Morphological Observations of Diplodia maydis on Synthetic and Natural Substrates as Revealed by Scanning Electron Microscopy

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Mycelial and spore morphology of Diplodia maydis were investigated by using scanning electron microscopy after growth on various media and natural substrates (oat and corn kernels, and corn husks). Of several specimen preparation methods studied, Parducz fixation followed by critical-point or freeze-drying gave adequate preservation for pycnidia, mycelia, and spores. Morphological characteristics were similar in rotary and reciprocal shaker cultures and differed from that found in stationary cultures in the amount of slime-like material produced and precipitated matter on the mycelial surfaces. In general, mycelial surfaces were smooth. Large areas of coalesced material were present in all samples examined. Slime-like material produced in liquid media appeared as a finely laced net, randomly appearing throughout the mycelia with bead-like structures present along the net. A fine netting also was observed interspersed among the spores inside the pycnidia obtained from oats. Slime-like material was observed to cover the pycnidia produced on oat and corn kernels. In the latter case, the spores were less protected by the outer slime-like covering. Thickened node-like structures were observed in mycelial mats produced in modified Fries 2 medium, on potato dextrose agar plates, and on infected oats. Round and ovate thickened node-like structures were observed in mycelium produced on corn kernels. In general, node-like structures were less abundant in mycelia from naturally infected substrates. Conidia were commonly rounded to tapered and two celled, with a distinctive ridged septum at the middle. Dried spores were collapsed in a characteristic flask-like fashion.

Diplodia maydis (Schw.) Lev is one of the major pathogens causing stalk rot (4) of corn (Zea mays L.), a complex disease resulting in an annual loss of more than 10^8 bushels of corn in the corn-growing areas of the world (8). Many studies concerning resistance or susceptibility of corn to D. maydis have been reported (see 4, 8), but only a few studies have been reported on the in vitro growth of D. maydis (1, 2, 6, 10, 11), and none of these at the ultrastructure level.

The paucity of information concerning the gross morphology of D. maydis led us to examine (via scanning electron microscopy) the ultrastructural morphology of D. maydis grown on laboratory media and natural substrates to lay the groundwork for further studies of host-parasite interaction.

MATERIALS AND METHODS

Media. Four types of liquid media and one agar plate medium were used for growth and comparison of D. maydis. Czapek medium (CPK) consisted of: sucrose, 30 g; NaNO₃, 3 g; KH₂PO₄, 1 g; KCl, 0.5 g; and FeSO₄, 0.01 g, per liter of water. Richard's original medium (MR1) consisted of: KNO₃, 0.1 g; KH₂PO₄, 5 g; MgSO₄·7H₂O, 2.5 g; FeCl₃, 0.02 g; and carboxymethylcellulose (CMC), 12 g, per liter of water. Modified Richard's medium (MR2) consisted of: yeast extract, 1 g; peptone, 5 g (Difco); KNO₃, 10 g; KH₂PO₄, 5 g; MgSO₄·7H₂O, 2.5 g; FeCl₃, 0.02 g; and glucose, 12 g, per liter of water. Modified Fries medium (MF2) consisted of: NH₄H₂PO₄, 5 g; NH₄NO₃, 1 g; KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.5 g; CaCl₂, 0.13 g; NaCl, 0.1 g; and sucrose, 30 g, per liter of water. Potato dextrose agar (PDA) consisted of...
potato infusion, 200 g; glucose, 20 g; and agar, 15 g, per liter of water.

**Growth conditions.** Cultures were maintained at 25°C on sterile oats inoculated with D. maydis and on potato dextrose agar plates. The inoculum for the liquid media and PDA plates was prepared by transferring a 5-mm plug of D. maydis grown for 4 days on PDA. Oats were inoculated with a few infected oat kernels from maintenance cultures of D. maydis. Spores were obtained from maintenance oat cultures by adding 10 ml of sterile distilled water into an inoculated oat flask, allowing spores to swell for 10 to 15 min with occasional gentle agitation of the flask, and decanting the suspended spores. For purposes of comparison, D. maydis was grown at 25°C in MR2 medium on a rotary shaker, a reciprocal shaker, and under stationary conditions. In other studies D. maydis was grown for 5 days at 25°C in CPK, MR1, or MF2 media. Corn kernels and corn husk tissue from field-grown plants infected with D. maydis were provided by A. L. Hooker.

