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CALCIUM OXALATE CRYSTAL PRODUCTION IN TWO MEMBERS OF THE MUCORALES

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Abstract

Calcium oxalate crystals are found in association with the sporangia of Mucor hiemalis and Rhizopus oryzae. Crystals observed in each species vary in morphology from simple crystals consisting of single spines in M. hiemalis to complex crystals with twin spines, sometimes three-parted, on a common base in R. oryzae. The early development of the crystals is similar in both species with a layer of the cell wall covering in the initial crystals. The spines of M. hiemalis rapidly emerge while the crystals of R. oryzae appear to remain covered with a layer of outer wall material. The crystals of both species become fully developed just prior to spore release. Details of crystal development are compared and possible mechanisms for crystal development are explored.

KEY WORDS: Mucor hiemalis, Rhizopus oryzae, calcium oxalate, scanning electron microscopy, sporangia, sporangiophore

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Introduction

Calcium oxalate formation occurs commonly in a variety of fungi (Hamlet and Plowright, 1877). Calcium oxalate has been found in association with the sporangia of the Mucorales, e.g., the spines on the sporangia of Mucor plumbeus and Cunninghamella echinulata (Jones et al., 1976) and the zygophores and sporangia of Mucor mucedo (Urbanus et al., 1978). The two reports confirm the presence of calcium oxalate dihydrate (weddellite) in the spines produced by these species.

The mechanism of formation of these crystals is unknown. Some reports of wood rotting fungi and soil fungi (Graustein et al., 1977; Cromack et al., 1979) suggest that oxalate is excreted and combined with external calcium to form crystals. In contrast, some studies of leaf litter fungi (Arnott, 1982; Horner et al., 1983) suggest the crystals may form internally. Urbanus et al. (1978) suggest the crystals formed in association with the zygophores of Mucor mucedo are covered by a layer of cell wall material.

This study expands observations of calcium oxalate crystals in the Mucorales and provides further evidence for the internal development of the crystals.

Materials and Methods

Cultures

Strains of Mucor hiemalis and Rhizopus oryzae were isolated from leaf litter and soil samples at three locations in Arlington, Texas. The fungi were grown on commercially prepared Sabouraud's Dextrose Agar (Scott Laboratories).

Crystal Isolation

Mature cultures grown in petri dishes were rapidly frozen in liquid nitrogen, and the sporangia were scraped from the frozen agar surface with a scalpel blade. The sporangia-crystal mixture was then transferred to a liquid nitrogen-cooled mortar and pestle and ground in a small portion of liquid nitrogen. The resulting mixture was used for microchemical analysis.

Microchemical analysis

Analysis was performed on whole sporangia as well as the powder extract described above. Reactions were observed at 400X with bright-field microscopy. Solubility tests (Frey, 1925; Pohl, 1965) were performed using the following reagents: 70% ethanol, 2% acetic acid, 4% sodium hydroxide, 4% and 60% sulfuric acid, 3% nitric acid, and 10% hydrochloric acid.

Scanning Electron Microscopy (SEM)

Cultures were observed by means of light microscopy to determine maturity of the cultures. Mature cultures were then vapor fixed for 4-6 h over 4% osmium tetroxide in 0.1 M phosphate buffer (pH 7.24) at 4° C. Specimens were allowed to air dry and portions of the culture were affixed to the sticky side of a short piece of clear tape. The tape was then inverted and attached to SEM stubs using double stick tape. Specimens were sputter coated in a Polaron E5100 sputter coater. Observations and photographs were made on a JEOL JSM-35C scanning electron microscope at 15 kV and 100 µA.

Results

Crystals found in association with sporangia of both Mucor hiemalis and Rhizopus oryzae have solubility characteristics consistent with calcium oxalate (Table 1). The crystals of these fungi show little or no birefringence under polarized light microscopy, though the small size of the crystals makes observation difficult.

