Initial Infection Processes by *Botrytis cinerea* on Nectarine and Plum Fruit and the Development of Decay

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ABSTRACT

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Infection of unwounded, detached plum and nectarine fruit by *Botrytis cinerea* was studied with light, fluorescence, and electron microscopy. Conidia in 25-μl drops of spore suspension germinated readily on the surface of green and mature fruit and penetrated the cuticle by means of a very thin infection peg formed from the inner appressorium wall. *B. cinerea* was unable to breach the cuticle of green fruit. The pathogen entered the substomatal cavity of green fruit, but further growth in the fruit tissue was not observed. Fluorescence microscopy studies indicated that hyphae confined to the stomata of green fruit may be localized by resistant reactions. In nectarines inoculated near picking-ripe stage, the majority of infection pegs had penetrated the cuticle. However, only a small number of attempted penetrations were successful on picking-ripe plum fruit. Successful penetration of both fruit types during the mature, susceptible phase was characterized by the presence of inter- and intracellular hyphae 24 h after inoculation. The fact that green fruit were resistant to decay and that no hyphae could be found in the epidermal cell layer of fruit during their resistant phase indicated that early field infections might be of lesser importance in postharvest *B. cinerea* rot.

Additional keywords: latent infection, postharvest decay.

Botrytis cinerea Pers.:Fr. is one of the major pathogens responsible for postharvest decay of stone fruit in the southwestern Cape Province of South Africa (12). The fungus commonly occurs on plum blossoms in local stone fruit orchards (33) and plays an important part in blossom blight (16). *B. cinerea* does not penetrate young nectarine and plum fruit via floral parts to establish latent infections (16), in contrast with fruit such as apple (35), strawberry (5), grape (21), pear (7), black currant (23), red raspberry (6), and cucumber (9). Postharvest decay is therefore more likely to be caused by field infection of developing fruit.

In general, wounded, artificially inoculated stone fruit are relatively resistant to decay by *B. cinerea* during the early developmental stages, but susceptibility increases as the fruit becomes physiologically more mature (14). However, no information is available on the infection and colonization of unwounded stone fruit by this pathogen. *B. cinerea* is one of the fungi whose spores are most frequently trapped in air (26,29), and it occurs worldwide on a variety of plants (18). Kobayashi (19) observed numerous conidial masses of the organism throughout the year on the fallen petals of 28 plant species belonging to 19 genera of 14 families. The southwestern Cape Province, a winter rainfall area, is well-known for its diversity of plant species. Inoculum for infection may therefore be readily available, and infection may occur throughout the growing season under natural orchard conditions.

This study describes the sequence of events accompanying germination of *B. cinerea* conidia in 25-μl drops of spore suspension on unwounded nectarine and plum fruit during different developmental stages. A preliminary report of this study has been published (15).

MATERIALS AND METHODS

Inoculum. A culture of *B. cinerea* isolated from naturally infected plum fruit was maintained in a lyophilized state. Inoculum was prepared by culturing the fungus on potato-dextrose agar (PDA) in petri dishes for 10 days at 22 C under a diurnal regime (12-h photoperiod). Spores were collected by inverting the sporulating culture over a petri dish containing sterile distilled water and gently tapping the bottom of the inverted half. This harvesting method avoided contamination of spores by nutrients from the medium. Spore counts were made with a hemacytometer, and suspensions were adjusted to approximately 1×10^5 spores per milliliter. Germination of conidia on water agar was examined at each inoculation to verify their viability (≥90%).

Inoculation of fruit. Unblemished fruit were collected during three consecutive seasons from fungicide-free trees of the plum cultivar Harry Pickstone and of the nectarine cultivar Sunlite. For each cultivar, 200 fruit with attached pedicels were collected at 2-wk intervals starting at the hard, green, unripe stage 9 wk before harvest until the picking-ripe stage. Fruit were surface disinfested by spraying with 70% ethanol, placed on sterile epoxy-coated steel mesh screens, and allowed to dry. In order to inoculate a specific area and be able to recognize the inoculated area at a later stage, a 1-cm-diameter circle was carefully drawn on the surface of the fruit with a soft-tipped koki pen (preliminary studies showed no phytotoxic effect), and a 25-μl drop of spore suspension was placed inside the marked area. A drop of sterile distilled water was used for control fruit. Treated samples were placed on screens and incubated in ethanol-disinfected Perspex (Cape Plastics) moist chambers (60 × 30 × 60 cm) at 22 C at a high relative humidity (≥93%) under a diurnal regime (12-h photoperiod).

