Biological Control of *Eutypa lata* on Grapevine by an Antagonistic Strain of *Bacillus subtilis*

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ABSTRACT

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An isolate of *Bacillus subtilis* from grapevine wood inhibited in vitro growth of *Eutypa lata*, the causal organism of dieback in grapevines. On potato-dextrose agar, the bacterium caused 91.4% inhibition of mycelial growth of *E. lata* and 100% inhibition of ascospore germination. Ascospore germination correlated negatively with time of exposure to *B. subtilis*. Malformation of hyphae of *E. lata* occurred in the presence of *B. subtilis*. At a concentration of ≥0.8 mg/ml, an antibiotic substance in an ethanol extract from *B. subtilis* totally inhibited germination of ascospores of *E. lata*. Thin-layer chromatography of crude antibiotic extract showed five bands, two of which inhibited mycelial growth of *E. lata*. Spraying a suspension of the bacterium on pruning wounds before inoculation with ascospores of *E. lata* significantly reduced infection as compared with the unsprayed, inoculated controls.

Additional keywords: antibiosis, *Vitis vinifera*.

Several *Bacillus* spp., including *B. subtilis* are antagonistic to plant pathogenic fungi and bacteria (4,5,7–9,19). *Bacillus* spp. produce at least 66 different antibiotics (14). Azad et al (1) found a number of rhizosphere bacteria, including a strain of *B. subtilis*, to be antagonistic in vitro to *Verticillium dahliae* Kleb. from potato. Several isolates of *B. subtilis* from healthy maple trees were antagonistic to *V. dahliae* in maples (10), whereas Schreiber and Ichada (9) reported a strain of *B. subtilis* antagonistic to *Ceratocystis ulmi* (Buisman) C. Moreau. We report here on an isolate of *B. subtilis* from grapevine (*Vitis vinifera* L.) wood, which was antagonistic to *Eutypa lata* (Pers:Fr) Tul. & C. Tul., the cause of dieback in grapevines. An extract from this strain also was tested for activity against *E. lata* in vitro and in vivo.

MATERIALS AND METHODS

The isolate of *B. subtilis* was found while isolating *E. lata* from grapevines with dieback symptoms at the Viticultural and Oenological Research Institute, Stellenbosch. Arms from a chenin blanc vine showing dieback symptoms were cut lengthwise to expose the lesions. Pieces of wood, 5 mm^2, were cut from the lesions, surface-disinfested in 0.5% sodium hypochlorite, placed on potato-dextrose agar (PDA), and incubated for 5 days at 25 C. In some petri dishes, bacterial contaminants were observed that showed inhibition of *E. lata*. These bacteria were streaked on PDA, and plates were incubated for 5 days at 25 C. Single colonies were transferred to Czapek-Dox broth (CDB) supplemented with 1% yeast extract and incubated for 3 days at 25 C on a rotary shaker. The ability of bacteria to inhibit *E. lata* was tested by placing a loopful of bacterial culture at a distance of 10 mm from actively growing mycelium of the fungus on PDA. Plates were incubated for 7 days at 25 C and examined for an inhibition zone. A loopful of bacterial cells from a colony showing the widest inhibition zone was cultured as before, and the bacterium was stored under liquid nitrogen as described by Leeson et al (15). This bacterium was used in all experiments. The bacterial isolate was identified by the API 50 CHB system (SA Montalieu, Vericieu, France) and verified by the methods of Harrigan and McCance (11) and Sneath (21).

Effect of bacteria on mycelium and ascospores of *E. lata*. *E. lata* and *B. subtilis* were transferred to PDA in 9-cm-diameter petri dishes as follows: A sterile glass ring (5- × 7-mm-diameter) was pressed lightly into the agar 12 mm from the edge of the petri dish. Ten microliters of *B. subtilis* grown in CDB (1×10^{10} cells per milliliter) was placed in the ring. A mycelial plug (7-mm-diameter) of an actively growing culture of *E. lata* was placed halfway (35 mm) between the bacterium and the opposite side of the petri dish. On each of six control plates, 10 μl of CDB was placed in the glass ring. Both experimental and control dishes were assigned according to a completely randomized design, with six replicates per treatment. Fungal growth away from and toward the bacterium was measured after mycelial growth reached the edge of the petri dish. The experiment was repeated and inhibition was calculated as a percentage, by subtracting the distance of mycelial growth toward the bacterium from the distance of mycelial growth away from the bacterium, dividing by the fungal growth away from the bacterium, and then multiplying by 100.