Table 1. Microchemical Analysis on Mucor hiemalis and Rhizopus oryzae

Treatment	Mucor	Rhizopus	Calcium Oxalate
70% ethanol	-	-	-
2% acetic acid	-	-	-
4% sodium hydroxide	-	-	-
4% sulfuric acid	+	+	+
60% sulfuric acid	+	+	+
3% nitric acid	+	+	+
10% hydrochloric acid	+	+	+

- = insoluble, + = soluble

Young sporangia of Mucor hiemalis have a smooth, sometimes bumpy, surface, and sporangiophores show no evidence of crystal formation (Fig. 1). As maturation continues, the crystals begin to appear tip first (Figs. 2 and 3), just protruding through the outer sporangial wall. Crystals usually develop uniformly on the sporangial surface, sometimes basipetally, but always within a short time span. After approximately 3-4 days, the crystals in maturing sporangia become fully developed and more numerous (Fig. 4).

As the sporangia age the external layer, along with the crystals, begins to mold around the spores beneath (Figs. 5 and 6). Close examination of the sporangia reveals long, pointed spines protruding from the outer sporangial wall (Fig. 7), with the wall molded to spores beneath it (Figs. 8 and 9). The entire process, from appearance of the sporangium to its complete encrustation with crystals, occurs over a period of 3-4 days with the first crystals appearing as early as the first day. This process of encrustation appears to be uniform among all sporangia and no mature sporangia were noted without at least some covered crystals present.

The individual spines reach a length of about 2 µm and are composed of a basal plate and a terminal spine. Their shape resembles a carpet tack (Fig. 9). The spine sometimes appears to be uniformly tapered but occasionally the spine has a central "joint" where it quickly tapers. In the mature spines it is difficult to demonstrate an overlying cell wall component with SEM (Fig. 9).

Changes in environmental conditions seem to have some bearing on the numbers of crystals produced. More crystals are produced in cultures grown in light than in darkness and at low (4-8° C) temperatures, though the development time is increased to 14-28 days.

Crystal production in Rhizopus oryzae is similar to that of Mucor hiemalis. The sporangia of young cultures show few crystals (Fig. 10) on a relatively smooth surface. During sporangial maturation, crystals appear to extrude through the outer sporangial layer (Figs. 11-13). The appearance of the crystals is rather rapid, occurring within 1-2 days of growth. As the sporangia mature the crystals become fully developed and much more numerous on the sporangial surface (Fig. 14). The individual crystal morphology is different from that of the spines found in Mucor (Fig. 15). The crystals have a flat rectangular base with two perpendicular spines rising from it.

Figures 1-9. SEM views of crystals on the sporangia of Mucor hiemalis.

Figure 1. Young sporangia showing absence of exposed crystals. Bar = 10 µm.

Figure 2. Early sporangium with newly exposed crystals. Bar = 10 µm.

Figure 3. Early emergence of crystals. Bar = 1 µm.

Figure 4. Maturing sporangium. Note increased number and length of crystals. Bar = 10 µm.

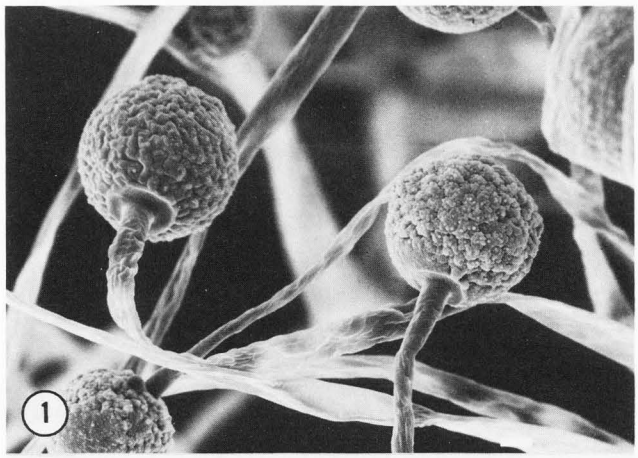
Figure 5. Mature sporangium showing molding to spores beneath. Bar = 10 µm.

Figure 6. Mature sporangium at spore release. Bar = 10 µm.

Figure 7. Closeup of spines in mature sporangium. Bar = 1 µm.

Figure 8. Detail view of spines molded to spore beneath. Bar = 1 µm.