Preparation of fruit for microscopy. *Light microscopy.* Infection of fruit by *B. cinerea* was studied by examining fresh and fixed fruit tissues. These were sampled at 1-h intervals for the first 24 h, thereafter at 3-h intervals until 48 h, and then at 24-h intervals until 10 days after inoculation. The sequence of events accompanying germination on the fruit surface was studied by examining hand-sectioned parts of the skin. The sections were prepared by placing one drop of a mixture of nail varnish and acetone (1:2, v/v) on each inoculated surface. The varnish was left to dry for approximately 6 h. The dried varnish was peeled off and stained with 0.01% (w/v) cotton blue in lactophenol. Thin hand-sectioned pieces of skin, comprising the cuticle, epidermis, and one or two cell layers, were also cut with a razor blade. The sections were placed with the inoculated part facing upwards on a glass slide and stained with 0.01% (w/v) trypan blue in lactophenol. Percent germination at each inoculation site

and type of structure produced on the germ tube ends were assessed for 100 randomly selected conidia (four replicate counts per site).

Pieces of tissue were removed from the marked areas on fruit, fixed in formalin-acetic acid-alcohol (FAA), dehydrated in tertiary butanol (3) in a Shandon automatic tissue processor, and embedded in Paramat extra (BDH Laboratory Supplies, Poole, England). Embedded material was sectioned at 10 μm with a Leitz rotary microtome, and the sections were attached to slides with chrome alum and dried on a slide warmer. Sections were stained with erythrosin and fast green (31).

Fluorescence microscopy. Fresh and FAA-fixed tissue sections (after washing in 0.1 N KH_2PO_4) were mounted in emersion oil or stained in 0.1% (w/v) aniline blue in 0.1 N KH_2PO_4. Conidial germination, fungal growth, and plant response were examined with the aid of a Zeiss Axioskop microscope equipped with an epifluorescence condenser, a high-pressure mercury lamp, Neofluor objectives, and Zeiss filters 02, 06, and 18. These sets include excitation filters G 365, BP 436/8, and BP 395-425, respectively. With this setup, conidia and fungal hyphae fluoresced blue (filter 02) or yellow (filter 06 and 18).

Scanning electron microscopy. Pieces of tissue fixed in FAA were dehydrated in a graded ethanol series and dried in a critical-point drier under CO_2. The specimens were mounted on stubs, gold-coated in a Giko IB-2 Sputter Coater, and viewed with an ISI 100-A SEM.

Transmission electron microscopy. Pieces of tissue were fixed for 2 h in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0, rinsed in 0.1 M cacodylate buffer, and postfixed for 2 h in chilled 2% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer. The specimens were then washed in buffer, dehydrated in ethanol, transferred to propylene oxide, and embedded in Spurr's resin (34). Ultrathin sections were cut with a diamond knife, using an LKB ultramicrotome. Sections were mounted on copper grids and stained (28) with 2% uranyl acetate for 20 min and with lead citrate for 8 min.

Sections (1.0–1.5 μm) of resin-embedded material, prepared as for transmission electron microscopy, were sectioned and mounted on glass slides. The sections were stained at 60 C for 1 min with 0.5% toluidine blue O in 0.1% (w/v) aqueous sodium carbonate and examined by light microscopy.

Lesion formation. Fruit not sampled for microscopic observations were examined for decay development 7 days after inoculation. Fruit were considered decayed when lesions were greater in diameter than the inoculum droplet and were centered on the inoculation site. Five replications of 20 fruit each were used to calculate percentage decay for each of the inoculation periods per year. The data were analyzed by a complete randomized design analysis of variance (ANOVA). Treatment means were compared by calculation of Student's *t* test.

