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THE RADIOMYCETACEAE (MUCORALES; ZYGOMYCETES). CALCIUM OXALATE CRYSTALS ON THE SPORANGIOLAR WALL AND AERIAL HYPHAE

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Abstract

Calcium oxalate crystals occur on the sporangiolar appendages and aerial hyphae of the two known genera (Hesseltinella, Radiomyces) of the mucoralean family Radiomycetaceae. In Hesseltinella the sporangiolar appendages are acicular with a polygonal, square, or hexagonal, discoid base. The appendage core consists of calcium oxalate and is composed of two portions: (1) a round, acicular spine with a more or less hexagonal base, and (2) a square, polygonal, or hexagonal, discoid base with a central perforation that generally contains the spine base. Both portions of the spine core appear to be readily separable. In species of Radiomyces the appendages are capitate with a crystal embedded in the distal, inflated end.

Hyphal crystals bearing a short spine are similar in morphology to the sporangiolar appendages and are regularly produced by Hesseltinella. Initially these crystals are embedded in the hyphal wall. First, the spine is extruded out. Then some of the wall material is lost, exposing the base, a process concurrent with dehiscence of the crystal spine. In Radiomyces, the young aerial hyphae are typically smooth but calcium oxalate crystals were observed on aging mycelia.

KEY WORDS: Radiomycetaceae, Radiomyces, Hesseltinella, Mucorales, scanning electron microscopy, calcium oxalate, sporangiole, hypha, fungi.

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Introduction

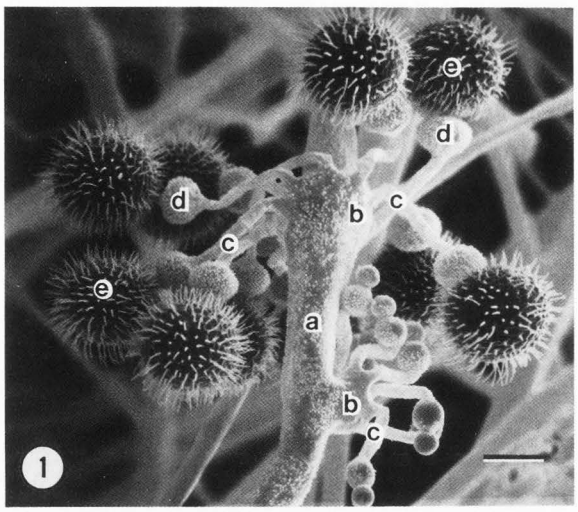
Calcium oxalate crystals are apparently widespread in plants (Franceschi and Horner, 1980), and they have been known in fungi for over 100 years (Hamlet and Plowright, 1877). Recently, the presence of calcium oxalate has been proven in Myxomycetes (Trichiales--Schoknecht and Keller, 1977), Basidiomycetes (Lycoperdales--Horner et al., 1985; Whitney and Arnott, 1986b), and Zygomycetes (Mucorales--Jones et al. 1976; Urbanus et al., 1978; Powell and Arnott, 1985; Whitney and Arnott, 1986a).

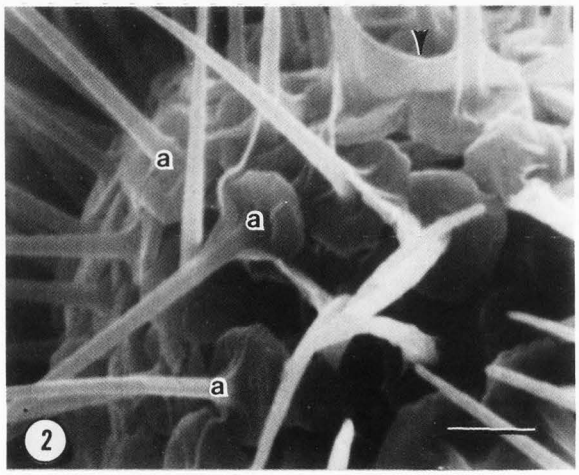
Similar crystals are present on the aerial hyphae and reproductive structures of many other members of the Zygomycetes (O'Donnell, 1979; Cole and Samson, 1979; Benjamin, 1979), except the sporangiospores (Young, 1985). One of these organisms, Hesseltinella vesiculosa Upadhyay (1970; O'Donnell, 1979), produces sporangiolar appendages very similar to those formed by species of Cunninghamella (Shipton and Lunn, 1980), one species of which [C. echinulata (Thaxt.) Thaxt. (Jones et al., 1976)] is known to produce calcium oxalate containing appendages. There are also spines on the reproductive branches and sporophores of H. vesiculosa. Two related taxa, Radiomyces embreei Benjamin (1960) and R. spectabilis Embree (1959)(Radiomycetaceae, Mucorales), also form sporangiolar appendages, but they are capitate and not spinose. Spines do not appear to be present on other aerial portions of these fungi, but crystals resembling those found on the zygophore of Mucor mucedo L: Fr. (Urbanus et al., 1978) are produced on the suspensors of Radiomyces embreei (Benny, unpublished).