Figure 9. Higher magnification of spines molded to spore beneath. Bar = 1 µm.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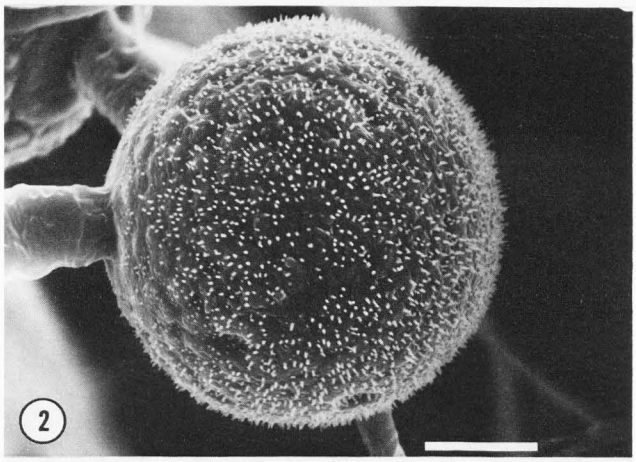
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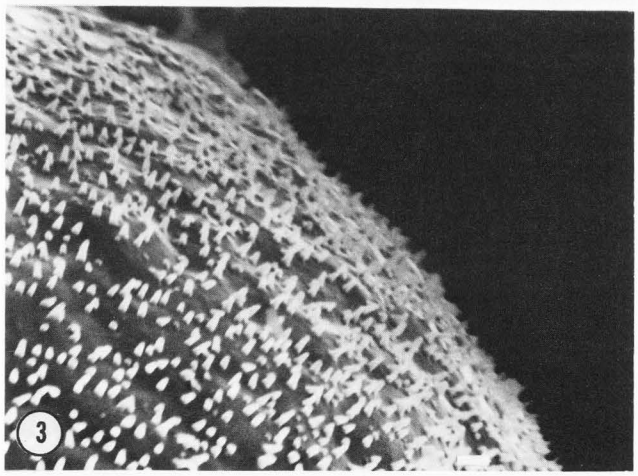
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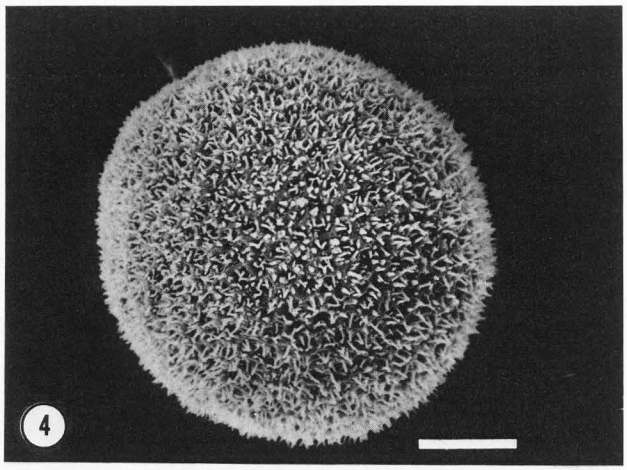
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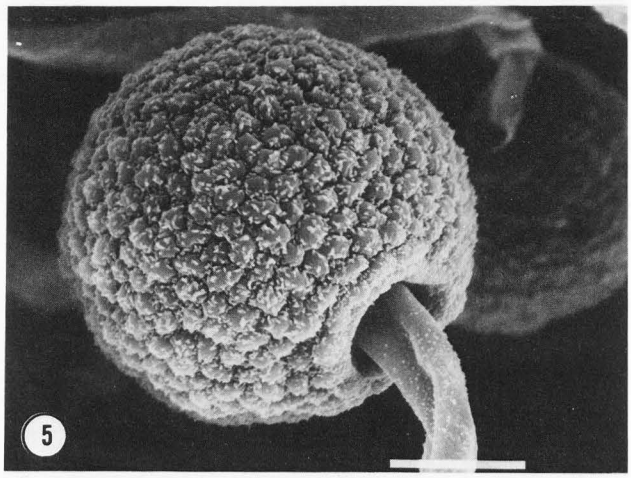
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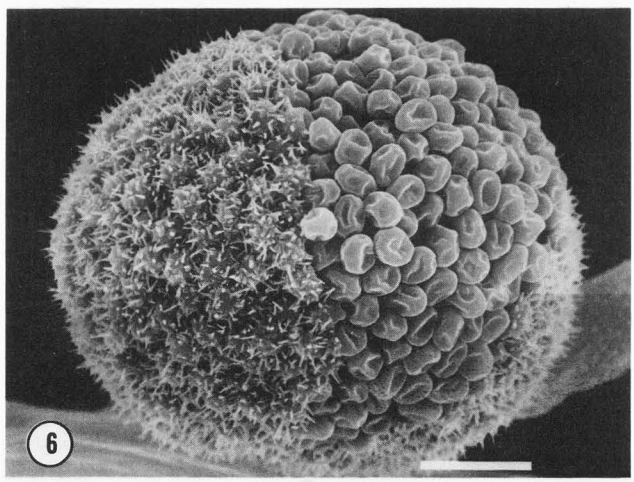