**Fixation and specimen preparation methods for scanning electron microscopy.** To determine a suitable fixation procedure for D. maydis mycelia and spores, the glutaraldehyde and Parducz fixation methods were tested. In glutaraldehyde fixation, mycelia and spores were immersed for 1 h in 0.1% s-collidine-buffered glutaraldehyde (Electron Microscopy Sciences), followed by three washes in buffered s-collidine and a subsequent 1-h fixation in 1% s-collidine-buffered glutaraldehyde, followed by 10 washes in s-collidine buffer. In Parducz fixation (9), mycelia and spores were immersed for 10 min in 6 parts of 2% OsO4 to 1 part of saturated HgCl2, followed by 10 washes in a buffered solution. Both fixatives were used alone as well as in conjunction with each other. Freeze-dried (Edwards Pearse tissue dryer) preparations were compared with air-dried preparations on all samples examined. Mycelia grown on PDA were compared by using freeze-drying, critical-point drying (Bomar 900EX or Denton critical-point drying apparatus), and air-drying.

**Scanning electron microscopy.** Fixed and dried D. maydis mycelia, infected corn kernels, corn tissue, and infected oat kernels were attached to Cambridge specimen stubs by using double sticky tape slightly melted with acetone. Spores were pipetted onto specimen stubs by the method of Murray and Campbell (7). Samples were rendered electrically conducting by evaporating a thin 40:60 palladium gold alloy coating onto the surface in a vacuum evaporator while the samples were simultaneously tilted and rotated to insure even coating. The specimens were examined in a Cambridge Stereoscan Mark 11A scanning electron microscope operated at 20 kV.

**RESULTS**

**Fixation results.** Glutaraldehyde fixation followed by Parducz fixation and freeze-drying gave the same morphological preservation as Parducz fixation alone followed by freeze-drying (compare Fig. 1a with Fig. 1c, illustrating well-preserved mycelia without collapse, and Fig. 1b with Fig. 1d, illustrating well-preserved spores). Glutaraldehyde fixation followed by freeze-drying resulted in mycelia and spore collapse. All air-dried samples and the samples unfixed and freeze-dried resulted in collapse of mycelia and spores as well as a matting of the mycelia and slime material.

**Freeze-drying versus critical-point drying.** In comparing preparation methods, mycelia were preserved equally well with fixation and freeze-drying (Fig. 2a and b) and fixation and critical-point drying (Fig. 2c and d). The advantages and disadvantages of each method are discussed by Boyd and Vesely (3) but, considering equal preservation, critical-point drying is of greater advantage because the drying time (45 min) is considerably less than that for freeze-drying (4 h).

As expected, air-dried mycelia, fixed or unfixed, resulted in collapse and matting (Fig. 2e).

**Rotary versus reciprocal shaker cultures versus stationary cultures.** D. maydis mycelia obtained from rotary shaker and from reciprocal shaker cultures were well preserved and had a similar overall appearance (Fig. 3a and b). D. maydis grown in stationary cultures, however, was found to be surrounded by slime-like material. The copious precipitate on the mycelia surfaces is illustrated in Fig. 4a and b, and a coalescence of the slime material is shown in Fig. 4c. Coalescence of slime-like material does not appear to be an artifact of fixation since unfixed controls showed the same type of coalescence.

**Comparisons on different growth media.** (i) **MR2 medium.** Figure 3a illustrates the mycelial organization in a mycelial pellet. Fixed and air-dried samples collapsed, and mycelia appeared to be matted. This is even more evident in samples which were air-dried without fixation.

Figure 4a illustrates the overall mycelial appearance after growth for 4 days in stationary cultures. Copious particulate matter is found on the surface of mycelia grown in stationary cultures (Fig. 4b); also apparent is the coalescence of slime-like material over the mycelia, as indicated by an arrow in Fig. 4c. This might be expected, as copious quantities of slime-like material are elaborated during the growth of D. maydis in liquid media (1, 2, 6). The greatest amount of the precipitate is found on the mycelia grown under stationary conditions.

Figure 5 shows a finely laced net randomly appearing throughout the mycelia with small bead-like structures interspersed along the net.
The amount of netting interlaced in the mycelia was well correlated with the amount of slime-like material elaborated by the fungus during growth.

The fungus grown in MR2 medium elaborated the least amount of slime-like material when compared with growth in MR1, MF2, and CPK media.