RESULTS

Histology. The sequence of events accompanying germination on the fruit surface was observed for a total of 150 sites for each fruit type during each year. These studies revealed no distinct differences in fungal behavior on green and senescing plum and nectarine fruit. Conidia germinated readily in the spore drops, and at least 70% had germinated 3 h after inoculation. Appressoria formed within 9 h when most germ tubes had reached lengths of 10–15 μm. A wide range of infection structures was observed, from protoappressoria (Fig. 1A), which formed after 9 h, to multicellular, lobate appressoria (Fig. 1B), which formed on longer germ tubes or hyphae. Protoappressoria and simple, hyaline appressoria (Fig. 1C) were the predominant infection structures formed. At 48 h, growth from some conidia was extensive. Secondary growth from primary appressoria was occasionally observed within 72 h of inoculation.

Surface observations on picking-ripe fruit indicated that infection had occurred within 72 h. Therefore, to follow penetration, infection sites on green and picking-ripe fruit were examined by light and transmission electron microscopy 36, 48, 72, 96, and 142 h after inoculation.

B. cinerea penetrated green and senescing plum and nectarine fruit either directly or through stomata. Direct penetration was by a very thin infection peg (Fig. 2A), 0.2–0.5 μm in diameter. More than one infection peg occasionally developed from simple appressoria (Fig. 2B). The first evidence of appressorial germination was a thinning in the center of the outer wall, which was in contact with the cuticle. This part of the outer wall subsequently disappeared, and an inner, electron-translucent cell wall passed through the opening in the outer wall to form a wedgelike tip (Fig. 2C) of the thin infection peg. The thin, threadlike infection pegs did not breach the cuticle of green fruit in any of the 50 sites studied by transmission microscopy. Small areas around and in advance of the pegs appeared to be more electron-translucent (Fig. 2D) than did the healthy cuticle. However, the underlying epidermal cell wall and cytoplasm remained unchanged and revealed no fluorescence associated with the presence of phenolics (blue, blue-green) or callose (yellow).