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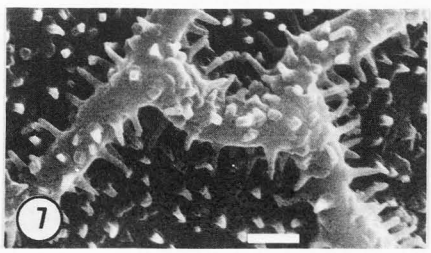
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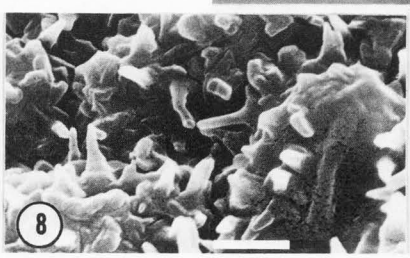
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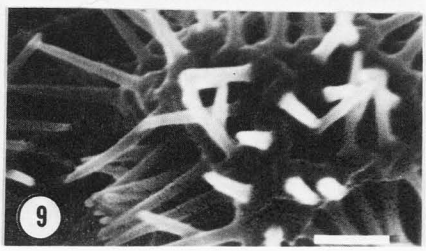
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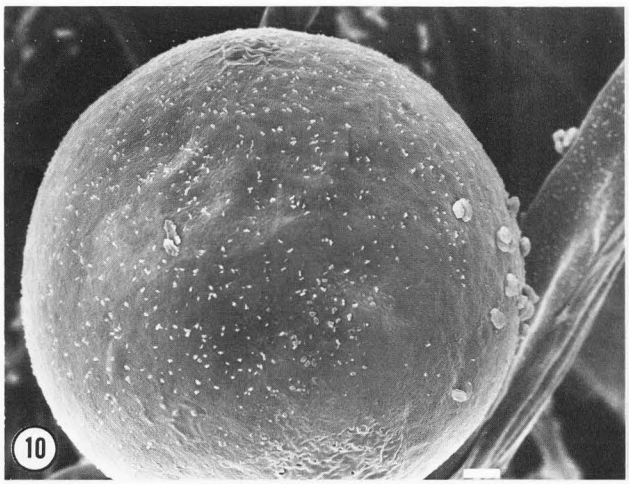
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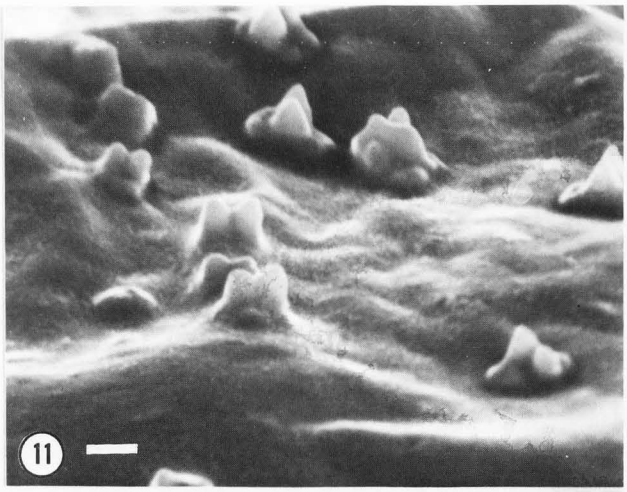
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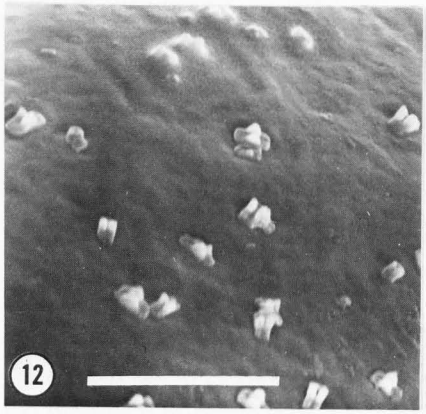
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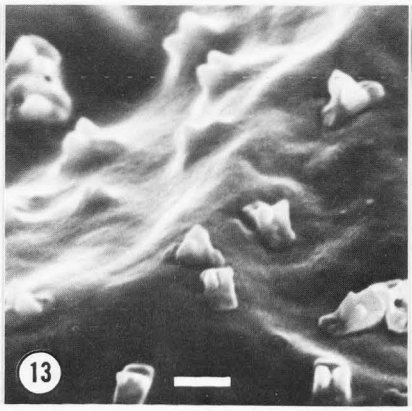
