Scanning EM Studies on the Colonization of Dent Corn by *Aspergillus flavus*

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ABSTRACT

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Colonization of silks and kernels of preharvest corn by *Aspergillus flavus* was examined by scanning electron microscopy (SEM). Silks of three physiological ages (green unpollinated, yellow-brown, brown) were examined 4, 8, and 24 hr after inoculation. The few conidia that germinated on unpollinated silks failed to colonize the silks. Conidia on yellow-brown silks germinated in 4–8 hr and extensively colonized the silks, especially near pollen grains, where thick hyphal mats produced numerous conidiophores. Indirect and direct penetration of silk was observed. Conidia germinated on brown silks but hyphal growth was sparse. SEM observation of split kernels from ears inoculated with *A. flavus* (inoculum applied to kernels) showed early hyphal growth localized in the tip cap.

Before the discovery of aflatoxins in the 1960s, reports of *Aspergillus flavus* Link ex. Fries in corn before harvest were concerned only with the role of the fungus as an ear rot organism (4,5,27,29). Following the isolation of aflatoxin and the identification of *A. flavus* as the toxin-producing fungus (24), *A. flavus* infection of corn has demanded more serious attention. Aflatoxin contamination was first considered to be a problem only during grain storage. This view changed in 1975, when Anderson et al (1) found aflatoxin contamination in corn sampled from fields 6 wk before harvest. Additional field surveys confirmed their finding (2,15,21,26,31) and also indicated that the likelihood of preharvest contamination was greater in the southern United States, where high temperatures and relative humidity may favor infection (1,12).

The exact nature by which *A. flavus* becomes established in developing corn ears is unknown. Jones et al (12) showed that kernel infection could occur as a result of atomizing an *A. flavus* spore suspension onto the exposed silks. Observations included heavy *A. flavus* sporulation on incubated detached corn silks and *A. flavus* growth and sporulation within kernels of silk-inoculated plants. These findings lent support to the possibility that *A. flavus* infected corn kernels by growing down the silks via the same path as the pollen tube (1,10,30). This study was initiated to investigate the nature of colonization of exposed silks by *A. flavus* and of entry of *A. flavus* into the corn kernel as observed with scanning electron microscopy (SEM) and light microscopy.

MATERIALS AND METHODS

Colonization of external silks. Dwarf dent corn (*Zea mays* L.) of a single cross hybrid (HY375br × OH43br) was grown in the greenhouse at 25–30 C. Approximately 0.5 ml of a spore suspension containing 10^6 spores per milliliter was sprayed from an atomizer onto the exposed silks at the green unpollinated stage, the yellow-brown stage, or the brown silk stage (Fig. 1A–C). The inoculum was prepared from 9-day-old cultures of *A. flavus* (NRRL 3357) grown on Czapek solution agar. Conidia were removed by flooding the cultures with 0.05% Triton X-100.

The inoculated ears were enclosed in plastic bags and covered with paper bags. The plants were moved into a warmer (32–38 C) greenhouse for incubation. Silks were sampled at 4, 8, and 24 hr after inoculation and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The silks were prepared for SEM by dehydration in a graded ethanol/Freon series, critical point drying in Freon 13, mounting on aluminum stubs, and gold coating with a Polaron No. 5000 sputter coater. Specimens were observed using an Etec Autoscan U-1 scanning electron microscope.

The experiment was repeated using another hybrid (Gaspé × W103) grown in controlled-environment chambers. The plants were grown under a daily regime of 9 hr at 26 C and 15 hr at 22 C. At the time of inoculation, the regime was changed to 9 hr at 34 C and 15 hr at 30 C. Plants were hand-pollinated in both experiments.

Infection of kernels. Dwarf dent corn of hybrid HY375br × OH43br was grown in pots in the greenhouse at 25–30 C. Four ears each were inoculated at milk, dough, and dent stages. Husks were pulled back, all silks were removed, and a concentrated suspension of isolate 5T (tan mutant obtained from K. E. Papa, University of Georgia) spores was applied to the kernel surfaces. Husks were replaced and secured with rubber bands, and each ear was enclosed in both a plastic and a paper bag. All inoculated plants were incubated at 30–35 C in a greenhouse. The plastic bag was removed 3 days after inoculation, and the ears were harvested 26 days after inoculation. Twenty kernels from one row on each ear were surface-sterilized for 3 min in 0.5% sodium hypochlorite and split lengthwise. One kernel half was plated on potato-dextrose agar and the matching half was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Kernel halves showing growth of the 5T mutant were grouped according to whether the fungus originated from the tip or from the crown. Corresponding fixed kernel halves were processed for SEM.

