Epifluorescence Microscopy of *Sphaerotheca fuliginea* Race 2 on Susceptible and Resistant Genotypes of *Cucumis melo*

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ABSTRACT

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Fungal growth and host response were investigated in leaves of susceptible, moderately resistant, and resistant genotypes of muskmelon with the aid of the fluorescent stain calcofluor, using ultraviolet epifluorescence microscopy. Fungal germination at 24 hr with a single germ tube proceeded equally well on all genotypes, but the production of the second and third germ tubes at 48 hr, hyphal growth, and sporulation were inhibited in resistant genotypes, probably because of the hypersensitive reaction of epidermal cells that was associated with germ tubes and hyphae. The possibility is raised that the resistant genotypes studied share a common gene with PI 124111 for resistance to race 2 of *Sphaerotheca fuliginea*.

Additional key words: genetics, powdery mildew.

Powdery mildew is a major disease of muskmelon all over the world. Three races of the fungal causal agent, *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll., were reported in the United States (9) and two, races 1 and 2, in Israel (4).

The muskmelon (*Cucumis melo* L.) line PI 124111 was reported to be resistant to races 1 and 2 of *S. fuliginea* in Israel and to races 1, 2, and 3 in the United States (4). The breeding line MR-1, derived from PI 124111, was reported to be resistant to all three races of the pathogen (12).

PI 124111 has been grown in our field plots on campus since 1978, along with the susceptible domestic cultivar Ananas-Yokneam (AY). While AY suffered heavy epiphytotics of powdery mildew each year, PI 124111 remained disease-free. Cohen and Cohen (3) studied the genetics and nature of resistance to powdery mildew race 2 in PI 124111. In crosses they made between PI 124111 and AY, they found that resistance is conferred by a partially dominant gene. Resistance in PI 124111 was attributed to reduced conidial germination of *S. fuliginea* on the leaf surface, reduced colony formation, and reduced sporulation in comparison to the susceptible AY (3).

The aims of this study were twofold: first, to further explore the interaction between *S. fuliginea* and *C. melo* PI 124111 with the aid of fluorescence microscopy and, second, to compare fungal growth on PI 124111 with that on other genotypes of *C. melo* resistant to powdery mildew.

MATERIALS AND METHODS

Plants. Various genotypes of *C. melo*—susceptible (AY and PMR-45), moderately resistant (Edisto-47, AY× PI 124111 F_1, and Seminole), and resistant (PI 124111, PI 124112, PMR-5, PMR-6, Male Sterile-1, and Dulce) to powdery mildew—were grown in the greenhouse (20–34 C) in 0.5-L pots containing sandy loam, one plant per pot. Once a week the plants were fertilized with a 1% solution of N, P, and K (20:20:20). Three weeks after sowing, at the 2-leaf stage, plants were transferred to growth chambers at 23 C for inoculation.

Pathogen. A culture of *S. fuliginea* race 2 collected from *C. melo* cv. PMR-45 (resistant to race 1 and susceptible to race 2) was used. This culture was maintained in a growth chamber at 23 C on PMR-45 plants.

Inoculation. One week after having been inoculated, PMR-45 plants were thoroughly shaken to dislodge conidia over the test plants. Conidial density was determined in leaf disks (2 cm in diameter) taken from the inoculated plants immediately after inoculation. The leaf disks were stained with calcofluor, and conidia counted with the aid of a fluorescence microscope (see below). Inoculated test plants were thereafter kept in a growth chamber at 23 C (60–70% RH) illuminated 12 hr a day with cool white fluorescent light at an intensity of about 100 $\mu E \cdot m^{-2} \cdot sec^{-1}$.

