Cytology and Histology

Histology of Infection of Wheat by *Tilletia indica*, the Karnal Bunt Pathogen

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ABSTRACT

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Infection of wheat by *Tilletia indica* was investigated using scanning electron microscopy and light microscopy. Recently emerged spikes were inoculated by placing them beneath inverted petri dish cultures that were discharging secondary sporidia. Apparent hyphal anastomosis was observed rarely on the glume surface. Germ tubes arising from secondary sporidia penetrated through stomatal openings of the glume, lemma, and/or palea. Growth of germ tubes toward stomata was common. Approximately 90% of germ tubes that penetrated beyond the stomatal ledges did not pass between the guard cells and failed to enter the substomatal chamber. During the early stages of infection, intercellular hyphae were present among parenchyma and chlorenchyma cells in the distal to midportions but not basal portions of the glume, lemma, and palea and were absent from the ovary, subovarian tissue, rachilla, and rachis. Later, hyphae had grown intercellularly toward the floret base to the subovarian tissue and had entered the pericarp of the ovary through the funiculus. Hyphae were found in the rachis only during the later stages of infection. The epidermis of the ovary was not penetrated, even after prolonged contact with germinating secondary sporidia.

Karnal bunt of wheat (also called partial bunt) is caused by *Tilletia indica* Mitra (= *Neovossia indica* (Mitra) Mundkur). When the fungus sporulates in the kernel, a black mass of teliospores forms which emits the odor of trimethylamine, characteristic of bunt fungi of wheat. The kernels usually are only partially consumed by the fungus, hence the name partial bunt, but in extreme cases the entire endosperm is converted into a sorus (5). Usually, only a few kernels per spike are affected and yield losses are very low; however, disease incidence of up to 89% of the kernels from some fields has been reported (15).

Since its first record in 1931 (17), the disease has spread from Karnal, Haryana, in northwestern India to almost all of the wheat-growing regions of India (14,25–27,30) and other parts of the Near East (6,16) and has been discovered recently in northwestern Mexico (9). The history of the disease in Mexico has been one of increasing severity and distribution (30). The threat to wheat-growing areas in the United States has led to the current quarantine of Mexican wheat (4) and to restrictions on the movement of wheat germ plasm from Mexico to the United States (1).

Although it has long been recognized that infection occurs during heading and that florets are infected locally (2,7,8,31) by airborne or waterborne sporidia (basidiospores) (3,20,22), the histological details of the early infection stages are still poorly understood. This study was conducted to determine how and where the fungus initially penetrates the host and how it reaches the site of sporulation.

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MATERIALS AND METHODS

Preparation of inoculum. Teliospores from infected kernels collected near Ciudad Obregón, Sonora, Mexico, were germinated on 2% water agar at 20 C (28). A culture of germinated teliospores was inverted over a petri dish of 2% potato-sucrose agar (PSA) for approximately 24 hr at 20 C. During this time allantoid secondary sporidia showered onto the PSA. The PSA plate then was incubated at 20 C and within 24–48 hr produced a white, floccose colony that actively discharged abundant secondary sporidia.

Inoculation. The susceptible wheat (*Triticum aestivum* L.), 'Oleson's Dwarf' was grown in a greenhouse at 25 C under natural light (about 13 hr per day) until just before anthesis (Zadoks 5.9 [32]). For inoculation, pots and plants were laid on their side, and the spikes were placed onto fresh water agar in a petri dish. Then the PSA plate containing the fungal colony, described above, was inverted over them. A slit was made in the side of the lower petri dish to accommodate each stem, and a 1-mm space was left between the two petri dishes for air exchange. Spikes were inoculated in an incubator for 24–48 hr at 20 C under continuous illumination from a 40-W cool-white fluorescent bulb. During this period, naturally liberated secondary sporidia showered down onto the spikes and the water agar to the extent that the inoculum

was macroscopic on the agar of the lower dish. Spikes were fixed immediately after inoculation and after the inoculated plants had grown in the greenhouse for 2, 4, 7, 9, and 13 days. Six inoculated spikes, which were grown to maturity for checks, had infection in 100% of the approximately 95 total kernels.

