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Behavior of *Pythium torulosum* Zoospores During Their Interaction with Tobacco Roots and *Bacillus cereus*

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Abstract. *Bacillus cereus* UW85 suppresses seedling damping-off diseases caused by Oomycetes and produces antibiotics that inhibit development of Oomycetes in culture. The goal of this study was to determine how UW85 and its antibiotics affected the behavior of an Oomycete, *Pythium torulosum*, in its interaction with plant roots. We studied tobacco seedlings inoculated with zoospores of *P. torulosum* and UW85 culture, culture filtrate, washed cells, antibiotics (zwittermicin A or kanosamine), purified from cultures of UW85, and UW030, a mutant of UW85 that does not suppress disease and does not produce the antibiotics. Microscopic observation revealed that all of the treatments inhibited zoospore activity around roots and encystment on roots. Treatment with UW85 culture, culture filtrate, zwittermicin A, or kanosamine delayed cyst germination and the elongation rate of germ tubes, whereas treatment with UW030 or washed UW85 cells did not. In an in vitro seedling bioassay of disease suppression, the antibiotics, zwittermicin A and kanosamine, suppressed disease singly or together, although UW85 culture suppressed disease more effectively than did the antibiotics. The results show that *B. cereus* cultures affect zoospore behavior in the presence of roots, and *B. cereus*-produced antibiotics, zwittermicin A and kanosamine, contribute to disease suppression and inhibition of germ tube elongation in the presence of the plant root.

Oomycetes are a favored group of organisms for the study of lower eukaryote cell biology in response to environmental stimuli [3, 4, 7, 17, 18, 20, 24, 25, 33–35]. Oomycete zoospores exhibit characteristic behavior in vitro in response to tactic stimuli (responses include chemotaxis, rheotaxis, geotaxis, and electrotaxis). Zoospores are also a major inoculum source for the diseases of plants caused by oomycete pathogens of the genera *Aphanomyces*, *Phytophthora*, and *Pythium*. Plant pathogenic Oomycetes cause seedling damping-off diseases of many plant species [2]. The asexual zoospores move in water films toward, and accumulate on, plant roots where they lose mobility and are induced to encyst [8, 10, 17, 22, 23, 27, 28]. The zoospore cysts germinate rapidly in response to certain amino acids [17], producing germ tubes that penetrate and infect roots [35]. Thus, the pre-infection processes, including zoospore taxis, encystment, cyst germination and orientation of the germ tube [5], all involve interactions between the Oomycete and the host plant, and may be targets for biocontrol agents that suppress diseases caused by Oomycetes [6, 17].

Our focus is on biological control agents found naturally among isolates of *Bacillus cereus* [36]. The best characterized of the *B. cereus* isolates with biological control activity is designated UW85, which was obtained from the rhizosphere of a healthy alfalfa plant [13]. UW85 enhances soybean nodulation [11] and suppresses Oomycete diseases of various plants [1, 12–14, 32]. In the laboratory, much of the disease suppressiveness of cultures of UW85 is associated with the noncellular fraction of the cultures. UW85 alters the ion concentration in culture filtrates, causing lysis in vitro of zoospores of *Phytophthora cactorum* [9, 37]. Culture filtrates of UW85 suppress alfalfa and tobacco seedling damping-off diseases [13, 14], and inhibit the development of *Phytophthora* sporangia [14]. The disease-suppressive activity of the culture filtrates is largely attributable to two antibiotics, zwittermicin A [15, 30, 31] and kanosamine [21, 30].

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Purified zwittermicin A reversibly inhibits elongation of germ tubes of *P. medicaginis* and, in separate experiments, suppresses disease of alfalfa seedlings caused by this pathogen [30].

No previous studies have focused on the influence of *B. cereus* and its antibiotics on the behavior of each developmental stage of the Oomycetes in the presence of a host plant. Here we report results of a microscopic study of the behavior of zoospores of *P. torulosum* with host roots after treatment with *B. cereus* cultures, culture filtrates, or purified antibiotics.

Materials and Methods

Bacterial growth. *Bacillus cereus* UW85 and UW030 were grown as described previously [13]. UW030 is a mutant that does not produce detectable zwittermicin A [30], or kanosamine [21, 30], and does not suppress damping-off of alfalfa. Four-day-old fully sporulated cultures of UW85 and UW030 (10^8 cells ml^{-1}) were used in all experiments. Concentrations of bacterial cells were determined by dilution plating on ½-strength tryptic soy agar (TSA). Filtrates of UW85 were prepared by centrifugation of 20 ml of the fully sporulated culture at 20,000 *g* for 12 min, and the supernatant was filtered through a 0.22-µm filter. The pellet was washed three times with sterilized water and resuspended in sterilized water; the resulting cell preparation was designated ''washed cells.'' The washed bacterial cells were diluted with sterilized, distilled water to a final concentration of 10^8 cells ml^{-1}.