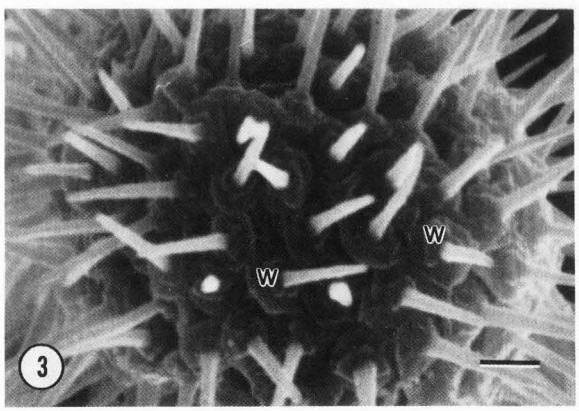
The purpose of this paper is to present the results of our study of the spines and crystals present on the aerial and reproductive hyphae, and sporangiolar appendages of Hesseltinella vesiculosa, Radiomyces embreei, and R. spectabilis.

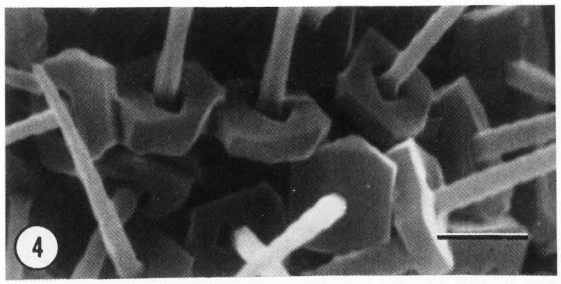
Materials and Methods

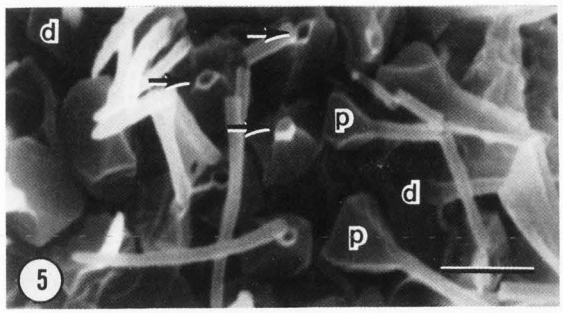
All of the fungi examined were grown on modified V8 juice agar [1 can V8 juice (177 ml), diluted to 1 liter with distilled water; calcium carbonate (powdered), 3 gm; agar, 15 gm], sterilized 40 min at 1.055 Kg/cm^2 pressure in an