(ii) MF2 medium. The mycelial pellet has a slightly different overall appearance when grown in MF2 medium in that it showed less regularity than found in MR2 medium. Typically, a film of secreted material overlying and stretched between the mycelia is apparent (Fig. 6a). Compared with cultures grown in MR2 medium, there is a greater abundance of bead-like structures interlaced throughout the mycelia (Fig. 6b and c), and larger thickened node-like structures are observed. The mycelia from cultures grown in MF2 and MR2 media both have a smooth surface with no discernible characteristic structures.
(iii) CPK medium. When grown in cpk medium, the mycelial pellet has a honeycomb-like appearance. In juxtaposition, one can see the honeycomb netting and smooth-surfaced mycelia (Fig. 7a); in certain areas, the honeycomb-like appearance takes on a more or less regular pattern (Fig. 7b).

Mycelia were of two types: those covered with particulate material and those with a smooth surface, relatively free of other material.

The bead-like structures and the coalescing of these structures noted in cultures grown in MR2 and MF2 media are also apparent in cultures grown in CPK medium (Fig. 8a and b). The thickened node-like structures observed in MF2-grown cultures were not observed in CPK-grown cultures.

(iv) MR1 medium. After 6 weeks of growth under stationary conditions in MR1 medium, the mycelial pellets were engulfed in a gel-like matrix; sporulation had occurred and the formation of bead-like structures and a coalescence of slime material were also noted. The mycelium appeared to be composed of a continuous series of bulges (Fig. 9) suggestive of intercalary spores, although these are not reported to occur in this organism.

(v) PDA medium. After 5 days of growth on PDA plates, coalescing of slime material on the smooth-surfaced mycelium, thickened nodes, and bead-like structures was observed. However, nodes and bead-like structures were not as abundant as in cultures grown in liquid media. This may be due to the relatively dry growth conditions in agar plate culture.

D. maydis spores are ovate, most commonly two-celled and slightly curved with rounded to tapered ends. Figure 10a illustrates the most commonly observed spore shape where a septum between the cells can be noted. Air-drying of D. maydis spores mimics the condition in which the spores are found in naturally infected tissues. Air-dried spores are generally flask shaped (Fig. 10c) or collapsed (Fig. 10b).

The average size of noncollapsed spores was 25 by 4 μm. Size calculations are only approximate due to the variable specimen to beam angles inherent in the scanning electron microscope.

(vi) D. maydis on oat substrate. On oats, D. maydis produces thousands of spores in small, black, flask-shaped pycnidia (Fig. 11a). Although the pycnidia may be globose, flask shaped, or irregular in shape, a typical pycnidium is illustrated in Fig. 11b and c. Pycnidia are filled with spores (Fig. 11b), and an opening (Fig. 11d) in the pycnidium for the dissem-
Fig. 3 (a) Typical appearance of D. maydis mycelial pellet obtained from rotary shaker and a reciprocal shaker. Parducz fixed and freeze-dried. Marker represents 10 μm. x24. (b) Typical appearance of D. maydis mycelia grown in rotary and reciprocal shaker cultures. Parducz fixed and freeze-dried. Marker represents 1 μm. x6,250. All cultures were grown in MR2 medium at 25°C.

Fig. 2. D. maydis grown in PDA plates. Parducz fixed, and dried as indicated. (a, b) Mycelia preservation in Parducz-fixed and freeze-dried samples. Markers represent: (a) 1 μm (x1,200); (b) 1 μm (x5,100). (c, d) Mycelia preservation in Parducz-fixed and critical point-dried samples. Markers represent: (c) 1 μm (x2,400); (d) 1 μm (x5,150). (e) Mycelia preservation in Parducz-fixed and air-dried samples. Note mycelial collapse and matting. Marker represents 1 μm. x2,100.
Fig. 4. Stationary-grown cultures of D. maydis, Parducz fixed and freeze-dried. (a) Note copious amount of particulate matter precipitating on mycelia. Marker represents 10 μm. ×1,000. (b) Note particulate nature of
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Fig. 5. D. maydis grown in MR2 medium, Parducz fixed and freeze-dried. Note the laced net randomly appearing throughout the mycelia, with small bead-like structures along the net indicated by an arrow. Note that in MR2 medium the fungus exhibits the least amount of beaded netting. Marker represents 1 μm. ×2,400.

