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Glucanolytic Actinomycetes Antagonistic to *Phytophthora fragariae* var. *rubi*, the Causal Agent of Raspberry Root Rot

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A collection of about 200 actinomycete strains was screened for the ability to grow on fragmented *Phytophthora* mycelium and to produce metabolites that inhibit *Phytophthora* growth. Thirteen strains were selected, and all produced β-1,3-, β-1,4-, and β-1,6-glucanases. These enzymes could hydrolyze glucans from *Phytophthora* cell walls and cause lysis of *Phytophthora* cells. These enzymes also degraded other glucan substrates, such as cellulose, laminarin, pustulan, and yeast cell walls. Eleven strains significantly reduced the root rot index when inoculated on raspberry plantlets.

Actinomycetes represent a high proportion of the soil microbial biomass (2). They have the capacity to produce a wide variety of extracellular hydrolases (15) that give them an important role in the decomposition of organic matter in the soil. In addition to their active function in decomposition, actinomycetes appear to be of importance among the microbial flora of the rhizosphere. Indeed, the last few years have seen different aspects of plant-actinomycete interactions studied extensively. Associations between actinomycetes and underground plant organs could be deleterious or beneficial for the plants. While some actinomycetes secrete herbicidal compounds (39) or cause diseases such as pox and common scab (22), others can symbiotically fix atmospheric nitrogen (4) or protect roots against fungal infections (44).

Actinomycete-fungus antagonism has been demonstrated for a wide variety of plant pathogens, such as *Alternaria* (9), *Rhizoctonia* (26, 35), *Verticillium* (42), *Fusarium* (36), and *Macrophomina* (17) spp. Moreover, actinomycetes of the genus *Streptomyces* have been used to commercially control plant diseases. For example, *Streptomyces* sp. strain 5406 has been used in China for more than 30 years now to protect cotton crops against soil-borne pathogens (46). More recently, Kemira Oy has developed a biofungicide that contains living *Streptomyces griseoviridis* cells to protect crops against *Fusarium* and *Alternaria* infections (20).

Evidence that diseases caused by fungus-like protoctistan *Phytophthora* and *Pythium* species can also be controlled by actinomycetes has accumulated. Broadbent et al. (8) and Knauss (19) have established that numerous streptomycetes can inhibit the growth of *Pythium* and *Phytophthora* spp. through the production of antibiotics. Furthermore, several experiments demonstrated that soil inoculation with specific streptomycete strains could significantly reduce damage caused by *Pythium* or *Phytophthora* species in ornamental (6, 7, 24, 47), legume (13), and horticultural (11, 38, 41) productions.

Phytophthora spp. were identified as causal agents of raspberry root rot in Canada for the first time in 1988 (40). Similarly, *Phytophthora* has also been associated with raspberry root rot in Australia (43), Chile (21), Europe (12, 27, 30), and the United States (45). In Canada, metalaxyl is the only authorized product to control *Phytophthora* disease in raspberry crops. Unfortunately, it has been demonstrated that *Phytophthora fragariae* can develop resistance against metalaxyl (29). Therefore, the ultimate goal of our research program is to develop a biocontrol method of root rot in raspberry crops.

The purposes of this study are (i) to propose a method based on the ability of actinomycetes to produce antifungal metabolites as well as glycosyl hydrolases for selecting biocontrol candidates, (ii) to verify the ability of the selected strains to protect raspberry roots against *P. fragariae* var. *rubi*, and (iii) to partially characterize the glucanolytic activity of the selected biocontrol agents.

