Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 27:1-9

The cell-free supernatant of *Pseudomonas protegens* CHA0 may not be sufficient to inhibit *Verticillium dahliae* growth

Samantha Krystal, Soomin Lee, Cameryn Meloche, Ariel Rosen

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY *Verticillium dahliae* is a fungal pathogen that lives in agricultural soil and causes the disease known as Verticillium wilt. This disease affects over 400 species of plants, including important food sources such as potatoes, peppers, and strawberries. Currently, broad-spectrum soil fumigation is the standard treatment for Verticillium wilt, but there is a need for more sustainable and environmentally friendly alternatives. Certain *Pseudomonas* species are ubiquitous in agricultural soil and have been proposed as biocontrol agents to replace or supplement traditional chemical fungicides. Fluorescent pseudomonads, in particular, characteristically produce an array of antimicrobial metabolites including 2,4-diacetylphloroglucinol, pyoluteorin, and hydrogen cyanide, which exhibit strong antifungal activity. Specifically, the fluorescent *Pseudomonas protegens* CHA0 has been shown to be a strong inhibitor of *Verticillium* spp. growth, likely due to its ability to produce 2,4-diacetylphloroglucinol. Here, we investigate the ability of intact *P. protegens* CHA0 to inhibit *V. dahliae* growth and evaluate whether *P. protegens* CHA0 secretions alone are sufficient to inhibit *V. dahliae* growth. Our results demonstrated that intact *P. protegens* CHA0 can inhibit *V. dahliae* growth, but suggest that bacterial secretions alone may not be sufficient for biocontrol. Findings from this study can provide insights into effective strategies for biocontrol of plant diseases.

INTRODUCTION

Verticillium dahliae is an economically important causative agent of Verticillium wilt, affecting over 200 plants in both temperate and subtropical climates, with the host range continuing to broaden (1–3). This fungal plant pathogen resides in the soil as microsclerotia, which can lie dormant for up to 14 years (1). When environmental conditions are favourable, the microsclerotia germinate into hyphae that infiltrate the roots of a susceptible host and interfere with water uptake and nutrient transport (1, 2, 4). Infected plants typically display chlorosis, necrosis (causing browning in a characteristic "V" shape), stunted growth, and wilted leaves (2). Since infected plants are no longer viable for sale, Verticillium wilt results in significant economic impacts, reducing the yield of several staple food, oil, and fibre crops (1). For example, it is the main cause of Potato Early Dying (PED), a disease that is responsible for 30% to 50% of potato yield reduction in North America per year (4, 5). Thus, research into sustainable and effective Verticillium wilt management strategies is important for food security and the protection of economically important crops.

Historically, fumigation with methyl bromide was the standard treatment for Verticillium wilt, but its use has been drastically limited due to widespread environmental impacts and the development of resistance in multiple pathogens (1, 6, 7). Most current strategies for Verticillium wilt focus on integrated pest management (IPM) to reduce the load of dormant *V. dahliae* in the soil (6). IPM is a systems-based strategy that combines pest management with established agronomic practices to minimize negative environmental impacts and promote agricultural sustainability (8). For example, one emerging IPM strategy is the use of rhizosphere microorganisms to combat fungal plant pathogens using biological control agents (BCAs). Biological control is defined as the inhibition or suppression of pathogens by living microorganisms (or their derivatives) through various mechanisms including nutrient competition, induction of resistance in the host plant, and direct antagonistic activity (7, 9).

Though the use of BCAs to combat fungal plant pathogens has been researched for over two decades, commercially available BCAs are still limited (2, 6). This lack of commercial availability is likely because *in vitro* studies are not representative of actual crop

Published Online: September 2022

Citation: Samantha Krystal, Soomin Lee, Cameryn Meloche, Ariel Rosen. 2022. The cell-free supernatant of Pseudomonas protegens CHA0 may not be sufficient to inhibit *Verticillium dahliae* growth. UJEMI 27:1-9

Editor: Andy An and Gara Dexter, University of British Columbia

Address correspondence to: https://jemi.microbiology.ubc.ca/

environments, which vary in climate, soil nutrient availability, and plant genetics (7). Moreover, like fungicides, overuse of BCAs can exert selective pressure on fungal pathogens and lead to the emergence of resistant strains (7). Currently, the most successful commercialized biopesticides have a broad host range and/or work in synergy with traditional fungicides to lower fungicidal dose and reduce selective pressure (10). For example, the fungicide Serenade® optimizes lipopeptides from *Bacillus subtilis* QST-713 to suppress a broad range of plant pathogens (10, 11). The commercialization and success of this biopesticide, although ineffective against Verticillium wilt, shows that BCAs can be isolated and optimized for widespread use.

