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Oak-Leaf-Litter Rhizomorphs from Iowa and Texas: Calcium Oxalate Producers

Harry T. Horner
Iowa State University, hth@iastate.edu

Lois H. Tiffany
Iowa State University

George Knaphus
Iowa State University

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Abstract
Unidentified basidiomycete rhizomorphs growing on oak-leaf litter (*Quercus alba*) in Iowa and in Texas (*Quercus gravesii*) displayed arrays of crystals associated with their hyphae. X-ray diffraction and birefringence analyses identified the crystals as a mixture of calcium oxalate-monohydrate and -dihydrate. The Iowa oak-leaf-litter rhizomorph crystals occurred in two forms: young hyphae displayed either small styloid-like crystals oriented in all directions along the hyphae; or large clusters of elongated styloid-like crystals surrounding the hyphae, with individual crystals in each cluster displaying pyramidal ends. Crystals associated with the Texas oak-leaf-litter rhizomorphs consistently covered all of the young hyphae and their tips with either small dagger-like crystals or thin, plate-like crystals whose margins were either smooth or finger-like. Some larger crystal masses were also composed of crystals with pyramidal ends. The dagger-like and plate-like crystals were tentatively identified as the monohydrate form based on their higher birefringence, whereas the crystals with pyramidal ends were identified as the dihydrate form based on their shape and lower birefringence. It is not known whether the two crystalline forms associated with the rhizomorphs are a function of the individual rhizomorphs, the litter source, the stage of crystal growth, or the ions present in the surrounding soil/ground water.

Keywords
calcium oxalate, crystals, oak-leaf litter, rhizomorphs

Disciplines
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Oak-leaf-litter rhizomorphs from Iowa and Texas: Calcium oxalate producers

Harry T. Horner1,2
Lois H. Tiffany1
George Knaphus1

1Department of Botany and 2Bessey Microscopy Facility, Iowa State University, Ames, Iowa 50011-1020

Abstract: Unidentified basidiomycete rhizomorphs growing on oak-leaf litter (Quercus alba) in Iowa and in Texas (Quercus gravesii) displayed arrays of crystals associated with their hyphae. X-ray diffraction and birefringence analyses identified the crystals as a mixture of calcium oxalate-monohydrate and -dihydrate. The Iowa oak-leaf-litter rhizomorph crystals occurred in two forms: young hyphae displayed either small styloid-like crystals oriented in all directions along the hyphae; or large clusters of elongated styloid-like crystals surrounding the hyphae, with individual crystals in each cluster displaying pyramidal ends. Crystals associated with the Texas oak-leaf-litter rhizomorphs consistently covered all of the young hyphae and their tips with either small dagger-like crystals or thin, plate-like crystals whose margins were either smooth or finger-like. Some larger crystal masses were also composed of crystals with pyramidal ends. The dagger-like and plate-like crystals were tentatively identified as the monohydrate form based on their higher birefringence, whereas the crystals with pyramidal ends were identified as the dihydrate form based on their shape and lower birefringence. It is not known whether the two crystalline forms associated with the rhizomorphs are a function of the individual rhizomorphs, the litter source, the stage of crystal growth, or the ions present in the surrounding soil/ground water.

Key Words: calcium oxalate, crystals, oak-leaf litter, rhizomorphs

INTRODUCTION

Calcium oxalate crystals are of widespread occurrence in both the plant and animal kingdoms, and among the fungi as well as in association with some of their symbiotic partners, such as lichens (Arnott and Pautard, 1970; Franceschi and Horner, 1980; Hodgkinson, 1977; Jackson, 1981; Lapeyrie et al., 1990; Wadsten and Moberg, 1985). There is increased interest in calcium oxalate-crystal-forming fungi, particularly because they are numerous in different kinds of soil environments (Cromack et al., 1979; Graustein et al., 1977). Certain fungi are pathogens on many economically important higher plants (Bennett, 1989; Bennett and Hindal, 1990; Punja et al., 1985; Punja and Jenkins, 1984).

Degradating fungi and other microorganisms are commonly associated with soil and plant litter (Arnott, 1982; Arnott and Fryar, 1984; Arnott and Webb, 1983; Horner et al., 1983; Whitney and Arnott, 1987). The soil/litter fungi composing an important group of organisms that may serve a number of significant functions besides breaking down detritus. Some of these fungi occurring as aggregates of parallel hyphae or cords are called rhizomorphs. Three recent reports show unidentified crystals associated with rhizomorph cortex hyphae (Cairney, 1990; Cairney and Clipson, 1991; Cairney et al., 1989).

