Effects of *Penicillium frequentans* and Its Antibiotics on Unmelanized Hyphae of *Monilinia laxa*

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ABSTRACT

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The effect of *Penicillium frequentans* and its antibiotics on the unmelanized mycelium of *Monilinia laxa* in culture has been investigated. An albino mutant of *M. laxa* obtained by ultraviolet radiation was utilized. Nonfungitoxic concentrations (10 ppm) of pyroquilon (1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one) were also used to inhibit melanin biosynthesis in the pathogen. *P. frequentans* and its antibiotics induced a progressive lysis on unmelanized hyphae of *M. laxa*. After 14 days of incubation, more than 60% of these hyphae contained empty cells. At day 35, many hyphae were partially lysed. When 10 ppm of pyroquilon was added to the media, crude antibiotics also induced lysis on 24-h-old germ tubes. Unmelanized colonies showed poor production of macroconidia and microconidia. The significance of these findings is discussed.

Additional keywords: brown rot, integrated control, melanin inhibitors, stroma.

Monilinia laxa (Aderhold & Ruhland) Honey causes twig blight and brown rot of stone fruits resulting in economically important losses. Cultural control of these diseases is not satisfactory, and chemical treatments present problems of resistance (30). Biological control of the fungus can be an alternative to fungicides. *Penicillium frequentans* Westling is a component of the resident mycoflora of peach twigs and flowers in Spain that controls peach twig blight (10). Antibiotics produced by *P. frequentans* are active against *M. laxa* spore germination and germ tube growth and may be related to the control of the pathogen (9). The antagonist and its antifungal substances induce the development of a stroma in *M. laxa* cultures (23). Pycnidiumlike structures and high production of melaninlike pigments are commonly observed in this stroma. Melaninlike pigments in walls of sclerotia, hyphae, or spores of several fungi have been related to increasing resistance to chemical and biological degradation and to protection against ultraviolet radiation (1,2,6,20,25,28). Hyaline spores or hyphae in soils are quickly killed and lysed, whereas melanized cells can survive for several years (1).

The objective of this study was to determine the contribution of melanins to the resistance of *M. laxa* against the antagonist *P. frequentans* by exposing mycelium of the pathogen with and without melanin deposits to the antagonist or its antifungal substances.

MATERIALS AND METHODS

Fungal cultures. Isolate 909 of *P. frequentans* (ATCC 908-81) was originally obtained from peach twigs in an experimental orchard in Madrid, Spain (21). The single-spore isolate of *M. laxa* (ATCC ZA-1) was collected from a commercial apricot orchard in Almonacid de la Sierra, Zaragoza, Spain. This isolate was used as the wild-type strain (WT). The albino mutant (TM81) was isolated from WT after mutagenic treatment with ultraviolet radiation. A spore suspension of WT (1×10^6 spores per milliliter) was prepared in Czapek broth (9). An aliquot (0.1 ml) of spore suspension was placed in each of 10 glass petri dishes (30 mm in diameter). Plates were subsequently exposed for 2.5 min to ultraviolet radiation from a germicidal lamp with a wavelength of 254 nm. After incubation for 7 days at 20–25 C on potato-dextrose agar (PDA) in petri dishes, mutants were visually screened by their colorless phenotypes. All fungi were maintained on PDA slants at 5 C. For conidial and mycelial production, the fungi were grown on PDA at 20–25 C in the dark.

Screening of chemicals for melanin inhibition. The effect of chemicals on melanization of *M. laxa* (WT) and the mycelial growth of both *M. laxa* and *P. frequentans* were tested in vitro (13). Technical grade samples of tricyclazole (5-methyl-1,2,4,-triazolo[3,4-*b*]benzothiazole) and pyroquilon (1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one) were supplied by Elanco, Indianapolis, IN, and Ciba-Geigy, Greensboro, NC, respectively. The natural plant product coumarin (1,2-benzopyrone) was purchased from Sigma Chemical Co., St. Louis, MO. Chemicals dissolved in methanol containing 1% Tween 80 were added to PDA after the medium was autoclaved. Doses tested were 0, 0.1, 0.5, 1, 10, 25, 50, 75, and 100 μg/ml. Four replicates were used for each concentration of chemical. Mycelial plugs of each fungus were cut from the margins of 7-day-old colonies actively growing on PDA and transferred either to PDA or to PDA amended with a chemical. Colony diameters of both fungi and the melanization of *M. laxa* (WT) were recorded every 7 days for 30 days. Inhibition of melanization was visually assessed by the absence of brown pigments in the reverse side of the colony. The complete experiment was repeated twice.