Microscopical examinations. The calcofluor staining technique reported earlier (5) for the pathosystem of *Peronospora tabacina* and tobacco was used. Leaf disks (1 cm in diameter) were removed from leaf 2 of the inoculated plants, 1, 2, and 7 days after inoculation. They were mounted on microscope slides with the adaxial surface uppermost, and 50 µl of aqueous 0.01% calcofluor solution (calcofluor white M2R New, disodium salt of 4,4-bis(4-anilino-6-diethylamino-*s*-triazin-2-ylamino)-2, 2-stilbene disulfonic acid; Polysciences, Warrington, PA) was applied to the surface of each disk. The disks were then covered with a glass coverslip. Conidial germination, fungal growth, and plant response were examined with the aid of a Zeiss standard epifluorescence microscope equipped with an Osram halogen lamp (HBO, 50 W), epifluorescence condenser IVFI, and filter set number 18. This set includes excitation filter BP395-425, dichromatic beam splitter FT 425, and barrier filter LP 450. Neofluor objectives 6.3/0.20, 16/0.40, 40/0.75, and 100/1.30 were used with CPL W10× eyepieces. With this setup, fungal hyphae and conidia fluoresced blue, and plant cells remained dark or emitted a weak red autofluorescence. Plant trichomes and physically injured cells also autofluoresced blue. Affected epidermal cells in resistant genotypes autofluoresced yellow.

To observe fungal penetration into plant epidermal cells, infected leaf disks, 1, 2, and 7 days after inoculation, were cleared by boiling in alcoholic lactophenol (phenol, 10 g; lactic acid, 10 ml; glycerine, 10 ml; and 96% ethanol, 20 ml) for 15 min and then washed twice with 96% ethanol and twice with deionized water. The leaf disks were then mounted on microscope slides with the adaxial surface uppermost and treated with calcofluor as described above. When examined with fluorescence microscopy, fungal hyphae and conidia fluoresced blue, and plant cells remained dark brown. Affected epidermal cells in resistant genotypes retained their yellow autofluorescence. Points of penetration of fungal hyphae into epidermal cells fluoresced blue, especially in leaf disks

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sampled 7 days after inoculation. Bright-field microscopy revealed the presence of circular holes at points of penetration into the epidermis.

To observe haustorial sacs inside epidermal cells, adhesive Scotch tape was attached to the adaxial surface of inoculated leaves, pressed lightly, and peeled off. Some hyphae and conidia were thus removed from the leaf surface. Peeled leaf pieces were cleared with lactophenol, mounted on microscope slides, and examined with bright-field microscopy. Points of hyphal penetration and haustorial sacs were clearly visible.

Fungal sporulation. Leaf disks (23 mm in diameter) were taken from leaf 2 of infected plants 10 days after inoculation and placed in 5 ml of formalin/acetic acid/ethanol (5:5:90). Conidia were brushed off the leaf disks with the aid of a small camel-hair brush, and the number of conidia was determined with the aid of a cytometer (five counts per disk).

Field observations. Various genotypes of *C. melo* were planted in microplots (5 × 10 m) on campus on 1 May and 1 June 1986. A source of infected PMR-45 plants was positioned in the middle of each plot, 1 wk after planting. Disease severity was visually assessed, 2 mo after planting, on a scale of 0 to 4 (0, no disease symptoms apparent; 1, 2, 3, and 4, about 25, 50, 75, and 100%, respectively, of the foliage affected with powdery mildew).

RESULTS

Disease development in the field. The occurrence of powdery mildew was assessed 2 mo after planting. AY, Topmark, Delicious 51, Planter's Jumbo, and Hale's Best Jumbo were all severely affected (rated 4 on a visual scale of 0–4). PMR-45, Edisto-47, and Rio Gold were moderately diseased (rated 2). No disease was recorded on Perlita, Male Sterile-1, Seminole, Dulce, PI 124111, PI 124112, PMR-5, and PMR-6. Seminole was slightly diseased (rated 1) 3 mo after planting.

Fungal sporulation in controlled environment. Sporulation on plants in growth chambers, 10 days after artificial inoculation with 70–160 conidia per square centimeter, is given in Table 1. The highest conidial yields were obtained from AY and PMR-45, a moderate yield from Edisto-47, low yields from AY × PI 124111 F_1 hybrid and Seminole, and very low yields from PI 124112, PMR-5, PI 124111, Male Sterile-1, Dulce, and PMR-6. In plants inoculated with 10–40 conidia per square centimeter, no sporulation was detected in the resistant genotypes; conidial yields in AY and PMR-45 were $41.7 \pm 21.4 \times 10^3$ and $19.7 \pm 11.6 \times 10^3$ conidia per square centimeter, respectively.

