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Aller au sommaire du numéro

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Résumé de l'article

On a étudié l'influence et les mécanismes d'action *in vitro* et *in vivoBacillus subtilissur* le *Colletotrichum trifolii*, un champignon causant l'anthracnose de la luzerne (*Medicago sativa*). Lors d'essais en chambre de culture, l'application du filtrat du bouillon nutritif du *B. subtilis* a réduit significativement l'incidence et la gravité de la maladie de respectivement 56 à 16% et de 2,0 à 1,2. L'application de cellules lavées du *B. subtilis* n'a pas entraîné une diminution de l'incidence et de la gravité de la maladie. Sur les folioles de la luzerne, le filtrat a causé une réduction de la germination des conidies, la lyse des conidies et une formation réduite d'appressoriums. *In vitro*, il a réduit la germination des conidies, induit la lyse des conidies et la formation de tubes germinatifs enflés. Un antibiotique de la famille des iturines, l'iturine D, serait l'agent actif dans la suppression du *C. trifolii* par le *B. subtilis.*

A note on the antibiotic properties of *Bacillus subtilis* against *Colletotrichum trifolii*

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The influence and mechanisms of action of *Bacillus subtilis* on *Colletotrichum trifolii*, a causal agent of anthracnose of alfalfa (*Medicago sativa*), were studied *in vivo* and *in vitro*. In growth room conditions, a cell-free culture filtrate of *B. subtilis* significantly reduced disease incidence and severity on alfalfa seedlings from 56% to 16% and from 2.0 to 1.2, respectively. Treatment of seedlings with washed cell suspensions of *B. subtilis* had no influence on disease. Applications of crude filtrate on alfalfa leaflets inoculated with *C. trifolii* were associated with reduced germination of conidia, lysis of conidia, and reduced formation of appressoria. Under *in vitro* conditions, crude filtrate reduced germination of conidia, and induced lysis of conidia and the formation of inflated germ tubes on germinating conidia. An antibiotic of the iturin family, iturin D, was tentatively identified as the active compound responsible for the suppressive effect of *B. subtilis* on *C. trifolii*.

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On a étudié l'influence et les mécanismes d'action *in vitro* et *in vivo* du *Bacillus subtilis* sur le *Colletotrichum trifolii*, un champignon causant l'anthracnose de la luzerne (*Medicago sativa*). Lors d'essais en chambre de culture, l'application du filtrat du bouillon nutritif du *B. subtilis* a réduit significativement l'incidence et la gravité de la maladie de respectivement 56 à 16% et de 2,0 à 1,2. L'application de cellules lavées du *B. subtilis* n'a pas entraîné une diminution de l'incidence et de la gravité de la maladie. Sur les folioles de la luzerne, le filtrat a causé une réduction de la germination des conidies, la lyse des conidies et une formation réduite d'appressoriums. *In vitro*, il a réduit la germination des conidies, induit la lyse des conidies et la formation de tubes germinatifs enflés. Un antibiotique de la famille des iturines, l'iturine D, serait l'agent actif dans la suppression du *C. trifolii* par le *B. subtilis*.

Anthracnose, caused primarily by *Colletotrichum trifolii* Bain & Essary, is a highly destructive disease of alfalfa (*Medicago sativa* L.) that is most prevalent in the southern and mid-Atlantic regions of the United States but also occurs in many other regions of the world (Elgin *et al.* 1981; Michaud *et al.* 1988). The pathogen is responsible for stand thinning (Jones *et al.* 1978), yield reduction (Elgin *et al.* 1981) and predisposition to winter injury (Barnes *et al.* 1969). In Canada, *C. trifolii* has been reported on alfalfa in eastern Ontario (Basu 1983) and on clover (*Trifolium* spp.) in Prince Edward Island (Willis 1965). In Ontario, anthracnose is caused primarily by *C. destructivum* O'Gara (Boland and Brochu 1989).

