Antifungal Substances Produced by *Penicillium frequentans* and Their Relationship to the Biocontrol of *Monilinia laxa*

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ABSTRACT

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Antibiotic substances produced by an isolate of *Penicillium frequentans*, a component of the resident mycoflora of peach twigs previously found to antagonize *Monilinia laxa*, were produced in potato-dextrose broth and isolated and partially purified from the cell-free medium by liquid:liquid partitions. Two active substances were partially characterized by thin-layer chromatography, infrared absorbance, and qualitative analysis and were named antibiotic A and antibiotic B. Both antibiotics were active against a wide range of plant pathogenic fungi including *M. laxa* and several components of the mycoflora of peach twigs. The ED_{50} values for the germination of spores and the germ tube growth of *M. laxa* were 0.31 mg/ml and 0.02 mg/ml, respectively, for antibiotic A and 4.84 mg/ml and 0.13 mg/ml, respectively, for antibiotic B. Both antibiotics were temperature-stable, while the activity of antibiotic A was lowered at basic pH. Antibiotic B was also more stable over time at room temperature than antibiotic A, conserving 30% of its activity against the germ tubes of *M. laxa* after 70 days. Both antibiotics showed significant inhibition ($p \leqslant 0.05$) of *M. laxa* on peach twigs.

Additional key words: antibiosis, brown rot disease, competition, phyllosphere.

Monilinia laxa (Aderh et Ruhl) Honey causes brown rot disease of stone fruit crops with economically important losses. The control of the disease is not satisfactory, and its treatment presents significant problems (16). Biological control of the fungus may be an alternative to chemicals. *Penicillium frequentans* Westling is a component of the resident mycoflora of peach twigs and flowers in central Spain. The potential for biocontrol of *M. laxa* with *P. frequentans* has recently been shown both in the laboratory (9) and in peach tree orchards (10). Based on preliminary data, an antibiosis mechanism was postulated for the antagonism between *P. frequentans* and *M. laxa* (11).

The purpose of the present study was to investigate the production of antifungal compounds by the isolate of *P. frequentans* isolated by us from peach twigs (9) and its involvement in the antagonistic action of *P. frequentans* against *M. laxa*.

MATERIALS AND METHODS

Fungal cultures. The 909 isolate of *P. frequentans* was originally taken from the phyllosphere of peach twigs (9) at an experimental orchard in Madrid, Spain. The monosporic isolate of *M. laxa* was collected from a commercial apricot orchard in Almonacid de la Sierra (Zaragoza, Spain). Both fungi were stored on potato-dextrose agar (PDA) slants at 5 C and were grown on PDA at 20–25 C in the dark for conidial and mycelial production.

Production of antifungal compounds by *P. frequentans*. To test for optimal production of antifungal compounds by the antagonist, conical flasks (250 cm^3) containing 150 ml of potato-dextrose broth (PDB) were each inoculated with three disks of mycelium, 8 mm in diameter, cut from the edges of actively growing colonies of *P. frequentans*. Flasks were incubated in the dark at 25 ± 1 C either in a stationary incubator or in a rotary shaker incubator at 150 rpm for 10, 20, and 30 days. Media were separated from the mycelia and spores by filtration through Whatman No. 1 filter paper in a Büchner funnel and by centrifugation at 15,000 *g* for 15 min. The culture filtrate was then sterilized by filtration through 0.22-μm Millipore membranes. The toxicity of these crude filtrates toward the germination of spores and the germ tube growth of *M. laxa* was bioassayed.

Isolation and partial purification of antibiotics. Crude preparations of antibiotics were obtained from crude filtrates of 20-day-old stationary cultures of *P. frequentans* by liquid:liquid partitions as described by Birkinshaw (1) with modifications (Fig. 1). At each purification step, relative antibiotic activity was bioassayed on the germination of spores and the germ tube growth of *M. laxa* and *Cladosporium cucumerinum* Ellis & Arth. *C. cucumerinum* was bioassayed for antibiotic activity as it allows the localization of active bands in chromatograms, since *M. laxa* cannot grow over the silica gel plates.

Partial characterization of antibiotics. The active substances were partially characterized by thin-layer chromatography (TLC), infrared absorbance (IR), and qualitative analysis. Silica gel plates were developed in equilibration tanks containing acetone:water (9:1, v/v) and examined under UV light. The plates were then sprayed with $SbCl_3$ saturated in chloroform and heated to 100 C for 10 min (6,12). Duplicate plates were bioassayed with *C. cucumerinum*. The IR spectra were obtained in chloroform with a Hilger D 209 (Chemical Faculty, Universidad Complutense, Madrid, Spain) spectrometer.