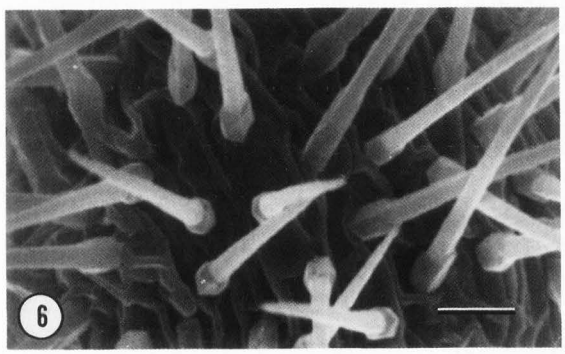


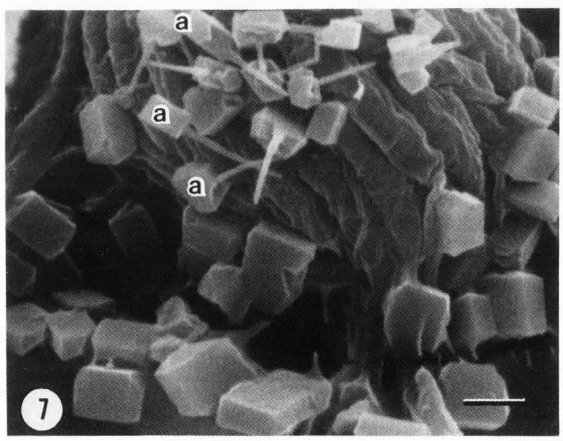












Figures 1-7. SEM of sporangiolar appendages of Hesseltinella vesiculosa. Figure 1. Fruiting head showing sporophore (a), vesicles (b), stalks (c), fertile vesicles (d), and sporangiola (e). Bar = 10 µm. Figure 2. Surface of a sporangiolum showing appendages (a) and remnants of sporangiola wall (arrowhead). Bar = 1.0 µm. Figure 3. Appendages with wall material (w) remaining around crystalline base. Bar = 1.0 µm. Figure 4. Hexagonal to polygonal appendage bases showing cavity containing shaft. Bar = 1.0 µm. Figure 5. Appendages with discoid (d) to pyramidal (p) bases showing that shaft (arrows) is usually same shape as subtending base. Bar = 1.0 µm. Figure 6. Appendage shafts showing their small square to polygonal bases. Bar = 1.0 µm. Figure 7. Appendages (a) with shafts and some without shafts. Bar = 1.0 µm.

autoclave and poured in 15 mm x 100 mm plastic Petri dishes. The fungus cultures were inoculated and then incubated at 22 °C (Hesseltinella vesiculosa--NRRL 3301) or 28 °C (Radiomyces embreei--RSA 984; R. spectabilis--RSA 1493) under a 12 h light/12 h dark cycle.

For SEM examination and X-ray microanalysis, the specimens were fixed in osmium vapors for 96 h and air dried according to the procedure of Quattlebaum and Carner (1980), mounted on aluminum stubs with double sticky tape, gold coated in a Hummer Jr. sputter coater, and examined with a Hitachi S-450 scanning electron microscope equipped with a Kevex X-ray analyzer.

Solubility of the crystals was tested using 2% acetic acid, 4% sodium hydroxide, 4% sulfuric acid, 3% nitric acid, and 10% hydrochloric acid. Reactions were observed at 400X and 1000X with bright field light microscopy (Powell and Arnott, 1985).

Results

Sporangiolar Appendages

Sporophores of Hesseltinella vesiculosa produce subterminal or lateral vesicles that bear several stalks that terminate in a fertile vesicle. Each fertile vesicle produces a single, pedicellate, multispored sporangiolum (Fig. 1). In nearly mature sporangiola, the remnants of the sporangiolar wall may hold the appendages together (Fig. 2), and some wall material may be present around the crystalline base (Fig. 3). After dissolution of the sporangiolar wall its crystalline core is exposed (Fig. 4). The sporangiolum is covered with acicular appendages that have a discoid, polygonal or hexagonal base (Figs. 2-4). The acicular shaft of the appendage appears rounded but its fractured face is polygonal, e.g., it has the same shape as the base (square or hexagonal)(Fig. 5). The shaft may fit within a central cavity of the crystalline base (Fig. 4). The appendage base may become enlarged and irregular in shape at maturity (Figs. 5, 7). The shaft base may be slightly larger than the shaft (Fig. 6) or small based shafts may be mixed with those having larger bases. The shaft base may be pyramidal (Fig. 5) or polygonal to square (Fig. 6) and mixed with shafts having discoid (Fig. 5) or other shaped bases (Figs. 6, 7). The appendage shaft is initially long and wide in comparison with the size of its base (Figs. 2, 4) but later becomes more slender and shorter in relation to base size (Fig. 7). Finally, the shaft disappears on a few crystals, but later most (Fig. 7) of the sporangiolar spines are replaced by crystals. The process of sporangiolar appendage development in Hesseltinella vesiculosa is summarized in Fig. 8.

Sporangiola of Radiomyces are covered with capitate appendages with a crystal present in the inflated end. The appendages lack a discoid base. In these fungi the appendage apices are globose to obovoid (Figs. 9, 10). In R. spectabilis (Figs. 10-12) the crystal in the appendage apex enlongates and widens (Fig. 11), but the appendages are still stipitate at this time. The crystal is irregular in shape at

