Ultrastructure and Mechanics of the Conidium-Conidiophore Attachment of Helminthosporium maydis

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ABSTRACT

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The structural and mechanical adaptations of *Helminthosporium maydis* for conidium dispersal were examined. The structure of the attachment of mature conidia to conidiophores was determined by thin-section electron microscopy of attached or partially detached conidia and their conidiophores. Serial section analysis showed that six of the eight mature conidia examined were attached solely by a narrow isthmus of wall material. The forces required for conidium removal were measured by centrifugation of leaf lesions collected in the field and induced to sporulate in the laboratory. Based on the observed structure of the isthmus and the measured forces, it was estimated that the material comprising the isthmus was about as strong as wood fiber. A less-frequent type of attachment, involving a finger of cytoplasm which extended from the conidiophore into the base of the conidium also was observed.

Additional key words: corn leaf blight, cell walls, dispersal, epidemiology.

The dissemination of certain plant pathogens, such as *Helminthosporium maydis,* which produce dry, aerial conidia, depends largely on wind for removal and dispersal of conidia from conidiophores. It was previously shown how the force required to detach conidia of *H. maydis* (2) can be related to the wind requisite for removal (3, 4). In this report, we further explore the mechanical properties of the conidium-conidiophore attachment by applying force at three different angles with respect to the attachment axis and relating that to the ultrastructure of mature attachment sites. A mechanical model, based on the present ultrastructural study and on the measured forces required for conidium removal, permits a reasonable estimate of the strength of the biomaterial which forms the attachment.

MATERIALS AND METHODS

Ultrastructural studies.—Dried corn (*Zea mays* L.) leaves with lesions caused by *Helminthosporium maydis* Nisikado and Miyake race T were cut across the lesions, and the cut edges were placed against sterile moistened filter paper in petri dishes. The dishes were incubated for 13 days at room temperature (~ 22 C) with continuous cool-white fluorescent illumination.

Filter paper bearing sporulating mycelium was cut into squares. Each square was dipped slowly, once or twice, into molten (~ 50 C) 2% water agar containing 0.2% "Tween-20" and either 1% glutaraldehyde (schedule A) or 1% acrolein (schedule B). The squares were then fixed for a further 15 minutes in 1% glutaraldehyde (schedule A) or acrolein-TAPO (7) (schedule B) in phosphate buffer at pH 7.2. Further processing involved an overnight soak in 4% unbuffered OsO_4, a similar soak in 0.5% aqueous uranyl acetate, acetone dehydration, propylene oxide rinses, and Epon-Araldite embedment.

Cells for electron microscopy were selected (1) and photographed in polymerized plastic by bright-field light microscopy prior to thin-sectioning. Serial sections, up to or beyond median, were obtained and photographed in each case. They were post-stained in lead citrate and viewed in a Philips EM 200 electron microscope. Some difficulty was experienced with the conidia falling out of the sections (9). This apparently was due to unsatisfactory embedment of the primary conidial wall layer. All other wall layers were well embedded. Six attachment sites (conidium and conidiophore attached or slightly separated) and two individual (detached) conidia were examined.

Centrifugation experiments.—Corn (*Zea mays* L., Clyde Black and Son, Inc., Ames, Iowa. Hybrid B37 Tms Ht × BIYA Ht) planted in the field 14 May 1975 was inoculated with *H. maydis* Nisikado and Miyake race T on 30 June. Infected leaves were excised from the plants in mid-August and incubated in moist chambers at 22 C for 15 hours in the dark. The leaves then were illuminated in the moist chambers with cool-white fluorescent light at an intensity in the visible spectrum of about one-tenth full sunlight. When needed, lesions were taken from the moist chambers, affixed to a sample holder, and centrifuged; conidium removal and size was assessed as described earlier (2). In that study the applied force gave a torque about the region of attachment and a compression force along the conidium-conidiophore axis. Here, by using three different orientations of lesions, and thus of erect

