Preinfectional Interactions Between *Helminthosporium oryzae* and Resistant and Susceptible Rice Plants

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ABSTRACT

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Comparisons were made of the prepenetration activities of *Helminthosporium oryzae* on plants of one susceptible, one intermediate, and two resistant rice cultivars. The percent germination of conidia on leaf surfaces was not significantly different between susceptible and resistant cultivars. On resistant plants, germ tube elongation was markedly increased. On susceptible plants, germ tubes were short, appressoria were produced early, and the hyphal mass per conidium was higher. Appressoria were mainly concentrated on bulliform cells and at junctures between epidermal cells. On polystyrene leaf surface replicas, appressoria formed equally well over all the cell types and were located mainly at cell junctures. Scanning electron microscopy showed that conidia, hyphae, and appressoria of *H. oryzae* were held in close contact with leaf surfaces by amorphous secreted sheathlike substances that adhered to the leaf surface, masked the wax crystals, spread some distance (5–15 μm) from the hyphae, and left imprints on leaf surfaces and their polystyrene replicas after the hyphae and the sheaths were removed.

Additional key words: brown leaf spot, *Cochliobolus miyabeanus*, *Oryza sativa*.

The physiology and pathogenicity of *Cochliobolus miyabeanus* Drechsler ex Dastur (*Helminthosporium oryzae* Breda de Haan) on rice (*Oryza sativa* L.) has been studied by a number of workers (18); however, the mechanism of disease resistance is not yet fully understood. Ganguly and Padmanabhan (7) recognized two types of resistance: resistance to penetration, and resistance to ramification after penetration. The former was attributed to mechanical characteristics of the epidermis and the latter to physiological defenses in the protoplasm. Mishra and Prasad (15) indicated that structural defenses might be related to resistance. However, Chattopadhyay and Chakrabarti (4) found no correlation between toughness of epidermal cells and resistance to the disease. Oku and Nakanishi (17) and Oku (16) suggested that phenolic compounds and a phytoalexinlike substance might be involved in resistance. Triveda and Sinha (21) found a fungitoxic substance in infected tissues. Scanning electron microscopical (SEM) observation of the stages of infection caused in rice leaves by *H. oryzae* was reported by Locci (14). Limited information is available on the histological aspects of infection, particularly at the stages between inoculation and infection.

Results of an inheritance study (8) suggested that as few as two major genes and some minor modifier genes may control resistance to infection by *H. oryzae* (8). These genes for resistance may be expressed as early as the prepenetration period of infection.

The objectives of this investigation were to examine morphological aspects of the preinfection interactions of *H. oryzae* on the leaf surfaces of resistant and susceptible rice plants and to attempt to identify resistance mechanisms, if any, at this stage of infection. This report describes the first quantitative SEM study of the conidial germination, infection structures, and hyphal growth of *H. oryzae* on resistant and susceptible rice plants.

MATERIALS AND METHODS

Plant materials. Two resistant cultivars, TN-1 (Taichung Native number 1) and Tetep; one intermediate cultivar, Starbonnet; and one susceptible cultivar, Dular, were used in these experiments. Seeds were treated with 0.6% sodium hypochlorite solution for 10 min, rinsed with distilled water three times, and germinated on moist filter paper. Germinated seeds were planted 0.5 cm deep in steam-sterilized soil in 15-cm-diameter plastic pots. The plants were watered with deionized water and fertilized with a $(NH_4)_2SO_4$-zinc chelate liquid fertilizer.

Inoculum and inoculation. Inoculum in all experiments consisted of conidia obtained from 15-day-old *H. oryzae* cultures on rabbit food agar medium (9). The *H. oryzae* cultures were washed with 10 ml of distilled water and filtered through two layers of cheesecloth to remove mycelial fragments. The conidial concentration was determined with a hemacytometer and adjusted to 1.5×10^4 conidia per milliliter. Tween-20 (0.1%, v/v) was added to the spore suspension. All operations were performed under aseptic conditions. Plants were inoculated 35 days after seeding by spraying the spore suspension on the leaves with a DeVilbiss atomizer. Inoculations in the greenhouse were made inside a humidity chamber maintained at 95–100% RH and ~30 C. After 24 hr, inoculated plants were removed from the chamber and placed on benches in the greenhouse. Disease severity index was determined by visual examination of size and number of lesions that had developed on inoculated rice leaves 15 days after inoculation. The disease index was rated on a scale of 0 to 5 with 0 representing highly resistant and 5 representing highly susceptible reactions.

Tissue preparation for microscopic observations. Leaf samples were collected at 5, 7, 10, 12, 15, 24, and 48 hr after inoculation.

Whole mounts were prepared by a modification of the method of Vance and Sherwood (22). Leaf pieces were cleared and stained by simmering for 3 min in aniline blue-lactophenol (1 ml lactic acid, 1 ml phenol, 8 ml ethanol, 4 mg aniline blue, and 1 ml distilled water) and mounted in lactophenol for observation (2).

Polystyrene leaf replicas were prepared according to the procedures reported by Wynn (27).

