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Pseudomonas species significantly inhibit growth of *Aphanomyces euteiches* mycelium *in vitro*

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SUMMARY Pea plants (*Pisum sativum*) are an important agricultural crop, and root rot is a major cause of low crop yield and economic loss. *Aphanomyces euteiches*, a common causative agent of pea root rot, is a potent and resilient pest due to its ability to produce long-lived oospores. Traditional methods of pathogen control, including crop rotation and pesticide use, have failed to reduce *A. euteiches* disease and illustrate the need for novel, sustainable, and non-toxic control methods. Several *Pseudomonas* strains native to the pea plant rhizosphere have documented anti-oomycete genes and function, and are a promising target for research on the biocontrol of *A. euteiches* in pea plants. Using growth inhibition assays that we developed, we observed that *Pseudomonas protegens* CHA0, *Pseudomonas* sp. NFACC39, and *P. protegens* PF-5 significantly inhibited *A. euteiches* mycelial growth *in vitro*. These findings support the potential of native *Pseudomonas* strains as a solution for *A. euteiches* root rot prevention and treatment, and highlight the necessity for further examinations into the role of specific anti-oomycete genes in the growth inhibition of *A. euteiches.*

INTRODUCTION

Pea plants are one of the oldest domesticated crops and a staple of the Canadian agricultural industry (1, 2). However, pea plants are susceptible to root rot, resulting in poor crop yield and economic losses (3). Common causative agents of root rot include *Aphanomyces euteiches*, *Rhizoctonia solani*, and several *Fusarium* species (3). *A. euteiches* is one of the most serious root rot pathogens due to its ability to produce long-lasting oospores that may persist for years in the soil environment (4). While methods such as seed treatment and crop rotation have been attempted to control *A. euteiches* root rot, minimal success has been found (5). Though the use of pesticides could, in theory, mitigate root rot, there is a constant arms race between the evolution of a pathogen's drug resistance and the discovery, production, and application of these chemicals (6). Thus, more sustainable, non-toxic, and effective strategies are urgently needed to combat root rot—and this is where microbiome manipulation shows potential as a therapeutic avenue (7).

Pseudomonas is a genus of aerobic, gram-negative Proteobacteria abundantly found in pea root rhizospheres. With 191 documented species, the genus is diverse, and several *Pseudomonas* species such as *P. protegens* CHA0, *P. protegens* PF-5, and *P.* sp. NFACC39, and *P.* sp. NFACC09 demonstrate antifungal and anti-oomycete capabilities (8-11). *P. protegens* CHA0 can inhibit *Pythium* growth, another oomycete and causative agent of root rot in pea plants (10). Some factors that contribute to anti-oomycete activity include 2,4-diacetylphloroglucinol (2,4-DAPG), a type-III secretion system (T3SS), syringomycin (SYR), and syringopeptin (SYP) (10-19). 2,4-DAPG is a secondary metabolite that induces membrane damage, alteration of the mitochondrial electron transport chain, and inhibition of vacuole ATPase activity in plant pathogens (10-15). T3SS have been found to activate virulence factors against pathogenic fungi such as adherence factor and biofilm production, has the potential to facilitate the injection of bacterial effector proteins into the cytoplasm of eukaryotic organisms, and interfere with cytoskeleton and cellular trafficking processes (16, 17). SYR and SYP act on plasma membranes to form ion-conducting channels resulting in electrolyte leakage and cell death of pathogens (18, 19). Based on preliminary experiments by the Haney Laboratory at the University of British Columbia and the protection ability of

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P. fluorescens N2E2, *P. fluorescens* WCS365, and *E. coli* DH5α against other pathogens, we will use these strains as positive and negative controls in our experiments. Given the presence of potential biocontrol genes in *Pseudomonas* strains, and known ability to control pathogenic fungi and oomycetes, we hypothesise that *Pseudomonas* strains have the potential to inhibit *A. euteiches*.

In addition to these identified virulence factors, it has been well-documented that *Pseudomonas* can behave differently as a group in comparison to its behaviour as a single individual bacterium (20, 21). This phenomenon of collective-based behaviour is caused by changes within the group that are induced by population-based factors, which may be dependent or independent of quorum sensing (QS). Population-based sensing and responding have been known to activate virulence factors such as biofilm production and T3SS expression (22). Thus, the population dynamics that *Pseudomonas* displays may aid or even activate its anti-oomycete genes, resulting in better biocontrol of *A. euteiches* in comparison to biocontrol abilities of bacteria that are not experiencing population-based changes, which is also explored in our experiment (21).

