Development of a Mutant Strain of *Sporothrix flocculosa* with Resistance to Dodemorph-Acetate

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ABSTRACT

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Several isolates of the powdery mildew biocontrol fungus *Sporothrix flocculosa* were screened for their resistance to the systemic fungicide dodemorph-acetate (Meltatox) by repeated exposure to increasing concentrations of the fungicide. Through this procedure, we obtained a new strain that was able to grow and form colonies on solid media amended with 300 µg of fungicide per ml, a concentration that exceeded the recommended dosage by 50% and totally inhibited the growth of the wild-type strain. This resistance trait was maintained after several subcultures on fungicide-free media. When tested for its biocontrol ability, the dodemorph-resistant strain was able to colonize conidia and mycelium of *Sphaerotheca fuliginea* as efficiently as the wild-type strain. In addition, the new strain was not hampered in its ability to control powdery mildew when applied in mixture with dodemorph-acetate, whereas the wild-type strain was unable to colonize the pathogen in the presence of the fungicide. This is the first report of resistance against dodemorph-acetate in a powdery mildew biocontrol agent and, to our knowledge, in a fungus. This new strain could have practical application in integrated control of rose powdery mildew.

Environmental concerns regarding the use of fungicides have called for the development of alternatives capable of eliminating or reducing dependence on chemicals for controlling plant diseases. Among strategies proposed to reduce fungicide use, the application of microbial pest control agents in integrated pest management systems has received much attention (14). However, microbial pest control agents are limited in their application by their susceptibility to fungicides currently used in agricultural systems. Attempts to overcome this problem have demonstrated that fungicide resistance of natural antagonists may be improved by selection or genetic means. For instance, new strains of *Trichoderma harzianum* (1,16), *T. viride* (15), *Talaromyces flavus* (11), *Gliocladium virens* (17), and *Epicoccum purpurascens* (22) that tolerate high concentrations of benomyl and other methyl benzimidazole carbamate fungicides have been isolated or developed.

The yeast-like fungus *Sporothrix flocculosa* Traquair et al. displays a strong antagonistic activity against several powdery mildew fungi (5,6,10). Recently, it was reported to control rose powdery mildew, *Sphaerotheca pannosa* var. *rosae*, under commercial conditions to a level similar to the fungicide dodemorph-acetate (Meltatox), a sterol biosynthesis inhibitor (SBI) used against rose powdery mildew (2). The morpholine compound dodemorph-acetate inhibits sterol biosynthesis, causing the accumulation of fecosterol and ergosta-8-en-3β-ol in *Ustilago maydis* and *Saccharomyces cerevisea* and fecosterol and ergosterol-8,22,24(27)-trien-3β-ol in *Botrytis cinerea* and *Penicillium expansum* (12). These results indicate the inhibition of $\Delta^8 \rightarrow \Delta^7$ isomerase. *S. flocculosa* is itself very sensitive to dodemorph-acetate, which prevents the integration of both biological and chemical approaches in a pest management scheme.

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To our knowledge, dodemorph-acetate is one of the few systemic fungicides for which cases of resistance have not been reported. The objectives of this study were to: i) screen for new biotypes of *S. flocculosa* resistant to dodemorph-acetate and ii) evaluate the antagonistic properties of the new phenotypes.

MATERIALS AND METHODS

Microorganisms. Single-spore isolates of *S. flocculosa* (SF-1) were obtained by reisolating the fungus from rose leaves that were submitted to weekly applications of a fungal suspension initiated from an isolate of *S. flocculosa* graciously provided by W. R. Jarvis, Harrow Research Station, Agriculture & Agri-Foods Canada. Stock cultures of the isolates were maintained at 4°C on yeast-malt-peptone-dextrose agar (YMPDA) before being transferred in shake cultures at room temperature to liquid YMPD.

Mildewed cucumber (*Cucumis sativus* L.) cv. Corona leaf disks (20 mm) were cut from fully expanded leaves grown in a glasshouse. The leaf disks were selected so that approximately 90% of the area was covered by mycelium and conidia of *Sphaerotheca fuliginea* (Schlectend.:Fr.) Pollacci.

Fungicide. Dodemorph-acetate (Meltatox, 400 g a.i./L) was obtained from Plant Prod Québec (Laval, Québec). This fungicide was available as an emulsion and was added directly to the liquid culture media at concentrations specified below. The recommended dosage for rose powdery mildew control is 200 µg/ml.