Qualitative analyses were carried out as described in Curtis et al (4) and Birkinshaw (1) by assaying several specific chemical reactions: reduction of $AgNO_3$ in NH_3 solution, decoloration of Br_2 in Cl_4C solution, precipitation with $NaHSO_3$, and Brady's test (12).

Bioassays. 1) In vitro: The toxicity of the crude filtrates, the different fractions from the purification procedure (Fig. 1) and of the aqueous solutions of crude antibiotics on the germination of spores, and the growth of germ tubes of *M. laxa* and *C. cucumerinum* was assayed in sterile Czapek broth (2 g of KNO_3, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 1 g of KH_2PO_4, and 30 g of sacarose in 1,000 ml of distilled water). Sterile glass slides were placed on glass

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15-mm-diameter petri dishes lined with moist filter paper. On a slide, a 15-μl droplet of spore suspension on Czapek broth was mixed with a 30-μl droplet of each treatment. Spore concentration and incubation time were 1×10^6 spores per milliliter and 8 hr for *M. laxa* and 1×10^6 spores per milliliter and 24 hr for *C. cucumerinum*. Controls and concentrations of extracted fractions were fixed in each experiment. Results were analyzed by analysis of variance and Duncan's multiple range test. Percentages of inhibition were treated by arc sine transformation to achieve homogenization of variances prior to statistical analysis. The results of the relative toxicity of different concentration levels of crude antibiotics on *M. laxa* were processed by the probit-analysis method (5). The effective doses (DE) were calculated in milligrams per milliliter. A test was done for parallelism according to the relative potency estimation method (5). When potency ratios of two lines were very similar, a studentized range test was performed (15) to confirm whether both lines were different.

The toxicity of compounds was also tested on *C. cucumerinum* to locate active bands in chromatograms. The plates were sprayed with a dense spore suspension of the fungus in Czapek broth and incubated for 48–72 hr at 25 ± 1 C in the dark under high humidity conditions.

2) In vivo: Healthy peach twigs, collected from active trees, surface sterilized as recommended by Sauer and Burroughs (13), were wounded by gently abrading their cuticle. Then, 5-μl-droplets of different treatments (aqueous solutions of crude antibiotic A, 1.48 mg/ml; crude antibiotic B, 6.7 mg/ml; benomyl, 0.25 mg/ml; spores of *P. frequentans*, 1×10^9 spores per milliliter, or distilled water in the controls) were applied to the wounds. Twigs were incubated for 8 hr at 20–25 C under high humidity conditions. Droplets were then collected and mounted with lactophenol blue to determine the toxicity of treatments to spore germination and germ tube growth of *M. laxa*.

All bioassays had four replicates, and independent assays were done at least twice. Fifty conidia or 25 germ tubes were considered in each replicate. A spore was considered germinated when its germ tube was 4 μm.

Effects of time, temperature, and pH on antibiotic activity. To test the relative loss of activity of the crude antibiotics against *M. laxa* with time, aqueous solutions (8.4 mg/ml) were maintained in the laboratory at room temperature for different time periods.

To test the influence of temperature and pH on the activity of the antibiotics, crude antibiotic A (1.5 mg/ml) or crude antibiotic B (6.8 mg/ml) was maintained for 30 min at temperatures ranging from 25 to 120 C, or their pH was adjusted from pH 1 to 10 with 0.1 N HCl or 0.1 N NaOH before performing bioassay against *M. laxa*.

Antifungal spectrum of antibiotics. The crude antibiotics were tested for activity against the fungi listed in Table 1 by the method described in McKeen et al (8) with modifications: 100 μl of 10^5–10^6 spore suspensions, or when necessary, fragmented mycelial suspensions of the fungi were spread on the PDA surface before placing a sterile Whatman No. 1 filter paper disk (15 mm in diameter) imbibed with 30 μl of diethyl ether solutions of each antibiotic (5.47 mg/ml) on the center of the plate. All fungi were tested four times. Control plates contained 30 μl of diethyl ether in the filter paper. Plates were incubated in the dark at 25 ± 1 C. Inhibition zones of the treated plates were measured after clearly visible fungal lawns had covered the control plates.