TABLE 1. Sporulation of *Sphaerotheca fuliginea* race 2 on susceptible and resistant genotypes of *Cucumis melo* at 23 C

Genotype	Class[w]	Conidia[x] (10^3 per square centimeter)
Ananas-Yokneam	S	71.1 ± 16.6 a[y]
PMR-45	S	70.6 ± 19.3 a
Edisto-47	S	43.9 ± 20.0 b
Ananas-Yokneam × PI 124111 F_1	MR	22.3 ± 11.8 c
Seminole	MR	16.0 ± 8.4 c
PI 124112	R	3.9 ± 4.6 d
PMR-5	R	2.1 ± 3.1 d
PI 124111	R	1.4 ± 2.4 d
Male Sterile-1	R	0.4 ± 0.8 d
Dulce	R	0.1 ± 0.2 d[z]
PMR-6	R	0.1 ± 0.2 d[z]

[w] S = susceptible, MR = moderately resistant, and R = resistant to powdery mildew.
[x] Inoculum density was 70–160 conidia per square centimeter of leaf surface. Conidial production was measured 10 days after inoculation with the aid of a cytometer, in 23-mm-diameter leaf disks taken from leaf 2 of four-leaf plants. Six plants were examined per entry.
[y] Data followed by different letters are significantly different at the 5% level, according to Duncan's multiple range test.
[z] Since no sporulation was observed with epifluorescence microscopy, these conidia may represent the original inoculum.

Fungal growth on susceptible and resistant genotypes. Repeated epifluorescence microscopical examinations showed that conidia produced their first germ tube (unbranched or bifurcate) in about 16–24 hr, regardless of whether they were placed on leaves of resistant or susceptible genotypes. The percentage of conidia germinating with a single germ tube, 24 hr after inoculation, ranged between 40 and 70 in different experiments, and no significant differences were noted between host genotypes. The production of the second and third germ tubes and the extension of the first one occurred within 48 hr, with significant differences emerging between host genotypes. A representative experiment, given in Table 2, shows that 42 and 47% of the conidia in a population developed two or three germ tubes on the susceptible genotypes, but only 6–19% did so on the resistant genotypes. At 72 hr after inoculation, an additional two or three germ tubes and extension and ramification of the first two were seen on susceptible genotypes. Such a development was rarely seen in resistant genotypes. By 96 hr discrete colonies and conidiophores bearing conidial chains (seven to nine conidia per conidiophore) were observed in susceptible genotypes. No conidiophores were seen in resistant genotypes at this stage. Profuse sporulation was observed on the susceptible genotypes a week after inoculation (Fig. 1F), but only very few conidiophores bearing conidia (three to seven per conidiophore) were seen in resistant ones (Fig. 1C), except in Dulce and PMR-6, on which no sporulation was seen (compare with Table 1).

Host response. Fungal growth and host cell response are shown in micrographs taken 1, 2, and 7 days after inoculation of the resistant PI 124111 (Fig. 1A–C) and the susceptible AY (Fig. 1D–F). A strong yellow autofluorescence of epidermal cells was observed in all resistant genotypes as early as 16–24 hr after inoculation with *S. fuliginea* (Fig. 1A). This fluorescence was not dependent on calcofluor staining and did not disappear after clearing with lactophenol. Autofluorescing epidermal cells were always associated with germ tubes or hyphae of the pathogen and did not show up in plants inoculated with nonviable conidia (killed by chloroform vapor) or in uninoculated plants. Trichomes did show blue autofluorescence in all genotypes, whether inoculated or not. The subjacent location of the yellow-fluorescing epidermal cells relative to germ tubes and hyphae (Fig. 1A–C) suggested that penetration of the fungus into these host cells had occurred. Penetrations into normal and yellow-fluorescing epidermal cells were difficult to detect at 1 day after inoculation. Penetrations into fluorescing cells were detectable at 7 days in calcofluor-stained tissue after clearing with lactophenol (Fig. 2C and F). No yellow-autofluorescing epidermal cells were seen in the susceptible AY and PMR-45, even on the 7th day after inoculation (Fig. 1D–F), although numerous penetrations and haustorial sacs were clearly seen in epidermal cells of these genotypes (Fig. 2A, B, D, and E). Some epidermal cells, especially in the center of the colony, did

