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Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9

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Streptomyces violaceusniger **strain YCED-9 is an antifungal biocontrol agent antagonistic to many different classes of plant pathogenic fungi. We discovered that strain YCED-9 produces three antimicrobial compounds with antifungal activity. These compounds were purified and identified, and included: AFA (Anti-Fusarium Activity), a fungicidal complex of polyene-like compounds similar to guanidylfungin A and active against most fungi except oomycetes; nigericin, a fungistatic polyether; and geldanamycin, a benzoquinoid polyketide highly inhibitory of mycelial growth of *Pythium* and *Phytophthora* spp. Antimicrobial assays were developed to estimate the production of each antibiotic independently. Medium composition had differential effects on the production of each metabolite. The hydrolytic enzymes chitinase and β-1,3-glucanase are also produced under induction by colloidal chitin and laminarin, respectively. Fungal cell walls induced the production of both enzymes. A potential for biological control of diseases caused by *P. infestans* was also suggested by strain YCED-9's strong *in vitro* antagonism towards pathogenic isolates of this fungus.**

Keywords: nigericin; geldanamycin; guanidylfungin A; chitinase; β-1,3-glucanase; *Streptomyces violaceusniger*; biocontrol

Introduction

Biocontrol of plant diseases caused by fungi has been achieved using microorganisms such as *Trichoderma* spp, *Gliocladium* spp, and *Pseudomonas* spp [10,40]. Bacterial strains belonging to the Actinomycetales have been described as potent antagonists of fungal pathogens under laboratory conditions [15], and in greenhouse and field experiments [14,22,33,41].

Two mechanisms have been proposed to explain inhibition of fungal pathogens in the rhizosphere by biocontrol agents. Antibiosis occurs when one or more diffusible compounds inhibit growth or developmental changes in the pathogen, impairing its ability to colonize the rhizosphere and establish disease. Mycoparasitism is a different process initiated by physical destruction of the fungal cell wall mediated by the action of hydrolytic enzymes produced by the biocontrol agent [1].

Many strains of *Streptomyces* are known to suppress fungal growth *in vitro* [12,15]. The organisms [14,22,33,41] or their products [17,33,34] have been used to suppress fungal plant diseases *in vivo*. Many antibiotics produced by actinomycetes have been used directly or assumed to be responsible for the biocontrol potential of the producing strain. Examples of such metabolites include macrolide benzoquinones [32], aminoglycosides [17,29], polyenes [30,33,34], and nucleosides [20]. *Streptomyces* are also known for their ability to produce extracellular enzymes active in fungal cell wall degradation, such as β-1,3-glucanases and chitinases [6,25]. The role of these enzymes in antifungal activity and biocontrol has been the subject of studies [9,19,23,25]. These hydrolases may be responsible for the mycoparasitic potential of some *Streptomyces* spp [38], and the partial suppression of plant diseases observed when natural soils are amended with fungal cell walls, chitin or laminarin [27].

The strain *Streptomyces violaceusniger* YCED-9 was isolated from soil by Crawford *et al* [12], and was selected for its potential to suppress damping-off disease of lettuce caused by *Pythium ultimum*, and for its ability to antagonize growth of many fungal strains *in vitro* and *in vivo* [11,12,36].

Here we report the purification and characterization of the antibiotics produced by strain YCED-9. In addition, we studied production of these antibiotics and production of chitinase and β-1,3-glucanase by strain YCED-9 in submerged culture. Data on the *in vitro* antagonism of strain YCED-9 towards *Phytophthora* spp strains are also presented.

Materials and methods

Microorganisms

The antimicrobial activity of fermentation supernatants and fractions obtained during purification was assayed using: *Candida albicans* ATCC 10231, *Candida albicans* ATCC 8657, *Bacillus subtilis* ATCC 6633, *Fusarium oxysporum* (lab stock), and *Pythium ultimum* No. 17 (lab stock). *Streptomyces lydicus* WYEC-108 (our laboratory, University of Idaho) was used for compatibility tests and studies of antagonism towards fungal pathogens.

A strain of *Phytophthora* sp provided by Dr Mark Roberts (Innovative BioSystems, Moscow, ID, USA) and two strains of *Phytophthora infestans* (No. 8 and No. 9) isolated and kindly provided by Dr Mike Thornton (University of

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Idaho Agricultural Station, Parma, ID, USA) were also used for studies of *in vitro* antagonism.

Streptomyces violaceusniger YCED-9, *Fusarium oxysporum*, *B. subtilis* ATCC 6633 and the *C. albicans* strains were stored as either cell or spore suspensions in 20% glycerol at −40°C. *Phytophthora* spp, *Pythium ultimum* and *S. lydicus* were stored on agar slants or plates at 4°C, and on agar slants under mineral oil at room temperature.

Antibiotics

Geldanamycin was kindly provided by Dr BE Williams (Upjohn Laboratories, Kalamazoo, MI, USA). Nigericin sodium salt was purchased from Sigma Co (St Louis, MO, USA).

Liquid media

Two basal media were selected after screening 12 media reported in the literature as useful for antibiotic production by *Streptomyces*. They were a complex medium SB [3], and a defined medium M2M, which was empirically developed for the cultivation of YCED-9. The composition of medium SB was: 10 g glucose (or 10 g starch); 2 g beef extract; 2 g yeast extract; 5 g bacto tryptose; 0.01 g $FeSO_4 \cdot 7H_2O$; distilled water to 1 L (pH = 7.2).

The composition of medium M2M was: 20 g starch (when added in substitution of starch, 20 g glucose and/or 10 g trisodium citrate); 5 g casaminoacids (vitamin free, Difco); 10 g $MgSO_4 \cdot 7H_2O$; 5 g NaCl; 0.05 g $CaCl_2 \cdot 6H_2O$; 4 g KH_2PO_4; 0.01 g $FeSO_4 \cdot 7H_2O$; 0.003 g $MnSO_4 \cdot H_2O$; 0.0025 g $CuSO_4$; 0.0044 $ZnSO_4 \cdot 7H_2O$; 0.00025 g $NaMoO_4 \cdot 2H_2O$; distilled water to 1 L (pH = 7.2).

The composition of medium M2 was the same as M2M, but contained 1.9 g L^{-1} $(NH_4)_2SO_4$ and 2.2 g L^{-1} $NaNO_3$ instead of casaminoacids.