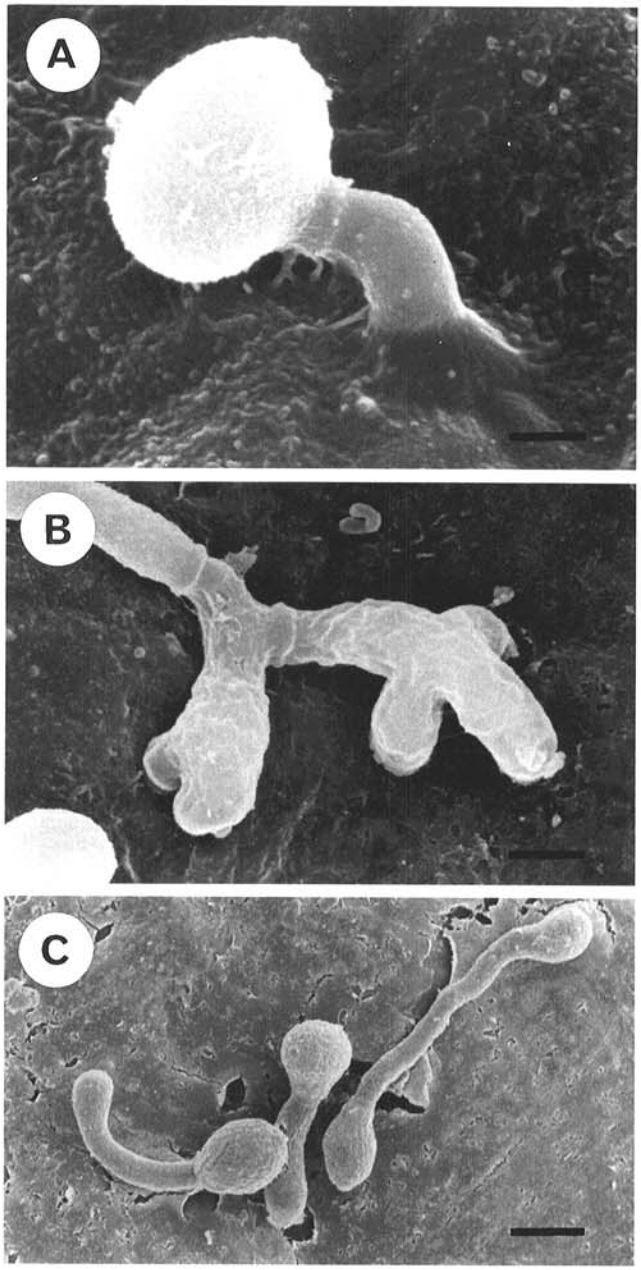

Fig. 1. Scanning electron microscope micrograph of appressoria formed by *Botrytis cinerea* on plum and nectarine fruit. **A,** Protoappressorium on plum 9 h after inoculation. Scale bar = 3.3 μm. **B,** Lobate appressoria on nectarine 48 h after inoculation. Scale bar = 4 μm. **C,** Simple, hyaline appressoria on nectarine 24 h after inoculation. Scale bar = 9 μm.

Germ tubes grew over stomata, or could penetrate them whether they were open (Fig. 3A) or closed (Fig. 3B). Direct penetration of the stomatal boundary cells was also observed. Infection hyphae penetrated stomata and grew down to the cells at the base of the stomatal chamber (Fig. 3C). Stomatal penetration eventually resulted in hyphae adhering to the stomatal base cells (Fig. 3D). However, on green fruit and on the majority of mature plum fruit, these hyphae remained confined to the outer wall of the stomatal base cells. Observations of fresh mounts and of FAA-fixed material indicated that stomatal boundary cells of colonized stomata from green fruit developed blue autofluorescence (indicative of phenolics), whereas cells surrounding the stomatal chamber fluoresced intensely yellow (possibly indicative of callose) 72 h after inoculation. These reactions were difficult to follow on senescing fruit due to apparent suberization of stomata.

Inspection of sound, inoculated nectarine and plum fruit under the stereo microscope revealed color changes at the inoculation site after 5 days. Lesion centers, which coincided with stomata, were a necrotic brown on green fruit (Fig. 4A) and were yellow-brown encircled with a decolorized zone on senescing fruit (Fig. 4B). Microscopic examination of fresh serial radial sections showed that only the fruit epidermis and the immediately underlying cells were affected. Although fluorescence microscopy observations indicated that the pathogen penetrated these stomata, no clear evidence of the presence of fungal cells could be found in the necrotic area. These changes were absent on fruit treated with sterile distilled water.

Successful penetration of both fruit kinds during the susceptible phase was characterized by the presence of inter- and intracellular hyphae 24 h after inoculation. In the case of direct penetration, invasion of the epidermal layer was observed at first in a lateral direction, with cell wall discoloration spreading to walls of neighboring cells. Hyphae were not always detected in discolored areas. Mycelium in early infections was largely restricted to the swollen epidermal cell walls and to the outermost five- to eight-cell layers of the mesocarp. Cell walls of the colonized epidermis and mesocarp either retained their normal appearance, thickened, or collapsed.

Lesion formation. Percentages of decayed fruit recorded during the three consecutive seasons are given in Table 1. No decay developed on nectarine fruit inoculated 7 and 5 wk before harvest. Decay incidences on fruit inoculated 3 wk before harvest were low but increased meaningfully during the last week before harvest. Plum fruit, on the other hand, were resistant to decay until the picking-ripe stage, when lesions developed on only a few inoculated fruit. No lesions formed on noninoculated fruit.

