BIOLOGICAL CONTROL 4, 125–131 (1994)

Phylloplane *Pseudomonas* spp. Enhance Disease Caused by *Colletotrichum coccodes* on Velvetleaf

W. G. D. FERNANDO, A. K. WATSON, AND T. C. PAULITZ[1]

Department of Plant Science, Macdonald Campus of McGill University, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9

Received April 27, 1993; accepted December 14, 1993

The effects of phylloplane bacteria on the conidial germination, germ tube growth, appressoria formation, and disease severity of the mycoherbicide agent *Colletotrichum coccodes* were investigated. Seven isolates of fluorescent *Pseudomonas* spp. and two other bacterial isolates were recovered from the mature leaves of velvetleaf (*Abutilon theophrasti*). Co-inoculation of *C. coccodes* with each of three isolates of *Pseudomonas* spp. significantly increased the number of lesions and disease severity caused by *C. coccodes* on velvetleaf seedlings in two growth chamber experiments, and also accelerated the appearance of symptoms. All bacterial isolates reduced conidial germination and germ tube branching of *C. coccodes* and three isolates increased the frequency of germ tubes with appressoria on cellophane membranes overlaid on leaf disks of velvetleaf. All isolates reduced conidial germination 30 h after co-inoculation with *C. coccodes* on detached leaves, but increased the relative frequency of dark-colored appressoria without germ tubes and decreased the total germ tube length. These results suggest that certain phylloplane bacteria may enhance the efficacy of mycoherbicides by stimulating the formation of appressoria and reducing the saprophytic preinfection mycelial growth of the pathogen on the phylloplane. This acceleration of infection may decrease the critical period of moisture required for infection in the field. © 1994 Academic Press, Inc.

KEY WORDS: ***Colletotrichum coccodes; Pseudomonas* spp.; biocontrol; velvetleaf; enhancement; conidia; germ tubes; appressoria.**

INTRODUCTION

Velvetleaf (*Abutilon theophrasti* Medic.) is a major problem weed in corn and soybean fields in the eastern parts of Canada and the United States (Warwick and Black, 1988). Control of this weed is difficult, because of its sporadic germination pattern and its general resistance to control tactics. Preemergence and postemergence herbicides are used to suppress velvetleaf, but a herbicide-resistant biotype of velvetleaf has been found, and alternative and complementary measures of control are needed (Andersen and Gronwald, 1987; Gronwald *et al.*, 1987; Ritter, 1986). The fungus *Colletotrichum coccodes* (Wallr.) Hughes, which causes a leaf-anthracnose on velvetleaf, has been tested and found to give effective biological control (Gotlieb *et al.*, 1987; Wymore *et al.*, 1988). One of the main constraints for effective biological control in the field has been that brief dew periods are not sufficient for the fungus to germinate and penetrate the host tissue. Researchers in the UK working on agricultural crops and different *Colletotrichum* spp. found that appressorial formation was increased in the presence of *Pseudomonas* sp. or cell-free siderophores (McCracken and Swinburne, 1979; Blakeman and Parbery, 1977; Slade *et al.*, 1986; Brown and Swinburne, 1981). After a conidium of *Colletotrichum* germinates on the leaf surface, a germ tube grows over the surface of the leaf, eventually forming an appressorium. The appressorium is a thick-walled attachment structure from which the fungus forms a penetration peg that pierces through the epidermal wall and initiates infection. Schisler *et al.* (1991) reported decreased shoot dry weight and number of leaves per plant of *Sesbania exaltata* Rydb. ex Hill co-inoculated with *Colletotrichum truncatum* (Schwein.) Andrus and Moore and several bacterial isolates.

The objective of this study was to investigate the possibility of enhancing and accelerating infection of velvetleaf by *C. coccodes* by co-inoculating the fungus with *Pseudomonas* spp. isolated from the phylloplane. We examined conidial germination and appressorial formation on cellulose membranes and intact leaves. Disease severity on seedlings was studied in the growth chamber. Preliminary results have been reported (Fernando *et al.*, 1992).

MATERIALS AND METHODS

Isolation of Pseudomonas spp. from the Phylloplane of Velvetleaf

Leaves of velvetleaf were collected from three different fields of corn and soybean in Ste-Anne-de-Bellevue,

[1] To whom correspondence should be addressed.