The production media used for antibiotic production were SB (glucose) and M2M (starch).

Fermentation

Production of antibiotics in liquid culture was performed in 2-L flasks containing 650 ml of either medium M2M or SB. The inoculum consisted of a spore suspension recovered from 12-day-old cultures of strain YCED-9 grown on sporulation agar (SB-agar). The spore suspension was washed with distilled water several times, centrifuged and then stored as a frozen stock in 20% glycerol. Inoculant was added at a rate of 3×10^7 CFU per 100 ml liquid medium. Cultures were shaken at 280 rpm and 28°C.

For experiments in which the effects of medium composition on antimicrobial production were studied, fermentations were carried in 250- or 500-ml flasks with a working volume of 1/5 of the flask capacity.

Biomass was expressed as dry weight of mycelium in 4-ml aliquots of well-mixed whole fermentation broth. The broth was centrifuged and the pellet washed with distilled sterile water three times. The pellet was then dried and weighed after equilibrating it to room temperature.

Isolation and purification of antibiotics

Cultures of strain YCED-9 grown in liquid media SB (96 h) or M2M (192 h) were used for the production of antibiotics. The broth was filtered through paper and the retained mycelium was air dried and frozen. The filtrate was lyophilized and stored at 4°C in the dark. Cultures grown in medium M2M were harvested at day 8 and the lyophilate of the filtrate was used for antibiotic purification.

Solvent extracts inhibitory to *Candida albicans* ATCC 8657, *C. albicans* ATCC 10231, *F. oxysporum*, *B. subtilis* ATCC 6633 or *P. ultimum* No. 17, were used for further fractionation and purification. Three main fractions called AFA (Anti-Fusarium Activity), ABA (Anti-Bacillus Activity) and AMA were screened for antimicrobial activity and purified.

Purification of ABA: The lyophilized supernatant of strain YCED-9 cultures grown in medium M2M was extracted with absolute methanol several times and dried under vacuum. The solids from the methanol extract were then solubilized in water and extracted with two parts of hexane:isopropanol 9:1. The organic phase was concentrated under vacuum at 35–40°C to dryness and fractionated again in a small volume of water-benzene 1:5. The process was repeated several times. The organic phase was concentrated to a few milliliters and the sample applied to a silica cartridge (Mega-bond elut, 12 cc per 2 g, Varian, Harbor City, CA, USA) equilibrated with benzene. The cartridge was then washed with methylene chloride, and the active compound eluted with methylene chloride:methanol 95:5. Fractions active *vs Bacillus subtilis* ATCC 6633 were pooled, concentrated under vacuum to dryness and resuspended in a small volume of hexane:isopropanol 9:1. A yellow precipitate was recovered by centrifugation and separated for purification of AMA, a second antibiotic. The soluble fraction was dried and applied to a new silica cartridge equilibrated with hexane. The activity corresponding to ABA was eluted with hexane:isopropanol 93:7. The fractions active against *B. subtilis* ATCC 6633 were concentrated to dryness, resolubilized in hexane isopropanol 8:2 and then chromatographed on preparative TLC silica plates developed with hexane isopropanol 8:2 as mobile phase. A white lipophilic band with $R_f = 0.56$ was eluted from silica with hexane:isopropanol 70:30 and the sample was concentrated to dryness *in vacuo*. The solids extracted from TLC were solubilized in a small volume of hexane. Subsequent slow evaporation of the solvent gave a white amorphous powder which was a pure preparation of ABA.

Purification of AFA: A 96-h-old culture of strain YCED-9 grown in medium SB was filtered and the mycelial cake air-dried at room temperature. The filtrate was lyophilized, resuspended in a small volume of water, and extracted with three volumes of water-saturated *n*-butanol. The butanolic phase was washed with Na_2CO_3 (0.16 M) and distilled water, and dried under vacuum. The solids extracted with butanol were then resuspended in a small volume of absolute methanol. A yellow precipitate (mostly AMA) was separated by centrifugation. The methanol extract was then dried under vacuum, resuspended in 80% water in methanol and refrigerated at 4°C overnight. A white precipitate was then recovered by centrifugation. This crude preparation was washed with cold water and solubilized in a small volume of methanol-water 7:3. Final purification was achieved by reverse phase HPLC using a semi-

preparative column (ODS-3, C-18, 5 μm, 100 A; 250×10 cm, Phenomenex, Torrance, CA, USA) with an eluant of methanol-water (Isocratic 0–10 min 65% methanol; 10–30 min gradient 65–76% methanol; 30–50 min isocratic 76% methanol). A peak containing a pure compound was eluted at minute 45.

The mycelial cake was extracted with 90% methanol in water, the extract was concentrated under vacuum to dryness and resolubilized in absolute methanol. The methanol soluble solids were concentrated under vacuum, washed with benzene and diethyl ether and processed in the same way as the supernatant sample for final purification.

Purification of AMA: The third antibiotic was purified from supernatants of fermentations in both SB and M2M media. The lyophilized samples were resuspended in water, extracted with water-saturated *n*-butanol and dried under vacuum. The solids were solubilized in a small volume of methanol:chloroform 80:20 and applied to a silica cartridge equilibrated with chloroform. A mixture of chloroform and methanol (95:5) eluted a yellow compound active against *C. albicans* ATCC strains. The active fractions were then concentrated to dryness under reduced pressure and resuspended in a small volume of hexane-isopropanol 9:1. A yellow precipitate, recovered by centrifugation was then washed with pure hexane and resolubilized in a small volume of hexane:isopropanol 1:1. Final purification was achieved by chromatography of the preparation using a silica cartridge with hexane and isopropanol as eluting solvents. A pure active compound was eluted with hexane:isopropanol 8:2. This preparation was analyzed by HPLC, a single peak was eluted from a normal phase analytical column at minute 20 (Microsorb-MV, 5 μm, 100 A, 4.6×250 mm, Rainin, Emeryville, CA, USA) using an isocratic method (mobile phase hexane:isopropanol 94:6; flow rate = 0.5 ml min^{-1}; detector set at 210 nm).

Analytical characterization of antibiotics

Mass spectrometry was carried out in a Hewlett-Packard spectrometer (5989A) using an LC-MS particle beam interface (Hewlett-Packard 59980B). Both electron impact (EI) and positive chemical ionization (PCI) mass spectrometric analysis were done for ABA and AMA. Fast atom bombardment (FAB) analyses of AFA were done in a Hewlett-Packard mass spectrometer.