A number of studies have found that *Pseudomonas* spp. are a potential biocontrol agent against Verticillium wilt (3, 6). *Pseudomonas* spp. constitute one of the many plant-beneficial bacteria that colonize the rhizosphere. Fluorescent pseudomonads in particular produce broad-spectrum antifungal and antimicrobial metabolites against many plant diseases, including Verticillium wilt (3, 6, 9). For instance, the fluorescent pseudomonad *P. protegens* CHA0 (P_DAPG) possesses the GacS/GacA two-component regulatory system that controls the production of bioactive secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, and hydrogen cyanide (HCN) (12–14). DAPG is a polyketide that disrupts the mitochondrial proton gradient in fungi, thereby preventing the production of ATP and inhibiting fungal growth (15). DAPG also contributes to the induction of systemic resistance in the host plant, which helps the plant resist fungal root colonization (16). Therefore, further investigation into the role of P_DAPG and its metabolites for fungal growth inhibition could be important for harnessing rhizosphere-mediated disease immunity as an alternative strategy to treat plant fungal diseases.

Although P_DAPG is known to secrete antifungal agents, it remains unclear whether these secretions alone are sufficient to inhibit fungal growth or if intact bacteria are required for inhibition (3, 17). For instance, a study by Naseby and colleagues demonstrated that the metabolite non-producing strain, *Pseudomonas* SBW25, possesses strong biocontrol activity by outcompeting pathogenic fungal strains for nutrients (18). Although current studies suggest P_DAPG may be an effective biocontrol agent, further research is needed to understand and exploit its antifungal properties (19). Here, we investigate if P_DAPG secretions alone are sufficient to inhibit *V. dahliae* growth and whether these metabolites are continuously produced or if their production is increased in the presence of fungi.

Since GacS/GacA knockout mutants of P_DAPG are deficient in metabolite production and have reduced antifungal activity against *V. longisporum* (3), we hypothesized that P_DAPG secretions alone may be sufficient to inhibit the growth of *V. dahliae*. Furthermore, because the DAPG and HCN-producing strain, *P. brassicacearum* LBUM300, overexpresses the DAPG biosynthetic operon when co-cultured with *Clavibacter michiganensis subsp. michiganensis* (20), we also hypothesized that P_DAPG secretions may be produced at higher levels in the presence of *V. dahliae* compared to when no fungi are present. If P_DAPG secretions are shown to be sufficient fungal inhibitors, future experiments could generate effective BCA-derived fungicides from metabolites in antifungal secretions, offering a promising alternative to treat Verticillium wilt.

METHODS AND MATERIALS

Preparation of Simulated Xylem Media. Simulated Xylem Media (SXM) was prepared as indicated in Supplemental Tables 1-3, adapted from a protocol provided by Dr. Gerhard Braus' laboratory at the Institute of Microbiology and Genetics, Göttingen Center for Molecular Biosciences (3). In summary, liquid SXM was prepared by adding 0.2% (w/v) of pectin and 0.4% (w/v) of tryptone to dH_2O. This solution was then autoclaved. Next, 2% (v/v) 50x AspA, 0.2% (v/v) anhydrous $MgSO_4$ and 0.1% (v/v) 1000x Trace Elements (TE) were filter sterilized with a 0.20µm filter and added to the autoclaved pectin-tryptone solution. Since AspA crystallizes at room temperature, it was re-solubilized in a 60ºC incubator before being added to the pectin-tryptone solution. For SXM plates, the same procedure was followed, with the addition of 2% (w/v) agar to the autoclaved solution. Liquid SXM, AspA, and anhydrous $MgSO_4$ were stored at room temperature. SXM plates and TE were stored at 4ºC.

***Pseudomonas protegens* CHA0.** P_DAPG was obtained on an LB plate from Dr. Cara Haney's laboratory at the Life Sciences Institute of the University of British Columbia. P_DAPG was then streaked onto an SXM plate for isolated colonies. The plate was wrapped in parafilm and stored at 4ºC for the duration of the study.

Generating a culture of *V. dahliae* spores. The procedure for cultivating fungal spores was adapted from a previously described method (3). *V. dahliae* was obtained from Dr. Cara Haney's laboratory on potato dextrose agar (PDA) wrapped in parafilm and stored at room temperature for the duration of the study. To prepare *V. dahliae* spores, a 5mm slice of *V. dahliae* was excised from the plate and placed into 50mL liquid SXM, where it was incubated at 25°C, 125rpm, for 5 days. After 5 days of incubation, spores were harvested through a cheesecloth, centrifuged at 3500rpm in an Avanti J-30I centrifuge at 4°C for 10 min and washed in 20mL dH_2O. The washing step was repeated and then the pellet was resuspended in dH_2O to cover the pellet. Spores were counted using a hemocytometer and diluted to a concentration of $1x10^6$ spores mL^{-1} with dH_2O.

Testing P_DAPG against *V. dahliae* growth using a zone of inhibition assay. The antifungal activity of P_DAPG was tested as described previously (3). $1x10^5$ *V. dahliae* spores were spread onto an SXM plate and 60µL of P_DAPG (OD_{600nm} = 1.0) was transferred to a 1.3cm-diameter hole in the centre of the plate. This procedure was repeated in triplicate for the experimental group and negative controls (*E. coli* DH5α and liquid SXM). The plates were incubated face-up at room temperature in the light for 4 days. The inhibition radius on each plate was calculated by measuring the diameter of inhibition, including the hole in the centre, and dividing the value in half.