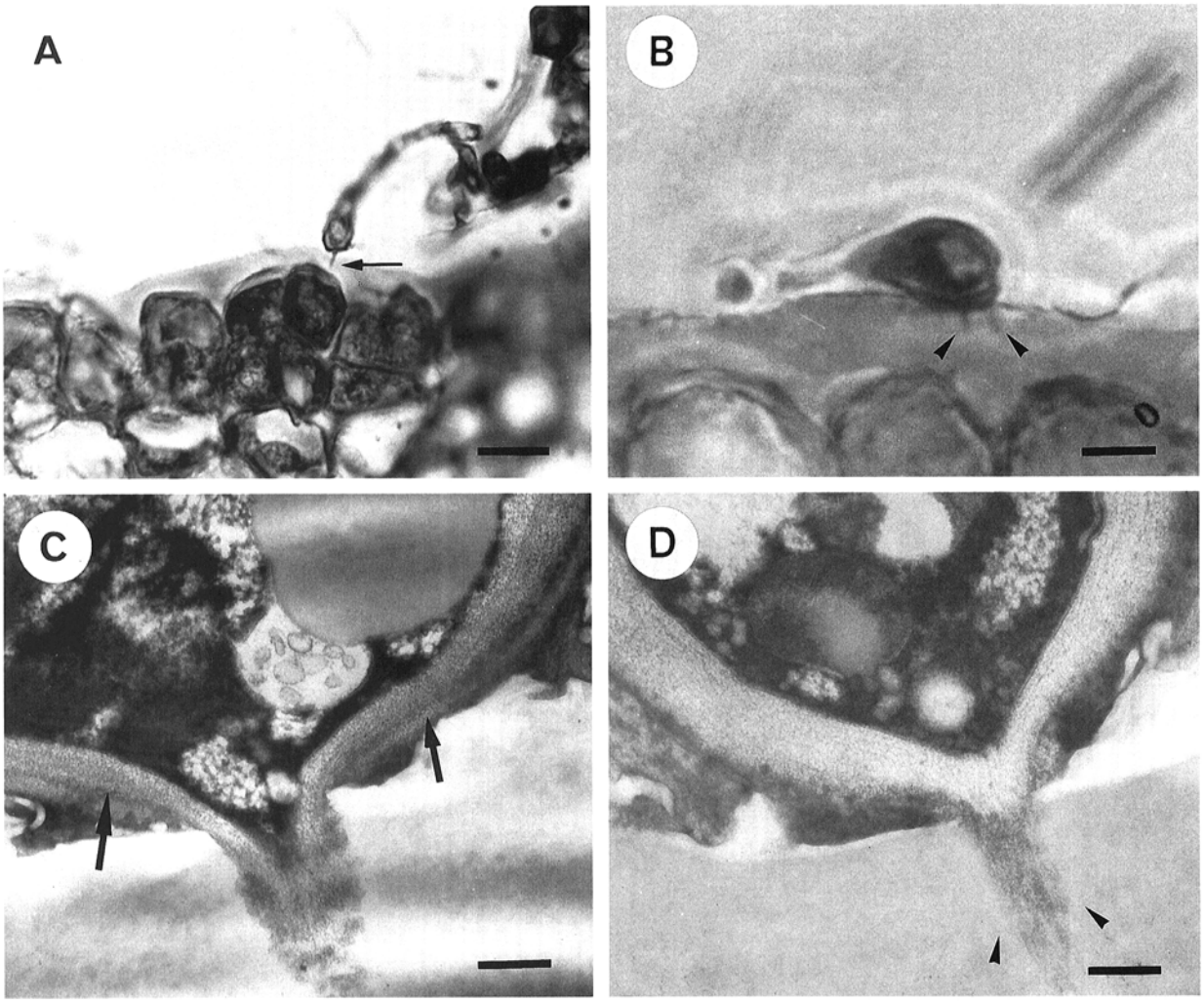

Fig. 2. Light and electron microscopy micrograph of the penetration of plum fruit by *Botrytis cinerea* during the resistant phase. **A,** Thin infection peg (arrow) formed in the cuticle 72 h after inoculation. Scale bar = 18 μm. **B,** Two infection pegs (arrows) formed by a simple, hyaline appressorium in the cuticle 96 h after inoculation. Scale bar = 8 μm. **C,** Wedgelike tip of the infection peg in the cuticle beneath the penetration point 96 h after inoculation. Note the inner wall passing through the outer wall (arrows) to form the tip of the infection peg. Scale bar = 0.2 μm. **D,** Thin infection peg formed 142 h after inoculation in the cuticle during the resistant phase. A small area around (arrows) and in advance of the peg appeared more electron-translucent than the intact cuticle. Scale bar = 0.5 μm.