TABLE 2. Germination of *Sphaerotheca fuliginea* race 2 on the adaxial leaf surface of susceptible and resistant *Cucumis melo* genotypes at 23 C

Genotype	Class[y]	Conidia developing two or three germ tubes at 48 hr (%, ± SD)
Ananas-Yokneam	S	47 ± 13 a[z]
PMR-45	S	42 ± 6 a
PI 124111	R	19 ± 12 b
PI 124112	R	10 ± 11 bc
Male Sterile-1	R	11 ± 2 bc
PMR-5	R	18 ± 16 bc
PMR-6	R	6 ± 4 c

[x] The calcofluor assay was conducted in leaf 2 of two-leaf plants, five plants per genotype, and 100 conidia were counted per plant, 48 hr after inoculation.
[y] S = susceptible, and R = resistant to powdery mildew.
[z] Data followed by different letters are significantly different at the 5% level, according to Duncan's multiple range test.

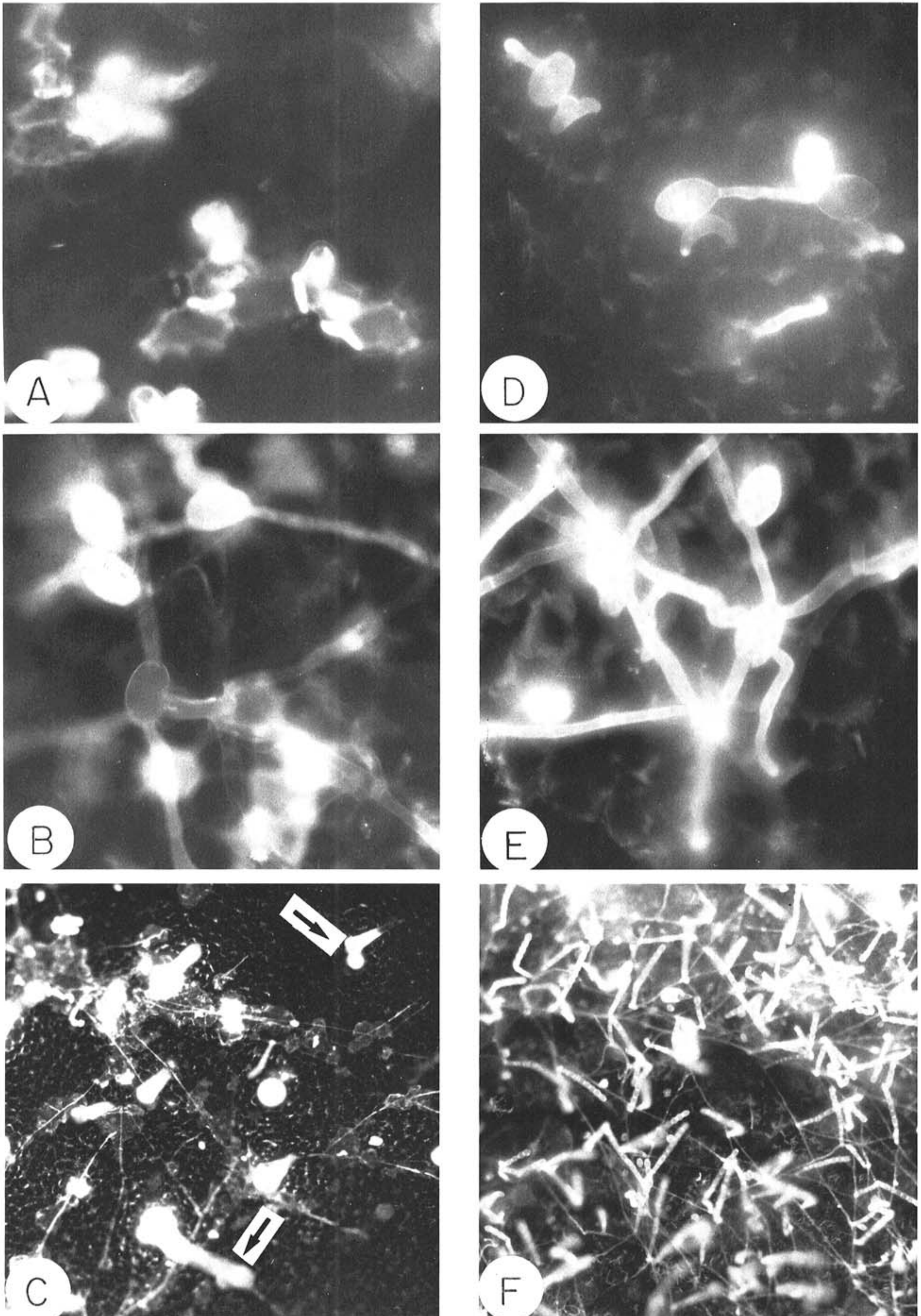

Fig. 1. Development of *Sphaerotheca fuliginea* race 2 on leaf surface of the resistant genotype PI 124111 (**A–C**) and the susceptible genotype Ananas-Yokneam (**D–F**), 1 day (A and D, ×450), 2 days (B and E, ×330), and 7 days (C and F, ×52) after inoculation. Photos were taken with the aid of an ultraviolet epifluorescence microscope after calcofluor staining, with Agfa ×R 100 i color film. Tissue photographed in A was first cleared with boiling lactophenol. Note the association of fluorescing epidermal cells with germ tubes in A and with hyphae in B and C. The arrows in C indicate trichomes.

