

# The Effects of *Fusarium graminearum* Infection on Wheat Kernels<sup>1</sup>

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## ABSTRACT

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A number of tests were conducted on scabby wheat to determine what damage was caused by fungal infection. Hard red winter wheat infected with *Fusarium graminearum* was graded into three categories based on appearance: 1) normal kernels appearing sound, of good color and weight; 2) lightly infected kernels of normal size but of light weight and color; and 3) heavily infected kernels that were shriveled and light colored. These wheat classes were then analyzed to detect fungal presence using light and electron microscopy, histochemistry, polyacrylamide gel electrophoresis of storage proteins, germination tests, and plating. The results of these tests revealed that the fungus is an aggressive invader destroying starch granules, storage

proteins, and cell walls. The fungus was most prevalent in aleurone and pericarp tissues, but hyphae were found throughout the starchy endosperm. The germ seemed to be spared infection except in heavily invaded kernels, however, the lightly infected kernels with apparently uninfected germs exhibited reduced germination and vigor. Microscopic examination of lightly infected kernels germinating revealed extensive invasion of the scutellum and embryonic axis, indicating renewed fungal growth during imbibition. The results of this study suggest that a visual inspection of scabby wheat kernels can be used to discern gross differences in infection.

Wheat scab, or head blight, is a fungal disease caused by *Fusarium graminearum* Schwabe. The fungus infects developing wheat panicles and results in kernels with varying degrees of infection. During the 1982 growing season much greater than normal amounts of wheat scab were reported for eastern Kansas and southeast Nebraska. Because of several local severe outbreaks of the disease and reports of high levels of the toxin deoxynivalenol (DON), there was a great deal of public concern and a reluctance of buyers to accept Kansas or Nebraska wheat. According to USDA estimates, less than 3.5% of the total hard red winter wheat crop was affected, however. We undertook a multifaceted research project to investigate the wheat scab problem. This report describes the structural and biochemical changes in wheat caused by the fungus.

## MATERIALS AND METHODS

### Samples

Wheat samples were from the 1982 growing season. The wheat was divided by hand into three categories based on gross morphological characteristics. Group 1 (normal) contained kernels normal in appearance, group 2 (light to moderately infected) kernels were normal in shape and size but light in color and weight, and group 3 (shriveled) kernels were small and shriveled. Wheat appearing normal had the highest protein, lowest ash, highest 1,000-kernel weight, and lowest levels of DON and ergosterol (a measure of fungal infection, Seitz et al 1979) (Table I). DON was measured by the methods of Pollmann et al (1985). Group 2 wheat had lower protein, higher ash, lower 1,000-kernel weight, and higher levels of DON and ergosterol than the normal wheat. Shriveled kernels had near normal protein and ash, very low 1,000-kernel weight, and extremely high DON and ergosterol levels (Table I).

### Light Microscopy

Wheat samples for histochemistry were fixed in 3% paraformaldehyde (w/v) and 3% glutaraldehyde (v/v) in 0.1M sodium dibasic and potassium monobasic phosphate buffer (Lillie 1954) for 1 hr at 21°C and 16 hr at 4°C. Samples were washed four

times in buffer at 21°C for a total of 2 hr and then dehydrated, infiltrated, and embedded as for electron microscopy (see next paragraph). Plastic thick sections (1- $\mu$ m thick) were cut with glass knives and affixed to slides at 80°C for 30 min. Protein was stained using the Coomassie Brilliant Blue method (Bechtel and Pomeranz 1978). Carbohydrates were stained by the periodic acid/Schiff's (PAS) reaction (Jensen 1962) with appropriate controls (Bechtel and Pomeranz 1981).

### Electron Microscopy

Wheat samples were fixed and washed in the same solutions as for light microscopy and postfixed in phosphate-buffered 1% osmium tetroxide for 2 hr at 21°C. Then the tissue was washed in water three times for a total of 30 min, dehydrated in a graded acetone series, and infiltrated and embedded in a low viscosity epoxy resin. Plastic sections 1- $\mu$ m thick were cut with glass knives; then thin sections were cut with a diamond knife, stained in 2% aqueous uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and viewed in a Philips EM201 electron microscope at 60 kV.

### Germination and Culture Conditions

Whole kernels (25 grains in each group) and dissected germs (10 in each group) were placed on sterile 2% agar in petri dishes at 27°C, and germination was monitored for one week. Germs were removed from surface disinfected kernels using the corner of a razor blade to pop the germ free from the endosperm. Microscopy revealed that the germ was removed between the scutellar epithelium and endosperm. Whole kernels and dissected germs were fixed after 72 hr according to the electron microscopy procedure. Germination vigor was considered low if seedling length was less than half and very low if less than one quarter that of uninfected kernels. *F. graminearum* that grew out of kernels was subcultured onto either potato-dextrose agar or 2% water agar with 1% (w/v) wheat flour added. Cultures were maintained at 25°C.