Considering the documented anti-oomycete activity of *Pseudomonas* strains and the abundance of *Pseudomonas* in the pea rhizosphere microbiome, we believe that *Pseudomonas* would be a logical place to test potential natural biocontrol agents against *A. euteiches* (8-10, 23). The aim of our study is to explore the mycelial *A. euteiches* growth inhibition potential of *P. protegens* CHA0*, P. protegens* PF-5*, P.* sp. NFACC09*,* and *P.* sp. NFACC39 *in vitro*. Since these strains express antifungal and anti-oomycete genes and were shown to inhibit fungal and oomycete growth *in vivo* and *in vitro,* we hypothesize that these strains will inhibit *A. euteiches in vitro*. Here, we show that single colonies of *P. protegens* PF-5 and *P.* sp. NFACC39, as well as lawn sections and streaks of *P. protegens* CHA0, significantly inhibit *A. euteiches* mycelial growth and suggests that these *Pseudomonas* strains are a potential *A. euteiches* biocontrol agent worth investigating *in vivo*.

METHODS AND MATERIALS

Strains and media preparation. All bacterial strains used in the mycelial growth inhibition assays are listed below in Table 1. *E. coli* DH5α was obtained from a sample of starter plates

TABLE. 1 Bacterial strains with corresponding genes of interest and relevance to the study.

Bacterial Strain	2,4-DAPG	SYR/SYP	T3SS	Relevance
E. coli DH5α	-	-	-	Negative control for Assay 1
P. fluorescens WCS365	+	-	-	Negative control for Assays 2-4
P. fluorescens N2E2	+	-	+	Positive control for Assays 1-4
P. protegens CHA0	+	-	-	Growth inhibition assay experimental strain
P. protegens PF-5	+	-	-	Growth inhibition assay experimental strain
P. sp. NFACC09	-	+	-	Growth inhibition assay experimental strain
P. sp. NFACC39	+	-	+	Growth inhibition assay experimental strain

from the MICB 401 laboratory at the University of British Columbia. The bacterial strains *P. fluorescens* WCS365, *P. fluorescens* N2E2, *P. protegens* CHA0, *P. protegens* PF-5, *P.* sp. NFACC09, and *P.* sp. NFACC39, as well as the oomycete *A. euteiches* were obtained courtesy of the Haney Laboratory at the University of British Columbia (11). All bacterial strains were streaked for individual colonies for Assay 1, and lawn plated for Assays 2, 3 and 4. The 4 different assays were performed to measure the capability for each bacterial strain to inhibit mycelial growth under various growth conditions. Assay 1 measures the zones of inhibition when picking a singular colony from a donor plate. Assays 2, 3, and 4 utilise lawn plating, with each assay having different placements for the bacterial strains to determine if altering the positions or inoculation techniques would affect the results obtained. All strains were grown at 23ºC on Potato Dextrose Agar (PDA: 39g/L commercial PDA powder, dH_2O) combined with Select Agar (12.5g/L, dH_2O) and stored at 23ºC. *A. euteiches* was also grown at 23ºC on PDA, but was wrapped in aluminium foil to protect it from light.

Mycelial growth inhibition assay 1. A co-culture of *A. euteiches* and one experimental bacterial strain were performed in triplicate, adapted from Wakelin *et al*. (24). A 0.75cm diameter plug of *A. euteiches* was excised from the edge of the growing source plate and placed in the centre of the petri plate. Then, a single colony was taken from a donor plate and dotted onto each side of the *A. euteiches* plug (Fig. 1A). These steps were repeated with each experimental and control bacterial strain. All plates were incubated in the dark for 5 days at 23°C. Following incubation, the zones of inhibition were measured between the closest edge of *A. euteiches* mycelial growth and bacterial colony using a ruler.