Hyphal morphology. A mycelial plug (7-mm-diameter) from a PDA culture of *E. lata* was transferred to the center of a petri dish, containing 15 ml of CDB, and incubated at 25 C. When mycelial growth on the surface of the liquid reached a diameter of about 10 mm, a loopful of bacteria from a 3-day-old CDB culture of *B. subtilis* was transferred to the dish, and the culture was incubated at 25 C for another 3 days. Hyphal strands at the edge of the fungal colony were removed and examined under a microscope for abnormalities. Cultures of *E. lata* in CDB without *B. subtilis* served as controls. Experimental and control dishes were arranged in a completely randomized design, with three replicates of each.

Ascospore germination. Vine wood containing perithecia of *E. lata* was washed under running tap water for 15 min. Tops of the softened perithecia were cut through with a sterile scalpel, and the contents of 10 perithecia were placed in a McCartney bottle (Lasec, South Africa) containing 5 ml of sterile distilled water. After vigorous shaking, the bottle was left for 1 hr and reshaken. Ascospores were counted with a hemacytometer and diluted to 1×10^4 spores per milliliter.

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Two experiments were conducted to determine the effect of *B. subtilis* on ascospores of *E. lata*. In the first experiment, inhibition of ascospore germination was determined on PDA in three petri dishes. Two wells (7-mm-diameter) were made 30 mm apart in the agar and 100 μl of the *B. subtilis* suspension (1×10^{10} cells per milliliter) in CDB was pipetted into each well. Control plates received 100 μl of CDB only. Three drops (approximately 30 μl each) of an ascospore suspension (1×10^4 spores per milliliter) of *E. lata* were pipetted around each well and spread in a radius of 15 mm around each well with a sterile glass rod. The experiment was repeated and both experimental and control dishes were arranged in a completely randomized design. After 24 hr of incubation at 25 C, spores occurring within 10 mm around each well were examined under a microscope for germination. A spore was considered to have germinated when the germ tube length was half the length of a spore.

In the second experiment, the antibiotic substance(s) produced by *B. subtilis* was extracted from a 4-day-old CDB culture of *B. subtilis* according to the method of McKeen et al (17). The ethanol extract was evaporated to dryness at 40 C in preweighed holders and the mass was determined. The antibiotic extract, redissolved in 100 ml of 80% ethanol, was pipetted into petri dishes just before the agar was poured, providing concentrations of 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/ml. Six plates were used for each concentration; control plates received only 150 μl of 80% ethanol. A few drops of the ascospore suspension of *E. lata* were spread over the agar with a sterile bent glass rod. Plates were arranged in a completely randomized design. After 24 hr of incubation at 25 C, 100 ascospores (not exceeding 30 per microscope field) were examined for germination at each extract concentration. The experiment was repeated and linear regression analysis was applied to the data.

Effect of exposure time to *B. subtilis* on ascospore germination. Ascospores from 10 perithecia obtained as before were placed in 12 McCartney bottles, each containing 2 ml of CDB in which *B. subtilis* had grown for 2 days. The contents of each set of four McCartney bottles (replicates) were centrifuged separately at 3.5 *g* for 15 min after exposure times of 24, 48, and 72 hr, respectively. The supernatant was decanted, and the sediment of bacterial cells and ascospores was washed three times by centrifugation with 10 ml of sterile distilled water. Two milliliters of sterile distilled water, containing 500 mg/L of chloromycetin to deactivate the bacterium, were added to each bottle. Spores were examined under a microscope for germination after 24, 48, and 72 hr of incubation at 25 C. The contents of 10 perithecia in 2 ml of sterile CDB, to which 2 ml of chloromycetin (500 mg/L) had been added, served as a control. Germination of ascospores was determined after 24 hr. Experimental and control bottles were arranged in a completely randomized design with four replications per treatment. The experiment was repeated.