RESULTS

Production of antifungal compounds by *P. frequentans*. Isolate 909 of *P. frequentans*, incubated in stationary regime in PDB, produced antifungal compounds that inhibited ($p \leqslant 0.05$) the germination of spores and the germ tube growth of *M. laxa* (Table 2). The production of these compounds was detected at 10 days by the inhibition of the germ tube growth of *M. laxa*. Maximal concentration of antifungal compounds in the filtrates was achieved at 20 days of incubation, when crude filtrates strongly inhibited both the germination of spores and the germ tube growth of the fungus. At 30 days, inhibition of *M. laxa* by the crude filtrates was decreased. No antifungal effect was observed in crude filtrates of the fungus when grown in shaking system.

Isolation and partial purification of antibiotics. Bioassays of the

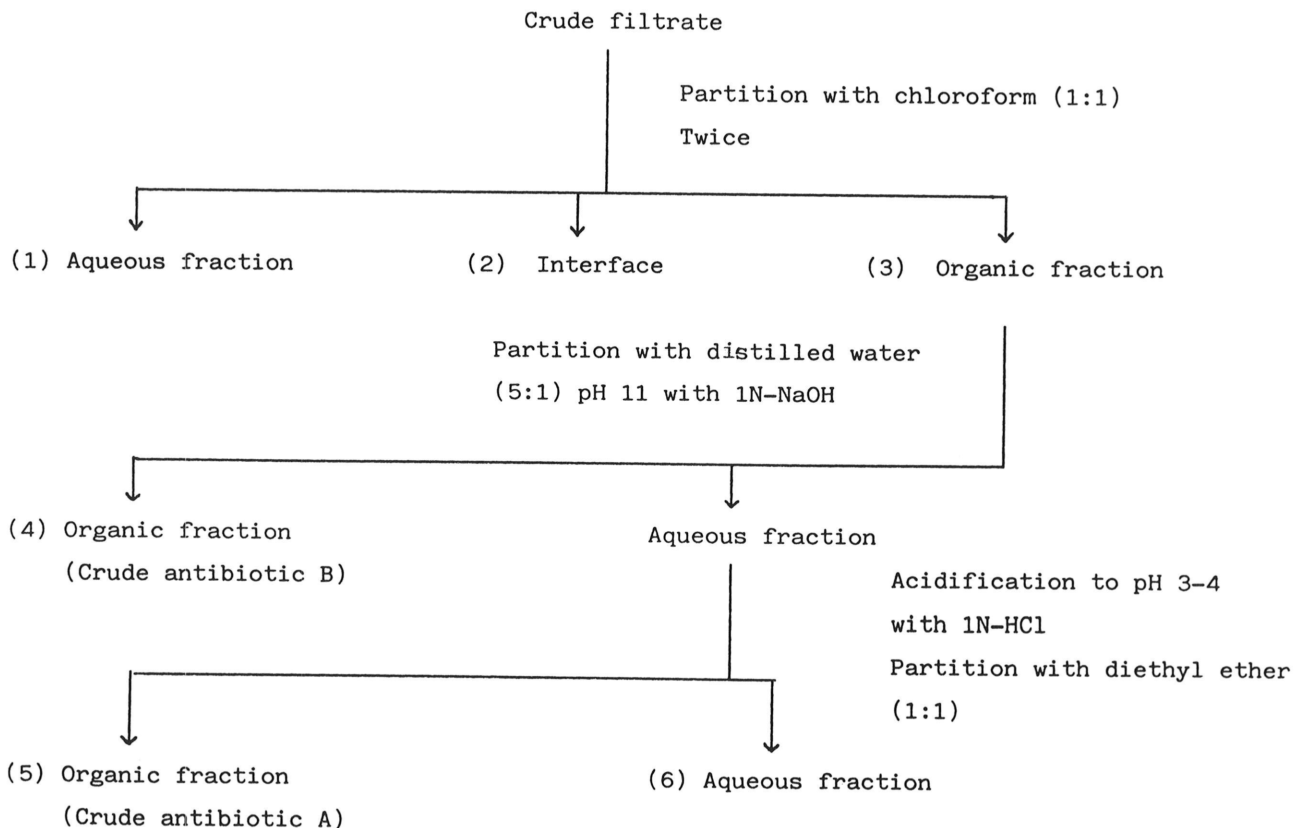

Fig. 1. Procedure of isolation and purification of antibiotics.

relative antibiotic activity of different fractions (Fig. 1) obtained from the extraction of the crude filtrate of 20-day-old stationary cultures of *P. frequentans* showed that fractions 3, 4, and 5 were the most toxic ($p \leq 0.05$) to *M. laxa*, and they were more active than the crude filtrate (Table 3). Only fractions 1 and 2 were inactive (Table 3).

