Suppression of Cottony Leak of Cucumber with *Bacillus cereus* Strain UW85

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ABSTRACT

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Bacillus cereus strain UW85 was previously shown to suppress seedling diseases caused by oomycetous fungi. In this study, we tested UW85 for suppression of cucumber fruit rot caused by *Pythium aphanidermatum*. To evaluate UW85, we developed an assay for rotting of cucumber fruits by *P. aphanidermatum*. Wounded fruits were each inoculated with a suspension containing 500 zoospores of the pathogen and incubated at 32 C for 48 hr. Undiluted culture of UW85, containing approximately 4.4×10^8 cells per milliliter, applied to fruit by one of three methods prior to inoculation with the pathogen, significantly reduced rotting ($P < 0.05$) compared with fruit treated with uninoculated medium or water prior to inoculation with *P. aphanidermatum*. Culture filtrates and suspensions of UW85 containing less than 1.1×10^8 cells per milliliter did not suppress fruit rotting. UW85 cells that were washed and resuspended in sterile medium did suppress rotting.

Pythium aphanidermatum (Edson) Fitzp. is the causal agent of cottony leak of cucumber (*Cucumis sativus* L.), a soft, watery rot followed by white fluffy growth of the fungus (1). Warm temperatures and high moisture contribute to the severity of cottony leak of cucumber. Wounded fruits are most likely to be infected, although *P. aphanidermatum* may directly penetrate fruit that are in contact with the soil. Cottony leak may become more prevalent as cucumber growers adopt mechanical once-over harvesting. This process involves narrower row spacing and higher plant densities, which result in the accumulation of moisture underneath the canopy, creating an environment conducive to infection of fruit by *Pythium*. Although infections by *P. aphanidermatum* are initiated in the field, major losses of fruit often occur after harvest during transport to a handling station. Infected and clean fruit are mixed during harvest and placed together in trucks or bins. If the cucumbers are not processed within 24–48 hr, the fungus spreads rapidly from infected to uninfected fruit, and a total loss may result (2).

Control of cottony leak has historically depended on rotation with grasses, gentle harvest and transport of fruit, and application of fungicides (8,15). Losses caused by cottony leak during transport can be reduced by cooling cucumber fruit to 10 C prior to shipment. However, these control measures can be difficult or expensive to use routinely. Biocontrol offers an attractive alternative for control of cottony leak. Other workers observed delayed symptoms of cottony leak of cucumber fruits dipped in sterile filtrates of the fungi *Acrophialophora nainiana* Edward and *Stachybotrys atra* Corda (14).

We previously described *Bacillus cereus* Frankland and Frankland strain UW85, which suppresses *Phytophthora* seedling diseases of alfalfa (7) and of tobacco (6) and lyses zoospores of *Phytophthora cactorum* (Lebert & Cohn) J. Schröt (4) and *Phytophthora medicaginis* Hansen & Maxwell (3). In this paper, we describe the first report of suppression of a postharvest disease by UW85.

MATERIALS AND METHODS

Zoospore inoculum. Isolates of *P. aphanidermatum* and *Phytophthora capsici* Leonian were maintained on V8 juice agar plates (4) at room temperature under 12 hr of fluorescent light and transferred weekly or stored on V8 juice agar slants at 4 C in the dark. Zoospores of *Phytophthora capsici* and *P. aphanidermatum* were generated as described by Ko and Chan (9) and Rahimian and Banihashemi (12), respectively. The highest concentrations of zoospores of *P.*

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aphanidermatum were produced by cultures that were between 7 and 10 days old. To obtain zoospores of *P. aphanidermatum*, the cultures were flooded and then placed in the dark at 30 C for 18–22 hr, the water was replaced, and the cultures were placed under fluorescent lights at 22 C for 4 hr. To estimate zoospore concentrations, suspensions were vigorously mixed with a Vortex mixer (Scientific Industries Inc., Springfield, MA) for 30 sec and incubated for 10 min to allow for encystment, and encysted zoospores were counted with a haemocytometer. Appropriate dilutions of zoospores were made with water.