MATERIALS AND METHODS

Culture conditions. *P. fragariae* var. *rubi* ML200 was usually grown at 22°C on V8 medium (33) or potato dextrose agar (PDA). Actinomycetes were maintained on YGM+ (18). A minimal medium (16) supplemented with different glucan sources at 0.5% and 0.1% yeast extract was used to assay the glucanolytic activity of the actinomycetes. The mycelium agar used to select biocontrol agents and to visualize glucanolytic activity was prepared as follows. *P. fragariae* var. *rubi* ML200 was grown in 25 ml of V8 broth for 7 days at 22°C. This culture was then autoclaved and centrifuged, and the pellet was rinsed three to four times with sterile water. The mycelium was then resuspended in 25 ml of sterile water and fragmented with a French press (12,000 lb/in^2). Three volumes of sterile water and agar at a concentration of 1.5% were then added to the fragmented mycelium suspension. The resulting mycelium agar was sterilized.

Screening of antagonistic actinomycetes. About 200 nonphytopathogenic actinomycete strains which were isolated from raspberry roots, potato rhizosphere, or soil were tested for their ability to grow and to produce clear zones on mycelium agar at 15°C. Actinomycete strains producing clear zones on mycelium agar were then analyzed for their activity against *Phytophthora* spp. at 15°C. For the antifungal activity test, actinomycetes were inoculated onto a V8 plate. A piece (1 cm^2) of V8 agar bearing a 14-day-old *P. fragariae* culture was inoculated 2 cm from the actinomycete inoculation spot. Zones of growth inhibition were recorded after 10 days of incubation. Actinomycete strains exhibiting production of both clear zones on mycelium agar and metabolites against the *Phytophthora* strain were considered antagonistic and were used in the biocontrol assay.

Biocontrol assay. In vitro-produced raspberry plantlets (cultivar Heritage; five-leaf stage) were planted in 12-cm-diameter pots containing sterilized loamy soil and grown for 14 days before bacterial inoculation. Ten plantlets were individually inoculated with each antagonistic actinomycete. Two control sets of 10 raspberry plantlets each were not inoculated.

Bacterial inocula were prepared by growing actinomycetes in YGM+ for 24 h at 30°C. A fraction of each culture (1 ml) was spread on a YME (32) plate, and the plates were incubated until sporulation occurred. Three pieces (10-mm

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TABLE 1. Actinomycete strains used in this study[a]

Strain(s)	Source	*Phytophthora* growth inhibition	Clearing zone produced on mycelium agar
EF-36, EF-39, EF-91, EF-94, EF-96	Potato rhizosphere	+	−
EF-6, EF-13, EF-16, EF-17, EF-18, EF-29, EF-37, EF-45, EF-100, EF-101, EF-105, EF-117, EF-119	Potato rhizosphere	−	+
DVX2, DVX3	Soil from raspberry plantation	−	+
EF-14, EF-22, EF-25, EF-27, EF-34, EF-43, EF-72, EF-76, EF-97	Potato rhizosphere	+	+
DVD1, DVD3, DVD4	Soil from raspberry plantation	+	+
N106	Soil from maple grove	+	+

[a] Strains producing no clear zone on mycelium agar and no inhibition of *Phytophthora* growth on V8 agar are not included in this table.

diameter) were cut from the agar cultures and set around the developing roots of each plantlet.

One week after bacterial inoculation, *P. fragariae* var. *rubi* ML200 was inoculated into the soil by the method of Nourrisseau and Baudry (30). Three pieces (10-mm diameter) were cut from *Phytophthora* cultures on V8 agar and added to the soil of raspberry plantlets infested by antagonistic actinomycetes. One set of raspberry plants was inoculated with *P. fragariae* (positive control), while another set was not (negative control). The plantlets were then incubated for 8 additional weeks and kept at 15°C on a 12-h photoperiod. Raspberry plantlets were sprayed with water every day, while the soil was kept constantly wet.

The severity of symptoms was recorded with a disease index ranging from 0 to 5 (24), where 0 represents a healthy plantlet and 5 represents a plantlet with chlorosis and necrosis on more than 80% of the surface of the leaves and the roots.