Field test for control of *E. lata*. *B. subtilis* was grown in CDB for 3 days at 25 C on a rotary shaker. Bacterial cells were centrifuged at 3.5 *g* for 20 min and mixed with mineral oil (B.P. cipron, manufacturer, BP SA (PTY) LTD, Cape Town, South Africa) to a concentration of 10^8 cells per milliliter. In the treatment with *B. subtilis*, the bacterium was grown in CDB for 3 days at 25 C and directly supplemented with 1% peptone and 1% sucrose before use to ensure a rapid buildup of bacterial cells in pruning wounds.

A 4-yr-old Riesling vineyard trellised to a Perold system was used; wounds were made on 2-yr-old wood by pruning. Treatments were applied with a household spraygun directly onto wound surfaces immediately after pruning until runoff. Treatments were benomyl 50WP at 10 g/L, benomyl (10 g/L) in mineral oil plus *B. subtilis* (10^8 cells per milliliter), benomyl (10 g/L) in mineral oil, antibiotic extract of *B. subtilis* (2 mg/ml), suspension of *B. subtilis* in CDB, and control (distilled water). Two additional trials of the same experiment were conducted in 1989, except that mineral oil was omitted and a treatment of *B. subtilis* + benomyl (0.5 g/L) was used.

Each wound was inoculated with 100 μl of a spore suspension (5×10^3 spores per milliliter) of *E. lata* 4 hr after treatment, and wounds were covered with aluminum foil to prevent dessication. Four wounds per vine on six vines were inoculated per treatment, and the treatments were replicated randomly in each of four blocks in a randomized block design. Wounds varied between 10 and 15 mm in diameter. Nine months after inoculation, cane pieces were cut 25 mm below the inoculated wound. These pieces were quartered lengthwise and placed on PDA after surface-disinfestation in 0.5% sodium hypochlorite for 5 min. After 5 days at 24 C, isolates were examined for typical mycelial growth of *E. lata*. Hyphal transfers were made to PDA and incubated under a combination of fluorescent and black light with a 12-hr photoperiod. *E. lata* was identified positively by the formation of stylospores. Data were subjected to a logit transformation before analysis of variance was performed; however, actual percentages are presented. The least significant difference test was used to compare treatment means.

Thin-layer chromatography. The antibiotic extract in 80% ethanol was applied as a band on a 20-cm^2 silica-gel plate containing a fluorescent indicator (Merck 5721, Merck & Co., Rahway, NJ). Plates were developed in ethanol/water (2:1, v/v), and the bands marked under ultraviolet light. The different bands and areas between them were scraped off separately, triturated with a mortar and pestle, and resuspended in 5 ml of 80% ethanol. Extracts were tested for inhibition of mycelial growth of *E. lata* by visually evaluating the inhibition effect.

RESULTS

Effect of bacteria on mycelium and ascospores of *E. lata*. *B. subtilis* strongly inhibited mycelial growth of *E. lata* on PDA (Fig. 1). Mycelial growth away from the bacterium was always 35 mm, whereas average growth toward the bacterial colony was 4.2 mm for a mean inhibition of 88%.

Mycelium morphology. After introduction of *B. subtilis* to CDB, little additional growth of *E. lata* was observed. Hyphal tips of the fungus became malformed, and hyphae were thickened and vacuolar compared with hyphae in the absence of the bacterium (Fig. 2). Many swellings occurred in the hyphae or at the tips of the hyphal strands, whereas normal hyphal walls were smooth with no swellings or vacuolation. No lysis of hyphae was observed.

Ascospore germination. Ascospores of *E. lata* did not germinate in the presence of *B. subtilis*, whereas an average of 68% germination was observed in the controls after 24 hr. In the presence of the bacterium, ascospores were swollen but did not germinate (Fig. 3A). In most spores, two vacuoles were visible (Fig. 3C and D). No such vacuoles were present in untreated control spores (Fig. 3B).

The mean germination of ascospores of *E. lata* in controls without antibiotic extract was 70%. Germination was reduced sharply ($b = -0.8386$) when the extract was added to the medium.