TABLE 1. Antibiotic spectrum of antibiotics[a]

Fungi[b]	Inhibition zone[c]		
	Antibiotic A	Antibiotic B	Control
Pathogens			
Ascochyta pisi Lib.[d]	++	++	−
Botrytis cinerea Pers. ex Fr.[e]	+++	++	−
Cladosporium cucumerinum Ellis & Arth	+	++	−
Fusarium oxysporum Schlecht. emend. Snyd. & Hans[d]	+	+	−
Fusarium solani (Mart.) Appel & Wollenw. emend. Snyd. & Hans[d]	+++	+++	+
Monilinia laxa (Aderh et Ruhl) Honey	++	++	−
Penicillium expansum Link ex F.S. Gray[e]	+++	+++	−
Rhizoctonia solani Kühn[d]	−	−	−
Verticillium dahliae Klebahn[d]	++	++	−
Components of the mycoflora of peach twigs			
Alternaria alternata (Fr.) Keissler	−	−	−
Aspergillus flavus Link ex Fries	+++	+++	−
Cladosporium herbarum (Pers.) Link ex S.F. Gray	+	+	−
Epicoccum nigrum Link	+	++	−
Mucor pusillus Lindt	−	−	−
Nigrospora sphaerica (Sacc.) Mason	−	−	−
Nitchkea sp.	+++	+++	−
Penicillium purpurogenum Stoll	−	−	−
Phoma glomerata (Corda) Wr. & Hochapf	+++	+++	+
Rhizopus stolonifer (Ehrenb. ex Fr.) Lind.	−	−	−
Sordaria sp.	−	−	−
Trichoderma viride Pers.	+++	+++	−
Trichothecium roseum (Pers.) Link	+	++	−

[a] 30 μl of crude antibiotic A and crude antibiotic B dissolved in diethyl ether to give a concentration of 5.47 mg/ml was assayed. The assay medium was potato-dextrose agar. Control treatment was 30 μl of diethyl ether. A 15-mm-diameter Whatman No. 1 filter paper imbibed with test solutions was placed on the center of plates.
[b] To produce a lawn in the assay plate, 100 μl of 10^5–10^6 spore suspensions or fragmented mycelial suspensions per milliliter were used as inoculum.
[c] −: no inhibition, +: little inhibition, ++: medium inhibition, +++: high inhibition.
[d] Culture obtained from J. Tello, Department of Plant Protection, I.N.I.A., Madrid, Spain.
[e] Culture obtained from I. Palazón, Department of Plant Protection, S.I.A., Zaragoza, Spain.

Inhibition of germination of spores and germ tube growth of *C. cucumerinum* followed the same pattern as that of *M. laxa* (data not shown).

Partial characterization of antibiotics. Fractions 3, 4, and 5 (Fig. 1), the most toxic towards *M. laxa* (Table 3), were partially characterized by specific chemical reactions and IR spectra.

Chemical reactions showed the presence of 1–3 dienes in fractions 3, 4, and 5 (decoloration of Br_2 in CCl_4 solution and a continuous purpureal-violet band parallel to the front at R_f values of 0.96–0.98 on TLC silica gel plates treated with $SbCl_3$ saturated in chloroform). Acetone groups were also detected (Brady's test positive and obtaining of a white precipitate with $NaHSO_3$) in these fractions. However, only fractions 3 and 5 reduced $AgNO_3$ in NH_3 solution, a test specific for the aldehyde group. Based on the intensity of these reactions, fraction 3 was judged to contain a mixture of fractions 4 and 5.

Fractions 4 and 5 were further characterized by IR spectroscopy (data not shown). There were strong similarities between the spectra of the two fractions. Bands due to alcoholic hydroxyl groups occurred at 3,400 cm^{-1} in both spectra, being more intense in fraction 4. Also, a band due to C = 0 stretching modes occurred in the 1,600–1,650 region. However, marked differences appeared in the region 1,000–1,200 cm^{-1}. In this region absorptions due to vibrational modes involving deformation of alcoholic OH groups occur, and while three fairly strong bands are present in both spectra (near 1,080 cm^{-1}, near 1,110 cm^{-1}, and near 1,150 cm^{-1}) variations in this frequency range were apparent.

Thus, two different active compounds were mainly present in fractions 4 and 5. We named antibiotic B the one in fraction 4 and antibiotic A the one in fraction 5.