Determination of doses of pyroquilon. The effects of different doses of pyroquilon on melanization of *M. laxa* (WT), antibiotic production by *P. frequentans,* and diametral growth of both fungi were assessed. Pyroquilon was tested at concentrations of 5–40 μg/ml in 0.5-μg increments. Control plates contained 1% methanol. Four replicates were used for each concentration of pyroquilon. Growth was measured as described above. The complete experiment was repeated three times. After incubation for 40 days, mycelium of *M. laxa* (WT) was recovered from each plate, lyophilized, and weighed. Melanin or melaninlike material was then extracted as described by Melgarejo et al (23).

A bioassay was used to assess the effect of pyroquilon on the production of antifungal compounds by *P. frequentans*. The fungus was grown in potato-dextrose broth (PDB) with or without pyroquilon. Mycelium disks, 8 mm in diameter, were taken from the periphery of 7-day-old cultures of *P. frequentans* grown on PDA and transferred to conical flasks (250 ml) containing 150 ml of medium. Flasks were incubated in the dark at 20–25 C for 20 days. Crude antibiotics were extracted from filtrates prepared as described by De Cal et al (9). The effect of antifungal compounds on germination and germ tube growth of *M. laxa* (WT) was bioassayed as described by De Cal et al (9). The same bioassay was used to test the toxicity of pyroquilon to the pathogen.

Effects induced by *P. frequentans* on mycelial growth of *M. laxa*. The effects of *P. frequentans* on the mycelium of *M. laxa* (WT) and the albino mutant (TM81) were studied in cocultures. Mycelial plugs taken from the margins of 7-day-old colonies on PDA were placed on PDA in 9-cm petri dishes approximately 5 cm apart as described by Melgarejo et al (21). PDA amended with 10 ppm of pyroquilon (PDA10P) was used for WT. There were four replicates of each combination. The plates were incubated for 50 days at 20–25 C in the dark. The inhibition of WT and TM81 by *P. frequentans* was evaluated by calculating the percentage of inhibition of radial growth every 7 days (23). The complete experiment was repeated three times. Samples of *M. laxa* mycelium adjacent to *P. frequentans* colonies were taken every 7 days and examined with a light microscope (400×).

Effects induced by crude antibiotics. The effects induced by crude antibiotics obtained from liquid cultures of *P. frequentans* on mycelia of both *M. laxa* (WT) and TM81 were studied on petri dishes as described by Melgarejo et al (23). *M. laxa* (WT) was grown on PDA and PDA10P, while only PDA was used for the mutant (TM81). Four plates were prepared of each combination. The complete experiment was repeated twice. Crude antibiotic solutions were heated to 100 C for 30 min before the assay was performed to eliminate possible side effects caused by enzyme action.

Effects of crude antibiotics were also assessed by using germ tubes of *M. laxa* (WT). Czapek's broth and Czapek's broth amended with 10 ppm of pyroquilon were used. Conical flasks (250 ml) containing 2 ml of medium were inoculated with 150 µl of a conidial suspension (2×10^6 spores per milliliter) of *M. laxa* (WT). Flasks were incubated at 20–25 C on a rotary shaker at 150 rpm for 24 h. After incubation, 2.1 mg of crude antibiotics per milliliter was added to half of the flasks for each of the two media. The flasks were then incubated in the dark at 20–25 C for 3 days. Four 15-µl aliquots were taken from each flask and examined with a light microscope (100 and 400×).

