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PATTERNS OF CALCIUM OXALATE CRYSTAL PRODUCTION BY THREE SPECIES OF WOOD DECAY FUNGI

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Abstract

Wood decay experiments using red spruce wood resting on moist soil were conducted to discern temporal and spatial patterns of calcium oxalate (CaOx) crystal production by three species of fungi over the course of decay. All three species produced crystals of calcium oxalate dihydrate, but not monohydrate, in and on wood. Over the course of decay, the production of CaOx crystals was shown to be heterogeneous in both space and time. The relative quantity, morphology and longevity of CaOx crystals varied among species. *Gloeophyllum (G.) trabeum* produced substantial quantities of "free" crystals; *Fomitopsis (F.) pinicola* produced encrusting crystals; and *Trichaptum (T.) abietinum* produced adhering crystals and druses. Paramorphic corrosion of crystals was observed most frequently in the brown rot fungus *G. trabeum* and not at all in the white-rot fungus *T. abietinum*. Often associated with crystal precipitations was the production of crystal surface-obscuring extracellular matrix. All the species observed produced CaOx crystals more consistently in or on soil than in wood. The study of crystal production patterns and crystal morphologies could yield important information about the microenvironmental conditions in wood during biodegradation and the mechanisms by which wood decay fungi decompose lignocellulose.

Key Words: Calcium oxalate, wood decay, extracellular matrix, crystal studies, crystal corrosion, microenvironment.

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Introduction

Oxalic acid is an extremely common fungal metabolite which under conditions of supersaturation frequently precipitates as calcium oxalate (CaOx) (Foster, 1949; Simkiss and Wilbur, 1989). The precipitation of CaOx by fungi has been described in the Ascomycete, Deuteromycete, Zygomycete and Basidiomycete classes of the true fungi (Horner *et al.*, 1983; Punja and Jenkins, 1984; Whitney and Arnott, 1986a, 1986b, 1987; Whitney, 1989). Despite the ubiquity of oxalate production and the precipitation of oxalate salts among the fungi, the role of oxalate in the adaptive physiology of these organisms is not well understood.

The decomposition of wood is a process necessary to the continued functioning of forested ecosystems (Dighton and Boddy, 1989; Jennings, 1990; Boddy, 1991). This process reduces the amount of organic material on the forest floor and returns mineral nutrients within the wood to the rooting zone for reassimilation into trees for growth and maintenance (Cromack *et al.*, 1975). Wood decomposition is carried out by a suite of organisms, the most important of which are the Basidiomycete fungi (Boddy, 1991). Two general categories of Basidiomycete wood decay are frequently described: brown-rot and white-rot decay. Both types of decay fungi are known to produce CaOx crystals (Dutton *et al.*, 1993; Takao, 1965), but the pattern of crystal production and its relative mechanistic importance, if any, in the decay process are not known.

Arnott and Webb (1983) described the production of CaOx crystals by an unidentified Basidiomycete growing on ponderosa pine. The morphology of the crystals was predominantly druses of interpenetrant bipyramids. These druses occurred at intervals along the length of the hyphae and thus potentially indicated specific sites at which oxalate was being generated or from which oxalate was exiting hyphae. In addition, the crystals were covered by a sheath, which after the crystals obtained a certain size, was ruptured, uncovering the crystal druses. Reconstruction of crystal development was possible, but a temporal pattern of overall crystal production over the

course of decay was not possible as the observations were made upon field samples.

CaOx crystal production has been observed and described in other litter-degrading fungi (Cromack *et al.*, 1979; Arnott, 1982; Malajczuk and Cromack, 1982; Arnott and Fryar, 1984; Whitney and Arnott, 1987; Jones *et al.*, 1992). The precipitation of CaOx in these instances occurred not only at certain points but also along the entire length of the hyphae. A sheath encasing the crystals was again observed in the majority of litter-degrading fungi examined.

The impact that this type of precipitation might have on calcium dynamics in the Oi and Oe (recognizable plant litter and partially decomposed litter, respectively) soil horizons has been a topic of speculation (Cromack *et al.*, 1977; Graustein *et al.*, 1977; Cromack *et al.*, 1979; Arnott, 1982). In the systems described thus far, calcium oxalate dihydrate (COD) is the dominant phase of the oxalate salt produced by litter-degrading fungi (Arnott, 1982; Arnott and Webb, 1983; Arnott and Fryar, 1984; Whitney and Arnott, 1987). COD is a more soluble form of CaOx than calcium oxalate monohydrate (COM). This fact could be of importance in assessing the impact of this biomineralization upon calcium dynamics in forest litter.

Little is known of the dynamics of CaOx crystal precipitation over the course of decay in the natural habitat of the wood decay fungi. The fungi's natural habitat includes both the soil and wood. The present study was undertaken in order to focus research on the production pattern, morphology, and potential turnover of CaOx crystals in wood decay Basidiomycetes; the task was accomplished through the examination of temporal and spatial patterns of CaOx crystal production by one white-rot and two brown-rot fungi within microcosms containing wood in soil contact. In addition, this study sought to explore the investigative potential of using crystal studies to reveal general mechanisms of wood decay.

Materials and Methods

A qualitative and comparative examination of CaOx crystal production by three species of wood decay fungi growing on wood was undertaken. The fungi were observed using light microscopy (LM) and scanning electron microscopy (SEM) over an extended decay period in soil block cultures. One isolate of the brown-rot fungus *Gloeophyllum (G.) trabeum* (Pers.:Fr.) Murill, three field isolates of the brown-rot fungus *Fomitopsis (F.) pinicola* (Sw.:Fr.) P. Karst and one field isolate of the white-rot organism *Trichaptum (T.) abietinum* (Dickson:Fr.) Ryvarden were used in single species soil block assays using red spruce wood. The field isolates were obtained from decaying red spruce logs in the fall of 1993 and the summer of 1994. *Trichaptum abietinum* and *F. pinicola* field isolate B were obtained from Kossuth, ME; *F. pinicola* field isolate A was from Howland, ME; and *F. pinicola* field isolate C was obtained from Crawford Notch, NH.

Culture conditions

Each fungus was inoculated into 32 Mason jars prepared in a manner modified from soil block assay procedures as described below (ASTM, 1994). Eight jars at each of four time points were observed for CaOx production: time 1, 3-5 weeks; time 2, 6-9 weeks; time 3, 10-16 weeks; time 4, 16-20 weeks. The range of the harvest time points increased with time due to the relative decay rates of wood blocks decomposed by species of fungi which have disparate wood decaying potentials.

Modified soil block assays were prepared by placing 300 ml of soil mixture into each of the 500 ml wide-mouth Mason jars (Ball Brand, Muncie, IN) used for the experiment. The soil mixture consisted of a 1:1:1 (by dry volume) ratio of Hyponex® (Hyponex, Marysville, OH) potting soil, fine vermiculite and peat moss. The soil mixture was moistened with deionized water, then autoclaved twice over a period of four days and inoculated with the five isolates of fungi. Blocks of spruce sapwood (2.5 cm x 2.5 cm x 1.0 cm) were added aseptically to the jars after colony establishment. Soil block cultures were maintained in the dark at room temperature as single species cultures until the time of harvest and observation.

Observation of crystal production patterns

Samples of mycelium growing on wood blocks at each of the four time points were placed on glass microscope slides and observed by LM with a Leitz Labrolux S photomicroscope (Leica Mikroskopie, Wetzlar, Germany). A minimum of four samples of mycelium from each of the wood blocks was mounted and examined: at least one from the upward block face, two from the sides of the block and at least one from the bottom surface of the block. Periodically, mycelium was aseptically removed from the surface of the wood blocks, allowing the fungus to reproliferate on wood. Hyphae, which had regrown over the wood, were then examined by LM. This process permitted some examination of crystals of known maximum age, thereby allowing a comparison between accumulated crystal material and recently produced crystal material. The quantity of CaOx crystals observed by LM was subjectively categorized: no crystals observed (0), sparse crystals observed (+), numerable crystals observed (++), innumerable crystals observed (+++). In instances where crystals were corroded, phase contrast, polarizing microscopy and acid solubility were used to confirm the nature of the observed crystal debris. Crystals observed by LM or SEM

Table 1. A comparison of diffraction pattern interplanar d spacings obtained from the mycelium of three wood decay fungi with that of COD on file.

Standard on File (I)†	*Fomitopsis pinicola*	*Gloeophyllum trabeum*	*Trichaptum abietinum*
6.18 (100)	6.21 strong‡	6.19 strong	6.18 strong
4.42 (30)	4.51 weak	4.43 weak	4.41 moderate
3.09 (10)	3.09 weak	none	3.11 weak
2.77 (65)	2.77 strong	2.77 strong	2.77 strong
2.41 (16)	2.42 weak	2.42 strong	2.41 moderate
2.24 (25)	2.25 moderate	2.25 weak	2.24 moderate
1.89 (16)	1.89 weak	1.90 weak	1.89 weak

†Relative intensity of reflection line ‡Subjective relative intensity of reflection line

were categorized as "large" if they were greater than 10 μm in width or length. Crystals were categorized as "small" if the were smaller than 10 μm in length or width. Crystals were categorized as adhering if they were distinctly outlined and clinging to particular hyphae. Crystals were categorized as encrusting if aggregates of crystalline material formed a crust over individual hyphae. Crystals were categorized as "free" if they were not adhering or encrusting any particular hypha.