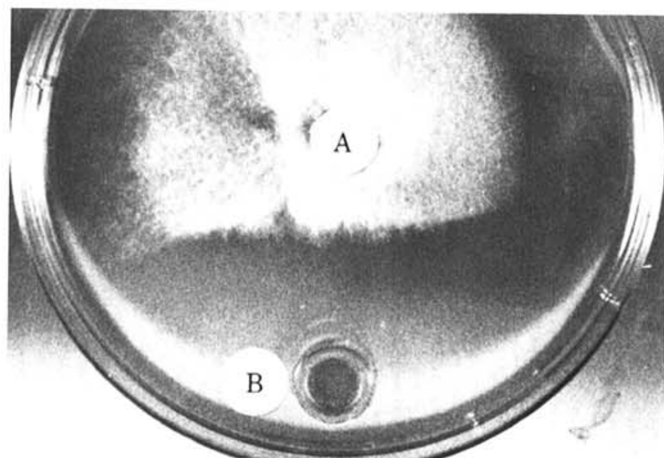

Fig. 1. Inhibition of *Eutypa lata* by *Bacillus subtilis*. **A,** *E. lata*. **B,** *B. subtilis*.

Germination of ascospores in which 0.5, 0.6, and 0.7 mg/ml of the antibiotic substance was added to the medium was 4.3, 3.3, and 1.3%, respectively; whereas, no germination occurred at or above a concentration of 0.8 mg/ml.

Effect of exposure time to bacteria on ascospore germination. Mean germination of ascospores in the absence of *B. subtilis* was 67.5%. Directly after exposure for 24, 48, and 72 hr, no germination of ascospores was observed. After incubation for 72 hr of the 24- and 48-hr exposed ascospores, a mean germination of 26.7 and 3% was determined, respectively. No germination of ascospores exposed for 72 hr was observed after incubation.

Field tests on control of *E. lata*. Infection of wounds with *E. lata* during 1988 was rather low (23%). During this period, only the antibiotic substance failed to give significant suppression, whereas only *B. subtilis* gave 100% suppression of the fungus (Table 1). In 1989, *B. subtilis*, *B. subtilis* + benomyl, and one test with the antibiotic substance (1989a) significantly suppressed the fungus as compared with the water control.

Thin-layer chromatography. Chromatography of crude antibiotic extract on silica gel plates yielded five bands under UV light, with R_f values 0.47, 0.55, 0.59, 0.65, and 0.75. Extractions from each of these bands and areas between bands showed that only the bands with R_f 0.55 and 0.59 inhibited mycelial growth.

DISCUSSION

The isolate of *B. subtilis* from dieback-affected grapevine wood produced at least two antibiotic substances in vitro that inhibited