Production of lytic enzyme activities by *P. frequentans*. *P. frequentans* was grown in 250-ml flasks containing 150 ml of PDB or PDB amended with pyroquilon at 10 ppm. Cultures were incubated in the dark at 20–25 C for 20 days. Culture exudates were obtained as described by De Cal et al (9). Three replicates were used. The lytic enzymes (1→3)-β-glucanase, (1→3),(1→4)-β-glucanase, (1→6)-β-glucanase, and polymethylgalacturonase were assayed by the hydrolysis of laminarin, lichenan, (1→6)-β-glucan (pustulan, obtained by the method of Lindberg and McPherson [17]), and pectin from apples. The reducing sugars were measured by the Somogyi (27) and Nelson (24) methods. One enzyme unit was defined as the amount that released 1.0 µmol of reducing substances as glucose equivalent per minute at 37 C. To determine chitinase activity, the colorimetric assay of Boller and Mauch (3) was followed with colloidal chitin as a substrate. The chitin was synthesized by acetylation of chitosan (Sigma) with acetic anhydride. The regenerated chitin was dissolved in cold hydrochloric acid and made colloidal by precipitation in deionized water. Product formation was not a linear function of enzyme concentration; therefore, activities were calculated for an enzyme concentration approaching zero. The amount of enzyme producing 1 µmol of *N*-acetyl-D-glucosamine equivalents per minute at infinite dilution was defined as 1 unit. Each value was the mean of at least two replicate assays performed at different culture-exudate dilutions. The complete experiment was repeated twice.

The contact zones between colonies of 60-day-old dual cultures (*M. laxa-M. laxa, M. laxa-P. frequentans,* and *P. frequentans-P. frequentans*) on PDA and PDA10P were assessed for (1→3)-β-glucanase and chitinase activity. Mycelial plugs from these zones were placed in the middle of petri dishes containing mineral medium (6 g of Na_2HPO_4, 4 g of KH_2PO_4, 2 g of $(NH_4)SO_4$, 0.2 g of $FeSO_4 \cdot 7H_2O$, 0.2 g of yeast extract, 0.6 mg of trace element solution, and 15 g of agar per liter) and 0.5 mg of chitin or laminarin per liter. There were five replicates of each combination. After 5 days of incubation at 20–25 C, the plates were examined for halo formation around the colonies, which indicated activity.

Statistical analyses. Data were subjected to the analysis of variance (29). Means were compared by a least significant difference test when treatments were found to be significant.

RESULTS

Tricyclazole and pyroquilon at 1 ppm inhibited pigmentation of *M. laxa* (WT) colonies. The color of *M. laxa* (WT) hyphae varied from light brown to orange, depending on chemical concentration. Coumarin did not produce any change in colony color at any concentration. Tricyclazole and pyroquilon at concentrations of 10 ppm and 35–50 ppm, respectively, significantly reduced the growth of *M. laxa* (WT) and *P. frequentans* ($P = 0.05$) after 7 days (Table 1). Measurements made up to 30 days after plating showed a very similar pattern. The *M. laxa* (WT) growth rate was higher on PDA amended with 1- to 100-ppm doses of coumarin than it was on PDA alone.

TABLE 1. Diameter (cm) of *Monilinia laxa* and *Penicillium frequentans* mycelia as affected by coumarin, tricyclazole, and pyroquilon on potato-dextrose agar after 7 days of incubation at 20–25 C

Chemical concentration (ppm)	*M. laxa*[a]			*P. frequentans*[a]		
	Coumarin	Tricyclazole	Pyroquilon	Coumarin	Tricyclazole	Pyroquilon
0	3.29	3.29	3.29	4.14	4.14	4.14
1	3.77	3.42	2.84	4.17	4.02	4.14
10	3.81	1.91	3.44	4.11	3.96	4.05
20	...[b]	...	3.46	...	...	...
25	4.20	...	3.16	4.17	...	...
30	...	...	3.44	...	...	...
35	...	...	2.04	...	...	...
50	3.50	1.14	2.02	4.01	3.45	3.84
75	3.76	...	...	4.09	...	...
100	3.67	1.35	1.96	3.99	3.46	3.94
LSD ($P = 0.05$)	0.35	0.50	0.81	NS[c]	0.12	0.15

[a]Values are the means of eight replicates.
[b]Not done.
[c]Not significant.