Concurrent with LM observations, wood blocks 0.5 cm^3 in size and weighing ca. 0.01 g each were removed from selected decaying blocks and prepared for SEM in one of three ways: air drying and mounting on stubs, fixation in 7% weight/volume (w/v) $HgCl_2$ in 70% ethanol with subsequent ethanol dehydration and critical point drying or direct quenching in liquid nitrogen followed by lyophilization for 10 hours at -70°C (Green *et al.*, 1990). Lyophilization was carried out in a FLEXI-DRY (FTS Systems, Stone Ridge, NY) lyophilizer and critical point drying was performed using a Samdri PVT-3 (Tousimis Research Corp., Rockville, MD) critical point dryer. Dried samples were mounted to pin stubs with either double-sided carbon sticky tape or with silver paste and coated with 30 nm of gold in a Conductavac sputter coater (Seevac, Inc., Pittsburgh, PA). Low resolution observations were made using an AMR-1000 SEM (AMRAY, Inc., Bedford, MA) operated at an accelerating voltage of 5 kV and a gun emission of 75-200 μA at a working distance of 2-10 mm. Higher resolution observations were made using a JEM 1200EM (JEOL USA, Peabody, MA) scanning transmission electron microscope operated at an accelerating voltage of 40 kV. Energy dispersive X-ray microanalysis was conducted using an Electroscan (Wilmington, MA) model E-3 environmental SEM (ESEM) equipped with a Noran Instruments (Middleton, WI) computer interface and operated at an accelerating voltage of 10 kV.

Table 2. Summary of CaOx crystal production by *Gloeophyllum trabeum* growing on red spruce wood.

Weeks →	3	8	13	17
Adhering†	+	0	0	0
Encrusting†	0	0	0	0
"Free"†	++	+++	+	+/0
Druses†	0	0	0	0
All Crystal Types†	++	+++	+	0
Crystal Corrosion	Yes	Yes	Yes	Yes
Soil Crystals	0	+	+	+
ECM‡	++	++	+	+
% wt. loss	13%	21%	39%	56%

†Subjective categorization of crystals as explained in **Materials and Methods**. ‡Extracellular matrix (ECM). Subjective categorization: 0: not observed; +: occasionally observed; ++: frequently observed; +++: thick and always observed.

Soil was examined for the presence of crystals by LM and SEM. Fungus colonized soil aggregates were either mounted directly to pin stubs or were prepared for SEM and ESEM by rolling soil aggregates over double sided carbon sticky tape and observing adhering crystalline material.

Identification of crystals

The identity of the presumptive CaOx crystals found

Table 3. Summary of CaOx crystal production by three isolates of *Fomitopsis pinicola* growing on red spruce wood.

Weeks →	5	8	14	20
Isolate A				
Adhering	+	+	0	0
Encrusting	++	+++	+++	+++
"Free"	+	0	0	0
Druses	0	0	0	0
All Crystal Types	+++	+++	+++	+++
Corrosion	No	No	No	No
ECM	+	+	+	+
Soil Crystals	++	+++	+++	+++
% wt. loss	37%	45%	59%	66%
Weeks →	**4**	**8**	**13**	**20**
Isolate B				
Adhering	+	+	0	0
Encrusting	++	++	+	0
"Free"	+++	+	0	0
Druses	0	0	0	0
All Crystal Types	+++	++	+	0
ECM	+++	++	+	+
Soil Crystals	++	+++	+++	+++
% wt. loss	24%	40%	59%	65%
Weeks →	**3**	**8**	**13**	**20**
Isolate C				
Adhering	+	+	0	0
Encrusting	++	+++	+++	+++
"Free"	+	0	0	0
Druses	0	0	0	0
All Crystal Types	+++	+++	+++	+++
Corrosion	No	No	No	No*
ECM	+	+	+	+
Soil Crystals	++	++	++	+++
% wt. loss	19%	39%	56%	61%

*However, degradation was observed at 35 weeks

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in association with fungal growth on wood was confirmed using crystal morphology, polarizing microscopy, acid solubility and X-ray powder diffraction. To date, there are no reported biomineral precipitations other than CaOx produced by polypore wood decay fungi on wood, and thus, crystal morphology, polarizing microscopy and relative acid solubility were used to distinguish the monohydrate and dihydrate phases of the presumed CaOx. COD crystals are often morphologically distinct from the monohydrate, have a much lower birefringence and are completely soluble in ca. 0.05% HCl after 12 hours, while essentially insoluble in acetic acid. COM is not completely soluble in 0.05% HCl after 12 hours. Both phases of CaOx fail to effervesce in any concentration of HCl. The chemical and phase identity of these crystals was confirmed using X-ray powder diffraction on mycelium containing presumptive CaOx crystals using a Supper® Debye-Scherrer (Supper, Natick, MA) camera fitted to a Philips diffractometer (Philips Electronic Instruments, Mahwah, NJ). Thirty-five millimeter film was exposed to Cu radiation at 15 mA and 35 kV for 24 hours, and the pattern was compared to patterns available on the Powder Diffraction File (Berry, 1974).

Table 4. Summary of CaOx crystal production by *Trichaptum abietinum* growing on red spruce wood.

Weeks →	3	12	20	28
Adhering	+	+	0	0
Encrusting	+	0	0	0
"Free"	0	0	0	0
Druses	+	++	+	+
All Crystal Types	+	++	+	+
Corrosion	No	No	No	No
Soil Crystals	0	0	0	0
ECM	+	+	+	+
% wt. loss	2%	13%	20%	26%

X-ray microanalysis was performed on crystals observed in the soil in order to determine the presence of the most dominant cation or cations in these crystals. A colorimetric oxalate oxidase based enzymatic assay (Sigma, St. Louis, MO) was used to confirm the relative abundance of total acid soluble oxalate in colonized soil above that of control soil.

Results

Overview

The crystals produced in wood by all three species of fungi were COD. X-ray powder diffraction pattern on the mycelium from all three species were similar to standard patterns (Table 1). The crystals were bipyramidal, had a low birefringence and were completely soluble in 0.05% HCl. All these characters are consistent with that of COD. COM does not form bipyramids, has a very high birefringence and is not completely soluble in 0.05% HCl after 8 hours.

In *G. trabeum* and isolate B of *F. pinicola*, LM monitoring of crystal production revealed an overall reduction in the prevalence of CaOx crystals associated with the mycelium growing on wood over time (Tables 2 and 3). In *T. abietinum* and two isolates of *F. pinicola*, there was a greater consistency of crystal production throughout the course of decay (Tables 3 and 4).