For SEM observations, leaf pieces were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 12 hr and postfixed in 1% osmium tetroxide in the same buffer for 1 hr. They were washed in the buffer three times after fixation. The specimens were dehydrated with 2,2-dimethoxypropane (19) and dried in a Denton critical-point drying apparatus with acetone as the

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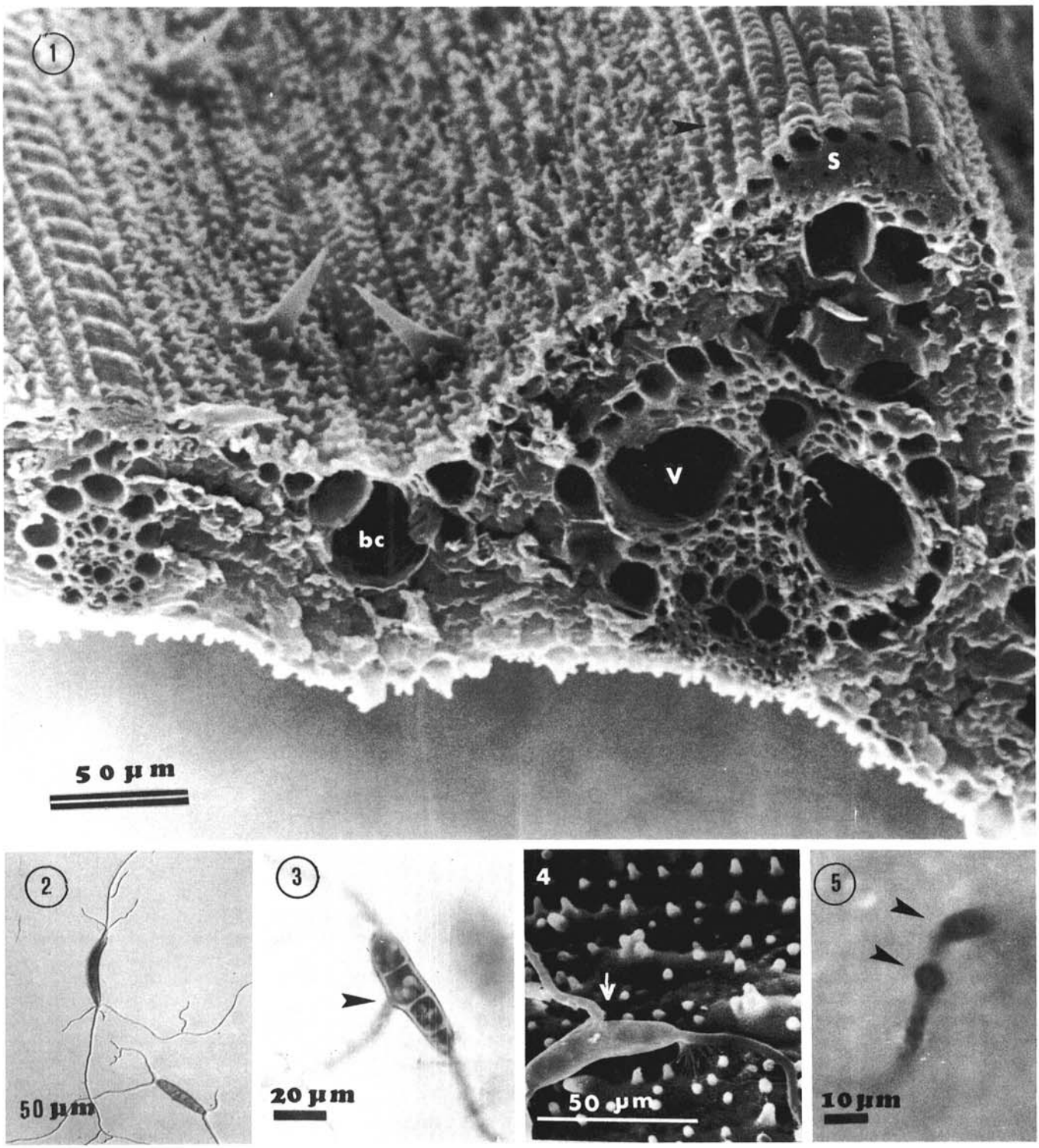
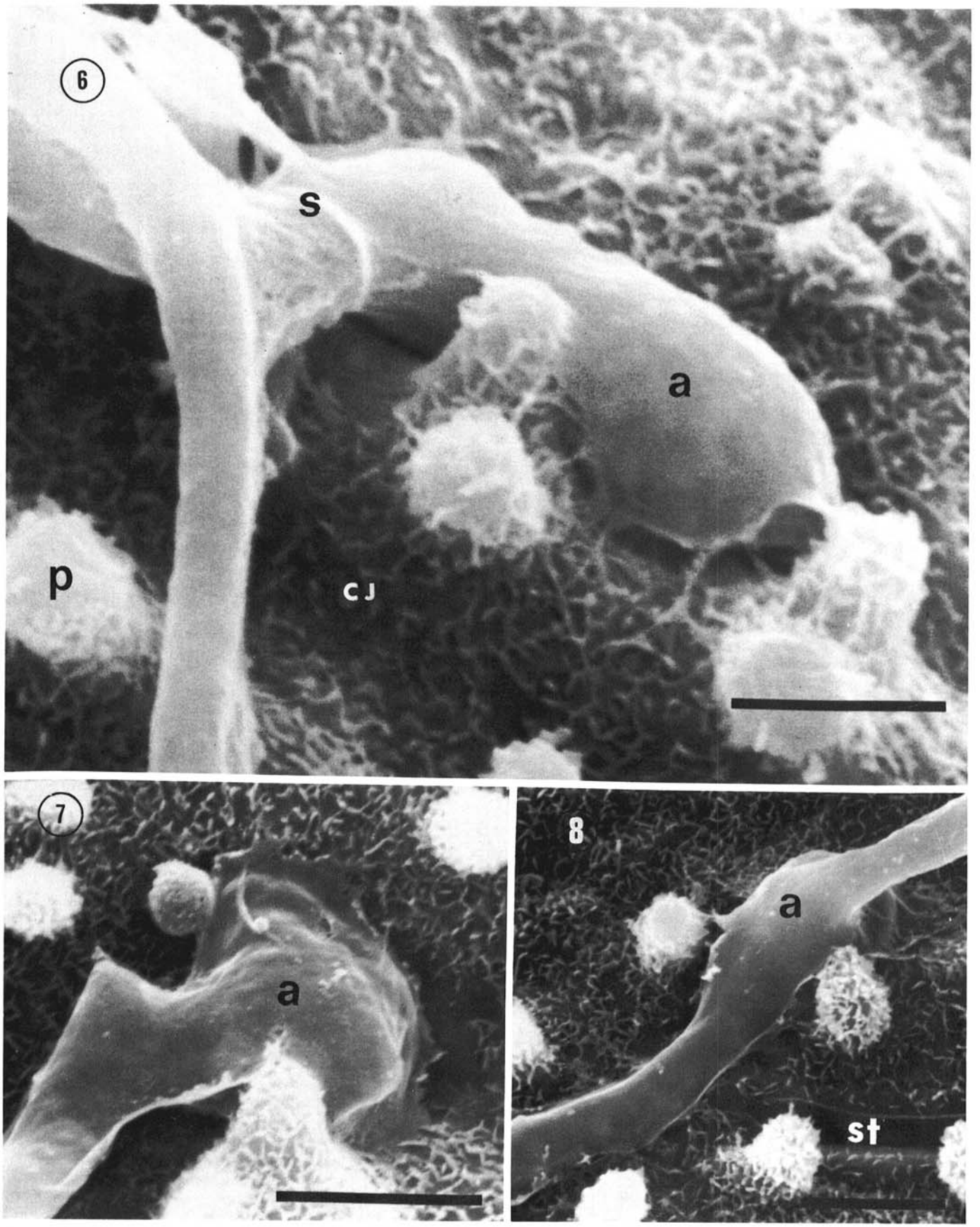
intermediate fluid and liquid nitrogen as the transition fluid. Polystyrene leaf replicas were processed by the method of Laane (13). All specimens were then mounted on stubs with silver paint and coated (0.01 μm [100 Å] thick) with gold-palladium in a Hummer I sputter coater. Examination and micrography were done with a Hitachi-Hiscan, Model S-500 scanning electron microscope. In each experiment, 25 conidia were examined for germination, length of germ tube when applicable, and the number of appressoria. Hyphal growth was determined by measuring the length of hyphae originating from each conidium.