Infrared spectrometry was done using a KSR thallium bromide window (Perkin-Elmer, Norwalk, CT, USA). AMA and geldanamycin were analyzed in $CHCl_3$. ABA and nigericin were analyzed in CCl_4. A KBr pellet was prepared for spectral analysis of AFA. Comparisons of pure preparations with the authentic antibiotics were done at similar concentrations. The spectra were obtained in a Perkin-Elmer FTIR spectrometer model 1600.

NMR spectrometry was performed on an IBM NR/300 NMR spectrometer. The spectra were obtained at 300 MHz for PNMR and at 75 MHz for ^{13}C NMR, in $CDCl_3$ for AMA and ABA, and in DMSO-d_6 for AFA.

UV-spectrophotometry was performed with a diode array Hewlett-Packard spectrophotometer model 8453. All analyses were done using methanol solutions.

Bioassay of antibiotics

The antibiotics were assayed using agar plates (15 ml) containing L-P medium [24]. Aliquots of fermentation supernatants, fractions from purifications, or purified compounds were placed on glass fiber discs. After drying, they were placed on agar plates seeded with *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231, *C. albicans* ATCC 8657 or *F. oxysporum* spores, all at a concentration of 5×10^7 CFU per 100 ml of medium. Growth inhibition halos were measured after 48 h of incubation at 30°C. In some experiments, the discs were placed 2.5 cm from a centrally located agar plug taken from a 3-day-old culture of *P. ultimum* No. 17. The plate was incubated at 30°C for 18 h. The radial growth of the colony towards the antagonizing material was then measured and compared with a control colony without antimicrobial agent present.

Quantitative measurement of the individual antibiotics was accomplished by preparing standard curves of inhibition as a function of concentration of the purified compounds. The functions were linearized and used for estimation of the concentration of each antibiotic.

Antagonism assays

The antagonism assays against *Phytophthora* sp strains were done using the same procedure described for *P. ultimum* No. 17; however, in addition to liquid samples, the antagonizing potential of colonies and agar plugs from solid cultures was also evaluated. The medium used was potato dextrose agar (PDA) at pH 7 (15 ml). Plates were inoculated with an agar plug from a week-old culture of the corresponding *Phytophthora* sp strain. A colony, an agar plug without actinomycete, or liquid culture supernatants of either strains YCED-9 or WYEC-108 in a glass fiber disc were placed 2.5 cm from the fungal inoculum. After 6 days incubation at 24°C, radial growth was evaluated. The level of inhibition (Dn) was defined in terms of the radius of growth at day 6 of the control culture (n_0). The control radius was subtracted from the one recorded for the antagonism test at the same day of the culture (n) and yielded a distance in which $Dn = n_0 - n$, expressed in cm, was an estimate of the level of inhibition. If $Dn < 0.5$, inhibition was rated (–); if $0.5 \leq Dn < 1$, inhibition was rated (+); if $1 \leq Dn < 2$, inhibition was rated (++); if $Dn \geq 2$, inhibition was rated (+++).

Enzyme production

Strain YCED-9 was cultivated in shake flask cultures using the M2 mineral salts base supplemented with 0.05% (w/v) casaminoacids and any of the following substrates: (1) 0.5% (w/v) of starch; (2) 0.5% colloidal chitin (kindly supplied by Dr Brinda Mahadevan of our laboratory); (3) 0.5% laminarin (Sigma Co, St Louis, MO); or (4) 5% (w/v) fungal cell wall preparation. The crude cell walls were prepared from *Fusarium oxysporum* cultures grown in potato-dextrose broth supplemented with yeast extract 0.1% (w/v). The mycelium was filtered, homogenized in a Waring blender, and thoroughly washed with distilled water. Microscopic analysis showed the presence of cell wall fragments. The preparation, at 45% solids, was autoclaved and then used in preparation of culture media. Chitinase activity was estimated using the *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchito-

biose assay [16,25]. In this method *p*-nitrophenol is released from the oligosaccharide upon hydrolysis. The chitinase activity units were defined as nmol of *p*-nitrophenol released min^{-1} of reaction ml^{-1} of supernatant at 37°C. Chitinase activity was estimated from a standard curve of *p*-nitrophenol absorbing at 405 nm. Boiled supernatant aliquots were used as blanks.

β-1,3-Glucanase activity was assayed by incubation of supernatants with laminarin at 50°C. The release of glucose residues was measured after 10 min, using a modified DNS method to estimate the concentration of reducing sugars [21]. Absorbance was measured at 540 nm and the glucose concentration was estimated from a standard curve. A unit of activity was defined as the μmol of glucose released min^{-1} of reaction ml^{-1} of supernatant at the referred temperature. Boiled supernatant aliquots were used as blanks.

Results

Production, purification and characterization of antibiotics

To produce and isolate the most active antimicrobial compounds, several liquid media were screened for antibiotic production, whose presence was monitored with the bioassay for bioactivity for fungal, yeast and bacterial strains [33]. Two media were selected on the basis of the bioactivity present in the culture broth against *B. subtilis* ATCC 6633; *C. albicans* strains ATCC 10231 and ATCC 8657, *P. ultimum* No. 17 and *F. oxysporum*. One, sporulation broth SB [3], is a complex medium. The second, M2M, is a chemically defined medium whose composition was empirically determined. M2M was used for subsequent physiological studies and production of antifungal compounds by strain YCED-9. The *Bacillus* and the fungal strains used in the bioassays were chosen based upon their differential sensitivities to the multiple antibiotic activities we knew to be present in culture supernatants. Supernatants and solvent extracts from M2M shake flask cultures gave a different spectrum of antimicrobial activity from those derived from cultures in medium SB. Supernatants from M2M broth supplemented with casaminoacids strongly inhibited *B. subtilis* ATCC 6633, but were poorly active against either *F. oxysporum* and *C. albicans*. In contrast, supernatants from SB broth were very active against the fungi, but poorly antagonistic towards the *Bacillus*. Supernatants and extracts from both media inhibited *P. ultimum* No. 17.