Spore germination of *M. laxa* (WT) was not inhibited in the presence of 5–35 ppm of pyroquilon ($P = 0.025$). However, 30 ppm of pyroquilon reduced the germ tube growth of the pathogen (Table 2).

When *M. laxa* (WT) was grown on PDA amended with 10 ppm of pyroquilon, hyphal wall deposits did not show any of the properties usually used to characterize melanins (1,18,25) or those specifically described for *M. laxa* by Melgarejo et al (23). The material recovered was insoluble in hot 0.5 M NaOH and cold 1 M Na_2CO_3, and the slopes of linear curves obtained by plotting the logarithm of absorbancy versus wavelength were very different from those of melanins (results not shown).

The production of inhibitory compounds by *P. frequentans* on PDB amended with 5–40 ppm of pyroquilon was not reduced. These compounds inhibited both germination and germ tube growth of *M. laxa* (WT) (Table 3).

P. frequentans and its antibiotics reduced significantly the growth of *M. laxa* (WT) on PDA plates (Table 4). Growth of either *M. laxa* (WT) on PDA10P or albino mutant (TM81) on PDA was not inhibited (Tables 4 and 5).

Effects induced by *P. frequentans* and its antibiotics on the hyphae of *M. laxa* (WT) on PDA10P or of TM81 on PDA differed from those observed for *M. laxa* (WT) on PDA (Fig. 1). After 50 days of incubation with *P. frequentans* or its antibiotics, *M. laxa* (WT) on PDA10P or TM81 on PDA did not develop a stroma or any type of pigmentation. After 7 days of incubation, some of the hyphae were lightly stained, and the protoplasm was frequently coagulated. After 14 days of incubation, more than 60% of these hyphae contained empty cells. At 35 days, many hyphae were partially lysed. These features were not apparent in control plates, where hyphae of *M. laxa* (WT) on PDA10P and of TM81 on PDA were hyaline and thin, as were those of *M. laxa* (WT) on PDA.

TABLE 2. Toxicity of pyroquilon to conidia of *Monilinia laxa*[a]

Pyroquilon concentration (ppm)	Germination (%)	Germ tube growth (μm)
0	90	52
5	91	53
10	88	47
15	80	43
20	82	45
25	79	41
30	76	32
35	87	29
40	56	29
LSD ($P = 0.025$)	17	17

[a]Data are the means of four replicates; 50 conidia or 25 germ tubes were considered in each replicate.

TABLE 3. Effect on germination and germ tube development of *Monilinia laxa* of crude antibiotics produced by *Penicillium frequentans* grown on potato-dextrose broth with increasing concentrations of pyroquilon[a]

Antibiotics + doses of pyroquilon (ppm)	Germination (%)	Germ tube growth (μm)
Control	90.0	52.2
Antibiotics (a)	3.5	23.4
(a) + 5	23.0	19.2
(a) + 10	19.0	11.7
(a) + 15	7.5	17.6
(a) + 20	4.0	23.9
(a) + 25	9.5	12.7
(a) + 30	4.0	18.7
(a) + 35	7.0	14.9
(a) + 40	3.5	14.6
LSD ($P = 0.025$)	8.5	10.2

[a]Data are the means of four replicates; 50 conidia or 25 germ tubes were considered in each replicate.

Colonies of *M. laxa* (WT) on PDA10P and of TM81 on PDA showed poor production of macroconidia. Microconidia were produced along the edges of the petri dishes but not in the contact zone of dual cultures with *P. frequentans* or its antibiotics. No pycnidiumlike structures were formed by WT or TM81 in these plates.

Crude antibiotics induced morphological changes in 24-h-old germ tubes of *M. laxa* (WT). Germ tubes were shorter, had more septa, and were more branched than those grown in Czapek broth without antibiotics. Similar effects were observed on germ tubes of *M. laxa* (WT) grown in Czapek broth amended with pyroquilon and crude antibiotics, but in this case formation of swelling, deformation, and aggregates of hyphae were also observed (Fig. 2). In other cases, cytoplasm escaped from hyphal cells, resulting in hyphae with areas of empty cells. Most of the hyphae were lysed. In contrast, in the control treatments, hyphae were hyaline, thin, and smooth (Fig. 2). Crude antibiotics exposed to high temperature had the same activity as unheated antibiotics.