Bacterial treatments. Fully sporulated cultures of UW85 and mutant strain UW030 (J. Handelsman and S. Raffel, *unpublished*), which lacks the ability to suppress Phytophthora damping-off of alfalfa, were grown as described by Handelsman et al (7), and cell concentrations were estimated by dilution plating on 50% tryptic soy agar. To prepare culture filtrates, cultures were centrifuged at 20,000 *g* for 10 min, and the supernatants were passed through a 0.45-μm filter. Cell suspensions were produced by resuspending the pellet to the original culture volume in sterile water or medium.

Cucumber fruit rot assay. A gynoecious inbred cucumber line, WI 3888, was used in all of the fruit-rotting experiments. In two experiments, five additional pickling cucumber cultivars were used. The additional cultivars were Pickle Mech, Calypso, XVC5834, Vlaspik, and Discover. Unblemished cucumber fruits, 3.0–4.8 cm in diameter, were harvested from greenhouse or field-grown plants. The fruits were rinsed with tap water, surface-disinfected by lightly spraying the fruit with 95% ethanol, and air-dried. The sides of the fruit were wounded by puncturing them at the center and near both ends with a 3-cm-diameter floral frog with 1-mm-diameter pins. Bacterial treatments were applied to the wounded cucumber fruits by one of three methods. Treatments (1.0 ml) were atomized onto fruit with an airbrush or swabbed onto fruit with a disposable wiping towel, or the fruit were dipped into the bacterial treatment. With each of the methods the bacterial treatment was applied until it ran off the surface of the fruit. Experiments quantifying the number of bacteria required for rot suppression and those involving small amounts of culture filtrates utilized the airbrush and swabbing methods. Experiments requiring large numbers of fruits utilized the dipping method. All of the methods resulted in similar rot suppression. After the fruits were dry, they were inoculated at the wounded sites with 50-μl aliquots containing 500 zoospores of *P. aphanidermatum*. This concentration of zoospores consistently caused infection at 32 C. Control fruits were treated with uninoculated medium or sterile water and inoculated with zoospores of *P. aphanidermatum*. The treatments were arranged in a completely randomized design. Groups of fruit were placed in metal or aluminum pans and sealed in a plastic bag. The pans were placed in an incubator at 32 C in the dark. The proportion of wound sites with visible mycelial growth after 48 hr was recorded for each fruit. Statistical analyses were performed on the arcsine square root transformation of the proportions with Statistical Analysis System (SAS) software (13).

Growth inhibition assay. An assay was developed to measure inhibition of fungal growth by UW85. *Phytophthora* was used in this assay instead of *Pythium* because it grew more slowly and produced more distinct zones of inhibition on the plates. A 0.1-ml suspension of zoospores of *Phytophthora capsici*, containing 1.0×10^4 zoospores, was spread evenly on the surface of a potato-dextrose agar (10) plate. Wells, 7 mm in diameter, were made in the agar with a sterile cork borer. Bacterial treatments to be tested were placed in the wells in 0.1 ml total volume. The plates were incubated at 22 C under fluorescent lights for 3 days, and the diameter of the clear zone was measured from the edge of each well.

RESULTS

Our objective was to evaluate the ability of UW85 to protect cucumber fruits from cottony leak. To do this, we developed an assay in which wounded fruit were inoculated with zoospores of *P. aphanidermatum*. In all treatments little rotting was observed 24 hr after inoculation (Fig. 1). By 48 hr after inoculation, 91% (standard error of the mean [SEM] = 4%, mean of 9 experiments [n] = 9) of the wounded sites on the control fruits treated with sterile water and then inoculated with *P. aphanidermatum* showed fungal growth and rotting.

