} and H_2O_2, associated to signalling processes and to a cross-linking reaction within the cell wall (Poovaiah and Reddy, 1987; Olson and Varner, 1993). So far, one can understand why plants deposit calcium oxalate crystallites in their tissues.

The only obvious difference between weddellite and whewellite (for a plant physiological process) exists in their precipitation route. The weddellite does not readily crystallize from solutions containing calcium and oxalate ions. Thermodynamically unstable, with a higher dissolution rate and a lower zeta potential than the monohydrate, the dihydrate undergoes a phase transformation into the more stable calcium oxalate monohydrate (Lepage and Tawashi, 1982). Anionic surfactants induce crystallization of dihydrate at the expense of monohydrate (Füredi-Milhofer *et al.*, 1995). Interactions of those surfactants with the calcium oxalate seem to inhibit phase transformation from weddellite to whewellite (Sikirić *et al.*, 1999). Now, considering that *Opuntia ficus indica* mucilage has high viscosity and behaves as a polyelectrolite (Trachtenberg and Mayer, 1982), it seems to behave towards calcium oxalate dihydrate as anionic surfactant.

Considering the whewellite (monohydrate) as the definite storage form, the dihydrate could be either a pre-storage, or a post-storage and premobilization form in which the calcium oxalate could be readily dissolved and directed to its use area. *Opuntia ficus indica* tissues could have stabilized the weddellite form using the surfactant properties of the mucilage, to inhibit the thermodynamically favoured phase transformation.

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