Isolation of new phenotypes. The occurrence of resistant phenotypes was screened by successive subcultures of isolates in liquid suspension containing increasing concentrations of dodemorph-acetate. Every 5 days, 2 ml of the culture suspension was transferred to fresh YMPD broth containing increasingly higher fungicide concentrations. The adaptation was initiated with a dose of 40 µg of dodemorph-acetate per ml that was increased gradually to 60, 80, 100 125, 150, 175, 200, and 250 µg a.i./ml. At the end of the adaptation process, fungal propagules that survived the treatments were maintained on YMPDA.

Toxicity assays. In vitro fungitoxic activity of dodemorph-acetate was tested on YMPDA. Dodemorph-acetate was added to the autoclaved medium, mixed with a magnetic stirrer, and dispensed (15 ml) into 9-cm petri plates. Disks of 7-day-old colonies from wild-type and resistant strains were placed on YMPDA amended or unamended with dodemorph-acetate. Five replicate plates were prepared for each phenotype and each fungicide concentration (0, 25, 50, 75, 100, 150, 200, and 300 µg/ml) tested. Cultures were incubated at 24°C, and the colony radius was measured after 8 days of incubation. The concentration at which mycelial growth rate was reduced by 50% (mean effective dose [ED_{50}]) was determined from dosage-response curves. The stability of the resistant strain was tested by reevaluating resistance after 10 serial transfers into fungicide-free liquid YMPD.

Biological properties. The resistant strains obtained from the experiments were tested for their ability to control powdery mildew. Five leaf disks of mildewed cucumber were placed on moist filter paper in 9-cm petri plates. The disks were sprayed lightly with one of the following: i) a cell suspension of *S. flocculosa* (SF-1) (10^6 CFU/ml); ii) a cell suspension of the putative resistant strain (SF-R_M) (10^6 CFU/ml); iii) a cell suspension of SF-1 in a mixture with 100 or 300 µg of dodemorph-acetate per ml; and iv) a cell suspension of SF-R_M in a mixture with 100 or 300 µg of dodemorph-acetate per ml. The petri plates were closed, sealed with Parafilm, and incubated at 24°C in mixed fluorescent and incandescent light at 30 mE m^{-2} s^{-1} for a 12-h photoperiod. Five leaf disks of mildewed cucumber were used for each treatment, and the experiment was repeated twice.

Effects of *S. flocculosa* treatments on *Sphaerotheca fuliginea* were scored as an arbitrary scale described by Jarvis et al. (10): 0 = no colonization of powdery mildew; 1 = 1 to 20%; 2 = 11 to 40%; 3 = 31 to 60%; 4 = 51 to 70%; and 5 = 81 to 100% dead colonies.

Scanning electron microscopy (SEM). Leaf-disk samples from each treatment, plus treatment with distilled water, were collected after 48 h and fixed with glutaraldehyde (3%, vol/vol) in 0.1 M sodium cacodylate at pH 7.2 for 16 h at 4°C, then rinsed three times with the same buffer and postfixed by immersion with osmium tetroxide (1%, wt/vol) for 2 h at 4°C in sodium cacodylate buffer. The samples were dehydrated in a series of ethanol solutions graded in 20% steps, after which they were mounted on aluminum studs and sputter-coated with gold to a thickness of about 20 nm and examined with a Cambridge Stereoscan 5-150 microscope (Cambridge Instruments, Cambridge, England) at 20 kV.

RESULTS

Isolation of resistant mutants. Exposure of different wild-type isolates of *S. flocculosa* to dodemorph-acetate strongly inhibited the development of fungal propagules even at low concentrations (40 µg/ml). The survival rate was low, and most isolates could not be recovered after the initial culture in fungicide-amended medium. The few isolates that showed growth at the lowest concentration were allowed further adaptation at 40 µg/ml before they were transferred to medium containing 60 µg/ml. Most isolates did not survive the series of adaptation steps. However, after 53 transfers, we obtained a fungal colony from one isolate that displayed sustained growth in a medium amended with dodemorph-acetate at 250 µg a.i./ml; it was designated as mutant SF-R_M.

When grown in a liquid medium amended with dodemorph-acetate, strain SF-R_M differed from the wild-type strain in morphological characteristics and sporulation (Fig. 1). These morphological differences were characterized by the formation of branched hyphae and chlamydospore-like structures (Fig. 1B). After 10 transfers into fungicide-free YMPD broth, SF-R_M exhibited the same level of resistance to dodemorph-acetate and the same growth characteristics.