Antifungal properties. The germination of spores and the growth of germ tubes of *M. laxa* were affected by antibiotics A and B. Figure 2A and B shows the probit-log concentration lines and the ED_{50} values for each antibiotic to the germination of spores and the germ tube growth of *M. laxa*. We found quite different values for the slopes of the four probit lines, and it is convenient to test for parallelism to compare the lines. The test for parallelism showed that data could be represented by parallel probit-log concentration regression lines (X^2 values for parallelism not significant with 1 df in all cases). Figure 2C–F shows: antibiotic B is more toxic ($p \leq 0.05$) to the germination of spores of *M. laxa* than antibiotic A, while the germ tube growth of *M. laxa* is more sensitive ($p \leq 0.05$) to antibiotic A than to antibiotic B, and the toxicity of antibiotic A to the germ tube growth of *M. laxa* is significantly greater ($p \leq 0.05$) than to the germination of spores of this fungus, while the toxicity of antibiotic B is similar for spores and germ tube growth.

The antibiotics were also toxic to *M. laxa* in detached peach twigs under laboratory conditions (Table 4). Both antibiotics significantly inhibited ($p \leq 0.05$) the spore germination and the

TABLE 2. Toxicity of culture filtrates of *Penicillium frequentans* on *Monilinia laxa*

Crude filtrates from different age cultures (days)	Incubation system[x]	Germination		Germ tube growth	
		Spores germinated[y] (%)	Inhibition[z] (%)	Germ tube length[y] (μm)	Inhibition[z] (%)
Control (potato-dextrose broth)		85 ± 3.8	···	80 ± 5.8	···
10	Stationary	83 ± 4.6	···	65 ± 6.3	19 a
	Shaking	86 ± 3.9	···	78 ± 1.7	···
20	Stationary	37 ± 1.5	57 a	4 ± 0.0	95 b
	Shaking	80 ± 4.5	···	83 ± 5.8	···
30	Stationary	62 ± 3.8	27 b	31 ± 2.7	62 c
	Shaking	87 ± 2.7	···	76 ± 3.4	···

[x] Incubation systems were in a stationary incubator or in a rotary shaker incubator at 150 rpm.
[y] Mean and standard deviation of four replicates; 50 conidia or 25 germ tubes were considered in each replicate.
[z] All values shown were different from that of control ($p \leq 0.05$). Means followed by the same letters are not significantly different by Duncan's multiple range test ($p \leq 0.05$).

germ tube growth of the pathogen, and their activity was similar to that obtained with the commercial fungicide benomyl. However, a concentrated spore suspension of *P. frequentans* inhibited ($p \leq 0.05$) *M. laxa* but to a lesser degree (Table 4).

The loss of activity of both antibiotics against *M. laxa* began to be significant ($p \leq 0.05$) at 14 days (Fig. 3). At this time, the activity of antibiotic A and antibiotic B on the germination of spores of the fungus decreased by 51 and 44%, respectively, and by 28 and 29%, respectively, on the growth of germ tubes. At 70 days, antibiotic A had lost almost all of its activity (90–95%), while antibiotic B conserved 30% of its activity on the germ tube growth of *M. laxa* (Fig. 3).

Activity of the antibiotics was not affected by changes of temperature in a water bath or after autoclaving (data not shown). Antibiotic B was also not affected by changes of pH, while maximum inhibition (95–100%) of spore germination and germ tube growth of *M. laxa* by antibiotic A was obtained at pH 1–5. Its activity then decreased significantly ($p \leq 0.05$), showing at pH 7, 64 and 66% of inhibition of spore germination and germ tube growth, respectively, and 13 and 26%, respectively, at pH 10.

The spectrum of action of the antibiotics is quite wide, since both inhibited the growth of 14 out of 22 fungi tested (Table 1). The fungi tested included important pathogens and the inhibition of growth obtained with the antibiotics against these fungi was similar to that against *M. laxa* (Table 1). The other fungi tested were components of the mycoflora of peach twigs (9). Some of them were also inhibited by both antibiotics while others were not (Table 1).

DISCUSSION

The production of antifungal compounds against *M. laxa* by *P. frequentans* in PDB in stationary regime begins about 10 days after incubation, at which time the crude filtrate is toxic to the germ tube growth of the pathogen (Table 2). Production continues until approximately 20 days, when inhibition reaches a maximum. Then, production may or may not stop but, in any case, the activity of the crude filtrate decreases. This can be attributed to a diminished rate of synthesis, or to an increased rate of metabolism of the antibiotics by the fungus, or a natural breakdown as in Figure 3. Alternatively, the change in activity of the crude filtrate could also be related to changes in pH, as we have observed that the pH of the filtrate of 20-day-old cultures is approximately 5, and that of 30-day-old cultures (and of shaking cultures) is higher than 7. Also, we have shown that the toxicity of antibiotic A is pH dependent. Oxidative reactions could also explain the lack of activity of filtrates from shaking cultures.

