Ultrastructural Effects of Ozone on the Host-Parasite Relationship of Botrytis cinerea and Pelargonium hortorum

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ABSTRACT

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Scanning electron microscopy (SEM) was used to observe effects of ozone on florist's geranium (*Pelargonium hortorum*) inoculated and noninoculated with the gray mold disease fungus, *Botrytis cinerea*. Greenhouse-grown geranium plants of cultivar Sincerity were exposed to either ozone-free air, 299 $\mu g/m^3$ [0.15 ppm (v/v)] ozone for 10 days for 6 hr/day, or 1,398 $\mu g/m^3$ (0.70 ppm ozone) for two 6-hr periods 24 hr apart. Plants inoculated with *Botrytis cinerea* were exposed to filtered air or ozone at 299 $\mu g/m^3$ (0.15 ppm) for two 6-hr periods 24 hr apart. Fresh leaf samples from each treatment were examined by SEM. Non-ozonized, noninoculated leaf tissues were distinguished from ozonized, chlorotic, prematurely senescent leaf tissues by SEM on the basis of cuticular features. Premature senescence was characterized by ruptured guard cells. Tissues subjected to 1,398 μg ozone/m^3 (0.70 ppm ozone) exhibited abaxial necrotic lesions; epidermal cells appeared smooth and without definite cell boundaries. Sporulation and germination of conidia, and hyphal penetration by, *B. cinerea* were readily observed by SEM on non-ozonized, inoculated geraniums. Neither hyphal penetration, sporulation, nor germination of conidia was observed on ozonized, inoculated leaves. A flocculence of unknown composition covered ozonized, inoculated specimens.

Additional key words: scanning electron microscopy, ozone injury.

Phytotoxic effects of ozone on vegetation have been described extensively (3, 4, 5, 13, 16). Few studies have dealt with the histopathological effects of ozone on plant species. Ledbetter et al. (11) described a dark stipple on ozone-treated leaf tissue of grape and avocado. Microscopically, the symptom coincided with thickened cell walls and collapse of palisade cells.

According to Hill et al. (6), leaf palisade cells in dicotyledonous plants are the first cell types to show injury from ozone. On grape (*Vitis* spp.), tobacco (*Nicotiana* spp.), and sugar beet (*Beta vulgaris*), palisade cells collapsed and became bleached after exposure to ozone. In the Gramineae, which lack palisade cells, mesophyll cells near the epidermal layer showed injury immediately after ozonization.

Few investigators have studied the effects of ozone on the ultrastructure of plant tissues. Thomson et al. (19) described ozone injury to the fine structure of palisade cells of bean leaves. Crystalline bodies in the chloroplasts and early changes in membrane ultrastructure of bean leaf mesophyll cells were studied in detail by Thomson et al. (20). Rufner et al. (17) found that chloroplasts of bean leaves treated with benomyl and exposed to ozone remained intact and were similar to those of nonexposed plants. The chloroplasts in benomyl-treated leaves were not disrupted like those in ozonized plants that did not receive the benomyl treatment. Swanson et al. (18) found that ozone induced shrinkage of tobacco chloroplasts. Pell and Weissberger (15) observed more rapid and extensive ozone-induced injury to paraveinal cells than to palisade or spongy mesophyll cells of soybeans.

Although the host-parasite relationships of *Botrytis cinerea* Pers. on a variety of genera have been the object of extensive research, few studies have described the ultrastructural relations. McKeen (12) described the fine structure of *B. cinerea* on *Vicia faba* leaves and concluded that enzymes degrade the cuticle, thus ruling out a purely mechanical mode of penetration.

Bessis (1) in a scanning electron microscopic (SEM) study described the mode of penetration by *Botrytis cinerea* and its parasitism on *Vitis* spp. Katumoto et al. (7) used SEM to study *B. cinerea* conidia formation, sclerotia morphology, conidia germination, and cuticular infection on *Fragaria* spp. The only published description of the ultrastructural effects of gaseous pollutants on host-parasite relation was that of Krause and Weidensaul (9). Because ozonization of *B. cinerea* conidia produced in vivo and in vitro decreased conidia germination, germ tube length, pathogenicity, and virulence (10), and ozonization of geranium leaves caused premature senescence as well as lesions (2, 8), an investigation was

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made: (i) to determine if SEM could be used to visualize the effects of low and/or high ambient ozone doses on geranium leaf surfaces, and (ii) to determine if SEM could be used to elucidate the interaction of ozone on the host-parasite relationship of the gray mold fungus and geranium.