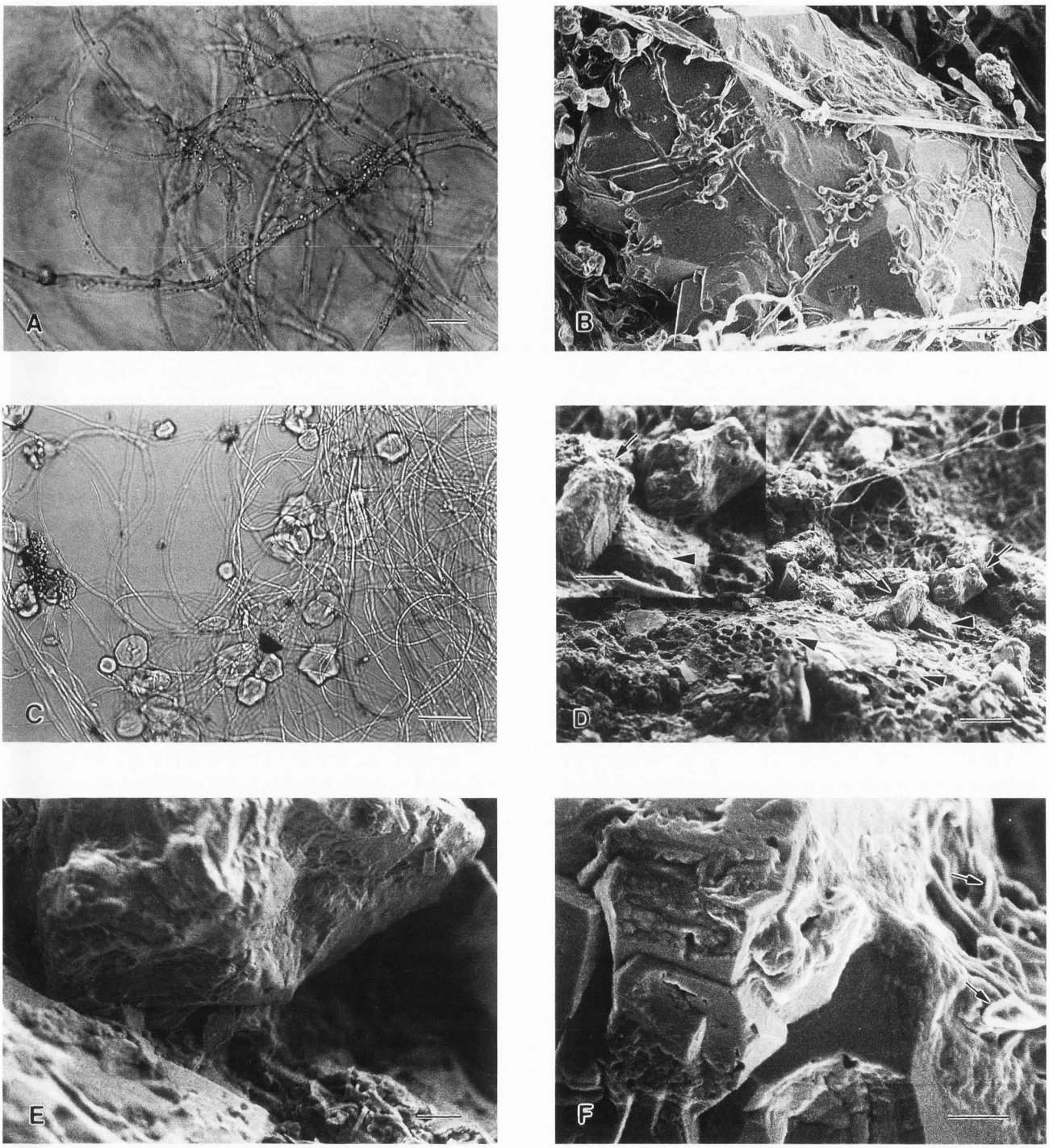

Figure 1. CaOx crystal production by *Gloeophyllum trabeum*. **(A)** Sparse crystals (granularities) of CaOx precipitated on the surface of hyphae at 3 weeks of decay. Bar = 10 μm. **(B)** Large (defined as > 10 μm), uncorroded "free" crystal with numerous collapsed hyphae evident on the surface. Little or no extracellular matrix (ECM) is visible. Bar = 10 μm. **(C)** Large, corroded "free" crystals present among the hyphae of *G. trabeum* in the early and middle stages of decay of red spruce blocks. Bar = 50 μm. **(D)** Scanning electron micrograph of large, corroded "free" crystals (arrows) during the early and middle stages of decay of red spruce blocks. Note the extensive ECM (arrowheads) and crystal corrosion (inset). Bar = 50 μm. Inset bar = 20 μm. **(E)** A more magnified image of corroded crystal. Bar = 5 μm. **(F)** The surface of a corroded crystal. Note hyphae growing on its surface (arrows). Bar = 2 μm.

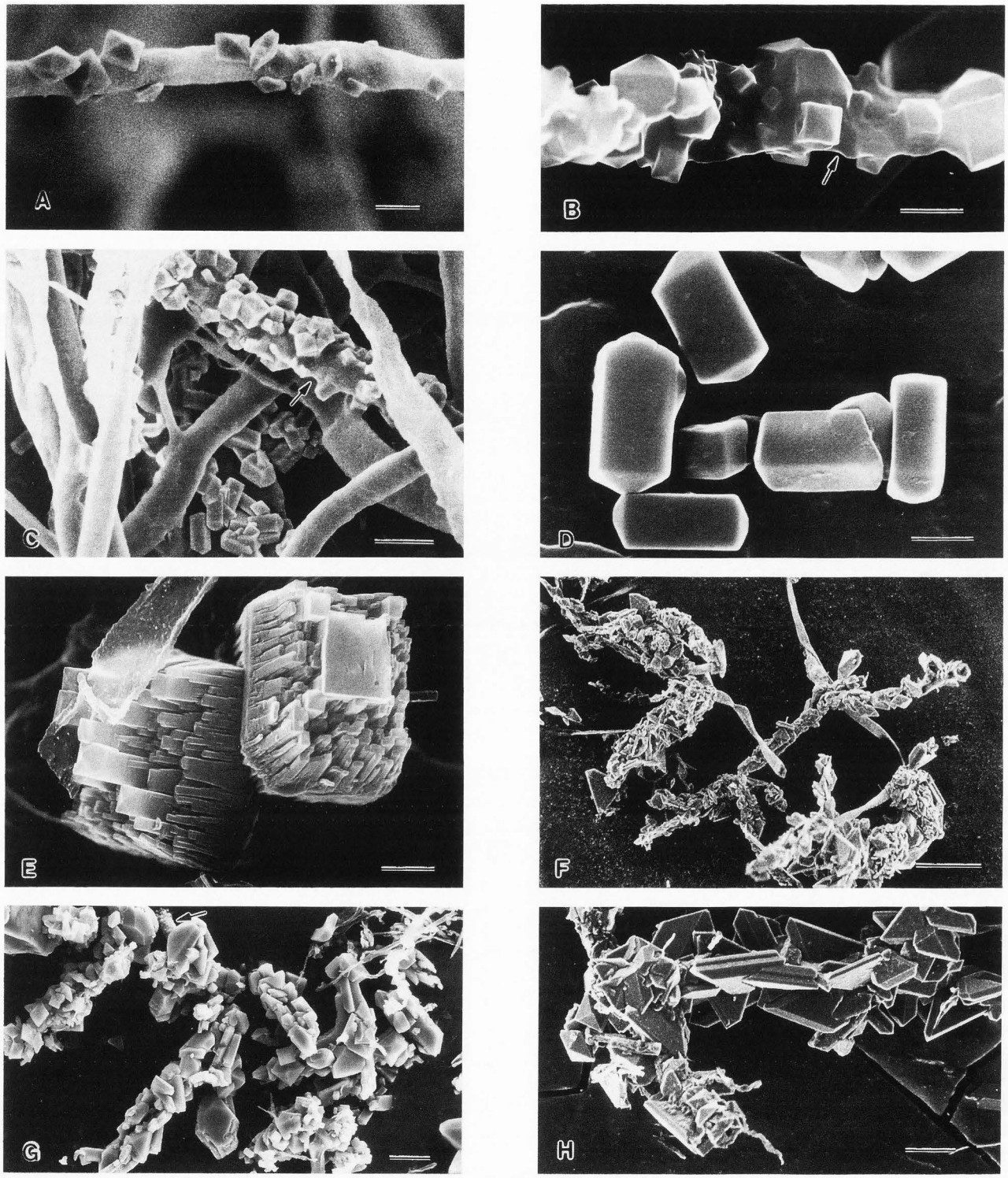

Figure 2. CaOx crystals produced by *Fomitopsis pinicola*. **(A)** Adhering crystals. Bar = 1 μm. **(B)** Encrusting crystals precipitated on hyphae. Note ECM (arrow). Bar = 1 μm. **(C)** Encrusting crystals precipitated on hyphae. Note ECM (arrow) and lack of crystals on other hyphae. Bar = 2 μm. **(D)** "Free" prismatic crystals precipitated on the wood cell wall. Bar = 0.5 μm. **(E)** Degraded crystals in very late stages of decay (35 weeks). Bar = 1 μm. **(F)** Hyphae isolated from soil which are mostly encrusted. Bar = 10 μm. **(G)** Heavily encrusted hyphae isolated from soil. Note hypha core encrusted with smaller crystals (arrow). Bar = 10 μm. **(H)** Branched, encrusted hypha showing small inner layer crystals and larger outer layer crystals. Bar = 5 μm.

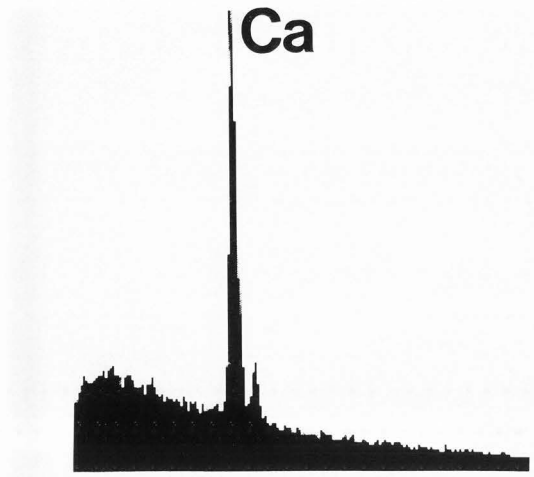

Figure 3. Representative energy dispersive X-ray microanalytical spectrum of encrusted hypha of *Fomitopsis pinicola* isolated from soil.

The continuous production or accumulation observed in isolates A and C of *F. pinicola* occurred in spite of decay rates (% weight loss) similar to isolate B and *G. trabeum*. These observations support the fact that some fungi accumulate oxalate while others do not, but does not support the popular notion that brown-rot fungi accumulate oxalate while white-rot fungi do not. In addition to differential crystal production over the course of decay, the three species of fungi precipitated these crystals differently within their microenvironment. *Gloeophyllum trabeum* precipitated mostly "free" crystals; *F. pinicola* produced mostly encrusting crystals; and *T. abietinum* precipitated predominantly adhering crystals and druses (Tables 2-4). All of the fungi produced CaOx crystals more consistently in space and time in or on the soil matrix than in or on the wood.

