

## Comparative Histopathology of *Botrytis squamosa* and *B. cinerea* on Onion Leaves

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### ABSTRACT

CLARK, C. A., and J. W. LORBEER. 1976. Comparative histopathology of *Botrytis squamosa* and *B. cinerea* on onion leaves. *Phytopathology* 66: 1279-1289.

*Botrytis squamosa* and *B. cinerea* had different prepenetration activities on onion leaves with and without exogenous nutrients. In water *B. squamosa* conidia germinated on the side closest to the nearest anticlinal wall juncture or stomate and penetrated immediately, whereas *B. cinerea* germinated randomly, did not produce appressoria, and did not penetrate the leaf. Addition of exogenous nutrients to inoculum resulted in greater superficial growth by *B. squamosa* prior to penetration and was requisite for formation of appressoria and penetration by *B. cinerea*. Development by both fungi with and without exogenous

nutrients was similar on leaf surface replicas and onion leaves. Postpenetration activities were the same for both pathogens. Both pathogens produced a cavity within each lesion as a result of collapse and separation of mesophyll and epidermal cells. Swelling of the outer periclinal epidermal wall was pronounced at all stages of lesion development. Pectinase C caused swelling of the outer periclinal epidermal wall, collapse of epidermal cells, and in some cases collapse of palisade parenchyma which mimicked histological changes associated with disease.

*Additional key words:* leaf surface replicas, *Allium cepa*, pectolytic enzymes, exogenous nutrients.

*Botrytis squamosa* Walker causes a leaf blight of onion (*Allium cepa* L.) and *B. cinerea* Pers. causes a leaf fleck (11). The isolate of *B. cinerea* used in this study did not produce symptoms when sprayed on leaves, although it macerated leaves when injected into the lacunae. Previous reports indicated that amending *B. cinerea* inoculum with exogenous nutrients stimulated pathogenic aggressiveness on other crops (2, 5, 7, 15, 29, 30). In preliminary studies, inoculation of onion leaves with *B. cinerea* conidia suspended in nutrients resulted in symptoms identical to those of *Botrytis* leaf blight. *Botrytis squamosa* produced leaf blight symptoms whether or not exogenous nutrients were added to the inoculum. The carbohydrate content of onion leaf diffusates was estimated to be from 7 to 20  $\mu$ g glucose equivalent/ml (9). Conidia of *B. cinerea* in vitro did not germinate in glucose concentrations below 99  $\mu$ g/ml and a concentration of 99 mg/ml was required for 100% germination (30). Other work indicates that *B. cinerea* is only partially dependent on exogenous nutrients for germination (8). Thus, the onion leaf surface probably is

deficient in nutrients required by *B. cinerea* (7, 15, 29).

Commercial pectinases can induce spotting of onion leaves similar to that of *Botrytis* leaf blight (13). Both *B. squamosa* and *B. cinerea* produce endopolygalacturonase when conidia are injected into the lacunae of intact onion leaves (12).

In this study, we have compared histologically the development of *B. squamosa* and *B. cinerea* on onion leaves after inoculation with or without exogenous nutrients. The effects of commercial pectinase on the histology of the onion leaf also were observed.

### MATERIALS AND METHODS

**Pathogen culture and inoculation.**—Cultures of *B. squamosa* (isolate 64a) and *B. cinerea* (isolate 61-34) were maintained by monoconidial transfers to slants of a complete medium (Difco Czapek-Dox broth, 35 g; agar, 15 g; Difco yeast extract, 2.5 g; Difco malt extract, 7.5 g; sodium nucleate, 10 mg; hydrolyzed casein, 250 mg; trace element stock solution, 1 ml; distilled water, 1,000 ml). Trace element stock solution contained:  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ , 723.5 mg;  $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$ , 203.0 mg;  $\text{H}_3\text{BO}_3$ , 2.0 mg;  $\text{H}_3\text{MoO}_3$ , 2.0 mg;  $\text{CuSO}_4$ , 2.0 mg; distilled water, 1,000

ml. Slants were incubated under fluorescent light (12-hour photoperiod) at 21 C. To obtain conidia of uniform age, 10 ml of sterile distilled water was rinsed over the surface of a slant which had been incubated 2-4 weeks. Aliquots (0.3 ml) of this suspension were spread over the surface of complete agar in petri dishes which were placed in darkness at 21 C for 1 day. *Botrytis squamosa* was incubated 6-7 days and *B. cinerea* 4-5 days under fluorescent and near-ultraviolet light (12-hour photoperiod) at 21 C. Conidia were collected by aspiration into flasks containing sterile glass distilled water to avoid contamination of the inoculum with nutrients from the medium.

Plants of the cultivar Elba Globe (3-5 months old, grown from seed) were inoculated. The plants were placed 24 hours prior to inoculation into a moist chamber at 21 C with 12 hours of fluorescent light per day and were misted overhead 5 seconds every 10 minutes. Conidial suspensions, adjusted to  $10^4$ - $10^5$  conidia/ml, were mixed with equal volume of glass distilled water or Czapek-Dox broth plus 0.10% yeast extract (nutrient solution). One drop of Tween-20 was added to 100 ml of inoculum and the plants were sprayed with approximately 5-10 ml per plant and were returned to the mist chamber.

**Collection of leaf diffusates.**—Onion leaf diffusate was obtained by atomizing glass distilled water onto leaves of onion plants which were placed in a mist chamber (described above), and after 24 hours the moisture on the surface was collected by vacuum suction and filter-sterilized by passage through a 0.22- $\mu$ m pore size Millipore filter.

**Pectinase treatment.**—Solid pectinase (0.5 g pectinase, C-grade, California Corporation for Biochemical Research, Los Angeles) was suspended in 100 ml of water, centrifuged, excess solid material was discarded, and the supernatant fluid was sprayed on excised leaf sections on moistened filter paper in glass petri dishes. The leaves were incubated 72 hours at 21 C under 12 hours of fluorescent light per day.

**Preparation of whole mounts.**—At 30, 48, 72, and 96 hours after inoculation sections of leaves (approximately  $1 \times 2$ -3 cm) with lesions were placed in petri dishes. The petri dishes were placed in a covered glass dish containing a paper towel saturated with formalin (37% formaldehyde) and the dish was sealed for 1-2 hours to fix the tissue. The fixed sections were floated on a clearing solution (chloral hydrate, 200 g; distilled water, 80 ml; 95% ethanol, 250 ml; Tween-20, 4 drops) for 24-48 hours (12 hours under a vacuum,  $-1.05$  kg/cm<sup>2</sup>). Leaf sections then were removed and 1 drop of cotton blue in lactophenol was placed on each. After 3-5 minutes the sections were rinsed in distilled water and mounted on microscope slides in 50% glycerin.