1049-9644/94 $6.00

Quebec. The nonsenescent leaves were removed from plants of two ages (young and mature) and three leaf classes (young, medium, and mature). In site 3, only leaves from older plants were collected since no young plants were present. One gram of leaf tissue was sonicated in 99 ml of sterile distilled water (Branson 1200, Branson Cleaning Equipment Company, Shelton, CT) for 30 s. This suspension was serially diluted and 0.1 ml was spread on half-strength nutrient agar amended with 100 ppm cycloheximide (Sigma Chemicals, St. Louis, MO). Plates were incubated at 26°C for 48 h. Representative colonies were selected and streaked on nutrient agar + 10% glycerol (NAGly) medium to obtain pure cultures. These bacterial isolates were further tested on King's B medium (King *et al.*, 1954) to detect fluorescent *Pseudomonas* spp. All bacterial isolates (fluorescent *Pseudomonas* spp. and nonfluorescent bacteria) were stored in 1 ml nutrient broth + 10% glycerol (NBGly) in 1.5-ml vials at −80°C. All seven strains of fluorescent pseudomonads isolated from the leaves were tested.

Preparation of Fungal Inoculum

A culture of *C. coccodes* isolate AG-3 (Gotlieb *et al.*, 1987) was used in all experiments. The *C. coccodes* was maintained on potato dextrose agar (PDA) at 26°C. Two 0.5-cm plugs from the actively growing margins of a colony were transferred to 100 ml of Richard's modified V-8 broth medium (Walker, 1980) and incubated at 25°C for 7 days on a rotary shaker (Lab-Line Orbital Shaker Model 3520, Melrose Park, IL) (250 rpm). The spores were harvested by filtering the culture through four layers of cheesecloth and centrifuging the filtrate at 2000*g* for 10 min. The supernatant was decanted and the spore pellet was resuspended in sterile distilled water. Spores were counted with a hemocytometer and adjusted to desired concentrations.

Preparation of Bacterial Inoculum

Bacterial isolates selected for experiments were streaked onto NAGly plates directly from the −80°C cultures and incubated at 26°C for 48 h. A loop of bacteria was transferred to 100 ml of NBGly and incubated on a rotary shaker overnight at 26°C, 150 rpm. The bacterial suspension (stationary phase) was centrifuged at 2000*g* for 15 min and the supernatant was decanted. The cell pellet was resuspended in 0.1 *M* $MgSO_4$ and centrifuged again. The pellet was resuspended in 0.1 *M* $MgSO_4$ and adjusted to the desired concentration based on absorbance at 640 nm in comparison with a standard curve developed for a strain of *Pseudomonas fluorescens*.

Effect of Bacteria on Disease Expression by C. coccodes

Velvetleaf seeds collected from the field were placed on moist filter paper in a glass petri dish and incubated at 5°C for 48 h, then 30°C for 24 h. Germinated seeds were sown, four per pot in 4-cm-diameter pots containing potting medium (Promix BX, Premier Brands Inc., Stamfort, CT). Seedlings were grown in growth chambers (14 h photoperiod, 300 $\mu E/m^2/s$, 24/20°C day/night temperature) and thinned to three per pot before use. In the first experiment, seedlings at the first leaf stage (1 week after germination) were inoculated with bacterial isolates. All seven fluorescent *Pseudomonas* spp. isolated from velvetleaf and two nonfluorescent bacterial isolates were selected as treatments; there was one untreated control. All plants were inoculated with *C. coccodes*. The bacterial suspensions were adjusted to 10^9 cells per milliliter, and Pelgel (Lipha-Tech, Milwaukee, WI) was added as a sticking agent at 0.1% concentration to the bacterial suspension. The cotyledons and the first leaf were immersed in the bacterial suspensions. The control was immersed into a 0.1% suspension of Pelgel. The pots were then covered with large plastic bags and placed in the growth chamber. The plastic bags were removed after 16 h, and the pots were kept in the growth chamber for a further 32 h. Forty-eight hours after bacterial inoculation, the pots including the control treatments were randomly arranged in a spray chamber (Research Instrument Manufacturing Co., Ltd., Guelph, Ontario, Canada) and inoculated with conidia of *C. coccodes* inoculum at 10^9 spores per square meter in 0.1% Pelgel at a rate of 500 liters per ha (2×10^6 spores per milliliter). The pots were immediately transferred to a dark dew chamber (Percival Model E-54 UDL, Boone, IA) for 18 h at 24°C and subsequently returned to the growth chamber and left for 2 additional weeks. Four replicates per treatment with three plants per replicate pot were arranged in a completely randomized design. The number of lesions and the disease severity on leaves were recorded. Disease severity was rated on a 0–4 scale (0 = no disease, 1 = 0–25% diseased area, 2 = 26–50% diseased area, 3 = 51–75% diseased area, and 4 = 76–100% diseased area or senescing leaves). The experiment was done three times.

To determine whether multiple inoculations of bacteria would further enhance disease, a second experiment similar to the above was conducted. In this experiment, a second inoculation of the bacteria and *C. coccodes* was carried out 10 days after the first applications and the plants were kept for 18 h in a dark dew chamber. *C. coccodes* was inoculated 1 day after the second bacterial treatment. The plants were at the fourth leaf stage when the second inoculation was done. The experiment was done twice. The disease severity rating was determined as above.