Gloeophyllum trabeum

In *G. trabeum*, early stages of decay, i.e., within the first 3 weeks, revealed the production of sparse amounts of crystals adhering to the surfaces of the hyphae (Fig. 1A). Often these crystals were acicular or prismatic. In addition, there were numerable, large, non-adhering bipyramidal crystals ("free" crystals) among intertwining hyphae (Fig. 1B). By 8 weeks of decay, no crystals were observed adhering to hyphae, but more and larger total "free" crystal material was present than at three weeks (Figs. 1C and 1D). Many of these "free" crystals exhibited paramorphic corrosion (Figs. 1C, 1D, 1E and 1F). Associated with the mycelium and crystalline material at these early time points was extensive sheath material that obscured surface features of both crystals and hyphae (Figs. 1D and 1E). Hereafter, this sheath will be referred to as extracellular matrix (ECM). Subjective determinations revealed that at subsequent time points of 13 and 17 weeks of decay, there was a reduction in the amount of total crystalline material present (Table 2). The few "free" crystals that were observed were frequently highly corroded.

Additional SEM observations of *G. trabeum* confirmed LM observations, particularly the lack of abundant crystals in the later stages of decay. By 12 weeks of decay, *G. trabeum* had begun to form sporocarps and functional hymenial layers. Observations of the hymenium revealed diminished oxalate production compared to earlier stages of decay in the wood blocks.

The soil upon which the red spruce blocks were resting was visibly colonized by *G. trabeum*. Light microscopic observations of soil rich in hyphae revealed the presence of sparse, uncorroded or scantly corroded bipyramid crystals. No encrusting crystals were observed at any time points or at any level of soil colonization.

There was significant spatial variability of CaOx production among replicates. It was not uncommon for the eight blocks of wood at one time point in this one specie to vary significantly from one another, e.g., at 13 weeks, four blocks showed significant levels of CaOx, three showed none at all, and one had modest CaOx production. In addition, within blocks, there was unpredictable spatial heterogeneity. Some portions of the block had extremely high numbers of crystals, whereas other areas of the block had no crystals at all. Such spatial heterogeneity occurred at millimeter distances from each other, e.g., 1.5-5.0 mm. When some surface mycelium was removed and permitted to regrow on the blocks, adhering crystals were often observed 7-10 days later, although no crystals of any kind could be observed in older mycelium of the same blocks.

Fomitopsis pinicola

Fomitopsis pinicola consistently produces oxalic acid in most artificial culture media (Connolly and Jellison, 1994b). In this study, *F. pinicola* produced a substantial quantity of CaOx crystals while growing on blocks of red spruce wood. Within the first three weeks of wood colonization, adhering and encrusting crystals were observed (Figs. 2A, 2B and 2C). Most frequently, the adhering crystals which were observed were small bipyramids that had precipitated on the surface of hyphae in the absence of obvious ECM (Fig. 2A). Encrusting crystals were observed more frequently than adhering crystals by LM and SEM, often filling the fields of view by LM. LM and SEM revealed that only certain sections of hyphae became encrusted while other sections of hyphae remained free of crystalline material (Fig. 2C).

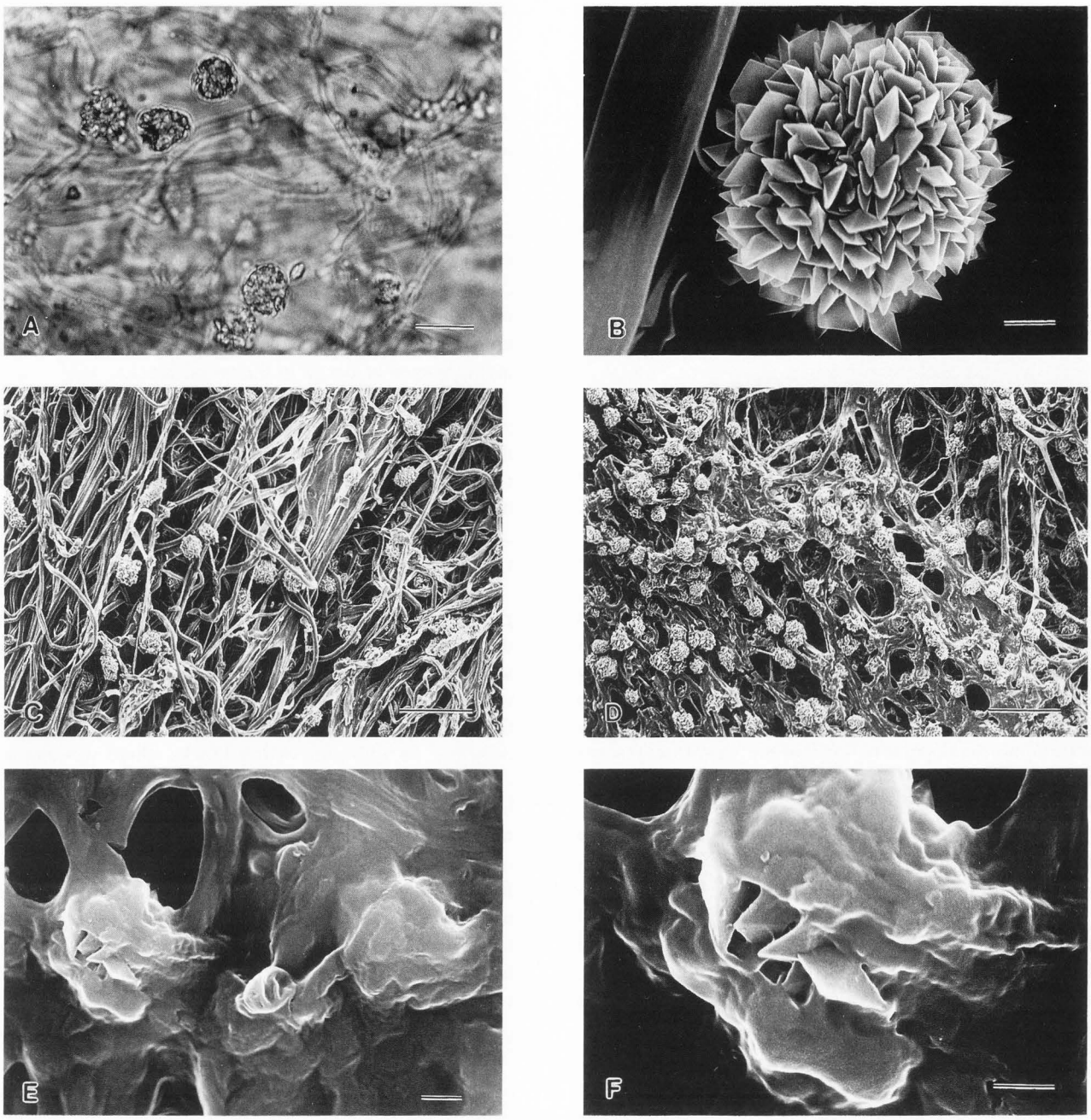

Figure 4. CaOx crystals produced by *Trichaptum abietinum*. **(A)** Typical druses of COD crystals which grow at irregular intervals along hyphae as viewed by LM. Bar = 10 μm. **(B)** A well-developed druse of COD crystals. Bar = 1 μm. **(C)** Mycelium growing on red spruce wood. Bar = 20 μm. **(D)** Mycelium in contact with soil containing more abundant druses. Extensive ECM is also visible. Bar = 20 μm. **(E)** A pair of druses partially or wholly obscured by ECM. Bar = 2 μm. **(F)** Magnified image of a partially obscured druse. Bar = 1 μm.

Frequently, albeit unpredictably, ECM was observed in association with encrusting crystals (Figs. 2B and 2C). Encrusting crystals of *F. pinicola* were most ordinarily bipyramidal or equant rods with pyramidal caps, were most often not twinned, showed no signs of paramorphic corrosion, and were smaller than 5 μm in all dimensions. Uncorroded "free" crystals were sometimes observed by LM and SEM, often in association with ECM. When "free" crystals precipitated on wood walls, they tended to be smaller and more prismatic than "free" crystals among mycelium (Fig. 2D). "Free" crystals which precipitated on wood walls were generally not