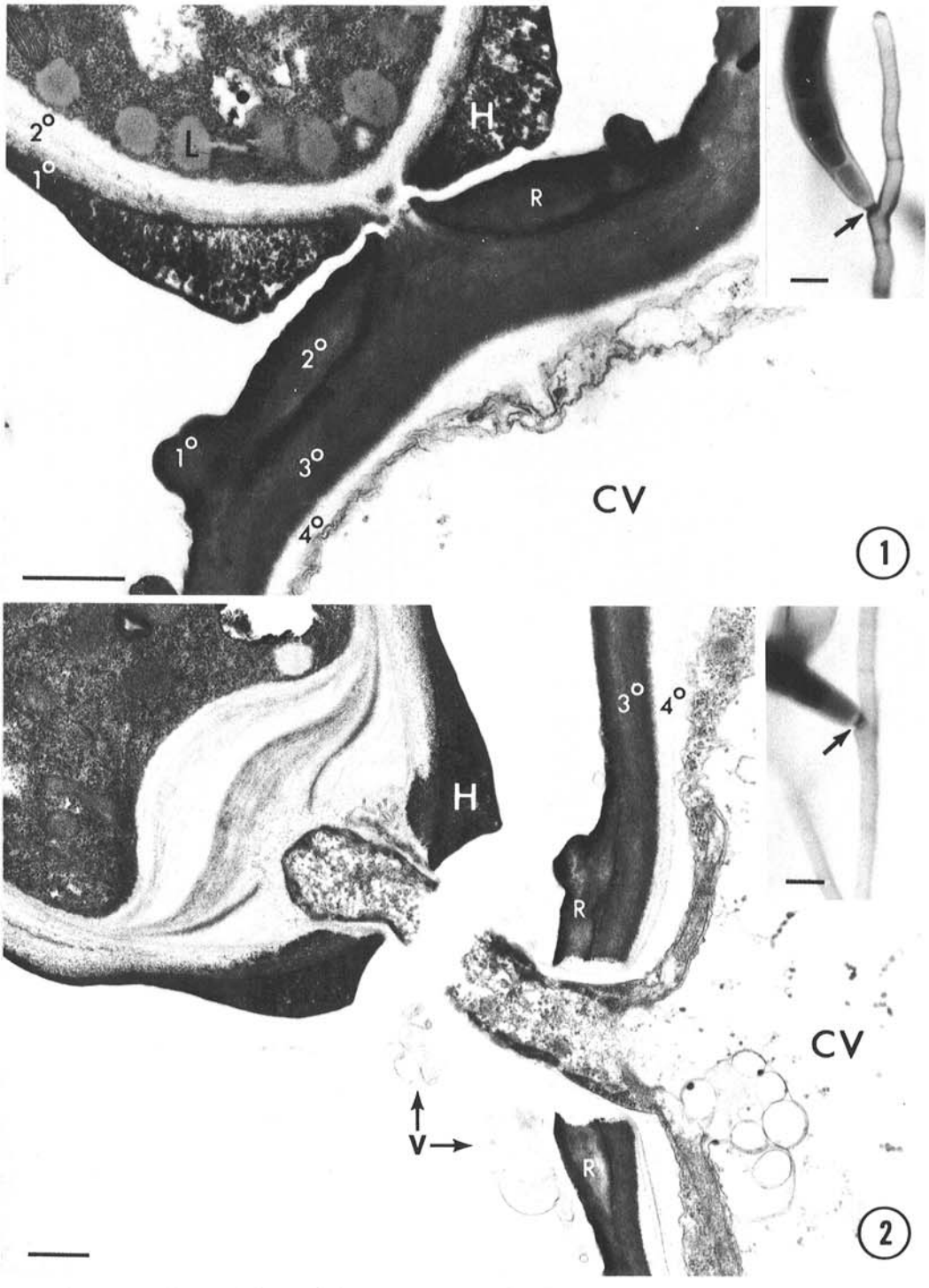

Fig. 1, 2. Micrographs of two conidium-conidiophore attachment sites of *Helminthosporium maydis* race T. **1)** An example of the usual wall-isthmus type of attachment. The conidium has been dislodged slightly (<0.1 μm) from the conidiophore; nevertheless, the relations between wall layers are obvious. The point of attachment, shown in median section, is an isthmus formed by the secondary and tertiary walls of the conidiophore and the primary and secondary wall layers, respectively, of the conidium. Note the regular arrangement of lipid bodies in the base of the conidium. Inset shows the same attachment site (arrow) as it appeared with light optics during the cell selection procedure. **2)** An example of the cytoplasmic type of attachment. A complete series of sections through the entire attachment was studied; a median section is shown. This conidium was also detached, but the figure clearly shows that a bridge of cytoplasm from the conidiophore occupied the region usually filled with wall material. Note the absence of lipid bodies in the conidium base, the enlarged secondary conidium wall, the altered staining pattern of walls adjacent to the cytoplasmic bridge, and the extracellular vesicles. Inset shows the same attachment site (arrow) as it appeared with light optics during the cell selection procedure. Glutaraldehyde-OsO_4 fixation. Scale bar calibration: electron micrographs = 0.5 μm; insets = 10 μm. Legend: CV = central vacuole, H = hilum, L = lipid body, R = annular ring, V = extracellular vesicles, 1° = primary wall, 2° = secondary wall, 3° = tertiary wall, 4° = quaternary wall.