Effect of Bacteria on Conidial Germination and Appressoria Formation

Cellulose membrane experiment. Cellulose membranes (dialysis membrane, Canlab, Baxter Co., Missis-

sauga, Ontario, Canada) were cut into 1-cm^2 pieces and autoclaved for 20 min in distilled water to remove any nutrients. The membranes were then placed on a detached first leaf of velvetleaf from a 12-day-old plant, and the leaf was placed on a moistened filter paper in a petri plate. Ten microliters of bacterial inoculum (prepared as described, but without Pelgel) at 10^8 cells/ml was spread on the membrane. The fungal inoculum (10^7 conidia per milliliter), prepared as above but without Pelgel, was misted onto the membrane immediately after the application of bacteria. The plates were covered and incubated in the dark with moist paper towels in an enclosed plastic box at 24°C for 24 h.

Intact leaf experiment. One-week-old plants grown in the growth chamber were inoculated as in the seedling assay experiments, except that both bacteria and fungi were inoculated on the same day (Day 7) without Pelgel being added to the inoculum solution. The plants were kept in a dark dew chamber for 18 h at 24°C and then transferred to the growth chamber for the rest of the experimental period.

Leaves or cellulose membranes were sampled at 4, 10, 24, and 30 h and transferred to a 50:50 mixture of glacial acetic acid and 95% ethanol. Pieces of absorbent cotton were soaked in a 50:50 mixture of glacial acetic acid + 95% ethanol and placed in plastic petri dishes (100 mm diameter). A filter paper was placed on top of the cotton. The leaves or membranes were placed on the filter paper and the plates were sealed with parafilm (American National Can, Greenwich, CT) (Fernando *et al.*, 1993). After 24–48 h, the cleared leaves or cellulose membranes were mounted on glass slides and stained with 0.01% cotton blue in lactoglycerine (1:2:1 water:glycerol:lactic acid, v/v). One hundred conidia on each sample were counted and classified as nongerminated, germinated with germ tube but no appressorium (Gt-a), or germinated with germ tube and appressorium (Gt + a). In addition, the number of appressoria without germ tubes (Awt) was also counted and expressed as a percentage of the total number of conidia. Video microscopy was used to measure the length of germ tubes and the number of branches. Slides were observed on an Olympus BHS phase-contrast microscope and images were recorded on videotape with a JVC TKS 300 black and white video camera and a Sony SVO 140 video recorder. Images were played back on a JVC TM 920 video monitor, where the length of the germ tubes and the number of branches were measured. A standard micrometer was also included on the video screen to provide a conversion factor for each magnification. Both experiments were repeated once.

In Vitro Mycelial Inhibition

All bacteria used in the above experiments were tested in plate assays to determine their potential to inhibit mycelia of *C. coccodes*. The pathogen was grown on PDA for 7 days, and 0.3-cm plugs were transferred to the center of nutrient agar plates. A 10-μl drop of bacterial inoculum was prepared as above and placed 3 cm away from the fungal plug on either side. Seven days after inoculation, the radii of fungal colonies were measured in the direction of the bacterial colony and away from the bacterial colony. Percentage of inhibition was calculated by the equation $100 \times (R1\text{-}R2)/R1$, where R1 is the maximum radius away from the bacteria and R2 is the radius directly opposite the bacteria.

Survival of a Pseudomonas Strain on Velvetleaf

The survival of bacteria on leaves of velvetleaf was studied, especially during the first 24 h when *C. coccodes* conidia would be expected to germinate and infect the tissue. A fluorescent *Pseudomonas* sp. (isolate Ps1), which showed a stimulation of conidial germination of *C. coccodes*, was selected. A bacterial suspension was prepared as described above. Spontaneous rifampicin-resistant mutants were selected on King's B medium (King *et al.*, 1954), amended with 100 ppm of rifampicin (Sigma), and were stored at −80°C in NBGly. A mutant Ps1R with a growth curve similar to that of the wild-type isolate was selected for the population study. Bacterial inoculum was prepared as described above, and 1-week-old leaves were dipped in this bacterial suspension (10^9 cells per milliliter). Plants were placed in a growth chamber, in a completely randomized design with five replicates per treatment per sampling time. The bacterial population densities on the leaf were sampled at 0, 4, 24, 30, 48, 240, 312, and 336 h. The cotyledons were weighed and placed in 9 ml sterile distilled water. The leaves were sonicated for 30 s and the suspension was serially diluted. One-tenth of a milliliter of each dilution was plated onto KB plates amended with 100 ppm rifampicin. Plates were incubated at 26°C for 72 h and single colonies were counted. A sample from uninoculated leaves at each sampling time served as a control treatment.