Glucanolytic activities in culture supernatants of antagonistic actinomycetes. The ability of antagonistic actinomycetes to produce β-1,3-glucanases, β-1,4-glucanases, and β-1,6-glucanases was tested in a minimal medium supplemented with laminarin (Sigma), cellulose (Fluka), and pustulan (Calbiochem), respectively. To assay β-1,4-glucanase activity, 250 μl of cellulose suspension (2.5 mg of cellulose per ml in 50 mM acetate buffer, pH 5.5) was mixed with culture supernatant and water to a final volume of 500 μl. The mixture was kept at 37°C for 1 h. The reaction was stopped by adding the first reagent for reducing sugar determination. The amount of reducing sugars released was determined by the Nelson-Somogyi procedure (37). β-1,3- and β-1,6-glucanases were assayed in the same way except that laminarin and pustulan were used as the substrate, respectively. The incubation with laminarin and pustulan dissolved in 50 mM phosphate-citrate, pH 7.0, lasted for 15 min at 50°C. One unit of glucanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per min in these conditions.

Chromatographic profile of glucanases produced by the actinomycete EF-14. Spores of EF-14 were inoculated into a medium containing (per liter) 8 g of yeast extract, 6 g of malt extract, 0.5 g of $K_2HPO_4 \cdot 2H_2O$, and 0.5 g of anhydrous KH_2PO_4, pH 6.5. After 24 h of incubation at 30°C, the mycelium was collected by centrifugation and washed with sterile 0.9% NaCl. A portion (1 ml) of the mycelium wet pellet was then inoculated into 100 ml of glucanase induction medium containing minimal salts (28) and 7.5 g (wet weight) of mycelium of *P. fragariae* var. *rubi* ML200. After 48 h of incubation at 30°C, the supernatant was collected by centrifugation. A portion (10 ml) of the supernatant was loaded onto a Sephacryl S-100 column (1.6 by 100 cm) previously equilibrated with 50 mM potassium acetate, pH 5.5. Fractions (5 ml) were collected, and glucanase activities were assayed in paired fractions for the capacity to release reducing sugars from the appropriate substrates.

Lytic activity assays. For estimation of lytic activity, a suspension of yeast cell walls was prepared as described by Rombouts et al. (34). Fractions from the chromatographic column (0.4 ml) were combined with 0.1 ml of yeast cell wall suspension and 0.5 ml of 50 mM potassium acetate buffer (pH 5.5). This mixture was incubated with continuous rotation at 30°C for 18 h, and then absorbance was measured at 450 nm. The lytic activity was calculated as described by Rombouts et al. (34).

The lytic activity of glucanases was also qualitatively verified on live *Phytophthora* cells. Extracellular glucanases were obtained by growing *Streptomyces* sp. strain EF-14 in mycelium broth for 72 h. The culture supernatant was collected by centrifugation. The supernatant was then dialyzed overnight against distilled water, and the β-1,3-glucanase activity of the supernatant was measured as described above. The *Phytophthora* mycelium used for the lytic test was obtained from 4-day-old V8 broth cultures. *Phytophthora* cultures were centrifuged, and the pellet was washed twice with distilled water. Crude enzyme solutions containing 1.5, 3.0, and 6.0 U of β-1,3-glucanases per ml were added to 50 mg of mycelium (wet weight) and incubated for 150 min at 22°C. The lytic assay on *Phytophthora* mycelium was carried out in triplicate.

RESULTS

Selection of antagonistic actinomycetes. Actinomycete strains exhibiting the ability to produce both clear zones on mycelium agar and metabolites against the *Phytophthora* strain on V8 agar were considered antagonistic (Table 1). A relatively high proportion (14%) of the 200 actinomycetes tested produced clear zones on mycelium agar. A smaller proportion of the actinomycete collection (6.5%) exhibited both properties, growth on mycelium agar and inhibition of *Phytophthora* growth. These antagonistic bacteria originated from potato rhizosphere (nine isolates) and from soil (four isolates). None of the actinomycete strains isolated from healthy or infested raspberry roots were found to be antagonistic.