conidia (Fig. 3-A, 64°; -B, 26°; and -C, 0°), with respect to centrifugal force, we were able to cause tension, rather than compression, along the conidium-conidiophore axis and study the possible effects of flexure of the conidiophore on conidium removal. Centrifugation in case C was limited by a tendency for the leaf sample to bulge. Not all conidia within a sample population were erect. The distribution in angles between the long axis of the conidium and the axis perpendicular to the leaf surface was estimated for each sample centrifuged and was measured from photomicrographs of selected samples. This could be accomplished because the projection in the plane of the photograph gave an estimate of the angle; the average ratio of conidium width to length was about 1:5.

RESULTS

Ultrastructure.—Fixation by either glutaraldehyde (schedule A) or acrolein-TAPO (schedule B) gave comparable results. Conidiophores generally had four wall layers (Fig. 1, 2). The secondary wall layer, which contained the annular ring, extended for some distance below the attachment site, but ended abruptly just above it. Concentrated in the region near the site of conidium formation was an amorphous, discontinuous, densely staining primary wall layer (Fig. 1).

Conidia had two wall layers. The primary wall was enlarged at the conidium base forming the hilum, which was particularly well-developed in these mature conidia. A net-like arrangement of irregularly thickened strands gave a sponge-like appearance to the hilum. Apparently the hilum is strong and rigid, since any pressure which may have been exerted on its outer rim due to removal during processing did not result in detectable compression of its structure. In the bases of some conidia, a densely staining layer and/or a ring of material was present in the secondary wall.

The most frequently encountered mature conidium-conidiophore attachment was composed solely of a narrow isthmus of cell wall material in which the secondary and tertiary walls of the conidiophore were continuous with the primary and secondary walls, respectively, of the conidium (Fig. 1). The wall fibrils in the isthmus seemed to pass uninterrupted between the conidiophore and the conidium. In one case, a few cytoplasmic remnants were found entrapped in the isthmus. This wall-type of connection was observed in four of the six attachment sites studied and was evident in the two detached conidia. The hilum did not appear to be connected to the annular ring. However, it seems likely that there was contact between these two structures at undisturbed, mature attachment sites, since the space between them (Fig. 1) apparently is the result of slight