Data Analysis

All experiments were arranged in a completely randomized design with four or five replicates and repeated at least once. Data were analyzed with one-way analysis of variance (ANOVA) and the means were separated using the LSD test ($P < 0.05$). Analysis was done using the Statistical Analysis System (SAS Institute, 1985). There was no significant interaction between trials and any of the variables measured. The variance of repeated trials was also compared with a Bartlett's Test of Homogeneity of Variances (Steel and Torrie, 1980), which showed no differences in variance between trials. The results of the first trial are presented for the cellophane membrane and intact leaf experiments.

TABLE 1

The Effect of Phylloplane Bacteria on Disease Caused by *C. coccodes* on Velvetleaf Seedlings

Treatment	Experiment 1[a]		Experiment 2	
	Lesions[b]	Severity	Severity[c]	Severity[d]
Ps1 + Cc[e]	12.39 a[f]	3.67 a	3.83 bc	3.75 ab
Ps2 + Cc	13.33 a	2.67 ab	4.71 a	4.75 a
Ps3 + Cc	12.65 a	2.33 abc	4.08 abc	3.15 b
Ps4 + Cc	9.47 ab	2.83 ab	3.71 bcd	3.99 ab
Ps5 + Cc	10.23 ab	2.66 ab	3.37 cd	3.83 ab
Ps6 + Cc	8.82 ab	2.25 bc	3.54 bcd	3.83 ab
Ps7 + Cc	5.50 ab	2.33 abc	3.75 bcd	3.49 ab
Nf8 + Cc	5.91 ab	3.50 ab	3.66 bcd	4.58 ab
Nf9 + Cc	7.15 ab	2.25 bc	4.12 ab	3.91 ab
Cc only	3.08 b	0.99 c	3.08 d	2.08 c
LSD	8.96	1.40	0.72	1.26

Note. LSD ($P = 0.05$). Ps1 to Ps7 are fluorescent *Pseudomonas* isolates; Nf8 and Nf9 are nonfluorescent bacterial isolates. Cc is *Colletotrichum coccodes*.

[a] Number of lesions and disease severity 14 days after inoculation with the pathogen.

[b] Number of lesions per leaf.

[c] Disease severity on cotyledons, 7 days after inoculation.

[d] Disease severity on third true leaf, with two inoculations of the bacterium in each treatment. Rating was done 24 days after inoculation with the pathogen.

[e] Bacteria were inoculated at 10^8 cells per milliliter and Cc at 10^9 conidia per square meter.

[f] Values within a column followed by the same letter do not differ significantly by LSD test at $P < 0.05$.

RESULTS

Of the more than 200 bacterial isolates examined, only 7 were fluorescent *Pseudomonas* spp. and these were isolated mostly from mature leaves.

Effect of Bacteria on Disease Expression by C. coccodes

When the bacteria were inoculated once, three *Pseudomonas* spp. significantly increased the number of lesions on the first true leaf. The disease severity after 14 days (experiment 1) was significantly greater in treatments inoculated with four of the *Pseudomonas* spp. isolates (Ps1, Ps2, Ps4, Ps5) and one nonfluorescent bacterium (Nf8) (Table 1). Symptoms on all bacteria-inoculated plants appeared 4–5 days earlier than in the treatment with *C. coccodes* only (data not shown, experiment 1). The nonbacterial control (*C. coccodes* only) had fewer lesions and lower disease severity than any of the treatments coinoculated with bacteria. In experiment 2 with two bacterial inoculations, the disease severity on the third true leaf was significantly increased by co-inoculation with bacteria (Table 1).

Effect of Bacteria on Conidial Germination and Appressoria Formation

Cellulose membrane experiment. All bacterial treatments significantly reduced the germination of *C. coccodes*, and there was a higher frequency of germ tubes without appressoria (Gt − a) in the *Colletotrichum*-only treatment (Table 2). In the *C. coccodes*-only treatment, there were significantly more branched germ tubes at 24 h, and the germ tubes were longer. There were no appressoria formed at 24 h in the treatment with Nf9 + *C. coccodes* or *C. coccodes* alone (control) (Fig. 1, Table 2). In treatments with Ps1, Ps5, Ps7, and Nf8, the frequency of germ tubes with appressoria (Gt + a) was significantly higher than in the treatment without bacteria.

Intact leaf experiment. The frequency of conidia with germ tubes was significantly less at all sampling times in the bacterial coinoculated treatments than in *C. coccodes*-only treatments (Table 3). The relative frequency of Awt was significantly higher in all bacterial coinoculated treatments than in the treatment without bacteria. We hypothesize that the original germ tubes from which the appressoria were formed had previously lysed, due to autolysis or heterolysis induced by the bacteria. Branching of germ tubes was observed only in the *C. coccodes* control treatment (data not shown). At 10, 24, and 30 h, conidia with germ tubes had branched 21, 33, and 60%, respectively. Germ tube length of the *C. coccodes*-only treatment was significantly greater than that in the bacterial co-inoculated treatments at 10, 24, and 30 h; values for 30 h are given in Table 3.