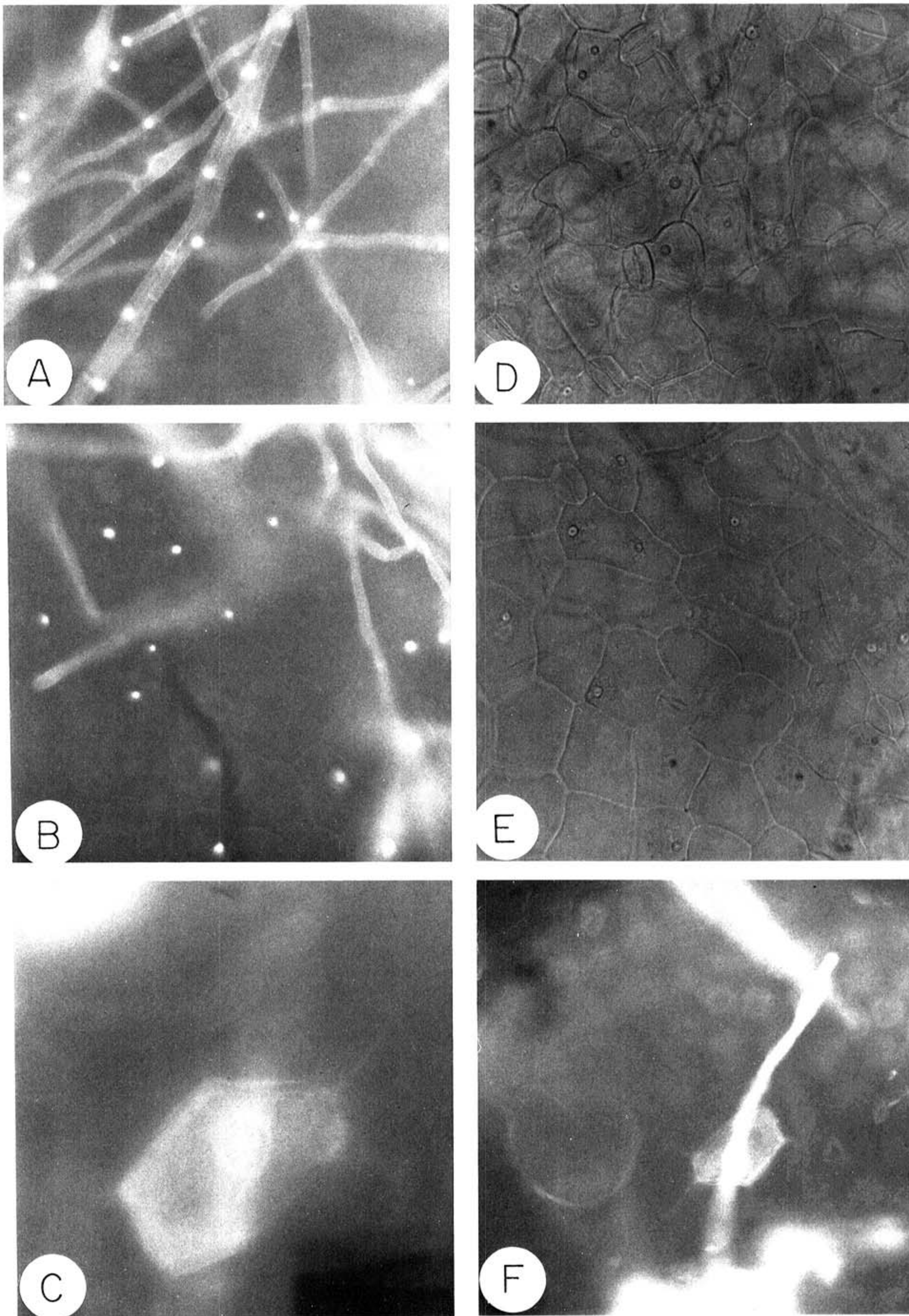

Fig. 2. Penetration of *Sphaerotheca fuliginea* race 2 into leaf epidermal cells of the susceptible genotype Ananas-Yokneam (**A, B, D, and E**, ×330) and the resistant genotype PI 124111 (**C**, ×825; **F**, ×330), 7 days after inoculation. A and D are matching photos taken under ultraviolet (A) and bright-field (D) microscopy, with calcofluor staining. Fluorescing points of penetration in A correspond with holes in epidermal cells in D. B and E are matching photos taken similarly after peeling with adhesive tape. Fluorescing points of penetration in B correspond with holes and haustorial sacs in epidermal cells in D. C and F show penetration into a fluorescing cell of PI 124111, under mixed ultraviolet and incandescent light. Specimens were cleared with lactophenol.

autofluoresce yellow in Edisto-47, the AY×PI 124111 hybrid, and Seminole a week after inoculation.

DISCUSSION

Despite extensive studies on the occurrence and inheritance of genes for resistance to powdery mildew in muskmelon (1,2,6–12), very little is known about their nature. Cohen and Cohen (3) showed that a partially dominant gene is responsible for the resistance of PI 124111 to race 2 of *S. fuliginea*. In this line, advanced stages of conidial germination, hyphal growth, and sporulation of *S. fuliginea* are suppressed (3).

The results presented in this paper show that the reduced fungal growth in PI 124111 is associated with a hypersensitive reaction of the host subsequent to a challenge inoculation with *S. fuliginea*. Leaf epidermal cells associated with germ tubes and hyphae showed extensive yellow autofluorescence. We postulate that epidermal cells produce yellow-fluorescing (probably phenolic) substances upon fungal penetration. The initial appearance of such autofluorescing cells was noticed as early as 16–20 hr after inoculation. Their number increased as the fungus continued to grow on the surface. This hypersensitive response of the host did not completely stop the growth of the fungus, which was able to produce a few conidia.