TABLE I  
Scabby Wheat Characteristics

Wheat	1,000-Kernel				
	Protein <sup>a</sup> (%)	Ash <sup>b</sup> (%)	Weight (g)	DON <sup>c</sup> (ppm)	Ergosterol <sup>d</sup> (ppm)
Normal	13.7	1.7	29.9	0.43	2.8
Lightly to moderately infected	11.8	2.0	25.6	22.7	29.0
Shriveled	12.3	1.8	13.1	68.7	103.0

<sup>a</sup> Determined by AACC method 46-11.

<sup>b</sup> Determined by AACC method 08-01.

<sup>c</sup> DON = deoxynivalenol, by the method of Pollmann et al 1985.

<sup>d</sup> Determined by the method of Seitz et al 1979.

<sup>1</sup> Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others not mentioned.

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### Polyacrylamide Gel Electrophoresis (PAGE)

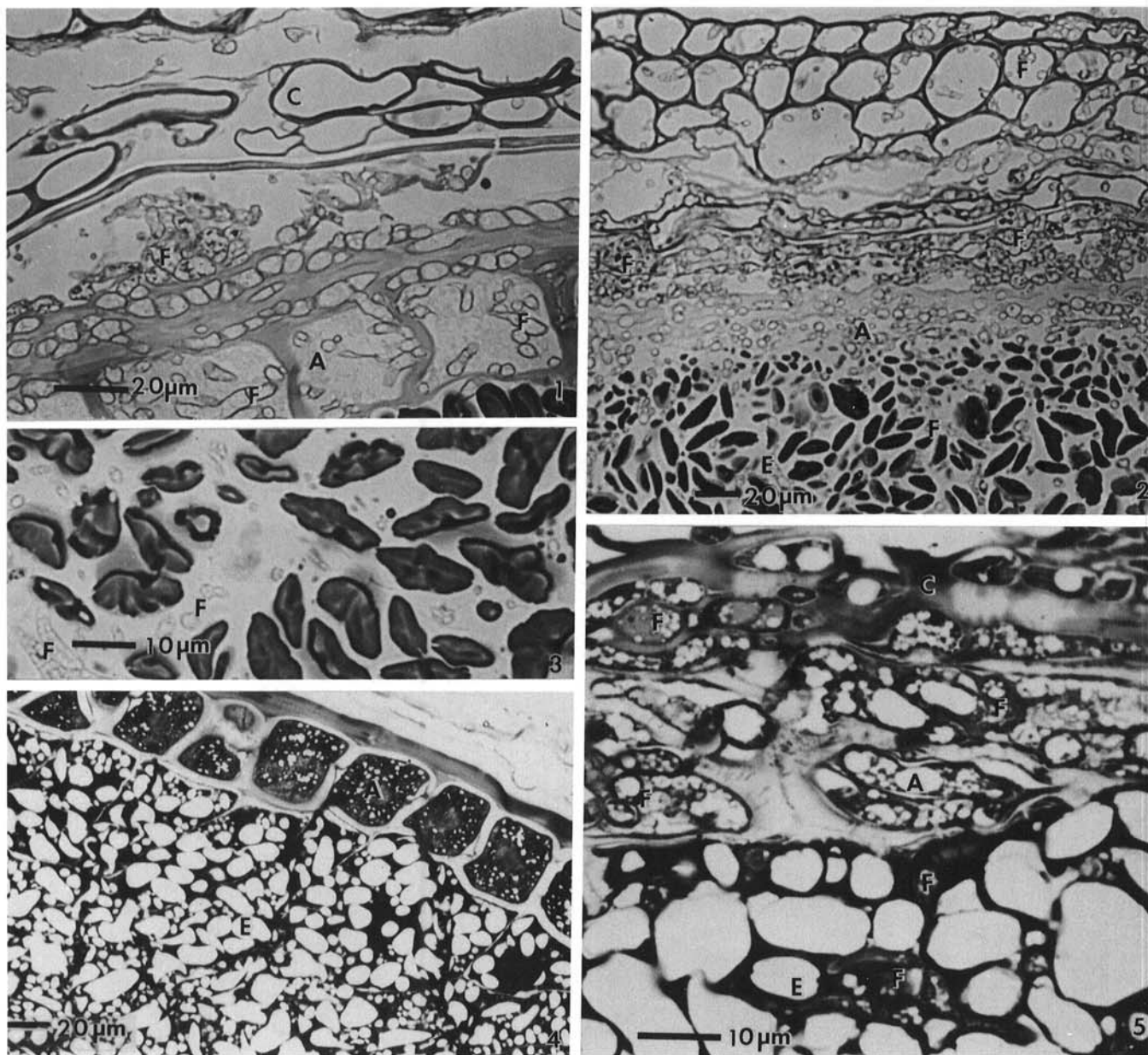
Ground wheat samples (30 mg) were mixed with 150  $\mu$ l of 2% sodium dodecyl sulfate (SDS) and 5%  $\beta$ -mercaptoethanol in 0.05 M Tris buffer at pH 6.8 for 5 min. The samples were then boiled for 3 min, another 150  $\mu$ l of extraction medium added, extracted for 1 hr at room temperature, and centrifuged. The clear supernatant was added to 50  $\mu$ l of 0.016% bromphenol blue in 30% glycerol. Extracts were subjected to electrophoresis on SDS polyacrylamide gels (Laemmli 1970). A 1.5-mm thick, 8–12% linear gradient slab gel was overlaid with a 3% stacking gel. Fifteen microliters of sample solution was used per sample well. Stacking and separation were conducted at a constant current of 7 and 17 mA, respectively, at 10°C. Molecular weight standards were lysozyme (14,400), soybean trypsin inhibitor (21,500), trypsinogen (24,000), pepsin (34,700), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase B (92,500). Gels were stained in 0.2% Coomassie Brilliant Blue R-250 stain in 8.6% acetic acid and 43% methanol for 5 hr, followed by destaining in 5% methanol and 7.5% acetic acid.