**Embedding.**—At 48 and 96 hours after inoculation, individual lesions were excised and fixed for 30 minutes in 4% glutaraldehyde in Pipes buffer [0.8 M piperazine-*N,N'*-bis(2-ethanol sulfonic acid) in 50 ml H<sub>2</sub>O, adjusted to pH 8.0 with 1N NaOH, and brought to 100 ml with H<sub>2</sub>O]. They then were placed in fresh fixative for 30 minutes, rinsed with four changes of Pipes buffer during 1 hour, postfixed in 2% osmium tetroxide for 1 hour, and then rinsed three times during 30 minutes with each of the following; Pipes buffer, distilled water, 25%, 50%, and 75% aqueous acetone. The sections were refrigerated in

the 75% acetone overnight in vials sealed with corks. The vials then were allowed to warm and dehydration was completed by rinsing three times during 60 minutes with 95% aqueous acetone followed by three rinses during 60 minutes with 100% acetone. The sections were rinsed six times with propylene oxide during 60 minutes and left in propylene oxide for an additional 60 minutes.

Spurr's (24) and 4:6 Epon resin (17) were used for embedding and the infiltration procedures were the same for both. The Spurr's was cured at 70 C for 16 hours and the 4:6 at 60 C for 48 hours.

**Sectioning.**—Sectioning was done at room temperature on an American Optical freezing microtome with single-edged razor blades. The face of the plastic block was warmed slightly prior to each cut. The sections (15 and 25  $\mu$ m in thickness) were transferred to a drop of distilled water on prewarmed (60 C) microscope slides.

**Preparation of replicas.**—Replicating kits (Ladd Research Industries, Inc., Burlington, VT) were used to prepare replicas of the onion leaf surface from plants acclimated to subdued light. 'Replicating solution' was brushed gently on the surface of a small area of leaf and a piece of 'replicating tape' was placed on the liquid. When hardened (approximately 15-60 seconds), the impression, adhering to the tape, was removed from the leaf and rinsed in Tween-20 (1 drop/100 ml distilled water) followed by distilled water. Positives were made by brushing clear finger nail lacquer over the surface of dry negative impressions. When hardened, the positives were removed with two-sided tape and affixed with the replica upward to a microscope slide. The slide and replica were rinsed in sterile glass distilled water and placed on a piece of bent glass rod in a petri dish containing a circle of moistened filter paper. Conidia suspended in sterile glass distilled water, nutrient solution, or onion leaf diffusate were pipetted onto the surface of the replicas and incubated at 21 C in darkness for 48 hours after which 1 drop of acid fuchsin (1% aqueous) was added.

## RESULTS

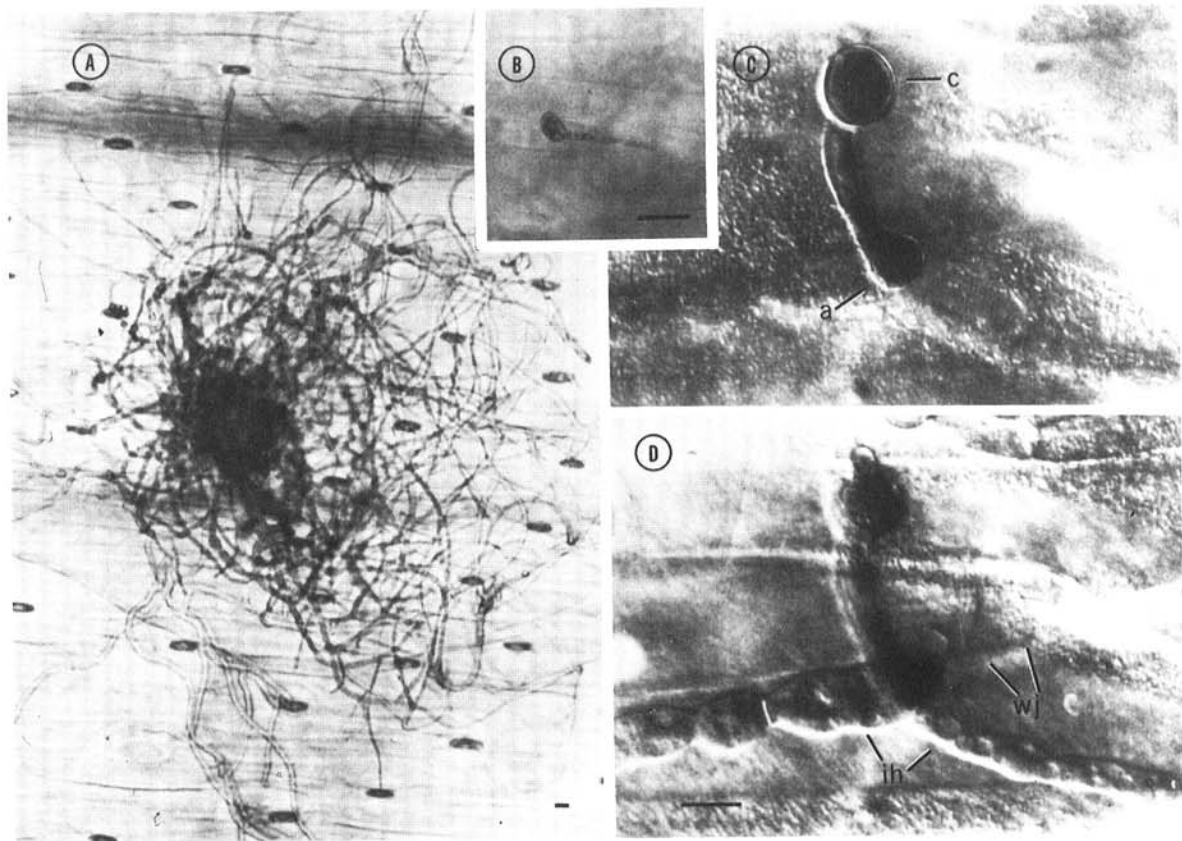
**Prepenetration activities.**—*Botrytis squamosa* and *B. cinerea* differed markedly in their prepenetration activities. Conidia of *B. cinerea*, sprayed in water onto onion leaves, germinated (60%), produced short germ tubes (36  $\mu$ m), but did not produce appressoria or penetrate (Fig. 1-B). Germ tubes emerged from all regions of the conidium regardless of proximity to anticlinal wall junctures or stomates. Germ tubes of conidia of *B. squamosa* (79% of the germinated conidia) emerged from the region facing the nearest anticlinal wall juncture or stomate (tropic germination) [Fig. 2-(A to C)] and grew toward this wall juncture. The germ tube apex functioned as an appressorium, deposited mucilage, and penetration occurred without formation of a septum [Fig. 3-(H to J)]. Such germ tubes averaged 6  $\mu$ m in length. This mode of direct penetration is called 'immediate penetration'. In some cases the conidium itself functioned as an appressorium without producing a visible germ tube [Fig. 2-(D to F) and 3-D]. Stomatal penetration was infrequent (Table 1). Conidia of *B. squamosa* near stomates produced germ tubes that emerged toward and grew into the stomatal aperture without forming appressoria [Fig. 2-(G to J)]. These means of penetration also

predominated in naturally formed lesions collected from different onion fields in New York.