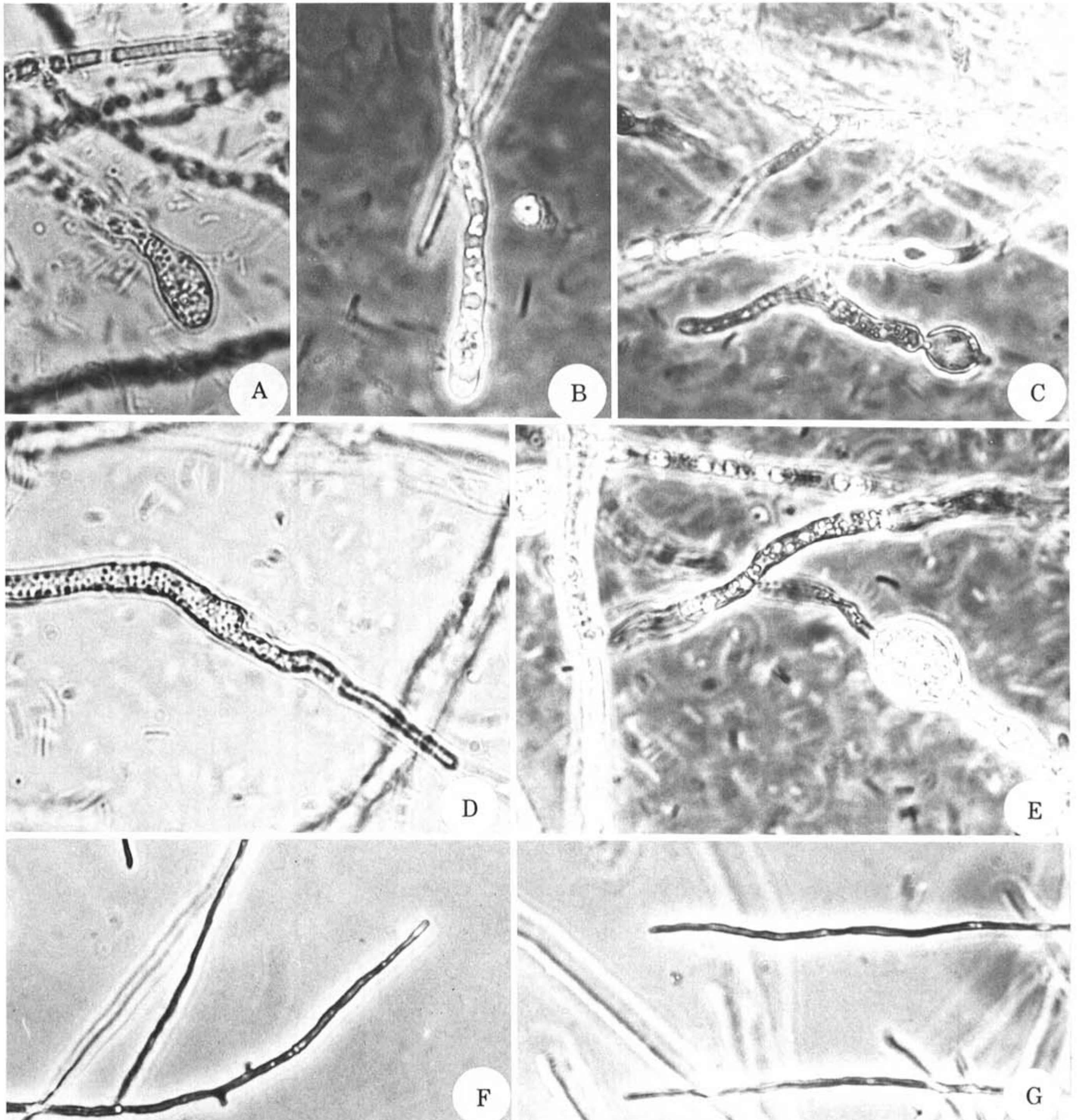

Fig. 2. A–D, Thickened hyphal tips and vacuoles in hyphae of *Eutypa lata* in the presence of *Bacillus subtilis*. **E,** Occurrence of bubbles in hyphae of *E. lata*. **F–G,** Normal hyphae of *E. lata* in the absence of *B. subtilis*.

mycelial growth and ascospore germination of *E. lata*. Mycelial malformation was observed and probably was due to the antibiotic substances interfering with normal growth processes. It is well known that strains of *B. subtilis* produce antibiotic substances that can influence the morphology of fungal mycelium (13,17–19). The inhibition of mycelium that occurred in this study is considered to be antibiosis, as defined by Baker and Cook (4), in which the toxic metabolite may penetrate a cell and inhibit its activity by chemical toxicity. The vacuolar appearance of the mycelium and ascospores probably was due to antibiotics produced by the bacterium, which may have penetrated and caused protoplasmic dissolution (12).