In Vitro Mycelial Inhibition

Bacterial isolates Ps1, Ps2, and Ps6 inhibited mycelial growth of *C. coccodes* by 21, 11.5, and 13%, respectively. The other strains did not inhibit mycelia on petri plates (data not shown).

Survival of Ps1R on Velvetleaf

The population density of Ps1R did not increase over time. It remained around 1.0×10^7 per gram of fresh leaf weight during the first 24-h period. By 30 h, it had decreased to 7.4×10^5 per gram of fresh leaf weight and by 14 days to 6×10^2 per gram of fresh leaf weight. No bacteria were detected in the noninoculated control (data not shown).

DISCUSSION

The most critical period in biocontrol of a weed in the field is the first 24 h following the application of the pathogen. The spores of the pathogen need free moisture on the leaves to germinate during this period. The second constraint is the latent period of the disease, the

TABLE 2

Germination and Appressoria Formation by Conidia of *C. coccodes* in the Presence of Phylloplane *Pseudomonas* spp. and Other Bacterial Isolates on Cellulose Membranes

Treatments	% Gt − a[a]				% Gt + a[b]	% Br[c]	Germ tube length (μm)
	4 h	7 h	10 h	24 h	24 h	24 h	24 h
Ps1 + Cc	4.66	0.00	8.66	58.00	6.66	0.00	15.00
Ps2 + Cc	2.66	15.32	8.00	45.34	3.34	0.00	7.00
Ps3 + Cc	0.66	0.66	0.66	17.34	1.32	0.00	9.00
Ps4 + Cc	4.66	2.66	6.00	18.00	2.00	0.00	9.00
Ps5 + Cc	8.00	10.66	4.66	64.66	6.00	0.00	13.00
Ps6 + Cc	0.00	6.66	14.66	24.00	2.66	0.00	17.00
Ps7 + Cc	0.00	1.32	4.66	18.00	4.66	0.00	12.00
Nf8 + Cc	0.66	2.00	5.34	25.34	7.34	0.00	35.00
Nf9 + Cc	0.66	4.00	9.34	18.00	0.00	4.00	21.00
Cc only	37.33	40.66	46.66	83.32	0.00	79.34	32.00
LSD	9.14	7.44	5.12	17.60	4.16	3.17	2.74

Note. LSD ($P = 0.05$). Ps1 to Ps7 are fluorescent *Pseudomonas* isolates; Nf8 and Nf9 are nonfluorescent bacterial isolates. Cc is *C. coccodes*, the bioherbicide agent.

[a] % Gt − a = % of germ tubes without appressoria/total number of conidia × 100.

[b] % Gt + a = % of term tubes with appressoria/total number of conidia × 100.

[c] % Br = number of germ tubes with branches/total number of germ tubes × 100. These results are from the first experiment.

period of time between inoculation and symptom expression. If the weed is not controlled at an early stage, it may quickly dominate the field and reduce crop growth. If the weed's growth is reduced at the initial stages by disease, the crop may out-compete the weed (Massion and Lindow, 1986; Paul and Ayres, 1987).

The foliar blight of *C. coccodes* occurred 4 to 5 days earlier on leaves treated with fluorescent *Pseudomonas* spp. than on nontreated leaves. The enhancement of disease by co-inoculation with epiphytic bacteria was also reported on *S. exaltata* (Raf.) Cory inoculated with *C. truncatum* (Schwein.) Andrus and Moore (Schisler *et al.*, 1991). However, in their work, none of the bacteria were identified as fluorescent pseudomonads and there seemed to be no correlation between the isolates that induced maximum disease and the isolates that stimulated the maximum number of appressoria.

On surfaces without bacteria, the conidia tended to form long, branched germ tubes with fewer appressoria. The presence of bacteria stimulated the formation of