Kernels were selected for presence of infection by the split kernel screening method, using silk-inoculated ears from three sources: DeKalb XL 394 grown in the field, HY375br × OH43br grown in the greenhouse, and Gaspé × W103 grown in controlled-environment chambers. Thirty infected kernel halves were fixed, embedded in Paraplast, and sectioned according to the method of Lawrence et al (14). Sections were stained with toluidine blue O, aniline blue in lactophenol, modified Conant's quadruple stain, or Harris' hematoxylin and orange G (9).

RESULTS

Colonization of external silks. *A. flavus* colonized all but green unpollinated silks. Only a few conidia germinated on the unpollinated silks 24 hr after inoculation, and they failed to establish significant mycelial growth. In marked contrast, conidia on the yellow-brown silks germinated in 4–8 hr. Germination occurred first nearest the pollen grains, and the hyphae spread rapidly across the silk, producing extensive growth and lateral branching (Fig. 2A). Similar hyphal growth patterns of aspergilli were observed on red pepper pods by Seenappa et al (25). Heavy colonization of the pollen grains accompanied by varying amounts

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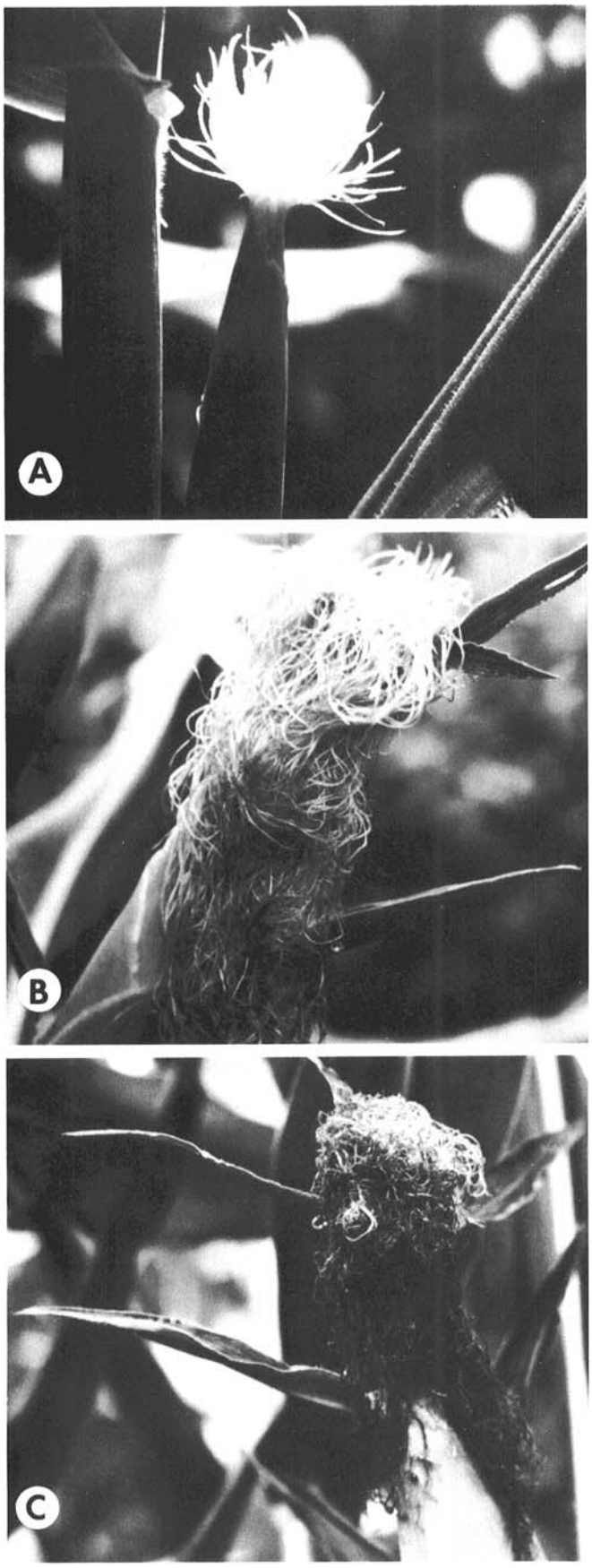