Recently, the potential for biological control of anthracnose using microor-

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ganisms that were originally selected for efficacy against other foliar plant pathogens was examined (Douville 1990; Douville and Boland 1989; Douville *et al.* 1990). Of 11 microorganisms that were evaluated for the ability to suppress anthracnose of alfalfa in greenhouse trials, one isolate of *Bacillus subtilis* (Ehrenberg) Cohn was shown to reduce the severity of disease on a susceptible cultivar. The objectives of this study were to investigate the influence and mechanisms of action of the observed suppression of *C. trifolii* by *B. subtilis*.

The isolate of *B. subtilis* used in this study was isolated from soil by Dr. B.H. MacNeil, University of Guelph. Agar cultures were grown on potato dextrose agar (PDA) at 22-25°C for 5-7 d in the dark. Liquid suspension cultures were grown by transferring inoculum from an actively-growing culture on PDA to 100 mL of potato dextrose broth (PDB) in a 250 mL erlenmeyer flask. Liquid suspension cultures were incubated for 48 h on a rotary shaker at 100 r min^{-1}. For experimental treatments, cell suspensions of *B. subtilis* were prepared by adjusting the concentration of cells to 1 x 10^8 cfu mL^{-1} using a spectrophotometer at 660 nm. Washed cell suspensions were prepared similarly except that cells were centrifuged and resuspended three times consecutively at 15 000 *g* for 20 min in a Sorvall RC2-B centrifuge. Cell-free crude filtrate was prepared from liquid suspension cultures by centrifugation at 15 000 *g* for 20 min in a Sorvall RC2-B centrifuge. The supernatant was then filtered through a 0.22 µm filter to obtain an undiluted cell-free crude filtrate of the bacterium. Unless indicated otherwise, all crude filtrates were adjusted to pH 7.2 before use.

Colletotrichum trifolii (race 1) was obtained from the American Type Culture Collection (# 42881) and was cultured on PDA at 22-25°C for 7-10 d. Conidia were harvested by washing the surface of the culture with sterile deionized water amended with 0.01% Tween 20 (Fisher Scientific). The concentration of conidia in the resulting suspensions was adjusted to 2 x 10^5 spores mL^{-1} using a haemocytometer.

Alfalfa seedlings (cv. Saranac) were grown for 21 d in a growth room maintained at 22-25°C with a 16 h d^{-1} photoperiod to study the influence of *B. subtilis* on disease incidence. In the first growth room experiment, 25 mL of washed cell suspension of *B. subtilis* and 25 mL of undiluted crude filtrate were individually applied to 48 plants. A control treatment of 25 mL of deionized water amended with 0.01% Tween 20 was also applied. In the second growth room experiment, 20 mL each of washed cell suspension and undiluted crude filtrate were applied to 60 plants. Immediately after application of the treatments, the plants were covered with plastic bags for 24 h to allow the bacterial population to stabilize. The bags were then removed and the plants were challenge-inoculated with 20 mL of 2 x 10^5 conidia mL^{-1} of *C. trifolii*. The plants were enclosed in plastic bags for an additional 48 h and were evaluated for disease incidence and severity 14 d following application of the pathogen. Treatments were arranged in a randomized complete block experimental design with eight and six replications in the first and second experiments, respectively. Seedlings were individually rated for disease severity on a scale of 1-5 where: 1 = healthy plants and 5 = dead plant (Ostazeski *et al.* 1969). The mean disease severity of plants within each replication was used to calculate a disease severity index (DSI) for each treatment. Disease incidence was expressed as the percentage of plants with disease symptoms compared to the number of plants evaluated. Disease severity and incidence values were transformed for statistical analysis using the arcsin square root transformation to improve normality and homogeneity of variances. Means were separated by a protected LSD test (Snedecor and Cochran 1980).