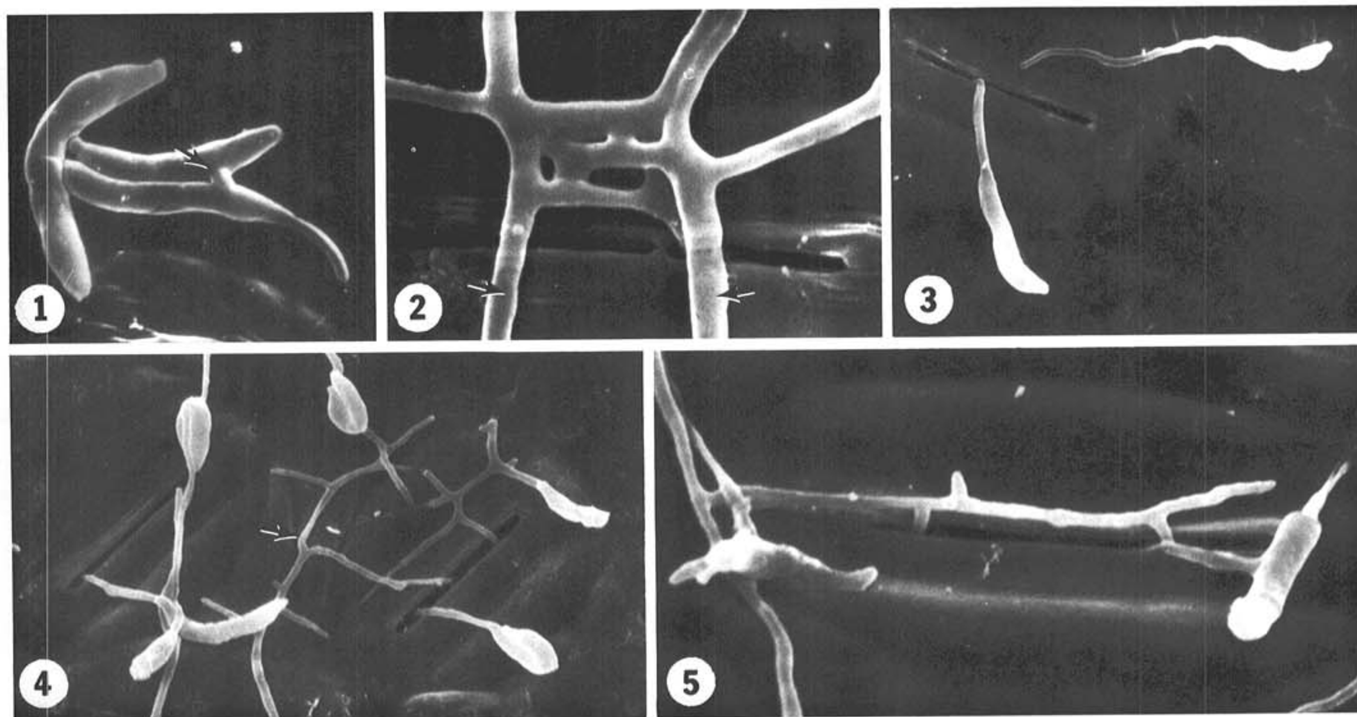
Two methods were used to place inoculum onto the ovary wall of florets at pre- or post-anthesis stages (Zadoks 6-7 [32]): 1) The glumes and lemmas were removed from the florets on one side of three spikes, which were then inoculated for three days, as described previously; or 2) the interiors of the individual florets of three spikes were flooded by hypodermic injection with an aqueous suspension of allantoid secondary sporidia (about 500,000 per milliliter) and then the plants were placed in a dew chamber for 1 day and grown in a greenhouse for 2 days. Three nascent kernels, 2–5 mm in length, were removed from the spikes inoculated with each technique and were prepared for examination with light microscopy as described later. Two kernels about 2 and 3 mm in length were transversely sectioned completely and four kernels 4–5 mm in length were sectioned transversely at a maximum of five levels along their length. A minimum of 60 serial sections were examined at each level.

Fixation. Florets that faced the fungal colony during inoculation were selected for fixation. For scanning electron microscopy, three spikes were flooded with 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at room temperature after 24 hr of inoculation. Then the outer glumes and portions of the rachis were removed from the spikes and immersed in the fixative for 2–24 hr, washed in buffer, immersed in 1% aqueous OsO_4 for 1 hr, dehydrated in an ethanol series, infiltrated with freon 113, and critical point dried using freon 13 as a drying fluid. The samples then were sputter-coated with gold and examined with an AMR 1000 scanning electron microscope (Amray Inc., Bedford, MA) at 10 Kv. Four glumes from each spike and three rachis portions were examined.

For light microscopy, florets (including a portion of the subtending rachis below the rachilla) and rachis samples from the midportion of spikes were removed and immersed in the buffered 2% glutaraldehyde. To aid fixation and embedment, the basal fourth of florets was cut off transversely and then each floret piece (that is, the basal portion and the upper three fourths of the glume, lemma, and palea, and the upper portion of the ovule, if it was large enough) was processed separately. The floret pieces were fixed for 2–24 hr, washed in buffer, and then immersed in 1% OsO_4 for 1 hr or were fixed for 24 hr in Formalin-acetic acid-ethanol (13). The samples then were dehydrated in an acetone series and embedded in low-viscosity resin (29). Approximately 60 transverse serial sections about 2.5 μm thick were cut with glass knives at intervals of about 0.75–1.5 mm along the length of the floret and rachis pieces. Each piece was sectioned at 3–5 levels. The basal portion of some floral parts was sectioned longitudinally for approximately 600–800 nearly consecutive sections. The sections were transferred to glass microscope slides using a wire loop, dried on a slide warmer at 45 C, and stained with 1% safranin-O in 30% ethanol followed by 1% aqueous methylene blue. The sections then were rinsed in deionized water to remove excess stain, air dried, mounted in immersion oil, and examined with a Zeiss Universal microscope (Carl Zeiss, Inc., Oberkochen, West Germany) or a Leitz Dialux 20 microscope (Ernst Leitz GMbH, Wetzlar, West Germany) at 400×. Fixatives and embedding resins were obtained from Polysciences, Inc., Warrington, PA.