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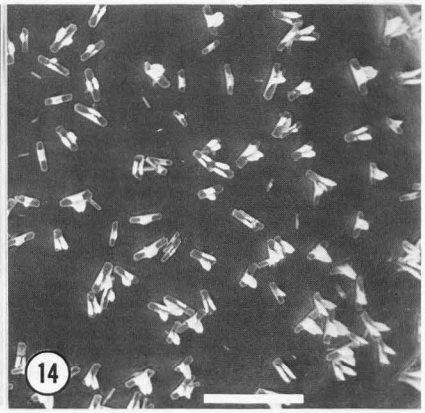
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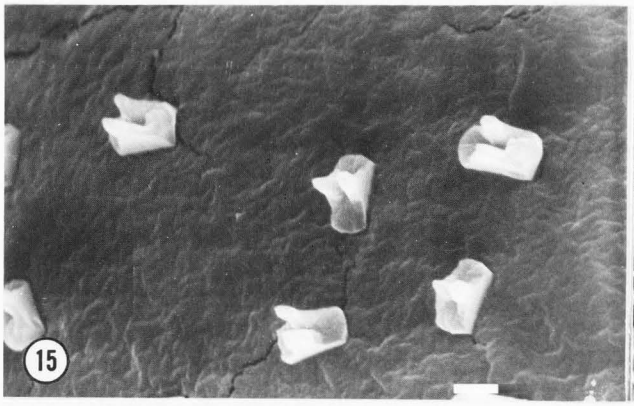
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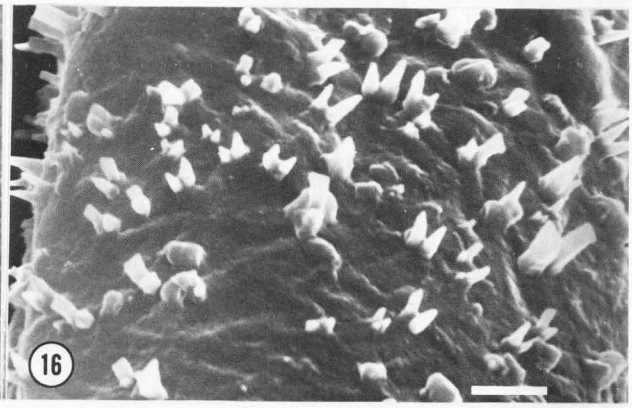
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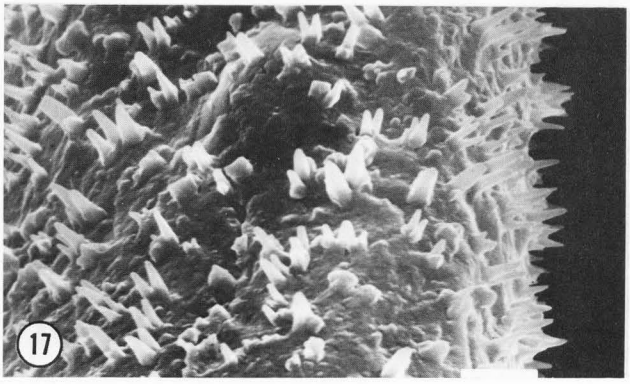
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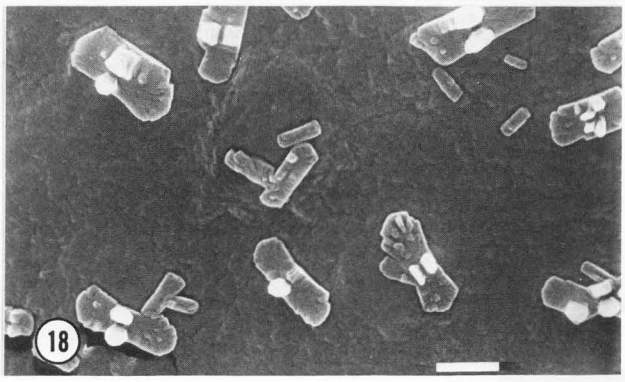
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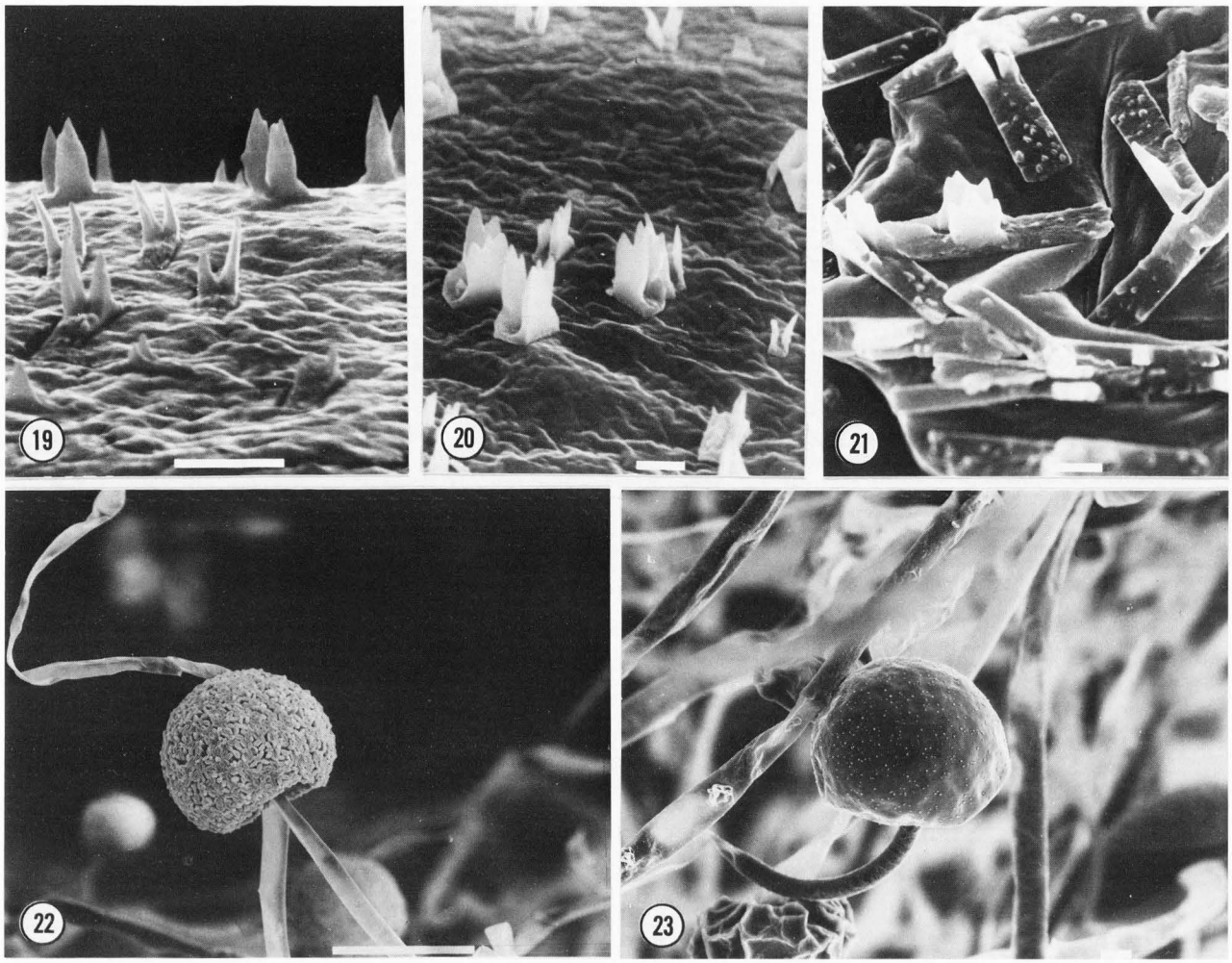