### RESULTS

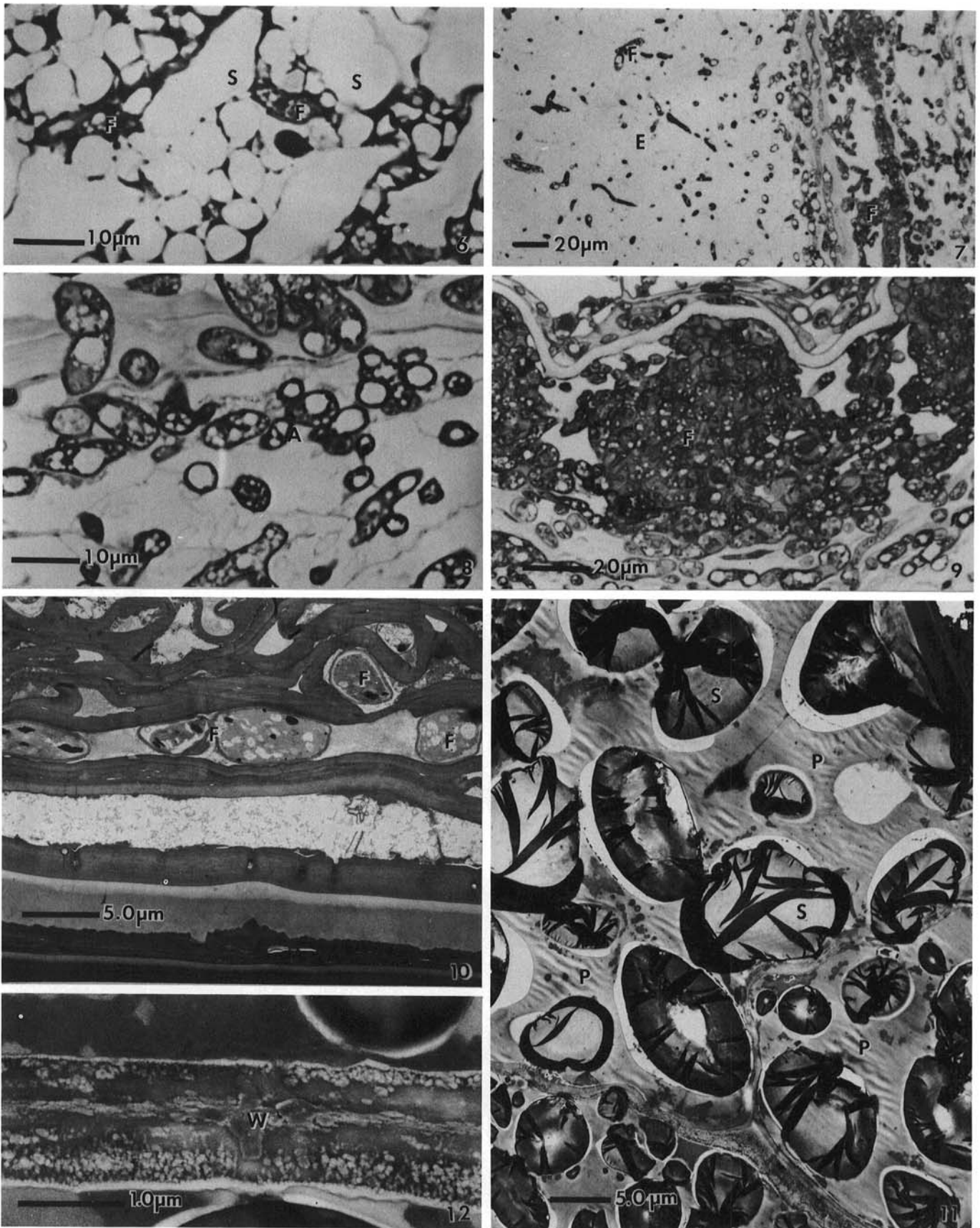
#### Light Microscopy

The PAS procedure for localizing carbohydrates showed that the sound wheat we examined did not have observable fungal hyphae (figure not shown). Light to moderately infected wheat showed numerous hyphae in the caryopsis coats, aleurone cells, and subaleurone region (Fig. 1). PAS staining did not reveal much fungus in the central endosperm because the large amount of starch that stained obscured the fungus. Shriveled kernels exhibited the greatest modification, with numerous hyphae