Research Article

Raphides in the Uncalcified Siphonous Green Seaweed, *Codium minus* (Schmidt) P. C. Silva

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The vacuole of utricles, the outermost cell layer of the siphonous green seaweed, *Codium minus*, had numerous single needles and needle bundles. The crystals composing each needle appeared arranged in a twisted configuration, both ends were pointed, and each needle was contained in a matrix or membrane; bundles of needles appeared enclosed by a matrix. Chemical and electron diffraction analysis indicated that the needles consisted of calcium oxalate. This is the first paper on terrestrial plant-like raphides in an alga.

1. Introduction

Bundles of acicular crystals of calcium oxalate formed in specialized cells, idioblasts, are termed raphides in embryophytes [1–3]. Only one bundle of needles occurs per idioblast; the needles are long; needle length from several key pacific economic plants had a mean minimum length of 43 μm [4]. Each needle in a bundle is enclosed in a membrane, the crystal chamber [5–7], and bundles are enclosed by a water-soluble organic matrix, termed the vacuolar matrix [5, 8]. Raphides can burst through mature idioblasts due to swelling of the large amounts of mucilage contained in the cell [9]. Single needles do not occur in terrestrial plants but only as a bundle of needles [9, 10]. Individual needles, raphide, are found as microfossils in soils [4].

Abundant evidence supports the role of these needles in deterring vertebrate and invertebrate herbivory [1, 3, 7, 11, 12]. Raphides abrade the mouth and digestive tract of terrestrial herbivores causing edema; in addition, grooves in the needles may inject noxious plant metabolites and bacteria into these grazers [2].

Defense against herbivory in the marine environment generally involves calcification of the outer surface of green, red, and brown seaweeds [13]. This hard outer surface of calcium carbonate not only increases the difficulty of getting to the soft inner tissue, but consumption of the hard outer

matrix can also alter the digestive pH, a deterrent for several herbivores [1, 13]. No alga has been found to have raphides. Where needles (acicular crystals) have been found, they appear singly, not in bundles of needles; they are surrounded by a vacuolar membrane or crystal chamber but not both, are generally very small, or reside individually in the cytoplasm and not the cell vacuole [14–18]. Single needles also occur in the cell vacuole of both lightly calcified parts of otherwise heavily calcified seaweeds [19–21].

We provide the first report of calcium oxalate crystals with all the characteristics that typify raphides of terrestrial plants. Large numbers of single needles and raphides (bundles of them) were found in the cell vacuole of an uncalcified green seaweed. The crystals in each acicular needle appeared twisted; individual needles were enclosed in a matrix or membrane, while bundles of needles were surrounded by a matrix. Single needles and raphides were located within the central cell vacuole and against the peripheral cytoplasmic layer.

2. Materials and Methods

Codium minus was collected on 13 July, 2003 from the Izu Peninsula, Honshu, Japan, fixed in copious 4% neutral formalin in sea water for several days, loosely wrapped in paper toweling saturated with fixative, placed in Ziploc bags,

FIGURE 1: Calcium oxalate needles and bundles of needles in *Codium minus*: (A) *C. minus*; (B) utricle with needle bundles and individual needles (arrows). (C) SEM of individual needles. (D) Individual needles and a needle bundle, the latter contained within a matrix or membrane (arrow); (E) and (F) TEM of needle tips showing twisting of crystal (E, brackets) and an apparent membrane about individual needles (arrows); (G). Needle bundle staining intensely for calcium oxalate (Yasue [22]). Scale bars: (A) ruler in mm; (B) 100 μm ; (C) and (D) 20 μm ; (E) and (F) 0.5 μm ; (G) 50 μm .

and shipped to the USA. Digital light micrographs of whole utricles were taken with an Olympus BX60. For scanning electron microscopy (SEM), utricles were rinsed in distilled water, and their contents were isolated onto stubs, air dried, and then visualized uncoated with a Jeol 5600LV scanning electron microscope.

For elemental analysis, samples with a similar preparation as above were analyzed uncoated using a Link/Oxford ISIS 300 (EDS) system ancillary to a Philips/FEI XL30 ESEM-FEG scanning electron microscope (SEM) operated in the environmental mode (1 to 10 torr.). Needles were analyzed so as to minimize beam interaction with the stub (an aluminum alloy containing copper, Figure 2).

For transmission electron microscopy (TEM), utricles were first rinsed in distilled water, and their contents were squeezed onto formvar-coated grids and examined, unstained with a Philips 300 electron microscope at 60 kV. Electron diffraction ring patterns were also obtained with the Philips 300 at 80 kV and 0 tilt.

