Production of Calcium Oxalate Crystals by Two Species of *Cyathus* in Culture and Infested Plant Debris

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Hyphae of *Cyathus striatus* and *C. olla* in culture and on infested plant debris were heavily encrusted with crystals. Scanning electron microscopy revealed that raphide- and styloid-shaped crystals were associated with the hyphae of *C. olla* in canola stubble and in culture. Bipyramidal crystals were also present in culture. Distinct raphide druses developed on *C. striatus* hyphae colonizing wood chips, but in culture most crystals were bipyramidal or other shapes. Energy-dispersive X-ray microanalyses, FT/IR spectroscopy, and ^{13}C NMR spectroscopy determined that these crystals were calcium oxalate. This is the first report of calcium oxalate crystal production by these fungi. This characteristic has implications towards decomposition of organic matter, biomineralization, nutrient cycling, and soil genesis.

Calcium is an essential plant nutrient and is accumulated in appreciable amounts in higher plants (Demarty *et al.*, 1984; Kirby and Pilbean, 1984). Fungi are potent biodegraders of plant debris and contribute significantly to the cycling of nutrients. They produce substantial quantities of organic acids, especially oxalic acid (Connolly and Jellison, 1995; Cromack *et al.*, 1977). Oxalic acid chelates with calcium and removes it from plant cell walls and membrane components (Anagonstakis, 1983). Accumulations of calcium oxalate crystals on the hyphae of basidiomycetes are commonly reported to be associated with decomposing wood and forest litter (Connolly and Jellison, 1995; Graustein *et al.*, 1977). Calcium oxalate formation by fungi has recently received much attention due to its importance in pathogenesis in plants, decomposition of plant organic matter, biomineralization, calcium cycling, and general role in the soil environment (Horner *et al.*, 1995; Dutton *et al.*, 1993; Connolly and Jellison, 1995; Bateman and Beer, 1965; Godoy *et al.*, 1977; Wang and Tewari, 1990).

Cyathus spp., commonly referred to as the bird's nest fungi, are frequently found growing on old wood and dead stems of plants (Brodie, 1975). The present study was undertaken to examine and identify the crystals produced by two bird's nest fungi, *C. olla* (Batch) ex. Pers. and *C. striatus* (Huds.) ex Pers. A preliminary report on a part of this work has been published (Tewari and Briggs, 1995).

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Materials and Methods

Cultures of C. striatus and C. olla

Cyathus olla was collected (September 1995) on the stubble of canola (*Brassica* spp.) at the Edmonton Research Station, University of Alberta, Edmonton, Alberta. *Cyathus striatus* was collected (August 1995) on landscaping wood chips at the University of Alberta, Edmonton, Alberta. Cultures from surface sterilized peridiola were grown on V8 juice Rose Bengal agar (V8 juice [Campbell Soup Co.] 200 ml, Rose Bengal 50 mg, Difco Bacto-Agar 20 g, $CaCO_3$ 3 g, distilled H_2O to 1 liter) and maintained at room temperature (approx. 22 °C) in the dark.

Scanning electron microscopy and energy dispersive X-ray microanalysis

Agar blocks from four-wk-old cultures of *C. olla* and *C. striatus*, pieces of canola residue colonized by *C. olla*, and wood chips colonized by *C. striatus* were vapor-fixed with 1% osmium tetroxide in water for 4 h, then air-dried overnight at room temperature. Samples were mounted onto scan-

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ning electron microscope (SEM) stubs and secured with Marivac colloidal carbon paint. The specimens were sputter-coated with approx. 1 nm layer of gold and examined in a Jeol JSM 6301 FXV SEM operated at 5 or 20 kV. Energy-dispersive X-ray microanalyses were conducted using a Link eXL energy dispersive X-ray system with a light element detector.