Fig. 1. Successive stages in senescence of external corn silks: **A,** Freshly emerged green-yellow silks; **B,** yellow-brown silks; **C,** brown silks.

of *A. flavus* mycelium on the silk surface was typical of growth on yellow-brown silks 24 hr after inoculation. The silks were penetrated both directly (Fig. 2B) and indirectly through cracks and intercellular gaps (Fig. 2C,D). In transverse sections of penetrated silks, hyphae were observed only in the parenchymatous tissue, with their growth oriented parallel to the silk axis (Fig. 2E). By 48 hr, conidiophores and conidia had formed over the pollen grains (Fig. 2F). Fungal growth on brown silks was comparatively scarce and often localized on pollen grains.

Stereomicroscopic observations of inoculated silks incubated on Czapek solution agar supported the SEM findings that sporulation occurred only on yellow-brown portions of the silks.

Infection of kernels. The 80 plated kernel halves from the milk, dough, and dent ears revealed tip infection in 4, 15, and 27 kernels, respectively, and crown infection in one, seven, and three kernels, respectively. Of the 11 kernel halves showing growth from the crown region, five had sporulation localized on the silk scar. Initial SEM examination of internal tissues of the matching kernel halves failed to reveal signs of infection. Because *A. flavus* or *Fusarium* had grown on the media from all of the plated halves of these kernels, further dissection was done to locate the hyphae. Removal of fragments of pericarp from various areas of the kernel also did not expose obvious fungal colonization. Both *A. flavus* and *Fusarium* mycelium were found in the tip cap (pedicel) only after it was partly broken away from the closing layer to expose more surface area (Fig. 3A). *A. flavus* hyphae were seen within the intercellular spaces, which make up a large proportion of the tip cap, and across the surfaces of the tip cap parenchyma cells, sclereids, and protoxylem elements (Fig. 3B,C). The kernels were not visibly damaged by infection, and no hyphae were observed inside the testa.

No fungal hyphae were seen in sections from the 30 kernels prepared for light microscopy.

DISCUSSION

Pollen seems to play a critical role in the establishment of *A. flavus* growth on external silks. Corn pollen is a rich source of carbohydrates, amino acids, and minerals (16,19,20) and acts as an excellent substrate for *A. flavus*. Conidiophores were rapidly and abundantly produced on pollen by inoculating *A. flavus* onto moistened pollen grains held at 34 C in the laboratory. Conidiophores were produced in less than 24 hr. *A. flavus* growth and sporulation on pollen of other plant species have been reported (18). The profound changes in physiology and structural integrity of the silks brought about by pollination begin their senescence, thus permitting the growth of *A. flavus*. Heslop-Harrison (7) reported that the first reaction of grass stigma cells to a compatible pollination occurs within 1–2 min of contact by the pollen tube tip. As the pollen tube progresses down the silk, vacuole contraction, cytoplasmic shrinkage from the wall, increased membrane permeability, and general necrosis of stigma cells follow closely. Such changes may decrease plant defenses and increase the levels of nutrients available to the fungus.

Although the point or points of initial penetration by *A. flavus* into the corn kernel have not been located in histological studies, indirect evidence is revealed in the literature. The manner in which *A. flavus* colonizes silks and follows their senescence down into the ear suggests the fungus might enter the kernel via the same path as the pollen tube, ie, through the stylar canal. Such a path has been proposed for fungi by Wolf et al (30) and for *A. flavus* by Jones (10). Anderson et al (1) have observed kernels with blue-green-yellow fluorescence in the crown area near the silk scar. Field observations from our study were made with this possibility in mind, but we noted that temporal aspects would limit fungal infection by this avenue. It seems that an abscission layer forms and the silk becomes detached from the kernel shortly after pollination, while the internal silks (silks enclosed by the husk) are often still green-yellow. This detachment usually occurs before the fungus has advanced down the silks to this location (S. F. Marsh, *unpublished*). Although the abscission layer normally forms promptly after pollination, sometimes a number of silks remain