RESULTS

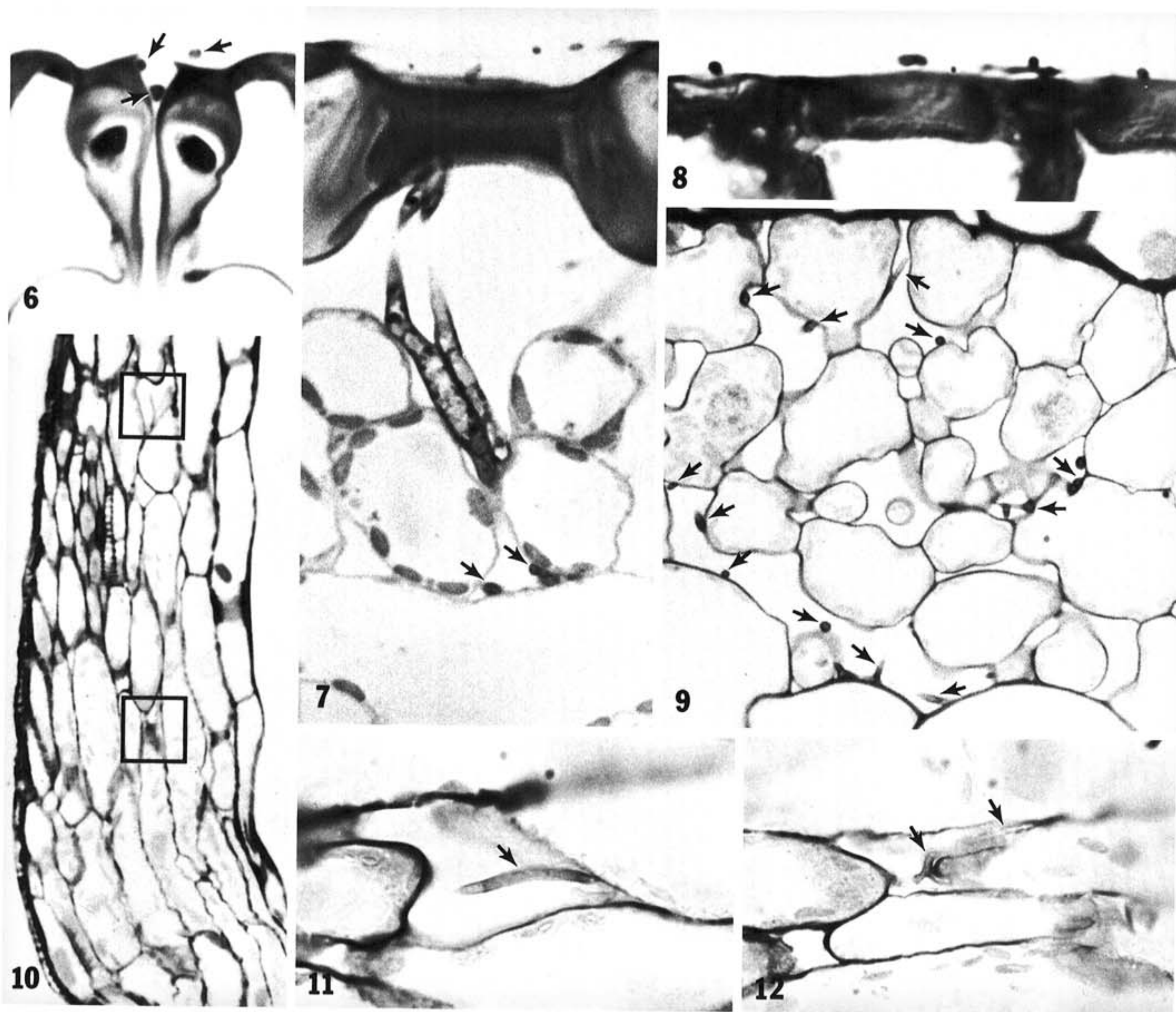
Hyphal penetration. Scanning electron microscopy showed numerous secondary sporidia, most of which had germinated, located primarily on the distal half of the abaxial surface of glumes. All hyphae observed on glumes could be traced back to a secondary sporidium. On four occasions, hyphae or secondary sporidia on the glume surface were observed that appeared to have undergone anastomosis (Figs. 1 and 2). However, numerous hyphae and sporidia on the surface of glumes contacted and/or grew parallel to each other but showed no evidence of fusion. Germ tubes arising from secondary sporidia often grew toward stomata (Figs. 3 and 4). The germ tubes entered stomatal openings directly or first grew parallel to the aperture and then produced perpendicular branches which entered the opening (Figs. 3–5).



Figs. 1–5. Scanning electron micrographs of secondary sporidia and hyphae of *Tilletia indica* on the surface of wheat glumes. **1,** Apparent fusion tube (arrow) between two of three secondary sporidia. 1,400×. **2,** Apparent fusion of hyphae near a stomatal aperture. All hyphae in the micrograph originated from two germ tubes (arrows) of separate secondary sporidia. 2,800×. **3,** Germ tubes from secondary sporidia growing toward and entering a stomatal opening. 800×. **4,** Secondary sporidia that have produced germ tubes which entered stomatal openings. One germ tube (arrow) has produced perpendicular branches. Some of the secondary sporidia have folded over onto themselves which is a response to low humidity. 800×. **5,** A germ tube oriented parallel to a stomatal aperture that has produced perpendicular branches which entered the opening. 1,700×.

Views of the surface of glumes that were fixed immediately after inoculation showed that germ tubes penetrated stomata beyond the stomatal ledges (Figs. 3–5). However, serially sectioned material fixed 2–9 days after inoculation showed that, of several hundred challenged stomata examined, about 90% of the time the hyphae were confined to the vestibule above guard cells (Fig. 6). Sometimes, all of 8–12 serially sectioned stomata within a sampling had hyphae in the vestibule but none had hyphae beyond the guard cells. Apparently in such instances the aperture between guard cells was closed tightly preventing hyphae from entering the substomatal chamber. The confinement was apparently not simply a delay before penetration because this phenomenon was observed in samples fixed 9 days after inoculation. Despite this exclusion, hyphae were commonly observed extending between the guard cells into the substomatal chamber (Fig. 7). Hyphae in the substomatal chamber usually were larger in diameter than hyphae on the epidermis of the glume or intercellular hyphae elsewhere (Fig. 7).