Testing cell-free supernatant against *V. dahliae* growth using a zone of inhibition assay. Cell-free supernatant (CFS) was prepared as described previously (3, 21). Briefly, a liquid culture of *V. dahliae* mycelia was grown by inoculating 170mL liquid SXM with $6.667x10^3$ *V. dahliae* spores and incubating at 25ºC, 150rpm, for 5 days. Next, *V. dahliae* mycelia were separated into three 10mL aliquots and then centrifuged at 4000 x g for 5 min. The supernatant was discarded and the pellet was resuspended in 8mL of fresh SXM. In parallel, an overnight culture of P_DAPG was grown in 5mL of liquid SXM at 25ºC and 150rpm, and then 1.5mL of this overnight P_DAPG culture was transferred to 150mL fresh SXM and incubated at 25ºC to an OD_{600nm}= 1. The P_DAPG culture was centrifuged at 4000 x g for 5 min and the supernatant was discarded. The resulting cell pellet was resuspended in fresh SXM to reach a final OD_{600nm} = 3. To prepare the CFS, 2mL of P_DAPG (OD_{600nm} = 3) was transferred into the 8mL resuspension of *V. dahliae* mycelia. In parallel, a 2mL aliquot of P_DAPG (OD_{600nm} = 3) was transferred to 8mL of fresh SXM. The liquid cultures were incubated at 25ºC, 150rpm, for 2 days, then centrifuged at 6000 x g for 10min at 4°C to pellet the cells. The supernatants were collected and passed through a 0.45µm filter, followed by a 0.20µm filter to remove any remaining cells. The above procedure was repeated in parallel for the negative controls (*E. coli* DH5α alone, *E coli* DH5α co-cultured with *V. dahliae, V. dahliae* alone, and liquid SXM alone). 60µL of CFS was then transferred to a hole in the center of an SXM plate spread with 1 x 10^5 *V. dahliae* spores and incubated for 4 days at room temperature in the light. The inhibition radii were then measured as described. The unused CFS was stored at -70ºC for the remainder of the study.

Statistical analyses. The inhibition radii of intact P_DAPG and CFS against *V. dahliae* growth were visualized as bar graphs using GraphPad Prism 9.3.0 (345) (GraphPad Software, USA). A two-tailed student's T-test was used to evaluate significant differences ($p < 0.05$).

Testing cell-free supernatant against *V. dahliae* growth using a direct contact assay. A liquid co-culture of *V. dahliae* and P_DAPG was prepared by adding 60µL of the overnight P_DAPG culture (OD_{600nm}= 1) and 1 x 10^5 *V. dahliae* spores to 5mL liquid SXM. In parallel, a liquid culture of P_DAPG was prepared by adding 60µL P_DAPG (OD_{600nm} = 1) to 5mL SXM. The cultures were incubated at 25ºC for 2 days at 150rpm. To prepare the CFS, the liquid cultures were centrifuged at 6000 x g for 10min at 4°C to pellet cells and the

supernatant was passed through a 0.45µm filter, followed by a 0.20µm filter, to remove any remaining cells. 1 x 10^5 *V. dahliae* spores were spread on a PDA plate and a groove was carved down the center of the plate. Once the spread of spores had dried, 120µL of CFS was evenly spread directly on top of half of the plate. The same procedure was repeated for the negative controls (filtered SXM and water) and positive control (intact P_DAPG, OD_{600nm} = 1). The plates were incubated for 4 days at room temperature in the light and inhibition was observed.

RESULTS

Intact *P. protegens* CHA0 inhibits *V. dahliae* growth. To investigate the ability of intact P_DAPG to inhibit *V. dahliae* growth, we conducted a zone of inhibition assay as described in the Methods section. Liquid SXM was used to culture both *V. dahliae* and P_DAPG and as a negative control, due to its high pectin content that mimics a natural plant habitat (3). As an additional negative control, we used a liquid culture of *E. coli* DH5α, since *E. coli* has been shown not to inhibit *Verticillium* spp. growth (3). All conditions were plated in three technical replicates. After a 4-day incubation, plates with intact P_DAPG displayed an average inhibition radius of 1.25 ± 0.05cm, whereas the negative control plates displayed no zones of inhibition (Figure 1). Statistical analysis showed significant differences between the negative controls and intact P_DAPG ($p < 0.0001$), indicating that intact P_DAPG can inhibit *V. dahliae* growth on SXM plates (Figure 1).