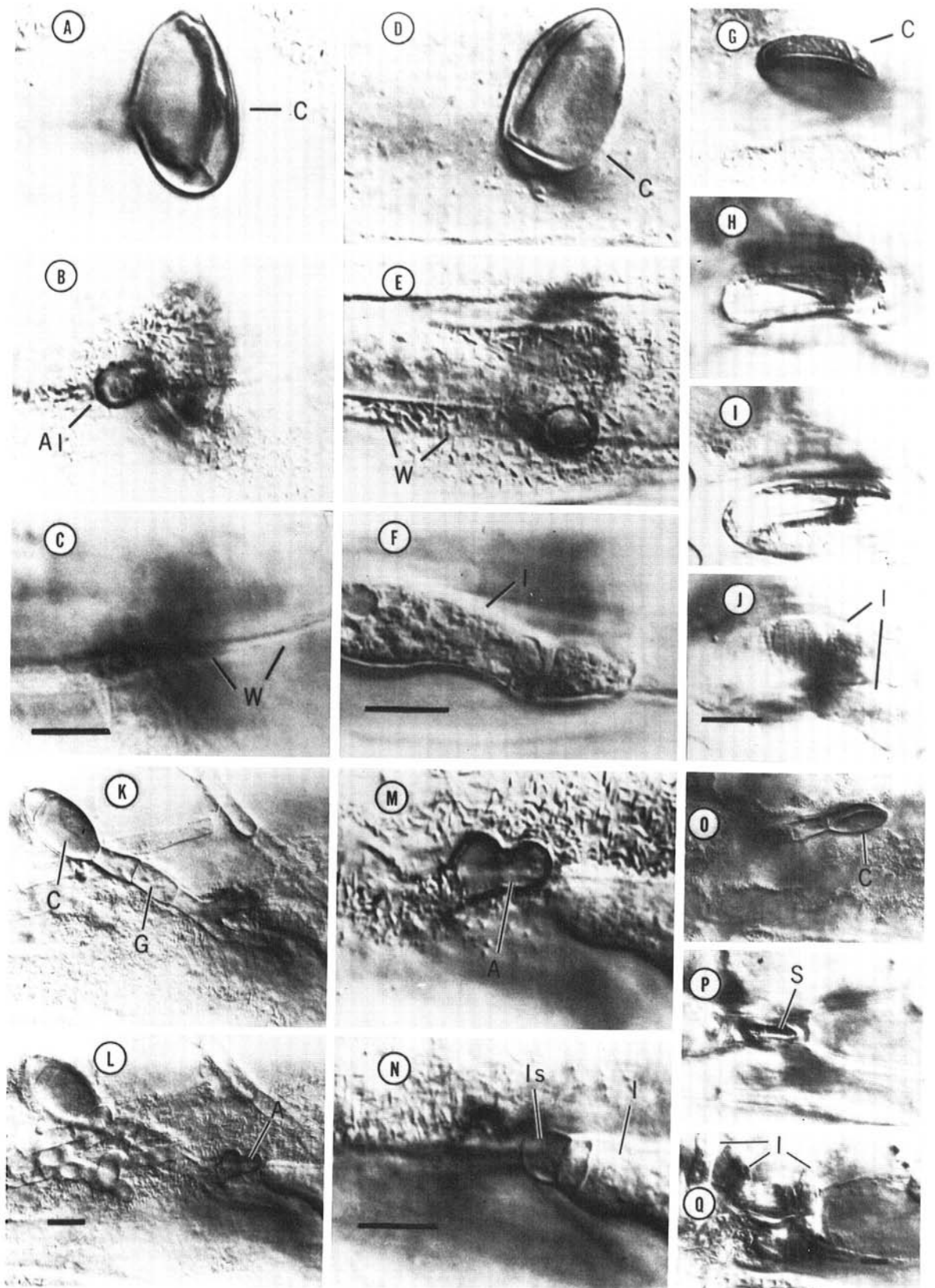
When transferred in nutrient solution onto onion leaves, conidia of *B. cinerea* germinated (germ tubes emerging randomly) and produced extensive hyphal growth (Fig. 1-A). Penetration occurred through stomates or the cuticle [Fig. 1-(C to D), 3-C, and Table 1]. Sometimes both occurred from the same or different conidia. There frequently were several sites of penetration associated with a single lesion. Conidia of *B. squamosa* produced germ tubes that emerged from either or both poles of the conidium (91% of the germinated conidia) regardless of positioning relative to anticlinal wall junctures or stomates [Fig. 2-(K to N)]. Superficial growth by *B. squamosa* (123  $\mu$ m) was not as extensive as for *B. cinerea* (Fig. 1-A) but both species often passed over anticlinal wall junctures before they formed appressoria over subsequent anticlinal wall junctures (77-84% of the appressoria) and penetrated. This form of direct penetration is called 'delayed penetration'. Mucilage was deposited around the appressorium (Fig. 3-F). When inoculated in nutrients both species frequently penetrated stomates without forming appressoria [Fig. 2-(O to Q) and 3-C].

**Penetration.**—Appressoria usually were delimited by septa and often the connecting superficial hyphae were not in contact with the leaf surface (Fig. 3-A). Penetration pegs of both species were narrow and below the limits of resolution with light microscopy. Shortly after penetration a septum was formed, isolating a small hyphal swelling within the outer periclinal wall of the epidermis (the 'intermediate swelling') (Fig. 3-B). Immediately adjacent to this swelling an enlarged infection hypha was produced. The intermediate swelling and the appressorium lacked cytoplasm 48 hours after inoculation. Superficial hyphae and appressoria stained intensely before penetration, but not afterward.