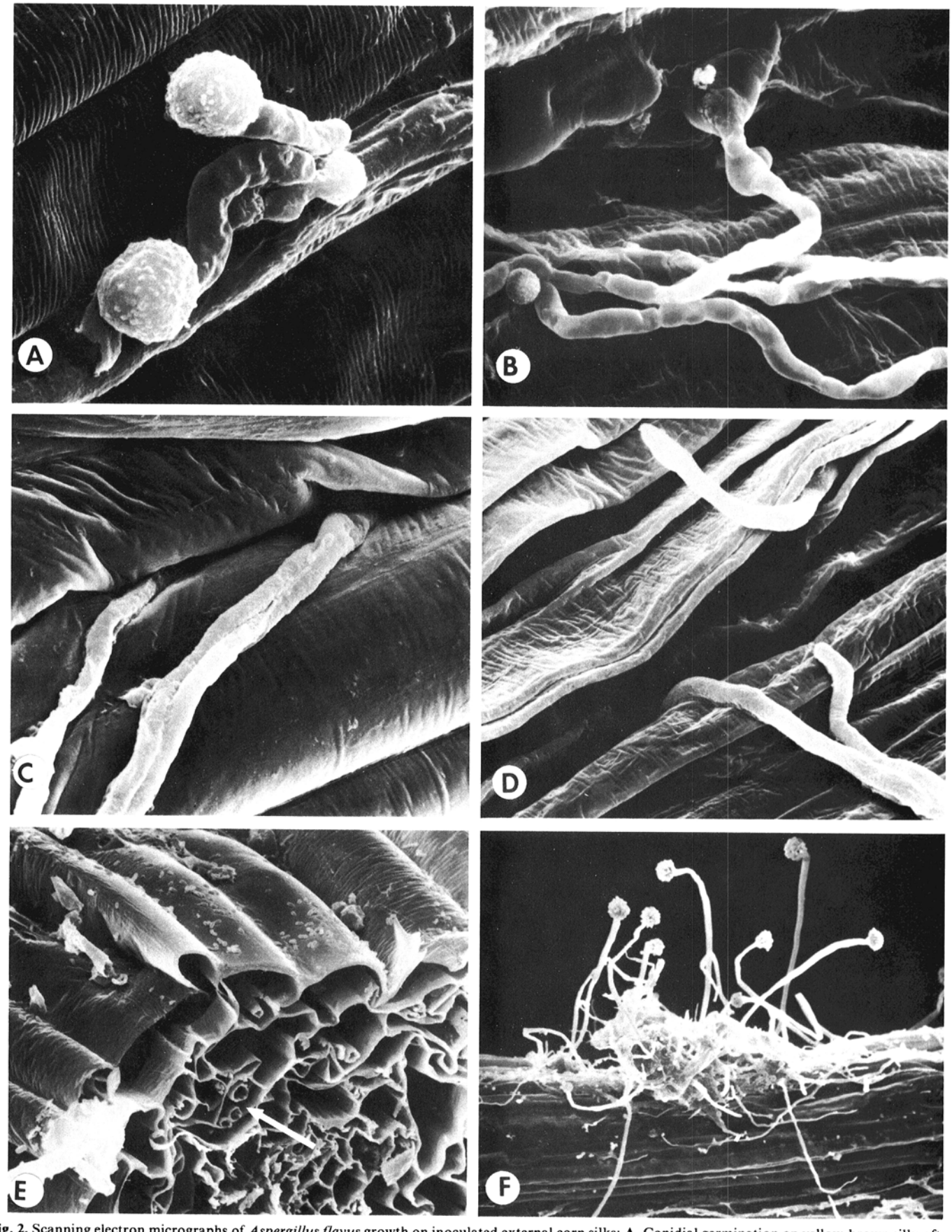

Fig. 2. Scanning electron micrographs of *Aspergillus flavus* growth on inoculated external corn silks: **A,** Conidial germination on yellow-brown silks after 4–8 hr of incubation (×2,500); **B,** direct penetration of yellow-brown silk (×2,200); **C,** indirect penetration of yellow-brown silk through intercellular gap (×2,300); **D,** indirect penetration through fold in silk (×2,2OO); **E,** transverse section of yellow-brown silk and internal *A. flavus* hyphae (arrow) (×1,100); **F,** sporulation on pollen grains clinging to silk (×200).