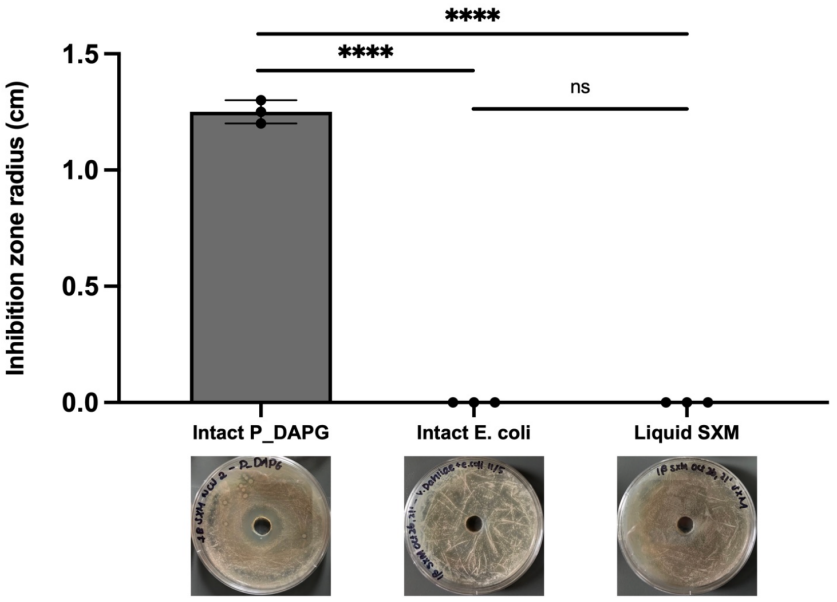


FIG. 1 Intact *P. protegens* CHA0 inhibits *V. dahliae* growth. 1 x 10^5 *V. dahliae* spores were spread on solid pectin-rich simulated xylem media (SXM). In three technical replicates, intact *P. protegens* CHA0 was plated in a hole in the centre of the plates. *E. coli* DH5α and liquid SXM were plated as negative controls. The plates were left for 4 days at room temperature face-up in the light. The inhibition radius on each plate was then measured and visualized using a barplot. The bars represent the means of the technical replicates and the whiskers indicate standard deviation (± 0.05cm). A two-tailed Student's T-test was used to calculate statistical differences (**** $p < 0.001$, ns = not significant). Representative plates for each condition are displayed beneath the diagram.

P. protegens CHA0 secretions alone do not inhibit *V. dahliae* growth. Next, to determine if P_DAPG secretions alone are sufficient to inhibit *V. dahliae* growth, we conducted the same zone of inhibition assay using CFS generated from a culture of P_DAPG, as described in the Methods section. To investigate whether P_DAPG secretions change when exposed to *V. dahliae,* we also tested inhibition of CFS generated from a co-culture of P_DAPG and *V. dahliae* mycelia. As negative controls, we used CFS generated from liquid cultures of SXM alone, *E. coli* alone, and *E. coli* co-cultured with *V. dahliae* mycelia. As a positive control, we used intact P_DAPG. All conditions were plated in three technical replicates. After a 4-day incubation, intact P_DAPG showed significant inhibition of *V. dahliae* growth (Figure 2; $p < 0.0001$). In contrast, we observed no inhibition on the plates with CFS generated from P_DAPG alone or P_DAPG co-cultured with *V. dahliae* to the *E. coli* negative control plates (Figure 2). These data suggest that P_DAPG secretions alone are not sufficient to inhibit fungal growth (Figure 2).

Since it is possible that P_DAPG secretions made no direct contact with *V. dahliae* in our zone of inhibition assay, we retested P_DAPG secretions against *V. dahliae* growth using the