Figures 10-23. SEM views of sporangia of Rhizopus oryzae.
Figure 10. Young sporangium with sparse crystals. Bar = 10 µm.
Figure 11. Early crystal development. Note covering of some crystals. Bar =1 µm.
Figure 12. Early sporangium with some exposed and some covered crystals. Bar = 1 µm.
Figure 13. Early sporangium with some exposed crystals. Bar = 1 µm.
Figure 14. Maturing sporangium with many exposed crystals. Bar = 10 µm.
Figure 15. Mature sporangium with fully exposed crystals. Note typical twin peaked crystal morphology. Bar = 1 µm.
Figure 16. Emerging spines showing similarity to spines of Mucor. Bar = 1 µm.
Figure 17. Emerging spines in side view. Note similarity to spines of Mucor. Bar =1 µm.
Figure 18. Top view of mature crystals. Note the twin nature of spines. Bar = 1 µm.
Figure 19. Maturing sporangium with emerging crystals. Note variance in morphology. Bar = 10 µm.
Figure 20. Mature crystals showing three-parted spines. Bar = 1 µm.
Figure 21. Mature sporangium with atypical crystal morphology. Bar = 1 µm.
Figure 22. Mature sporangium just prior to spore release. Note total loss of outer layer and crystals. Bar = 100 µm.
Figure 23. Mature sporangium with outer layer intact. Note slight molding to spores beneath. Bar = 10 µm.