All glumes, lemmas, and paleas examined showed hyphal penetration through stomata at some point along their length. However, hyphae were not observed penetrating the lower fourth of the glume and lemma or the rachis. Stomata were highly concentrated in longitudinally oriented rows located near the keel, above the linear areas of chlorenchyma cells of the glume and lemma. The number of stomata gradually decreased toward the base to the extent that very few were observed on the basal fourth of glumes and lemmas. Numerous stomata were on the epidermis of the rachis but there were very few secondary sporidia that were either deposited or retained in these areas after processing for microscopy. Stomata of the palea were oriented in rows adjacent to the keeled areas and were present until just above their attachment to the floret base. Penetration sites that were closest to the base of the ovary occurred through stomata at the basal portion of the palea. Hyphae commonly penetrated the basal portion of awns, which have numerous stomata. Hyphae penetrated only between stomatal guard cells and there was no evidence of direct penetration or localized dissolution of the epidermis beneath hyphae on glumes (Fig. 8).



Figs. 6–12. Establishment and growth of *Tilletia indica* hyphae in wheat florets. **6,** One section of a complete series through a transversely sectioned stoma of a glume, showing transversely sectioned hyphae above and below the stomatal ledges. The hyphae (arrows) were confined to the vestibule above the guard cells in this and all other sections of this stoma. 1,990×. **7,** Two hyphae have penetrated into the substomatal chamber of a longitudinally sectioned stoma of a glume. Other intercellular hyphae also are present (arrows). 1,730×. **8,** Hyphae on the surface of a glume fixed 9 days after inoculation. 1,270×. **9,** Numerous intercellular hyphae (arrows) among chlorenchyma cells of a glume fixed 2 days after inoculation. 790×. **10,** One of a series of longitudinal serial sections through the basal portion of a glume fixed 4 days after inoculation. The glume had a hypha (in squares and shown in Figs. 11 and 12) that terminated in the subovarian tissue in adjacent sections. 305×. **11 and 12,** High magnification of the hypha (arrows) located within the squares of Figure 10. The micrographs are oriented 90° counterclockwise from that shown in Figure 10. 1,400×.

Location of hyphae in spikes during infection. After penetration, hyphae grew intercellularly among parenchyma and particularly chlorenchyma cells of glumes, lemmas, and paleas. In the palea, hyphae were observed only in the keeled areas. Approximately 2–8 hyphae usually were observed at any particular level of sectioning of the glumes, lemma, and palea, but occasionally more than 20 hyphae were observed in glumes (Fig. 9).

Two days after inoculation, intercellular hyphae were present in the upper to midportions of the glume, lemma, and palea, and rarely at the base of the palea. Hyphae were not present elsewhere in the floret (Table 1) or in the rachis at this time. Of five florets fixed 4 days after inoculation, three had hyphae in the upper to lower portions of the glume, lemma, and palea but not elsewhere; a portion of one floret, that was serially sectioned longitudinally, had a longitudinally oriented hypha that was followed through the basal fourth of the glume (Figs. 10–12) to a termination point in the subovarian tissue (that is, tissue between the rachis and the chalaza) but had no hyphae elsewhere in the subovarian tissue, ovary, or rachis; and in one floret, hyphae were present in the glume, lemma, palea, and subovarian tissue but absent in the ovary and rachis. Florets fixed 7, 9, and 13 days after inoculation (Table 1) had hyphae in all floral parts except the stamens and anthers. Hyphae were observed in the secondary stalk of the rachilla as early as 4 days after inoculation.

Longitudinal serial sections of the basal fourth of florets fixed 9 days after inoculation showed hyphae that were longitudinally oriented and continuous through the glume, lemma, and/or palea to the subovarian tissue and were present from the subovarian tissue to the base of the ovary (Figs. 13–34). Hyphae that were followed through the floret base had penetrated at a point above the basal fourth of the floret except for one that penetrated near the base of the palea. Hyphae were numerous (usually > 20) in the subovarian tissue and funiculus and were relatively sparse (Fig. 35) to prolific (Fig. 36) in the pericarp at the base of the ovary at this stage. If hyphae were present in the distal portions of the ovary, they were always present in the basal portions.

Samples of the rachis dissected from near the middle of spikes were transversely sectioned at a minimum of two levels that were about 1.5 mm apart. Hyphae were not observed in four rachis samples fixed 2 days after inoculation or in three rachis samples fixed 4 days after inoculation. However, rachis samples from six spikes fixed 7, 9, and 13 days after inoculation had from 2 to 25 intercellular hyphae (Figs. 37–39). All hyphae found in the rachis were located among cortical cells and none were present in the chlorenchyma adjacent to the epidermis, indicating that the hyphae grew to the rachis from some other point rather than penetrating it.

Hyphae were never observed in the ovary or rachis without the presence of hyphae in the base of the glume, lemma and/or palea, and the subovarian tissue. Hyphae in the subovarian tissue apparently grew toward the ovary and entered the pericarp through the funiculus. Sections of six nearly mature, highly infected kernels (three each, fixed 13 and 18 days after inoculation) showed that hyphae were restricted to the pericarp by the highly lignified seed coat (Fig. 40) that covers the endosperm and embryo. Teliosporogenesis was observed only in samples fixed at least 13 days after inoculation (Fig. 40).