Crystal isolation

To collect crystals, 10 ml of distilled water were added to 20 four-wk-old cultures of *C. olla* and *C. striatus*. The mycelium was scraped from the agar surface and the water and hyphal fragments were collected in a 250 ml Erlenmeyer flask. The slurry was filtered through 5 sheets of cheesecloth and the filtrate spun at 1748 g for 10 min at 20 °C in a Sorvall GLC-1 (10 cm rotor) table-top centrifuge. The supernatant was discarded and 10 ml of distilled water were used to resuspend/wash the pellet. The samples were respun and this washing procedure was repeated three times. The pellet was dispersed in 20 ml of distilled water and frozen to await chemical analysis.

FT/IR and ^{13}C NMR spectroscopy

The suspension was lyophilized using a Labconco 4.5 Freeze Dryer. This yielded a white crystalline powder which was relatively insoluble in water, hexane, dichloromethane, ethyl acetate, and methanol. An FT/IR spectrum of this powder was obtained using a Nicolet Magna 750 FT/IR with a Nic-Plan IR microscope. The powder was dissolved in deuterium chloride (20% solution in D_2O obtained from Aldrich Chemicals) and a ^{13}C NMR spectrum was obtained using a Bruker AM-300 NMR spectrometer (300 Mhz). Calcium oxalate (Fisher Scientific) was used for comparison with the unknown sample.

Results

In both *C. olla* and *C. striatus*, the hyphae in culture and in natural substrata were present either singly or were organized into mycelial cord-like structures. In both species, many hyphae were heavily encrusted with crystals which rendered them almost unidentifiable. However, in both species most of the superficial aerial hyphae in culture were virtually free of crystal deposits. Many pieces of canola stubble colonized with *C. olla* were soft and macerated and the xylem elements showed irregularly-shaped holes and other forms of structural damage (Fig. 1). In contrast to this, the wood chips colonized by *C. striatus* did not reveal any macroscopic signs of maceration but did display obvious structural deterioration as hyphae were associated with weak areas and hyphal impressions which were present on the surface of xylem elements, indicative of enzymatic activity.

In *C. olla*, in culture and in canola stubble a compact layer of raphide crystals, approximately 6.0 μm in thickness, was present along the length of hyphae (Fig. 2). Styloid crystals were also associated with this fungus but were not as abundant as the raphide type. Both these crystal shapes are characteristic of the monohydrate form of calcium oxalate (Frey-Wyssling, 1981). When grown in culture, bipyramidal crystals (approx. 2.5 x 2.5 μm) typical of the polyhydrate form of calcium oxalate were immersed in agar and were associated with some hyphae. Most hyphae, however, were heavily encrusted with raphide and styloid crystals.

The crystal morphology was appreciably different in *C. striatus*. When grown in culture, the hyphae of this species were heavily encrusted with bipyramidal (approx. 2.0–6.0 x 2.0–6.0 μm), styloid (approx. 3.0 x 0.5 μm), and variably-shaped crystals (Figs. 3 and 4). On wood chips, the hyphae were encrusted with raphide druses (approx. 5.5–6.0 μm) which were evenly spaced along the length of individual hyphae (Figs 5 and 6).

The FT/IR spectrum of the crystal preparation revealed absorbance bands at 3600–3000 and 1620–1320 cm^{-1} which closely matched those obtained for a sample of calcium oxalate. The ^{13}C NMR spectrum presented a single carbon resonance at 160.9 ppm which was characteristic of the carbonyl carbon resonance for oxalate. On the basis of these spectral data the crystal preparation was identified as a salt of oxalic acid. X-ray microanalyses revealed a strong peak for the presence of calcium (Fig. 7; Table I). Therefore, the crystals were identified as calcium oxalate on the basis of these three parameters.