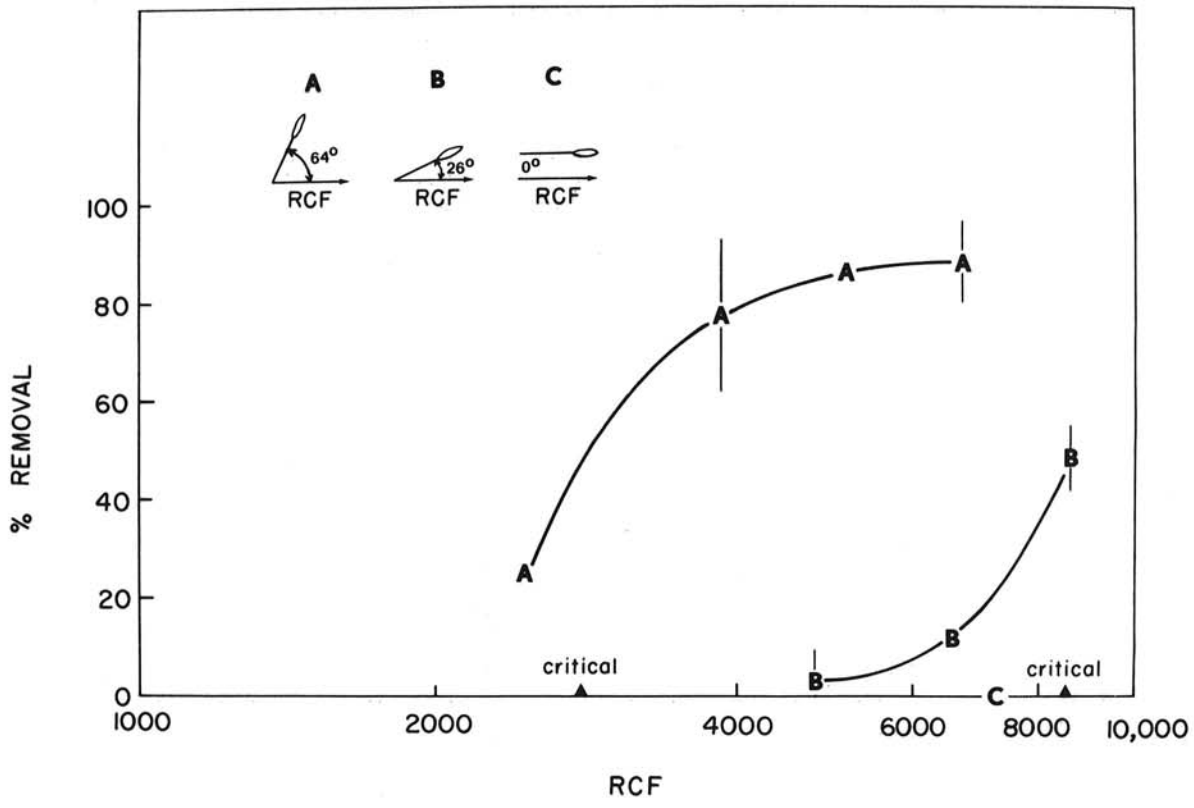

Fig. 3. Percent removal, by centrifugation, of conidia of *Helminthosporium maydis* race T vs. relative centrifugal force (RCF). RCF is W^2r/g, where W is the angular velocity of centrifugation, r is the radius of gyration, and g is the acceleration of gravity. The three orientations of the RCF vector in relation to an erect conidum are shown schematically (top). The vertical bars through data points represent the standard deviation of replicate samples. The "critical" points on the abscissa are the RCF's which cause 50% removal of conidia for A and B, respectively.

separation that occurs during preparation. The ultrastructure of the hilum was quite different from that of the annular ring (Fig. 1).

Two other conidia were attached to their conidiophores by means of cytoplasm-cell wall bridges (Fig. 2). Both of these attachments were uniquely characterized by (i) a bridge of cytoplasm bounded by the quaternary conidiophore wall, (ii) a secondary conidium wall greatly enlarged at the base, (iii) no orderly accumulation of lipid bodies at the base of the conidium, (iv) an altered staining pattern and density of the walls of both the conidium and the conidiophore (except the quaternary wall) in a zone bordering the cytoplasmic bridge, and (v) extracellular vesicles in the region of attachment.

Mechanics.—Figure 3 shows the removal of conidia having different orientations (A through C) with respect to the centrifugal force vector as a function of relative centrifugal force (RCF). The relative centrifugal force, RCF, is the centrifugal force normalized by the weight of the particle; e.g., RCF = 1,000 corresponds to a force on the conidium equivalent to 1,000 times its own weight.

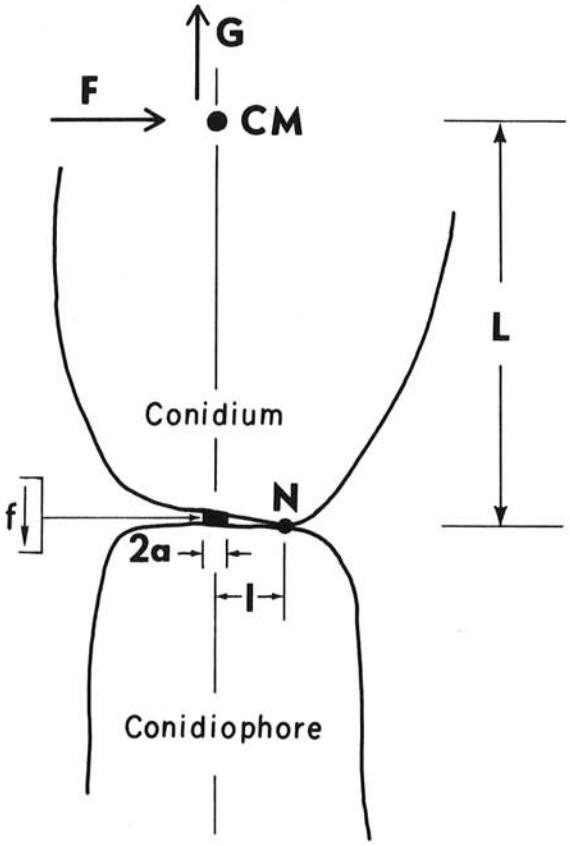

Fig. 4. A sketch of the mechanical model of conidium removal. Vector force F is the component of RCF acting perpendicular to and G the component of RCF acting parallel to the long axis of the conidium. CM indicates the center of mass of the conidium (here taken to be at the mid-length, L), a is the radius of the isthmus connecting the conidium to its conidiophore and l is the radius of the hilum. The stress in the isthmus was determined by considering bending moments about the point N (see text).