Antibiotics A and B were the only significant antifungal compounds present in the crude filtrates of *P. frequentans*, isolate 909. This was shown by testing the biological activity of the crude filtrates and of the fractions 3, 4, and 5 from the extraction procedure (Fig. 1) against *C. cucumerinum* on TLC silica gel plates. Only active bands parallel to the front at R_f values of 0.96–0.98 were observed. These bands were also the only ones that we observed in duplicate plates when sprayed with $SbCl_3$.

The partial characterization of the antibiotics shows that antibiotics A and B should be two substances closely related in structure with 1–3 dienes, acetone, and alcoholic hydroxyl groups in their molecules, being the presence of an aldehyde group in antibiotic A the main difference between them. An antifungal and weakly antibacterial substance called frequentin was described as a product of an unusual strain of *P. frequentans* found abundantly in acid heath soils (4). Recently, another isolate of *P. frequentans* from olive fruits was shown to produce frequentin and was highly antagonistic to *Geotrichum candidum* (12). In liquid cultures of these *P. frequentans* isolates, another secondary metabolite (2), palitantin, was described, with no appreciable antifungal activity. Birkinshaw (1) obtained crude frequentin and palitantin by a liquid:liquid partition procedure similar to that followed here to antibiotics A and B (Fig. 1). The IR spectra of antibiotics A and B has similarities to those given in Curtis and Duncanson (3) for frequentin and palitantin, respectively. Thus, antibiotics A and B seem to be frequentin and palitantin, respectively, although at present we cannot assure this and further work on their purification and characterization now in progress is needed.

Whatever the antibiotics were, we demonstrate here their toxicity to *M. laxa* and 14 other fungi (Fig. 2, Table 1). The inhibition of the germination of spores of *M. laxa* by antibiotic B was significantly higher than by antibiotic A (Fig. 2). Antibiotic B also presents an uncommon characteristic in being as toxic to spores as to germ tube growth of *M. laxa*. The ED_{50} values of the antibiotics for the germination of spores and for the germ tube growth of our isolate of *M. laxa* are higher than the ED_{50} values of benomyl to *M. fructicola* (1 μg/ml) (14) or vinclozolin and iprodione to *M. laxa* (5 μg/ml) (7). However, we cannot forget that we are working with crude antibiotics and their purified forms could be more or less active.

The antibiotics were also toxic to *M. laxa* in detached peach twigs under laboratory conditions in the same degree as benomyl (Table 3). However, under the same conditions, spores of *P. frequentans* inhibited the pathogens to a lesser degree, probably because they have not had time enough to produce the antibiotics. We can postulate that the mechanism of antibiosis should be efficient in the biocontrol of *M. laxa* by *P. frequentans*. However, our evidence for the role of antibiosis in nature is obviously tentative, pending the technically difficult task of isolating the antibiotics at inhibitory concentration levels from fungal treated twigs. This work is also in progress.

TABLE 3. Toxicity of different compounds on *Monilinia laxa*

	Germination		Germ tube growth	
Compound[x]	Spores germinated[y] (%)	Inhibition[z] (%)	Germ tube length[y] (μm)	Inhibition[z] (%)
Control	80 ± 3.6	···	32.3 ± 2.8	···
Culture filtrate	52 ± 2.0	34 a	12.9 ± 3.3	60 a
Fractions 1	82 ± 2.0	···	35.7 ± 4.3	···
2	81 ± 1.7	···	28.2 ± 1.7	···
3	2 ± 0.5	97 c	0.0 ± 0.0	100 c
4	2 ± 0.4	97 c	0.0 ± 0.0	100 c
5	31 ± 3.5	61 d	9.2 ± 0.5	71 d
6	70 ± 2.2	12 e	18.3 ± 4.6	43 e

[x]Compounds are : culture filtrate and different fractions obtained from the liquid: liquid partition procedure described in Figure 1. Concentration of compounds in the crude filtrate were maintained in fractions. Control was sterile distilled water.

[y]Mean and standard deviation of four replicates; 50 conidia or 25 germ tubes were considered in each replicate.

[z]All reported differences from control are significant ($p \leq 0.05$). Means followed by the same letters are not significantly different ($p \leq 0.05$) by Duncan multiple range test.