Hyphae on the ovary surface. Hyphae were never observed on the epidermis of the ovaries after intact spikes were inoculated. Hyphal penetration of the epidermis of the ovary was not observed even after ovaries were inoculated directly (Fig. 41). Six ovaries, ranging from 2 to 5 mm in length at the time of fixation, were inoculated directly and serially sectioned partially or entirely. Despite luxuriant hyphal growth on the surface of the ovaries, the epidermis and stigmas were not penetrated.

DISCUSSION

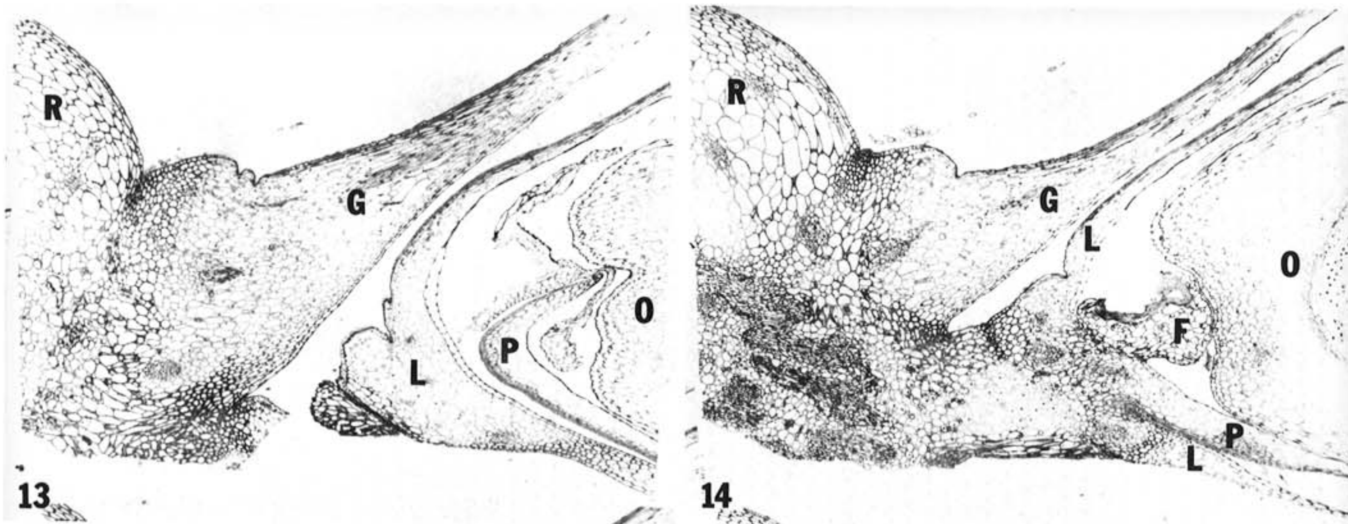
Karnal bunt was first considered to be systemic and seedborne as in common bunt, caused by *Tilletia tritici* (Bjerk.) Wint. and *T. laevis* Kühn. However, Mitra (18,19) and Mundkur (21) were unsuccessful in producing the disease by inoculation of seed in numerous experiments that were performed over a period of several years. Mundkur (22) demonstrated that a low level of disease incidence could be induced by inoculating spikes with teliospores, which indicated that infection of florets was local and produced by airborne inoculum at the time of heading. Later Bedi et al (3) confirmed this by demonstrating that high disease incidence results after applying sporidia to the spikes during anthesis and that the disease is prevented by bagging heads from the boot stage to maturity.

Previous studies on the mode of penetration and establishment of *T. indica* hyphae have concluded that the fungus penetrates the cuticle of the epidermis of the glume, lemma, or palea, passes

TABLE 1. Location of hyphae within wheat florets at different stages of disease development

Days after inoculation (no.)	Floret part[b]	Samples sectioned (no.)	Percent of samples that had hyphae at:[a]						
			Tip			Midportion			Base
2	G	5	80	60	80	40	0	0	0
	L	5	40	40	0	0	20	0	0
	P	5	0	40	40	60	20	20	0
	S	5						0	0
	O	5						0	0
4	G	5	100	100	80	80	60	60	60
	L	5	80	80	100	40	40	20	0
	P	5	0	20	20	60	60	60	60
	S	5						40	20
	O	5						0	0
7	G	3	66	100	66	100	100	100	100
	L	3	33	66	33	66	33	33	33
	P	3	0	33	66	66	66	33	33
	S	3						100	100
	O	3					66	66	66
9 and 13[c]	G	6	66	83	83	83	100	100	100
	L	7	57	86	86	86	100	100	100
	P	4	25	50	75	100	100	100	100
	S	5						100	100
	O	5					100	100	100