The activity of lytic enzymes in liquid cultures of *P. frequentans* was similar when the antagonist was grown in each medium. (1→3)-β-Glucanase activity at 20 days was 380–510 mU in PDB or PDB10P. Total chitinase activity was very low, and it was maximal in PDB (73 mU). The average standard deviation was less than 10% of the mean in both assays. No activity of (1→3),(1→4)-β-glucanase, (1→6)-β-glucanase, or polymethylgalacturonase was detected.

Mycelial plugs from the contact zones between colonies from the dual cultures *M. laxa-P. frequentans* and *P. frequentans-P. frequentans* on PDA and PDA10P were able to grow on solid medium containing laminarin or chitin as the sole carbon source. All of these disks presented some level of (1→3)-β-glucanase and chitinase activity. In contrast, no lytic enzymes were found from mycelial disks of the dual culture *M. laxa-M. laxa* on either medium.

DISCUSSION

Melaninlike pigments on hyphal walls of *M. laxa* (WT) in dual cultures with *P. frequentans* or its antibiotics increase the resistance of the pathogen to autolysis induced by exposure to anti-

TABLE 4. Percent inhibition of radial growth of wild type of *Monilinia laxa* (WT) by *Penicillium frequentans* (PF) and its crude antibiotics (a) at 20 days in dual culture on potato-dextrose agar (PDA) and PDA + 10 ppm of pyroquilon (PDA10P)

Dual culture	Inhibition[a] (%)
WT-WT/PDA	0.0
WT-PF/PDA	19.0
WT-WT/PDA10P	0.0
WT-PF/PDA10P	7.7
(a)-WT/PDA	18.2
(a)-WT/PDA10P	4.7
(a)-WT/PDA10P[b]	0.0
LSD ($P = 0.005$)	7.8

[a]Data are the means of four replicates.
[b]Crude antibiotic solutions were exposed to 100 C for 30 min before the dual culture was performed.

TABLE 5. Percent inhibition of radial growth of albino mutant (TM81) by *Penicillium frequentans* (PF) and its crude antibiotics (a) at 20 days in dual culture on potato-dextrose agar

Dual culture	Inhibition[a] (%)
TM81-TM81	5.7
TM81-PF	0.0
TM81-(a)	0.0
LSD ($P = 0.05$)	6.63

[a]Data are the means of four replicates.

biotics. Brammall (4) suggested that *Trichoderma* spp. induced hyphal melanization in *Rhizoctonia solani.* Melgarejo et al (21,23) described the presence of an induced stroma in the contact zone of *M. laxa-P. frequentans* dual cultures. The black or dark brown color of this stroma was caused by the presence of melaninlike pigments in the walls of outer hyphae. The inhibition of *M. laxa* (WT) mycelium growth by *P. frequentans* and its antibiotics was also observed. However, in dual cultures with *P. frequentans* or its antibiotics, *M. laxa* (WT) on PDA10P or TM81 on PDA did not develop any dark stromata, and their growth was not inhibited. Thus, the melanization was responsible for growth inhibition of the pathogen but prevented the mycelial lysis of *M. laxa* (WT). Melanized hyphae of *Colletotrichum lagenarium* are resistant to enzymes involved in cell wall lysis (14,15). Huang and Kokko (12) found that the degradation of the melanized rind cells of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* was slow compared with that of the nonmelanized cortical and medullary cells.

Although *P. frequentans* produces the lytic enzyme (1→3)-β-glucanase, the lytic effects observed in *M. laxa* cannot be caused by this or other enzymes, because enzymatic activity of the crude antibiotic solution was inactivated by heat. Therefore, lysis of mycelia of *M. laxa* seems to be an autolytic phenomenon. Reyes and Lahoz (26) described a balance between synthesis and lysis of the wall polymers of *Sclerotinia fructigena* during growth, the equilibrium being displaced toward wall degradation (beginning of autolysis) when synthesis stops for some reason. Antibiotic-induced autolysis has been reported for the pathogens *Glomerella cingulata, Helminthosporium victoriae* (complete autolysis), and *Fusarium solani* f. sp. *phaseoli* (incomplete autolysis) (19).