Appressoria were formed from *B. squamosa* conidia in water relatively soon (8-12 hours) after inoculation, but it was sometime before infection hyphae were evident in the outer periclinal wall of the epidermis. The intermediate swelling, however, usually was not visible in whole mounts. The intermediate swelling was small and lacked cytoplasm 48 hours after inoculation (Fig. 3-F), but contained cytoplasm prior to formation of the infection hypha (Fig. 3-E). Sometimes infection hyphae were produced prior to lesion formation. In other instances, lesions were produced before infection hyphae became

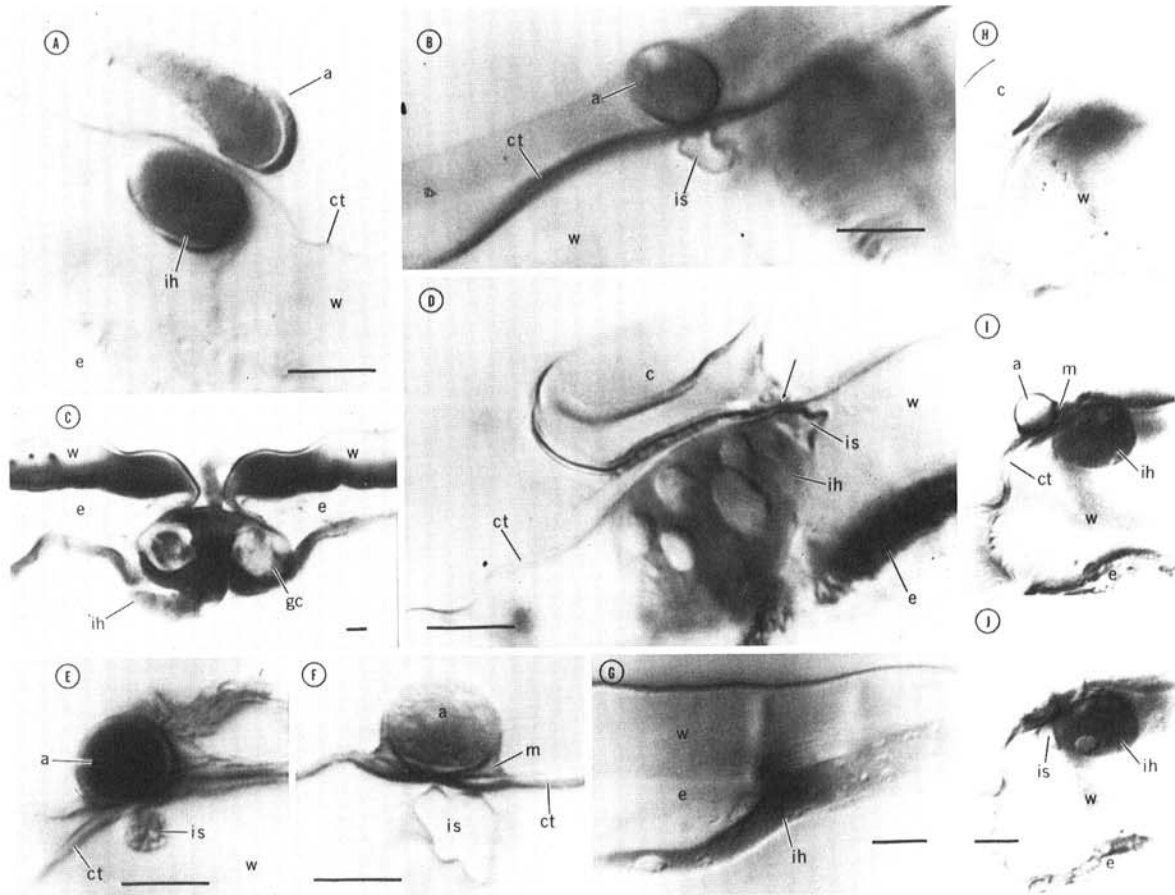


**Fig. 1-(A to D).** Development of *Botrytis cinerea* on onion leaf surface. **A)** Bright field micrograph after 48 hours of superficial colonization by *B. cinerea* (in exogenous nutrients). **B)** Bright field micrograph of a germinated conidium of *B. cinerea* (in water 48 hours after inoculation). **C and D)** A sequence of Nomarski interference micrographs in two different focal planes of a conidium of *B. cinerea* which had germinated (in exogenous nutrients), formed an appressorium, penetrated over an epidermal anticlinal wall juncture and formed infection hyphae within the outer periclinal wall of the epidermis. (Scale bars = 10  $\mu$ m, a = appressorium, c = conidium, ih = infection hypha, and wj = anticlinal wall juncture).



evident. Once formed, however, the infection hyphae grew very rapidly. The exact time of penetration was not determined.

**Postpenetration activities.**—Postpenetration development was similar for both pathogens and was not altered by exogenous nutrients. Following cuticular



**Fig. 3-(A to J).** Cross sections of infected onion leaves. **A)** Appressorium produced by *Botrytis cinerea* (in exogenous nutrients) and an infection hypha within the outer periclinal wall of the epidermis. **B)** Intermediate swelling formed following penetration by *B. cinerea* (in exogenous nutrients). **C)** Penetration of a stomate by *B. cinerea* (in exogenous nutrients). **D)** Conidium of *B. squamosa* (in water) which germinated and formed an appressorium. Penetration occurred immediately underneath the appressorium and an intermediate swelling and infection hypha formed within the outer periclinal wall of the epidermis. The approximate site of penetration is indicated by the arrow. **E)** Appressorium produced by *B. squamosa* (in exogenous nutrients). Note the intermediate swelling containing cytoplasm and deep staining of the appressorium. **F)** Typical appressorium and intermediate swelling produced by *B. squamosa* (in exogenous nutrients). **G)** An infection hypha of *B. squamosa* has grown from within the outer periclinal wall of the epidermis down between epidermal cells. **H to J)** A sequence in different focal planes showing penetration by *B. squamosa*. The conidium germinated laterally and the germ tube apex formed an appressorium. Following penetration, an intermediate swelling and infection hypha were produced within the outer periclinal wall of the epidermis. A-B and D-J are Nomarski interference contrast micrographs. C is a bright field micrograph. A-C, E-F, and H-J were taken at 48 hours, G was taken at 72 hours, and D was taken at 96 hours after inoculation. (Scale bars = 10  $\mu$ m, a = appressorium, c = conidium, ct = cuticle, e = epidermal cell lumen, gc = guard cell, ih = infection hypha, is = intermediate swelling, m = mucilage, and w = outer periclinal wall of the epidermis).