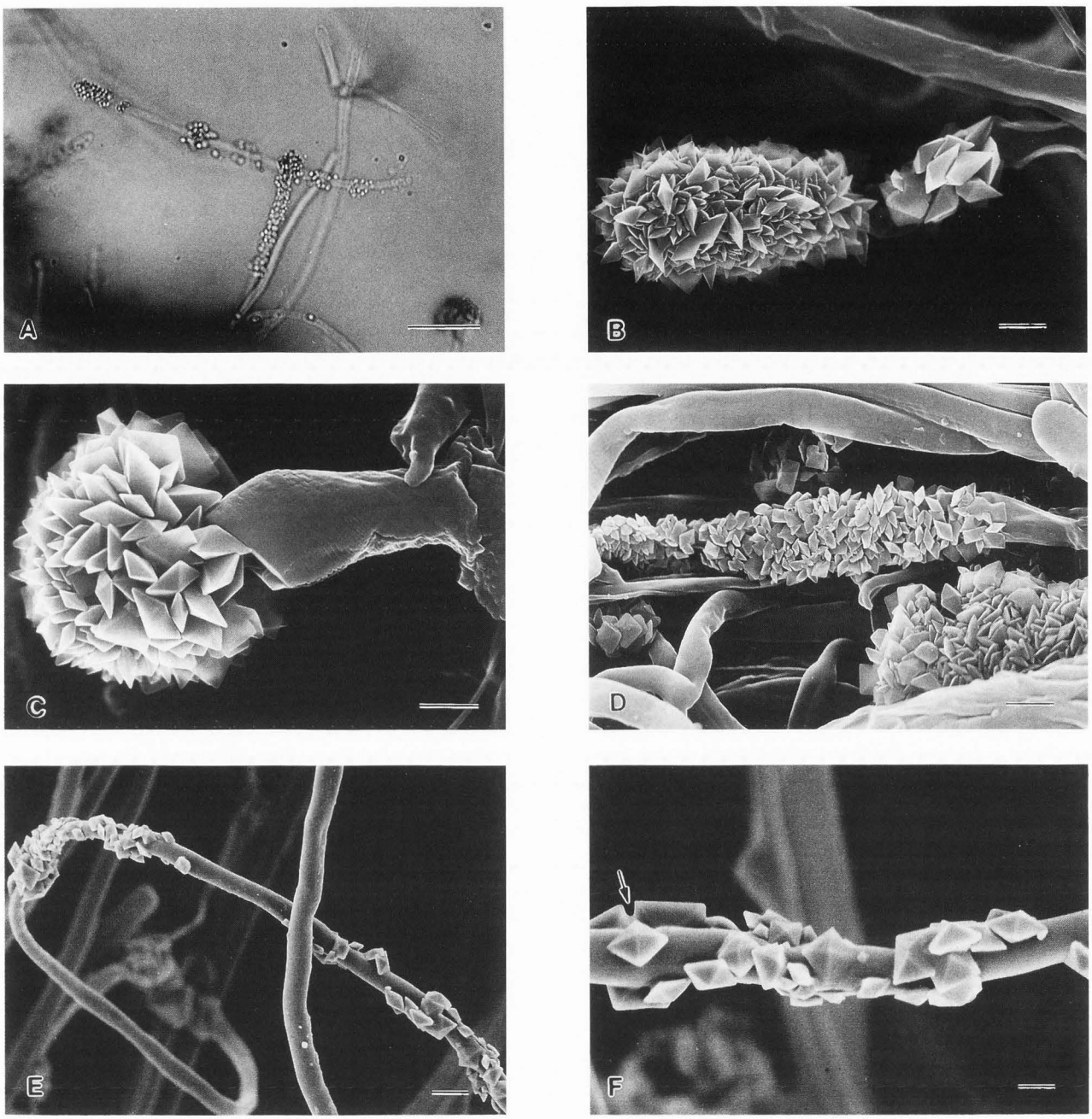

Figure 5. CaOx crystals produced by *Trichaptum abietinum*. **(A)** LM of encrusting crystals growing on the ends of hyphae. These hyphal ends have become encrusted to form terminal druses. Bar = 20 μm. **(B)** An elongate terminal druse. Bar = 2 μm. **(C)** A more spherical terminal druse. Bar = 1 μm. **(D)** Encrusting crystals with an intermediate terminal druse-like appearance. Bar = 2 μm. **(E)** Adhering and encrusting crystals on a hypha. Bar = 2 μm. **(F)** Adhering crystals with ECM (arrow). Bar = 1 μm.

associated with ECM. In very late stages of decay, e.g., 35 weeks of decay, some crystal degradation was observed (Fig. 2E). Degradation along presumptive planes of crystal weakness resulted in oppositely oriented styloids with pyramidal caps which converged at the midpoint of the c-axis of the crystal.

Although *F. pinicola* appeared to be a prolific producer of crystals, not all isolates showed the same pattern of production (Table 3). All three isolates produced a similar quantity of total crystalline material in the early stages of decay, but isolate B produced more "free" crystals and far more ECM than isolates A and C.

Isolate B produced very few or no crystals at all in later stages of decay, whereas isolates A and C continued to produce crystals steadily during the decay experiment. *Fomitopsis pinicola* colonized soil more thoroughly than did *G. trabeum*. LM observation revealed that soil colonized by *F. pinicola* contained a substantial amount of "free" crystals and encrusting crystals. "Free" crystals and encrusted hyphae were not observed in visually uncolonized soil and uninoculated control soil. SEM observation showed that although hyphae became heavily encrusted, not all sections of the same hypha were (Fig. 2F). This is similar to what was observed in the wood environment. Encrusting crystals on hyphae in wood tended to be rather uniform in size and morphology, but those found on hyphae in the soil matrix showed a much greater range in size and form (Figs. 2F, 2G and 2H). Crystals which precipitated on hyphae in the soil often formed very thick crusts around hyphae, sometimes in two discernible layers: one consisting of very small intercalating crystals and an outer layer of crystals which were much larger and more morphologically diverse. Crystals in the soil were conspicuously and consistently euhedral, uncorroded and not associated with ECM.

Many of the crystals observed from the soil are presumed to be COD based upon morphology, the presence of high levels of acid soluble oxalate in the soil as determined by colorimetric assay, and from energy dispersive X-ray microanalysis which showed strong calcium peaks but no peaks for other cations such as magnesium (Fig. 3). However, the diversity of crystal forms within the inoculated soil makes it likely that oxalate crystals other than COD also precipitated. Uncolonized soil did not possess any crystals and had barely detectable levels of oxalate by colorimetric assay.

Isolates A and B of *F. pinicola* rapidly proliferated through the soil matrix in the soil block assay, whereas isolate C grew more slowly into the soil. Among the three isolates of *F. pinicola*, differences in the abundance of the crystals observed in soil by LM is likely related more to isolate differences in soil colonization than to relative oxalate production within the soil.

Trichaptum abietinum

The white-rot fungus *T. abietinum* produced less crystalline oxalate than the brown rot fungi *G. trabeum* or *F. pinicola* in the early stages of decay but continued to produce and accumulate oxalate throughout decay (Table 4). *Fomitopsis pinicola* and *G. trabeum* produced "free" crystals to varying degrees, but *T. abietinum* produced only crystals in contact with hyphae: adhering, encrusting and druses.

The dominant crystal precipitation in *T. abietinum* consisted of druses of crystals which had precipitated at random intervals at specific points along hyphae (Figs. 4A and 4B). These druses were observed throughout the course of decay but were more abundant in mycelial cords which were in contact with the soil than those associated with the top of the spruce wood block (Figs. 4C and 4D). The majority of the crystal druses produced by *T. abietinum* ranged in size from 3-7 μm and consisted of long bipyramidal crystallites which did not show frequent obvious twinning (Fig. 4A). Spacing of the druses along hyphae was more compact at the higher druse frequencies present in mycelial cords and in mycelium near the soil. Although ECM was evident among hyphae (Fig. 4D), ECM did not frequently cover druses although sometimes it did (Figs. 4E and 4F).

Trichaptum abietinum also produced a significant number of cystidial-like structures, hereafter referred to as terminal druses (Arnott, 1995) which consisted of hyphal tips which became encrusted with bipyramidal CaOx crystals (Figs. 5A, 5B and 5C). The shape of the terminal druses varied from more elongate structures (Figs. 5A and 5B) to spherical rosettes (Fig. 5C). Occasionally, some mid-sections of hyphae were encrusted by crystals in a manner similar to elongated terminal druses (Fig. 5D). Sometimes, *T. abietinum* produced adhering and encrusting crystals (Figs. 5E and 5F). Few or no "free" crystals were observed in *T. abietinum*. As was observed in *F. pinicola*, both adhering and encrusting crystals were precipitated along certain sections of hyphae and not along other portions of the same hypha (Figs. 5D, 5E and 5F). Both adhering and encrusting crystals were bipyramidal; no prismatic crystals were observed. Our observations indicate that the majority of adhering crystals precipitate on the surface of hyphae in the presence of a thin ECM. Adhering and encrusting crystals were observed more frequently in *T. abietinum* than in *G. trabeum* but much less frequently that in *F. pinicola*. Unlike the corrosion of "free" crystals which was observed in *G. trabeum*, there was no paramorphic corrosion of the crystals in druses or along the hyphae in mycelium of *T. abietinum*.

Although there was mycelial contact with the soil, *T. abietinum* did not proliferate through the soil to the degree that *G. trabeum* and *F. pinicola* did. The majority of *T. abietinum* growth was limited to the wood and no crystals were observed in the soil (Table 4).

Discussion

Many recent studies have focused on the role of oxalate in the process of wood decay and in the metabolic strategy of wood decay fungi (Bech-Anderson, 1987; Espejo and Agosin, 1991; Ma *et al.*, 1992; Dutton *et al.*, 1994; Evans *et al.*, 1994; Itakura, *et al.*, 1994; Kishi *et al.*, 1994). It has long been suspected that oxalate may be involved in the cyclic reduction of iron which could

continue to generate hydroxyl radicals which are thought to be involved in the wood degradative process (Schmidt *et al.*, 1981; Hyde and Wood, 1995). Green *et al.* (1991, 1992, 1993) and Shimada *et al.* (1992) have suggested that oxalic acid in sufficient quantity could be a causative agent or facilitator in the physico-chemical hydrolysis of cellulose microfibrils. Ma *et al.* (1992) have hypothesized a role for oxalate in the regulation of lignin degradation in white-rot fungi by the noncompetitive inhibition of lignin peroxidase. The production of oxalic acid may also function primarily as an efficient and convenient carbon waste disposal mechanism in the carbon-rich and nitrogen-poor wood substrate (Shimada *et al.*, 1992). It is very likely that oxalate performs multiple adaptive functions simultaneously (Micales, 1995).