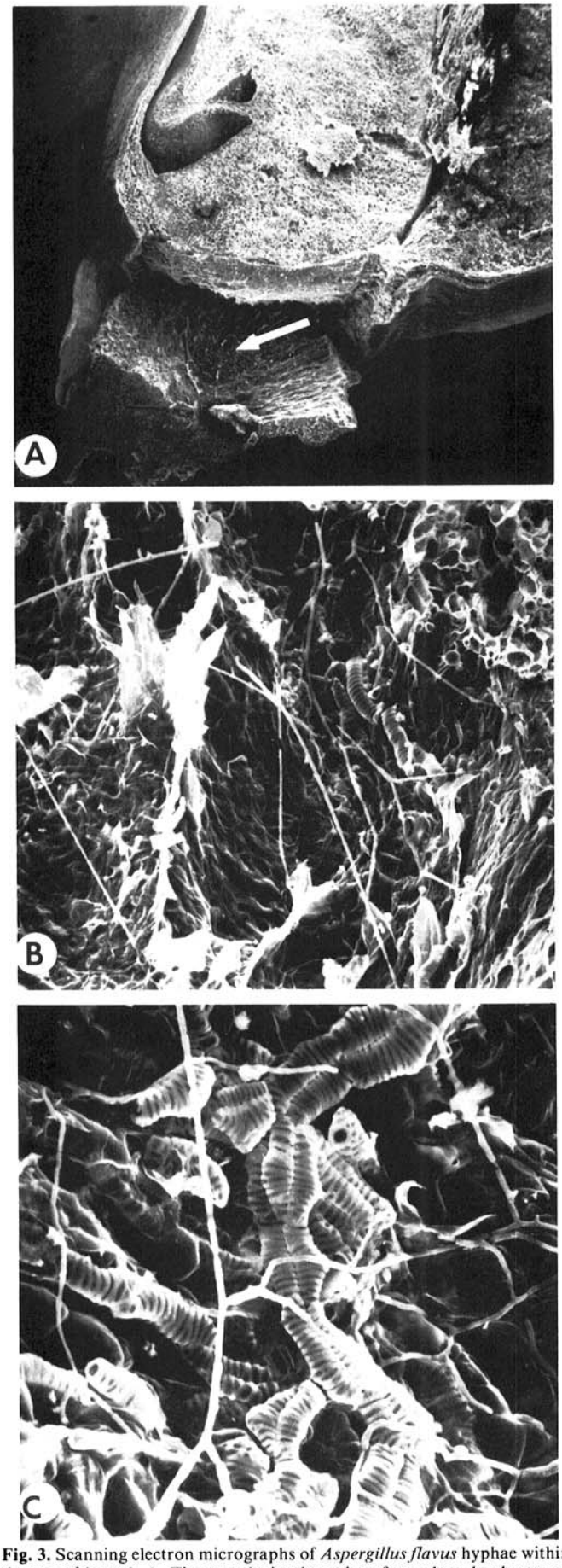

Fig. 3. Scanning electron micrographs of *Aspergillus flavus* hyphae within tip cap of kernel: **A,** Tip cap–closing layer interface where hyphae were found (arrow) (×20); **B** (×300) and **C** (×700), growth within intercellular spaces and across parenchyma cells.