In some ways each of the two spines in this case bear a strong similarity to the spines of Mucor hiemalis in that they have an expanded basal portion attached to a flat plate (Figs. 16 and 17). Rather than being attached in the center of the flat plate, however, each spine is attached at the opposite edge of the basal plate (Fig. 18). As the culture ages some of the spikes occasionally show some variation in shape (Figs. 19-21), though most remain pointed. Prior to spore release a sporangium loses its outer layer along with the associated crystals (Fig. 22). The loss of the outermost layer is also seen if ethanol is used to dehydrate in the preparation for critical point drying. This can be contrasted with mature sporangia with the outer layer intact (Fig. 23). Some molding onto the underlying spores can be noted in these older sporangia but not as pronounced as that in Mucor (Fig. 5).

Most hyphae of both Rhizopus oryzae and Mucor hiemalis show no crystals, but sporangiophores of both have crystals on their surface (Figs. 24 and 25). The morphology of these crystals is somewhat similar to that found on the sporangia.

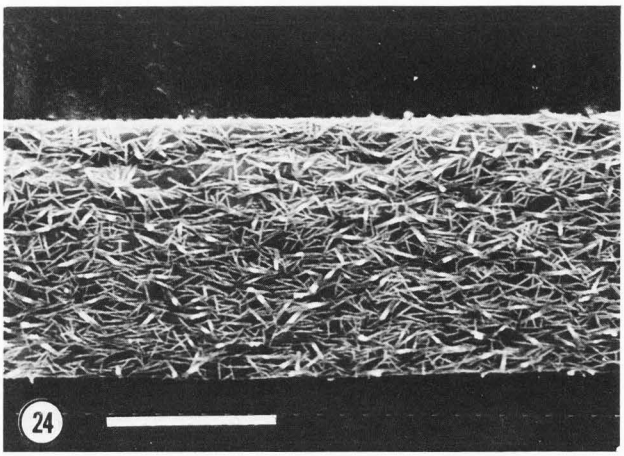

Figure 24. SEM view of mature sporangiophore of Rhizopus oryzae. Bar = 10 µm.

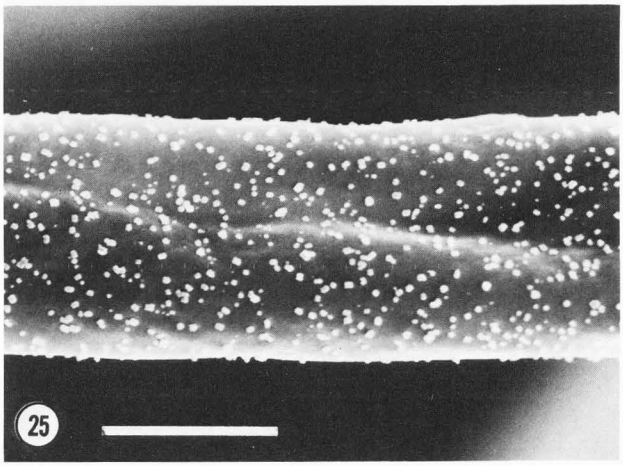

Figure 25. SEM view of mature sporangiophore of Mucor hiemalis. Bar = 10 µm.