Biocontrol assay. Antagonistic strains were tested for their ability to protect raspberry plantlets against *Phytophthora* infections. Most of the antagonistic actinomycetes protected raspberry plantlets, but the level of protection varied among the antagonistic strains. Five of these antagonistic strains (EF-72, EF-22, EF-34, EF-14, and EF-97) reduced disease symptoms (measured by the root rot index) to levels that could not be significantly distinguished from those of the uninfected raspberry plants, the negative controls (Table 2). Six other actinomycete strains (EF-76, EF-27, EF-43, DVD3, EF-25, and DVD4) were less efficient as biocontrol agents, even though they still caused a significant reduction in the severity of the symptoms. Indeed, the root rot index was decreased from 4.98 in the positive controls (raspberries inoculated only with the pathogenic *Phytophthora* strain) to 2.67 to 3.22 for the plants treated with these six actinomycetes. Inoculation of raspberry roots with the remaining two antagonistic strains, N106 and DVD1, did not significantly reduce the severity of the symptoms caused by *P. fragariae* var. *rubi* (Table 2).

Glucanolytic activity associated with antagonistic bacteria. All of the antagonistic actinomycetes produced β-1,3-, β-1,4-, and β-1,6-glucanases when grown on laminarin, cellulose, and pustulan, respectively. However, glucanolytic activities varied considerably among the isolates, ranging from 0.015 to 2.21 U/ml for β-1,3-glucanases and from 0.001 to 0.035 U/ml for β-1,4-glucanases. The difference in β,1-6-glucanase production was less pronounced, ranging from 0.072 to 0.109 U/ml (Table 3). The glucanases secreted by the antagonistic actinomycetes also efficiently degraded glucans from *Phytophthora* cell walls,

TABLE 2. Effect of bacterial inoculation on root rot caused by *P. fragariae* var. *rubi* ML200

Inoculum	Root rot index[a]
None (control)[b]	0.89 a
ML200 + actinomycete EF-72	1.63 ab
ML200 + actinomycete EF-22	1.67 ab
ML200 + actinomycete EF-34	2.25 ab
ML200 + actinomycete EF-14	2.56 abc
ML200 + actinomycete EF-97	2.56 abc
ML200 + actinomycete EF-76	2.67 bc
ML200 + actinomycete EF-27	2.78 bc
ML200 + actinomycete EF-43	3.11 bc
ML200 + actinomycete DVD3	3.11 bc
ML200 + actinomycete EF-25	3.22 bc
ML200 + actinomycete DVD4	3.22 bc
ML200 + actinomycete N106	3.33 cd
ML200 + actinomycete DVD1	4.22 cd
ML200	4.98 d

[a] Index values accompanied by the same letter do not differ significantly (Duncan's test, $P < 0.05$).

[b] The root rot index of the negative control is not zero because such young raspberry plantlets occasionally exhibit some necrosis on their basal leaves.

since bacterial growth on mycelium agar resulted in clearing of the opaque medium.

The extracellular proteins produced by strain EF-14 after 48 h of contact with sterilized mycelium of *P. fragariae* var. *rubi* ML200 were separated by size-exclusion chromatography on Sephacryl S-100 (Fig. 1) to estimate the number of glucanases produced by this strain. Three peaks of β-1,4-glucanase and one peak of β-1,6-glucanase activity were detected. A second very weak peak of β-1,6-glucanolytic activity was found in fractions 42 to 44. Two peaks of β-1,3-glucanase activity were found; they may correspond, however, to at least three or even four different molecular species, considering their asymmetric shape.