To evaluate the effect of the bacterial treatments, cultures and filtrates were compared with the uninoculated culture media control, and cells resuspended in water were compared with the sterile water control. In 15 of 18 experiments, undiluted cultures of UW85 significantly ($P < 0.05$) reduced rotting of fruits inoculated with *P. aphanidermatum* compared with uninoculated medium (Fig. 2). Across all 18 experiments, 77% (SEM = 5%) of the wounded sites rotted when treated with uninoculated medium in contrast with 35% (SEM = 7%) that rotted on fruits treated with UW85 culture. We determined by serial dilutions the number of bacterial cells required to protect fruits from rotting by *P. aphanidermatum*. In two experiments evaluating serial twofold dilutions from 2 to 16, suspensions containing less than 1.1×10^8 cells per milliliter did not significantly ($\alpha = 0.05$) reduce rotting. The UW85 culture filtrate did not significantly reduce rotting (Table 1). We conducted two experiments to further evaluate the role of the culture filtrate in rot suppression; Figure 1 is representative of the results of the two experiments. The culture filtrate and cells resuspended in sterile water reduced rotting by 1% (SEM = 20%, $n = 2$) and 34% (SEM = 25%, $n = 2$), respectively, whereas the complete culture reduced rotting by 90% (SEM = 14%, $n = 2$). When the cells were separated from the

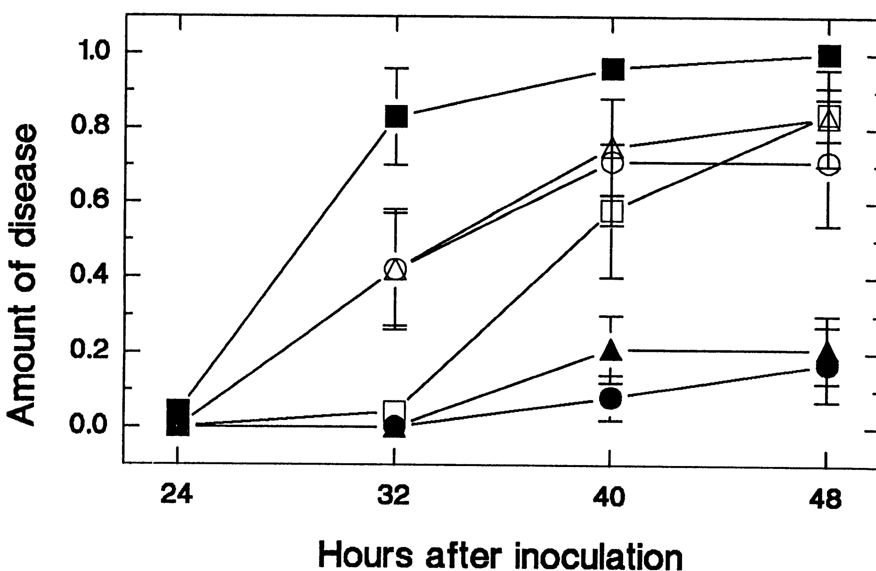

Fig. 1. Effect of *Bacillus cereus* UW85 bacterial treatments on disease progress. Mean proportion of wound sites with visible mycelial growth at 8-hr intervals after inoculation with *Pythium aphanidermatum*. Each point represents the mean of eight fruit. Vertical bars are the standard error of the mean. ● = UW85 culture, ○ = UW85 culture filtrate, △ = UW85 cells resuspended in sterile water, ▲ = UW85 cells resuspended in sterile medium, □ = sterile medium, and ■ = sterile water.

culture and resuspended in sterile medium, rotting was reduced by 88% (SEM = 18%, $n = 2$). We tested the bacterial treatments from the fruit rot assay simultaneously in an assay for inhibition of fungal growth. The mutant, UW030, produced a significantly smaller zone (0.88 mm) in the fungal inhibition assay compared with UW85 (4.25 mm, LSD = 0.92) and, in a total of nine experiments, did not significantly reduce rotting compared with the uninoculated medium controls. Across all treatments in the experiments listed in Table 1, the size of the clear zone in the growth inhibition assay correlated ($r = 0.77$) with the level of protection on fruit.

We evaluated UW85 on five additional field-grown cucumber cultivars in two experiments. UW85 significantly ($P < 0.0001$) reduced rotting in all cucumber cultivars (Table 2) in both experiments. Rotting of the untreated cucumber fruit did not significantly differ among the cultivars, and the interaction between UW85 culture treatment and cucumber cultivar was not significant.