MATERIALS AND METHODS

Plant materials.—Six-mo-old rooted cuttings of *Pelargonium hortorum,* Bailey 'Sincerity', were grown in a greenhouse in plastic pots in a soil-peat-perlite (2:1:1, v/v) mixture in charcoal-filtered air at 18-24 C and 16-hr photoperiod. The plants were watered as needed and fertilized every 14 days with 20-20-20 fertilizer (8g/pot).

An isolate of *B. cinerea* from a natural infection on a geranium plant was cultured on V-8 juice agar. Inoculation and re-isolation of the fungus from geranium demonstrated the pathogenicity of the isolate, which produced large, brown, water-soaked, foliar and stem lesions and blossom blight.

Inoculation procedures were similar to those used by Krause and Weidensaul (10). The inoculum of *B. cinerea* was prepared by flooding each 10-day-old culture plate with 15 ml of sterile, deionized, distilled water containing two drops of Tween-20. Sealing tissue sheets were placed tightly over the tops of the flooded culture plates and the lids were replaced. The entire plate then was agitated to dislodge the conidia. The contents of several plates were pooled and collected in a 500-ml sterile Erlenmeyer flask and the resulting spore suspension was filtered through four layers of sterile gause to remove mycelial fragments. The spores then were washed in sterile, deionized, distilled water and separated from the liquid by means of a membrane filter (2-μm pore size) to remove any remaining nutrients from the original culture medium. The conidia were resuspended in sterile, deionized, distilled water to obtain an inoculum suspension containing 7,000 to 8,000 spores/ml. Only fresh conidial suspensions were used for inoculations.

Plants were inoculated in a chamber under a fine mist sprayed at 10-min intervals for 3 sec. Approximately 10 ml of conidial suspension were sprayed on each plant. Individual plants then were incubated in darkness for 72 hr at 22 C.

Experimental treatments.—The geranium plants were subjected to various treatments as follows: (i) plants grown in a greenhouse in charcoal-filtered air; (ii) plants grown in a chamber in charcoal-filtered air without ozone exposure; (iii) plants ozonized with 299 $\mu g/m^3$ (0.15 ppm, a low ambient level) for 10 days at 6 hr/day; (iv) plants ozonized with 1,398 $\mu g/m^3$ (0.70 ppm, a high ambient level) for two 6-hr periods on successive days; (v) plants previously inoculated and placed in a ozone-free chamber for 2 days; and (vi) plants previously inoculated with *B. cinerea* and then ozonized for two 6-hr periods at 299 $\mu g/m^3$ (0.15 ppm) in exposure chambers. Each treatment was replicated three times. Each of six exposure chambers (102 × 102 × 76 cm) was monitored continuously for light intensity (21,520 lux at the leaf surface), temperature (24 C), relative humidity (80%), and ozone concentration. Chamber air was completely exchanged approximately three times per min. All chambers were equipped with a misting system that was activated once each hour for 6 sec. Ozone concentrations were monitored with a Model 1100 McMillan ozone meter.

Preparation of samples for microscopy.—Fresh leaf samples (2 mm^2) from each treatment were fixed immediately for 18 hr in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 C. Then the tissues were washed in a buffer solution, postfixed in OsO_4 (1% in phosphate buffer, pH 7.2) and dehydrated in 35%, 50%, 70%, 85%, 99%, and 100% (three times) ethanol (30 min). Specimens were critical-point dried in CO_2 (14), with amyl acetate (15 min, 3X) as an intermediate fluid, coated with carbon and Pd-Au on a tilting, rotary stage in a vacuum evaporator, and then examined on a Cambridge-Imanco S4-10 Stereoscan scanning electron microscope at 5 kV. Coated and uncoated fresh tissue also was examined with SEM.

RESULTS

Non-ozonized plants.—The SEM observations of untreated greenhouse-grown plants in ozone-free air revealed morphological characteristics similar to those described by Parsons el al. (14). The abaxial leaf surfaces showed trichomes, stomata, and epidermal cells with a high degree of regularity and turgidity, with cuticles of uniformly smooth surface, and with characteristic topography (Fig. 1). Guard cells were shaped regularly with no indication of shrinkage (Fig. 2). Non-ozonized leaf samples from plants grown under exposure chamber conditions for 2 wk appeared slightly chlorotic. When this tissue was examined with SEM, epidermal cells retained their integrity, but the cuticle appeared wrinkled, especially the basal trichome cells (Fig. 3). The cuticle above the guard cells was flaccid but the outer stomatal wall (white arrows) appeared rigid (Fig. 4) and was similar to that of guard cells of untreated greenhouse grown plants.