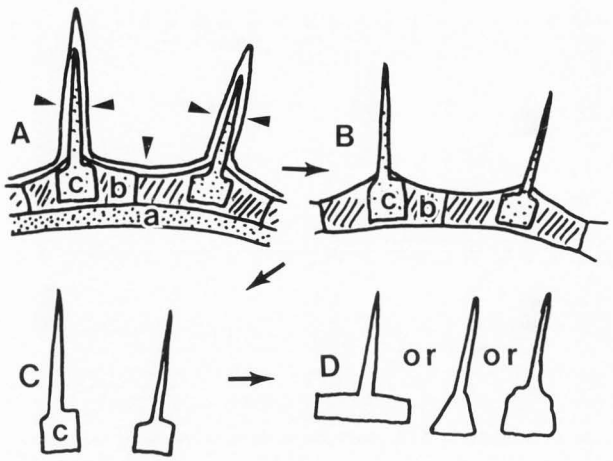


Figure 8A-D. Stages in sporangiolar appendage development in Hesseltinella vesiculosa. A. Appendages with sporangiolar wall (a) and covering membrane (arrowheads) still intact. B. Appendages after sporangiolar wall and covering membrane have deliquesced. C. Appendage after noncrystalline portion of the base has disappeared. D. Some of the morphological variation in the crystalline portion of the appendage. (A-D not drawn to scale). Legend: a = sporangiolar wall; b = noncrystalline portion of the appendage base; c = crystalline portion of the appendage; arrowheads = membranous cover of appendages.

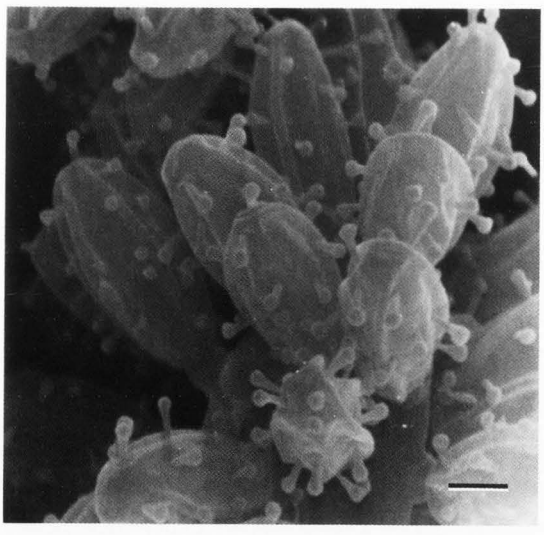
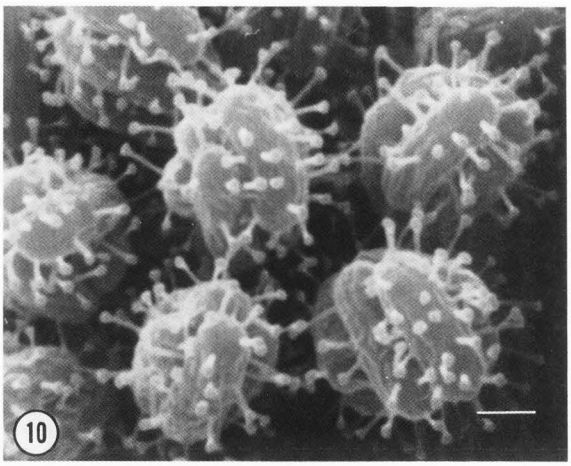
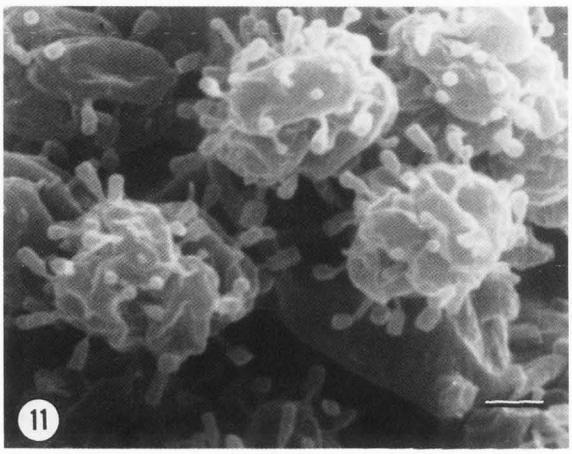
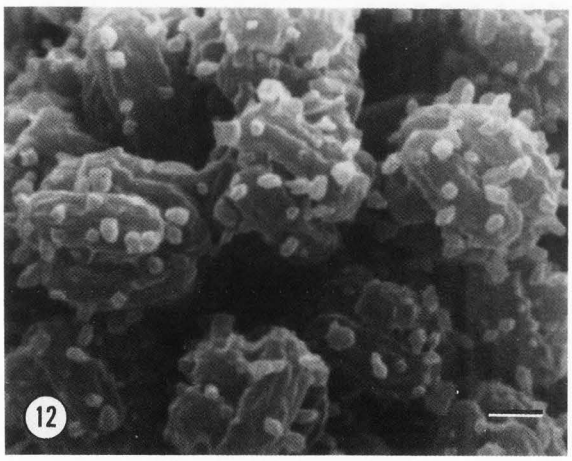


Figure 9. SEM of Radiomyces embreei sporangiola showing typical capitate appendages. Bar = 1.0 μm.