For transmission electron microscopy, the specimens were fixed and dehydrated as described for SEM. After the dehydration was completed, the excess of 2,2-dimethoxypropane and reaction by-products was decanted, and an equal volume of absolute acetone was added, followed by another change of fresh absolute acetone. To embed them in Spurr's low-viscosity resin medium, the specimens were carried through mixtures with increasing plastic-acetone ratios of 1:3, 1:1, 3:1, and, finally, into pure plastic. Specimens were then transferred to fresh plastic in molds and polymerized in a 70 C oven for 8 hr or more. Thin sections were cut



Figs. 1–5. Scanning electron micrograph of a healthy rice leaf of TN-1 showing vacuolated bulliform cells (bc), vessels (v), sclerenchyma cap (s) and papillae (arrow) on leaf surface. **2–5,** Early stages of leaf surface activities of *Helminthosporium oryzae* on rice leaves. **2,** Germination of conidia in distilled water on a glass slide. **3,** Germination occurred from both end cells and middle cells (arrow). **4,** SEM micrograph of germination of a conidium on a leaf surface of TN-1. Note also the germination of the middle cell (arrow). **5,** Appressoria (arrows) were initiated terminally on germ tubes.



Figs. 6–8. Formation of appressoria by *Helminthosporium oryzae* on leaf surface of rice cultivar Dular. **6,** An oval-shaped appressorium (a) was formed over the cell juncture (cj) with papillae (p) on both sides. The appressorium (a) was delimited from the hypha by a septum (s). **7,** An appressorium (a) was formed and sheath accumulate around the appressorium. **8,** A mature appressorium (a) with a secondary hypha. The stomate (st) did not stimulate appressorium formation. All scale bars represent 5 μm.

with glass knives in an LKB-2088 ultramicrotome. They were stained in uranyl acetate for 5 min and in lead citrate for 10 min. Sections were then examined and photographed with a Hitachi Hu-11-A electron microscope.

RESULTS

Gross anatomy of rice leaves. Salient features of the anatomy of a rice leaf are shown in Fig. 1. Vascular bundles are located between thick-walled, nonliving sclerotized sclerenchyma cells, most of which are fibers. Parenchyma cells are positioned between the vessels. The rice leaf has a typical gramineous epidermis composed of four main cell types: long cells, silica cells, cork cells, and bulliform cells. The latter are large, thin walled, highly vacuolated cells located between veins (6). Bulliform cells form bands, usually several cells wide, that parallel the veins. In cross section, bulliform cells appear as a fan; the median cells are usually the largest and somewhat wedged-shaped. The rice leaf surface is characterized by the presence of papillae. These are epidermal cell projections arranged in rows along the long axis of the leaf.