Soil/litter fungi that produce oxalate may bind calcium and other cations as a means of mineral retention and nutrition within the surface soil microecosystem for already established plants and their successors (Cromack et al., 1979; Graustein et al., 1977). Furthermore, the oxalic acid produced by some of these fungi may be involved in the breakdown of soil particles and, thus, may play an important role in soil genesis (Cromack et al., 1979). These functions, as well as others not yet identified (Benny and Khan, 1988; Horner et al., 1983, 1985a, b; Powell and Arnott, 1985), suggest that further knowledge of any kind about the diversity of fungi producing oxalic acid, oxalate, and calcium oxalate crystals in the soil/litter microenvironment is warranted.

To our knowledge, no previous study has specifically identified the crystals associated with litter rhizomorphs (Cairney and Clipson, 1991). Therefore, the present study, a part of a larger field study which deals with degrading fungi, serves to extend the kinds and ranges of fungi producing calcium oxalate crystals in association with soil, leaf, and other plant litter, in general, and discusses some implications of this association.
MATERIALS AND METHODS

Unidentified basidiomycete rhizomorphs, with no visible fruiting bodies, colonizing oak-leaf litter (Quercus alba L. (Location 1) and Q. gravesii Sudw. (Location 2)) were collected from two geographically diverse locations: Location 1, Forest Lake Camp, Wapello County, southeastern Iowa; and Location 2, Chisos Mountains, Big Bend National Park, Texas. Both collections showed crystals covering the hyphae over large portions of the leaf surfaces in the litter.

The leaves were air dried and brought back to the laboratory where they were further observed with a dissecting microscope and/or a scanning electron microscope (SEM). Small square segments (ca 2–4 mm²) of the leaves containing the surface rhizomorphs were removed from the leaves with razor blades. The segments were placed onto double-stick tape already attached to brass specimen holders. All edges of each segment and tape were cemented to each holder with silver paint. After attachment, an approximately 15-nm layer of palladium-gold (80:20) was deposited on the leaf segments using a Polaron 5100 Sputter Coater. The segments were then observed with either a JEOL 1200-EX scanning transmission electron microscope (STEM; operated at 40–80 kV) with attached KEVEX Delta IV X-ray energy dispersive elemental analysis system, or a JEOL JSM-35 SEM (operated at 15 kV). The segments were scanned to determine variations in the rhizomorphs growth patterns, crystal shapes and element(s) present. Electron micrographs and elemental maps (not shown) were made using Polaroid type 665 positive/negative film and Ilford RC print paper.

Determinations of the crystallographic forms present in the two collections were made by observing their shape and degree of birefringence with a polarizing microscope, using a KEVEX energy dispersive X-ray microanalysis system, and analyzing the crystals of the air-dried rhizomorphs by X-ray diffraction analysis.

With the latter technique, the rhizomorphs with crystals were scraped from the leaf surfaces with clean razor blades onto glass slides coated with Vaseline. These preparations were mounted in a Debye-Shearer X-ray powder camera and exposed to nickel-filtered CuKα radiation. Peaks were obtained and compared to American Society for X-ray Standard file cards for calcium oxalate-monohydrate, calcium oxalate-dihydrate, calcium carbonate, and cellulose.

RESULTS

Oak-leaf litter from both locations displayed abundant growths of unidentified basidiomycete rhizomorphs over much of the leaf surfaces. In some instances, the hyphae appeared to have just emerged through the leaf epidermis, whereas in other instances larger hyphae and cords covered portions of the leaf surface area. In all cases, hyphae with clusters of crystals were present along with hyphae that were completely encrusted with crystals. There were differences in the appearance, shape and distribution of the crystals within and between each location. The crystals from both locations were shown to consist of both calcium oxalate-monohydrate and -dihydrate based on X-ray diffraction and birefringence analyses. No other crystallographic forms were identified in the X-ray diffraction analyses. Three major peaks for each crystallographic form were identified as well as an additional 7–12 minor peaks for calcium oxalate-dihydrate and 4–6 minor peaks for calcium oxalate-monohydrate (data not shown). Calcium was the dominant crystal element identified using X-ray energy dispersive elemental analysis. Differences in birefringence were noted.