Figures 10-12. SEM of Radiomyces spectabilis sporangiola. Figure 10. Typical capitate appendages on immature sporangiola. Bar = 1.0 µm. Figure 11. Stalked appendages showing that crystals are growing wider and longer. Bar = 1.0 µm. Figure 12. Irregularly shaped (appendage) crystals on wall of mature sporangiola after appendage stalks disappear. Bar = 1.0 µm.

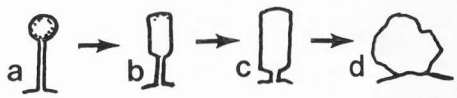
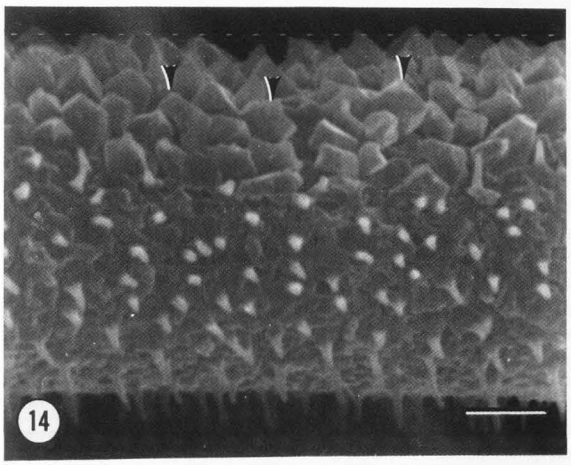
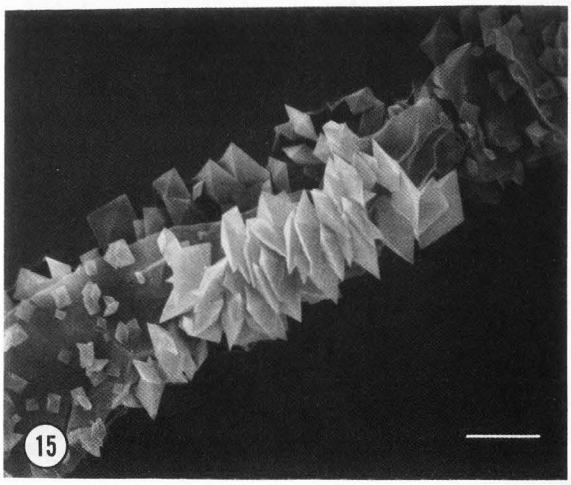


Figure 13a-d. Stages in sporangiolar appendage ontogeny in Radiomyces spectabilis. a. Capitate appendage after sporangiospores are mature; b. Elongation of crystal in appendage tip; c. Continued enlargement of appendage crystal; d. Irregular crystal from appendage resting on sporangiolar wall. Note that stalk is present in a-c but has disappeared in d.





Figures 14, 15. Crystals on vegetative hyphae of Hesseltinella vesiculosa (Fig. 14) and Radiomyces spectabilis (Fig. 15). Figure 14. A young hypha showing crystal spines and crystals (arrowheads). Bar = 1.0 µm. Figure 15. Crystals on mature hyphae. Bar = 5.0 µm.

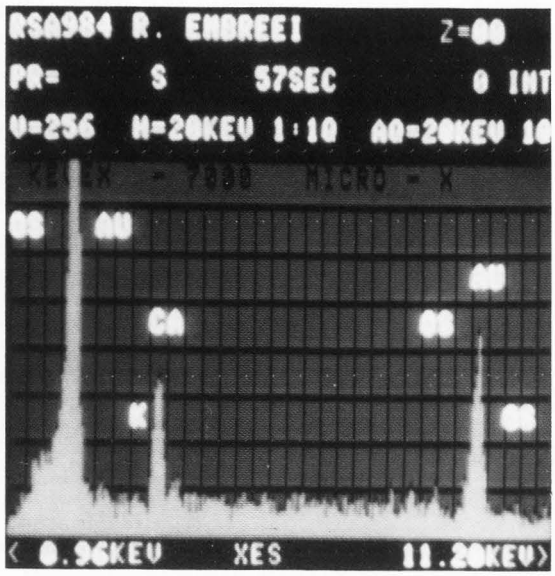


Figure 16. X-ray microanalysis of crystals on young hyphae of R. embreei.