Analysis of the mixture of antimicrobial compounds was done by bioautography. The anti-*Fusarium* activity (AFA) was shown to be extractable with water-saturated butanol. Its chromatographic mobilities on silica TLC plates were as follows: methanol: $R_f = 0.51$; chloroform:methanol 1:1: $R_f = 0.23$; butanol:acetic acid:water 60:20:20: $R_f = 0.54$. The anti-*Bacillus* activity (ABA) was extracted with benzene, diethyl ether and ethyl acetate and had very different chromatographic mobility:methanol: $R_f = 0.68$; chloroform:methanol 1:1: $R_f = 0.95$; and butanol:acetic acid:water 60:20:20: $R_f = 0.9$. A third compound, AMA, co-purified with ABA in several solvent systems containing chloroform and methanol in different combinations. AMA was successfully separated from ABA in hexane:isopropanol 8:2. This product was a bright yellow compound and extractable from cultures of strain YCED-9 grown in either M2M or SB, using chloroform, *n*-butanol and ethyl acetate.

AFA was purified from both supernatants and mycelium derived from cultures of strain YCED-9 grown in medium SB. Extracts of supernatants with water saturated *n*-butanol or methanolic extracts of mycelial cake were followed by solvent fractionation and chromatography on silica columns. TLC and HPLC-MS analysis of crude preparations of AFA extracted from the mycelial cake revealed the presence of several compounds with related structural properties as analyzed by EI-MS, but with different UV profiles. In particular, a yellow compound, unstable after purification in C18-HPLC but stable in dry solvents, showed a characteristic UV absorption with maxima at 289, 334, 354 and 377 nm which is typical of the hexaene class of polyene antibiotics. Separation of this component by HPLC resulted in very low antifungal activity associated with a mass signal of 1046 as determined by laser desorption-MS. The active preparations from both mycelium and supernatant had either no or very low UV absorption. Preparations from the supernatant showed the presence of a major component with UV absorption in the range of 210–220 nm. Final purification of this major component was achieved by RP-HPLC. Preparations obtained from semipreparative HPLC inhibited both *F. oxysporum* and *C. albicans* ATCC 10231. This product was soluble in methanol, DMSO and aqueous solutions of lower alcohols. It was insoluble in water and most other solvents. FAB MS analysis yielded a molecular weight of 1129.7. Elemental analysis, IR, ^{13}C and proton NMR data were consistent with a chemical structure very similar to the polyene-like antibiotic guanidylfungin A (Figure 1) [33], a member of the macrocyclic lactone antibiotics. Guanidylfungins are a heterogeneous group of related compounds with similar spectra [33], and they are difficult to separate. The spectrum of pure AFA was identical to that reported for guanidylfungin A.

Extracts of M2M-grown mycelium had insignificant antibiotic activity; therefore, antibiotics produced in M2M broth were recovered only from supernatants. ABA was isolated from supernatants of cultures of strain YCED-9 grown in medium M2M, and its antimicrobial activity measured using *Bacillus subtilis* ATCC 6633. Due to its low absorption in the UV region, and its poor resolution by reverse phase liquid chromatography, ABA was purified by extensive solvent fractionation. The final purification was achieved by preparative TLC. ABA inhibited *B. subtilis* but

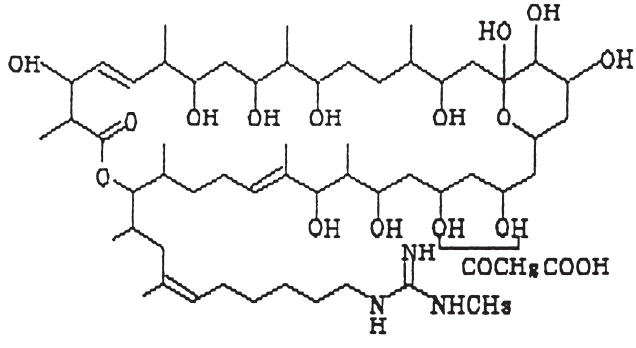

Figure 1 Structure of guanidylfungin A (from [35]).

also showed a weak fungistatic activity. IR analysis revealed strong absorption between 2800 and 3000 cm^{-1}. PCI-MS analysis gave a MW = 746 (Figure 2). Subsequent mass spectrometry, IR, PNMR and ^{13}CNMR analysis, in parallel with analysis of authentic standard, confirmed its identity as the sodium salt of the polyether nigericin [8,18].

AMA was isolated from M2M supernatants and its activity followed by bioassay against *P. ultimum* No. 17. The compound was extracted with *n*-butanol, dried and precipitated in methanol, and finally precipitated from several washes with hexane:isopropanol. A yellow crude preparation was chromatographed twice in different solvent systems. This product was very soluble in chloroform, moderately soluble in methanol, and had a distinctive UV spectrum with maxima at 205, 254 and 305 nm in methanol (Figure 3). UV, IR, ^{13}CNMR and mass spectra were identical to those obtained with an authentic standard of the antibiotic geldanamycin [13].

Quantification of antibiotic production in different media

Standard curves of antimicrobial activity *vs* antibiotic concentration were made using *B. subtilis* ATCC 6633, *F. oxysporum* and *P. ultimum* No. 17, and purified AFA, nigericin and geldanamycin respectively. *B. subtilis* ATCC 6633 was inhibited by as low as 0.05 μg of nigericin, which caused a larger inhibition halo than the one detected with 400 μg of either geldanamycin or AFA. Geldanamycin was the only strain YCED-9 antibiotic active towards *P. ultimum* No. 17. AFA was the only compound that inhibited germination of *F. oxysporum* spores in agar. By assaying dilutions of supernatants of strain YCED-9 liquid cultures against the indicator strains, we estimated the individual concentrations of the three antibiotics (Table 1). The time course of production of AFA, nigericin and geldanamycin in cultures of strain YCED-9 is presented in Figure 4. Two media, M2M and SB, were compared for time courses of antibiotic and biomass production, and for pH of the culture broth over an 8-day period. Biomass and pH changes were similar in the two media, but antibiotic production was not. In medium M2M, both nigericin and geldanamycin were produced in higher concentrations than in medium SB. The opposite was the case for AFA which was almost nine times more concentrated in cultures grown in SB as opposed to M2M. To better understand what kind of general nutrient effects were taking place in these media, we also used an M2 basal medium, which had only inorganic nitrogen (medium I, Table 1) and compared it with the medium containing casaminoacids (M2M, medium III) and one containing both casaminoacids and inorganic nitrogen (medium II). The addition of inorganic nitrogen to M2M caused a decrease in both volumetric and specific production of AFA, nigericin and geldanamycin, even though the biomass production in medium II was about twice that present in M2M (medium III). Only nigericin was detected in medium IV, which contained citrate instead of starch, even though biomass yield was double that reached in medium II. The final pH was much more alkaline than in other cultures. Glucose and starch had differential effects on antibiotic production, depending on the medium composition. Medium V contained glucose instead of starch, but the medium composition was otherwise identical to medium II. In medium V, production of nigericin and AFA increased moderately, but geldanamycin production increased almost 40-fold. Complex medium SB containing glucose (medium VI) had a much lower production of nigericin than SB containing starch (medium VII). In contrast, geldanamycin production was much higher in SB-glucose than in SB-starch.