Other microscopical examinations showed that resistance to race 2 in PI 124112, PMR-5, PMR-6, Dulce, and Male Sterile-1 is also associated with a hypersensitive response similar to that described in PI 124111. The difference observed in the capacity of the fungus to sporulate on the various resistant genotypes indicates that hypersensitivity is a major but not the sole mechanism responsible for resistance to race 2 of the fungus. All genotypes showing a strong hypersensitive reaction supported a reduced hyphal growth and severely inhibited sporulation. AY and PMR-45, which showed no fluorescing epidermal cells throughout the first week after inoculation, supported profuse sporulation. Interestingly, Edisto-47, AY × PI 124111 F_1, and Seminole, which supported moderate to weak sporulation, showed some fluorescing epidermal cells in the center of the fungal colonies.

According to Bohn and Whitaker (2) resistance in PMR-5 and PMR-6 is governed by the partially dominant gene *Pm*2. These lines have PI 78374 in their pedigree together with additional sources of resistance to race 2, such as PI 79376l1. No information is yet available on the genes for resistance to race 2 in PI 124112, Male Sterile-1, and Dulce. It is possible that they share a common gene with PI 124111 against race 2 of *S. fuliginea*. This possibility is being investigated.

The calcofluor epifluorescence technique reported here was found to be useful for rapid characterization of resistance to powdery mildew in muskmelon genotypes. It may be adapted easily for similar purposes in other fungal-plant pathosystems.

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