TABLE 1. Site of appressorium formation by *Helminthosporium oryzae* on resistant and susceptible rice cultivars and on leaf replicas

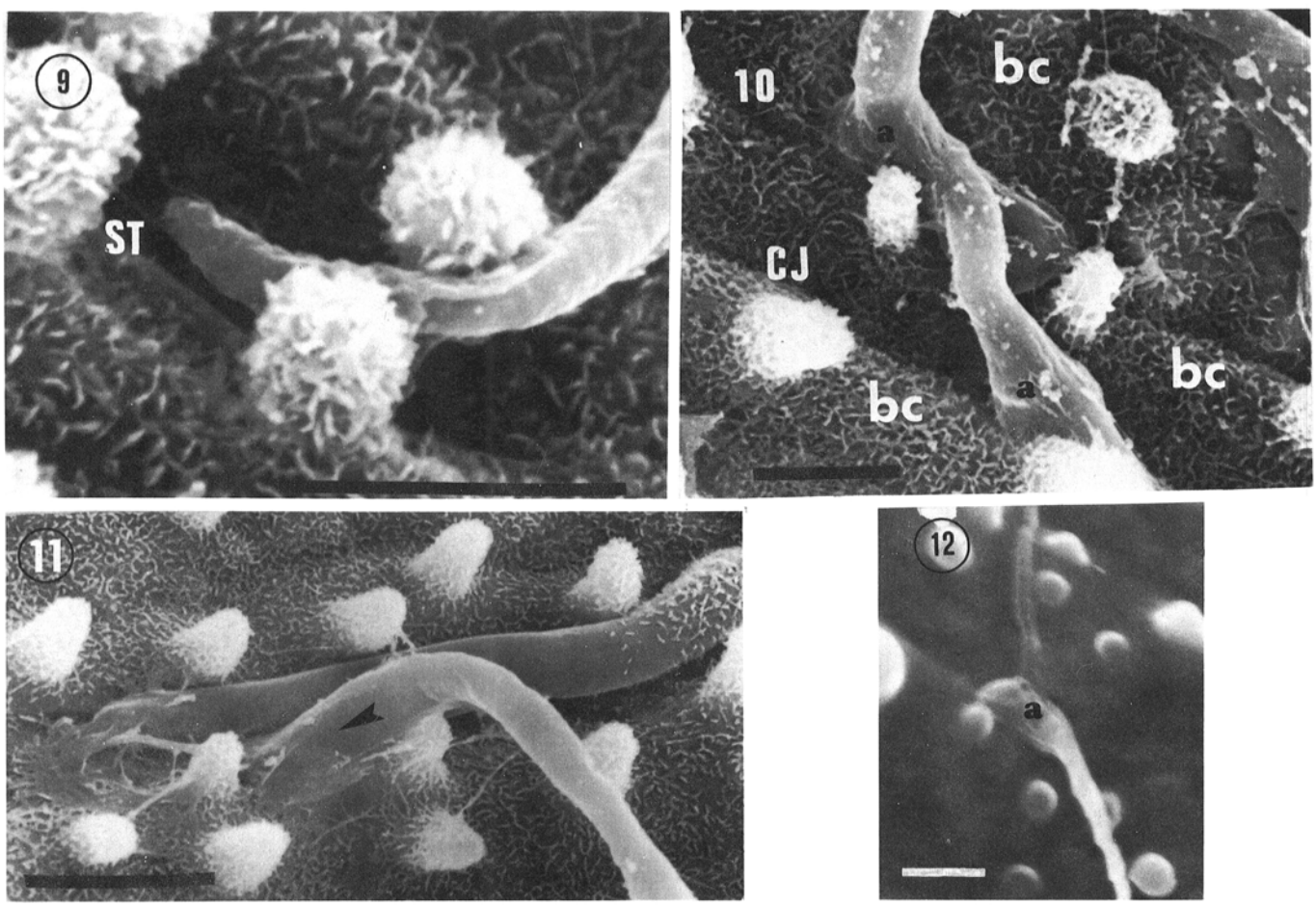
	Percentage of appressoria formed[a]			
	Template leaf		Leaf replica	
Epidermal structure	TN-1	Dular	TN-1	Dular
Bulliform cell	84.8	77.1	25	25
Long cell	5.8	14.7	30	35
Short cells (siliceous and cork)	9.4	8.2	40	40
Stomata	0.0	0.0	5	0
Trichome	0.0	0.0	0	0

[a]A total of 25 appressoria were observed in each cultivar of template leaf and leaf replica.

Prepenetration activities. The primary infection process consisted of conidial germination, germ tube elongation, formation of appressorial initials, maturation of appressoria, and formation of secondary hyphae.

Disease severity index for TN-1, Tetep, Starbonnet, and Dular were 1.0, 1.5, 3.0, and 4.0, respectively. Conidia germinated ~2.5 hr after inoculation. Regardless of the resistance level of the host, the percentage of conidia germinating was not significantly different, $P = 0.05$ (Fig. 13). Germination was lowest on the susceptible cultivar Starbonnet.