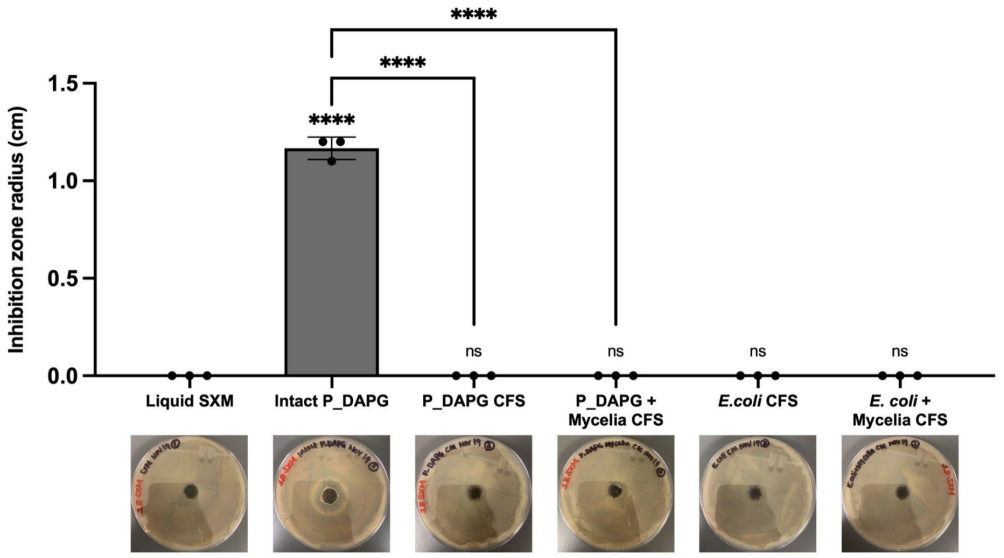


FIG. 2 *P. protegens* CHA0 secretions alone may not be sufficient to inhibit *V. dahliae* growth. Cell-free supernatant (CFS) was generated by collecting and filter sterilizing the supernatant from a culture of *P. protegens* CHA0 (P_DAPG) and a co-culture of P_DAPG and *V. dahliae* mycelia. 1 x 10^5 *V. dahliae* spores were spread on solid pectin-rich simulated xylem media (SXM). In three technical replicates, the generated CFS was plated in a hole in the centre of the plates. As negative controls, liquid SXM and CFS generated from cultures of *E. coli* DH5α and *E. coli* DH5α co-cultured with *V. dahliae* mycelia were plated in the plates' centres. The plates were left for 4 days at room temperature face-up in the light. The inhibition radius on each plate was then measured and visualized using a barplot. The bars represent the means of the technical replicates and the whiskers indicate standard deviation (± 0.06cm). A two-tailed Student's T-test was used to calculate statistical differences (**** $p < 0.001$, ns = not significant). Differences to the liquid SXM control are indicated directly on top of the bars. Differences to the positive intact P_DAPG control are indicated with connecting lines. Representative plates for each condition are displayed beneath the diagram.

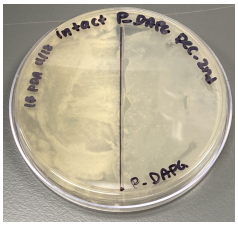
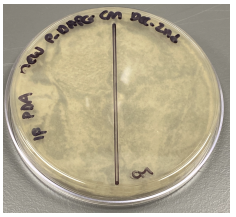
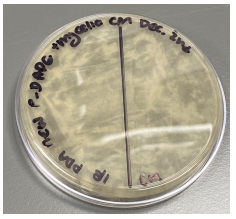
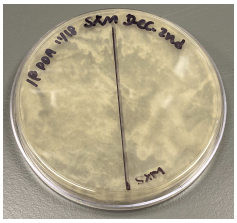
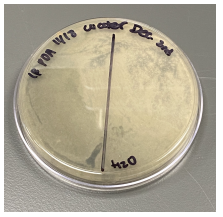
direct contact assay described in the Methods section. For negative controls, we repeated the assay using either filtered liquid SXM or water, to account for potential disturbance of *V. dahliae* spores that occurs when plating CSF. In addition, intact P_DAPG was plated as a positive control. Each condition was plated in three technical replicates. After a 4-day incubation, we observed no fungal inhibition on the experimental or negative control plates, while the positive control plates displayed a lawn of P_DAPG without any fungal growth (Table 1). Taken together, these results suggest that P_DAPG secretions alone may not be sufficient to inhibit *V. dahliae* growth.

DISCUSSION

Our study investigated the potential of intact P_DAPG and P_DAPG secretions to inhibit *V. dahliae* growth. We found that intact P_DAPG inhibited *V. dahliae* growth, but that P_DAPG secretions alone did not inhibit growth under the specific conditions of our assays.

Intact *P. protegens* CHA0 inhibits *V. dahliae* growth. We demonstrated that intact P_DAPG can inhibit *V. dahliae* growth when plated on SXM spread with *V. dahliae* spores. After 4 days of incubation at room temperature, we found a significant *V. dahliae* growth inhibition radius on each experimental plate. These results were as expected because P_DAPG has been shown to inhibit *Verticillium* spp. using different assays (3). Specifically, Harting *et al* found that P_DAPG could reduce *V. longisporum* in a microfluidic device from 100% (control) to 6%. Further, the authors found that the colonization of *V. dahliae* on *Arabidopsis thaliana* roots was reduced from 100% (control) to 8% when pre-treated with P_DAPG (3). Taken together, our results support existing research showing that P_DAPG can inhibit *V. dahliae* growth.

Table 1. Even in direct contact, *P. protegens* CHA0 secretions alone may not be sufficient to inhibit *V. dahliae* growth. Cell-free supernatant (CFS) was generated by collecting and filter sterilizing the supernatant from a culture of *P. protegens* CHA0 (P_DAPG) and a co-culture of P_DAPG and *V. dahliae* spores. 1 x 10^5 *V. dahliae* spores were spread on solid potato dextrose agar (PDA) plates. In three technical replicates, the generated CFS was plated directly on top of half the plate of spores (right side of the plate), with the other half acting as a negative control for itself (left side of the plate). As additional negative controls, filtered liquid SXM or water were dispensed on top of half the plate of spores, and intact P_DAPG was plated as a positive control. Once the plates dried, they were left upside down at room temperature in the light for four days. Representative plates for each condition are displayed in the table.

Intact P_DAPG	P_DAPG CFS	P_DAPG + mycelia CFS	Liquid SXM	Water
				

***P. protegens* CHA0 secretions alone may not inhibit *V. dahliae* growth.** In our zone of inhibition assay, CFS generated using P_DAPG alone and P_DAPG co-cultured with *V. dahliae* mycelia did not inhibit *V. dahliae* growth. We expected to observe some fungal inhibition from secretions alone because GacS/GacA knockout mutants of P_DAPG had reduced antifungal activity against *V. longisporum*, suggesting that antifungal secretions are important in inhibiting fungal growth (3). The lack of inhibition seen in our study may be due to a low concentration of metabolites in our CFS. Additionally, when generating the cultures used to make the CFS, P_DAPG did not reach the desired OD_{600nm} of 3, as described in the Methods section (3). Therefore, the bacteria to fungi ratio may have not been optimal for P_DAPG to produce antifungal secretions. Furthermore, the mycelial culture used to generate the CFS was overgrown and therefore, there may not have been enough nutrients remaining in the media to sustain further growth. Another possible explanation is that as the CFS diffused into the agar, its concentration decreased with increasing distance from the centre of the plate. Thus, it is possible that the CFS was at a much lower concentration than the amount originally plated in the central hole, thereby posing no direct challenge to the fungus.