To determine the influence of *B. subtilis* on the pathogen, leaflets from plants sprayed with water (control) and with crude filtrate of *B. subtilis* were examined 48 h after inoculation with *C. trifolii*. Leaflets were placed on a glass microscope slide, covered with a coverslip, and stained with lactophenol-cotton blue. Conidia of *C. trifolii* were observed at random under 400 X magnification and the percentage of germinated conidia was determined. A conidium was considered germinated if the germ tube was longer

than one-half the length of the conidium. The percentage of total conidia (ungerminated + germinated) and the percentage of germinated conidia that produced appressoria were also determined. At least 100 conidia were evaluated in each repetition of the control treatments and at least 60 conidia in each of the crude filtrate treatments.

Aliquots of the crude filtrate were diluted with sterile distilled water to final concentrations of 0, 3.3, 13.3, 33.3 and 100% to assay the activity of the crude filtrate *in vitro*. Each dilution was adjusted to pH 7.2. Experimental treatments consisted of 10 µL of each of the crude filtrate dilutions mixed with 10 µL of a 2 x 10^5 conidia mL^{-1} suspension of *C. trifolii* on 2% water agar. A control treatment consisted of 10 µL deionized water mixed with 10 µL of spore suspension of *C. trifolii*. One-hundred conidia in each of four replications were evaluated for the percentage of germination, morphological abnormalities, and cytological damage after 6 and 18 h of incubation. Additional observations were made after 48 and 72 h of incubation.

Crude filtrate was fractionated according to molecular weight in Sephadex G-25 gel (Nap-25, Pharmacia). The fractionation range was 1000-5000 Da. Fractions of 0.5-1.5 mL were collected and individually bioassayed using the method described previously. Control treatments consisted of conidia of *C. trifolii* in water or in crude filtrate.

To concentrate and extract active compounds, 1.2 L of the crude filtrate were precipitated by adjusting to pH 2.5 with 3 *M* HCl (Gueldner *et al.* 1988; Vanittanakom *et al.* 1986). The precipitate was collected by centrifugation at 10 000 *g* for 10 min and then extracted twice with 100% ethanol. The extract was centrifuged at 4000 *g* for 5 min and the precipitate was discarded. The supernatant was concentrated to a volume of approximately 10 mL by evaporation at 60°C.

The concentrated solution was spotted onto silica gel-60 F-254 TLC strips (Mandel Scientific). Each strip was developed in solvent A (chloroform:methanol:water 65:25:4) for a distance of 15 cm. The TLC strip was then cut horizontally in sections of 1 cm. For the detection of biological activity, each section was placed at a distance of 4 cm from a 6-mm-diam agar plug of *C. trifolii* on a 5-cm-diam plastic Petri dish containing PDA medium. The presence of a clear zone of inhibition in the developing colony of *C. trifolii* was monitored over a period of several days.

The concentrated filtrate was also examined with two-dimensional chromatography using solvents A and B (butanol: acetone:water 4:6:1). Compounds were detected using an UV lamp at 254 nm, and by application of a solution of ninhydrin that was then developed at 110°C for 10 min to detect free amino groups (Winkelmann *et al.* 1983). Compounds which are insoluble in water, such as lipids, were detected by spraying the plates with water (Bobitt 1963). Rf values of detected compounds were compared with published Rf values of antibiotics obtained under similar experimental conditions (Besson and Michel 1988; Ebata *et al.* 1969; Schreiber *et al.* 1988; Vanittanakom *et al.* 1986). Rf values were considered to be the same if they were within 0.02 units. All experiments were conducted at least twice.

Applications of washed cells of *B. subtilis* to 3-wk-old seedlings of alfalfa did not result in a significant reduction of anthrac-

Table 1. Effect of washed cells and crude filtrate of *Bacillus subtilis* on incidence and severity of anthracnose of alfalfa[a]

Treatment	Incidence	Severity[b]
Control (water)	56 a	2.0 a[c]
Washed cells	67 a	2.1 a
Crude filtrate	16 b	1.2 b

[a] Three-wk-old alfalfa seedlings (cv. Saranac) were sprayed with 25 mL of crude filtrate or a washed suspension of 1 x 10^8 cfu mL^{-1} of *B. subtilis*. Seedlings were then challenge - inoculated 24 h later with 20 mL of 2 x 10^5 conidia mL^{-1} of *C. trifolii*.