DISCUSSION

Our results indicate that UW85 culture significantly reduced rot of cucumber fruit by *P. aphanidermatum*. A mutant of UW85, which produced only small clear zones in a fungal growth inhibition assay, did not suppress rotting of cucumber fruits. This suggests that an extracellular product, produced by UW85 and not by the mutant UW030, might be responsible for rot suppression and fungal growth inhibition. The same extracellular factor may be important for suppression of fruit rot and alfalfa damping-off, since UW030 does not suppress either disease. However, filtrates of cultures of UW85 did not substantially reduce rot, and most of the activity was associated with the cellular fraction of the culture. Suppression of rot by the UW85 culture appears to be greater than the additive effects of washed cells suspended in water and filtrate components. The results suggest that extracellular products accumulated during growth in culture are not sufficient for suppression of fruit rot. Interestingly, cells separated from the filtrate and resuspended in sterile medium suppressed rot in a way similar to that of the complete culture. These results contrast with previous work showing that sterile filtrates of UW85 cultures protected alfalfa seedlings from damping-off (7). Cells resuspended in sterile medium provided better disease suppression than cells resuspended in water in both the cucumber and alfalfa systems (Handelsman and Raffel, *unpublished*). Perhaps UW85 spore germination is enhanced in the presence of the medium. Enhanced disease suppression might result from promotion of germination of UW85 spores by medium, since spore germination does not occur in sterile water. Both the extracellular factor in the culture filtrate and biologically active *Bacillus* cells are required for optimum disease suppression.

Suppression of Pythium fruit rot on cucumbers is one of many properties of the biological control agent *B. cereus* strain UW85. It has been previously reported to lyse zoospores of *Phytophthora cactorum* (4) and *Phytophthora medicaginis* (3), reduce damping-off of alfalfa in the lab and field (7), reduce infection of hydroponically grown tobacco (6), enhance nodulation in soybean (5), and reduce the effects of *Sclerotinia* on peanuts (11). In this work, we found activity against a new pathogen, *P. aphanidermatum*, on a new host, cucum-

Table 1. Effect of *Bacillus cereus* strains on cucumber fruit rotting caused by *Pythium aphanidermatum*

Treatment	Proportion of wound sites rotted (mean)
UW85 culture	0.39 a[z]
UW85 cells	0.50 ab
UW85 culture filtrate	0.95 c
UW030 culture	0.78 bc
UW030 cells	1.00 c
UW030 culture filtrate	1.00 c
Medium	0.78 bc
Water	0.83 bc

[z] Each value represents the mean of six fruits per treatment with three wound sites per fruit. Means followed by the same letter do not significantly differ ($P < 0.05$) as determined by protected LSD test (LSD = 0.243). Treatments were applied to runoff by swabbing with a disposable towelette.

Table 2. Effect of *Bacillus cereus* UW85 culture on rotting of six cucumber cultivars caused by *Pythium aphanidermatum*.

	Proportion of wound sites rotted (mean)			
	Treatment			
	Experiment A		Experiment B	
Cultivar	SDW[y]	UW85	SDW	UW85
Pickle Mech	0.13[z]	0.00	0.80	0.00
Discover	0.17	0.00	0.73	0.03
Vlaspik	0.18	0.07	0.90	0.03
Calypso	0.33	0.04	0.97	0.13
XVC 5834	0.37	0.00	0.97	0.10
WI 3888	0.43	0.00	0.93	0.07

[y] SDW = sterile distilled water; UW85 = undiluted culture.
[z] Each value represents the mean of 10 fruits with three wound sites per fruit. Treatments were applied by the dipping method. The effect of UW85 treatment was significant across cultivars ($P < 0.0001$) in both experiments.