found throughout the kernel (Fig. 2). In these heavily infected kernels, starch granules showed large amounts of apparent enzymatic degradation as well as association with numerous hyphae (Fig. 3).

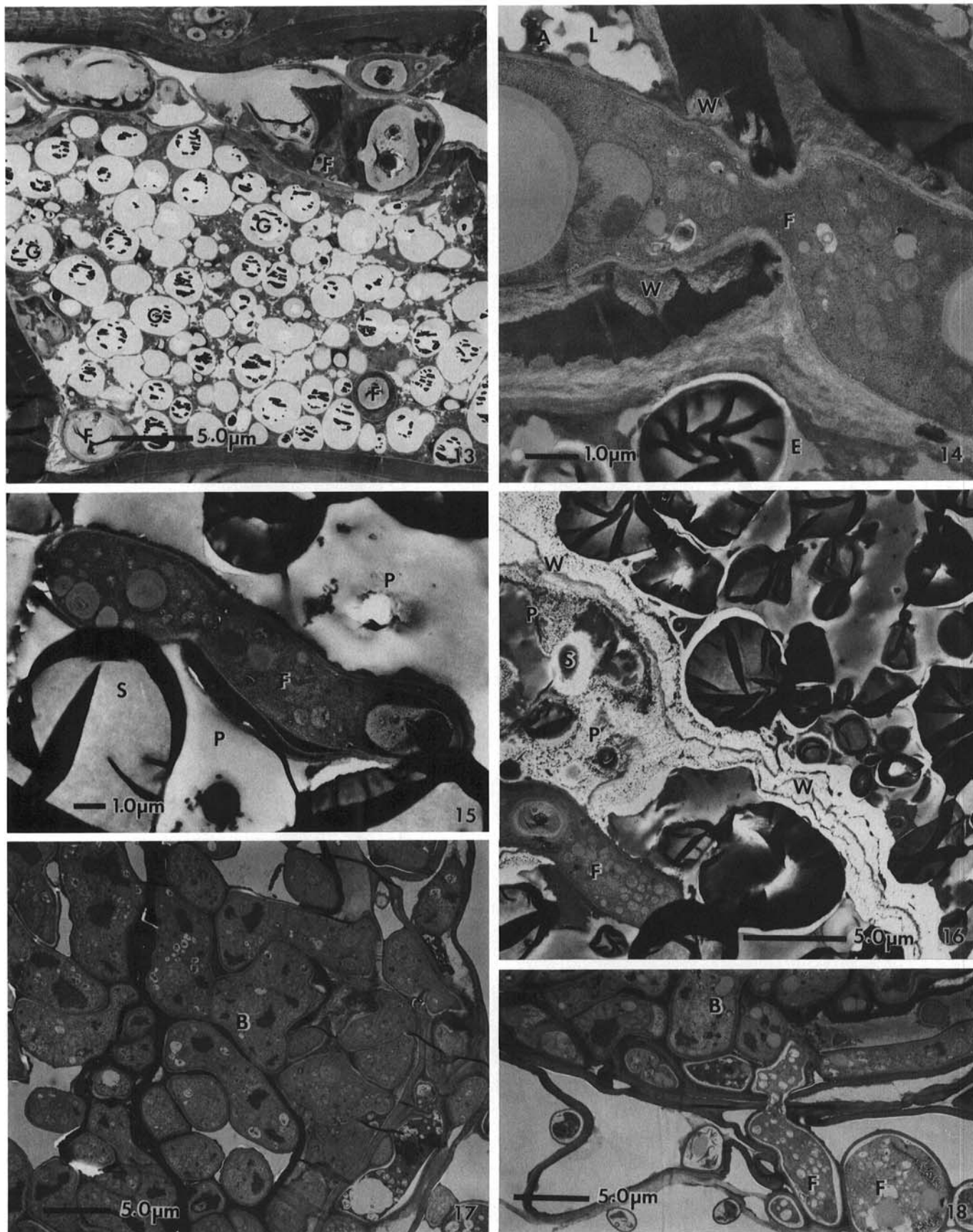
The Coomassie Brilliant Blue method for staining protein was used to demonstrate degree of infection as well as storage protein modification. The kernels appearing sound showed no signs of fungal invasion in either the caryopsis coats, aleurone layer, or starchy endosperm (Fig. 4). The moderately infected wheat showed