Germ tubes emerged mostly from the two end cells of conidia (Fig. 2). However, germ tubes were also observed emerging from middle cells as seen with the light microscope (Fig. 3) and SEM (Fig. 4). Throughout this paper, germ-tube length refers to the length of the germ tube from the conidium to the first appressorium. The length of germ tubes varied with cultivars (Fig. 13). Longer germ tubes were observed on the resistant cultivars TN-1 and Tetep, whereas germ tubes were shorter on susceptible cultivars. At about 4 hr after inoculation, club-shaped appressorial initials formed terminally on germ tubes (Fig. 5). Length of germ tubes was inversely related to percentage of appressorium formation during the first few hours after inoculation. The conidia germinating on resistant cultivars TN-1 and Tetep had long germ tubes and a low percentage of appressorium formation compared with those on the more susceptible cultivars Starbonnet and Dular at 10 hr after inoculation (Fig. 13). Orientation of germ tubes



Figs. 9–12. Colonization of leaf surfaces of rice cultivar Dular by *Helminthosporium orzae.* **9,** The hypha neither penetrated nor formed an appressorium on the stomate (st). **10,** Appressoria (a) were formed mostly on cell junctures (cj) between the bulliform cells (bc). **11,** Penetration without a well-defined appressorium (arrow). **12.** An appressorium (a) of *Helminthosporium oryzae* formed at a cell juncture on a polystyrene rice leaf surface replica. All bars represent 5 μm.

seemed to be random. However, appressoria were formed over bulliform cells (Table 1, Fig. 10). Appressoria varied greatly in size and shape. Some were relatively large, well differentiated and round or club-shaped (Figs. 5–8). Mature appressoria were often delimited from the hyphae by a septum (Fig. 6). Secondary hyphae emerged from the first appressorium and often produced one or more additional appressoria (Figs. 5 and 10). The number of appressoria produced by each conidium was determined with SEM at different time intervals after inoculation. Early in the prepenetration period, numbers of appressoria per conidium differed little between resistant and susceptible cultivars (Fig. 14). However, beyond 8 hr after inoculation, more appressoria were formed on Dular and Starbonnet; the total number of appressoria per conidium at 15 hr was about 2.5 times that on the resistant cultivars. The number of appressoria formed was significantly correlated to the disease severity index of the host (r values were 0.957, 0.945, 0.793, and 0.974 at 7, 10, 12, 15 hr, respectively, after inoculation). Hyphal growth was also more vigorous on susceptible cultivars (Fig. 15). Hyphal growth was significantly correlated with the number of appressoria produced ($r = 0.964$) and with the disease severity index of the host ($r = 0.929$) 15 hr after inoculation (Fig. 15).

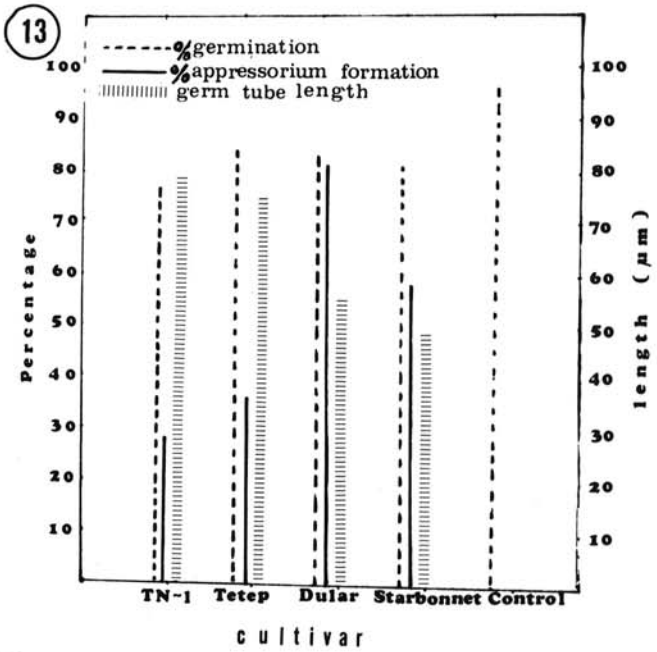
The fungus formed appressoria mainly over bulliform cells (Figs. 6 and 10). Of the appressoria produced, 84.8 and 77.1% were formed over bulliform cells on TN-1 and Dular, respectively (Table 1). SEM observations indicated that the fungus did not form appressoria over or enter through the stomata and trichomes (Figs. 8 and 9). Very rarely, penetration appeared to have been accomplished without a well-defined appressorium (Fig. 11). Another commonly observed site of appressorium formation was the groovelike junctures between epidermal cells (Figs. 6 and 10). On leaf replicas, the fungus formed appressoria on cell junctures (Fig. 12) and the sites of appressorium formation were less specific (Table 1).