weakly attached to the kernels. Mycelium was occasionally observed growing from the silk attachment site; the majority of the evidence, however, points toward initial penetration occurring at the tip (pedicel) region of the kernel. We think that *A. flavus* mycelium that arrives at the silk attachment site usually grows onto the adjacent pericarp and colonizes the kernel surface rather than actually penetrating the abscission layer into the kernel (S. F. Marsh, *unpublished*). Sporulation of *A. flavus* from plated, surface-sterilized, undamaged or slightly cracked kernels was reported by Fennell et al (6) to occur most often from the germ (68%), next from the tip cap (50%), and least from the endosperm (12%). In a similar study, Rambo et al (22) surface-sterilized, split, and plated undamaged kernels. The frequency of *A. flavus* sporulation from the kernel parts was 54% in the germ, 38% in the endosperm, and 8% in both germ and endosperm. *A. flavus* mycelium and sporulation have been observed in undamaged kernels split without being plated onto media. Fennell et al (6) reported massive colonization and sporulation in the germ and also small masses of mycelium in the space external to the closing layer. Jones et al (11) showed several photographs of *A. flavus* mycelium and sporulation in the germ region. In scanning electron micrographs of mature corn kernels removed from the ear and then inoculated with *A. flavus*, Tsuruta et al (28) showed infection occurred via the tip cap. Scanning electron micrographs in our study of preharvest infection also show mycelial growth in the tip region.

The tip cap and germ regions are common areas for colonization by some other fungi. Manns and Adams (17) found *Fusarium* and *Cephalosporium* growth often localized in the tip cap. Branstetter (3) also found infection of the tip to be most common and stated further that if a kernel is found to be infected in any region, the tip will invariably contain the fungus. The most common location of internal seed infection of five ear-infecting fungi in Illinois was in the tip cap, followed by the germ, floury endosperm, and horny endosperm (13). Johann (8) found that hyphae of *Diplodia zeae* most frequently penetrated the suberized membrane of the testa at its thinner spots, over the embryo, and at its junction with the closing layer in the kernel tip. Although the closing layer is quite resistant to fungal penetration, Johann (8) suggested that hyphae can traverse the hilum before the closing layer is formed or hyphae may pass around the ends of the closing layer in cases of delayed or incomplete junction of the closing layer with the suberized membrane of the testa. Salama and Mishricky (23) suggested that immature corn kernels become infected with *Fusarium moniliforme* through this same area, which they referred to as the placento-chalazal region.

Our observations have shown that *A. flavus* conidia are most likely to germinate on and colonize exposed silks that are in the yellow-brown stage of senescence. We have also provided evidence that preharvest invasion of corn kernels occurs via the tip cap.

LITERATURE CITED

1. Anderson, H. W., Nehring, E. W., and Wichser, W. R. 1975. Aflatoxin contamination of corn in the field. J. Agric. Food Chem. 23:774-782.
2. Anonymous. 1971. FDA recalls corn meal, bread mix allegedly tainted by toxin. Southwest Miller 50:26.
3. Branstetter, B. B. 1927. Corn root rot studies. Mo. Agric. Exp. Stn. Res. Bull. 113:1-80.
4. Butler, F. C. 1947. Ear, cob, and grain rots of maize. Agric. Gaz. N.S.W. 58:144-151.
5. Eddins, A. H. 1930. Corn diseases in Florida. Fla. Agric. Exp. Stn. Bull. 210:1-35.
6. Fennell, D. I., Bothast, R. J., Lillehoj, E. B., and Peterson, R. E. 1973. Bright greenish-yellow fluorescence and associated fungi in white corn naturally contaminated with aflatoxin. Cereal Chem. 50:404-414.
7. Heslop-Harrison, J. 1979. Pollen-stigma interaction in grasses: A brief review. N.Z. J. Bot. 17:537-546.
8. Johann, H. 1935. Histology of the caryopsis of yellow dent corn, with reference to resistance and susceptibility to kernel rots. J. Agric. Res. 51:855-883.
9. Johansen, D. A. 1940. Plant Microtechniques. McGraw-Hill, New York. 523 pp.
10. Jones, R. K. 1979. The epidemiology and management of aflatoxins