Zwittermicin A and kanosamine preparation. Zwittermicin A and kanosamine were purified from fully sporulated cultures of UW85 as described previously [21, 30, 31]. Zwittermicin A and kanosamine were dissolved in sterilized, distilled water to a final concentration of 0.1 µg ml^{-1} of zwittermicin A, 0.2 µg ml^{-1} of kanosamine, or as a mixture of zwittermicin A (0.1 µg ml^{-1}) and kanosamine (0.2 µg ml^{-1}). The antibiotics were used in both zoospore behavior observation experiments and tobacco seedling mortality tests.

Zoospore preparation. *Pythium torulosum* was maintained on V8-juice agar plates at 24°C and transferred weekly [9, 32]. Zoospores of *P. torulosum* were produced by the methods of Ko and Chan [19] and Rahimian and Banihashemi [26] with modifications. Seven- to ten-day-old cultures were used to obtain high concentrations of zoospores [32]. The culture and agar in a petri plate were cut in half, and one-half was transferred to an empty petri plate. Both plates were flooded with 20–25 ml of sterilized, distilled water and incubated in the dark at room temperature (22°–24°C) overnight. The following morning the water was replaced, and the culture was placed under fluorescent light at room temperature for 3 h to induce zoospore release. Zoospore concentrations were estimated with a hemacytometer, and the appropriate dilution was made with sterilized water to a final concentration of 10^5 zoospores ml^{-1}.

Tobacco seedling preparation. Tobacco (*Nicotiana tabacum* Xanthi nc) seeds were soaked in 20% bleach for 20 min, followed by two 5-min rinses with sterilized distilled water. The seeds were then placed in petri plates on Whatman #2 filter paper moistened with 2.0 ml of 1/4-strength Hoagland's solution [16]. The plates were incubated for 10 days in a 24°C growth chamber with a 12-h photoperiod. The tobacco seedlings were either transplanted to 0.5% water agar plates (40 seedlings per plate) for the mortality tests [1] or transferred to 24-well microtiter plates for zoospore behavior experiments.

Observations of zoospore behavior on root surfaces. Two tobacco seedlings were transferred to each well of microtiter plates, and 10^5 zoospores of *P. torulosum* were added to each well. Treatments were applied in a total volume of 50 µl. Treatments included UW85 (cell culture, culture filtrate, or washed cells) and UW030 (cell culture). Uninoculated tryptic soy broth (TSB) medium and sterilized water were used as controls. The plates containing inoculated and treated seedlings were placed under the dissecting microscope (Olympus, Model HSZ) with a total magnification of 150×. The behavior of the zoospores in some experiments was continuously observed under the microscope; in other experiments zoospore behavior was recorded with a video camcorder (Sony, Model 3CCD-IRIS) connected to a video recorder/monitor (Panasonic, Model AG-513A). The video camera recorded a 0.75-mm-diameter field of view around the plant root. In this view area, zoospore movement or taxis (expressed as the number of moving zoospores $time^{-1}$), zoospore encystment, time of cyst germination, and growth rate of germ tubes (germ tube length $time^{-1}$) were recorded. The observation for the growth rate of germ tubes was continued until the germ tubes were too long to be measured (more than 250 µm). For zoospore encystment measurements, two seedlings were removed from each treatment at 0, 5, 10, 15, and 20 min after inoculation, rinsed with sterilized water, and sonicated with an ultrasonic cleaner (Branson, Model 2200) for 40 s in 1 ml of water. The number of cysts was determined with a hemacytometer. There were three experiments, containing different treatments; each experiment was a complete randomized block design with three replicates, and each experiment was repeated three times. Statistical analysis was performed with the Statistical Analysis System (SAS) program [29].

Tobacco seedling mortality test. Two days after transplanting, 40 seedlings were inoculated with 5×10^5 zoospores of *P. torulosum*. Then, 50 µl of UW85 culture (10^8 cfu ml^{-1}), zwittermicin A, kanosamine, or a mixture of zwittermicin A and kanosamine was pipetted into the petri plates. The plates were then swirled to ensure a uniform suspension. Sterilized distilled water was used as a control. The inoculated seedlings were grown in the same conditions as described above. This experiment was a complete randomized block design with three replications, and it was repeated three times. Ten days after inoculation, seedling mortality was determined. Statistical analysis was performed with the Statistical Analysis System (SAS) program [29].