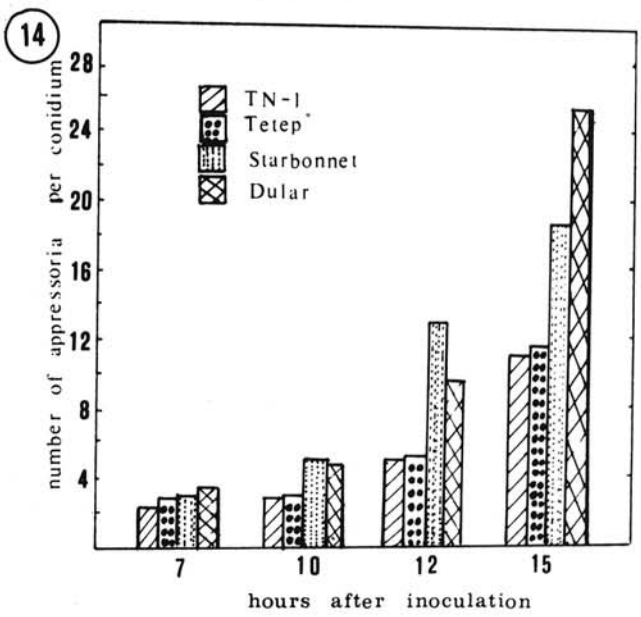
Extracellular sheaths. Fungal structures on rice leaf surfaces often had sheathlike substances associated with them. These sheaths interacted with the cuticle surface. Sheaths were observed on the undersides of germ tubes where they emerged from the conidia (Fig. 4). As germ tubes elongated, sheaths were found associated with the developing hyphae. The sheath material was often attached to papillae (Figs. 16 and 17). At the later stage of hyphal growth, sheaths were found on appressoria (Fig. 18) and hyphal tips (Fig. 19). Wax crystals were found adhering to the underside of the sheath when an appressorium was detached (Fig. 20). When hyphae were removed by treating the leaf surface with molten gelatin, an imprint was observed showing a hyphal track with the wax crystals removed (Fig. 21). Sheaths were also observed on hyphal tips growing on the polystyrene leaf replicas (Fig. 22). This observation suggests that the sheaths were secreted by the pathogen.

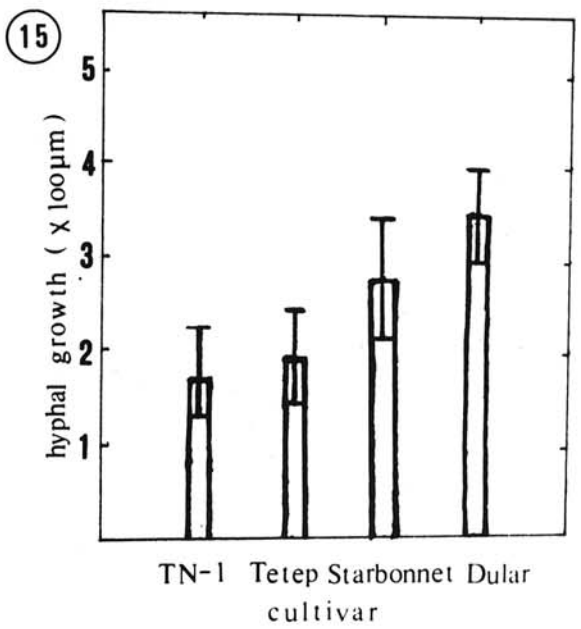
The existence of such a sheath on hyphae of *H. oryzae* was confirmed and demonstrated using transmission electron microscopy. The extracellular sheath formed an electron dense matrix outside the fungal cell wall. The matrix was composed of amorphous substances (Fig. 23). The mechanism by which the matrix accumulated was not determined.

DISCUSSION

This paper is the first ultrastructural report of the leaf surface activities of *H. oryzae* on resistant and susceptible rice cultivars. Horino and Akai (10) and Locci (14) reported similar