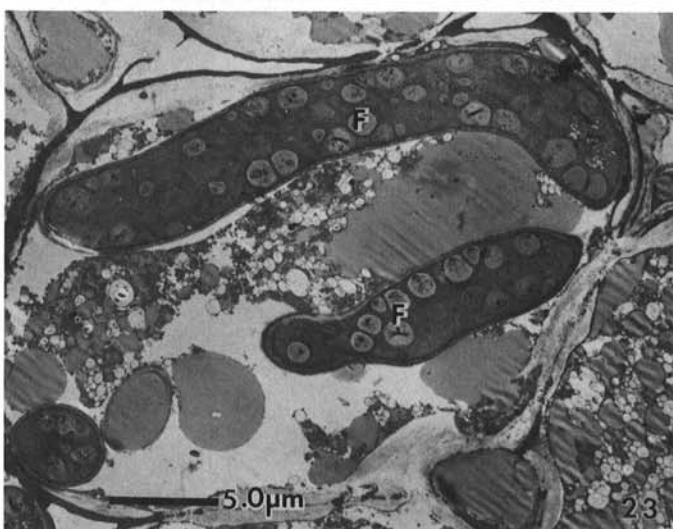
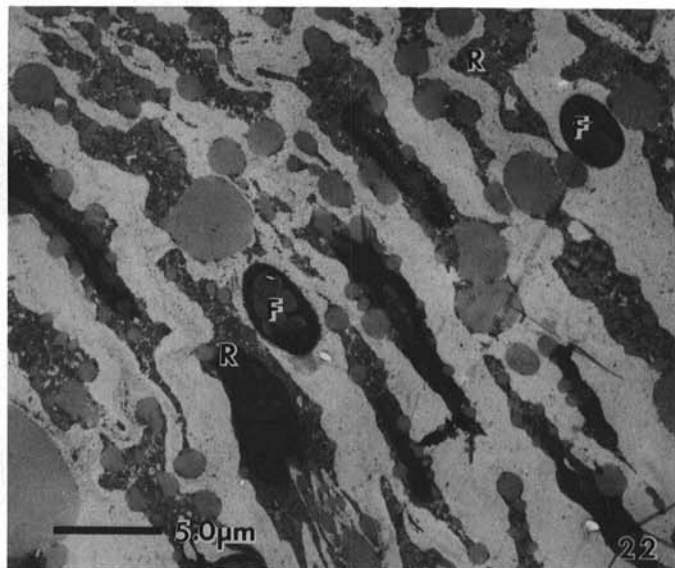
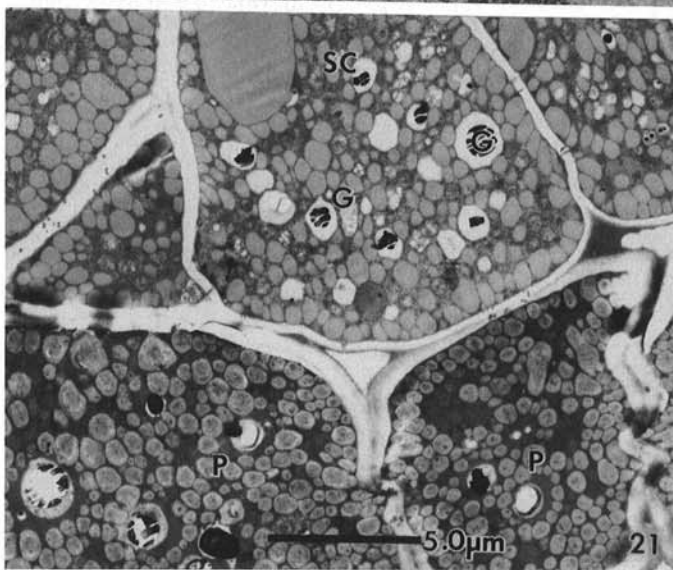
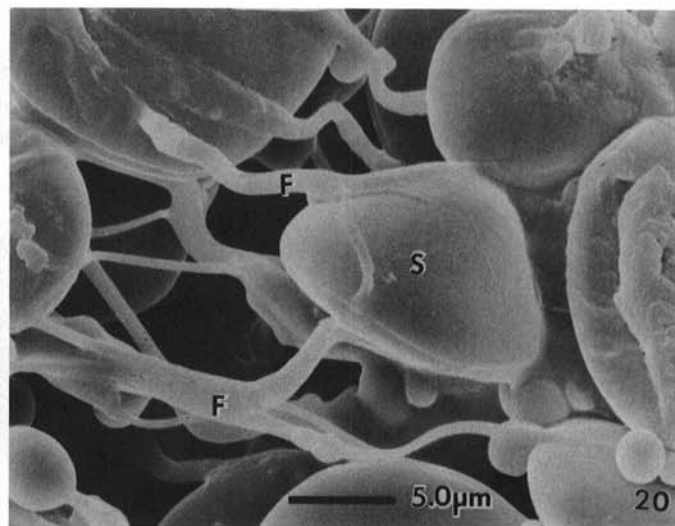
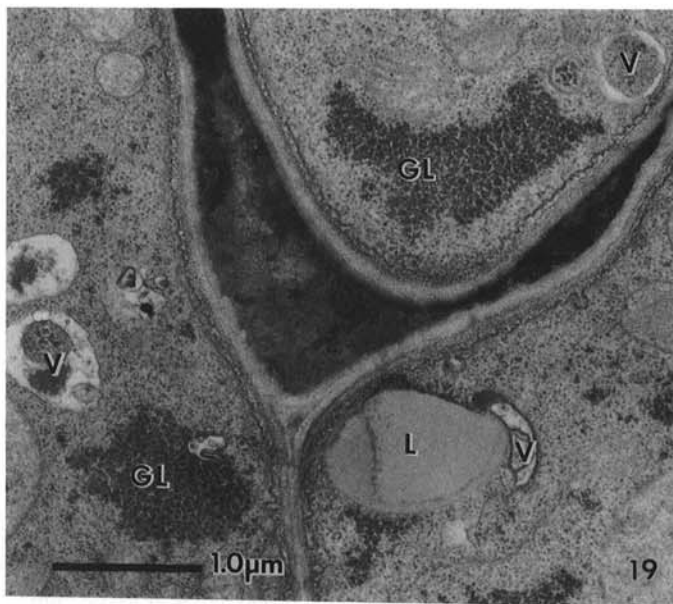
**Fig. 1.** Light to moderately infected wheat has numerous hyphae (F) in the caryopsis coats (C) and aleurone layers (A). Fungal cell walls stain dark with periodic acid/Schiff's (PAS) procedure. **Fig. 2.** Shriveled kernel stained with PAS reagent reveals much fungus (F) and damaged starch in starchy endosperm (E). A discernable aleurone layer is lacking (A). **Fig. 3.** PAS-stained central endosperm of a shriveled kernel showing fungus (F) associated with darkly stained and damaged starch. **Fig. 4.** Normal wheat stained with Coomassie Brilliant Blue showing lack of fungus in aleurone (A) and starchy endosperm (E). **Fig. 5.** Moderately infected wheat shows numerous fungal hyphae (F) in caryopsis coats (C), aleurone (A), and subaleurone endosperm (E).