Results

The objective of the study was to determine the effect of *B. cereus* and its antibiotics zwittermicin A and kanosamine on the behavior of zoospores on plant roots. To do this, we compared the effects of *B. cereus* (strain UW85) culture, culture filtrates, washed cells, UW030 (a mutant of UW85 that does not produce zwittermicin A or kanosamine in culture), and the two purified antibiotics applied singly and together.

Zoospore movement. Addition of UW85 cell culture, culture filtrate, or UW030 cell culture significantly reduced the movement of zoospores around the tobacco roots (Fig. 1a). Washed cells and unwashed cells of UW85 had an immediate effect on the movement of zoospores. Purified zwittermicin A, kanosamine, and the mixture of zwittermicin A and kanosamine significantly reduced zoospore movement (Fig. 1b).

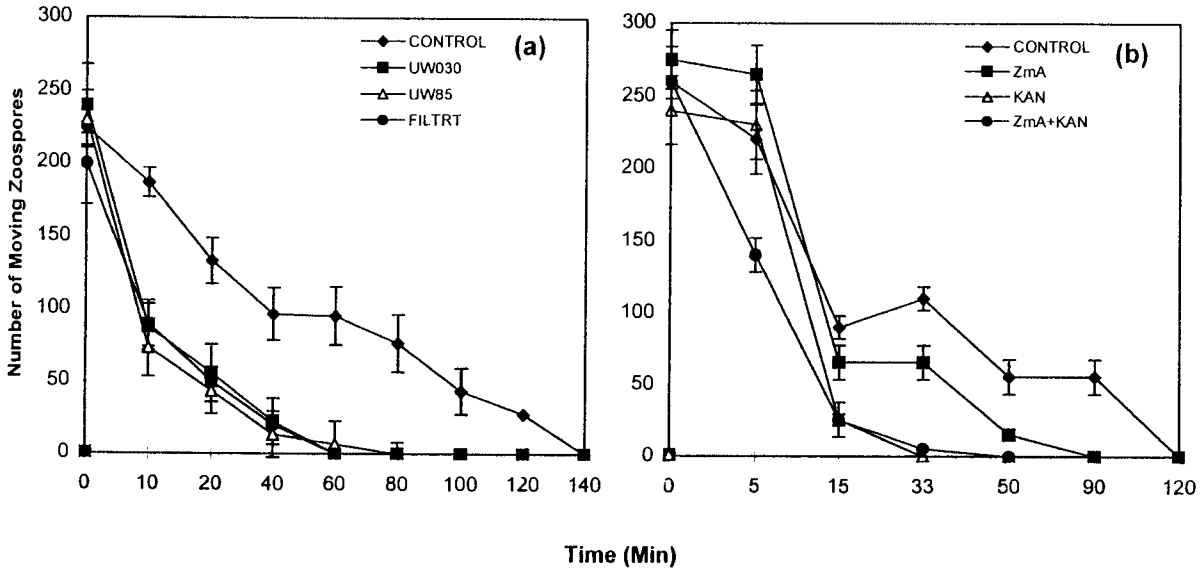

Fig. 1. Movement of zoospores of *Pythium torulosum* around tobacco roots. Panels (a) and (b) depict data from separate experiments. Each point represents the mean of observations of nine roots. Vertical bars indicate standard error of the mean; where a bar is not visible, it is within the symbol. FILTRT, UW85 culture filtrate; ZmA, zwittermicin (0.1 μg ml^{-1}); KAN, kanosamine (0.2 μg ml^{-1}); CONTROL, tryptic soy broth. Each value represents the number of zoospores per viewing field of the video camera (0.75 mm diameter).