The pH of wood and various defined media used in physiological experiments is often significantly lowered by the activity of brown-rot decay organisms (Green *et al.*, 1992, 1994; Chen and Jellison, 1994). In defined media, these reductions in pH are concomitant with increases in oxalate concentration (Dutton *et al.*, 1993; Green *et al.*, 1994) implying a role for oxalate in reducing the pH of the substrate to optimal levels for attack upon the wood. Other recent work continues to refine our understanding of the dynamics of oxalate production and accumulation by these fungi in artificial defined media (Dutton *et al.*, 1993, 1994; Itakura *et al.*, 1994).

Acid neutralization and calcium interference avoidance mechanisms have been proposed as adaptive functions for the precipitation of oxalate as CaOx crystals (Bech-Anderson, 1987; Whitney and Arnott, 1987; Whitney, 1989). The production of CaOx may also function as a means of mineralizing exchangeable calcium which could interfere with the fungal controlled reduction of ambient microenvironmental pH (Connolly and Jellison, 1994a).

The abundance of crystals during early colonization of wood, such as occurred in *G. trabeum* and *F. pinicola*, indicates that these brown-rot fungi produce significant quantities of oxalic acid in the initial stages of decay, much of which is precipitated as CaOx at a distance from the hyphae as "free" crystals. This is consistent with the research of others working with brown-rot physiology in defined media (Itakura *et al.*, 1994).

The production of "free" crystals instead of adhering and encrusting crystals may be indicative of the environmental conditions into which the oxalate is delivered. Oxalic acid excretion into a more acidic milieu will push oxalic acid towards the monovalent anion and the diprotic acid. In such circumstances, CaOx precipitation would not be favored. More alkaline conditions would push oxalate to the oxalate divalent anion and would favor precipitation with calcium. It is therefore possible that adherent and encrusting crystals are indicators that the immediate hyphal environment is sufficiently alkaline to push oxalic acid to the divalent anion and precipitate crystals immediately upon excretion. If the immediate microenvironmental pH was more acidic, oxalate could diffuse further from the hyphae, within the ECM, and precipitate at a distance. This would result in the production of "free" crystals. It is also possible that "free" crystals result instead of adhering crystals when critical nucleation factors are absent on the surfaces of the hyphae. Such nucleation factors might be present in the hyphal ECM and could determine where in the mycelium precipitation will occur.

An alternative explanation for the precipitation of "free" crystals relative to adhering and encrusting crystals could be calcium concentration or calcium flux around the immediate area of the hypha. High levels of calcium or high calcium flux in the vicinity of the hypha would result in adhering or encrusting precipitation. Diminished calcium in the vicinity of the hypha would result in the diffusion of oxalate away from the hypha with precipitation occurring as "free" crystals. Also, the precipitation of "free" crystals may be an indication of available free moisture in which the oxalate can diffuse.

If a preponderance of large "free" crystals, rather than encrusting crystals, is an indicator of microenvironmental pH, then *G. trabeum* produced the most acidic microenvironment of the fungi in this study. *Gloeophyllum trabeum* produced no encrusting crystals and produced abundant and large "free" crystals in the early stages of decay. *Fomitopsis pinicola* produced substantial quantities of encrusting crystals as well as occasional "free" crystals and therefore might have had the next most acidic microenvironment. *Trichaptum abietinum* produced few or no "free" crystals and may therefore have produced the least acidic microenvironment.

Evidence for an acidic milieu, at a distance from the hyphae in *G. trabeum*, in later stages of decay and wood colonization is perhaps supported by the substantial and consistent crystal corrosion observed in this fungus. It is possible that after "free" crystals have initially precipitated in higher pH microenvironments, they become corroded by the controlled maintenance of more acidic conditions later in decay. These acidic conditions would protonate oxalate sufficiently to prevent its reprecipitation. In this case, there would be an accumulation of oxalic acid and monovalent oxalate in the mycelium. The much later appearance or absence of crystal corrosion in the other fungi could be an indication that these organisms do not cultivate acidic conditions to the same degree as *G. trabeum*.

An alternative explanation for the corrosion of crystals by *G. trabeum* may have less to do with low pH conditions than with the slow desorption of ions from crystal surfaces that can occur even at higher pH levels,

albeit more slowly than at lower pH levels. Ions liberated from crystal surfaces could reprecipitate elsewhere in the mycelium to form new euhedral crystals if all thermodynamic and kinetic conditions were to allow it. The corrosion and disappearance of crystals by *G. trabeum* suggests that at higher pH levels, any desorbed oxalate would be degraded upon its release. Oxalate degradation by *G. trabeum* would be consistent with the results of Espejo and Agosin (1991) who observed rapid oxalate oxidation in semisolid cultures inoculated with this species. The possible oxalate degradation in this soil block study could be oxalate decarboxylase catalyzed (Micales, 1995) or iron facilitated (Espejo and Agosin, 1991). If the latter mechanism were operative, a biologically derived compound would be required to supplant the important role of light in the iron-mediated oxidation of oxalate.

It is also possible that the corrosion and disappearance of crystals is indicative of a more acidic mycelial environment as well as oxalate degradation. In this case, there would not be accumulation of oxalate in the mycelium despite a continued low pH.

The absence of crystal corrosion in *T. abietinum*, a white-rot organism which might presumably have a high oxalate decarboxylase activity, may be the result of an insufficiently low microenvironmental pH to facilitate crystal dissolution.

Many of the crystals which were found encrusting hyphae precipitated along certain portions of a hypha and not along the entire length. This suggests that either oxalate is being exported at these sites at particular times in the ontogeny or physiological status of the hypha, or that the microenvironmental conditions of that section became favorable for CaOx precipitation, e.g., nucleation factors, elevated pH and available calcium.

Punctated crystal precipitation has been observed in various fungi and environments (Graustein *et al.*, 1977; Arnott and Webb, 1983; Whitney and Arnott, 1986b; Connolly and Jellison, 1995). This precipitation at intervals along hyphae raises interesting questions about how these druse formations are controlled by these fungi. Export of oxalate from the cells at designated loci is one possible explanation.

The tips of hyphae are the actively growing portion of the fungus thallus and are often thought to be sites of extracellular enzymatic delivery and nutrient absorption. It is therefore interesting that in *T. abietinum* hyphal tips can differentiate to form encrusted terminal druse structures. The implicit regulation of crystal production, which yields encrusted tips and uniformly organized druses, suggests that CaOx production is more than a convenient disposal of metabolic waste in this fungus.

The occurrence of occasional encrusted sections on the hyphae of *T. abietinum* is also a potentially interesting example of oxalate biomineralization. These encrustations could form one of two ways. It is possible that a hypha could begin to develop an elongated terminal druse, but then "switches back" to active hyphal tip elongation. This would leave an encrusted section as the hypha elongated beyond the crystals. Alternatively, these encrustations could develop as they do in *F. pinicola*: by the accumulation of adhering crystals to the point of encrustation. Either scenario requires differential regulation of genes or gene products.

It is an interesting observation that all of the examined fungi which produce CaOx crystals do so more consistently and apparently at higher levels in the soil than on wood. Although the spatial and temporal heterogeneity of crystal occurrence in soil is less than in wood, the representative crystal morphologies are more diverse. Bipyramids and rods of COD are the dominant and consistent morphologies observed within the wood environment; but in this study, a range of crystal types was observed within the soil. A far greater number of interpenetrant twins, needle-like and plate-like crystals was observed in soil. The consistency of crystal shape within the wood may be evidence for the capacity of the fungi to maintain relatively stable conditions within the wood, whereas the more complex and heterogeneous matrix of the soil may surpass the environmental control abilities of the fungi. High levels of exchangeable calcium could stimulate CaOx production in fungi (Connolly and Jellison, 1994a), and it could be the greater calcium exchange capacity of the soil matrix that stimulates oxalate production.

Conclusions

Examination of the temporal and spatial occurrence of CaOx crystals produced by the three species of fungi in this study revealed several general patterns: (1) the production of CaOx crystals within a species is heterogeneous in both space and time; (2) the relative quantity, morphology and longevity of CaOx crystals varies among isolates and species; (3) all oxalate crystal producing species investigated showed the tendency to produce larger, morphologically more diverse and a greater number of crystals in soil than within wood; and (4) the observation that encrusting crystals are limited to certain segments of hyphae suggests that oxalic acid production may be related to the developmental or physiological stage of hyphae, and the environmental circumstances.

In the future, more rigorous crystal studies could focus on what crystal morphology might reveal about the microenvironment that these fungi create. Although it will be challenging to interpret future observations, the crystals produced by wood decay fungi potentially represent a physical record of the microenvironment in which

they formed. Interpreting microenvironmental conditions from the patterns of crystal production and morphology of crystallites has potential promise in contributing to an understanding of the physico-chemical environment in which wood decay occurs. Whether CaOx crystal production by these fungi is the result of decay or is participating in effectuating wood decay, understanding the patterns of crystal production could be important in elucidating mechanisms of lignocellulose decomposition.