Direct contact of *P. protegens* CHA0 cell-free supernatant with *V. dahliae* does not inhibit fungal growth. To address pitfalls encountered in the CFS zone of inhibition assay, we dispensed the CFS directly on top of the *V. dahliae* spores spread evenly on PDA plates to put any secreted metabolites in direct and uniform contact with the fungus. This assay enabled us to plate a higher volume of CFS than the well-based assay, leading to potentially higher metabolite concentration. Further, we generated new CFS to address the overgrown mycelia and low OD_{600nm} challenges experienced when making the CFS in the first assay. However, even with these adjustments, we observed no inhibition of fungal growth by CFS alone.

Experiments by Harting *et al.* suggest that antifungal secretions alone may not be sufficient biocontrol agents (3). For example, in testing P_DAPG mutant derivatives against *V. longisporum* in a microfluidic device, the authors found that an absence or an upregulation of the structural gene for DAPG did not drastically affect the antifungal activity of the mutants. Harting *et al.* also observed that both wild-type P_DAPG and GacA/GacS mutant P_DAPG strains were able to reduce *V. dahliae* colonization on *Arabidopsis thaliana* roots, suggesting that there are GacA/GacS-independent antifungal properties (3). Thus,

Pseudomonas spp. may protect against fungal pathogens independent of, or in synergy with, antifungal compounds.

The type III secretion system (T3SS), in *Pseudomonas* spp. may be another reason metabolites alone are not sufficient inhibitors of fungal growth. A study by Rezzonico *et al.* found that the genes for the T3SS in *P. fluorescence* KD (KD) are induced in the presence of the oomycete *Pythium ultimum* (22). The T3SS is utilized by the bacterium to secrete virulence factors that target *P. ultimum* and protect cucumber plants from infection. When the T3SS gene was inactivated, the biocontrol activity of KD against *P. ultimum* was reduced. Although there is little information regarding the T3SS in P_DAPG, this mechanism may explain the importance of the bacteria being intact and in close physical contact with its target to secrete the necessary metabolites for fungal inhibition (22).

Another reason the bacteria may need to be intact to inhibit fungal growth is nutritional competition, as both *V. dahliae* and P_DAPG utilize pectin as a carbon source (3). For instance, analysis of fungal mRNA production by Harting *et al.* showed that when *V. longisporum* is co-cultured with P_DAPG, the fungus significantly downregulates genes involved in pectin degradation (3). Although this downregulation may simply be a result of reduced fungal growth, it may also be due to nutrient starvation in the presence of P_DAPG, which in turn lowers pectinolytic gene expression by *V. longisporum*.

Taken together, our results suggest that P_DAPG secretions alone may not be sufficient to inhibit the growth of *V. dahliae*. Our results suggest that other factors, such as a T3SS and nutrient competition, likely play a key role in reducing fungal growth.

Conclusions In conclusion, we tested the biocontrol activity of intact P_DAPG and P_DAPG secretions alone, and observed inhibition on the plates with intact P_DAPG but not on the plates with CFS. Therefore, our results suggest that P_DAPG inhibits *V. dahliae* growth but that P_DAPG secretions alone are not sufficient to inhibit growth of *V. dahliae.* However, although our assays were sufficient for testing antifungal activity, they may not be representative of natural habitats. For example, previous studies have demonstrated that *V. longisporum* can distinguish between SXM and plant xylem sap (25), suggesting that this medium may not be ideal for understanding real-life applications. Further, Harting *et al.* found that although *Pseudomonas* N2E2 had the strongest inhibitory effects against *V. dahliae* growth on SXM plates compared to other strains, it showed the weakest effect against fungal root colonization (3). *In vitro* analyses are suitable to detect BCAs that secrete antifungal metabolites, but cannot easily detect or measure other mechanisms of biocontrol. Thus, the importance of metabolites may be artificially inflated (9). This inflation is supported by gene expression data that demonstrate that antifungal metabolites may only play a minor role, if any at all, in fungal growth inhibition *in vivo* (26). Taken together, the potential for antifungal secretions as effective BCA-derived fungicides needs to be further explored to provide insights into effective biocontrol strategies against plant diseases.

Future Directions In the future, the zone of inhibition assay we employed could be used to test other *Pseudomonas* strains against *V. dahliae,* and/or intact P_DAPG could be tested against other plant pathogens, such as *Aphanomyces euteiches* (23). Testing other *Pseudomonas* strains could provide more insight into the most effective strain of *Pseudomonas* to combat Verticillium wilt and using knockout strains could provide more clarity on the importance of various antifungal metabolites. Testing P_DAPG against other fungi could demonstrate its role in protecting plants from a wider range of phytopathogens.