[a] Approximate level of sectioning.
[b] G = glume; L = lemma; P = palea; S = subovarian tissue; O = ovarian tissue.
[c] Data represent two to four samples from each sample date.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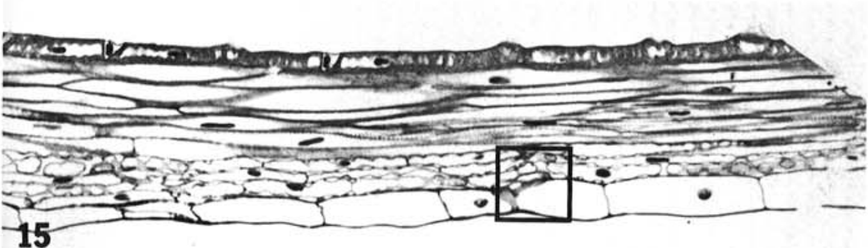
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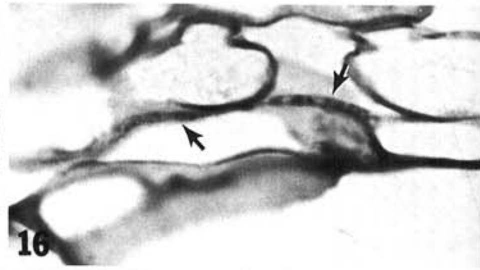
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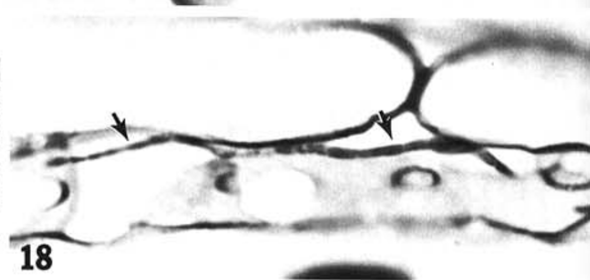
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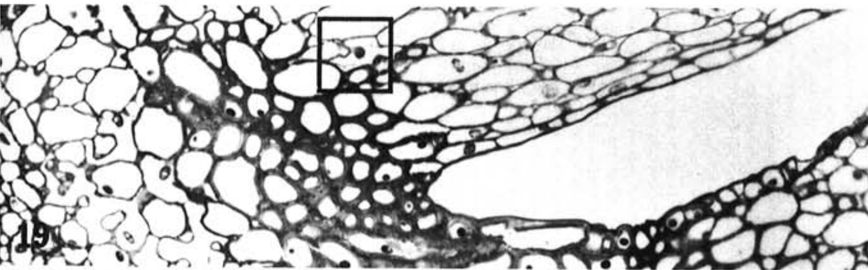
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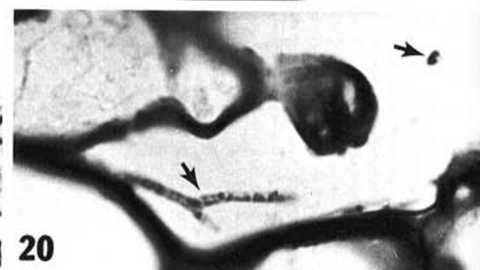
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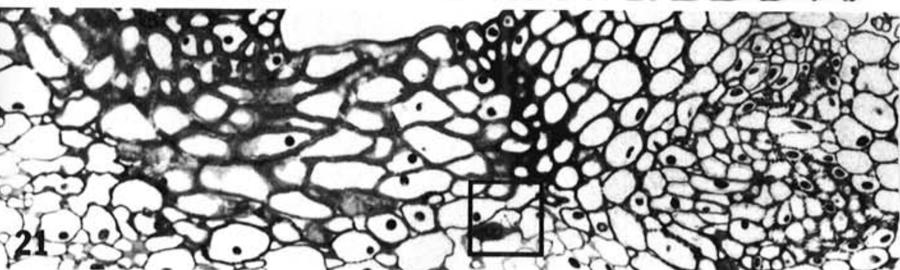
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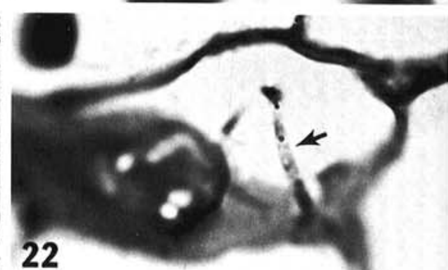
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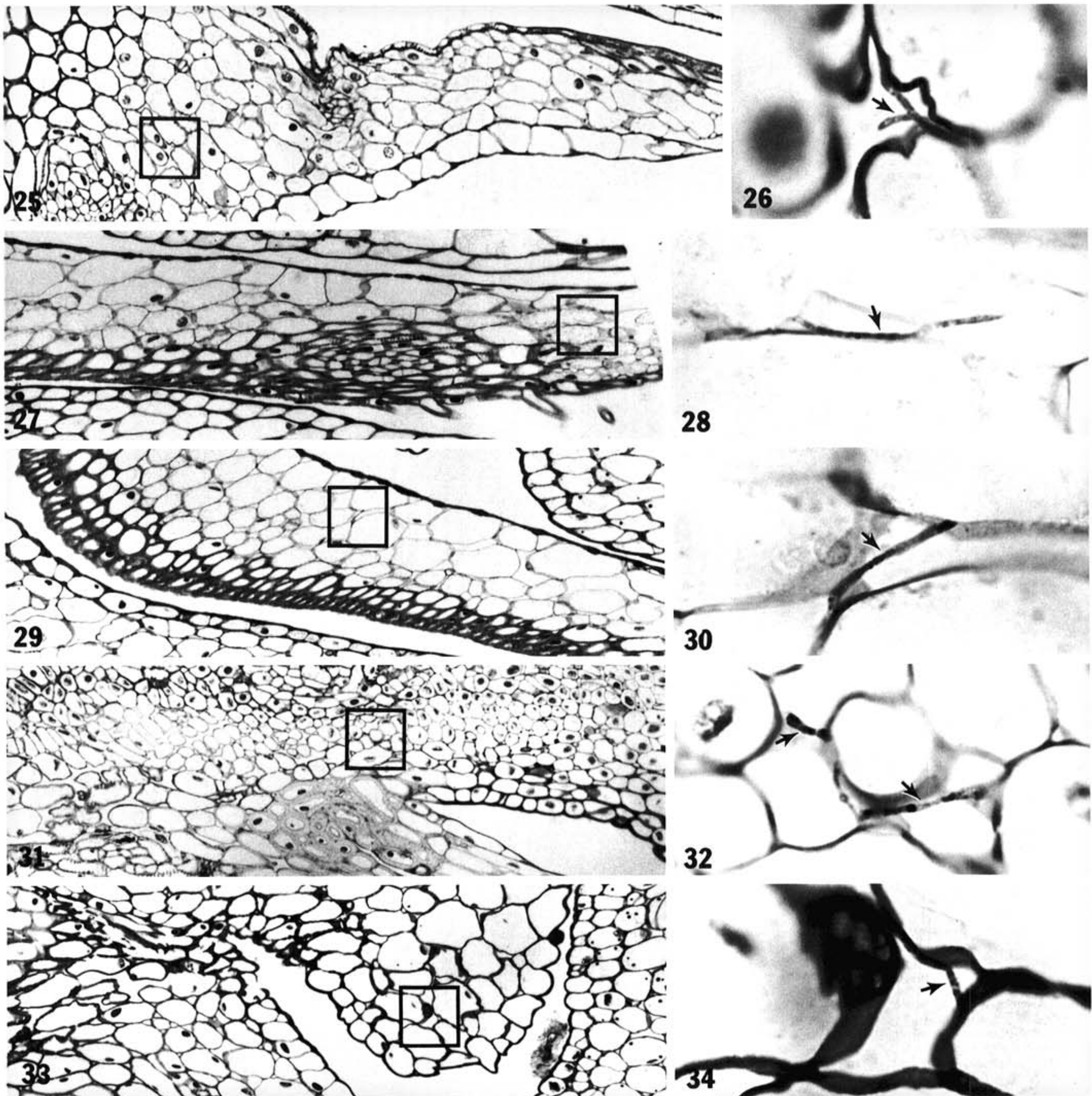