Another feature that distinguished between the effect of *P. frequentans* and its antibiotics on melanized and unmelanized mycelium of *M. laxa* was that sporogenesis and formation of pycnidiumlike masses of microconidia in culture was greater on melanized mycelium. Melanin synthesis is often closely associated with cellular differentiation in certain fungi (1,7). Furthermore, Hegnauer et al (11) suggested that melanogenesis in fungi is restricted to certain developmental stages, such as sporogenesis, aging of hyphae, and formation of sclerotia. Microconidia in the brown rot fungi may have a spermatial function, while macroconidia are the main propagules of the fungus (5). Hence, melanins play an important role in disease development.

The potential for biocontrol of *M. laxa* with *P. frequentans* has been shown in the laboratory (21), in experimental peach plots (22), and in commercial orchards (10). De Cal and Melgarejo (8) demonstrated that the albino mutant (TM81) was unable to infect peach twigs. Also, pyroquilon interfered with colonization of host cells by *M. laxa.* The results of this work suggest a new approach for integrated control of *M. laxa:* the possibility of exploiting the melanin-inhibitory activity of pyroquilon (interfering with colonization and sporogenesis) combined with the biological control of *P. frequentans* (producing autolysis of unmelanized mycelium). Further breakdown of the remaining unmelanized hyphae of *M. laxa* could be obtained by extracellular