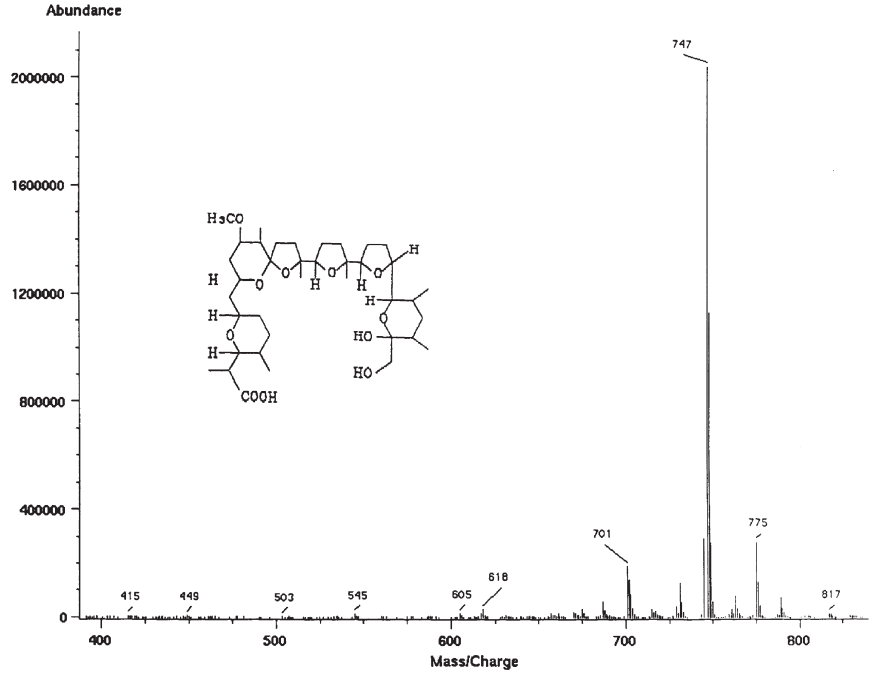

Figure 2 Mass spectrum of purified ABA from *Streptomyces violaceusniger* YCED-9 cultures, showing a spectrum identical to that of authentic nigericin standard.

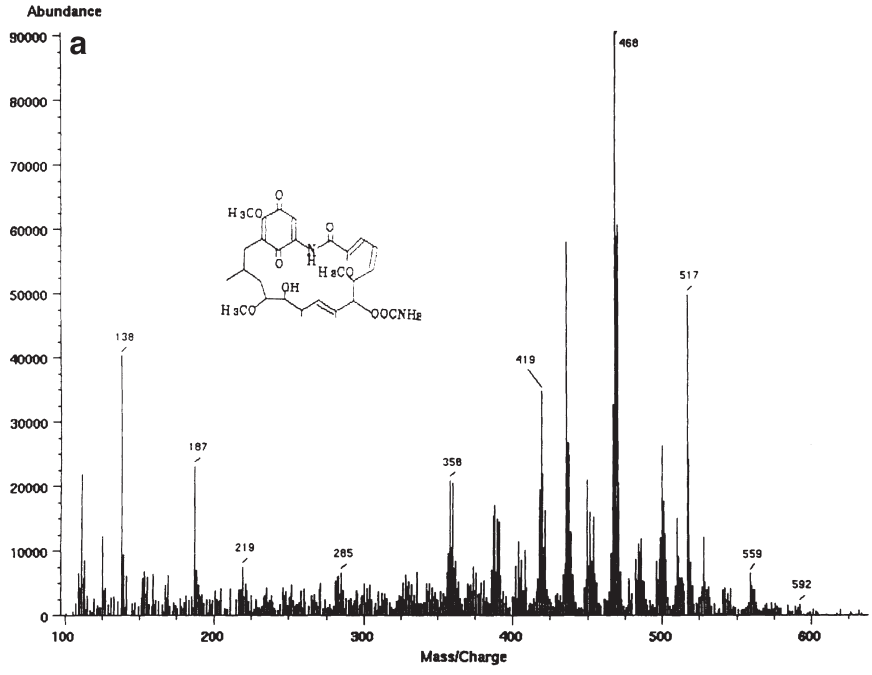

Figure 3 (a) Mass spectrum and (b) UV spectrum of purified AMA from *Streptomyces violaceusniger* YCED-9 cultures, showing spectra identical to authentic geldanamycin standard.

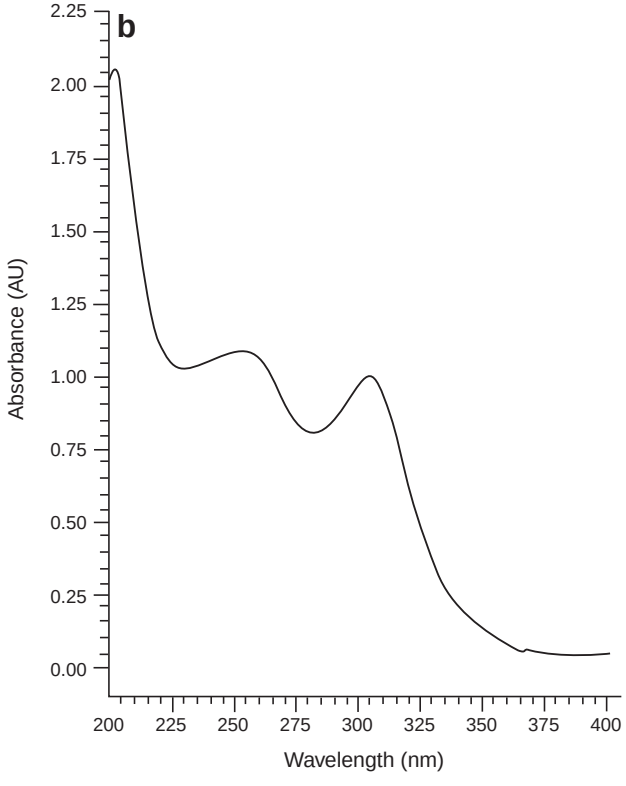

Figure 3 Continued.