**Fig. 6.** Moderately infected wheat central endosperm stained with Coomassie Brilliant Blue (CBB) showing fungus (F) between starch granules (S). **Fig. 7.** Shriveled kernels stained with CBB show loss of stainable storage protein in endosperm (E). Fungus (F) stains densely. **Fig. 8.** The aleurone layer (A) is lacking in CBB-stained shriveled kernels. **Fig. 9.** Shriveled kernels possess bundles of mycelium (F) in the caryopsis coats region. **Fig. 10.** Occasionally transmission electron microscopy revealed hyphae (F) in caryopsis coats of wheat appearing sound. **Fig. 11.** Starchy endosperm of sound wheat did not contain any observable fungus. Starch (S) and storage protein (P) appeared normal. **Fig. 12.** High magnification of starch endosperm cell wall (W) of normal wheat.



**Fig. 13.** Transmission electron micrograph (TEM) of aleurone cell from moderately infected kernel showing lack of protein around globoids (G) and fungus (F) within the cell. **Fig. 14.** Fungus hyphae (F) penetrating into subaleurone endosperm (E) from aleurone cell (A). Note fused lipid droplets (L) and pockets of digestion in endosperm cell wall (W). **Fig. 15.** TEM of moderately infected kernel central endosperm shows fungus (F) embedded in storage protein (P) and in close association with starch (S). **Fig. 16.** TEM of portion of starchy endosperm where fungus (F) has partly digested starch (S), storage protein (P), and endosperm cell walls (W). **Fig. 17.** Bundles of fungus hyphae (B) make the bulk of the shriveled wheat's caryopsis coats. **Fig. 18.** The mycelial bundles (B) differed morphologically from the fungus (F) located in the rest of the shriveled kernel.