and other mycotoxins. Pages 381-392 in: Plant Disease. An Advanced Treatise. Vol. 4. How Pathogens Induce Disease. J. G. Horsfall and E. B. Cowling, eds. Academic Press, New York. 496 pp.
11. Jones, R. K., Duncan, H. E., and Hamilton, P. B.. 1981. Planting date, harvest date, and irrigation effects on infection and aflatoxin production by *Aspergillus flavus* in field corn. Phytopathology 71:810-816.
12. Jones, R. K., Duncan, H. E., Payne, G. A., and Leonard, K. J. 1980. Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. Plant Dis. 64:859-863.
13. Koehler, B. 1942. Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. J. Agric. Res. 64:421-442.
14. Lawrence, E. B., Nelson, P. E., and Ayers, J. E. 1981. Histopathology of sweet corn seed and plants infected with *Fusarium moniliforme* and *F. oxysporum*. Phytopathology 71:379-385.
15. Lillehoj, E. B., Fennell, D. I., Kwolek, W. F., Adams, G. L., Zuber, M. S., Horner, E. S., Widstrom, N. W., Warren, H., Guthrie, W. D., Sauer, D. B., Findley, W. R., Manwiller, A., Josephson, L. M., and Bockholt, A. J. 1978. Aflatoxin contamination of corn before harvest: *Aspergillus flavus* association with insects collected from developing ears. Crop Sci. 18:921-924.
16. Linskens, H. F., and Pfahler, P. L. 1973. Biochemical composition of maize (*Zea mays* L.) pollen. III. Effects of allele X storage interactions at the waxy (wx), sugary (su) and shrunken (sh) loci on the amino acid content. Theor. Appl. Genet. 43:49-53.
17. Manns, T. F., and Adams, J. F. 1923. Parasitic fungi internal of seed corn. J. Agric. Res. 23:495-524.
18. Nair, P. K. K., and Srivastava, V. 1977. A study of fungal infection of pollen grains of *Cereus tetragonus*. Indian Phytopathol. 30:229-232.
19. Pfahler, P. L., and Linskens, H. F. 1971. Biochemical composition of maize (*Zea mays* L.) pollen. II. Effects of the endosperm mutants, waxy (wx), shrunken (sh) and sugary (su) on the carbohydrate and lipid percentage. Theor. Appl. Genet. 41:2-4.
20. Pfahler, P. L., and Linskens, H. F. 1974. Ash percentage and mineral content of maize (*Zea mays* L.) pollen and style. I. Genotypic effects. Theor. Appl. Genet. 45:32-36.
21. Rambo, G. W., Tuite, J., and Caldwell, R. W. 1974. *Aspergillus flavus* and aflatoxin in preharvest corn from Indiana in 1971 and 1972. Cereal Chem. 51:848-853.
22. Rambo, G. W., Tuite, J., and Crane, P. 1974. Preharvest inoculation and infection of dent corn with *Aspergillus flavus* and *Aspergillus parasiticus*. Phytopathology 64:797-800.
23. Salama, A. M., and Mishricky, A. G. 1973. Seed transmission of maize wilt fungi with special reference to *Fusarium moniliforme* Sheld. Phytopathol. Z. 77:356-362.
24. Sargeant, K., Sheridan, A., and O'Kelly, J. 1961. Toxicity associated with certain samples of groundnuts. Nature 192:1096-1097.
25. Seenappa, M., Stobbs, L. W., and Kempton, A. G. 198O. *Aspergillus* colonization of Indian red pepper during storage. Phytopathology 70:218-222.
26. Shotwell, O. L. 1977. Aflatoxin in corn. J. Am. Oil Chem. Soc. 54:216A-224A.
27. Taubenhaus, J. J. 1920. A study of the black and yellow molds of ear corn. Tex. Agric. Exp. Stn. Bull. 270:3-38.
28. Tsuruta, O., Gohara, S., and Saito, M. 1981. Scanning electron microscopic observations of a fungal invasion of corn kernels. Trans. Mycol. Soc. Jpn. 22:121-126.
29. Tuite, J. 1961. Fungi isolated from unstored corn seed in Indiana in 1956-1958. Plant Dis. Rep. 45:212-215.
30. Wolf, M. J., Buzan, C. L., MacMasters, M. M., and Rist, C. E. 1952. Structure of the mature corn kernel. II. Microscopic structure of pericarp, seed coat, and hilar layer of dent corn. Cereal Chem. 29:334-347.
31. Zuber, M. S., Calvert, O. H., Lillehoj, E. B., and Kwolek, W. F. 1976. Preharvest development of aflatoxin B_1 in corn in the United States. Phytopathology 66:1120-1121.