**Fig. 2-(A to Q).** Sequences of Nomarski interference contrast micrographs taken in different focal planes of the same field of view of whole mounts of leaves inoculated with *Botrytis squamosa*. **A to C)** Lateral, tropic germination by a conidium (in water). The germ tube apex has functioned as an appressorium over the anticlinal wall juncture of the epidermis. **D to F)** A conidium (in water) has formed an appressorium without producing a visible germ tube. The infection hypha in F was in the outer periclinal wall of the epidermis. **G to J)** Tropic germination of a conidium (in water) and stomatal penetration. **G)** shows the conidium, **H)** the lateral germ tube, **I)** the germ tube growing through the stomatal aperture, and **J)** the infection hyphae formed beneath the epidermis. **K to N)** Polar germination of a conidium and delayed penetration following inoculation (in nutrients). M and N show higher magnification of the appressorium, the intermediate swelling, and the infection hypha. **O to Q)** Polar germination and stomatal penetration (in nutrients). (Scale bars = 10  $\mu$ m, A = appressorium, Al = germ tube apex functioning as appressorium, C = conidium, G = germ tube, I = infection hypha, Is = intermediate swelling, S = stomatal aperture, W = outer periclinal wall of the epidermis).

penetration, infection hyphae ramified within the outer periclinal wall of the epidermis. The first cells of the infection hyphae were 10-15  $\mu$ m in diameter, but cells produced subsequently were 5  $\mu$ m in diameter. Infection hyphae occurred in all layers of the wall and often continued to ramify within the wall until 48-96 hours after inoculation when penetration between epidermal cells occurred after the anticlinal walls had collapsed and the middle lamella had dissolved (Fig. 3-G). Intramural hyphae often branched and grew around stomatal apertures (Fig. 4-B).

Following stomatal penetration, enlarged infection hyphae were formed beneath the epidermis (Fig. 2-F, 2-Q, and 3-C) and ramified (primarily intercellularly) before entering the lacuna. Eventually hyphae emerged through stomatal apertures (Fig. 4-G) and later differentiated into conidiophores.

**Effects on the onion leaf.**—A healthy onion leaf is illustrated in cross section in Fig. 5-C. Both pathogens produced a cavity at the center of each lesion (Fig. 4-C). Cavities were produced by collapse of epidermal and mesophyll cells 30-48 hours after inoculation, but the outer periclinal wall of the epidermis remained continuous and unbroken over the cavity. Cells collapsed at a distance from infection hyphae (Fig. 4-D). The cavity was the same width (approx. 1-2 mm) as the initial lesion.

Both pathogens caused the outer periclinal epidermal wall to swell to two to three times (12-26  $\mu$ m) normal thickness (9  $\mu$ m) and occasionally layers of the cell wall were separated (Fig. 5-A and B). Swelling occurred at all stages of pathogenesis and well in advance of hyphae. Birefringence of the wall was not altered.

As lesions enlarged (72-96 hours after inoculation) epidermal and mesophyll cells continued to collapse but

did not separate and thus the cavities did not enlarge (Fig. 4-H). The cuticle began to separate from the epidermis. Prior to collapse, host cells became brown and granular in nature and were plasmolyzed (Fig. 4-I). At 96 hours after inoculation cubic crystals were observed on and within tissues infected by *B. squamosa* or *B. cinerea*, but not in uninfected tissue (Fig. 4-E).

**Effects of pectinase-C.**—Seventy-two hours after pectinase-C was sprayed on onion leaves, spots were produced and the outer periclinal wall of the epidermis had marked swelling (17  $\mu$ m). The epidermal cells and occasionally the palisade parenchyma became brown, granular, and collapsed (Fig. 4-J and 5-D).

**Development on leaf surface replicas.**—Development of *B. squamosa* and *B. cinerea* hyphae on replicas of the onion leaf surface was similar to that on the onion leaf. In water, those conidia of *B. cinerea* that germinated produced short germ tubes, but did not form appressoria. Germ tubes of *B. squamosa* conidia emerged (usually laterally) toward anticlinal wall junctures and did not grow beyond the wall juncture. The germ tube apex appeared similar to those on the leaf which functioned as appressoria (Fig. 4-A). Incubation in onion leaf diffusate increased the frequency of germ tube emergence toward the anticlinal wall junctures (Table 2). In nutrients, both species grew extensively on the replica surface, often passed over anticlinal wall junctures, and formed appressoria over subsequent anticlinal wall junctures (Fig. 4-F). Percentages of appressoria formed over wall junctures were the same on replicas as on leaves (Table 2). Stomatal apertures were entered but not as frequently as on the leaf.

On microscope slides in nutrients or water, germ tubes emerged, primarily from the poles of *B. squamosa* conidia

TABLE 1. Percent of lesions resulting from different types of penetration of onion leaves by *Botrytis squamosa* and *B. cinerea* inoculated in water (H<sub>2</sub>O) or exogenous nutrients (N)<sup>a</sup>

Pathogen	Treatment	Percent of lesions				
		Immediate <sup>b</sup>	Delayed <sup>c</sup>	Stomatal	Delayed + Stomatal	Other Combinations <sup>d</sup>
<i>B. squamosa</i>	H <sub>2</sub> O	61.3	0.9	11.7	1.8	24.3
	N	4.7	21.0	46.8	16.7	10.8
<i>B. cinerea</i>	H <sub>2</sub> O	0.0	0.0	69.2	16.5	14.3
	N	0.0	16.9	60.8	11.4	10.9