Ozonized plants.—Samples of tissues exposed to low ambient ozone (299 $\mu g/m^3$ = 0.15 ppm for 10 days) appeared to have mottled areas, but none of the flecking or stippling characteristically associated with ozone injury. Observations with the SEM revealed certain ultra-structural changes of leaf surface topography. Epidermal cells appeared flaccid and most trichomes had collapsed at the base (Fig. 5, white arrows). The most obvious feature of low ambient ozone exposure was the rupture of guard cells (Fig. 6, white arrows). In Fig. 7, a ruptured guard cell (white arrows) was adjacent to an apparently intact guard cell (black arrows) on which the outer stomatal lip (clear arrows) was wrinkled, indicating additional injury.

Samples of tissue subjected to high ambient ozone (1,398 $\mu g/m^3$ = 0.70 ppm for two 6-hr periods) exhibited necrotic lesions, but only on the abaxial surface. Examination of lesions by SEM revealed that the abaxial epidermis had lost its integrity and lacked definite cell boundaries (Fig. 8, black arrows). However, outside the lesions, the epidermal cells appeared unaffected (Fig. 8, white arrows) and turgid. Stomatal configurations of leaves exposed to a high ambient ozone were ruptured like those of geranium leaves exposed to low ambient ozone (299 $\mu g/m^3$) for 10 days at 6 hr/day (Fig. 6-7).