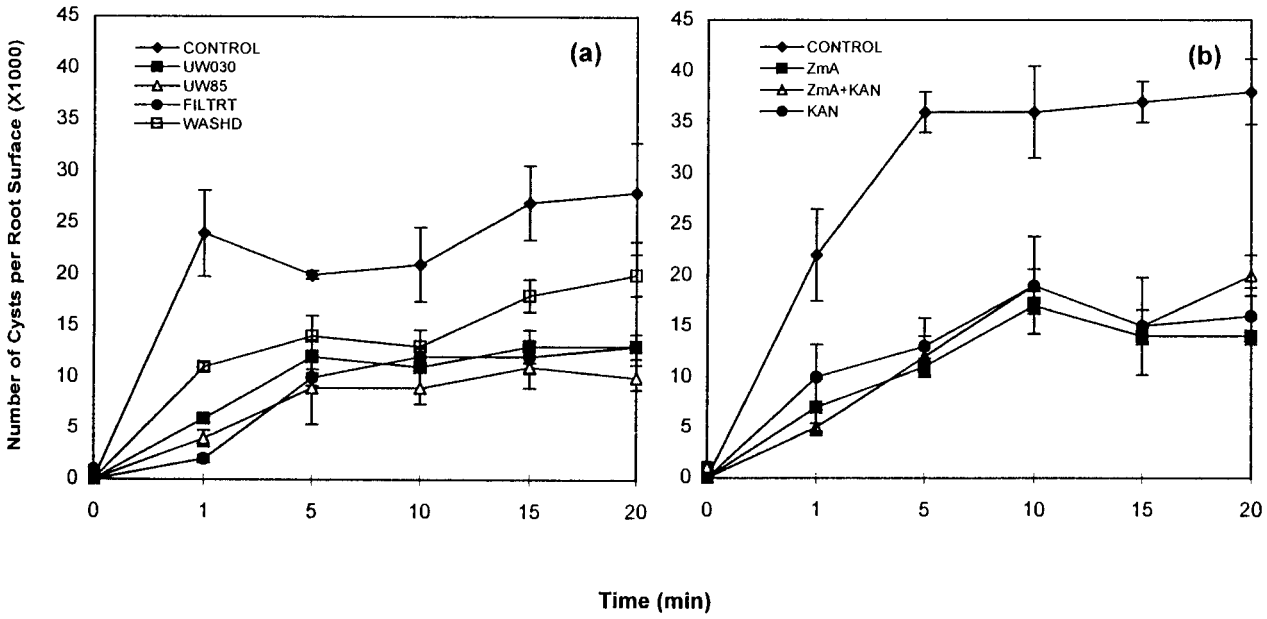

Fig. 2. Cysts of *Pythium torulosum* on roots of tobacco. Panels (a) and (b) depict data from separate experiments. Each point represents the mean of observations of nine roots. See Fig. 1 legend for treatments and WASHD: UW85 culture-washed cells.

Zoospore encystment and attachment to roots. UW85 cell culture, culture filtrate, washed cells, and unwashed cells reduced encystment of zoospores of *P. torulosum* on tobacco roots (Fig. 2a). Purified zwittermicin A, kanosamine, and the mixture of zwittermicin A and kanosamine also reduced zoospore encystment on roots, but the mixture did not show a significantly greater effect than either antibiotic alone (Fig. 2b). UW030 reduced encystment of zoospores on roots, although UW85 cell culture consistently showed the highest inhibitory effects compared with other treatments. Since UW030 cultures contain no detectable zwittermicin A or kanosamine [30], these results suggest that zwittermicin A and kanosamine may contribute to, but are not the only factors that are responsible for, the inhibition of zoospore encystment on roots.