DISCUSSION

Inoculation experiments with *B. cinerea* on detached, unwounded Harry Pickstone plum and Sunlite nectarine fruit in different stages of development, and subsequent histological observations, confirmed the resistance of green fruit and the susceptibility of mature fruit (14). Direct penetration was by a very thin infection peg formed from the inner appressorium wall. Upon appressorial germination, the infection peg appeared wedgelike. The marginal alteration of the cuticle around the infection peg indicates restricted cutinase activity only. This mode of penetration differs from that of *B. cinerea* in bean (22) and tomato (30), where the plasmalemma covers the blunt infection peg and the enzymatic degradation of the cuticle is pronounced. During the resistant phase, the penetration peg grew for various distances into the cuticle but did not breach it. Successful penetration occurred within 24 h after inoculation during the susceptible phase.

There are several reports of resistance of stone fruit to infection by *Monilinia* spp. and *Alternaria alternata*. In studies of brown rot of fruit of sweet cherry, cuticle and cell wall thickness were correlated with longer incubation periods and a lower incidence of infection caused by *Monilinia fructicola* (4). Adaskaveg et al (1,2) showed that fruit of peach cultivars resistant to *M. fructicola* had a thicker cuticle and a denser epidermis than did susceptible cultivars and correlated resistance to delayed penetration and longer incubation periods for infection. Active host responses were also reported on green fruit. The fruit tissue of apricots responded to *M. fructicola* (37) by the death of cells around the point of infection, suberization of walls of surrounding living cells, and accumulation of phenolic compounds; whereas resistance of plum fruit to *Monilinia laxa* was ascribed (32) to periderm formation, suberization, and gum deposits at the infection site. Larsen et al (20) suggested that *A. alternata* elicits a hypersensitive host response on apricots. Our fluorescence microscopy observations gave no indication of an active host response at sites where *B. cinerea* tried to penetrate the intact cuticle of green and senescing nectarine and plum fruit, but our observations gave positive reactions for phenolics and callose at stomata on green fruit penetrated by the pathogen. This suggests that some factor(s) of the green fruit skin restricted direct penetration, as was found with *B. cinerea* on green grape berries (17), and that the underlying host cells can respond actively to *B. cinerea*. The reddish epidermal cell coloration and necrotic centers at the stomata observed on resistant fruit might be the outcome of such an early host reaction.

In general, green stone fruit are less susceptible than mature fruit to infection by *B. cinerea* (14), although in this study, plum fruit at picking-ripe stage were nearly as resistant as were green fruit. This shows that factors governing resistance are negated at an earlier developmental stage in nectarine than in plum fruit. One such factor might be the development of microcracks. Cuticular microcracks on the surface of senescing nectarine fruit (10,11) might provide alternative sites for penetration by *B. cinerea,* as has been observed for *M. laxa* and *Rhizopus stolonifer* (24,25), and for *B. cinerea* on grape (27).

Our data relate to detached fruit kept in moist chambers, a situation that could lead to greater susceptibilty to *B. cinerea*