TABLE 4. Toxicity of different treatments on *Monilinia laxa* in peach twigs

	Germination		Germ tube growth	
Treatment[x]	Spores germinated[y] (%)	Inhibition[z] (%)	Germ tube length[y] (μm)	Inhibition[z] (%)
Control	88 ± 3.27	···	63.39 ± 7.55	···
Antibiotic A	17 ± 6.53	89 a	19.63 ± 8.22	69 a
Antibiotic B	34 ± 2.00	61 a	41.40 ± 1.58	50 a
Spore-suspension of *Penicillium frequentans*	70 ± 6.39	21 b	55.98 ± 2.89	12 b
Benomyl	24 ± 8.41	72 a	11.50 ± 1.02	82 a

[x]Treatments are : Antibiotic A (1.48 mg/ml), Antibiotic B (6.7 mg/ml), benomyl (0.25 mg/ml), and aqueous spore suspension of *P. frequentans* (1×10^9 spores/ml).

[y]Mean and standard deviation of four replicates; 50 conidia or 25 germ tubes were considered in each replicate.

[z]Means followed by the same letters in each column are not significantly different ($p \leq 0.05$) by Duncan's multiple range test.

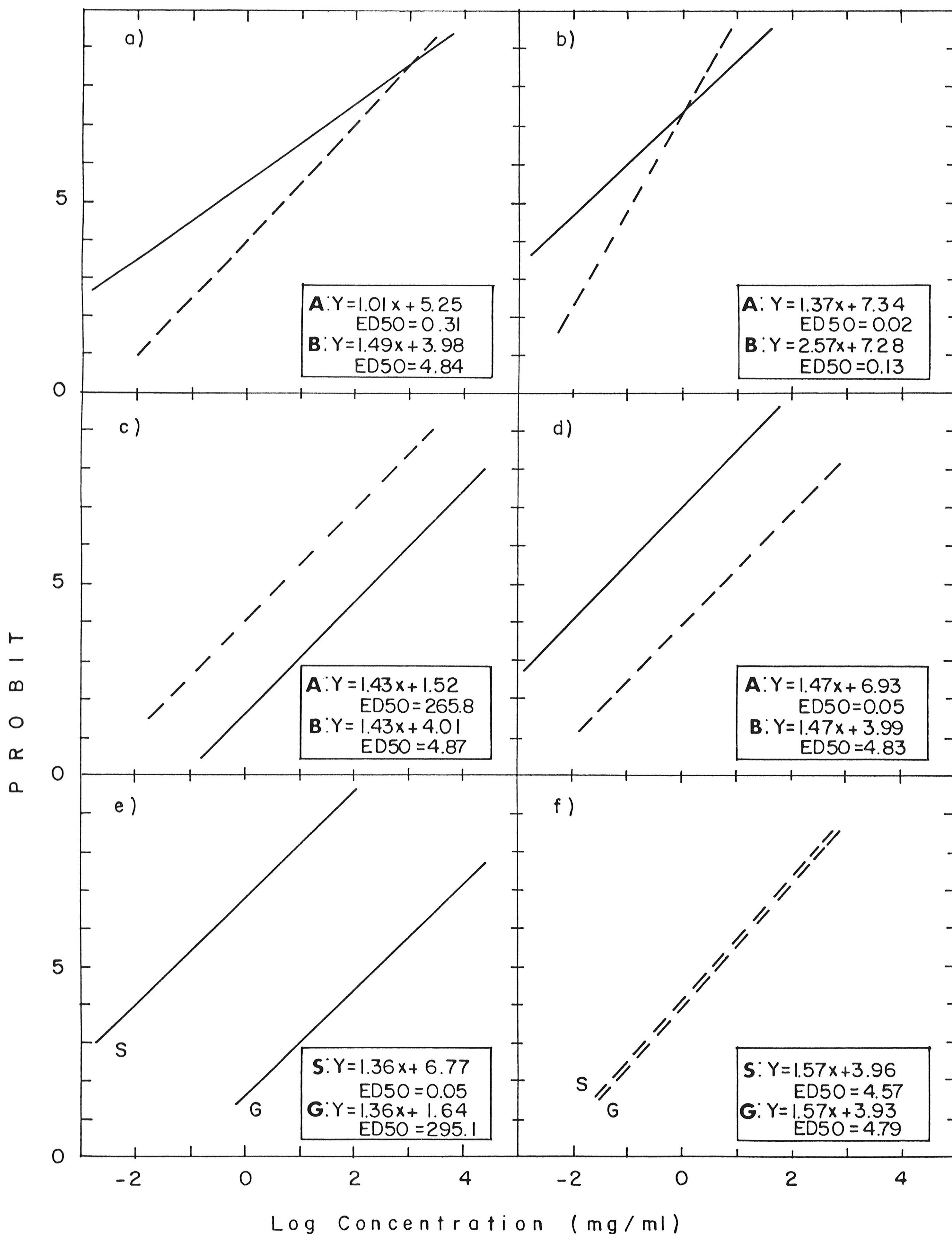

Fig. 2. Toxicity of antibiotics to *Monilinia laxa* represented as probit-log concentration regression lines and ED_{50} (mg/ml). ____ A, _ _ _ B. **A,** Toxicity of antibiotic A and antibiotic B to germination of spores of *M. laxa*. **B,** Toxicity of antibiotic A and antibiotic B to germ tube growth of *M. laxa*. **C and D,** Parallel regression lines fitted for toxicity of the antibiotics to the germination of spores and to the germ tube growth of *M. laxa*, respectively. **E and F,** Parallel regression lines fitted for toxicity of antibiotic A and antibiotic B (respectively) to the germination of spores (G) and to the germ tube growth (S) of *M. laxa*. X^2 for parallelism not significant in all cases. Significant differences ($p \leqslant 0.05$) exist among lines in cells c, d, and e.