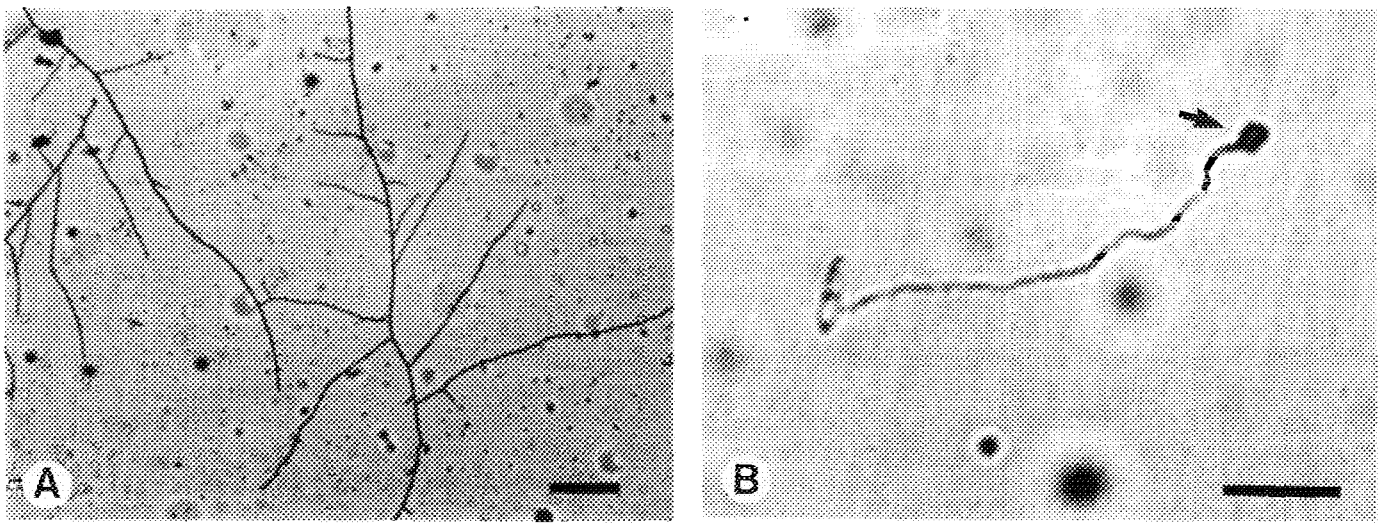

FIG. 1. Germination of conidia of *C. coccodes* on cellulose membranes 24 h after inoculation. (A) Germ tubes ending in numerous branches in *C. coccodes*-only treatment. (B) Germ tube without branches, but ending in an appressorium (arrow) in *C. coccodes* treatment co-inoculated with Ps2 bacterium. Bar, 25 μm.

TABLE 3

Germination and Appressoria Formation by Conidia of *C. coccodes* in the Presence of Phylloplane *Pseudomonas* spp. and Other Bacterial Isolates on Intact Leaves of Velvetleaf

	4 h	10 h		24 h			30 h			
Treatment	% Gt[a]	% Gt	% Gt + a[b]	% Gt	% Gt + a	% Awt[c]	% Gt	% Gt + a	% Awt	Germ length (μm) 30 h
Ps1 + Cc	3.1	11.7	3.2	11.9	9.9	68.1	5.4	2.9	75.2	8.7
Ps2 + Cc	2.0	10.6	0.3	16.1	12.8	50.5	9.3	4.6	59.9	12.8
Ps3 + Cc	4.4	10.2	0.5	15.9	11.1	48.4	6.8	3.9	53.4	8.1
Ps4 + Cc	7.3	13.8	3.7	16.3	7.9	35.8	8.3	4.0	43.3	8.8
Ps5 + Cc	3.1	14.0	7.4	20.8	13.4	63.3	8.1	2.9	60.4	12.4
Ps6 + Cc	5.1	8.6	1.2	15.9	10.5	57.2	15.4	5.3	53.7	11.2
Ps7 + Cc	1.2	9.9	0.2	15.8	8.5	37.5	10.0	5.2	45.8	10.8
Nf8 + Cc	1.9	10.7	2.3	11.3	5.6	36.8	12.1	6.4	49.5	7.8
Nf9 + Cc	7.5	19.6	3.1	10.9	3.9	58.1	8.9	3.6	59.2	9.9
Cc only	30.3	32.9	0.1	57.4	7.9	13.6	48.8	8.4	24.4	18.5
LSL	4.53	4.18	1.83	6.45	3.35	9.14	4.42	1.87	7.66	1.3

Note. LSD ($P = 0.05$). Ps1 to Ps7 are fluorescent *Pseudomonas* isolates; Nf8 and Nf9 are nonfluorescent bacterial isolates. Cc is *C. coccodes*.

[a] % Gt = total number of germ tubes/total number of conidia × 100.

[b] % Gt + a = number of germ tubes with appressoria/total number of conidia × 100.

[c] % Awt = number of appressoria without germ tubes/total number of conidia × 100. These results are from the first experiment.

appressoria on intact leaves and on cellulose membranes. Appressoria are vital for the infection process. Appressoria may play a role in survival (Emmett and Parbery, 1975); the fungus may survive for long periods in the leaf as melanized appressoria, until environmental conditions become more favorable for disease development. Appressorial formations were more numerous on intact leaves than on cellulose membranes, although the latter were in contact with leaf exudates. Parbery and Blakeman (1978) also found that six species of *Colletotrichum acutatum* Simmonds developed more appressoria on leaves than on glass. In our work, the bacterial treatments that most increased disease in greenhouse experiments induced the most Awt.