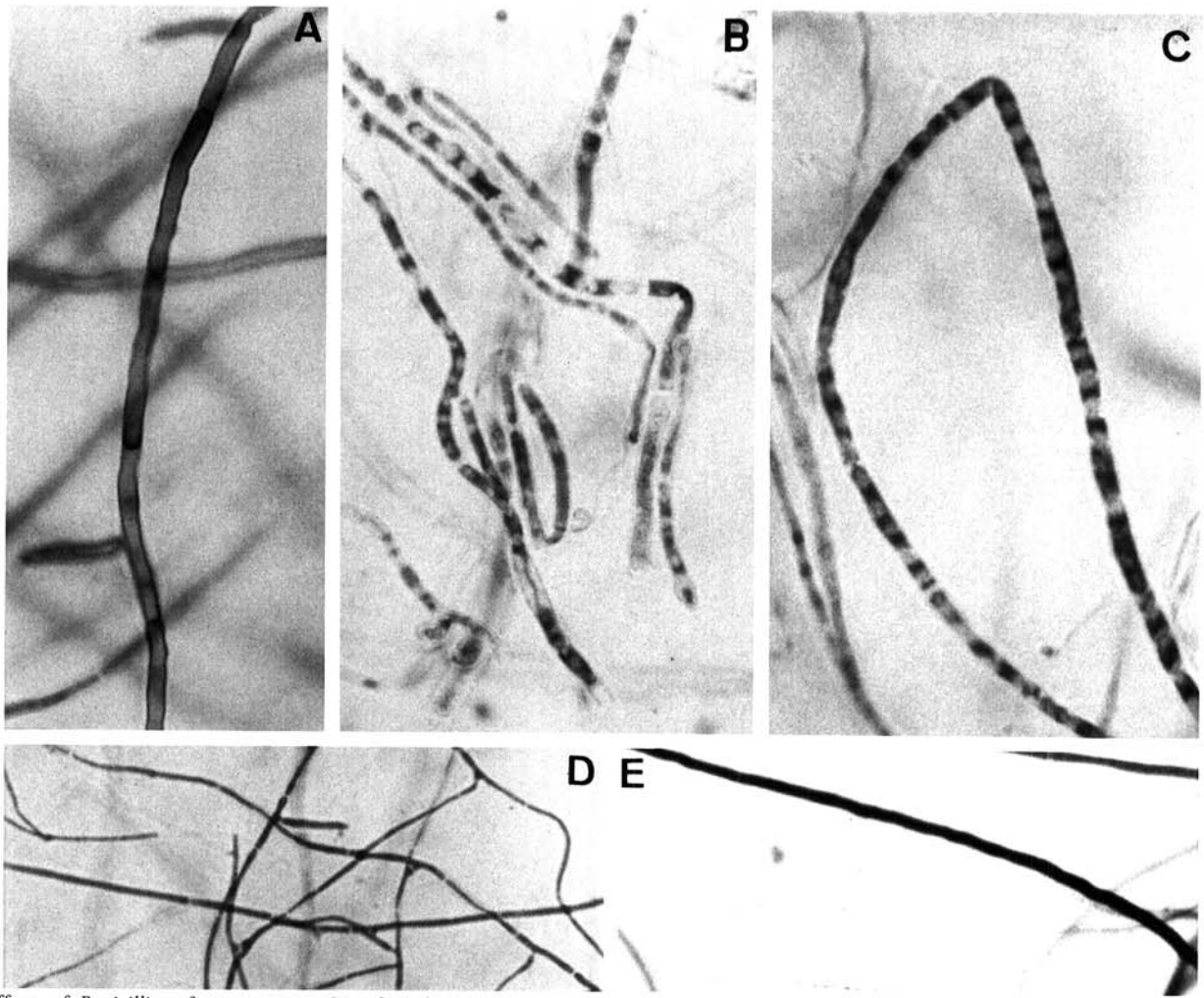

Fig. 1. Effects of *Penicillium frequentans* on *Monilinia laxa* wild type (WT) and albino mutant (TM81) mycelium observed under a light microscope. **A,** WT mycelium from a 20-day-old dual culture with *P. frequentans* on potato-dextrose agar (PDA) (400×). **B,** WT mycelium from a 20-day-old dual culture with *P. frequentans* on PDA amended with 10 ppm of pyroquilon. Empty cells appear along the hyphae (400×). **C,** TM81 mycelium from a 14-day-old dual culture with *P. frequentans* on PDA. Protoplasm is separated into segments (400×). **D,** WT mycelium from a 20-day-old culture on PDA (100×). **E,** TM81 mycelium from a 14-day-old culture on PDA. Hyaline hyphae after staining (400×).

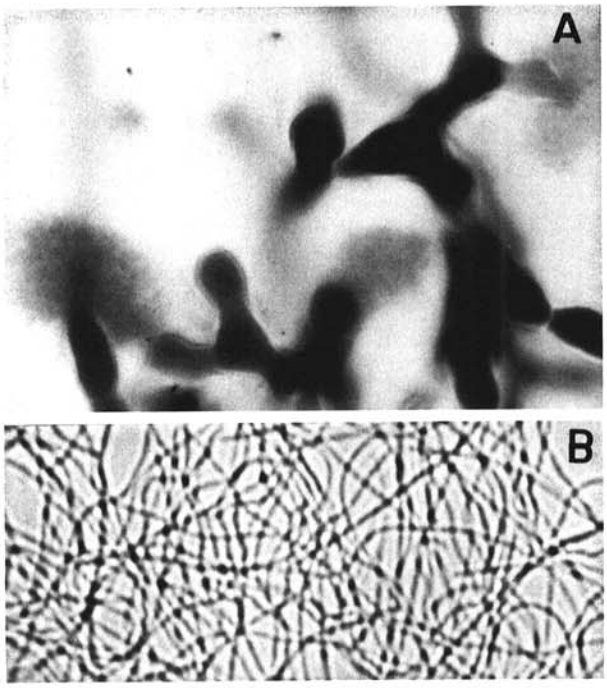

Fig. 2. Optical microscopic observations of hyphae of *Monilinia laxa* wild type grown on Czapek broth amended with 10 ppm of pyroquilon. **A,** Three days after treatment with 2.1 mg of crude antibiotics of *Penicillium frequentans* per milliliter (600×). **B,** Untreated (100×). Treatment was applied to 24-h-old germ tubes.

lytic enzymes from other combined microorganisms such as *P. purpurogenum,* which produces high (1→3)-β-glucanase activity (16).

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