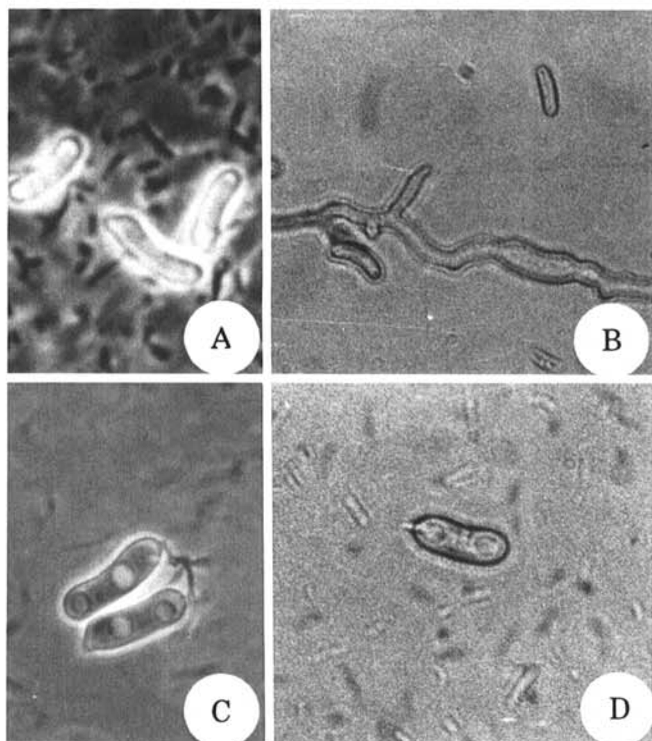

Fig. 3. A, Ungerminated, swollen ascospores of *Eutypa lata* in the presence of *Bacillus subtilis*. **B,** Germinated ascospores in the absence of *B. subtilis*. **C–D,** Vacuoles in ascospores in the presence of *B. subtilis* (×400).

TABLE 1. The influence of different treatments on infection of grapevine pruning wounds by *Eutypa lata* (1988–1989)

	Wounds infected (%)[z]		
Treatment[y]	1988	1989a	1989b
Control (water)	22.9	39.2	45.0
Antibiotic substance (2 mg/ml)	5.0	12.5 x	22.5
Benomyl (10 g/L)	4.0 x	27.5	29.2
Bacillus subtilis (10^8 cells/ml) + benomyl + mineral oil	3.0 x	...	...
B. subtilis + benomyl (0.5 g/L)	...	1.6 x	3.2 x
Benomyl (10 g/L) + mineral oil	1.0 x	...	...
B. subtilis	0.0 x	4.1 x	3.1 x

[y] Treatments were applied to 4-yr-old Riesling grapevines immediately after pruning 2-yr-old wood. The antibiotic substance was an ethanol extract from a 4-day-old broth culture of *B. subtilis*; benomyl concentrations indicate product. Four hours after treatment, wounds were inoculated with an ascospore suspension of *E. lata* (5×10^3 spores per milliliter). Percentage of infection of the wounds was determined 9 mo later by placing cane pieces (taken 25 mm below the wound) on PDA.

[z] Ninety-six wounds examined per treatment in 1988, and 120 wounds examined for each treatment in 1989; ··· = not tested. Means within columns followed by the letter x differ significantly from the control at $P = 0.05$ according to the least significant difference test. Percentages were subjected to a logit transformation for statistical analyses.

The failure of ascospores of *E. lata* to germinate after a 72-hr exposure to *B. subtilis* indicated that the antibiotic substance(s) produced by *B. subtilis* is not only fungistatic but also fungicidal to ascospores of the fungus. Suppression of conidial germination of several fungi by *B. subtilis* has been reported (3,8,9,17,20).

In field tests, biological control of *E. lata* was obtained with *B. subtilis*. Baker et al (2) reported control of bean rust with a strain of *B. subtilis*, and Hall et al (10) found several isolates of *B. subtilis* that reduced stem colonization of silver maple by *V. dahliae*. Control was obtained with the antibiotic substance(s) in our 1989a test, but it was relatively less effective compared with *B. subtilis* and *B. subtilis* + benomyl. Suppression with benomyl was insignificant in the 1988 and 1989b tests, but *B. subtilis* + benomyl gave significant control. It has been reported that benomyl can control Eutypa dieback in grapevines at a concentration of 20 g/L (16). The need for this high concentration may be similar to that reported by Carter and Price (6) who found that benomyl concentration in apricot wounds declined to such a level that it was no longer effective against *E. lata* 1 hr after application.

Five bands, with R_f values of 0.47, 0.55, 0.59, 0.65, and 0.75, separated when the crude extract was developed in thin-layer chromatography. Only two of these, with R_f values 0.55 and 0.59, inhibited *E. lata*. Crude extract from the *B. subtilis* strain used by McKeen et al (17) separated into four biologically active bands, with R_f values 0.48, 0.55, 0.60, and 0.67, which inhibited *Monilinia fructicola* G. Wint. Honey. The 0.55 band, and possibly the other three bands, correspond with the bands of our strain, except the 0.75 band.

The use of *B. subtilis* as a biocontrol agent against *E. lata* may be an economical way to suppress the disease. The form in which the bacterium can be applied commercially needs further research.

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