Discussion

This study indicated that *C. olla* and *C. striatus* are crystal-forming fungi and effectively sequester

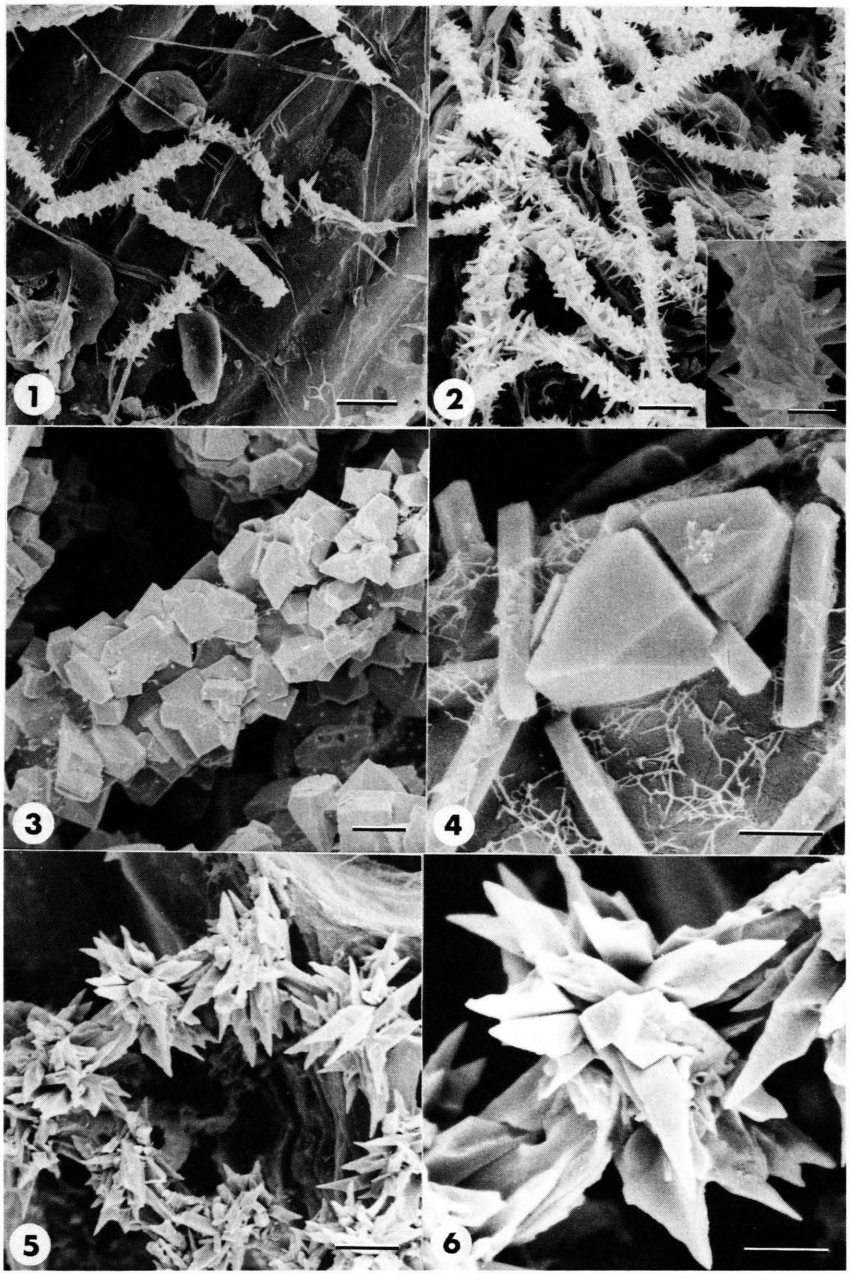

Fig. 1. SEM of the basal part of canola stem colonized by *Cyathus olla*. Note the compact layer of raphide crystals on the hyphae, and irregularly-shaped holes on the surface of xylem elements. Scale, 10 μm.
Fig. 2. SEM of *Cyathus olla* hyphae from culture encrusted with raphide, styloid, and bipyramidal crystals. Note the higher magnification of a raphide-encrusted hypha. Scale, 10 and 2 μm, respectively.
Fig. 3. SEM of *Cyathus striatus* hyphae from culture heavily encrusted with bipyramidal crystals. Note that crystal deposition has rendered the hyphae unidentifiable. Scale, 5 μm.
Fig. 4. Higher magnification SEM of bipyramidal and styloid crystals on the hyphae of *C. striatus* from culture. Scale, 1 μm.
Fig. 5. SEM of crystal-encrusted hyphae of *C. striatus* colonizing wood chips. Note the raphide druses evenly spaced along the length of the hyphae. Scale, 2 μm.
Fig. 6. Higher magnification SEM of a raphide druse from Fig. 5. Scale, 1 μm.