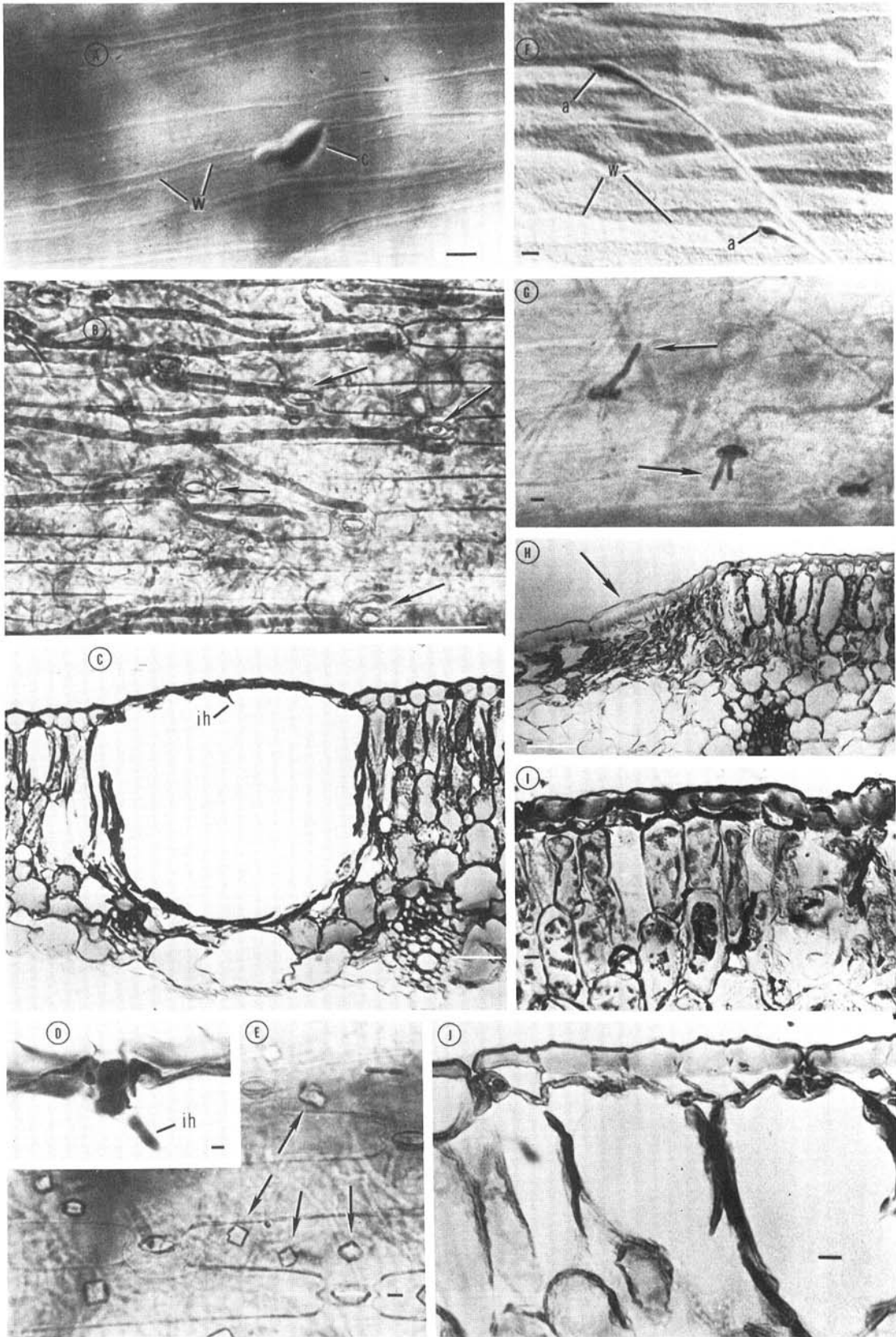
<sup>a</sup>N = 50% Czapek-Dox broth plus 0.05% yeast extract.

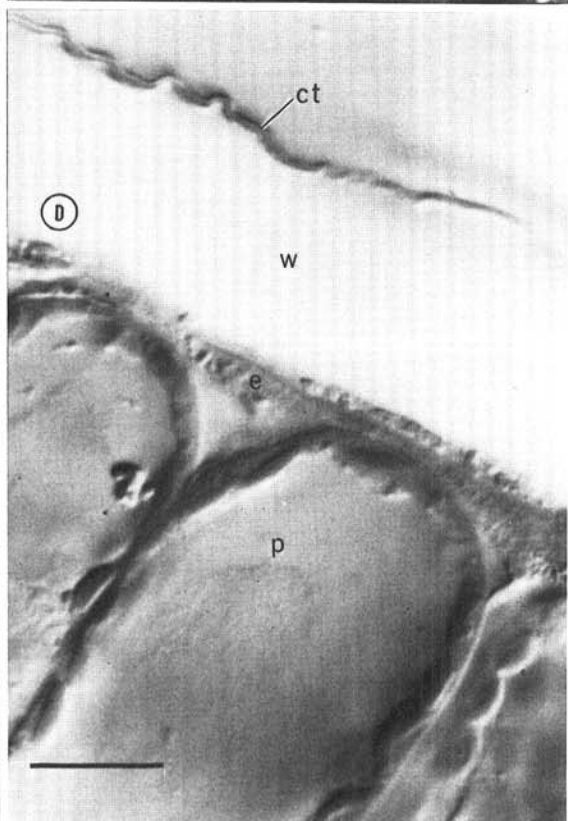
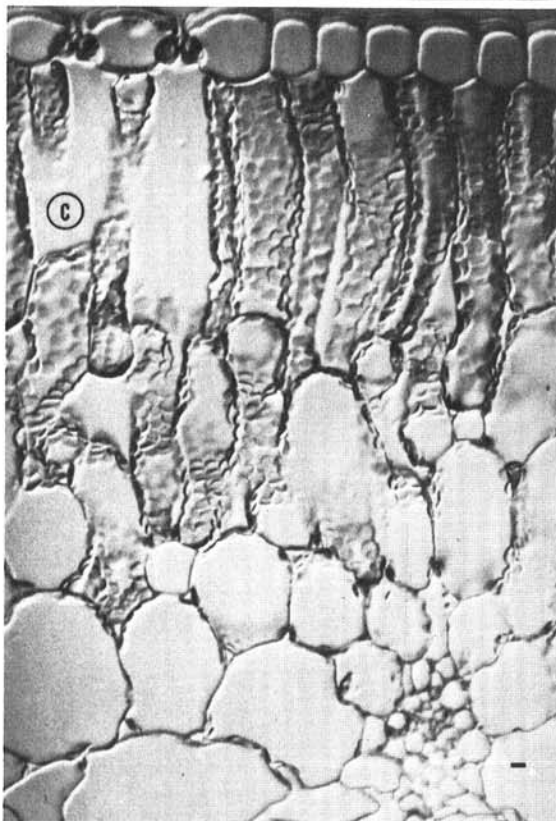
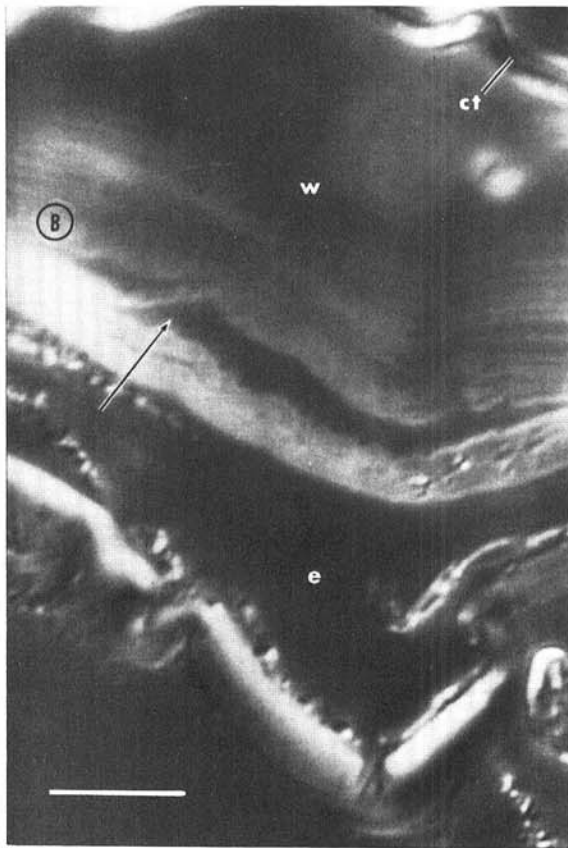
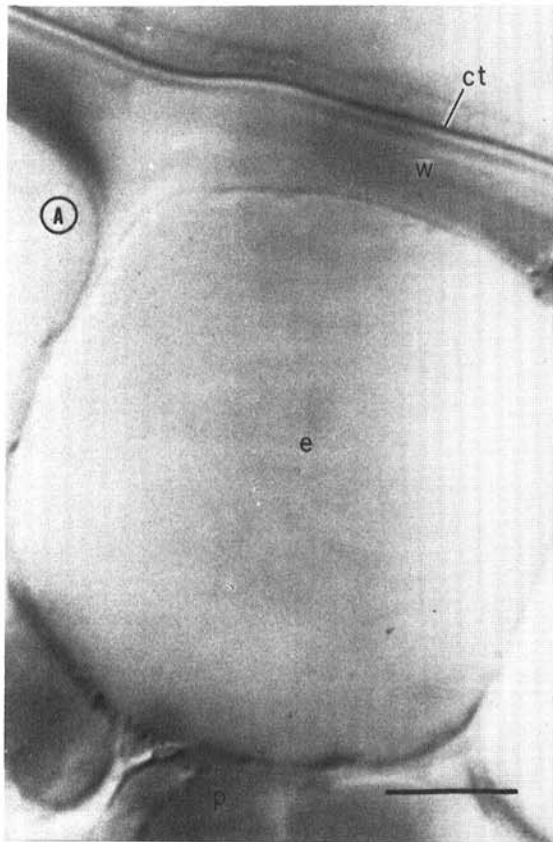
<sup>b</sup>Direct penetration occurred at anticlinal wall juncture nearest conidium.