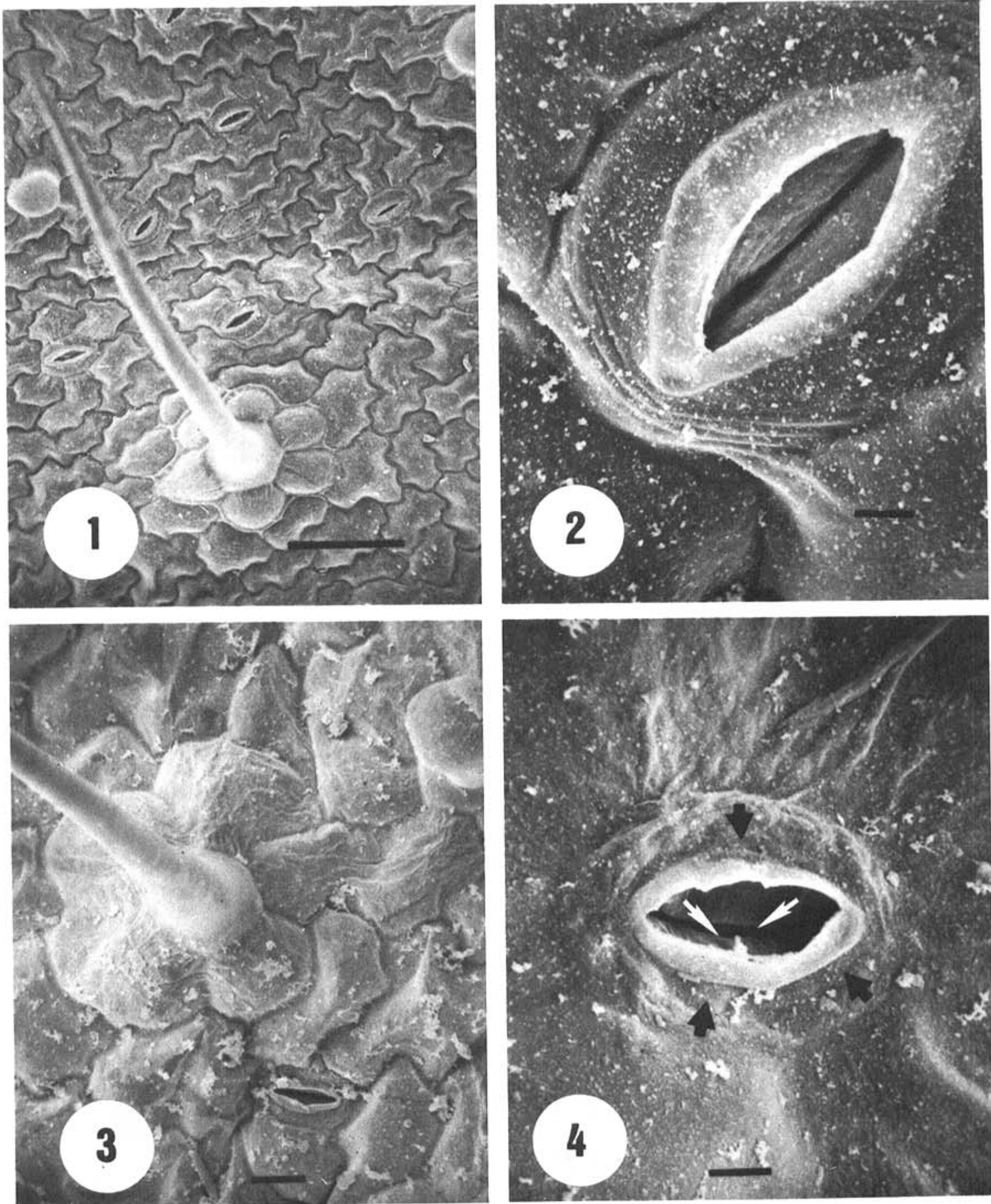

Fig. 1-4. Scanning electron micrographs of noninoculated, non-ozonized geranium leaves greenhouse grown in charcoal-filtered air. **1)** Abaxial surface showing the regularity of epidermal cell shape and the smooth cuticle (bar = 100 μm). **2)** Regularly shaped, turgid guard cell (bar = 5 μm). (3-4) Naturally senescing (chlorotic), noninoculated geranium leaves grown in an exposure chamber in charcoal-filtered air. **3)** Note the turgid epidermal cells but wrinkled appearance of the cuticle especially at basal trichome cells (bar = 20 μm). **4)** Stoma with flaccid appearing guard cells (black arrows) but with rigid outer stomatal lip (white arrows, bar = 5 μm).

SEM studies of the host-parasite relationship.—Examination of *B. cinerea*-infected geraniums not exposed to ozone showed that the fungus appeared 'normal' and that growth, sporulation, and penetration apparently were occurring when the tissue was fixed. Mycelial growth appeared vigorous, and conidia production was evident (Fig. 9). Mycelia and conidia were turgid, normal, and nondistorted. Apparently viable mycelium covered the leaf surfaces of non-ozonized, inoculated plants. Hyphal penetration through stomata occurred frequently (Fig. 10).