About 50% of these conidia in orientation A were removed at RCF = 2,800 and about 10% appeared to be nonremovable, as observed previously (2). For orientation B, 50% removal did not occur until RCF was increased to 8,600. Finally, for C, no conidia were removed at RCF up to 7,300. In general, removal increased with increasing applied force in agreement with previous results (2).

The ultrastructural (Fig. 1) and mechanical (Fig. 3) information facilitates an estimate of the ultimate strength of the material comprising the isthmus. Effects of the various force vectors on the connecting wall isthmus are shown schematically in Fig. 4. The centrifugal force, taken to act at the conidium's center of mass (CM), can be resolved into component vectors directed parallel (G) and perpendicular (F) to the conidium. The perpendicular component of force (RCF sin θ) generates a bending moment about point N. The equilibrium condition for the moments is given by Stippes et al. (10):

$$F \cdot L - f \cdot l = 0 \qquad \text{(Eq. I)}$$

where the moment arm L is the distance from the region of attachment to the conidium's CM, f is the force induced in the isthmus resisting the bending due to F, and l is the moment arm through which f acts. The stress in the isthmus is given, approximately, by f divided by the cross-sectional area of the isthmus (πa^2; see 2a in Fig. 4), in which f equals $F \cdot (L/l)$ from equation (I). Therefore, the critical stress causing fracture σ_c, or, the ultimate tensile stress the isthmus can withstand is:

$$\sigma_c = \left[\frac{F_c}{\pi a^2}\right]\left[\frac{L}{l}\right] \qquad \text{(Eq. II)}$$

where F_c is the critical force causing fracture; its determination will be explained below. We wrote the right hand side of equation (II) as two terms to emphasize that the stress in the isthmus due to the force component F is much larger [$L/l \simeq 40$] than the stress due to tension caused by component G which would be of the order of magnitude of the first term alone.

Because of the variation in conidium size and angle distribution, not all conidia experience the same force at the same RCF. When allowance is made for this variation in mass and angle the critical force for removal or, equivalently, the ultimate strength of the attachment, varies little from conidium to conidium (2). Therefore, the critical RCF for orientation A and B occurs at the 50% removal point; for A this RCF is 2,800 and for B it is 8,600 (Fig. 3). Furthermore, from Fig. 3 it is clear that the smaller the proportion of RCF which is represented by the F component the greater is the critical RCF.

To determine F_c for equation (II), we multiplied the critical RCF in case A by sin θ and by the weight of the average conidium. Assuming the density of the conidium to be 1.0, $F_c \simeq 0.028$ dynes. From micrographs of medium sections (e.g. Fig. 1) through the four examples of the wall isthmus type of attachment, the mean radius of the isthmus, a, was calculated to be about 0.19 μm and the half-width of the hilum, l, about 0.99 μm. Inserting these values and a mean conidium mid-length of 39.9 μm into equation (II) gives $\sigma_c \simeq 9.9 \times 10^8$ dynes/cm^2. For

comparison, this is about the strength of wood fiber (8).

DISCUSSION

The wall layers observed in both the conidia and the conidiophores generally were similar to those already described for this and related species, though designation of the different layers varies (5, 6, 9, 11). Dissimilarities were noted, and some appeared to be caused by differences in staining properties of specific wall layers. The quaternary layer of the conidiophore wall, as described here, has not been reported previously. Our results support the description by Brotzman et al. (5) of conidium-conidiophore wall relationships in *H. maydis* in early stages of conidiogenesis. At mature attachment sites the continuity between the primary wall layer of the conidium and the secondary wall layer of the conidiophore is a well-defined, thin-walled, hollow cylinder, in contrast to the corresponding, yet poorly defined, continuity in early conidiogenesis. The continuity between the secondary wall of the conidium and the tertiary wall of the conidiophore is more substantial. Apparently, maturation is accompanied by (i) extensive development of the hilum, (ii) increased staining density of the tertiary wall layer of the conidiophore, and (iii) development of primary and quaternary wall layers in the conidiophore.