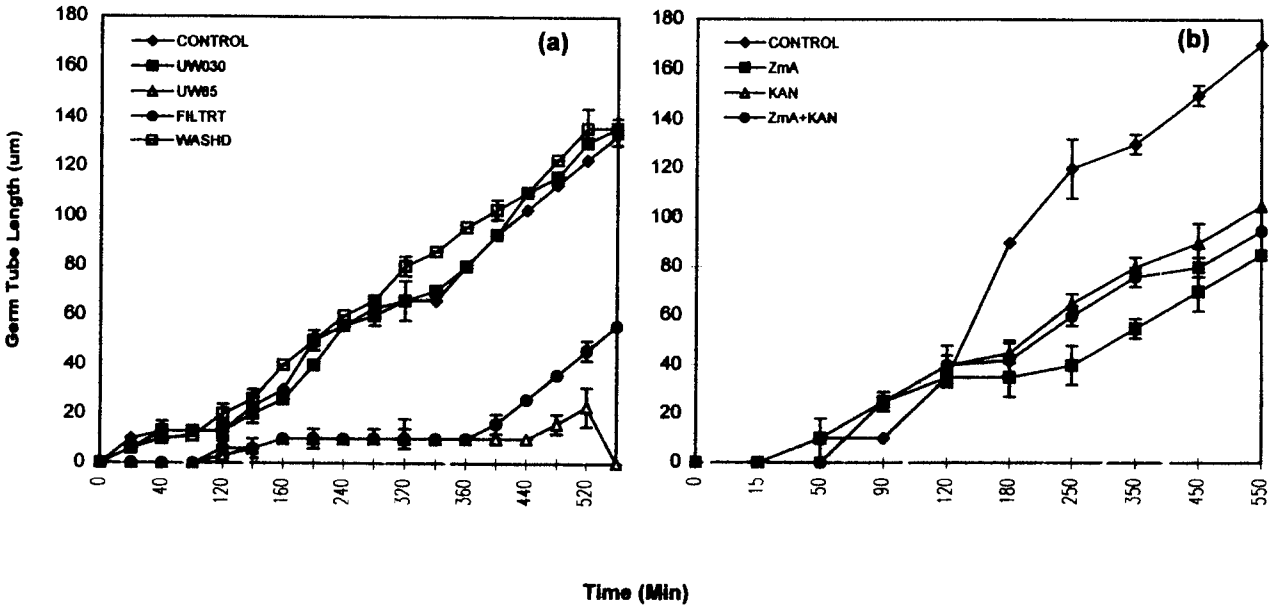

Fig. 3. Germination and germ tube growth from cysts of *Pythium torulosum* on tobacco roots. Panels (a) and (b) depict data from separate experiments. See Fig. 1 legend for treatments. Values represent the mean of 20–30 measurements in a single view area (0.75 mm diameter).

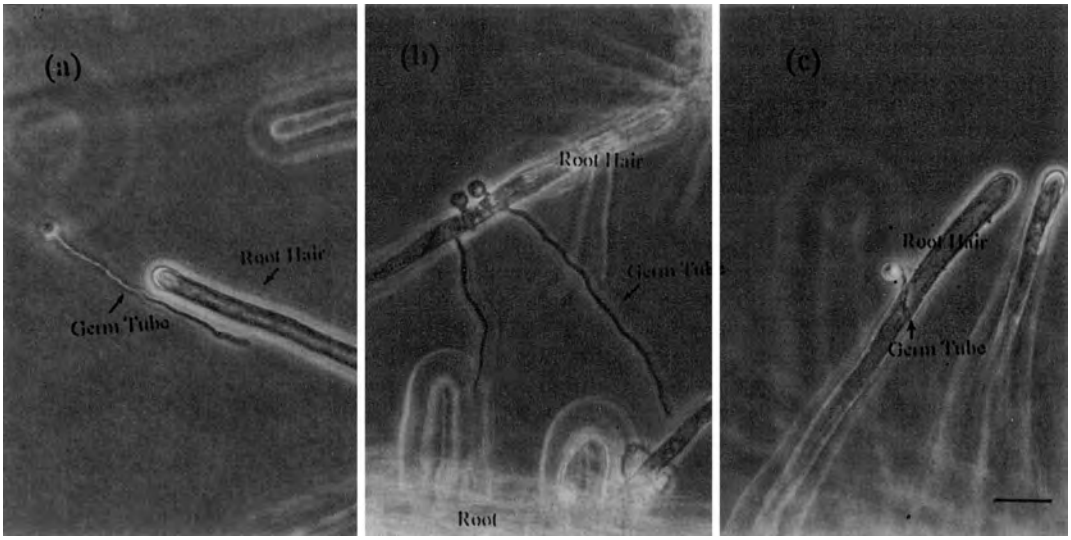

Fig. 4. Germinating cysts of *Pythium torulosum* on tobacco roots 4 h after inoculation. Panels (a) and (b), control (tryptic soy broth); panel (c), treated with 0.1 μg ml^{-1} of zwittermicin A. Bar, 30 μm.

Time of cyst germination and growth rate of germ tubes. Cyst behavior on the 0.5-mm section of the root tip and around the root tip was observed both by continuous observations under the microscope and on recordings made by video microscopy. Treatment with UW85 cell culture and culture filtrate delayed cyst germination of *P. torulosum* for 2 h, and the germ tube growth rate was lower than in the controls for another 4 h. In the other treatments and controls, germination occurred at about 0.3 h after inoculation (Fig. 3a). Six hours after inoculation, however, the growth of germ tubes in the UW85 culture filtrate increased, and by 9 h after inoculation reached a similar rate of growth as in the other treatments (Fig. 3a). In contrast, in the UW85 cell culture treatment, 9 h after inoculation, the germ tubes and the ungerminated cysts were lysed (Fig. 3a). Purified zwittermicin A inhibited cyst germination and germ tube growth (Figs. 3b and 4), whereas effects of high concentrations of kanosamine were not detectable until 4 h after inoculation (Fig. 3b). Visual inspection showed that the orientation of the germ tubes was not influenced by any treatments in the experiment.