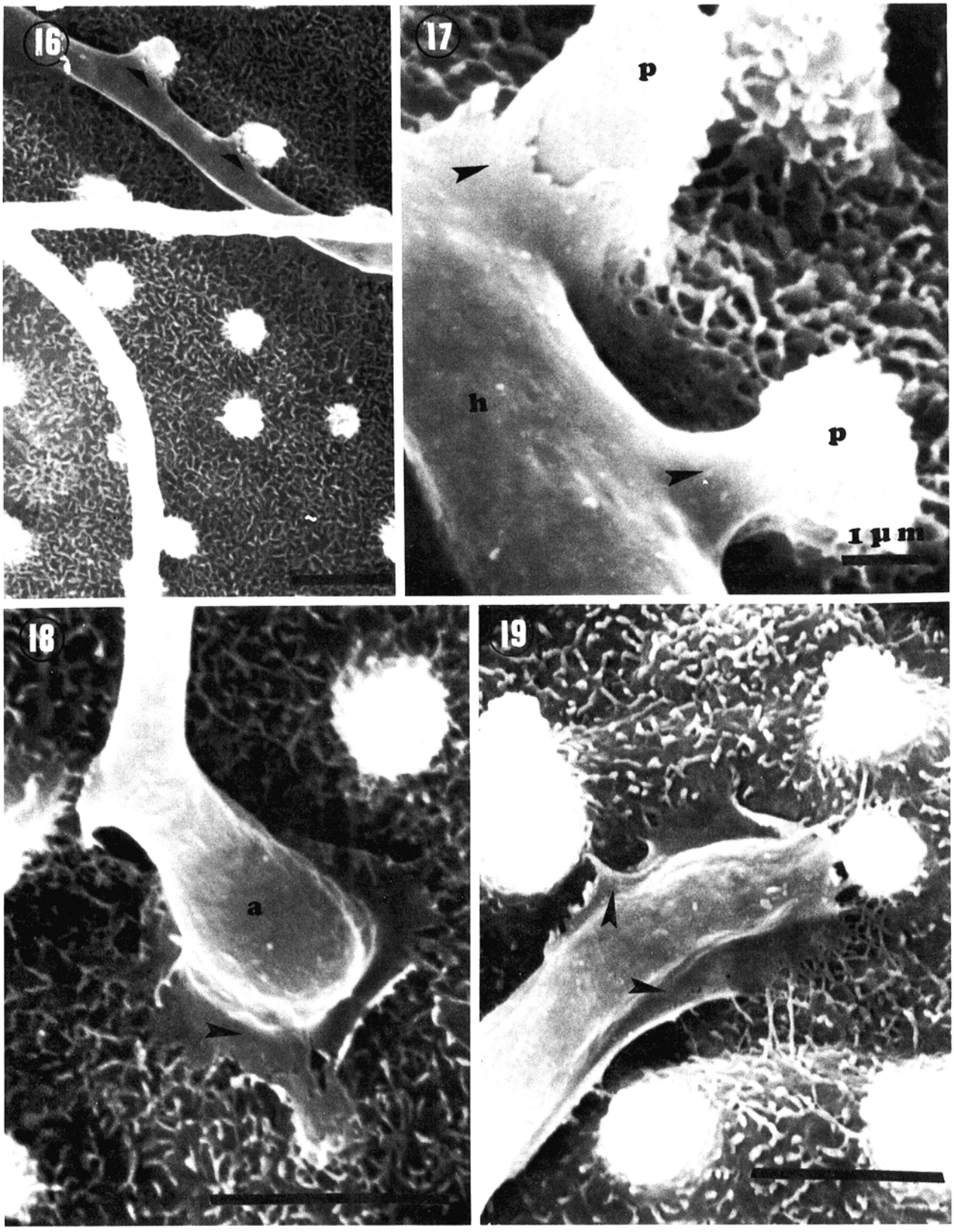




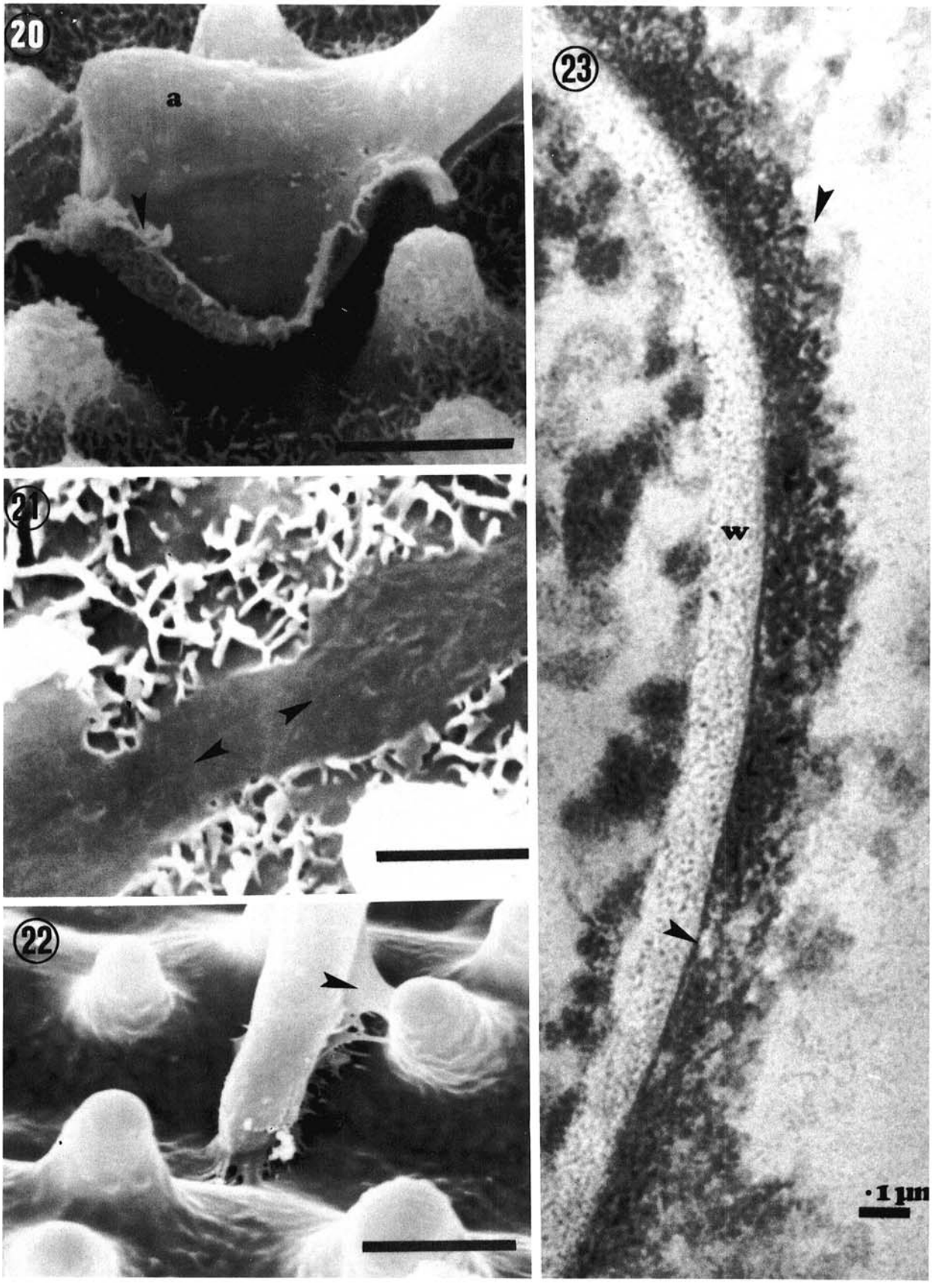


Figs. 13–15. Conidial germination, appressorial formation and hyphal growth of *Helminthosporium oryzae* on leaves of resistant and susceptible rice cultivars. **13,** Conidial germination, percentage of appressorium formation and length of germ tubes of *H. oryzae* on resistant and susceptible rice cultivars at 10 hr after inoculation. **14,** Appressorium formation by *H. oryzae* on resistant and susceptible rice cultivars. **15,** Hyphal growth of *H. oryzae* on resistant and susceptible rice cultivars at 15 hr after inoculation. Vertical bars represent standard errors at data points. All experiments were repeated twice with 25 observations in each experiment.

prepenetration activities of *H. oryzae* on coleoptiles of rice seedlings. These similarities suggest that seedlings and mature plants may have comparable resistance mechanisms in the prepenetration period. An inheritance study of resistance to the disease (8) also indicated that screening for mature plant resistance to *H. oryzae* could be done at the seedling stage. This suggests that on resistant cultivars, the longer germ tubes, fewer appressoria, and poor hyphal growth could be indicators of resistance mechanisms prior to infection. The results of the present study also confirmed the observations made by Locci (14) in 1969. With low-resolution



Figs. 16–19. Scanning electron micrographs of extracellular sheaths of *Helminthosporium oryzae*. **16,** Hyphae on leaf surface of rice cultivar Dular were attached to the papillae with sheaths (arrows). **17,** Close-up extracellular sheaths (arrows) on the hypha (h). Note that the sheaths attached to the papillae (p). **18,** The sheath (arrow) was formed a considerable distance around the appressorium (a). **19,** An extracellular sheath (arrows) was also formed around a hyphal tip. Unless otherwise specified, all bars represent 5 μm.



Figs. 20–23. Scanning and transmission electron micrographs showing interactions of extracellular sheaths with epicuticular wax crystals on rice cultivar Dular. **20,** The sheath (arrow) of the appressorium (a) curled up revealing the adhesion of the wax adhered to the underside of the sheath (arrow). **21,** An imprint (arrows) was revealed after the hypha was removed with molten gelatin. **22,** A sheath (arrow) was also found on a hyphal tip growing on the leaf surface replica. **23,** A transmission electron micrograph of the extracellular sheath. This micrograph shows the matrix (arrows) outside the hyphal cell wall (w). All bars represent 5 μm, unless specified.

SEM he observed an adhesive matrix associated with hyphae and appressoria and the absence of stomatal penetration (14).