AFA production was good in both media, though higher in the SB-glucose medium. Overall, the best medium for production of nigericin was VII. For AFA it was VI, and for geldanamycin, it was III or VI.

Production of enzymes

Cultures of YCED-9 in medium M2 (with 0.05% (w/v) casaminoacids and inorganic nitrogen) were supplemented with either starch, colloidal chitin or laminarin at a final concentration of 0.5% (w/v), or with 5% (w/v) of a preparation of cell walls derived from *Fusarium oxysporum* mycelia. Figure 5 shows chitinase and β-1,3-glucanase activities in supernatants of 6-day-old cultures grown in these media. Strain YCED-9 produced extracellular chitinase in media containing chitin or fungal cell walls as carbon sources, while β-1,3-glucanase was produced in media containing laminarin or fungal cell walls. Chitinase production was induced by laminarin, but only to one half of the level present in chitin medium. Correspondingly, β-1,3-glucanase production was induced by chitin, but to a much lower level than those in laminarin- or fungal cell wall-containing media. Induction of chitinase production by laminarin and other polysaccharides has also been reported for another biocontrol agent, *Streptomyces lydicus* WYEC-108 [25]. Chitinase and β,1-3-glucanase activities in supernatants of cultures grown in a medium containing starch were 20- to 50-fold lower than in media with the corresponding inducer. However, after 12 days growth in starch media, chitinase and β-1,3-glucanase activities were 107 and 203 units ml^{-1} respectively, an increase evidently associated with cell lysis.

In vitro antagonism towards Phytophthora spp—compatibility with other antagonists

Colonies of strain YCED-9, culture filtrates, and plugs taken from cultures grown on agar were used to measure antagonism of strain YCED-9 against one *Phytophthora* spp strain and two *P. infestans* strains isolated from an infested potato field in southern Idaho. *S. lydicus* WYEC-

Table 1 Growth and antibiotic production by strain YCED-9 in different liquid media at day 4

Medium composition		pH	Antibiotic concentration (mg L^{-1})			DCW (g L^{-1})
			nigericin	AFA	geldanamycin	
I	M2 starch, iN[a]	6.3	2.2	2.0	0	3.1
II	M2 starch, iN, cas[b]	5.3	0.3	9.7	0.1	7.5
III	M2 starch, cas	6.4	18.7	89.9	293.1	3.1
IV	M2 citrate, cas	9.0	1.1	0	0	10.5
V	M2 glucose, iN, cas	6.4	13.6	49.0	38.5	3.1
VI	SB glucose	6.0	2.8	450.9	317.8	3.4
VII	SB starch	7.3	50.8	312.4	31.9	2.0

[a]iN = inorganic nitrogen.
[b]cas = casaminoacids.

108, another biocontrol organism isolated by Crawford *et al* [11,12,25] is also active against *Phytophthora* strains. The antagonism of strain YCED-9 against *P. infestans* No. 8 is shown in Figure 6. Data on the antagonism of strains YCED-9 and WYEC-108 or their fungitoxic products against three *Phytophthora* strains are shown in Table 2. Strain WYEC-108 supernatants from the media used for strain YCED-9 had no activity against either *Pythium* or *Phytophthora* strains (data not shown), though the solid medium potato dextrose agar (PDA) was suitable for production of anti-*Phytophthora* compounds by strain WYEC-108 (Table 2). Strain YCED-9 was very antagonistic towards the *Phytophthora* (Table 2). Experiments were also run to determine if the two actinomycete strains were compatible. They are mutually antagonistic.

Discussion

The strain *S. violaceusniger* YCED-9 was isolated for its ability to suppress damping off of lettuce caused by *P. ultimum*. Due to its strong antagonism towards fungal strains belonging to different taxonomic groups, it was suspected to produce more than one fungitoxic product. The results demonstrate that strain YCED-9 produces at least three antimicrobial compounds active against fungi. One of these products, AFA, is a complex of polyene-like compounds related to the guanidyl-containing macrocyclic lactone antibiotics. The major component of the secreted antibiotics active *vs F. oxysporium* was found to be either identical or very similar to guanidylfungin A. It is also possible that the activity detected against *F. oxysporium* was partially due to the other compounds related to this component (like guanidylfungin B [35]), but their concentration and potency, as detected in the supernatant, were very small. AFA seems to be associated to a hexaene complex when isolated from mycelium. Interestingly, scopafungin (synonym of niphimycin) was originally isolated from endomycin, a hexaene complex with similar UV properties to those of the active mycelial preparation from strain YCED-9 [4]. Although no report exists of a structure for hexaenes, their weak antibacterial properties [7] and the fact that they were secreted extracellularly [26] make them different from classical polyenes. These characteristics might be explained by the presence of active compounds like the macrocyclic lactone antibiotics in the preparations with hexaene spectral properties. We were not able to compare our products with hexaenes reported in the literature, since they were not available from any of the original sources [7]. The other antimicrobials produced by strain YCED-9 were nigericin and geldanamycin, two common polyketides. In a screening for microorganisms producing natural products with herbicidal activity, a strain of *Streptomyces hygroscopicus* was reported to produce both nigericin and geldanamycin [18]. No herbicidal activity has been observed in our experiments *in vivo*. In other studies, a strain belonging to the same species of *Streptomyces* was reported to produce geldanamycin in culture and in soil. This strain was also able to biocontrol diseases caused by *Rhizoctonia solani* [32]. A different strain of *S. hygroscopicus* has been recently reported to produce geldanamycin and elaiophyllin [2].