[b] Mean disease severity index of 108 plants that were evaluated 14 d after pathogen inoculation using a 1 to 5 scale where: 1 = healthy plant and 5 = dead plant.

[c] Means were transformed with an arcsin square root transformation for statistical analyses. Means within a column followed by the same letter are not significantly different ($P = 0.05$) according to a protected Least Significant Difference (LSD) test.

Table 2. Effect of crude filtrate of *Bacillus subtilis* on the germination and infection processes of *Colletotrichum trifolii* on alfalfa[a]

Variable	Treatment	
	Filtrate	Control
Number of conidia	124	242
Lysis of conidia	yes	no
Germination (%)[b]	49.8	66.4
Inflated germ tubes (%)[c]	1.0	0
Appressorium formation (%)[d]	16.5	68.3
Appressorium formation - total (%)[e]	6.5	44.5
Appressorium color	dark	dark
Appressorium morphology	round	round
Appressorium position	near or on junction of cells	

[a] Numbers are the means of observations from two repetitions of the experiment.

[b] The percentage of germination was determined on well-stained conidia only.

[c] The percentage of inflated germ tubes was determined on the number of germinated conidia.

[d] The percentage of appressorium formation was determined on germinated conidia only.

[e] The percentage of total appressorium formation was determined on 100 well-stained conidia (ungerminated + germinated).

nose disease incidence or severity (Table 1). However, applications of crude filtrate obtained from the same isolate of *B. subtilis* did significantly suppress disease incidence from 56% in the control treatment to 16% in the filtrate treatment (71% reduction). Disease severity was also suppressed from 2.0 in the control treatment to 1.2 in the filtrate treatment (Table 1).

Antibiotics present in the crude filtrate of *B. subtilis* appeared to be responsible for the suppression of anthracnose of alfalfa. In particular, the crude filtrate of *B. subtilis* reduced the percentage of conidia that germinated from 66.4% on control plants to 49.8% on plants sprayed with filtrate (Table 2), and reduced the percentage of germinated conidia that developed appressoria from 68.3% on control plants to 16.5% on plants sprayed with filtrate (Table 2). In addition, the crude filtrate was associated with lysis of ungerminated conidia.

The filtrate of *B. subtilis* affected several stages in the germination of conidia of *C. trifolii* and the infection of susceptible plants. The percentage of germinated well-stained conidia decreased logarithmically with increasing concentration of the culture filtrate of *B. subtilis* (Figure 1a). This decrease in the percentage of germination was observed after 6 h (R^2=0.95)

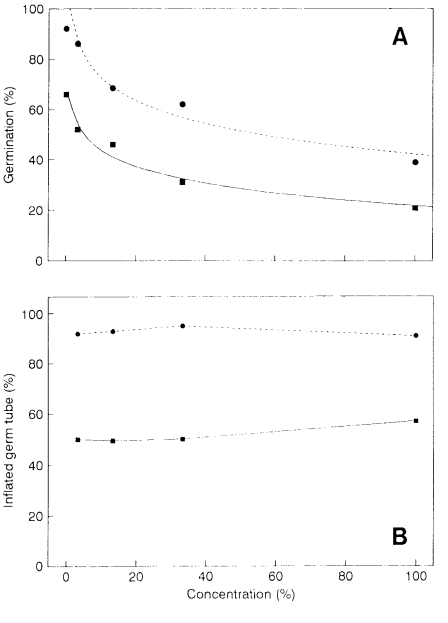

Figure 1. Influence of concentration of crude filtrate of *Bacillus subtilis* on *a*) germination of conidia of *Colletotrichum trifolii* and *b*) the percentage of conidia of *C. trifolii* with inflated germs tubes. The relationship was determined by mixing 10 µL of each concentration of crude filtrate with 10 µL of a 2 x 10^5 conidia mL^{-1} suspension of *C. trifolii* on water agar. (■ = 6 h of incubation, ● = 18 h of incubation).

and after 18 h (R^2=0.96). The percentage of germinated conidia in the undiluted crude filtrate was 20.5% after 6 h and 38% after 18 h. Corresponding values for the control treatments after 6 and 18 h were 65.7 and 92.2%, respectively.