Solubilities of the needles were tested by exposing whole specimens to 1.0 N hydrochloric acid, 5.25% sodium hypochlorite (commercial bleach), or 67% aqueous acetic acid [16, 19, 22]. The Yasue [22] method for calcium oxalate

localization was done according to that described by Pueschel [16] using dithiooxamide (Sigma Chemical) which results in calcium oxalate inclusions staining densely black.

3. Results

The thallus of *Codium minus*, a pulvinate Codiaceae, had a mean diameter of 30 mm (± 8.1 , 21; SD, N; Figure 1(A)). Needles and raphides (needle bundles) were found in all terminal utricles in such numbers that they caused a glistening hue to the utricle under low magnification (Figure 1(B)). They were absent from the medullary filaments that compose the mass of the central portion of the thallus. Both ends of the needles were drawn out into a point. Sizes ranged from 31 to 72 μm long (55.1 ± 0.7 ; 12 mean, SD, N) by 0.5 to 1.3 μm (0.7 ± 0.3 ; 12 mean, SD, N) wide (Figures 1(B), 1(C), and 1(D)). All needles in needle bundles, raphides, were oriented in the same direction, forming a large compound needle (Figures 1(D) and 1(G)). Optical section found that individual needles and bundles were located throughout the large central vacuole of the utricle as well as against the cytoplasm lining the cell wall. Light microscopy of raphides found a membrane or matrix surrounding the

FIGURE 2: Energy dispersive X-ray spectrum (EDS) of a needle (a) and stub (b). Emission intensity (counts) is plotted versus energy (keV) characteristic for various elements (Al: aluminum; C: carbon; Ca: calcium; Cu: copper; O: oxygen; S: sulfur).

entire structure (Figure 1(D)). TEM showed a membrane-like matrix surrounding the tips of individual needles (Figure 1(F)); this membrane became obscure in the needle body. Crystalline globular material that composed the needle appeared to be arranged in a helical pattern (Figure 1(E), brackets). Under the SEM, however, the needles appeared smooth and uniform, and no globular substructure was apparent (Figure 1(C)).

Chemical tests found that needles failed to dissolve after 2 hrs in cold or warm (70°C) acetic acid (67%) or Clorox (5.25%). Hydrochloric acid (1 N) dissolved the needles in 5 to 10 min but without release of any gas. The Yasue [16, 22] method for localization of calcium oxalate found both single and compound needles outside of the utricles stained densely (Figure 1(G)), at times with a precipitate about them; needles within utricles did not stain.

EDS-SEM of the needles found strong signals for calcium, while signals for copper and aluminum appeared to originate from the stub (Figure 2). Electron diffraction patterns for the needles appeared to more closely match, those for calcium oxalate than those for calcium carbonate (Table 1).

4. Discussion

This is the first paper on embryophyte-type raphides (bundles of acicular needles) in an alga. Raphides apparently dissociated within the vacuole of the utricle forming quantities of single needles. Single needles and needle bundles were found in the vacuole of utricles of *Codium minus* in such numbers that these cells glisten under low magnification. EDS-SEM found strong signals for calcium, while electron diffraction analysis suggested that the needles consisted of calcium oxalate. This was further supported by various chemical tests. Needles were found only in the utricles (the

TABLE 1: D-spacing from electron diffraction of needles from *Codium minus* and for calcium oxalate [19] and standards (JCPDS, 1986) for calcium carbonate as calcite (24–27°C; 5–586).

Needles	Oxalate	CaCO ₃	CaCO ₃
2.767	2.75	2.83	2.845
2.434	2.4	2.495	2.495
1.695	1.69	1.6259	1.626

outer most, green layer of *Codium* species) and were not found in the internal portion of the plant composed of colorless intertwined medullary filaments. But in this group of seaweeds, there are no cross-walls; thus, utricles and medullary filaments share a common cytoplasm [23].

Raphides in embryophytes are formed in idioblasts, specialized cells that form crystals of various size, morphology and composition [3]. Idioblasts that form raphides are located in all portions of terrestrial plants and have an enlarged nucleus, a dense cytoplasm, and a small to absent vacuole [4, 7]. A small vacuole appears and is the site for crystal nucleation; the vacuole membrane and cell itself markedly expand during growth of the crystal [6]. The vacuole membrane, vacuolar matrix, appears granular at maturity under the scanning electron microscope and is apparently water soluble [5]. Idioblasts that give rise to raphides produce a single bundle of multiple, parallel-aligned, needle-like crystals [5, 11]. In some cases, needles within a bundle can be disorganized [3]. Each needle of a bundle, raphide, is contained within an additional membrane, the crystal chamber, which apparently dictates both the shape and precipitation of the crystal itself [6]. Raphides at maturity are often contained in very large cells, the raphides consuming most of the idioblast, while mucilage frequently fills the remaining available space [9, 10]. Swelling of the mucilage causes the cell to burst ejecting the raphides

to the outside of the plant [9]. A single needle, raphide, furthermore, refers to needles outside the plant or microfossils found in soils [3, 4].