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References

ASTM (1994) Standard method of accelerated laboratory test of natural decay resistance of woods. (D 1413-76). In: 1994 Annual Book of ASTM Standards. Sect. 4. Vol. 04.10. American Society for Testing and Materials, Philadelphia, PA. pp. 218-224.

Arnott HJ (1982) Calcium oxalate (weddellite) crystals in forest litter. Scanning Electron Microsc **1982**; III: 1141-1149.

Arnott HJ (1995) Calcium oxalate in fungi. In: Calcium Oxalate in Biological Systems. Khan SR (ed.). CRC Press, Boca Raton, FL. pp. 73-111.

Arnott HJ, Fryar A (1984) Raphide-like fungal crystals from Arlington Texas compost. Scanning Electron Microsc **1984**; IV: 1745-1750.

Arnott HJ, Webb MA (1983) The structure and formation of calcium oxalate crystal deposits on the hyphae of a wood rot fungus. Scanning Electron Microsc **1983**; IV: 1747-1758.

Bech-Anderson J (1987) The dry rot fungus, growth and treatment. International Group on Wood Preservation Document no. IRG/WP/2389. IRG Secretariat, Box 5607, S-114 86 Stockholm, Sweden.

Berry L (ed.) (1974) Selected Powder Diffraction Data for Minerals. 1st ed. The Joint Committee on Powder Diffraction Standards, Swarthmore, PA. p. 534.

Boddy L (1991) Importance of wood decay fungi in forest ecosystems. In: Handbook of Applied Mycology. Arora DK, Rai B, Mukerji KG, Knudsen GR (eds.). Marcel Dekker, New York. pp. 507-540.

Chen Y, Jellison J (1994) Regulation of hyphal sheath formation and iron-chelator production by the brown rot fungi *Gloeophyllum trabeum* and *Postia placenta*. International Group on Wood Preservation Document no. IRG/WP/94-10074. IRG Secretariat, Stockholm.

Connolly JH, Jellison J (1994a) Oxalate production and calcium oxalate accumulation by *Gloeophyllum trabeum* in buffered cultures. International Group on Wood Preservation Document no. IRG/WP/94-10075. IRG Secretariat, Stockholm.

Connolly JH, Jellison J (1994b) Calcium oxalate crystal production by three brown rot fungi. Phytopath **84**: 542 (abstract).

Connolly JH, Jellison J (1995) Calcium translocation, calcium oxalate accumulation, and hyphal sheath morphology in the white-rot fungus *Resincium bicolor*. Can J Bot **73**: 927-936.

Cromack K, Todd RL, Monk CD (1975) Patterns of basidiomycete nutrient accumulation in conifer and deciduous forest litter. Soil Biol Biochem **7**: 265-268.

Cromack K, Sollins P, Todd RL, Fogel R, Todd AW, Fender WM, Crossley ME, Crossley DA (1977) The role of oxalic acid and bicarbonate in calcium cycling by fungi and bacteria: Some possible implications for soil animals. Ecol Bull **25**: 246-252.

Cromack K, Sollins P, Graustein W, Speidel K, Todd AW, Spycher G, Li CY, Todd RL (1979) Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum*. Soil Biol Biochem **11**: 463-468.

Dighton J, Boddy L (1989) Role of fungi in nitrogen, phosphorus, and sulphur cycling in temperate forest ecosystems. In: Nitrogen Phosphorus and Sulfur Utilization by Fungi. Boddy L, Marchant R, Read DJ (eds.). Cambridge University Press, Cambridge, U.K. pp. 269-297.

Dutton MV, Evans CS, Atkey PT, Wood DA (1993) Oxalate production by Basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol **39**: 5-10.

Dutton MV, Kathiara M, Gallagher IM, Evans CS (1994) Purification and characterization of oxalate decarboxylase from *Coriolus versicolor*. FEMS Microbiol Lett **116**: 321-326.

Espejo E, Agosin E (1991) Production and degradation of oxalic acid by brown rot fungi. Appl Environ Microbiol **57**: 1980-1986.

Evans CS, Dutton MV, Guillén F, Veness RG (1994) Enzymes and small molecular mass agents

involved with lignocellulose degradation. FEMS Microbiol Rev **13**: 235-240.

Foster JW (1949) Chemical Activities of Fungi. Academic Press, New York. pp. 326-350.

Graustein WC, Cromack K, Sollins P (1977) Calcium oxalate: Occurrence in soils and effect on nutrient and geochemical cycles. Science **198**: 1252-1254.

Green F, Larsen M, Highley T (1990) Ultrastructural morphology of the hyphal sheath of wood decay fungi modified by preparation for scanning electron microscopy. Biodeterioration Research **3**: 311-325.

Green F, Larsen MJ, Winandy JE, Highley T (1991) Role of oxalic acid in incipient brown rot decay. Mater Org **26**: 191-213.

Green F, Larsen MJ, Hackney JM, Clausen CA, Highley TL (1992) Acid mediated depolymerization of cellulose during incipient brown-rot decay by *Postia placenta*. In: Fifth International Conference on Biotechnology in the Pulp and Paper Industry. May 27-30, 1992. Kuwahara M, Shimada M (eds.). Uni Publishers, Tokyo, Japan. pp. 267-272.

Green F, Hackney JM, Clausen CA, Larsen MJ, Highley TL (1993) The role of oxalic acid in short fiber formation by the brown-rot fungus *Postia placenta*. International Group on Wood Preservation Document no. IRG/WP/93-10028. IRG Secretariat, Stockholm.

Green F, Larsen MJ, Highley TL (1994) Hemicellulosic induction of oxalic acid in *Postia placenta*. International Group on Wood Preservation Document no. IRG/WP/94-10060. IRG Secretariat, Stockholm.

Horner HT, Tiffany LH, Cody AM (1983) Formation of calcium oxalate crystals associated with apothecia of the discomycete *Dasyscypha capitata*. Mycologia **75**: 423-435.

Hyde SM, Wood PM (1995) A model for attack at a distance from hyphae based on studies with the brown rot *Coniophora puteana*. International Group on Wood Preservation Document no. IRG/WP/95-10060. IRG Secretariat, Stockholm.

Itakura S, Hirano T, Tanaka H, Enoki A (1994) Relationship between degradation of wood, cellulose or lignin-related compounds and production of hydroxyl radicals or accumulation of oxalic acid in cultures of brown-rot fungi. International Group on Wood Preservation Document no. IRG/WP/94-10062. IRG Secretariat, Stockholm.

Jennings DH (1990) The ability of basidiomycete mycelium to move nutrients through the soil ecosystem. In: Nutrient Cycling in Terrestrial Ecosystems. Harrison AF, Ineson P, Neal OW (eds.). Elsevier, Amsterdam, Netherlands. pp. 233-245.

Jones D, McHardy WJ, Wilson MJ, Vaughan D (1992) Scanning electron microscopy of calcium oxalate on mantle hyphae of hybrid larch roots from a farm forestry experimental site. Micron Microsc Acta **23**: 315-317.

Kishi K, Hiroyuki W, Marquez L, Dunford HB, Gold MH (1994) Mechanism of manganese peroxidase compound II reduction: Effect of organic acid chelators and pH. Biochemistry **33**: 8694-8701.

Ma DB, Hattori T, Akamatsu Y, Adachi M, Shimada M (1992) A novel equation for the noncompetitive inhibition of the Li-P catalyzed reactions by oxalic acid. In: Fifth International Conference on Biotechnology in the Pulp and Paper Industry, May 27-30, 1992. Kuwahara M, Shimada M (eds.). Uni Publishers, Tokyo, Japan. pp. 321-326.

Malajczuk N, Cromack K (1982) Accumulation of calcium oxalate in the mantle of ectomycorrhizal roots of *Pinus marginata* and *Eucalyptus marginata*. New Phytol **92**: 527-531.

Micales JA (1995) Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. Mater Org **29**: 177-186.

Punja ZK, Jenkins SF (1984) Light and scanning electron microscopic observations of calcium oxalate crystals produced during growth of *Sclerotium rolfsii* in culture and in infected tissue. Can J Bot **62**: 2028-2032.

Schmidt CJ, Whitten BK, Nicholas DD (1981) A proposed role for oxalic acid in non-enzymatic wood decay in brown-rot fungi. Proc Am Wood Pres Assoc **77**: 157-164.

Shimada M, Akamatsu Y, Ma DB, Takahashi M (1992) New biochemical aspects of oxalic acid production and decomposition by wood destroying fungi. In: Fifth International Conference on Biotechnology in the Pulp and Paper Industry, May 27-30, 1992. Kuwahara M, Shimada M (eds.). Uni Publishers, Tokyo, Japan. pp. 273-278.

Simkiss K, Wilbur KM (1989) Biomineralization: Cell Biology and Mineral Deposition. Academic Press, San Diego. pp. 111-113.

Takao S (1965) Organic acid production by basidiomycetes. I. Screening of acid-producing strains. Appl Microbiol **13**: 732-737.