Discussion

Members of the Mucorales make convenient subjects for the study of calcium oxalate formation in fungi. They are common inhabitants of soil and leaf litter and are readily grown in vitro on common media. Their rapid growth allows the study of complete development in a short time span.

The question of whether the crystals are formed external to or within the layer of cell wall remains unknown. Graustein et al. (1977) suggests that oxalic acid is excreted through the cell wall and combines with external calcium ions in the environment to form the crystals. Conversely, Arnott (1982) and Horner et al. (1983) give evidence that the crystals are produced internally and are covered by a layer of cell wall. Our observations favor the latter mechanism in the organisms in this study. In Mucor hiemalis there is a short period where crystals are absent or very sparse followed by the appearance of the tips of the crystals across the entire surface of the sporangium. Likewise in Rhizopus oryzae there is a period where crystals are absent followed by rapid appearance on the surface. Clearly, in this organism there is a period where the crystals are covered by a thin layer of cell wall. It appears that as the sporangia mature the outer layer of the cell wall is stretched over the crystals. This type of crystal development is known in higher plants (Horner and Franceschi, 1978).

During early development of sporangia the outer layer covering the crystals can be removed by treatment with alkali. Urbanus et al. (1978) described a similar situation in zygophores of Mucor mucedo and speculated that this outer layer may consist of carbohydrate material. This seems likely since the cell wall material of Mucor mucedo is known to contain glucuronic acid (Datema et al., 1977). Current views on the ultrastructure of the fungal cell wall consider it to be a network of fibrils with the spaces filled by matrix polymers such as carbohydrates or glucuronic acid (Rosenberger, 1976). Such a structure can serve as a sink for reserve materials (Zonneveid, 1972) or possibly for metabolic end products (Dennis, 1949; Hodgkinson, 1977): Urbanus et al., 1978). It seems possible that calcium oxalate, formed as an end product of metabolism, is excreted through the plasma membrane (Gentile, 1954) and crystallized within the carbohydrate layer. Later, as the outer layer is stretched thin by sporangial growth, the crystals become exposed.

Still, many basic questions regarding the metabolic pathways of oxalate production and mechanisms controlling crystal development and shape are yet unanswered. While some evidence presented here suggests that crystal production in these fungi is internal, additional studies utilizing transmission electron microscopy are needed to investigate crystal origin.

Acknowledgements

We thank Dr. K. Whitney and Ms. W. R. Riza for help in preparation of this manuscript. This work was carried out in the Electron Microscope Laboratories of The University of Texas at Arlington and was supported in part with funds from the Graduate School, The University of Texas at Arlington.

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Discussion with Reviewers

H.T. Horner: Will you be able to grow enough sporangia crystals in culture to carry x-ray diffraction analysis in order to determine the hydration form?

Authors: To date this has not been possible. The extremely small size of the crystals makes isolation of large enough aggregates to test difficult. It is possible that some sort of concentration technique such as centrifugation may provide sufficient material.

H.T. Horner: Have you attempted to add calcium to the culture medium to see if it increases crystal production?

Authors: Calcium chloride was added to basal media in increments of a tenth of a gram per liter up to one gram. No effect on numbers of crystals was noted on either species. Attempts are being made to formulate a calcium free medium to test for the effect of lack of calcium, but total lack of calcium has yet to be achieved.

W.C. Graustein: In dilute abiotic solutions, weddellite, calcium oxalate monohydrate, is observed to form when the temperature is about 5° C. Whewellite, calcium oxalate monohydrate, is the form that precipitates from solution at higher temperatures. Did you observe any difference in the morphology of the crystals produced by the fungi that you cultured at low temperatures?

Authors: Individual crystal morphology remained unchanged in both species at low temperatures. The arrangement of crystals appeared more dense as temperatures were lowered. We speculate that this difference may be due to changes in physiology at lower temperatures.