Location 1, Iowa.—Rhizomorph crystals were of two types on the leaf surface hyphae: either they were in individual clusters consisting of more elongate styloid-like crystals with pyramidal ends (bipyramidal crystals; Fig. 1); or they were short, blunt, styloid-like crystals within the hyphae and stretching their young hyphal walls in all directions (Figs. 2–4). The large clusters were dispersed among the older hyphae on the leaf surfaces and did not encrust all of the hyphae (Figs. 1, 5, 6). Each individual crystal in a cluster, at higher magnification (Fig. 7), showed pyramidal ends and low birefringence. The smaller styloid crystals showed higher birefringence.

Location 2, Texas.—Many young rhizomorphs hyphae appeared to have just emerged through the leaf surfaces at the time of collection. Their hyphal tips occurred individually (Fig. 8) or in small groups (Fig. 9). The tips were completely encrusted with small crystals (Fig. 10) or broader, flatter crystals coming to a point (Fig. 11), or sometimes, plate-like crystals with smooth margins (Fig. 12). Crystals covering the hyphae away from the tips either appeared dagger-like (Fig. 13) or plate-like with finger-like margins (Fig. 14). All of the dagger-like and plate-like crystals seemed to be associated with younger hyphae of the rhizomorph.

In some regions of the leaves the hyphae displayed a mixture of crystals which consisted of bipyramids, daggers and plates (Fig. 15). In other regions, the hyphae were completely encrusted with bipyramidal crystals (Fig. 16). These latter crystals showed a lower birefringence than the dagger-like and plate-like crystals.
DISCUSSION

The results of this study of rhizomorph crystals add to a small but increasing body of knowledge about both nonpathogenic and pathogenic fungi which produce significant amounts of oxalic acid, oxalate, and calcium oxalate crystals during some phase(s) of their life cycles. This knowledge is especially interesting with respect to fungi that inhabit soil, detritus and other organic substrates.

The unidentified basidiomycete rhizomorphs observed growing on oak-leaf litter in Iowa and Texas display a mixture of calcium oxalate-monohydrate and -dihydrate crystals associated with most of the leaf surface hyphae, based on X-ray diffraction and birefringence analyses. These crystallographic analyses represent the first such specific identification of crystals on rhizomorphs that we are aware of (Cairney, 1990; Cairney and Clipson, 1991; Cairney et al., 1989).

The individual crystals and crystal clusters display differences in crystallographic form with respect to how they are associated with what we interpret as the younger and older hyphae. The two hydration forms of calcium oxalate seem to be divided between the younger, emerging or developing hyphae (calcium oxalate-monohydrate) or the older hyphae (calcium oxalate-dihydrate). In some cases, the two forms are mixed.

Verrecchia et al. (1993) summarized the various crystal morphologies and hydration forms they interpreted as occurring during fungal filament biomineralization. They showed both dagger-like and raphide-like crystals making up Quaternary calcretes. They believed the dihydrate form of the crystals developed first and later converted into the monohydrate form of the crystals. However, the prevalent forms of the young rhizomorph crystals in this study seem to be the monohydrate form. Calcium oxalate in the former hydration form is consistent with crystals identified by X-ray diffraction and other chemical methods associated with the majority of fungi analyzed to date (Arnott, 1982; Cromack et al., 1979; Graustein et al., 1977; Horner et al., 1985b; Punja and Jenkins, 1984; Whitney and Arnott, 1987) even though some of these studies have identified both forms being present. These latter reports did not distinguish between young and older hyphae. We believe that our rhizomorph samples were collected at times when the rhizomorphs were in the process of forming crystals and already had produced many crystals. The two different hydration forms could represent changes taking place in the formation and maturation of the crystals; i.e., the monohydrate form is produced first, and it is eventually converted to the dihydrate form.

Development of these two forms could also be the result of the ions present in the water associated with the soil/litter, changes in pH, temperature, and overall diurnal fluctuations in moisture. These possibilities are difficult, if not impossible, to determine in the natural microenvironment. Any laboratory studies would be complicated by the problem of being able to reproduce the field conditions under which the rhizomorphs were growing at the time of crystal formation. In the case of the Iowa rhizomorphs, the calcium oxalate crystals occur as small styloid-like crystals which appear inside the young hyphae causing their walls to be distended. The larger crystal clusters consist of styloids with pyramidal ends. Similar, small styloid-like crystals were also observed by Arnott (1982) and Whitney and Arnott (1987), growing on an unidentified forest litter fungus and on Agaricus bisporus, respectively. As mentioned previously, styloid-like crystals were found on Quaternary calcretes by Verrecchia et al. (1993).