In re-examining the biocontrol activity of secretions alone, it would be useful to check for the presence of antifungal metabolites in the CFS before testing it against fungal growth. Bonsall *et al.* have outlined a procedure for isolating and identifying DAPG from both broth and agar cultures, as well as from plant root rhizospheres (24). This procedure is also suitable for the extraction of other antifungal metabolites, including phenazines and pyoluteorin. It would also be worth concentrating the CFS via vacuum drying to ensure there are high enough concentrations of the antifungal metabolites to have an effect on fungal growth.

If CFS is shown to work against *V. dahliae* growth at a higher concentration, the CFS could be treated with proteolytic agents or pH changes to see how these factors affect antifungal activity. Further, the CFS could be fractionated and/or subjected to GC-mass

spectrometry to identify both novel and known antifungal agents. Different fractions could then be combined to look for synergies. If CFS lacks biocontrol activity even at higher concentrations, intact P_DAPG could be treated with a bacteriostatic antibiotic before plating it against *V. dahliae* to determine if the bacteria need to be growing to cause inhibition. This would help clarify the role of nutritional competition in the inhibition of fungal growth.

Harting *et al.* used mRNA sequencing to observe changes in the fungal transcriptome when *V. longisporum* was co-cultured with P_DAPG (3). In the future, sequencing of P_DAPG mRNA could be conducted to provide insight into the regulation of antifungal genes in the presence versus absence of fungi. This experiment could help identify novel antifungal agents. Furthermore, sequencing of fungal mRNA could be repeated using fungal strains co-cultured with CFS to determine if CFS alone is sufficient for reducing fungal growth.

ACKNOWLEDGEMENTS

We thank the Department of Microbiology and Immunology at the University of British Columbia for funding this study. We would also like to thank Jade Muileboom, Dr. David Oliver, and Braydon Black for their guidance and support. Further, we would like to thank Dr. Cara Haney for sharing her expertise on this topic and for providing us with *V. dahliae* and P_DAPG, as well as Alexandra Nagel for providing the recipe for the simulated xylem media. Finally, we express our appreciation to the rest of the MICB 401 teaching team and our peers for their advice and support.

CONTRIBUTIONS

Co-authorship should be considered equal for this manuscript. S.K. devised the project idea. C.M. worked on the Abstract and created the figures. C.M. and S.L. worked on the Introduction section. A.R. and S.K. worked on the Methods section. S.L. and S.K. worked on the Results section, and S.K. worked on the Discussion section. C.M. helped with the Conclusion. All authors equally carried out the experiments and helped revise the manuscript.

REFERENCES

1. **Carroll CL, Carter CA, Goodhue RE, Lawell C-YCL, Subbarao KV.** 2018. A Review of Control Options and Externalities for Verticillium Wilts. Phytopathol. 108:160–171.
2. **Fradin EF, Thomma BPHJ**. 2006. Physiology and Molecular Aspects of Verticillium Wilt Diseases Caused by *V. dahliae* and *V. albo-atrum*. Mol. Plant. Pathol. 7:71–86.
3. **Harting R, Nagel A, Nesemann K, Höfer AM, Bastakis E, Kusch H, Stanley CE, Stöckli M, Kaever A, Hoff KJ, Stanke M, deMello AJ, Künzler M, Haney CH, Braus-Stromeyer SA, Braus GH.** 2021. *Pseudomonas* Strains Induce Transcriptional and Morphological Changes and Reduce Root Colonization of *Verticillium* spp. Front. Microbiol. 12:652468.
4. **Molina OI, Tenuta M, El Hadrami A, Buckley K, Cavers C, Daayf F.** 2014. Potato Early Dying and Yield Responses to Compost, Green Manures, Seed Meal and Chemical Treatments. Am. J. Potato Res. 91:414–428.
5. **Davis JR, Huisman OC, Everson DO, Schneider AT.** 2001. Verticillium Wilt of Potato: A Model of Key Factors Related to Disease Severity and Tuber Yield in Southeastern Idaho. Am. J. Potato Res. 78:291–300.
6. **Deketelaere S, Tyvaert L, França SC, Höfte M.** 2017. Desirable Traits of a Good Biocontrol Agent against Verticillium Wilt. Front. Microbiol. 8:1186.
7. **Ons L, Bylemans D, Thevissen K, Cammue BPA**. 2020. Combining Biocontrol Agents with Chemical Fungicides for Integrated Plant Fungal Disease Control. Microorganisms 8:1930.
8. **Jack CJ, Ellis JD.** 2021. Integrated Pest Management Control of *Varroa destructor* (Acari: Varroidae), the Most Damaging Pest of (*Apis mellifera* L. (Hymenoptera: Apidae)) Colonies. J. Insect Sci. 21.
9. **Köhl J, Kolnaar R, Ravensberg WJ**. 2019. Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. Front. Plant Sci. 10:845.
10. **Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L, Stewart A.** 2012. Have Biopesticides Come of Age? Trends Biotechnol. 30:250–258.
11. **Marrone PG.** 2002. An Effective Biofungicide with Novel Modes of Action. Pest Outlook 13:193–194.
12. **Bull CT, Duffy B, Voisard C, Défago G, Keel C, Haas D. 2001.** Characterization of Spontaneous *gacS* and *gacA* Regulatory Mutants of *Pseudomonas fluorescens* Biocontrol Strain CHA0. Antonie van Leeuwenhoek 79:327–336.
13. **Yan Q, Lopes LD, Shaffer BT, Kidarsa TA, Vining O, Philmus B, Song C, Stockwell VO, Raaijmakers JM, McPhail KL, Andreote FD, Chang JH, Loper JE.** 2018. Secondary Metabolism and Interspecific Competition Affect Accumulation of Spontaneous Mutants in the

GacS-GacA Regulatory System in *Pseudomonas protegens*. mBio https://doi.org/10.1128/mBio.01845-17.