In addition, the percentage of germinated conidia that formed appressoria was reduced by the presence of the filtrate. No disturbance of orientation, morphology, or colour of appressoria was observed. It appears, therefore, that the germ tubes were primarily affected in their ability to produce appressoria. It is clear that germ tubes were affected by the crude filtrate as approximately 50% of the germinated conidia had inflated germ tubes (20-30 μm in diam) *in vitro* after 6 h, and 90% after 18 h. The concentration of crude filtrate did not have a significant effect on the percentage of germinated conidia that developed inflated germ tubes (Figure 1b). Although inflated hyphae were rarely observed on leaves, it is possible that active substances, while not inducing inflated germ tubes on leaf surfaces, disrupted the development of germ tubes sufficiently to decrease the formation of appressoria. Induction of inflated germ tubes by *B. subtilis* has been reported against several fungi, including *Sclerotiorum cepivorum* (Backhouse and Stewart 1989), *Colletotrichum falcatum* Went (Vasudeva *et al.* 1958) and *Alternaria daturae* Peck (Rai 1975).

The fungicidal influence of filtrate of *B. subtilis* is supported by a 50% reduction in the number of conidia that were well stained on filtrate-treated plants. Ungerminated, non-staining conidia without cytoplasmic contents were commonly observed, especially after 48 h exposure to filtrate. In addition, lysis of conidia was observed during the *in vivo* test. Similarly, Baker *et al.* (1985) observed that the cytoplasm of about 30% of the conidia of *Uromyces appendiculatus* (Pers.:Pers.) Ung [syn.: *U. phaseoli*] had ballooned after application of *B. subtilis* on plants.

Two active fractions that suppressed germination of conidia were obtained following Sephadex column chromatography of crude filtrate but only one active compound from the combined 4.5-9.5 mL of eluate was detected using a bioautogram on PDA following thin-layer chromatography of semi-purified crude extract. The identification of antibiotic compounds within crude filtrate of *B. subtilis* by pH precipitation, ethanol extraction, and thin layer chromatography produced 10-12 detectable bands. However, when individual bands were assayed for activity against *C. trifolii*, a zone of inhibition of 10 mm was evident only for the compound corresponding to Rf values of 0.18-0.22 in solvent A. This compound was ninhydrin negative and water positive.

Similarly, crude filtrate was examined for potential antibiotic compounds using two-dimensional, thin-layer chromatography in solvents A and B. The active compound identified in the previous studies had Rf values of 0.17-0.20 in solvent A and 0.33-0.38 in solvent B. The compound was ninhydrin negative and water positive.

The Rf values obtained for the active compound identified in the crude filtrate of *B. subtilis* are similar to values obtained for a known antibiotic produced by *B. subtilis*, iturin D (Rf = 0.18 in solvent A, 0.35 in solvent B). None of the remaining compounds that were detected on the TLC studies possessed Rf values similar to other known antibiotics produced by *B. subtilis* (e.g. other iturins, bacillomycins, BS1, bacilysin or mycosubtilin). Iturin D is also characterized as ninhydrin negative, water positive, and active against filamentous fungi (Besson and Michel 1987). Therefore, we conclude that at least one of the active components of crude filtrate of *B. subtilis* used in these studies is the antibiotic iturin D.

It is possible that the second active compound from the combined 1.0-3.5 mL of eluate was lost or degraded during the pH precipitation and ethanol extraction purification. Another possibility is that the activity of the second active compound is nutrient dependent and therefore would be less inhibitory in the bioautogram on PDA, which is a nutrient-rich medium.

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