S. violaceusniger YCED-9 produced three different polyketides, both in submerged culture and in agar (data not shown). These three compounds exert their inhibitory action in different ways [5]: geldanamycin like other ansamycins, interferes with RNA biosynthesis, and has been reported to exhibit strong antitumour activity. Nigericin is an ionophore which forms complexes with monovalent cations promoting an electrically neutral exchange diffusion of cations. The major component of AFA may also inhibit oxidative phosphorylation, like the related compound scopafungin [31]. In laboratory tests, we have found that strain YCED-9 is antagonistic *in vitro* to a large number of fungal pathogens [36], yet the only compound of the three that is antagonistic to *Pythium* spp, *Phytophthora* spp, and presumably other oomycetes, is geldanamycin. Production of this ansamycin may also be related to the biocontrol poten-

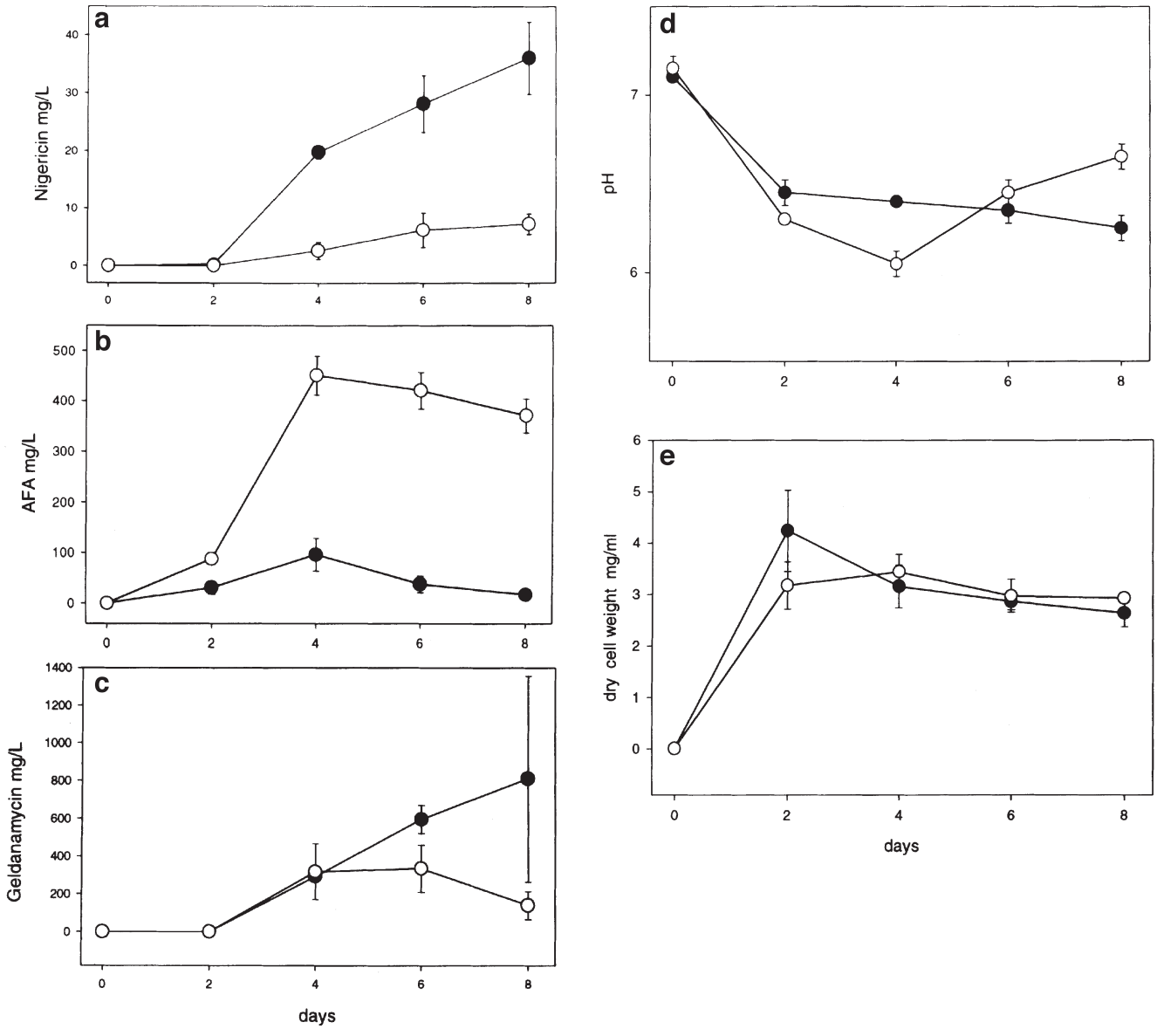

Figure 4 Production of the antibiotics nigericin (a), AFA (b), and geldanamycin (c), by *Streptomyces violaceusniger* YCED-9 cultures in liquid media M2M (●) and SB (○). Changes in biomass (d) and pH (e).

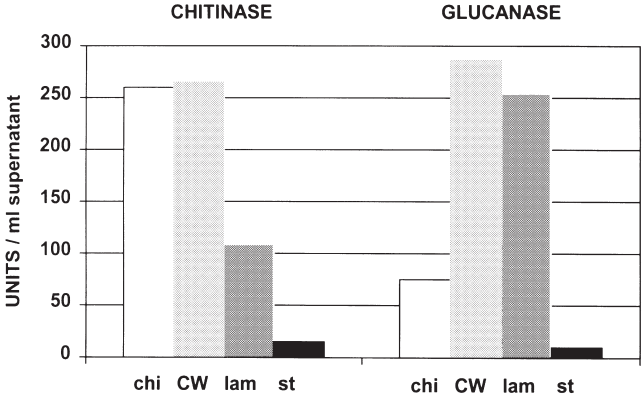

Figure 5 Production of extracellular chitinase and β-1-3 glucanase in M2 medium (containing casaminoacids, inorganic nitrogen, and supplemented with different carbon substrates: colloidal chitin (chi); fungal cell wall (cw); laminarin (lam); and starch (st).

tial of strain YCED-9 in the suppression of damping off of lettuce [12].