Toxicity assays. The ED_{50} values for the inhibition of mycelial growth of the wild-type and resistant strains by dodemorph-acetate were 75 and 220 µg/ml, respectively. The comparative effect of dodemorph-acetate on radial growth of wild-type and resistant strains of *S. flocculosa* is presented in Figure 2. When inoculated on a fungicide-free medium, both strains produced subglobose to obovoid, rough-walled secondary conidia and displayed a similar growth rate (Fig. 2). In the presence of dodemorph-acetate at a concentration as low as 50 µg/ml, the wild-type strain (SF-1) was strongly inhibited, and growth was completely stopped at 150 µg/ml (Fig. 2). The resistant strain was not affected in its development on potato-dextrose agar containing 100 µg of dodemorph-acetate per ml. In addition, although the mutant could grow on medium amended with dodemorph-acetate at 300 µg/ml, this concentration was lethal to the parent strain (Fig. 2).

Biological properties. When tested for its ability to colonize powdery mildew, resistant strain SF-R_M displayed the same ability as the wild-type strain (Fig. 3). It induced complete collapse of the conidia, conidiophores, and hyphae of *Sphaerotheca fuliginea* within 48 h of application. In addition, strain SF-R_M was just as effective at parasitizing *Sphaerotheca fuliginea* when applied in a mixture with dodemorph-acetate (100 µg/ml). In fact, the colonization rate after treatment with SF-R_M + dodemorph-acetate at 100 µg/ml was not significantly different from that after treatment with SF-1 or SF-R_M alone (Fig. 3).

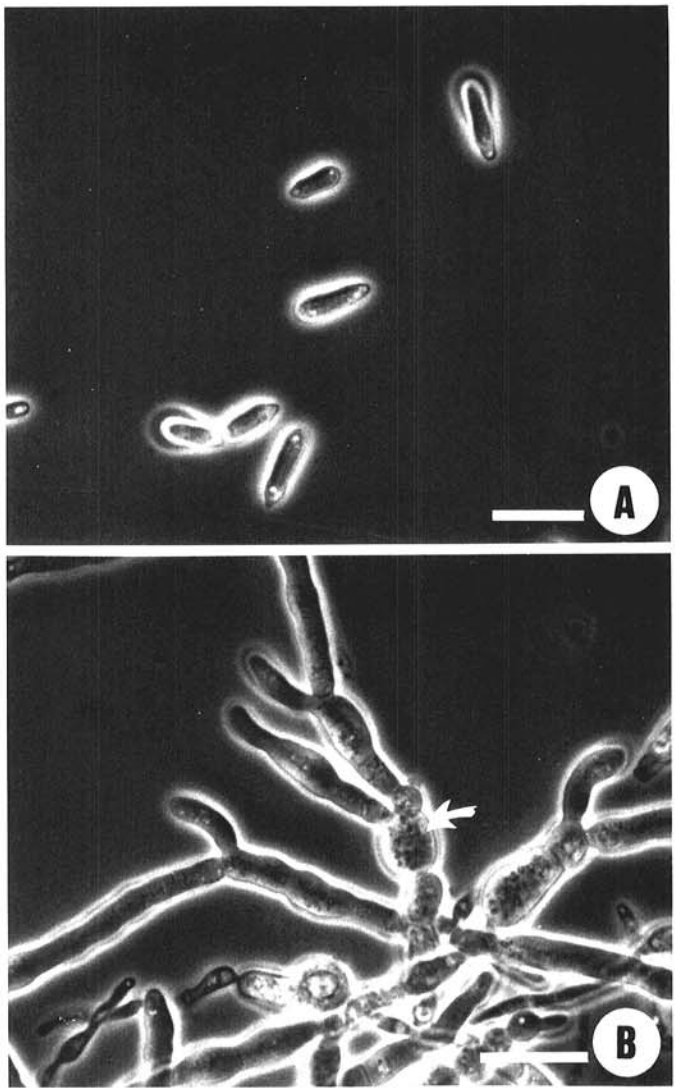

Fig. 1. A, Typical conidia formation of *Sporothrix flocculosa* in liquid culture. **B,** Development of the dodemorph-resistant strain of *S. flocculosa* grown in liquid culture amended with the fungicide dodemorph-acetate (300 µg a.i./ml). A chlamydospore-like structure has formed (arrow). Bars = 10 µm.