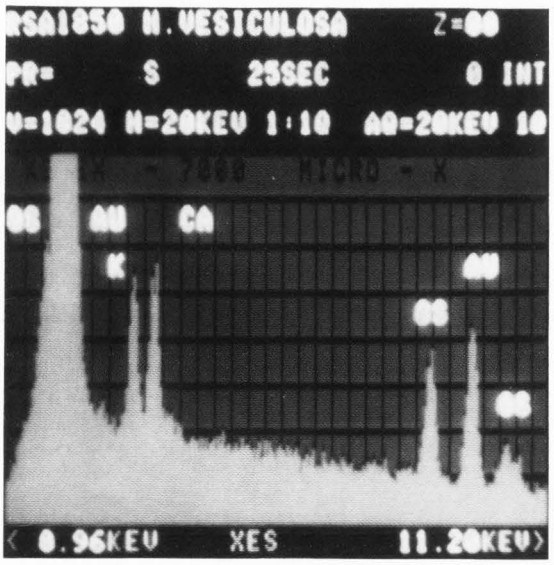


Figure 17. X-ray microanalysis of crystals on young hyphae of Hesseltinella vesiculosa.

maturity (Fig. 12), a stage in which the stipe is broken or disappears and the crystal lies on the sporangiolar surface. The sequence of sporangiolar appendage ontogeny in R. spectabilis is summarized in Fig. 13.

Crystals on Aerial Hyphae

Aerial hyphae (Fig. 14) of Hesseltinella vesiculosa are generally covered with spiny appendages which, like sporangiolar appendages, usually have a discoid base. Initially the crystal spines protrude through the wall but later only crystals are visible (Fig. 14). In older hyphae the spines usually have disappeared. Distinct crystals are produced in some portions of the mature aerial hyphae of Radiomyces embreei but at maturity they are present only on the surface. Many crystals were observed on the hyphae of R. spectabilis (Fig. 15). These crystals appeared bipyramidal which is the characteristic morphology of calcium oxalate dihydrate (Khan and Hackett, 1986).

X-ray microanalysis.

X-ray microanalysis of the appendages and crystals on the sporangiola and aerial hyphae of both Radiomyces and Hesseltinella showed the presence of calcium. In Radiomyces calcium was present only at the capitate tips of the sporangiolar appendages while in Hesseltinella the core of the entire appendage is composed of calcium except for the crystals on Radiomyces aerial hyphae of all of the crystalline structures of Radiomyces (Figs. 16, 17) and Hesseltinella contained potassium in addition to calcium. Appendages of the mature sporangiola and hyphae were larger and displayed a larger peak for calcium than younger sporangiola and hyphae.

Crystal Solubility

Both the sporangiolar spines and hyphal crystals of Hesseltinella dissolved in 3% nitric acid, 4% sulfuric acid, and 10% hydrochloric acid. They did not dissolve in 2% acetic acid or 4% sodium hydroxide. The sporangiolar spines of Radiomyces spp. were too small to visualize by light microscopy.

Discussion

Both Hesseltinella and Radiomyces have calcium containing appendages or distinct crystals on their sporangiola and distinct calcium crystals on their aerial hyphae. Morphological characteristics of crystals on aerial hyphae of Radiomyces and solubility characteristics of the appendages and crystals produced by Hesseltinella vesiculosa indicate that they are composed of calcium oxalate.

Hesseltinella vesiculosa is similar to several other zygomycete taxa (e.g., Backusella, Dichotomocladium, Gilbertella, Helicostylum, Pilaira, Thamnidium, and Thamnostylum) in that crystal-containing spines and/or crystals are present on the sporangium and/or sporangiolum or merosporangium, and fruiting mycelium (sporophore) or aerial hyphae (Benny and Benjamin, 1976; O'Donnell, 1979; Whitney and Arnott, 1986a). Many members of the Kickxellaceae (Coemansia, Dipsacomyces, Kickxella, Linderina, Martensiomyces) have external spines on all aerial structures (hyphae, fertile branches, sporocladia, pseudophialides)(Benjamin, 1958, 1959; Young, 1970b; Benny and Aldrich, 1975; O'Donnell, 1979) that appear to arise from the inner, chitinous wall layer (Benny and Aldrich, 1975) except in the merosporangiospores where the spines are embedded in the spore wall (Young, 1968a, 1970a, 1971, Benny and Aldrich, 1975) Many other taxa produce crystals or spines on the surface of the

sporangium, sporangiolum, or fertile vesicle but not on any other aerial structures (e.g., Choanephora, Cunninghamella, Mycotypha, Pilobolus, Utharomyces)(Buller, 1934; Brain and Young, 1979; Cole and Samson, 1979; O'Donnell, 1979; Kirk and Benny, 1980; Jeffries and Young, 1983; Benny et al., 1985). Only a few taxa appear to not form spines and/or crystals on any aerial structures (e.g., Basidiobolus, Zychaea; Cole and Samson, 1979; O'Donnell, 1979).