In contrast, the mycelial growth on inoculated leaves

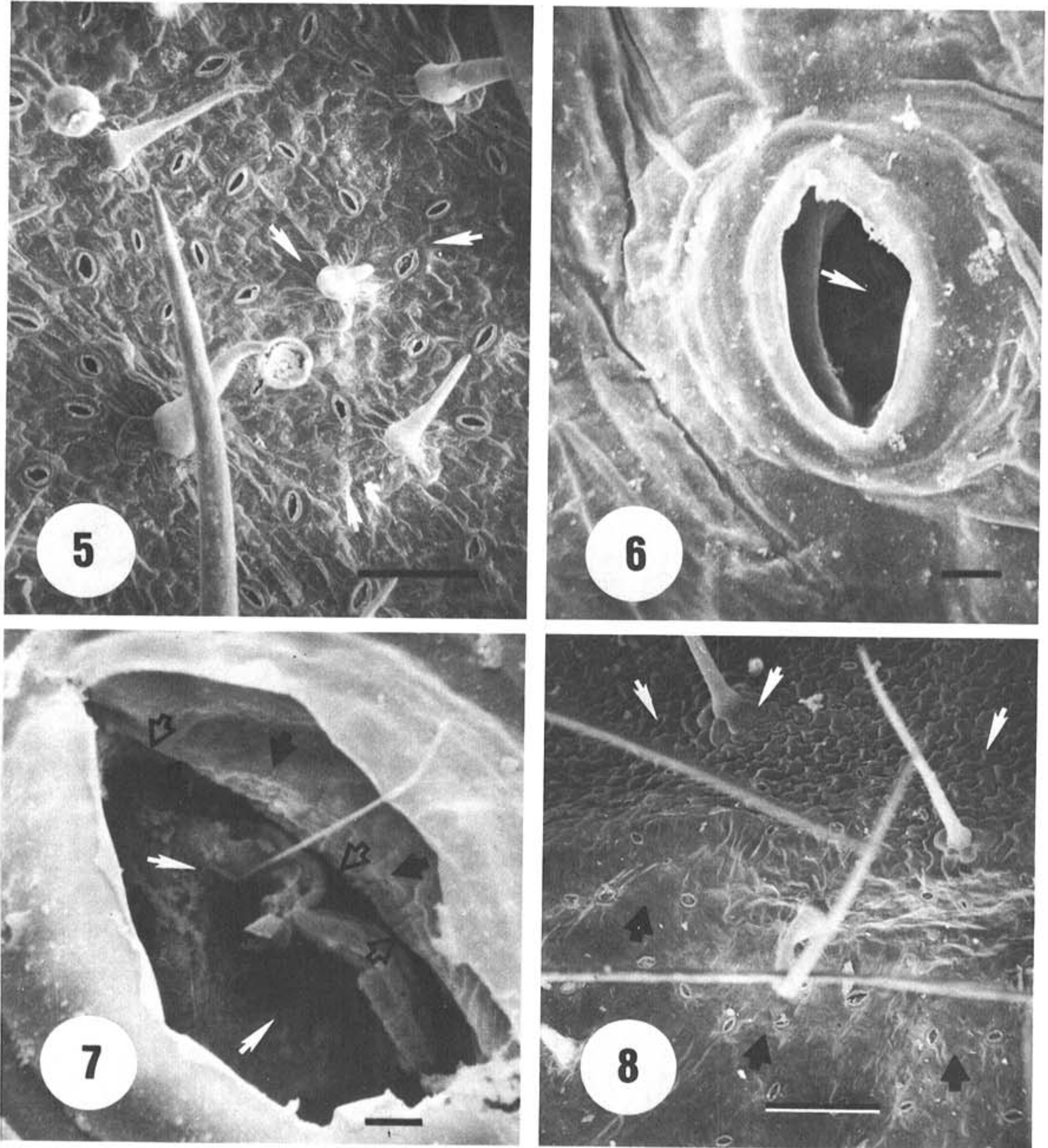

Fig. 5-8. Scanning electron micrographs of an ozone-induced prematurely senescing geranium leaf (299 $\mu g/m^3$ for 10 days). **5)** Flaccid epidermal and basal trichome cells, see white arrows (bar = 100 μm). **6)** White arrow indicates an apparently ozone-induced ruptured guard cell within a stoma (bar = 5 μm). **7)** Ruptured guard cell (white arrows) adjacent to an apparently intact guard cell (black arrows) but with a wrinkled outer stomatal lip (clear arrows bar = 1 μm). **8)** Necrotic lesion induced by ozone (1,398 $\mu g/m^3$ for two 6-hr periods). Note the loss of cell integrity: cells lack definite cell boundaries within the lesion (black arrows) although outside of the lesion the epidermal cells appeared turgid and nonsymtomatic.

ozonized with 299 $\mu g/m^3$ for two 6-hr periods on successive days was evenly distributed over both leaf surfaces, but it appeared flaccid and shrunken (black arrows) and was not as prevalent as that occurring on non-ozonized samples. A heavy flocculence (white arrows) covered both host and parasite tissues (black arrows) on ozonized samples (Fig. 11-12). Since the plant cells apparently were turgid, flocculence was not considered a product of host cytolysis. In many cases, mycelial growth (Fig. 13, black arrow), unlike that on non-ozonized tissue, was suspended above the leaf surface and was not in intimate contact with the cuticle. Conidiophores appeared constricted (black arrows) and flocculence covered the conidia (clear arrows, Fig. 14). Ozonized *Botrytis cinerea* on geranium leaf samples appeared adversely affected (i.e., flaccid and shrunken). Scanning electron microscopy of ozonized samples did not reveal germination of conidia or stomatal penetration, both of which were visible on non-ozonized specimens.

DISCUSSION

Scanning electron microscopy can be a useful tool for studying the effects of ozone on host-parasite interactions and may be used for diagnosing ozone injury to plants. The slight chamber effect (mild chlorosis) that was detected with SEM on non-ozonized plants grown in exposure chambers had been shown previously to be statistically insignificant (8). Guard cells that had ruptured in response to low ambient ozone (299 $\mu g/m^3$ for 10 days at 6 hr/day) appeared to be primary sites of ozone phytotoxicity. Ruptured guard cells could have resulted from the action of ozone on cellular membranes which was characterized by Pell and Weissberger (15). Further experimentation must be performed to define the specific mode of action of ozone on geranium. At ozone exposure levels of 1,398 $\mu g/m^3$ (0.70 ppm) for two 6-hr periods, SEM revealed a compartmentalization of necrotic lesions on geranium leaves. The cells within the lesion apparently lost integrity completely, whereas cells outside the lesion appeared unaffected.