The rapid induction of appressoria may explain the increase in lesion number and disease severity. However, contrary to our findings, Williamson and Fokkema (1985) found that two common phyllosphere yeasts, *Sporobolomyces roseus* Kluyver and Niel and *Cryptococcus laurentii* var. *flavescens* (Kuff.) Skinner, reduced the lesion density and necrosis on *Zea mays*, caused by *Colletotrichum graminicola* (Ces.) Wils. These yeasts had no effect on conidial germination, superficial mycelial growth, or appressorial formation. Lenne and Parbery (1976) postulated that hyphal lysis of *Colletotrichum gloesporioides* (Penz.) Penz. & Sacc. by phylloplane bacteria was responsible for appressoria without connecting germ tubes. However, we observed that in treatments without bacteria, 24% of the appressoria did not have germ tubes at 30 h, suggesting autolysis by the fungus itself. Blakeman and Parbery (1977) found that appressoria were stimulated by limitations in nutrient availability induced by competing bacteria. High population densities of fluorescent *Pseudomonas* in our experiment may limit the carbon and nitrogen available to *C. coccodes* for saprophytic mycelial growth on the phylloplane. When nutrients are limited, the fungus may respond by autolysis of germ tubes and formation of appressoria and subsequent penetration pegs and by more rapid induction of the parasitic phase of fungal growth. This relationship between the saprophytic and parasitic phase of *C. coccodes* is shown by the negative correlation between appressorial formation and germ tube length and branching. Swinburne (1976) also reported a negative correlation between appressoria formation and germ tube length of *Colletotrichum musae* (Berk. & Curtis) Arx on banana leachate. Increases in appressoria formation may also be due to competition for iron by *Pseudomonas* spp. that produce iron-chelating siderophores (Loper and Buyer, 1991). Swinburne and Brown (1983), working with *C. musae* on banana, found that appressoria formation was greater when 2,3-dihydroxybenzoic acid (DHBA), an iron chelator, was added than in the water treatment. When DHBA-Fe was added, this effect was lost. Progressive lesions were also significantly more in the DHBA treatment, compared to the water treatment. Some of the bacteria that increased disease severity also produced zones of inhibition of mycelial growth *in vitro*, suggesting the formation of antibiotics. These antibiotics may also stimulate the formation of appressoria by stressing the fungus to produce these melanized survival structures. Lenne and Brown (1991) found that several phylloplane *Pseudomonas* spp. and *Bacillus* spp. inhibited both mycelial growth on

agar and germination of conidia of pathogenic and weakly pathogenic isolates of *C. gloeosporioides* (Penz.) Penz. & Sacc. on leaves of *Stylosanthes guianensis* (Aubl.) Sw. However, they did not consider the relationship between reduction of germination and appressoria formation. The bacteria may also produce toxins that predispose the leaves to infection by *C. coccodes*. However, in experiments where seedlings were treated with the *Pseudomonas* strains alone, we did not observe any lesions or symptoms on the plants (data not shown).

Our results reinforce the hypothesis that some phylloplane microbes, especially the *Pseudomonas* spp., influence pathogenic foliar fungi. This knowledge has been exploited to control or reduce the effects of disease-causing fungi (Windels and Lindow, 1985). However, some microbes also enhance disease and these could be utilized to optimize the efficacy of mycoherbicides applied in the field by reducing the critical period of time prior to infection, when the fungus is sensitive to environmental constraints.

Time of application of the bacterium in the field will be an important factor in influencing efficacy of the bioherbicide. Under natural conditions, young leaves may not support a population of appressoria-stimulating microbes. Inundative inoculation with these microbes at an early stage may render young seedlings more susceptible to the mycoherbicide. Formulations of the fungus and the bacterium have to be developed either together or separately to achieve maximum efficacy. Knowledge of the mode of action of these appressorial-stimulant microorganisms will be very useful in the development of an effective biological weed control strategy.

ACKNOWLEDGMENTS

This work was supported by a grant from Martinex Science, Inc., Montreal, Canada and the Natural Sciences and Engineering Research Council of Canada. We gratefully acknowledge the technical assistance of C. Hoffert and P. Masilamany. We also thank Dr. W. E. Sackston for reviewing the manuscript.

REFERENCES

Andersen, R. N., and Gronwald, J. W. 1987. Noncytoplasmic inheritance of atrazine tolerance in velvetleaf (*Abutilon theophrasti*). *Weed Sci.* **35,** 496–498.

Blakeman, J. P., and Parbery, D. G. 1977. Stimulation of appressorium formation in *Colletotrichum acutatum* by phylloplane bacteria. *Physiol. Plant Pathol.* **11,** 313–325.

Brown, A. E., and Swinburne, T. R. 1981. Influence of iron and iron chelates on formation of progressive lesions by *Colletotrichum musae* on banana fruits. *Trans. Br. Mycol. Soc.* **77,** 119–124.

Emmett, R. W., and Parbery, D. G. 1975. Appressoria. *Annu. Rev. Phytopathol.* **13,** 147–167.