The lytic character of the glucanases was tested on yeast cell walls and on live *Phytophthora* cells. The lytic activity in various chromatographic fractions was estimated semiquantitatively by the turbidometric test, with yeast cell walls as the substrate. Peaks of turbidometric activity were found in fractions 25 and 26, 31, and 37 and 38 (data not shown). They did not coincide

TABLE 3. Glucanolytic activities of actinomycete strains grown on different glucan sources[a]

Strain	Activity (U/ml)		
	β-1,3-Glucanase	β-1,4-Glucanase	β-1,6-Glucanase
EF-14	0.339	0.350	0.109
EF-22	0.067	0.009	0.072
EF-25	0.284	0.001	0.075
EF-27	0.365	0.006	0.071
EF-34	0.015	0.015	0.087
EF-43	0.110	0.027	0.083
EF-72	0.461	0.018	0.085
EF-76	1.128	0.002	0.072
EF-97	0.224	0.023	0.083
DVD1	1.108	0.014	0.077
DVD3	1.901	0.006	0.073
DVD4	2.085	0.001	0.075
N106	1.821	0.003	0.078

[a] Bacteria were grown for 7 days in the presence of laminarin or cellulose or for 4 days in the presence of pustulan as the sole carbon source to assay β-1,3-, β-1,4, and β-1,6-glucanase activity, respectively.

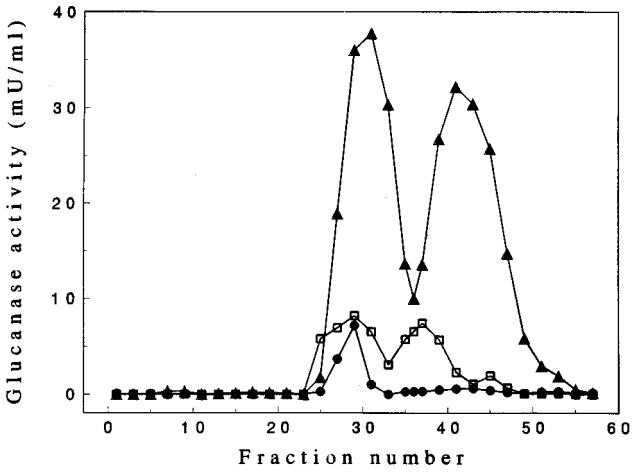

FIG. 1. Size-exclusion chromatographic profile of β-1,3- (▲), β-1,4- (□), and β-1,6-glucanolytic (●) activities from a culture supernatant of strain EF-14 grown in the presence of fragmented *Phytophthora* mycelium.

with a single peak of glucanase activity, suggesting that the lysis of yeast cell walls is the result of cooperation between different enzymes. The enzymes contained in the culture fluid of strain EF-14 were also shown to exert lytic activity on living mycelium of *P. fragariae* var. *rubi* ML200 (Fig. 2). Fungal mycelium lysis increased with increasing concentrations of β-1,3-glucanase. The culture fluid of strain EF-14 lost its lytic properties after being boiled for 10 min. This lytic activity could not be attributed to antibiotics present in the supernatant because even after intensive dialysis, this supernatant retained its full lytic activity.

DISCUSSION

Several mechanisms by which biocontrol agents reduce plant diseases have been proposed. These mechanisms include production of antibiotics and toxic products (14), secretion of hydrolytic enzymes (10, 23), production of siderophores (31), and niche exclusion (44). Screening for biocontrol agents often relies on one criterion, such as inhibition of pathogen growth or parasitism of pathogen structures. In this report, the antagonistic microbes were simultaneously selected for two traits often associated with biocontrol agents, the ability to hydrolyze the cell walls of the pathogen and the production of metabolites active against *Phytophthora* spp. Thirteen actinomycete strains exhibited both properties and were considered antagonistic to *Phytophthora* spp. All of the antagonistic strains except N106 belong to the genus *Streptomyces* (unpublished data); strain N106 belongs to the genus *Nocardioides* (25).