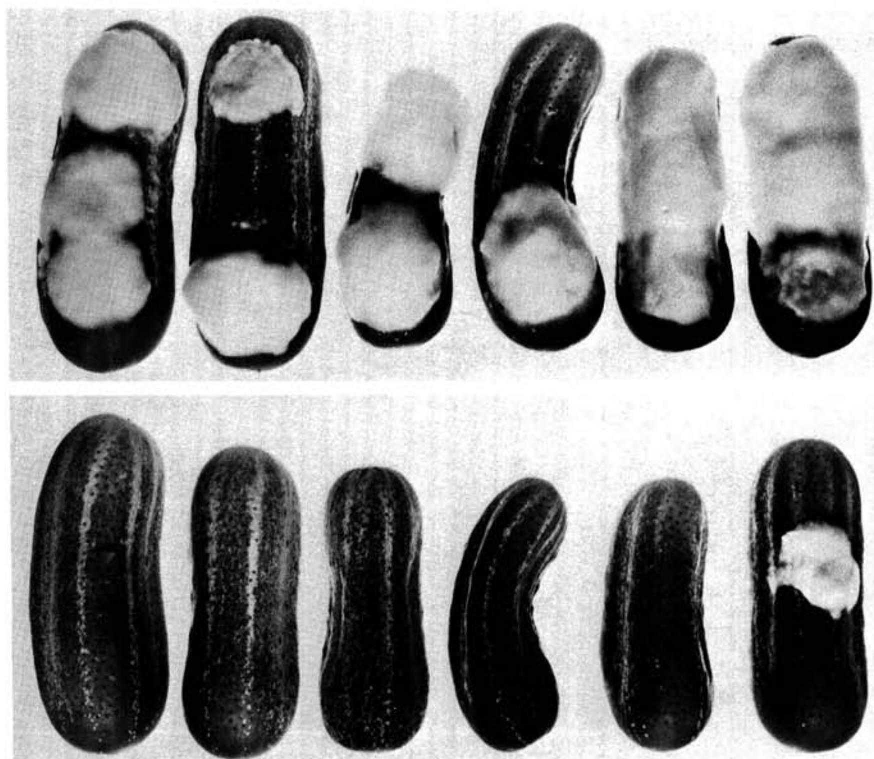

Fig. 2. Wounded cucumber fruit inoculated with 500 zoospores of *Pythium aphanidermatum*. Treated with uninoculated medium (top) and *Bacillus cereus* UW85 culture (bottom).

ber. In this study we observed biocontrol activity on fruit surfaces at high temperature (32 C), in contrast to previous work that focused on activity on roots in the soil or hydroponic systems at moderate temperatures (24 C). Therefore, UW85 appears to have various properties that are beneficial to plants under diverse conditions.

Application of UW85 to cucumber fruits may be an alternative to existing disease control measures for cottony leak. Fungicide applications to protect fruit are expensive, undesirable close to harvest, and difficult to apply to fruit underneath the leaf canopy. Genetic resistance to other fruit-rotting organisms (e.g., *Rhizoctonia*) has been reported (16); however, the skin of resistant fruit was tough and netted, characteristics unacceptable for pickling fruit. The application of biological or chemical agents is a potential control strategy for cottony leak. The rapid screening method presented in this report could be used to evaluate resistant germ plasm or chemical and biological agents for control of Pythium fruit rots. *B. cereus* UW85 is especially attractive as a biological control agent because it occurs naturally in the soil, because cells can be produced easily in large quantities, and because the cells remain viable throughout extensive storage. In other attempts to exploit biocontrol, a difficulty has been maintaining sufficient populations of the biocontrol agent for disease suppression. In contrast, control of postharvest fruit disease may provide an unusual opportunity for biocontrol, since the time during which cucumber fruits must be protected from rotting after harvest is short compared with that of other plant diseases that develop throughout the growing season. Application of a biocontrol agent immediately after cucumber harvest may ensure that high populations will be present on the fruit surface during the time when disease spreads.

Future work needs to address biocontrol activity under standard storage conditions to determine appropriate methods of application and the number of bacteria required for disease suppression. Under our experimental conditions, a high concentration of cells was required for inhibition of *P. aphanidermatum*. This might have been because conditions for infection were optimal, in that zoospores were applied directly to wounds, and fruit were placed in environmental conditions conducive to infection. Fewer cells may be required to protect fruit under field conditions. If the high level of disease control observed in the laboratory can be duplicated during storage, UW85 may be a useful alternative for control of Pythium fruit rots of cucumber.

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