In mixture with 300 μg of fungicide per ml, a dosage 50% higher than the recommended dosage for powdery mildew on roses, the resistant strain displayed the same pattern of colonization as the wild-type strain and was only slightly delayed in its efficiency, especially after 48 h (Fig. 3). On the other hand, when the wild-type strain was applied in mixture with dodemorph-acetate, it was unable to maintain its development in the presence of the fungicide, and its colonization of powdery mildew was extremely limited, especially in combination with 300 μg of dodemorph-acetate per ml (Fig. 3).

SEM. SEM observations of powdery mildew colonies treated with water or dodemorph-acetate showed that fungicide treatment affected the hyphal integrity of the fungus, resulting in collapse of conidiophores and conidia (Fig. 4B). The colonies treated with water were turgid, with well-formed reproductive structures (Fig. 4A). When *Sphaerotheca fuliginea* was treated with inoculum of SF-R_M, the hyperparasite developed abundantly over the pathogen whether it was sprayed alone (Fig. 4C) or in mixture with the fungicide at 100 and 300 μg/ml (Fig. 4D and F). In contrast, a mixture of the wild-type strain of *Sphaerotheca flocculosa* with dodemorph-acetate resulted in the death of the hyperparasite (Fig. 4E).

DISCUSSION

With the current scheme of integrated control of plant diseases, the use of natural and chemical fungicides is a very appealing strategy to optimize both disease control and protection of the environment. However, this approach presupposes that the biocontrol agent is resistant to the fungicide used to control the particular disease. In the case of powdery mildew, previous work had been done to suppress *Sphaerotheca fuliginea* with biocontrol agents alternated with SBI fungicides (7,19,20). In this paper, we present the first report of the development of a biocontrol agent, and possibly of a fungus, resistant to the fungicide dodemorph-acetate registered for the control of rose powdery mildew.

Dodemorph-acetate is a morpholine fungicide that blocks the $\Delta^8 \rightarrow \Delta^7$ isomerase (12). In a previous study, one polyene-resistant mutant was derived by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidisine; this mutant contained some Δ^8-sterols, indicating a block at the $\Delta^8 \rightarrow \Delta^7$ isomerase (18). In our case, the new dodemorph-resistant strain of *S. flocculosa* was obtained through mass selection after repeated exposure to the fungicide. This approach previously yielded SBI-resistant strains of *P. italicum* and *Monilinia fructicola* (4,13) and *U. maydis* (21). In our case, 53 transfers were necessary to obtain the mutant. It is believed that the new biotype probably was obtained as a result of spontaneous mutation. This suggestion is supported by the fact that no gradient of resistance was observed among the other isolates tested and the fact that dodemorph resistance was maintained after several transfers on a fungicide-free medium.

The new biotype grew at rates similar to those of the wild-type strain when it was grown in fungicide-free solid YMPD. However, the morphology of the SF-R_M strain was somewhat different when grown in the presence of the fungicide. The formation of highly branched hyphae and chlamydospore-like structures have been observed in a polyene-resistant mutant of *U. maydis* (Erg2) (defective in ergosterol biosynthesis) exposed to polyene antibiotics (9). This phenomenon is probably attributable to a stress reaction by the fungus to the presence of the fungicide.

Our results showed that the SF-R_M strain could colonize *Sphaerotheca fuliginea* as efficiently as the wild-type strain. Hyphae and conidia of *Sphaerotheca fuliginea* were collapsed and plasmolyzed after colonization by SF-R_M, in a manner similar to the one reported in previous studies with *S. flocculosa* (6,7,8,10). This result suggests that the new biotype did not lose its characteristics as a biocontrol agent.

Previous studies have shown that dodemorph-acetate and other morpholine fungicides can cause damage to plants. Indeed, dodemorph-acetate induced a drastic leakage of betacyanin from disks of beet roots and an efflux of electrolytes from disks of bean leaves and sections of barley leaves (3). Phytotoxity also was reported on rose plants treated with dodemorph-acetate (2). Thus, obtaining a dodemorph-resistant strain that possesses a strong biocontrol ability may be useful in integrated pest management programs to maximize the effects of dodemorph-acetate on rose powdery mildew in greenhouses and to decrease the phytotoxity effects. In fact, with the combination of resistant strain and fungicide, less chemical and fewer sprays may be required for adequate disease control in commercial greenhouses, and better disease control may be obtained than when either product is used alone. In addition, the development of a do-