The definition of raphides as described above for embryophytes, therefore, requires the presence of several traits: needles developing in a specialized cell (idioblast) with an enlarged nucleus and dense cytoplasm; needles occurring in bundles; large needle size, one bundle per cell; a crystal chamber about each needle; a vacuolar matrix about a bundle of needles. As mentioned above, no paper on raphides in marine or fresh water algae meets these requirements including those found in *C. minus*, the current paper. But the needles in *C. minus* do meet the following traits for raphides: they have a mean length greater than the minimum mean length of raphides in many pacific economic plants [4]; they are composed of calcium oxalate; the needles occur in bundles, each bundle surrounded by a matrix (vacuolar matrix?); a crystal chamber apparently surrounds each needle. But raphides in embryophytes occur singly per cell, while each utricle has several needle bundles. But the whole thallus of *Codium minus* is a single cell as cross-walls are absent. The traits of *C. minus*, that do not meet the definition of terrestrial raphides are, therefore, several bundles per cell, the cell also being multinucleate. Is there a connection between production of needle bundles in the multinucleate, siphonous alga, *C. minus*, and evolution of raphides in land plants?

Dawes [24] and Leliaert and Coppejans [25] found abundant calcium oxalate needles (35–55 μm in length) in the vacuole of all cells in the endemic Australian coenocytic green alga, *Apjohnia laetevirens* Harvey. Calcium oxalate crystals have been recorded for other noncalcified algae. Pueschel [16] found small, cruciate crystals in the peripheral cytoplasm but not within the vacuole of the fresh water green alga, *Spirogyra. Antithamnion*, a marine red alga, had single needles up to 30 μm in length in the peripheral cytoplasm [15, 18]. The length of the needles in *Antithamnion*, though shorter than those in *C. minus*, suggested that they may adversely affect herbivores [18], but the bipyramidal morphology of crystals (apparently surrounded by an organic matrix = the vacuolar membrane?) in *Chaetomorpha*, a marine green seaweed, probably negates their role in grazer defense [26]. Other papers [14, 15, 17] also describe needles, in some cases more than 100 μm long. All of these papers describe, however, only single needles, not bundles of them and, in addition, may describe the presence of a crystal chamber or vacuolar matrix but not both.

The acicular shape of raphides appears to be a critical component of plant defense against herbivory [7, 11]. These acicular crystals may also bear barbs or grooves [27]. Arnott and Webb [11] found that raphides in grape (*Vitis*) are twinned (indicated by one end being forked, the opposite pointed), the twinning occurs along the long axis of the raphide, and the crystal lattice rotates around the twin axis. This rotation of the crystal lattice structure is thought to enhance crystal stability and growth [11]. The calcium oxalate needles described for *C. minus* share similarities to raphides described for grape. (1) Needles in *C. minus* and those in grape have a similar range in length, but those in

C. minus were approximately half as wide. Raphides in grape, as mentioned above, are twinned crystals, and the width of one twin is approximately, that of the nontwinned needle in *C. minus* (both ends of which were pointed). (2) The crystals composing the needle appear to be twisted (twisting in *C. minus*, however, appeared to occur at a greater frequency than that in grape). (3) Each needle in *C. minus*, as mentioned above, is enclosed in a crystal chamber, a bundle of needles in a vacuolar matrix.

The crystal chamber surrounding each needle in a bundle appears to share many similarities with the silicalemma of diatoms [23]. Both are single-membrane sacks, each apparently provides an internal surface for crystal nucleation and deposition, and both determine the mature crystal morphology [23]. The silicalemma deposits the highly characteristic, taxonomically important, silicon frustule of diatoms, while the crystal chamber appears to be involved with calcium deposition as either carbonate or oxalate in the shape of a needle or more elaborate shape.

Raphides within noncalcified green seaweeds is one possible mechanism for defense against herbivory. Another is the lack of the production of a chemical attractant for grazers. Prince and Leblanc [28] found that *Codium fragile* did not produce an attractant for *Strongylocentrotus droebachiensis*, the green sea urchin. Green sea urchins would graze on *C. fragile* only if they happen to come across it. On the other hand, the brown kelp, *Laminaria saccharina*, produces a strong attractant for green sea urchins resulting in urchin barrens where once kelp beds were dominant [28].

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