**Fig. 19.** High magnification of hyphae in the mycelial bundles showing glycogen (GL), autophagic vacuoles (V), and limited amount of lipid (L). **Fig. 20.** Scanning electron micrograph of air-dried, shriveled kernel showing interaction of fungus (F) with starch (S). **Fig. 21.** Ungerminated moderately infected wheat kernel has scutellar epithelial cells (SC) that lack protein matrix around globoids (G) in the protein bodies, whereas the parenchyma cells (P) appear normal. **Fig. 22.** Germinated moderately infected kernel has highly damaged radicle. Most of the cells of the radicle (R) are disrupted by the fungus (F). **Fig. 23.** Scutellum parenchyma heavily infected by fungus (F) after moderately infected kernels were germinated for 72 hr. **Fig. 24.** Ungerminated shriveled kernel germ has extensive fungal (F) invasion in the radicle.

numerous hyphae in the caryopsis coat, aleurone, and subaleurone layers (Fig. 5). The Coomassie Brilliant Blue stain also clearly depicted the fungus in the central endosperm (Fig. 6). A major change observed in the heavily infected kernels was loss of stainable storage protein; most of the protein staining in these shriveled kernels was fungal (Fig. 7). Shriveled kernels did not possess a discernible aleurone layer (Fig. 8), and the region of caryopsis coats was occupied by a mass of mycelium that had begun to differentiate, possibly into sporogenous hyphae (Fig. 9).

### Electron Microscopy

Transmission electron microscopy of grain appearing sound revealed an occasional fungal hypha in the pericarp region (Fig. 10). Hyphae were not observed in aleurone cells (figure not shown) or in the starchy endosperm (Fig. 11). Endosperm cell walls were intact, with no visible modifications (Fig. 12). Sections from light to moderately infected kernels showed the aggressive nature of *F. graminearum* (Figs. 13 and 14). Aleurone cells contained large numbers of hyphae that apparently removed the protein matrix from the aleurone grains and caused lipid bodies to fuse (Fig. 14). Cell walls penetrated by the fungus were marked with numerous small digested pockets in the cell wall (Fig. 14). Sections through the starchy endosperm revealed the fungus embedded in the storage protein matrix (Fig. 15). One of the most obvious changes caused by the fungus was dissolution of starchy endosperm cell walls and parts of the starchy endosperm (Fig. 16).

Shriveled kernels had little structure remaining that resembled normal kernels. Fungal tissue constituted most of the external part of the kernel (Figs. 9 and 17). This fungal tissue appeared as bundles of morphologically distinct hyphae that differed from those found in the other parts of the kernel (Fig. 18). The hyphae in the bundles contained numerous autophagic vacuoles, masses of glycogen, and limited amounts of lipid droplets (Fig. 19). The starchy endosperm seemed to have lost most of the storage protein, and the starch granules were greatly digested (Figs. 3 and 20). Scanning electron microscopy of shriveled kernels clearly revealed the close interaction between fungus and starch granules as well as the lack of storage protein matrix (Fig. 20).

### Germination

Germination results on whole kernels and on dissected germs are shown in Table II. Sound grain had the highest germination rates for both whole grain and isolated germ, although only the isolated germ showed signs of the fungus. Nearly all the light to moderately

infected kernels and all of the shriveled kernels were infected (Table II). The germination rates and seedling vigor of the infected grains were greatly reduced over those of the sound kernels.

Microscopy of sound kernels germinated for 72 hr revealed no fungal invasion in the scutellum (figures not shown). Hyphae were not observed in the germ of ungerminated lightly infected kernels. These ungerminated kernels showed few fungal-caused effects in the germ; most of the damage was limited to the scutellar epithelium where the aleurone grains (protein bodies) had lost the protein matrix (Fig. 21). When these kernels were germinated for 72 hr, fungal hyphae were found in most parts of the germ including the radicle (Fig. 22) and scutellar parenchyma (Fig. 23). Ungerminated shriveled germs showed extensive invasion by *F. graminearum* (Fig. 24). When placed on the germination medium, mycelium grew extensively and all of the germ became infected. Secondary invaders, primarily gram-negative rods, were frequently observed in the imbibed, shriveled kernels.

### PAGE

SDS-PAGE was conducted on wheat samples to determine the effects of infection on the electrophoretic mobilities of the proteins. In general, the greater the infection, the less intense the staining of high molecular weight components (Fig. 25). Lightly infected kernels specifically lacked a band between the 66,200 and 92,500 molecular weight markers. Lightly infected kernels also showed a smearing of bands at the lower molecular weights. This smearing became very prominent in extracts from shriveled kernels (Fig. 25).

### Culture Effects

Analysis for DON was conducted on the mycelium and the agar in which the fungus was growing. Fungus grown on and in wheat flour agar medium yielded high concentrations of DON (61.5 ppm) as well as the medium (70.6 ppm), whereas fungus grown on and in potato-dextrose agar produced no detectable levels of DON.