Bulliform cells have also been reported as the usual entrance point for *Pyricularia oryzae*, the causal agent of the blast disease of rice (11). In the Gramineae, bulliform cells are unusual epidermal cells that, according to some botanists, cause the rice leaf to fold up during conditions of water stress. The radial walls of these cells are thin (6) and the outer walls remain in a pectic-cellulosic state long after the other epidermal cells become lignified (5). Early research on blast disease showed that the high degree of penetration in bulliform cells was correlated with the low degree of mechanical toughness of this outer wall (25). Later workers found lower concentrations of chlorogenic acid (a fungitoxin) in the bulliform cells than in other epidermal cells (25). They suggested that this could explain the lower resistance of bulliform cells to penetration. The present investigation showed that the fungus preferentially formed appressoria over the bulliform cells. As early as the 18th century, deBary noted that fungi formed appressoria on cell junctures (20). Since then, numerous workers have reported cell junctures as favorable sites for appressorium formation by pathogenic fungi (5,12,20,26). Blakeman (3) and Allen (1) proposed that appressorium formation was primarily controlled by genotypes whose expression may require a specific conducive environment. *H. oryzae* consistently formed appressoria over cell junctures on leaf replicas. This suggests that a physical stimulus may play an important role in appressorium formation. Junctures of bulliform cells offer a chemical environment and groovelike topography that may stimulate the formation and maturation of appressoria.

According to Wheeler (23) an extracellular mucilaginous sheath was observed by Blanchard on *Helminthosporium maydis* hyphae on corn leaves. Wheeler suggested that the fungal sheaths might actually represent a rupture of the host cuticle during specimen preparation and associated these sheaths with unidentified matrix materials within the infected corn leaf. The following evidence in the present investigation suggests a different interpretation: SEM revealed no ruptures of the cuticle; sheaths were found on both resistant and susceptible cultivars, so they were not likely to be related to the unidentified matrix material within the infected, resistant corn plant in Blanchard's study; and these sheaths were also found on leaf surface replicas, indicating that they were secreted by the pathogen. Wheeler (24) reported that *H. maydis* and *H. victoriae* have extracellular sheaths associated with their hyphae. Thus, such sheaths may be common among species within the genus *Helminthosporium*. The present investigation showed that the sheath adheres to wax crystals and might enable the fungus to attach to the leaf surface and facilitate infection.

This study indicated that resistance mechanisms were operative during the prepenetration period, but that these were not the major disease resistance mechanisms. Further histological studies are necessary to identify additional resistance mechanisms that are operative both during and after penetration.

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