Antibiotic production has often been associated with the biocontrol ability of actinomycetes (11). In this study, we showed that a high proportion (72%) of the actinomycetes inhibiting *Phytophthora* growth by producing antifungal metabolites also secreted glucanases active on *Phytophthora* cell walls. Even though most actinomycetes secrete chitinases, glucanases, or other extracellular hydrolases, no correlation has been established between production of hydrolytic enzymes and the biocontrol ability of actinomycetes. However, a correlation between enzyme production and the ability to suppress plant disease has been suggested for another biocontrol agent by Chernin et al. (10), who showed, using a gram-negative biocontrol agent, *Enterobacter agglomerans*, that its ability to protect plants against diseases caused by *Rhizoctonia solani*, a fun-

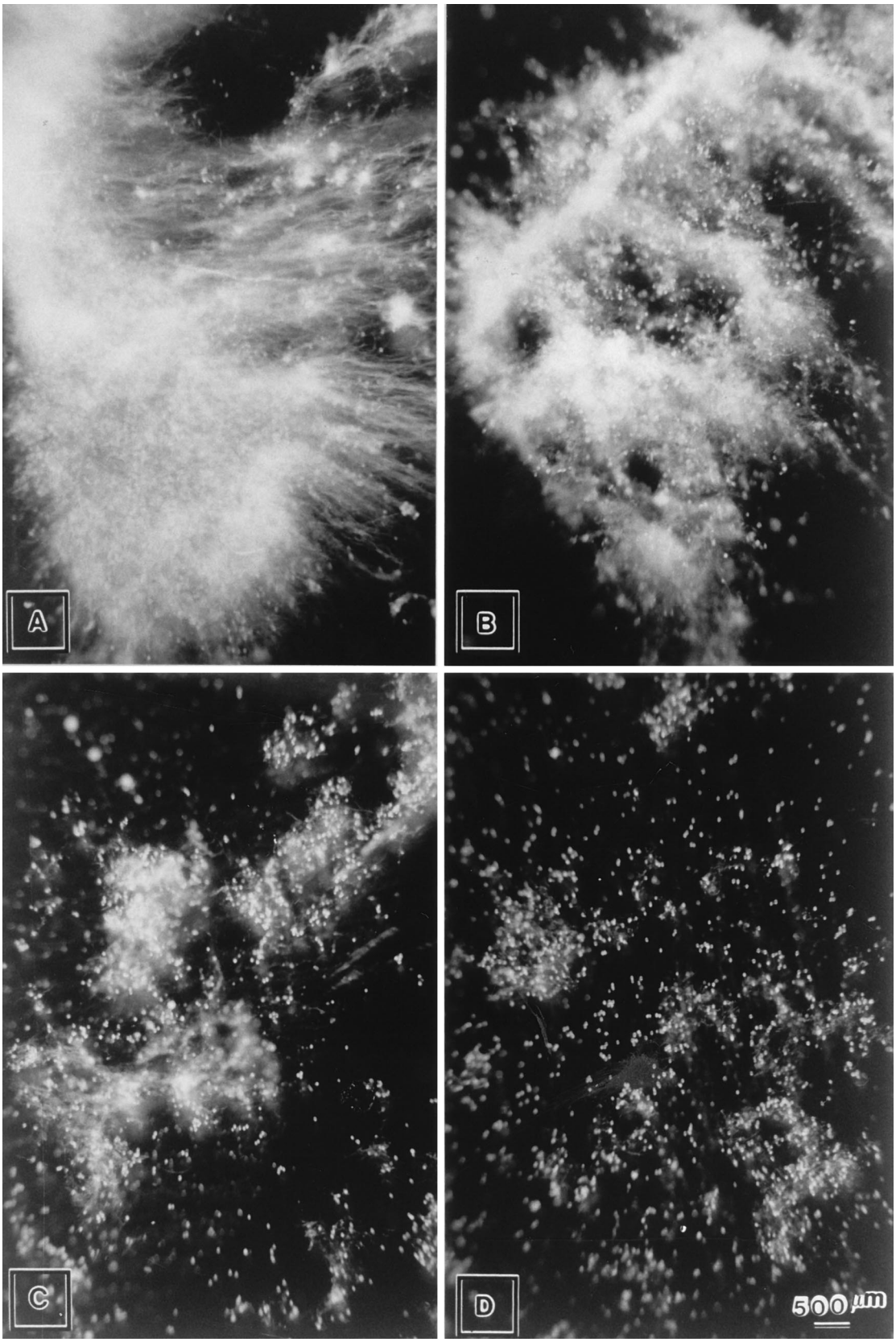

FIG. 2. Micrograph of *Phytophthora* mycelium incubated at 22°C for 150 min in the absence (A) or in the presence of crude enzyme extracts having 1.5 (B), 3.0 (C), or 6.0 (D) U of β-1,3-glucanase activity per ml.

gus with chitinous cell walls, was lost by making it defective in chitinase production.

In the present study, biocontrol strains were selected for their ability to produce clearing zones on a medium consisting of fragmented *Phytophthora* mycelium. *Phytophthora* cell walls do not contain a significant amount of chitin. Instead, β-glucans constitute 80 to 90% of the wall dry weight. Cellulosic β-glucans generally represent less than 30% of the *Phytophthora* wall dry weight, whereas noncellulosic β-glucans account for more than 60% of the weight. The noncellulosic glucan is a highly branched β-1,3-glucan, with β-1,6 links at the branching residues (3). The antagonistic actinomycetes that produced clearing zones on mycelium agar were shown to produce glucanases cleaving β-1,3, β-1,4, and β-1,6 links. Moreover, separation of glucanases by size-exclusion chromatography suggests that more than one enzyme or enzyme form is associated with each type of glucanolytic activity. The antagonistic actinomycetes thus possess a battery of enzymes which potentially allow very effective lysis of *Phytophthora* cell walls. These glucanases hydrolyzed other glucan-containing substrates, such as yeast cell walls, laminarin, pustulan, and cellulose. Furthermore, they were also proved to be active on live *Phytophthora* mycelium, which they lysed in the absence of any antibiotic activity, indicating that they might play a significant role in plant disease suppression.