DISCUSSION

Glutaraldehyde fixation followed by Parducz fixation and critical-point or freeze-drying resulted in the same morphological preservation as Parducz fixation alone followed by critical-point or freeze-drying. Mycelia and spores collapsed if treated with glutaraldehyde fixation followed by freeze-drying or air-drying, or prepared by freeze-drying or air-drying without fixation. From these results, we decided to routinely use the double-fixation procedure for field studies (glutaraldehyde fixation in the field followed by Parducz fixation and critical-point or freeze-drying after returning to the laboratory) and eliminating the glutaraldehyde fixation when sampling D. maydis from laboratory cultures. The collapse in mycelia, spores, and pycnidia observed in the air-dried field collections (husks and kernels of corn) are believed to be real and not induced by a fixation procedure.

Major variations in fungal morphology were not observed due to culture conditions (rotary, reciprocal, or stationary liquid culture) or media, although the greatest amount of precipitate was observed on mycelia from stationary liquid culture. Apparently, the slime-like material is dispersed by movement in rotary and reciprocal shaker culture. Fine-lace netting with beads was found in all synthetic cultures. Minor differences appeared in the amounts of slime-like material observed and in the presence or absence of thickened nodes. Also apparent in MR1 medium was cell-like bulging of the mycelia. These cell-like bulges are suggestive of intercalary spores; however, these are not reported to occur in this organism, and no cross-walls (indicative of intercalary spore formation) are found in thin-section studies (Murphy, unpublished data).

From this study, it appears that the degree of coalescence of slime-like material progresses as follows: the formation of bead-like structures (Fig. 6b); progressive accumulation of these precipitate on mycelia at a higher magnification. Marker represents 1 μm. ×5,000. (c) Note coalescence of slime material. Marker represents 10 μm. ×180. All cultures were grown in MR2 medium at 25 C.
Fig. 6. *D. maydis* grown in MF2 medium, Parducz fixed, and freeze-dried. (a) Note the thickened film of secreted material overlying and stretched between the mycelia. Marker represents 10 μm ×1,000. (b) Note the delicate bead-like structures interlaced throughout the mycelia along a laced netting. Compare with Fig. 5, where in MR2-grown samples only very sparse bead-like structures are observed. Marker represents 10 μm ×1,000. (c) Higher magnification illustrating bead-like structures. Marker represents 1 μm ×5,000. (d) Note thickened node-like structures which appear much larger than bead-like structures. Marker represents 1 μm ×2,000.
Fig. 7. *D. maydis* grown in CPK medium, Parducz fixed and freeze-dried. (a) In juxtaposition, smooth-surfaced mycelia and the honeycomb netting can be observed. Marker represents 10 μm. ×620. (b) Regular pattern of honeycomb-like netting. Marker represents 10 μm. ×600.
Fig. 8. *D. maydis* grown in CPK medium. Parducz fixed and freeze-dried. (a) Abundance of bead-like structures. Compare with Fig. 5 and 6b. Marker represents 10 μm. ×1,200. (b) Coalescence of bead-like material into a film overlying and stretched between mycelia. This observation compares with that found in MF2-grown cultures (Fig. 6a). Marker represents 10 μm. ×980.
structures (Fig. 8a and b); formation of thickened film of secreted material overlying and stretched between the mycelia (Fig. 6a); and finally a coalescence of the slime-like material (Fig. 4a). We believe that the same type of progression results in matting and coalescence of mycelia in the pycnidium. It should be realized that these suggestions are made from studying hundreds of micrographs, and the coalescence sequence is only briefly represented in the five micrographs listed.

The slime-like material appears to be different from the material precipitated on the mycelia. However, it may be that the two arise from the same metabolic pathways since both seem to occur simultaneously.

Why the great variation in amounts of slime-like material observed with the culture media tested is not known. It may be that the slime-like substance produced by D. maydis plays a role in the prevention of desiccation under normal conditions. One variation that did occur in the mycelial organization in the cultures was the formation of a honeycomb netting of mycelia in CPK medium.

Why thickened nodes were observed in MF2 and PDA media and were not found in MR2, CPK, and MR1 media is also not known. Again, in thin-section studies (Murphy, unpublished data), cross-walls are not found. Perhaps the nodes represent storage material in the hyphae and occur only when certain metabolic products are in excess, depending on the food source. The amount of slime secreted by the fungus does not seem to be correlated with the presence or absence of thickened nodes.

When comparing morphology of D. maydis on artificial versus natural substrates, the biggest difference appears to be in the formation of pycnidia. On natural substrates, these occurred along vascular bundles of the corn husk and along the oat and corn kernels. The tightness of the pycnidium was least on corn kernels and greatest on infected oats. The presence of fine lace netting with beads on infected oats but not on corn kernels or husks, and the lack of thickened nodes in fungal samples from infected corn husks but not corn kernels or oats may indicate significant nutritional and environmental differences resulting in physiological differences in spores of the pathogen. Similarly, there may be differences in these characteristics when host-pathogen responses with a wide variety of cultivars and pathogen isolates are studied.