Table I. Percent composition of elements monitored in crystals from *Cyathus striatus* and *Cyathus olla*.

Element	Emission line monitored	Energy range of window [keV]	% Elemental composition			
			C. olla		C. striatus	
			mean[a]	range	mean[a]	range
Oxygen	K_α	0.403–0.623	48.459	43.213–54.307	37.921	27.877–51.341
Chlorine*	K_α	2.443–2.763	0.760	0.077– 1.141	0.528	0.446– 0.586
Potassium*	K_α	3.123–3.483	0.476	0.329– 0.552	0.365	0.266– 0.458
Calcium	K_α	3.503–3.863	50.068	44.023–54.899	61.063	47.704–71.054

[a] Means from three replicates.
* Traces of elements detected from the V8 juice based medium.

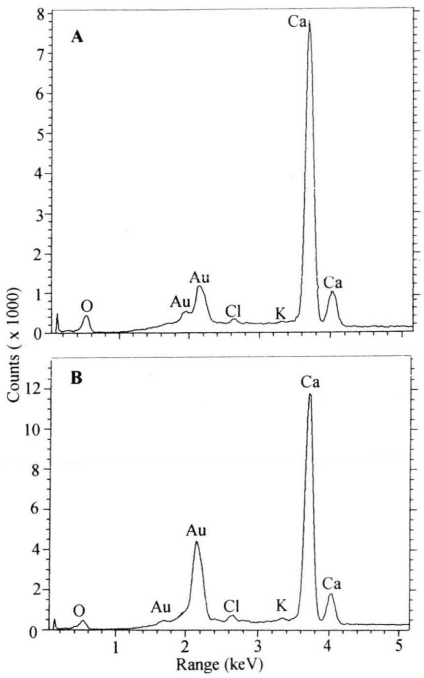

Fig. 7. X-ray spectra of crystals from **(a)** *C. striatus* and **(b)** *C. olla*. Note the oxygen and calcium peaks. Gold is present due to the specimen coating, and the chlorine and potassium are detected from the background V8 juice based medium.

calcium from their substrata, indicating an active role in decomposition. To our knowledge, this is the first report of calcium oxalate crystal production by these fungi. Crystal formation is an indicator of biodegradation of natural substrates. Oxalic acid secretion by fungi results in the sequestration of calcium from the cell walls and other components of the substratum (Rao and Tewari, 1987; Punja and Jenkins, 1984; Wang and Tewari, 1990; Bateman and Beer, 1965; Cromack *et al.*, 1977; Dutton *et al.*, 1993). Oxalate acts synergistically with fungal pectinases (Bateman and Beer, 1965). This results in a weakened wood structure and increased pore size which is conducive to further degradation by allowing penetration of lignocellulolytic enzymes secreted by the fungus (Dutton *et al.*, 1993).

The crystals were identified as being of either the monohydrate or polyhydrate form of calcium oxalate (Frey-Wyssling, 1981). Crystal morphology is dependent on environmental factors which influence crystal structure and stability (Frey-Wyssling, 1981). The crystal encrusted mycelial cord-like structures present on the surface of the substrates appear to be similar to rhizomorphs. They have been reported in *C. striatus* (Townsend, 1954), but to the best of our knowledge, not in *C. olla*. Histological examination and determination of the growing point is required before these structures can be classified as true rhizomorphs or mycelial cords.

Crystal formation is an important quality of *C. olla* and *C. striatus*. Calcium oxalate crystals are a reservoir of calcium for the ecosystem, and more importantly, oxalate in solution increases the effective solubility of iron and aluminum in soil. Oxalate is also a chelator of these two metals, and as such improves the availability of phosphorus for uptake by plant roots (Graustein *et al.*, 1977). Based on these properties, *C. olla* and *C. striatus* may make significant contributions to nutrient cycling and plant nutrition in addition to their active roles as decomposing basidiomycetes.

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