The sporangiolar appendages of H. vesiculosa resemble those formed by species of Cunninghamella (Young, 1968b; Cole and Samson, 1979; O'Donnell, 1979; Shipton and Lunn, 1980; Lunn and Shipton, 1983). The development of sporangiolar spines, as demonstrated in Cunninghamella echinulata by Cole and Samson (1979), indicates that the acicular spine is formed first, usually initially appearing when the sporangiolum is relatively young. The appendage base becomes distinct after the sporangiolum attains its mature size.

In R. embreei crystals on the aerial hyphae which contain calcium are covered by wall material (Fig. 16), but only exposed crystals were observed on R. spectabilis mycelium. Urbanus et al. (1978) also observed various types of crystals on different portions of the same organism and they postulated that crystallization was influenced by differences in the chemical environment in the cell wall. Powell and Arnott (1985) speculated whether the crystals are produced external or internal to the cell wall, believing that they initially were formed inside the wall. This does appear to be true for the crystals on the aerial hyphae of Radiomyces embreei.

It is possible that calcium oxalate crystals are produced to protect the fungus from having a toxic reaction to calcium and/or oxalate (Whitney and Arnott, 1986a). The morphology and arrangement of crystals on spore bearing structures on these and other mucoralean fungi would indicate that their presence may be an adaption for optimal modes of spore dispersal. A result of the crystal formation may be to produce a hydrophobic environment around the spore-bearing structure by trapping air between the crystals and/or appendages (Buller, 1934; Ingold and Zoberi, 1963; Ingold, 1971). Sporangiola, whether containing one, two, or many sporangiospores, are dislodged and dispersed intact by air currents (Zoberi, 1985). The outer layer of the spore wall deliquesces allowing displacement of the sporangiolar appendages. A great enough displacement of the sporangiolar spines would prevent air entrapment which would allow water to contact the sporangiospores, eventually resulting in their germination.

The presence of hyphal spines and disrupted sporangiolar appendages, aided by the geometry of reproductive structures and their subtending hyphae, might promote the presence of a sufficient amount of condensed water on localized areas of hyphae and/or post-dehiscence sporangiola to allow some calcium oxalate to dissolve and produce a saturated solution. The calcium oxalate in the shafts and bases of the sporangiolar appendages of H. vesiculosa are in equilibrium with the surrounding calcium oxalate-saturated solution, but because the spines have a higher surface or Gibb's free energy due to a larger surface-to-mass ratio than the base structures, the base crystals will grow at the expense of the appendage shaft length and diameter. Eventually only crystals will remain where the specialized sporangiolar appendages were originally present. This would also be true for the hyphal spines of H. vesiculosa. These structures will eventually recrystallize to form distinct crystals on the hyphal surface. The process will continue as long as there is sufficient water on the surface and, therefore, smaller crystals (high surface area to mass) will disappear at the expense of producing large crystals (low surface area to mass)(personal communication, D. G. Rands). Theoretically, only a single crystal would be produced as a result of this process proceeding to a state of equilibrium. The resulting decrease in spine or appendage shaft length, or their disappearance, would obviously change the morphology of hyphal crystal and sporangiolar appendages. The use of old cultures might lead to erroneous observations on the morphology of crystals produced by these and other fungi. We suggest that actively growing cultures be fixed in osmium vapors since crystal morphology might conceivably change in the fluids used in most fixation-dehydration processes and/or if a senescing culture, covered with condensed water, is used.

In species of Radiomyces (Embree, 1959; Zoberi, 1985) air currents may dislodge and blow the sporangiolum away after the subtending pedicel breaks or, probably less commonly, in R. spectabilis the sporangiospores can be released if the sporangiolar wall cracks (Cole and Samson, 1979). Air currents would promote dispersal of the sporangiola some distance away from the original growth site. These latter means of spore and/or sporangiole dehiscence may occur before the sporangiolar wall is mature. When the multispored sporangiola of R. spectabilis are wetted they burst open releasing the spores (Embree, 1959; Zoberi, 1985), a process preceding germination.