No direct correlation between the level of glucanolytic activity and the ability of a specific strain to reduce the root rot index was established. However, one should consider that glucanolytic activity was estimated in liquid cultures with a purified substrate such as cellulose, pustulan, or laminarin. The ability of the antagonistic strains to degrade *Phytophthora* cell wall in soil does not necessarily reflect hydrolysis ability on purified glucan substrates in liquid cultures.

It has been reported previously that actinomycetes produce extracellular β-1,3- and β-1,4-glucanases (5, 15). However, this is the first report describing β-1,6-glucanase activities associated with actinomycetes. Indeed, few microorganisms have been characterized as producers of β-1,6-glucanase (1, 34). Rombouts et al. (34) showed that β-1,6-glucanases played a crucial role in the lysis of yeast cells. This enzyme, by debranching the glucan frame, could facilitate the lysis of *Phytophthora* cell walls. The levels of β-1,6-glucanolytic activity in the antagonistic actinomycetes growing on pustulan corresponded to those associated with other β-1,6-glucanolytic microorganisms (34).

In this study, we established that the production of fungal metabolites and the ability to degrade *Phytophthora* cell walls are good markers for the selection of biocontrol actinomycetes. Possibly, secretion of enzymes and production of antifungal metabolites interact synergistically in disease suppression mechanisms. For example, antifungal metabolites could prevent *Phytophthora* growth and impair its defense mechanisms, while glucanases, by degrading *Phytophthora* cell walls, would contribute to pathogen cell lysis and also supply additional nutrients to the antagonistic population. Further studies are in progress to determine the role of glucanase production and antifungal activity in root rot suppression on raspberry.

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