14. **Wang D, Lee S, Seeve C, Yu JM, Pierson LS, Pierson EA.** 2013. Roles of the Gac-Rsm Pathway in the Regulation of Phenazine Biosynthesis in *Pseudomonas chlororaphis* 30-84. Microbiol. Open 2:505–524.
15. **Troppens DM, Dmitriev RI, Papkovsky DB, O'Gara F, Morrissey JP.** 2013. Genome-Wide Investigation of Cellular Targets and Mode of Action of the Antifungal Bacterial Metabolite 2,4-Diacetylphloroglucinol in *Saccharomyces cerevisiae*. FEMS Yeast Res. 13:322–334.
16. **Weller DM, Mavrodi DV, van Pelt JA, Pieterse CMJ, van Loon LC, Bakker PAHM.** 2012. Induced Systemic Resistance in *Arabidopsis thaliana* Against *Pseudomonas syringae* pv. *tomato* by 2,4-Diacetylphloroglucinol-Producing *Pseudomonas fluorescens*. Phytopathol. 102:403–412.
17. **Hernández-León R, Rojas-Solís D, Contreras-Pérez M, Orozco-Mosqueda MaDC, Macías-Rodríguez LI, Reyes-De La Cruz H, Valencia-Cantero E, Santoyo G.** 2015. Characterization of the Antifungal and Plant Growth-Promoting Effects of Diffusible and Volatile Organic Compounds Produced by *Pseudomonas fluorescens* Strains. Biol. Control 81:83–92.
18. **Naseby DC, Way JA, Bainton NJ, Lynch JM.** 2001. Biocontrol of *Pythium* in the Pea Rhizosphere by Antifungal Metabolite Producing and Non-Producing *Pseudomonas* Strains. J. Appl. Microbiol. 90:421–429.
19. **Nadeem S, Naveed M, Ayyub M, Yahya Khan M, Ahmad M, Zahir Z.** 2016. Potential, Limitations and Future Prospects of *Pseudomonas* spp. for Sustainable Agriculture and Environment: A Review. Soil Environ. 35:106–145.
20. **Paulin MM, Novinscak A, Lanteigne C, Gadkar VJ, Filion M. 2017.** Interaction between 2,4-Diacetylphloroglucinol- and Hydrogen Cyanide-Producing *Pseudomonas brassicacearum* LBUM300 and *Clavibacter michiganensis* subsp. *michiganensis* in the Tomato Rhizosphere. Appl. Environ. Microbiol. 83:AEM.00073-17.
21. **Ndagano D, Lamoureux T, Dortu C, Vandermoten S, Thonart P.** 2011. Antifungal Activity of 2 Lactic Acid Bacteria of the *Weissella* Genus Isolated from Food. J. Food Sci. 76:M305–M311.
22. **Rezzonico F, Binder C, Défago G, Moënne-Loccoz Y.** 2005. The Type III Secretion System of Biocontrol *Pseudomonas fluorescens* KD Targets the Phytopathogenic Chromista *Pythium ultimum* and Promotes Cucumber Protection. Mol. Plant-Microbe Interact. 18:991–1001.
23. **Godebo AT, Germida JJ, Walley FL.** 2020. Isolation, Identification, and Assessment of Soil Bacteria as Biocontrol Agents of Pea Root Rot Caused by *Aphanomyces euteiches*. Can. J. Soil Sci. 100:206–216.
24. **Bonsall RF, Weller DM, Thomashow LS.** 1997. Quantification of 2,4-Diacetylphloroglucinol Produced by Fluorescent *Pseudomonas* spp. In Vitro and in the Rhizosphere of Wheat. Appl. Environ. Microbiol. 63:951–955.
25. **Leonard M, Kühn A, Harting R, Maurus I, Nagel A, Starke J, Kusch H, Valerius O, Feussner K, Feussner I, Kaever A, Landesfeind M, Morgenstern B, Becher D, Hecker M, Braus-Stromeyer SA, Kronstad JW, Braus GH.** 2020. *Verticillium longisporum* Elicits Media-Dependent Secretome Responses with Capacity to Distinguish Between Plant-Related Environments. Front. Microbiol. 11:1876.
26. **Koch E, Ole Becker J, Berg G, Hauschild R, Jehle J, Köhl J, Smalla K.** 2018. Biocontrol of Plant Diseases is Not an Unsafe Technology! J. Plant Dis. Prot. 125:121–125.