One of the most striking SEM observations was the presence of the flocculent material on inoculated, ozonized leaves but not on inoculated, non-ozonized leaves. This flocculence, which apparently was related to ozone exposure, may have been one of the factors associated with protection against pathogenesis observed by Krause and Weidensaul (10). The absence of flocculence on non-ozonized geranium leaves infected with *B. cinerea* and its absence on ozonized geranium leaves not infected with *B. cinerea* suggests: (i) that ozone in the presence of *B. cinerea* may have stimulated the host to exude leachates on the plant surface, or (ii) that ozone may have stimulated production of flocculence by *B. cinerea*. Since ozonized mycelia were not observed in contact with leaf surfaces, it is possible that the flocculence impeded fungal penetration mechanically or enzymatically.

Lack of stomatal invasion by *B. cinerea* in ozonized leaves was consistent with another observation that ozone protected geraniums against *B. cinerea* infection (8). Ozone may have caused decreased infection by directly reducing the virulence and pathogenicity of the fungus since only a few constricted conidiophores and no germinating conidia were observed on inoculated, ozonized plants. Although ozone-induced inhibition of *B. cinerea* sporulation and germination both in vivo and in vitro had been verified (10) by conventional techniques (i.e., spore germination and re-infectivity tests), we used SEM to visualize and verify the former conclusions.

Whatever physiological mechanisms cause

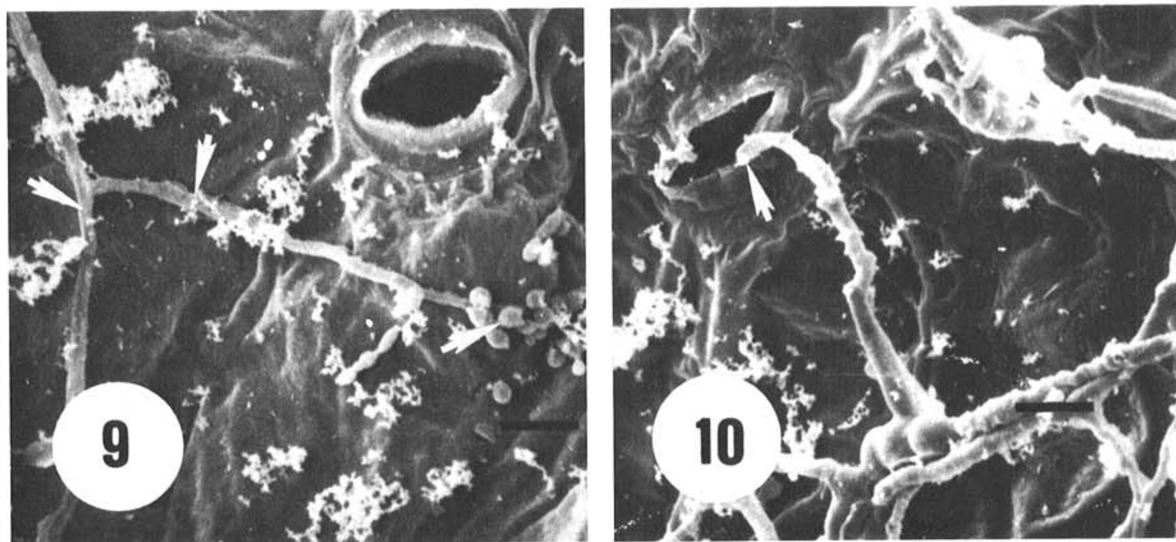

Fig. 9-10. Scanning electron micrographs of non-ozonized geranium leaves inoculated with *Botrytis cinerea* bar = 10 μm. **9)** Turgid, 'normal'-appearing mycelium and conidia (white arrows). **10)** Hyphal penetration of stoma (white arrow).