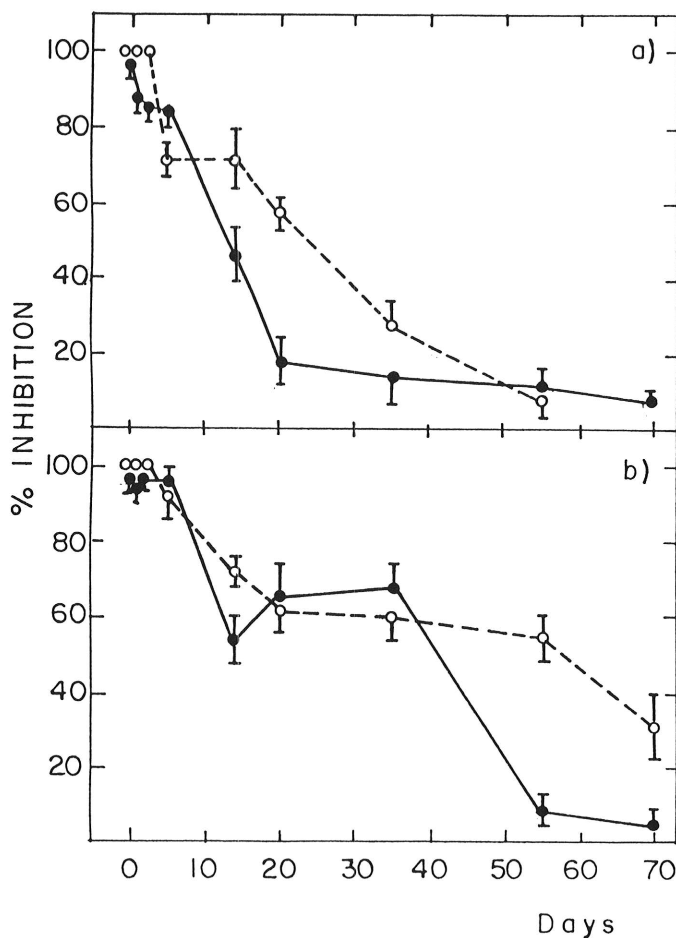

Fig. 3. Loss of activity of antibiotic A (a) and antibiotic B (b) with time. Points and brackets represent mean and standard error of four replicates of percentage of inhibition of spore germination (--•--) and of germ tube growth (--o--) of *Monilinia laxa*. Significant differences are reported text.

Because antibiotics appear to have a role in the biocontrol of *M. laxa* by *P. frequentans* their stability is crucial. Both antibiotics were shown to be stable at a wide range of temperatures. As a rule, antibiotic B is more stable and less influenced by the external media than antibiotic A, conserving 30% of its activity on germ tube growth after 70 days at room temperature as compared with 10% for antibiotic A. In addition, pH changes did not affect antibiotic B while the activity of antibiotic A decreased in alkaline conditions.

Another important aspect concerning the mechanisms involved in the biocontrol of *M. laxa* by *P. frequentans* in peach twigs is the relationship among the microorganisms present in the phyllosphere of twigs. Thus, we have tested the activity of the antibiotics on several fungal components of the mycoflora of peach twigs (9). Some of them were inhibited by antibiotics A and B; this may favor the growth and development of *P. frequentans* and, thus, enhanced the biocontrol of *M. laxa*, if antibiotics were produced by the antagonist in the twigs.

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