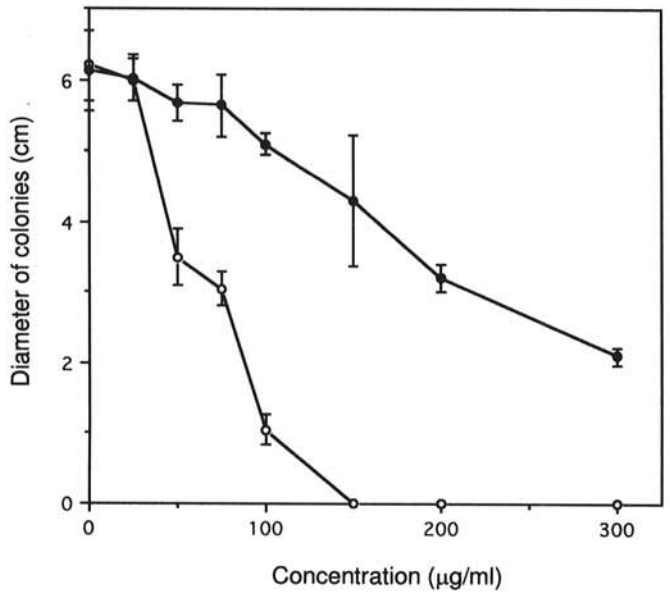

Fig. 2. Growth of *Sporothrix flocculosa* (SF-1) and dodemorph-resistant mutant (SF-R_M) after incubation on yeast-malt-peptone-dextrose agar amended with various concentrations of the fungicide dodemorph-acetate. O indicates wild-type strain; ● indicates resistant strain.

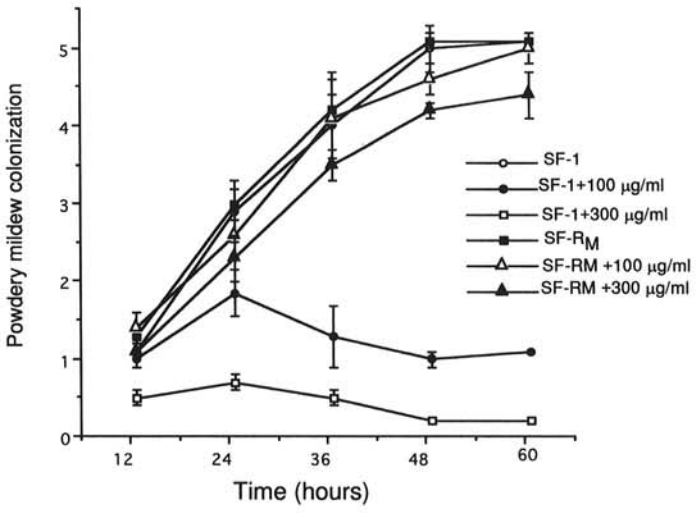

Fig. 3. Colonization of *Sphaerotheca fuliginea* by *Sporothrix flocculosa* wild-type strain SF-1 and dodemorph-resistant strain SF-R_M applied alone or in combination with two dodemorph-acetate fungicide concentrations. Points represent the mean colonization rate with standard deviation bars. Colonization was scored as an arbitrary scale, in which: 0 = no colonization of powdery mildew; and 1 = 1–20%; 2 = 11–40%; 3 = 31–60%; 4 = 51–70%; and 5 = 81–100% dead powdery mildew colonies.