<sup>c</sup>Direct penetration occurred following extensive superficial growth.

<sup>d</sup>Immediate + delayed; immediate + stomatal; immediate + delayed + stomatal.

**Fig. 4-(A to J).** A) Lateral, tropic germination by a conidium of *Botrytis squamosa* (in water) on an onion leaf surface replica. B) Hyphae growing within the outer periclinal wall of the epidermis 1-2 mm from the point of penetration. Arrows indicate areas where hyphae have grown around stomates. C) Cross section of an onion leaf infected by *B. squamosa* showing cavity formation. D) Enlargement of 4-C showing infection hypha associated with cavity. E) Whole mount of an onion leaf infected by *B. cinerea* showing cuboid crystals (arrows) (possibly oxalic acid) found only in infected leaves. F) Appressoria formed by *B. squamosa* (in nutrients) over anticlinal wall juncture regions of an onion leaf surface replica. G) Whole mount with hyphae of *B. squamosa* (arrows) emerging through stomates. H) Cross section of a leaf infected by *B. squamosa* 96 hours after inoculation. The outer periclinal wall of the epidermis was swollen in all areas. Epidermal and mesophyll cells nearer the point of penetration (toward the left) were brown, granular, and collapsed. The cuticle had separated from the epidermis in the region marked by the arrow. I) Cross section of a leaf infected by *B. squamosa* 96 hours after inoculation. The palisade parenchyma although not brown, granular, or collapsed, had partially plasmolyzed. J) Cross section of an onion leaf sprayed with commercial pectinase. The outer periclinal wall of the epidermis was swollen and the epidermal and palisade cells were brown, granular, and collapsed. (Scale bars: black = 10  $\mu$ m; white = 100  $\mu$ m, a = appressorium, c = conidium, and w = anticlinal wall juncture region).







(Table 3). Onion leaf diffusate increased the frequency of lateral germination and appressorium formation (Table 3).

### DISCUSSION

The ability of *B. squamosa* to immediately penetrate the leaf reduced the energy expended on superficial growth and reduced dependency on exogenous nutrients for prepenetration activities. The addition of large amounts of exogenous nutrients to the inoculum induced greater superficial growth. The addition of exogenous nutrients enabled *B. cinerea* to overcome the inability to penetrate immediately by increasing the amount of superficial growth. This increased the probability that hyphae randomly would encounter the usual sites of penetration of onion leaves (anticlinal wall junctures and stomates). Stimulation of lesion formation by exogenous nutrients may be caused by other factors: (i) greater production of pectolytic enzymes and/or oxalic acid which act in advance of the pathogen to reduce resistance of the susceptible (23); or (ii) nutrients may provide energy needed for appressorium formation or for penetration.

Ward (28) showed that when saprophytic nourishment was not necessary for infection of lily by *Botrytis* spp. the conidia produced were larger than when nutrients were required. The same is true in the present system since conidia of *B. squamosa* ( $1,861 \mu\text{m}^3$ ) are considerably larger than those of *B. cinerea* ( $660 \mu\text{m}^3$ ).

Greater frequency of penetration at anticlinal wall junctures has been demonstrated before (19). It appears that (in part) the two species differ in their ability to penetrate because of differences in the ability to germinate toward anticlinal wall junctures and penetrate immediately. The propensity of *B. cinerea* conidia to germinate tropically has been demonstrated (14).

The distribution of 'preferential polar pathways' (regions of the cuticle permeable to polar compounds) in the cuticle of the onion leaf has been demonstrated (21). These areas were concentrated over the anticlinal wall junctures of the epidermis. Possibly polar compounds may emanate from the anticlinal wall junctures and induce a chemotropic response by the pathogens.