Figs. 13–34. Longitudinal sections of a series through the basal portion of a wheat floret fixed 9 days after inoculation with *Tilletia indica*. Serial sections showed that hyphae were present and continuous throughout the floret. **13 and 14,** Sections of the basal portion of the floret. The section in Figure 13 is about 85 sections above that in Figure 14. A portion of the ovary of this floret is in Figure 35. R = rachis; G = glume; L = lemma; P = palea; F = funiculus; O = ovary. 32×. **15–24,** Various portions of the floret, and high magnification of hyphae (arrows) within the area delimited by the squares. **15 and 16,** The tip of the glume. **17 and 18,** The midportion of the glume. **19 and 20,** The base of the glume. **21 and 22,** The area between the base of the glume and the base of the lemma. **23 and 24,** The base of the lemma. **25 and 26,** The base of the lemma. **27 and 28,** The tip of the palea. **29 and 30,** The base of the palea. **31 and 32,** Opposite the base of the palea. **33 and 34,** The funiculus. Low magnifications at left = 170×; high magnifications at right = 1,400×.

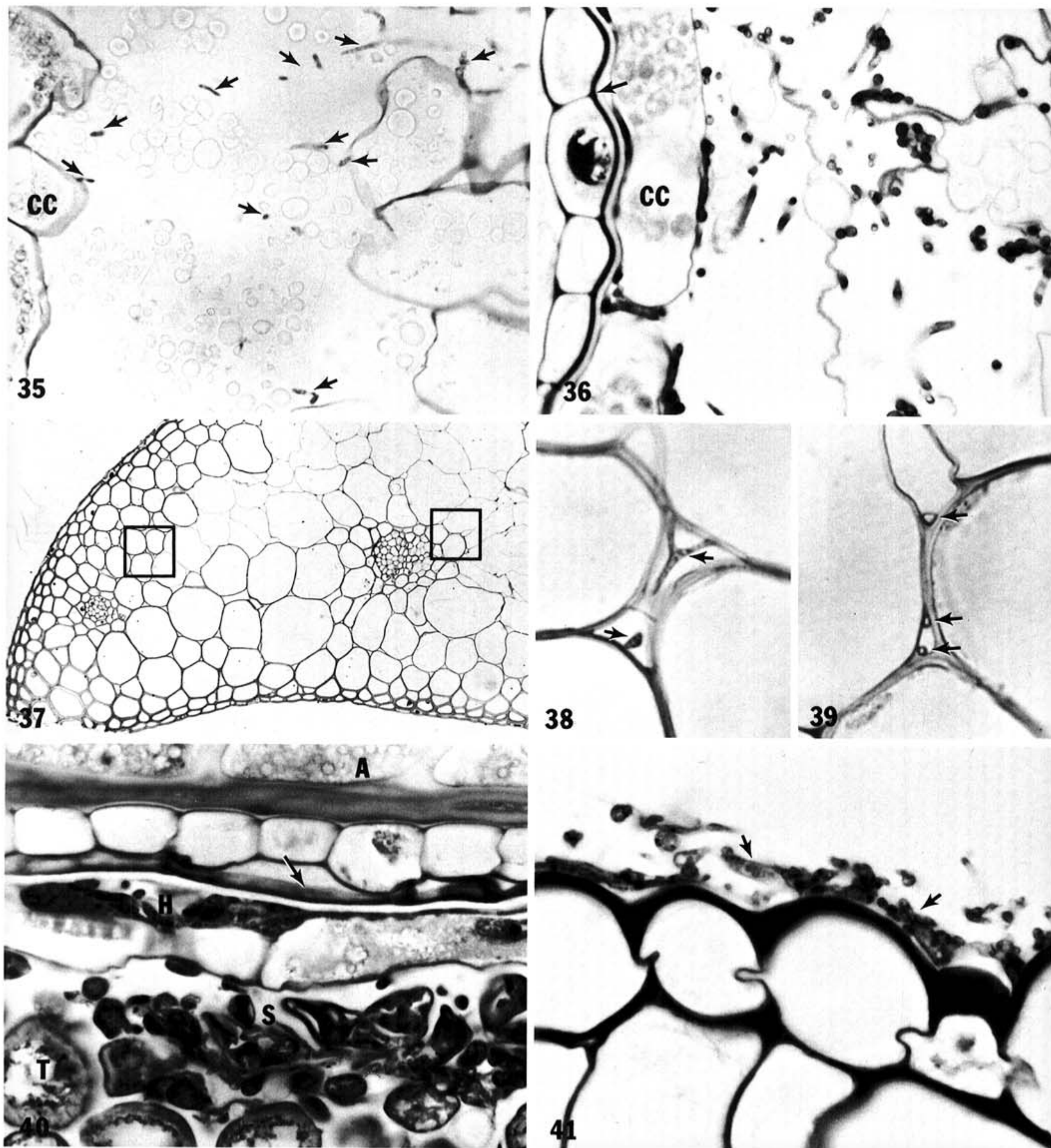
transversely through these organs, and then penetrates the epidermis of the ovary (11,23) or that the ovary wall can be penetrated directly from inoculum in the floral cavity (11). Other hypotheses on infection also involve direct penetration by the fungus through the epidermis of the ovary (25). According to these hypotheses, the fungus would penetrate the ovary at random sites, which is incompatible with the fact that sporulation (17,18,23) and hyphal invasion of the ovary always begin at its base. Munjal and Chatrath (23) considered the environment at the base to be more favorable for teliosporogenesis.