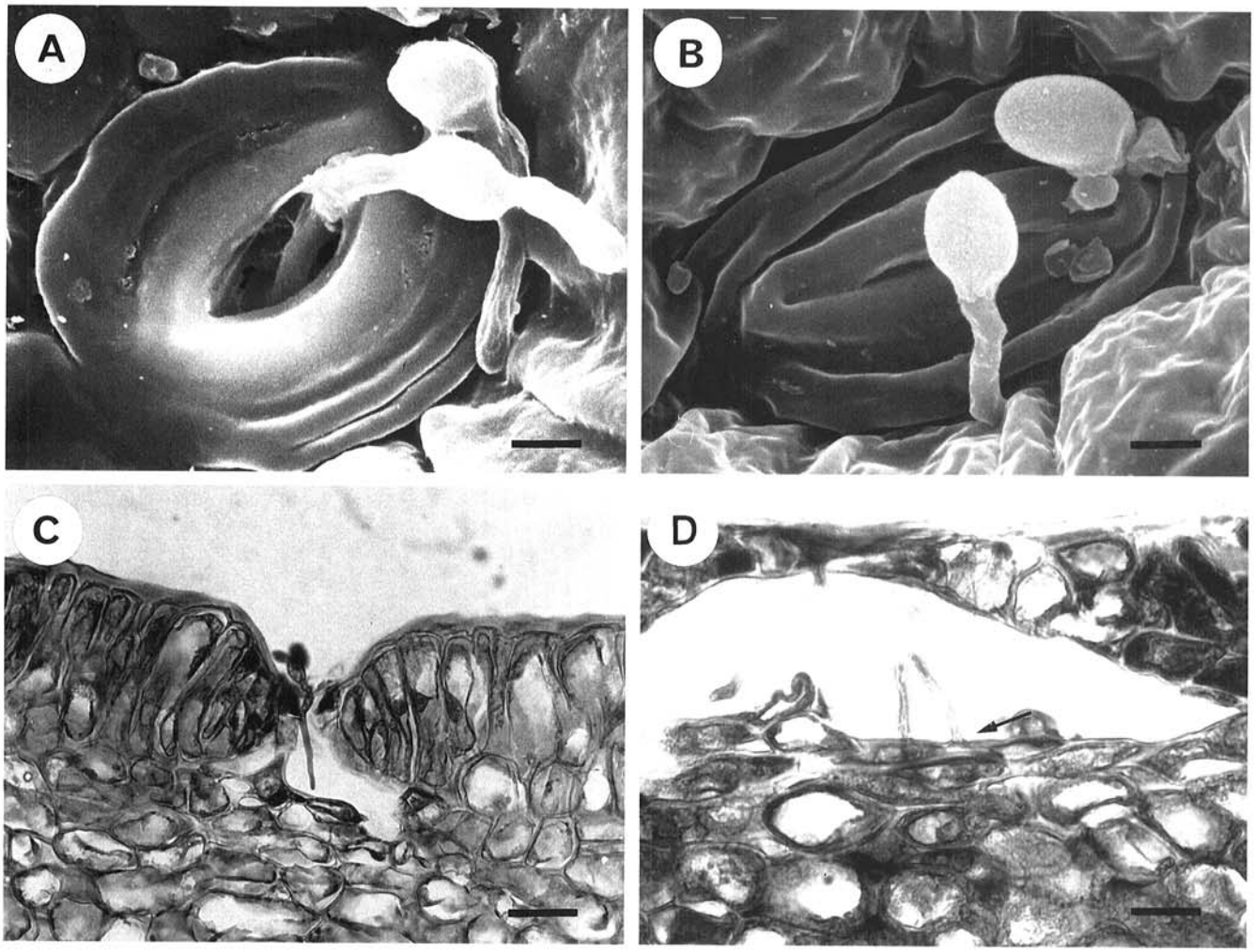

Fig. 3. Light and electron microscopy micrograph of stomatal penetration of plum and nectarine fruit by *Botrytis cinerea*. **A and B,** Germ tube entering an opened and a closed stoma on nectarine, respectively, 36 h after inoculation. Note the penetration of stomatal boundary cells. Scale bar = 7 μm. **C,** Infection hyphae entering a stoma on nectarine fruit. Scale bar = 36 μm. **D,** Hyphae adhering to the stomatal base cells (arrow) after entering a closed stoma on plum fruit. Scale bar = 18 μm.