The topography of leaf surface replicas alone provided sufficient stimulation to many conidia of *B. squamosa* to

TABLE 2. Frequency of entry of stomates and formation of appressoria on onion leaf surface replicas<sup>a</sup> by *Botrytis squamosa* and *B. cinerea* in water (H<sub>2</sub>O) or nutrient solution (N)<sup>b</sup>

Pathogen	Treatment	Germinated conidia		Appressoria	
		Entering stomates (%)	Forming appressoria (%)	Over anticlinal wall juncture (%)	Over epidermal cell lumen (%)
<i>B. squamosa</i>	H <sub>2</sub> O	6.9	22.0	78.8	21.2
	N	13.3	46.0	82.7	17.3
<i>B. cinerea</i>	H <sub>2</sub> O	0.9	0.0	...	...
	N	29.7	54.5	71.0	29.0

<sup>a</sup>Positive of leaf surface made with finger nail lacquer.

<sup>b</sup>N = 50% Czapek-Dox broth plus 0.05% yeast extract.

TABLE 3. Frequency of different types of germination by *Botrytis squamosa* conidia on onion leaf surface replicas and microscope slides when suspended in water, onion leaf diffusates, or nutrient solution

Treatment	Expt.	Germinated conidia					
		Replica			Microscope slide		
		Polar <sup>a</sup> (%)	Lateral <sup>b</sup> (%)	Tropic <sup>c</sup> (%)	Polar <sup>a</sup> (%)	Lateral <sup>b</sup> (%)	Forming appressoria (%)
Water	I	17.7	82.3	59.4	92.0	7.2	40.3
Water	II	60.5	39.5	11.4	96.9	2.5	6.3
Leaf diffusates	II	27.5	72.5	40.7	83.4	15.7	28.3
Nutrients	I	81.7	18.3	7.3	94.8	4.2	30.3
Nutrients	II	71.3	28.7	4.6	90.8	7.9	2.7

<sup>a</sup>Polar = germ tubes emerged from one or both poles of the conidium.

<sup>b</sup>Lateral = germ tubes emerged from other than the conidial poles.

<sup>c</sup>Tropic = germ tubes emerged from region of conidium facing the closest anticlinal wall juncture or stomate.

Fig. 5-(A to D). Micrographs of cross sections of onion leaves. A) Nomarski interference contrast micrograph of a healthy onion leaf epidermal cell. B) Nomarski interference contrast micrograph of an epidermal cell in a leaf infected by *Botrytis cinerea* 96 hours after inoculation. Outer periclinal wall is swollen and laminae of the wall have separated (arrow). The remainder of the cell is partially collapsed. C) Nomarski interference contrast micrograph of a healthy onion leaf. D) Nomarski interference contrast micrograph of the epidermis of an onion leaf 72 hours after treatment with commercial pectinase showing swelling of the outer periclinal wall and collapse and granulation of the remainder of the cell. (Scale bars = 10  $\mu\text{m}$ , ct = cuticle, e = epidermal cell lumen, p = palisade parenchyma, and w = outer periclinal cell wall of the epidermis).

germinate toward anticlinal wall junctures. Behavior of both species on leaf surface replicas does not explain the factors affecting stomatal penetration. *Botrytis squamosa* failed to penetrate stomates in water on the leaf because immediate penetration was effected before stomates were encountered. Entry of stomatal apertures on replicas occurred only when hyphae randomly encountered the stomates. The faithfulness of the replicas to the ultrastructure of the leaf surface waxes was not determined and thus the actual leaf may differ somewhat. *Alternaria porri* can be induced to form appressoria in response to isolated onion leaf wax (1).

The fact that direct penetration by both pathogens was from appressoria differs from reports that appressoria were not produced by *B. cinerea* (3, 4) or that appressorial production was variable (16, 18). As has been recorded, cytoplasm appeared to migrate from superficial hyphae through the intermediate swelling into the infection hyphae which might function as a measure of conservation on the part of the pathogens (20, 28).

Ramification of infection hyphae of *B. cinerea* within the outer periclinal wall of the epidermis has been reported previously (4, 20, 21). Since penetration occurred over anticlinal wall junctures, it would seem that less physical resistance might have been encountered had the hyphae grown between epidermal cells into intercellular spaces. The orientation of cellulose microfibrils may determine the observed course of growth.

Two distinct histological phases were evident during leaf blight development: (i) cavity formation which appeared coincidentally with macroscopic spotting and (ii) subsequent collapse and general degeneration of adjacent tissues concomitant with chlorosis and hydrosis. Cavities were formed soon after penetration and often in advance of infection hyphae, suggesting extreme sensitivity of the susceptible to the pathogens. Cavities frequently were formed well before infection hyphae were visible. Possibly penetration had occurred and the intermediate swelling had formed. Leaves of *Eucharis* sp. were penetrated by *B. cinerea* only after turgor had been reduced by creating a 'window' by artificially scraping away the sub-epidermal tissues of the susceptible (6). The cavity produced by *B. squamosa* and *B. cinerea* in onion leaves in effect removes sub-epidermal tissue and is thus homologous to the window and might have a similar function.

Pectolytic enzymes and organic acids have been implicated in the pathogenesis of *B. cinerea* (10, 23). The causal role of pectolytic enzymes in symptom production in *Botrytis* leaf blight of onion has been determined (13). In the present study, commercial pectinase caused many of the same histological effects of the disease (swelling of the outer periclinal wall of the epidermis; browning, granulation, and subsequent collapse of epidermal and mesophyll cells). It was not unexpected that pectolytic enzyme activity alone would account for the swelling and loosening of the wall. Fluorescent labelled polygalacturonase from *Pseudomonas cepacia* can penetrate into all regions of onion root tissue and cells (25). Thus, these enzymes may have more than merely a macerating effect as is suggested by the observation that the crude preparations used in this study caused browning and granulation of cells. Pectolytic enzymes may also

affect the susceptible prior to penetration. Verhoeff and Warren (27) have demonstrated endopolygalacturonase activity in ungerminated conidia of *B. cinerea*. The effect of externally applied enzymes on onion leaf tissue is illustrated by the present study. Pectolytic enzymes produced prior to penetration might alter the physical structure of the susceptible and increase leakage of nutrient substances.

Although exact timing of penetration relative to cavity formation and lesion development was not determined, it appears that since the initial development by *B. squamosa* remains superficial, it also remains vulnerable to external factors for a time after lesion production as was suggested previously (22). Because the pathogens may form lesions without appreciable development within the host, latent infections as described by Verhoeff (26) for other systems do not appear to occur on onion leaves.

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