The Texas rhizomorph crystals occur as daggers or thin plates along the young hyphae as well as at their tips. The crystals on the older hyphae are bipyrimald calcium oxalate-dihydrate. This mixture of crystals, composed of two different crystallographic forms, again, suggests that the crystals may be under the control of the same phenomenon as the Iowa rhizomorphs, but for unknown reasons they occur as different shapes.

Small, paired, plate-like calcium oxalate crystals, of unknown hydration form, with one to three finger-like projections, were observed by Powell and Arnott (1985) growing on the surfaces of sporangia of Rhizopus. Based on their developmental study, they showed the finger-like projections remaining throughout development, to maturity. These latter observations sug-

Figs. 1–7. Scanning electron micrographs of calcium oxalate crystals associated with unidentified basidiomycete rhizomorphs found on Quercus alba leaf litter collected in southeastern Iowa (Location 1). 1. Low magnification of segment of a leaf showing clusters of crystals and associated hyphae. Bar = 50 μm. 2. Portion of hypha with internal, short styloid-like crystals. Bar = 5 μm. 3. Similar image to Fig. 2. Bar = 1 μm. 4. Crystals are covered by hyphal wall. Bar = 1 μm. 5. Different associations of crystal clusters associated with hyphae. All individual crystals are rod-like. Bar = 10 μm. 6. Crystal cluster amongst hyphae showing individual rod-like crystals of different sizes. Bar = 10 μm. 7. Portion of a crystal cluster consisting of individual crystals with pyramidal ends. Bar = 5 μm.
gest one hydration form and our interpretation of the finger-like projections indicate they are only present during the early growth stages of the crystals.

Other shapes and forms of calcium oxalate crystals have been shown associated with a wide variety of fungi at different stages in their life cycles. They are: druses (spherical clusters of individual crystals; Arnott and Webb, 1983; Cromack et al., 1979; Graustein et al., 1977; Horner et al., 1983; Whitney and Arnott, 1986); short bipyramids (Horner et al., 1985a); flattened pyramids, prismatic and bipyramids (Punja et al., 1985; Punja and Jenkins, 1984); and spines (Benny and Khan, 1988). Some of these different crystal shapes may be developmentally transient and yet their shape(s) and locations on the fungi seem to be species specific. Their characteristic shapes suggest that they are under the influence of the genetics and physiology of the fungus, and possibly certain environmental conditions such as pH, temperature and the ions available to them, either through the detritus or soil/ground water. These interpretations are supported by studies on calcium oxalate crystals produced artificially (Cody and Horner, 1984) or by plant tissue culture (Kausch and Horner, 1982).

In order for the degrading fungal hyphae (Thompson, 1984) to be active in the production of crystals, there must be adequate moisture and a conducive temperature range. The ability of the fungi to metabolically produce oxalic acid and/or oxalate also is a necessary step in the process of crystal formation. Availability of water within and outside the hyphae (Jennings, 1984) allows for ion transport to occur, particularly calcium. These factors provide the necessary ingredients for calcium oxalate formation which apparently occurs interior to the fungal hyphae initially (Whitney and Arnott, 1987, and the present study) and later on the surfaces of the hyphae. These conditions seem consistent with respect to the formation of the rhizomorphs crystals observed in this study and suggest that variations in availability of environmental ions and daily fluctuations also may have caused the differences observed in the rhizomorphs crystal forms from both Iowa and Texas.

The fact that many litter fungi produce calcium oxalate crystals is undeniable. These fungi are programmed to produce crystals which are bound to them until they die. At that time the crystals may be dissolved, weathered, or degraded by soil microorganisms, returning the component ions to the surface soil/water layer. We agree with the earlier studies of Arnott and Webb (1983), Cromack et al. (1979), and Graustein et al. (1977) that suggest this cycling of calcium, oxalate, and oxalic acid, as well as other associated ions, may be very significant to the retention of biologically important ions in the surface soil/water layer for plant nutrition, and for soil genesis. A better understanding of how these fungi, and other microorganisms, contribute to the welfare of this poorly understood soil microecosystem worldwide is warranted.

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LITERATURE CITED