Whitney KD (1989) Systems of biomineralization in the fungi. In: Origin Evolution and Modern Aspects of Biomineralization in Plants and Animals. Crick RE (ed.). Plenum Press, New York. pp. 433-441.

Whitney KD, Arnott HJ (1986a) Morphology and development of calcium oxalate deposits in *Gilbertella persicaria* (Mucorales). Mycologia **78**: 42-51.

Whitney KD, Arnott HJ (1986b) Calcium oxalate crystals and basidiocarp dehiscence in *Geastrum saccatum* (Gasteromycetes). Mycologia **78**: 649-656.

Whitney KD, Arnott HJ (1987) Calcium oxalate crystal morphology and development in *Agaricus bisporus*. Mycologia **79**: 180-187.

Discussion with Reviewers

H.T. Horner: Based on your study, do you believe the ability of soil borne fungi to produce oxalate and calcium oxalate is of major environmental importance?
Authors: It is difficult to conclude from this study alone that oxalate production in soil borne fungi is of major environmental importance. The culture conditions of these experiments approximate the material habitat of wood decay fungi but eliminate the other biotic factors such as mites, non-basidiomycete fungi, plants and bacteria. As such, it is only a model. What is clear from our results is that soil can be very effective in stimulating CaOx precipitation. Although it has not been examined sufficiently, we know that oxalate is found in soils and that it precipitates as the calcium salt frequently (Graustein *et al.*, 1977; Fox and Comerford, 1990). This oxalate could be important in plant-host and pathogen interactions in agricultural soils, in root-nutrient dynamics in the rooting zone of forests and in the microbial ecology of all soils. There is evidence that much of the oxalate in soils and litter horizons is derived from fungi (Cromack *et al.*, 1975, 1979), and there is strong theoretical support for the idea that the unique physicochemical properties of oxalate, along with the K_{sp} of CaOx phases, might contribute to the heterogeneity of soil nutrient availability.

H.T. Horner: It is interesting that some of the fungi produce crystals at specific intervals along their hyphae. How would you go about determining this positioning with respect to hyphal structure? Is it important?
Authors: It cannot be emphasized enough how the very pervasiveness of CaOx precipitation is a testimony to its importance to these fungi (Arnott, 1995). The punctated nature of druse distribution along the hyphae of many fungi has a more implicit importance to these fungi. The specific points at which the druses grow are very likely to be either channels or pumps from which oxalate is exported from the cell. These may also be sites of calcium export. This would mean that this oxalate biomineralization is a highly regulated cell function; such functions are rarely neutral adaptive features and would surely be lost if they reduced the organisms' fitness. Cytolocalization of these pumps would be both an interesting and important undertaking, and could be achieved by a combination of pulse-chase transmission electron microscopy experiments and patch clamping.

H.T. Horner: You indicate that CaOx is more widespread than expected. What do you perceive is the evolutionary significance of oxalate formation within the fungi? What about other organisms?
Authors: In spite of the ubiquity of oxalate occurrence among the fungi, we know little about the adaptive significance of oxalate production, accumulation and particularly, biomineralization. The common nature of oxalate production suggests its synthesis is either a primitive physiological process or one that evolved independently several times. The authors cannot speculate on its adaptive significance to animals. In vascular plants, it is likely that it functions most often as a mechanism to sequester accumulated calcium from the transpiration stream after all exchange sites in the cell wall have been exhausted. Among the fungi, its adaptive significance is probably very diverse, with oxalate often performing more than one important function simultaneously in an organism. The potential role that oxalic acid and its precipitation plays in controlling microenvironmental pH is probably the most universally important among the fungi. As already discussed, among the wood decay fungi, oxalate is likely to have a direct or indirect role in effecting wood decay. We think it is less likely that oxalate increases the fitness of wood decay fungi by precipitating out calcium that might interfere with intracellular processes or by affording these organisms with an efficient carbon waste disposal system.

M.V. Dutton: Would enzymes degrade a crystal? Do they not require soluble oxalate?
Authors: It is likely that oxalate decarboxylase requires solvated oxalate ions or oxalic acid as the substrate. Dihydrate crystals could begin to dissolve in lower pH conditions, yielding corrosion features. The dissolved oxalate could then be degraded enzymatically. At higher pH levels, crystals could still lose oxalate ions off the surface of the crystals by desorption. The most significant difference between these two would be the rate at which they would occur and the degree of corrosion that one could observe. Although Espejo and Agosin (1991) did not observe CaOx clearing in *G. trabeum*, they did not investigate the ability of this fungus to degrade CaOx crystals that the fungus itself can produce. Our laboratory has observed accumulation of CaOx crystals by *G. trabeum* during colonization of half strength malt agar. The accumulation of crystals was followed by the rapid and complete disappearance of such crystals *in situ* in the agar as the colony approached maturity. Hundreds of prismatic crystals per cubic centimeter of agar completely disappeared within 5 days in these cultures.

V. Franceschi: What is the concentration of calcium in the soil and soil solution of these cultures?
M.V. Dutton: Is the greater number of crystals and greater consistency of their production in the soil due to the fact that there is more calcium in the soil than in the wood?
Authors: There was ca. 200 μmol/g exchangeable cal-

cium present in the culture soils and ca. 20 μmol/g calcium present in the wood. The soil solution phase calcium was 1.5 mmol/g. The fact that there is more calcium in the soil than in the wood used for these experiments is probably not the most important factor responsible for our results. It is very likely that the matrix effects and soil reaction are even more important than the calcium levels. The results of these soil block experiments have not been duplicated in our laboratory using any liquid, semisolid or agar medium containing levels of calcium even three times greater than that found in the soil used for the soil block experiments. More important than the calcium content or concentration is the amount of exchangeable calcium with sites that can adsorb hydrogen ions. The exchange of calcium for hydrogen at soil exchange sites in various soil matrices would render these soils resistant to the acidification of microenvironments by oxalate where it was produced by hyphae. Fungi might therefore respond to the hydrogen ion sinks and coincident buffering of the soil by producing larger amounts of oxalate (Connolly and Jellison, 1994a). It is likely this complex, matrix-dependent buffering that gives rise to the increase in CaOx and more variable crystal morphology in the soils. This exchange capacity buffering may also explain the greatly diminished or absent crystal corrosion among soil crystals.

M.V. Dutton: The diverse range of crystals in soil suggests that not all the crystals are CaOx.
Authors: The vast majority of crystal shapes observed in the soil are consistent with various reports of CaOx morphology. To our knowledge, this is the first report of so many crystal shapes being produced simultaneously by one organism. The precipitation of other oxalate salts is also very possible, but the precipitation of carbonates or phosphates, for example, is very unlikely.

M.V. Dutton: Can crystals embedded in ECM be called "free" crystals?
Authors: A "free" crystal is one which is not adhering to or encrusting a hypha. These "free" crystals could be precipitated soon after the fungus has produced the oxalate or as a result of reprecipitation from the dissolution of other crystals. Our extensive observations lead us to the conclusion that adhering and encrusting crystals are derived from the oxalate produced by the hypha with which they are associated and not as a result of reprecipitation. The ECM could be extremely important in nucleating both adhering crystals and "free" crystals. Although matrix embedded "free" crystals may have grown within this matrix, the oxalate ions responsible for their growth are likely to have come from the mycelium and not one specific hypha.

M.V. Dutton: Would not oxalate degrading enzymes decompose oxalate before it had time to precipitate?
Authors: Oxalate degrading enzymes would not necessarily be produced at the same time that the fungus is precipitating oxalate. These two processes would be separated in space-time during wood colonization. These fungi could colonize wood and produce oxalate in the initial phases and then later, after crystals have precipitated, produce oxalate degrading enzymes. Colony ontogeny and substrate utilization are key to this dynamic.

M.V. Dutton: You mention that you removed the mycelium from the wood blocks and allowed the hyphae to regrow over the blocks. What is the significance of this approach?
Authors: Observation of mycelium that has regrown over the blocks allowed us to observe "free," adhering, or encrusting crystals of known maximum age. This permitted us to distinguish between crystals that had newly formed versus those that were simply remnants from earlier precipitations. Since it is clear that there is a dynamic of crystal formation, accumulation and dissolution, a method was needed to distinguish between CaOx precipitation in the later stages of decay and those crystals that are not degraded in some fashion after they precipitate in the early stages of decay. This also allowed us to distinguish at what time-points during decay the different categories of crystals, i.e., "free" crystals, adhering crystals and encrusting crystals, were produced. Without doing this it would be impossible to see these differences. It is true, however, that newly formed crystals do not necessarily indicate that the fungus is still actively producing oxalic acid. What it shows is that oxalate is present and that the kinetic and thermodynamic conditions allow for CaOx to precipitate.

M.V. Dutton: Three isolates of *F. pinicola* were used, but only the species is referred to in your figures. Are the results in the figures representative of all three isolates?
Authors: The micrographs shown (Fig. 2) are representative for all three isolates. The micrographs shown are a mixture from the three isolates of *F. pinicola*. The degradation in very late stages of decay was only observed in isolate C.

Additional Reference

Fox TR, Comerford NB (1990) Low-molecular-weight organic acids in selected forest soils of the southeastern USA. Soil Sci Soc Am J **54**: 1139-1144.