To our knowledge, there are no previous reports of biocontrol agents of the genus *Streptomyces* whose biocontrol properties are associated with any of the macrocyclic lactone antibiotics. Other strains have been shown to produce antibiotics of the polyene family. A *Streptomyces griseus* strain and its polyene antibiotic faeriefungin, a pentaene, suppressed asparagus root diseases caused by pathogenic *Fusarium* spp strains *in vitro* [33]. Aureofungin, a polyene of the heptaene family has been used in agriculture to control fungal diseases [34]. Another heptaene is apparently associated with the antifungal properties of *Streptomyces griseoviridis*, an isolate from Finnish peat moss that shows biocontrol activity [29]. Even though polyenes are among the most active antifungal compounds found in nature, their use in agriculture is limited by the fact that they are not inhibitory towards pathogens belonging to the

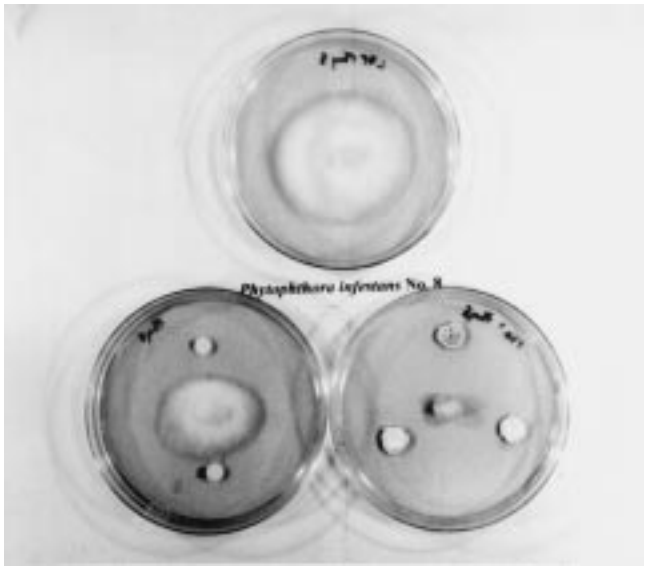

Figure 6 Antibiotic compounds produced by *Streptomyces violaceusniger* YCED-9 on solid medium are antagonistic to pathogenic *Phytophthora infestans* strain No. 8. Top: control plate of *P. infestans* No. 8. Bottom left: 40 μl of supernatant of broth cultures from cultures of strain YCED-9 grown in M2M medium (top) and SB liquid medium (bottom). Bottom right: colonies of *S. violaceusniger* YCED-9 antagonistic to the fungal colony.

Pythiaceae family of oomycetes. Neither *Phytophthora* spp nor *Pythium* spp are sensitive to nystatin, pimaricin and other polyenes [37]; however, cultures of the strain YCED-9 grown on solid or in liquid media produced in large amounts the antibiotic geldanamycin. This compound has been previously associated with the biocontrol activity of a *Streptomyces hygroscopicus* strain [32], as well as the herbicidal activity of a different isolate of the same species [18]. Geldanamycin, as previously reported [13], inhibited the growth of *Pythium ultimum* and *Phytophthora* spp colonies *in vitro* at concentrations as low as 2.5 μg ml^{-1}. The biocontrol activity shown by strain YCED-9 in the original screening [11] may be related to the production of geldanamycin in the rhizosphere of lettuce plants. The third compound, nigericin, is one of the few polyethers with fungistatic activity. Although it inhibited growth of many different fungal species *in vitro*, it was not active against *Pythium ultimum* strains. Requirements for growth and secondary metabolite production by cultures of strain YCED-9 were minimal. Production of nigericin was achieved in liquid cultures of strain YCED-9 in the chemically defined medium M2, in addition to several pigmented non-active products. This low nutritional requirement may explain its prevalence in soil and rhizosphere, as well as its ability to control fungal diseases of turfgrasses (Trejo-Estrada, Rivas-Sepulveda and Crawford, unpublished results). The effects of medium composition on the production of individual compounds may not have any correlation with their production in the rhizosphere, but it is posible that the general nutritive value of soil and root exudates of different plants will affect growth and secondary metabolism, and either enhance or decrease the antifungal potential of the actinomycete. The effects of different mineral salts on the production of these three polyketide antibiotics are under study. In addition to pH, and growth rate, nutrients such as glucose, ammonium, phosphate and trace metals have been described as important factors in the regulation of secondary metabolism in actinomycetes [28].

Production of the hydrolytic enzymes chitinase and β-1,3-glucanase was strongly inducible by the corresponding carbohydrate and by fungal cell walls. The role that these enzymes play in the biocontrol process has been studied for other biocontrol agents [25,39]. No studies have been made to determine if strain YCED-9 is a mycoparasite, but the induction of the production of chitinase and β-1,3-glucanase by fungal cell walls is relevant if such a mechanism is considered. Both chitinolytic [25] and glucanolytic [39] strains of *Streptomyces* have been associated with biocontrol of diseases caused by members of the Pythiaceae family. In the last report, glucanolytic strains of actinomycetes were able to antagonize a pathogenic *Phytophthora fragariae* strain both *in vitro* and *in vivo*, suppressing a root rot disease of raspberry. The production of chitinase was also induced by laminarin, and production of β-1,3-glucanase was induced by colloidal chitin. Crossed induction may be a general pattern that includes other glucans. But it is plaus-

Table 2 *In vitro* antagonism of *Streptomyces violaceusniger* YCED-9 and *Streptomyces lydicus* WYEC-108 against *Phytophthora* species

Antagonist	Level of inhibition		
	Phytophthora sp	*P. infestans* No. 8	*P. infestans* No. 9
Strain YCED-9			
Colonies	++	++	++
M2M supernatant	+++	+++	+++
SB supernatant	+	+	+
Agar plug SB-agar	+++	+++	+++
Strain WYEC-108			
Colonies	+	n.d.	n.d.
Agar plug PDA agar	+	n.d.	n.d.

Level of inhibition: see Materials and Methods.
SB = sporulation broth.
PDA = potato dextrose agar.
n.d. = not done.

ible that a common intracellular process triggers the production of the enzymes involved in degradative processes associated with mycoparasitism. The absence of a constitutive production of the hydrolytic enzymes relevant to a mycoparasitic process, and the abundant production of antibiotics in liquid cultures of strain YCED-9, point to secondary metabolites as the products most likely involved in the biocontrol activity shown by the actinomycete. Moreover, the presence of multiple antifungal metabolites with different spectra of activity, and whose production may respond to changes in the environment, ensure the adaptation of the strain to a competitive rhizosphere where its populations may well be established by the exclusion of other bacterial and fungal competitors sensitive to their antimicrobial metabolites.

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