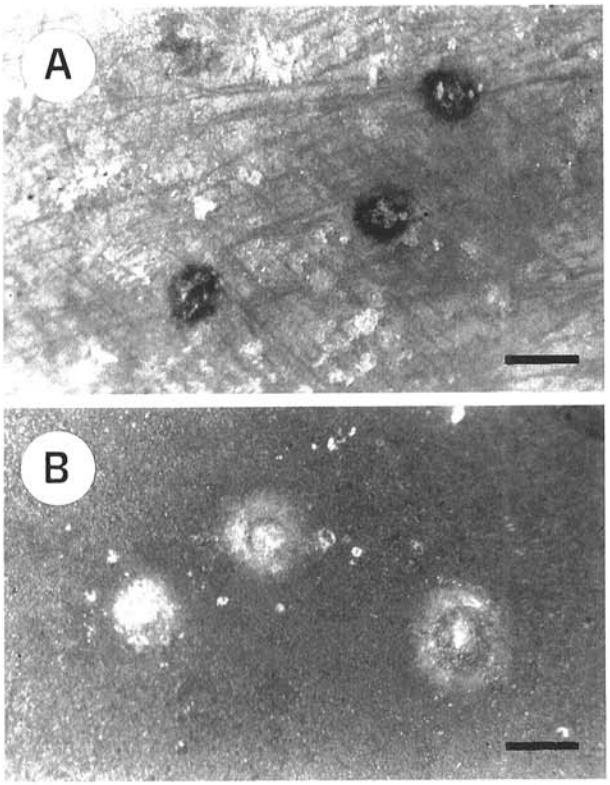

Fig. 4. Microscopic lesions formed on plum fruit 5 days after inoculation with *Botrytis cinerea*. **A,** Necrotic brown lesions on green fruit, associated with stomata. Scale bar = 100 μm. **B,** Yellow-brown lesions encircled with a decolorized zone, formed on mature picking-ripe plum. Scale bar = 80 μm.

TABLE 1. Percentage of Sunlite nectarine and Harry Pickstone plum fruit showing decay[x] 7 days after inoculation with *Botrytis cinerea* at different development stages during three consecutive seasons

	Nectarines			Plums		
Inoculation[y]	1988[z]	1989[z]	1990[z]	1988[z]	1989[z]	1990[z]
7	0 e	0 d	0 d	0 c	0 c	0 b
5	0 e	0 d	0 d	0 c	0 c	0 b
3	5 d	1 d	4 d	0 c	0 c	0 b
2	20 c	18 c	18 c	0 c	1 c	0 b
1	87 b	71 b	77 b	5 b	6 b	2 b
0	91 a	82 a	88 a	8 a	13 a	7 a

[x]Inoculated fruit samples were kept in moist chambers at 22 C. Fruit were considered decayed when lesions were greater in diameter than the inoculum droplet and were centered on the inoculation site.
[y]Weeks before harvest.
[z]Values within a column followed by the same letter do not differ ($P = 0.05$) according to Student's t test.

than that of fruit attached to trees. The single-drop inoculation of fruit in the laboratory also differs from natural inoculation in the orchard, where a smaller amount of inoculum may be deposited simultaneously at several sites on a fruit surface. The latter process could alter the host response to infection and hence the estimate of susceptibility. In the orchard, the frequent runoff of inoculum-containing raindrops would promote faster drying of fruit and a lower incidence of infection than might be expected from laboratory-inoculated fruit. In the latter instance, drops deposited on fruit remained undisturbed for longer periods, which could enhance germ tube and hyphal growth, thereby facilitating its chance of contacting suitable sites for penetration, such as stomata and microcracks.

Postharvest diseases are the result of two kinds of infections: infections resulting from handling injuries sustained during or after harvest, and latent infections occurring during the growing season (8). In the latter case, the pathogen penetrates the subepidermal layer of the developing fruit, and its further growth is then arrested until the fruit ripens (36). As no hyphae of *B. cinerea* could be found in the epidermal cell layer of nectarine and plum during their resistant phase, early field infections might be of lesser importance in this case than in other rots. However, additional research is needed to determine the prevalence of stomatal invasion under natural conditions, and to determine the outcome on senescing fruit of infections arrested in earlier infection of the substomatal cavity. Viable hyphae arrested in the substomatal cavity of green fruit may resume growth when the fruit ripen, as was recently reported for *M. fructicola* on apricot (37), or when fruit are injured or bruised during harvest and handling. It was previously shown (13) with *B. cinerea* that wounding was necessary for infection of plum.

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