## DISCUSSION

The results of this study clearly show the dramatic effects of the wheat scab fungus, *F. graminearum*, on wheat kernels. The fungal infection occurs in the field and can greatly affect the quality of harvested wheat. The exact timing of fungal infection is not known, although the flowering period is suspected to be important (Sutton 1982). Other evidence indicates that the initial infection occurs via the anthers (McKay and Loughrane 1945, Strange and Smith 1971) and that susceptibility decreases rapidly after the soft dough stage (Sutton 1982). Our data, based on structural studies of mature wheat, indicate that the fungus does much of its damage between the second and third week after anthesis. We arrived at this conclusion from two lines of reasoning. First, when the infected kernels were imbibed during the germination study, they all enlarged to about the same size as sound kernels. This suggested that the kernels were shriveled due to shrinkage caused by either nondeposition of or depletion of storage reserves. Wheat kernels normally reach maximum size and shape during the second week after anthesis. Therefore, the wheat kernel must have grown to mature size before the fungus affected development. The second

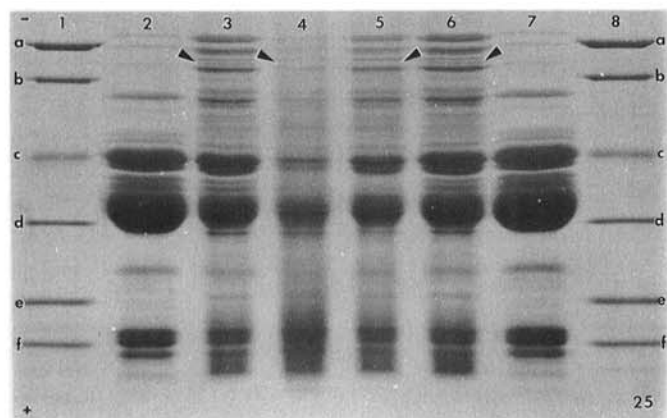


Fig. 25. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole wheat kernel extracts. Lanes 1 and 8 are molecular weight markers: (a) phosphorylase B (92,500), (b) bovine serum albumin (66,200), (c) ovalbumin (45,000), (d) carbonic anhydrase (31,000), (e) soybean trypsin inhibitor (21,500), (f) lysozyme (14,400). Lanes 2 and 7 are 70% ethanol extracts of sound kernels. Lanes 3 and 6 are SDS- $\beta$ -mercaptoethanol extracts of sound kernels. Lanes 4 and 5 are SDS- $\beta$ -mercaptoethanol extracts of shriveled kernels and lightly infected kernels, respectively. Note lack of band in infected kernels (arrows).

TABLE II  
Germination of Scabby Wheat

Wheat	Percent Germinated	Percent Scab Infected	Seedling Vigor
Normal			
Whole	100	0	High
Germ	90	30	Low
Lightly to moderately infected			
Whole	20	92	Low
Germ	40	100	Low
Shriveled			
Whole	0	100	None
Germ	10	100	Very low

line of reasoning involves the germ. In cereals the germ normally does not undergo much differentiation during the first week after anthesis but then quickly develops and matures during the second week. Our structural studies found little evidence of any fungal invasion in the germ except in the most heavily infected kernels. We certainly cannot be sure that these germs were free of fungus using structural studies as a guide. In fact, the plating data (Table II) showed fungal presence in the germ of all the infected kernels. This would indicate that the germ was infected. Our explanation of this apparent paradox is that the fungus was present in the caryopsis coat that surrounds the germ, and which we have shown to be present in even lightly infected kernels. The germ is probably fungus free, but upon hydration the fungus readily attacks the germ tissues from the caryopsis coats. Both lines of reasoning indicate that the fungus invaded the wheat and did most of its damage at or about the time of germ maturation, two to three weeks after anthesis.

The reduced germination rates and seedling vigor can be explained by the structural studies. The fungus apparently did little damage to the germ during kernel development. Upon imbibition, however, the fungus became active and attacked the germ (possibly because during imbibition the germ absorbs water first). This explains why many of the seedlings that germinated showed low vigor as well as the presence of hyphae on the coleoptile. We suspect that even lightly infected kernels will show reduced germination and vigor under field conditions because the fungus was internal.

The manner of invasion by *F. graminearum* contrasts with that by storage fungi. Storage fungi are typically thought to invade the germ first (Christensen and Lopez 1963, Tsuruta et al 1981). The scab fungus invades the pericarp and aleurone first and penetrates cell walls quickly to enter the starchy endosperm. This aggressive invasion results in hyphae being distributed throughout the endosperm, where the fungus digests storage protein and starch. The digestion reveals itself as band smearing in SDS-PAGE and as irregular surfaces of starch granules in microscopy. The presence of hyphae throughout the kernel also explains the fact that the DON toxin was found in all the mill streams (Young et al 1984; Seitz et al 1985). Similar data were also obtained for peanuts contaminated with aflatoxin, in which both toxin and fungus were distributed throughout the seed tissue (Lee et al 1967).

The gross morphological appearance of infected kernels is a good indication of the extent of fungal infection and amount of toxin. Shriveled kernels have the greatest damage and highest levels of DON (68.7 ppm). Kernels that are light in color and weight will have less fungus and toxin present (22.7 ppm). Kernels with more normal color and weight will have the least amount of toxin. By making simple visual inspections of wheat samples one can obtain a rough idea of the degree of infection and DON levels.

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