Fernando, W. G. D., Watson, A. K., and Paulitz, T. C. 1992. Interactions of phylloplane bacteria in the enhancement of disease caused by the bioherbicide *Colletotrichum coccodes* on velvetleaf. *Phytopathology* **82,** 1081. [Abstract].

Fernando, W. G. D., Watson, A. K., and Paulitz, T. C. 1993. A simple technique to observe conidial germination on leaf surfaces. *Mycologist* **7,** 188–189.

Gotlieb, A. R., Watson, A. K., and Poirier, C. 1987. First report of *Colletotrichum coccodes* on velvetleaf. *Plant Dis.* **71,** 281.

Gronwald, J. W., Andersen, R. N., and Yec, C. 1987. Enhanced atrazine metabolism in an atrazine tolerant velvetleaf (*Abutilon theophrasti* Medic.) biotype. *Plant Physiol.* **83,** 135. [Abstract].

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44,** 301–307.

Lenne, J. M., and Brown, A. E. 1991. Factors influencing the germination of pathogenic and weakly pathogenic isolates of *Colletotrichum gloeosporioides* on leaf surfaces of *Stylosanthes guianensis*. *Mycol. Res.* **95,** 227–232.

Lenne, J. M., and Parbery, D. G. 1976. Phyllosphere antagonists and appressorium formation in *Colletotrichum gloesporioides*. *Trans. Br. Mycol. Soc.* **66,** 334–336.

Loper, J. E., and Buyer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. *Mol. Plant–Microbe Interact.* **4,** 5–13.

Massion, C. L., and Lindow, S. E. 1986. Effects of *Sphacelotheca holci* infection on morphology and competitiveness of Johnsongrass (*Sorghum halepense*). *Weed Sci.* **34,** 883–888.

McCracken, A. R., and Swinburne, T. R. 1979. Siderophores produced by saprophytic bacteria as stimulants of germination of conidia of *Colletotrichum musae*. *Physiol. Plant Pathol.* **15,** 331–340.

Parbery, D. G., and Blakeman, J. P. 1978. Effect of substances associated with leaf surfaces on appressorium formation by *Colletotrichum acutatum*. *Trans. Br. Mycol. Soc.* **70,** 7–19.

Paul, N. D., and Ayres, P. G. 1987. Effects of rust infection of *Senecio vulgaris* on competition with lettuce. *Weed Res.* **27,** 431–441.

Ritter, R. L. 1986. Triazine resistant velvetleaf and giant foxtail control in no-tillage corn. *Proc. Northeast. Weed. Sci. Soc.* **40,** 50–52.

SAS Institute, 1985. "SAS Language Guide for Personal Computers." SAS Inst. Cary, NC.

Schisler, D. A., Howard, K. M., and Bothast, R. J. 1991. Enhancement of disease caused by *Colletotrichum truncatum* in *Sesbania exaltata* by coinoculating with epiphytic bacteria. *Biol. Control* **1,** 261–268.

Slade, S. J., Swinburne, T. R., and Archer, S. A. 1986. The role of a bacterial siderophore and of iron in the germination and appressorium formation by conidia of *Colletotrichum acutatum*. *J. Gen. Microbiol.* **132,** 21–26.

Steel, R. G. D., and Torrie, J. H. 1980. "Principles and Procedures of Statistics: A Biometrical Approach," p. 633. McGraw-Hill, New York.

Swinburne, T. R. 1976. Stimulants of germination and appressoria formation by *Colletotrichum musae* (Berk. & Curt.) Arx. in banana leachate. *Phytopathol. Z.* **87,** 74–90.

Swinburne, T. R., and Brown, A. E. 1983. Appressoria development and quiescent infections of banana fruit by *Colletotrichum musae*. *Trans. Br. Mycol. Soc.* **80,** 176–178.

Walker, H. L. 1980. *Alternaria macrospora* as a potential biocontrol agent for spurred anoda: Production of spores for field studies. *U.S. Sci. Educ. Adm. Adv. Agric. Tech. South. Ser., No. 12.* p. 5.

Warwick, S. I., and Black, L. D. 1988. The biology of Canadian weeds. 90. *Abutilon theophrasti*. *Can. J. Plant Sci.* **68,** 1069–1085.

Williamson, M. A., and Fokkema, N. J. 1985. Phyllosphere yeasts antagonize penetration from appressoria and subsequent infection of maize leaves by *Colletotrichum graminicola*. *Neth. J. Plant Pathol.* **91,** 265–276.

Windels, C. E., and Lindow, S. E. 1985. "Biological Control on the Phylloplane," p. 169. American Phytopathol. Soc. St. Paul, MN.

Wymore, L. A., Poirier, C., Watson, A. K., and Gotlieb, A. R. 1988. *Colletotrichum coccodes:* A potential bioherbicide for control of velvetleaf (*Abutilon theophrasti*). *Plant Dis.* **72,** 534–538.