There was no evidence in mature attachment sites that a septum had been present during conidiogenesis, as was reported for a related fungus (6). Septum formation probably would have resulted in partial interruption of the continuity of wall fibrils in the mature isthmus. We did not find evidence of such a discontinuity in our material. If a septum was present during early conidiogenesis, it was masked at mature attachment sites.

How the hilum develops in contact with the surface of the annular ring without becoming connected to it is an interesting problem. Several observations point to differences between these two structures. The secondary layer of the conidiophore wall is already well-developed before the hilum starts to form (5, 6), and it would seem that a new wall forming against an older one would be less likely to lead to interconnections than would two new ones developing simultaneously. The two wall layers in question were shown here to be quite distinct ultrastructurally. Chemical and/or structural differences may account for the fact that the entire primary wall of the conidium, including the hilum, was more difficult to embed in the plastic than were the other wall layers studied. One can argue that the hilum is quite different from the secondary wall layer of the conidiophore and that fusion between the two would not necessarily be expected to occur. In fact, the secondary conidiophore wall layer may *prevent* fusion of the annular ring with the hilum and thus maintain a relatively weak, tenuous attachment of the conidium.

The origin of the cytoplasmic type of attachment is not clear, but at least two possibilities may be envisaged; (i) the cytoplasmic channel present during early conidiogenesis may never have been occluded in the usual manner, or, (ii) after normal occlusion of the cytoplasmic channel, a finger of conidiophore cytoplasm may have grown through the wall isthmus into the conidium base. The latter possibility seems most logical in view of the marked cell wall alterations involved; it appears that the conidiophore cytoplasm enzymatically etched its way into the conidium. The process could function in weakening of the conidium-conidiophore attachment, thereby facilitating detachment.

The observed range of applied force required for removal of conidia probably is a function of three factors. First, the different types of attachment would probably account for some variability. Second, conidia were not uniformly sized but had a mean volume of 9×10^3 μm^3 with a standard deviation of 30% of the mean. This broadens the range of force necessary for removal, since larger conidia experience more force than smaller ones at a given RCF. Third, not all conidia were erect; nearly half were at an angle of between 10 and 25 degrees from the axis perpendicular to the leaf surface. This distribution in conidium angle would cause a widening of the range of force required for removal by varying the proportion of the RCF which is represented by the F component (Fig. 4).

Figure 3 shows that the smaller the proportion of RCF which is represented by the F component the greater is the critical RCF. However, the relationship is apparently not proportional, since the critical RCF increases faster than F decreases as θ is reduced: for case A versus case B, the ratio of the critical RCF's (8,600/2,800) is 3.1, whereas ($\sin 64° / \sin 26°$) is 2.1. This apparent discrepancy could be caused by bending of the conidiophores, which would tend to align them somewhat with the centrifugal force and further reduce F. For instance, a mere 11° bend in the conidiophores at removal would eliminate the discrepancy since $\sin (64°-11°)/\sin (26°-11°) \simeq 3.1$. Indeed, a calculation based on a long beam approximation (10) for the conidiophore and taking a Young's modulus of elasticity for wood shows that the amount of bending of the conidiophores would be about 12°. Earlier results (2) also are in accord with these principles.

Our model assumes that the bending moment around point N (Fig. 4) produces the critical stress in the isthmus. However, a more complex stress distribution could exist. The RCF acting on the eccentricity of the conidium [i.e., when the center of mass is not on the axis of torsion (10)], gives rise to a moment in torsion. If the conidium were free to rotate, this moment would provide for a shear stress comparable in magnitude to the stress developed in bending. However, because of the very narrow gap between the conidium and the conidiophore, and because the moment due to F forces the two surfaces into contact around point N, it is doubtful that twist occurs.

Helminthosporium maydis appears to have interesting adaptations which facilitate conidium dispersal. The connecting isthmus is strong enough to support more than 2,000 times the weight of the relatively large, mature conidium. This would assure that mature conidia remain attached until wind sufficient for dispersal occurs (2, 4). The small radius of the isthmus together with subsequent development of the hilum apparently creates a pivot point about which a bending moment can be generated. Rigidity of the hilum then would assure that wind would exert sufficient stress on the isthmus to remove the conidium.

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