In the present study, hyphae were never observed to penetrate the cuticle of floral parts even after direct inoculation. It appears that *T. indica* is incapable of direct entry through the cuticle. Longitudinally oriented hyphae were present in the basal portions of the glume, lemma, and palea at a time when hyphae were absent in the ovary and points below (that is, subovarian tissue and the rachis). Furthermore, if hyphae were present in the ovary, they also were present in the subovarian tissue. This evidence indicates that hyphae enter the base of the ovary after first growing intercellulary through the glume, lemma and/or palea, and possibly the rachis to the subovarian tissue and then through the funiculus to the ovary.

Several investigators have reported that hyphae of *T. indica*

invade the endosperm (15,23) or embryo (15,18,20,25), whereas others have reported that the endosperm (5,14,17,19,24) or embryo (5,12,23,24) are free from hyphae. Most of these reports, however, are not results of detailed histopathological studies. In the present study, the embryo and the endosperm of highly infected kernels were protected from hyphal invasion by the lignified seed coat that covers both of these tissues.

Investigations of the spatial relationship between infected spikelets have demonstrated that infection is local rather than systemic and that the fungus can invade adjacent spikelets from an initial penetration point (2,7,8,31). The nature of this secondary spread is considered aerial (2) or systemic (7,8), although hyphae previously had not been identified in the rachis. In the present study, hyphae were observed in the rachis between infected



Figs. 35–41. Tissue of wheat spikes inoculated and/or infected with *Tilletia indica*. **35 and 36,** Hyphae adjacent to cross cells (CC) in the periderm of two florets fixed 9 days after inoculation. **35,** Longitudinal section from the same floret as that in Figures 13–34 showing relatively few hyphae (arrows). 590×. **36,** Transverse section showing numerous hyphae. Note the developing seed coat (arrow). 1,125×. **37,** A portion of a transversely sectioned rachis from a sample fixed 13 days after inoculation. The rachis contained 25 hyphae. 135×. **38 and 39,** High magnification of hyphae (arrows) located within the squares of Figure 37. The figures are oriented 90° counterclockwise from that in Figure 37. 1,400×. **40,** Sporogenous hyphae (S) and developing teliospores (T) in the pericarp of a wheat kernel that was fixed 13 days after inoculation. The fungus is limited to the pericarp by the seed coat (arrow). Densely packed hyphae (H) are present adjacent to the seed coat. The aleurone layer (A) is at the top of the micrograph. 1,075×. **41,** Hyphae and secondary sporidia (arrows) on the epidermis of a transversely sectioned ovary that was inoculated directly with secondary sporidia. Penetration of the epidermis did not occur. 1,490×.

spikelets during the later stages of infection, showing that the rachis is a likely pathway for spread from initial infection sites.

Concentration of sporidia on the upper portions of the glume surface in these experiments might have resulted from uneven deposition, possibly caused by electrostatic forces. The lack of sporida may be the reason why penetrations were not observed in the rachis. Because numerous stomata were observed on the rachis, it seems likely that penetrations could occur here if the inoculum were present.

Because *T. indica* is a heterothallic organism (10), primary or secondary sporidia or hyphae of compatible mating type must fuse to form the pathogenic dikaryon; however, it is not known when this occurs. Because primary sporidia of *T. indica* do not fuse on the promycelium following teliospore germination (10), fusion probably occurs at some time after sporidia reach the plant. Although there was some evidence of anastomosis on the glume surface in this study, it was far too rare to be considered the normal mode of dikaryon formation. Conclusive evidence of the initiation of the pathogenic dikaryotic phase will require elucidation of the nuclear condition of hyphae during the infection process.

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