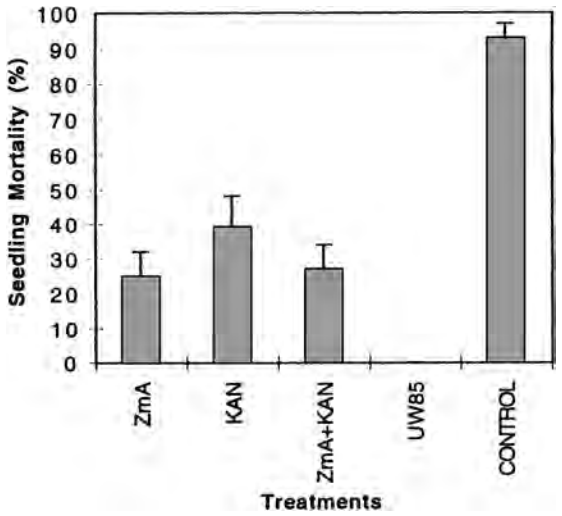

Fig. 5. Damping-off of tobacco caused by *Pythium torulosum*. Each value represents the mean of observations of 40 plants. Vertical bars indicate standard error of the mean; where a bar is not visible, it is within the symbol.

Seedling mortality test. Treatment with UW85 cultures fully suppressed *P. torulosum*-induced damping-off on tobacco seedlings. Zwittermicin A, kanosamine, and a mixture of the two antibiotics also suppressed the disease (Fig. 5).

Discussion

The results reported here indicate that UW85 and its antimicrobial metabolites possess a previously undescribed biological activity, which is inhibition of cyst germination and germ tube growth on roots. Biological activities of UW85 cultures reported previously have been accounted for by either the ionic changes in the growth medium effected by UW85 [9], or by the antibiotics, zwittermicin A and kanosamine, produced by UW85 [21, 30]. Here we show that significant effects on zoospore movement around roots and encystment on them were associated with washed cells and with cultures of UW030, the antibiotic-deficient mutant. Either the cells contain significant internal concentrations of the antibiotics that were not previously detected, or, more likely, they have an activity that is different from those reported previously.

Those treatments that contained zwittermicin A provided the strongest inhibition of germination of the cysts and growth of germ tubes of *P. torulosum*. These data suggest that the germination and germ tube growth of *P. torulosum* cysts may be the important pre-infection stages with which *B. cereus* UW85 interferes. After several hours of inhibition, the germ tubes in the treatment with UW85 culture filtrate started to grow again (Fig. 3a). This result, in the absence of UW85 cells, may indicate a destruction of the biological activity of the antibiotics in these treatments with time, perhaps by metabolism due to the plant or to seed-associated microorganisms in the system. In treatments with UW85 cell culture, however, the germ tubes still grew slowly, and the germ tubes and ungerminated cysts were eventually lysed by the culture. This result may be due to continuous growth, sporulation, and zwittermicin A and kanosamine production by UW85 cells in the medium, and may also be due in part to the high ammonia and low calcium in UW85 culture filtrates, which were previously shown to lyse *Phytophthora* zoospores [9].

In the presence of either *B. cereus* UW85 or UW030, fewer moving zoospores of *P. torulosum* were found around the root, and fewer encysted zoospores were found on the root surface than in the untreated control. Cyst germination was delayed, germ tube growth was slowed, and less disease was observed. Since UW030 does not produce detectable antibiotics in culture, these results suggest that factors other than antibiotic production may contribute to the effect of *B. cereus* on zoospore behavior. There may be other metabolites that we have not yet identified, or the presence of the plant root may induce UW030 to produce antibiotics despite its failure to do so in culture. Previous work showed that alfalfa seedlings stimulate production of zwittermicin A and kanosamine by UW85 in culture [21]. Similarly, tobacco may enhance production of known metabolites, or it may induce production of previously unidentified factors that contribute to suppression of disease. Our ongoing work directed to understanding the effect of plant factors on metabolite production and gene expression in *B. cereus* will contribute to an understanding of this plant–microbe interaction. Similarly, understanding the effect of *B. cereus* on expression of plant genes may elucidate plant responses to the biocontrol agent that are central to limiting infection by Oomycetes.

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