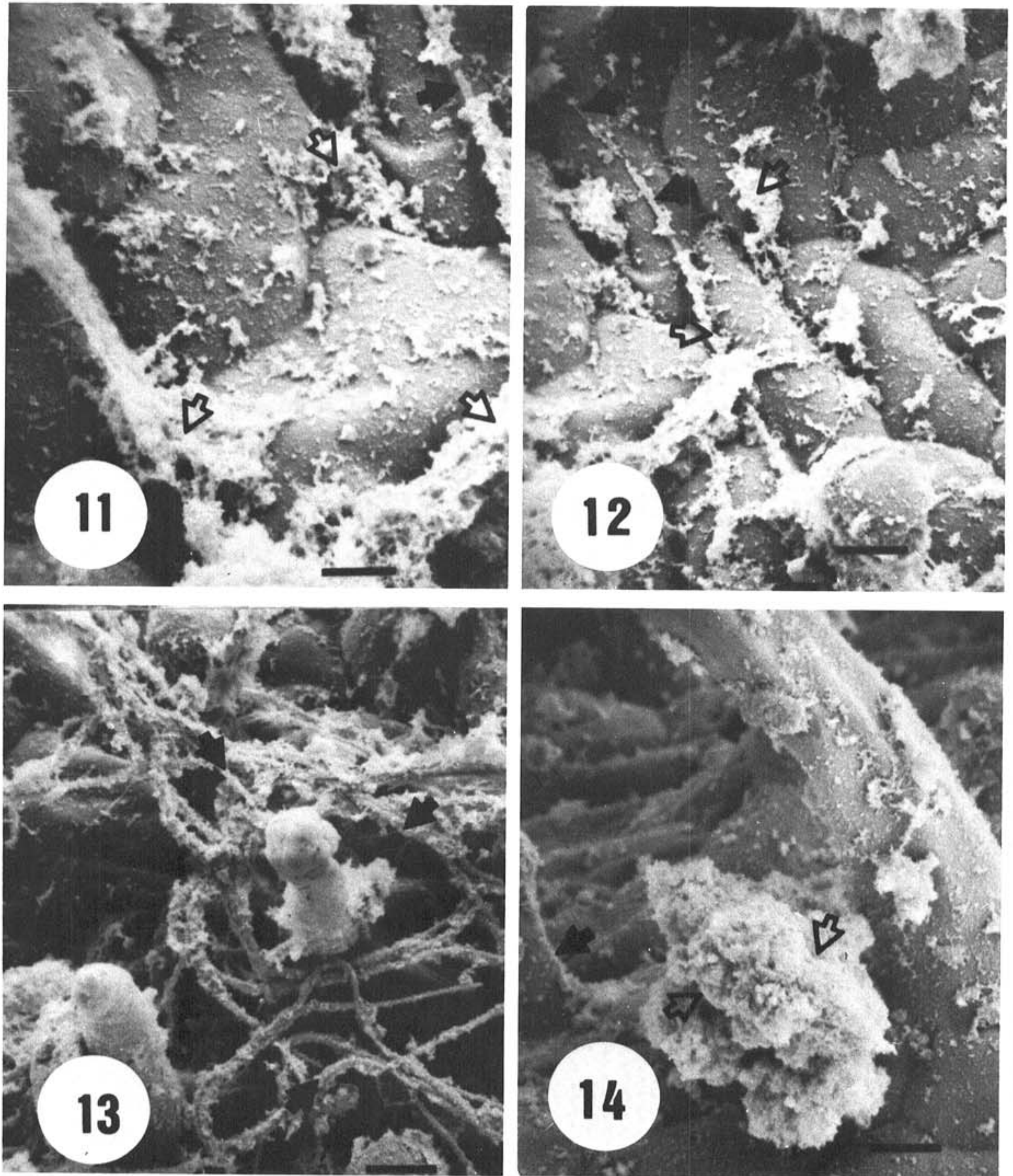

Fig. 11-14. Scanning electron micrographs of ozonized (299 $\mu g/m^3$ for two 6-hr periods on successive days) geranium leaves previously inoculated with *B. cinerea*. **11-12**) Mycelial growth appearing flaccid and shrunken (black arrows) with a flocculence (white arrows) covering both the fungal and epidermal cells which appeared to be turgid (bar = 20 μm). **13**) Mycelia (black arrows) growing above the leaf surface and not in intimate contact with epidermis (bar = 20 μm). 14) Flocculence (black arrows) covering a constricted conidiophore and conidia (clear arrow) at the base of a trichome (bar = 10 μm).

morphological changes in the host and parasite in the presence of ozone, both organisms are clearly affected. Although ozone injury occurs, extracellular materials which may limit infection could be produced by the host in response to ozone and the fungus.

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