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Discussion with Reviewers

H. J. Arnott: How can crystals aid in spore distribution?.

Authors: Tightly arranged calcium oxalate crystals trap air and prevent water from coming in contact with the surface of a wall. In the case of a sporangial wall this would prevent its dissolution and would result in the dispersal of intact sporangiola. When the sporangiola come in contact with water the spores would be released.

H. J. Arnott: How do you think Ca++ arrives at the site of crystallization? How do you think the oxalate ion gets to the site of crystallization?

Authors: The presence of Ca++ and oxalate above a certain concentration in the cell probably activates the mechanism responsible for their

removal. Both Ca++ and the oxalate ions are excreted through the cytoplasmic membrane after they have reached a predetermined concentration in the cytoplasm. These ions then probably concentrate in specific regions of the hyphal or sporangiolar wall because of the presence of enzymes or other essential molecules. After calcium oxalate has reached the saturation point in this system then the crystals are formed.

J. Wattendorff: Would animals be dissuaded from feeding on fungi bearing calcium oxalate crystals and spines?
Authors: We could not find a study of this topic in the literature. Perhaps calcium oxalate would prevent a small animal, e.g., a mouse or shrew, from feeding on a fungus. Mites or insects, depending on the species, might avoid a fungus bearing calcium oxalate crystals. Other factors may also play a role in predator defense, e.g., the coprophilous species of the ascomycete genus Chaetomium (von Arx, Guarro, and Figueras, Beih. Nova Hedwigia 84:1-162. 1986) produce ornamental perithecial hairs (Hawksworth and Wells, Mycological Papers 134: 1-24. 1973) that prevent predation by fly larvae (Wicklow, Trans. Brit. Mycol. Soc. 72: 107-110. 1979). Wicklow and Yocom (Trans. Brit. Mycol. Soc. 78: 29-32. 1982) later demonstated that the number of fly larvae present directly correlated with the decrease in the number of coprophilous fungal species. Even if a fungus is eaten by an animal some of the spore bearing structures or the spores themselves may survive. Each spore is capable of producing a new thallus. A thallus can produce a large number of spores.

H. Horner: What do you think develops first, the spine or the base? Why?
Authors: A study of spine development on the sporangiola of Cunninghamella echinulata by Cole and Samson (1979) shows that the shaft appears before the base is visible and, therefore, is noticed first. This, however, does not mean that the shaft forms first. We would guess that the appendage base forms in the cell wall and then the spine is produced later. This cannot be substantiated, however, because these structures are too small to be readily observed with the SEM. Perhaps this could be investigated using TEM.

H. Horner: Is it possible to use the shape and location of the crystals as a taxonomic character in the genera you studied?
Authors: The morphology and location of crystals is different for the two genera studied. In Hesseltinella crystals are present on all aerial structures whereas in Radiomyces they are normally found only in the sporangiolar appendages, especially in young fungi. The sporangiolar crystals are different in the two genera, acicular with a flattened base in Hesseltinella and crystalline within the appendage apex in Radiomyces. These criteria correlate well with other morphological features that are more easily observed under lower powers of the light microscope (Embree, 1959; Benjamin, 1960; Upadhyay, 1970).

H. Horner: Is it possible to reduce or eliminate the calcium oxalate crystals by growing the fungi on defined media with no calcium or low concentrations of calcium?
Authors: We did not investigate this in Hesseltinella or Radiomyces but this has been done on Cunninghamella spp. (Shipton and Lunn, 1980, p. 489). The effects of differing calcium concentrations were not consistent in all of the isolates that they studied. Low calcium concentration often resulted in the formation of aberrant spines and high concentrations often resulted in the production of abnormal sporangiolar appendages. Whether this is the case in Radiomyces and Hesseltinella remains to be investigated but the sporangiolar spines may change in both numbers and morphology under certain conditions.

K.D. Whitney: The V-8 juice agar you used in this study contained 3 gm/liter calcium carbonate (about 0.03M, not counting calcium in the juice). This is a rather high calcium concentration, especially since most mycological media rarely call for added calcium. Any comments on potential effects of these calcium levels on the results obtained in your study?
Authors: Calcium concentration does regulate spine morphology as discussed in the question above. Miller (Phytopathology 45: 461-462. 1955) devised V8 juice agar as a general purpose medium for fungi and bacteria. We use V8 juice agar as a general culture medium and those fungi that sporulate well on it do not appear to be aberrant. The concentration of Ca++ derived from calcium carbonate may not be that high since this chemical is not very soluble in this culture medium. The morphology of Hesseltinella and Radiomyces does not appear to be altered when grown on V8 juice agar, and the crystal distribution appears normal when compared to cultures grown on other media.