Regardless of these possibilities, from our study we suggest that there should be little morphological differences expected in the pathogen mycelia, spores, and pycnidia. Although scolecospores have been reported (5) in certain
Fig. 10. _D. maydis_ spores. Specimen preparation as indicated. (a) _D. maydis_ spore, Parducz fixed and freeze-dried. Most commonly appearing spores are two celled, slightly curved, with rounded to tapered ends. Note the septum between the two cells. Marker represents 1 μm. ×4,500. (b) _D. maydis_ spores, air-dried without fixation, illustrating collapsed shape found commonly on naturally infected tissue. Marker represents 1 μm. ×4,500. (c) _D. maydis_ spore, air-dried without fixation, illustrating flask-shaped spore, commonly found on naturally infected tissue. Marker represents 1 μm. ×4,500.

Fig. 11. _D. maydis_-infected oats. (a) Randomly occurring pycnidia developing on oat surface. Note the variation in size and shape. Also indicated by an arrow are empty pycnidia which have oozed out their spores from within. Marker represents 100 μm. ×21. (b) Top view of pycnidial fructification. Marker represents 10 μm. ×130. (c) Side view of pycnidial fructification. Marker represents 10 μm. ×250. (d) Pycnidial opening illustrating spores within. Marker represents 10 μm. ×250. (e) Higher magnification of Fig. 11d illustrating spores inside of pycnidium. Marker represents 1 μm. ×1,250.

Fig. 12. _D. maydis_-infected oats. (a) Interior of pycnidium illustrating fine-laced netting interspersed between the spores. A few bead-like structures are present as indicated by arrow. Marker represents 10 μm. ×660. (b) Interior of pycnidium showing spores to be engulfed in a thick gel-like matrix. Marker represents 1 μm. ×1,250. (c) Interpycnidial mycelia. Marker represents 1 μm. ×1,250. (d) Thin film overlying and stretching between mycelia, as was also observed in mycelia grown in synthetic media. Marker represents 1 μm. ×1,350. (e) A more extensive coalescing of slime material similar to that observed in synthetic media. Marker represents 10 μm. ×875. (f) Thickened node-like structures, as also observed in cultures grown in MF2 and PDA. Marker represents 1 μm. ×2,500.
Fig. 13. *D. maydis*-infected corn husks. (a) Naturally infected corn husks showing surface dotted with pycnidia in a relatively random distribution. Marker represents 100 μm. ×20. (b) Naturally infected corn husks showing surface dotted with pycnidia. Note that pycnidia appear to be closely associated with vascular bundles. Marker represents 100 μm. ×24. (c) Typical two-celled, flask-shaped spores found in pycnidia from naturally infected substrates. Marker represents 1 μm. ×2,100. (d) Collapsed mycelia with a smooth surface. Marker represents 1 μm. ×2,100.
FIG. 14. *D. maydis* on corn kernel substrates. (a) General overall appearance of *D. maydis* grown on corn kernel substrate. Note random distribution of infection. Marker represents 100 μm. ×26. (b) Pycnidium observed on infected corn kernel. Note looseness of mycelia-covered pycnidia. Compare with pycnidia found in oats and infected corn husks. Marker represents 10 μm. ×230. (c) Low magnification micrograph illustrating typical nodes found in all corn kernel samples examined. Marker represents 10 μm. ×575. (d) Higher magnification micrograph illustrating typical round and ovate nodes found in samples from all six areas. Marker represents 1 μm. ×2,300.
strains of *Diplodia*, and including *D. maydis*, none were observed in the material studied.

From this study, we have enough morphological data about this pathogen to identify it if observed in corn stalk tissue infected with *D. maydis*. With the scanning electron microscopy preparation methods now available, and with knowledge about the morphological differences in various synthetic and natural environments of the pathogen, several other investigations of *D. maydis* can be undertaken. Of particular interest now is the documentation and clarification of the stalk rot process as *D. maydis* penetrates its natural host and spreads from cell to cell within the stalk, over kernels, and through the husk tissue. Included in this is the need to study the digestion process and changes in morphology when production of cellulolytic enzymatic enzymes are stimulated and their release occurs (1, 2).

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