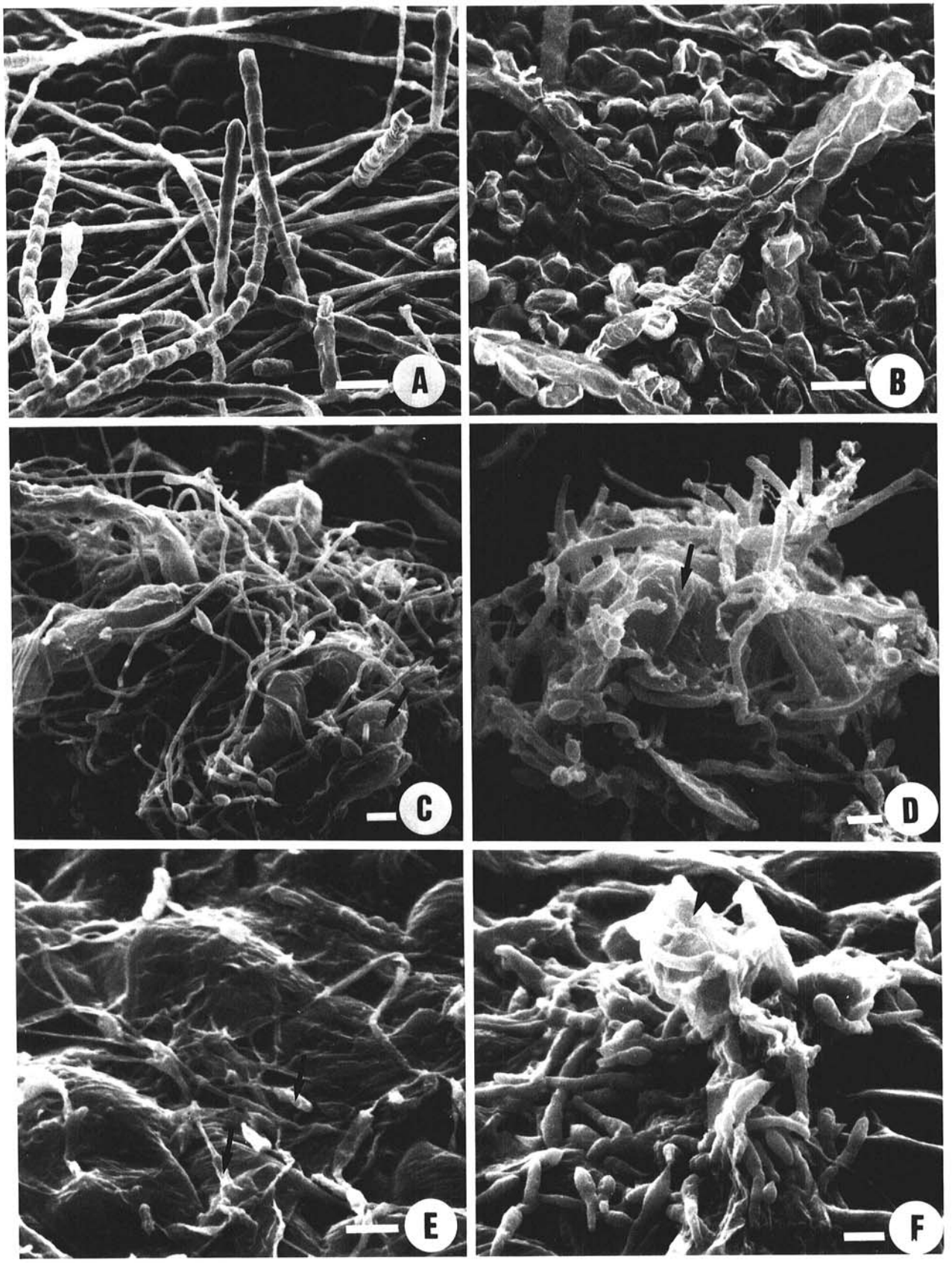

Fig. 4. Scanning electron micrographs of powdery mildew hyphae and conidia 48 h after treatment with **A,** water (control). Mildew hyphae are turgid with well-developed conidiophores; **B,** dodemorph-acetate fungicide (300 µg/ml); **C,** dodemorph-resistant strain of *Sporothrix flocculosa* (SF-R_M) alone. Conidia of powdery mildew are collapsed (arrow). **D,** SF-R_M in mixture with fungicide (100 µg/ml). *Sphaerotheca fuliginea* is abundantly colonized (arrow) by SF-R_M; **E,** wild-type strain in mixture with dodemorph-acetate (100 µg/ml). Mycelia and conidia of the antagonist are collapsed (arrows); and **F,** SF-R_M in mixture with dodemorph-acetate (300 µg/ml). Structural integrity of SF-R_M is intact and SF-R_M has colonized collapsed conidia of the pathogen (arrow). Bars = 10 µm.

demorph-resistant strain of *S. flocculosa* with improved biocontrol characteristics may be a key to the practical use of this biocontrol agent in disease management.

This study presents the first report of resistance against the fungicide dodemorph-acetate by a fungus, in this case the powdery mildew antagonist *S. flocculosa*. This new strain of *S. flocculosa* (SF-R_M) maintained its biocontrol properties and was as effective at colonizing powdery mildew when used alone or in combination with dodemorph-acetate. These results could lead to practical integrated control of powdery mildew diseases, especially on roses.

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