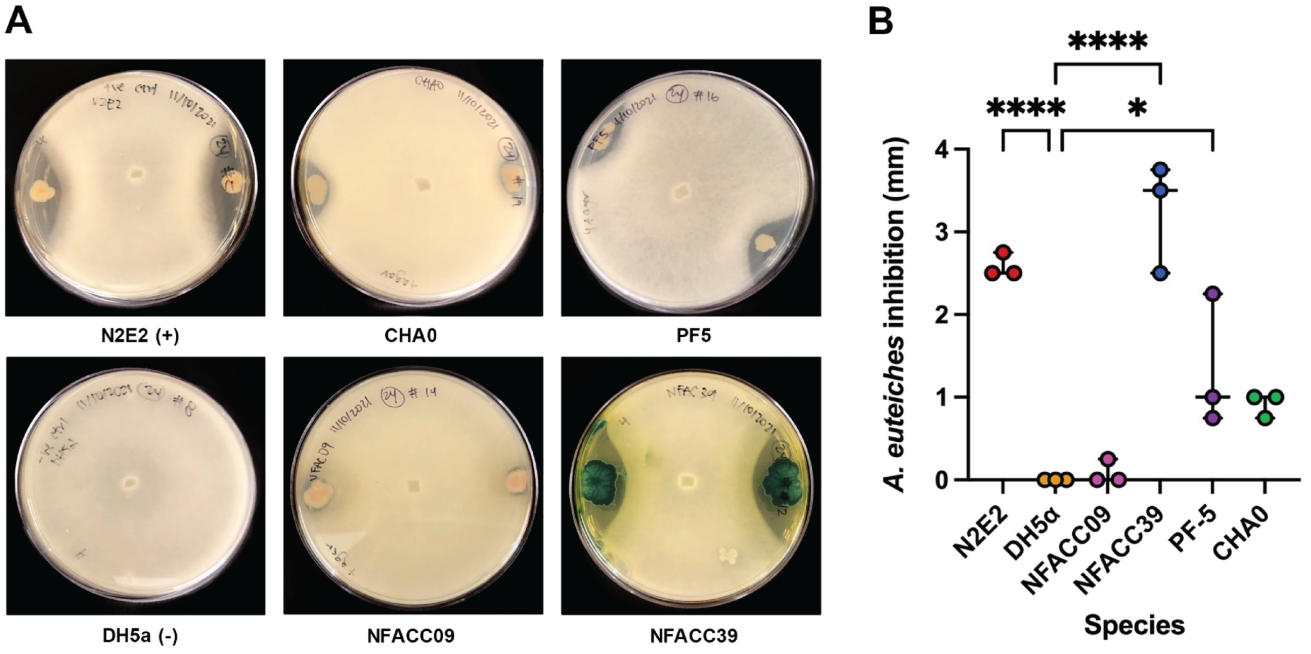

FIG. 1 *P.* sp. NFACC39 and *P. protegens* PF-5 colonies significantly inhibited *A. euteiches* growth *in vitro.* A) Representative images of co-culture Assay 1. Plates were grown at 23ºC in the dark for 5 days. B) Inhibition zones (mm) of each bacterial strain. Ordinary one-way ANOVA and Dunnet's multiple comparisons test, **** $p < 0.0001$, * $p < 0.05$. Error bars represent 95% confidence intervals.

Mycelial growth inhibition assay 2. A co-culture of *A. euteiches* and one experimental bacterial strain were performed in triplicate. Using a Pasteur pipette, a 0.75cm diameter plug of *A. euteiches* was excised from the edge of the growing source plate and placed at one third of the petri plate. Then, a 0.75cm diameter section of confluent bacterial lawn was placed in a 0.75cm diameter well at the second third of the plate (Supplemental Fig. 1A). These steps were repeated with each experimental and control bacterial strain. All plates were incubated in the dark for 5 days at 23°C. Following incubation, the zones of inhibition were measured between the closest edge of *A. euteiches* mycelial growth and bacterial colony using a ruler.

Mycelial growth inhibition assay 3. Co-cultures of *A. euteiches* and one experimental bacterial strain were performed in triplicate. A Pasteur pipette was used to excise a 0.75cm diameter plug of *A. euteiches* from the edge of the growing source plate, and it was placed in the centre of the petri plate. Two 0.75cm diameter sections of confluent bacterial lawn were placed upside down (bacterial lawn contacting the co-culture plate) on each side of the *A. euteiches* plug (Fig. 2A). These steps were repeated with each experimental and control bacterial strain. All plates were incubated in the dark for 5 days at 23°C. Following incubation, the zones of inhibition were measured between the closest edge of *A. euteiches* mycelial growth and bacterial colony using a ruler.

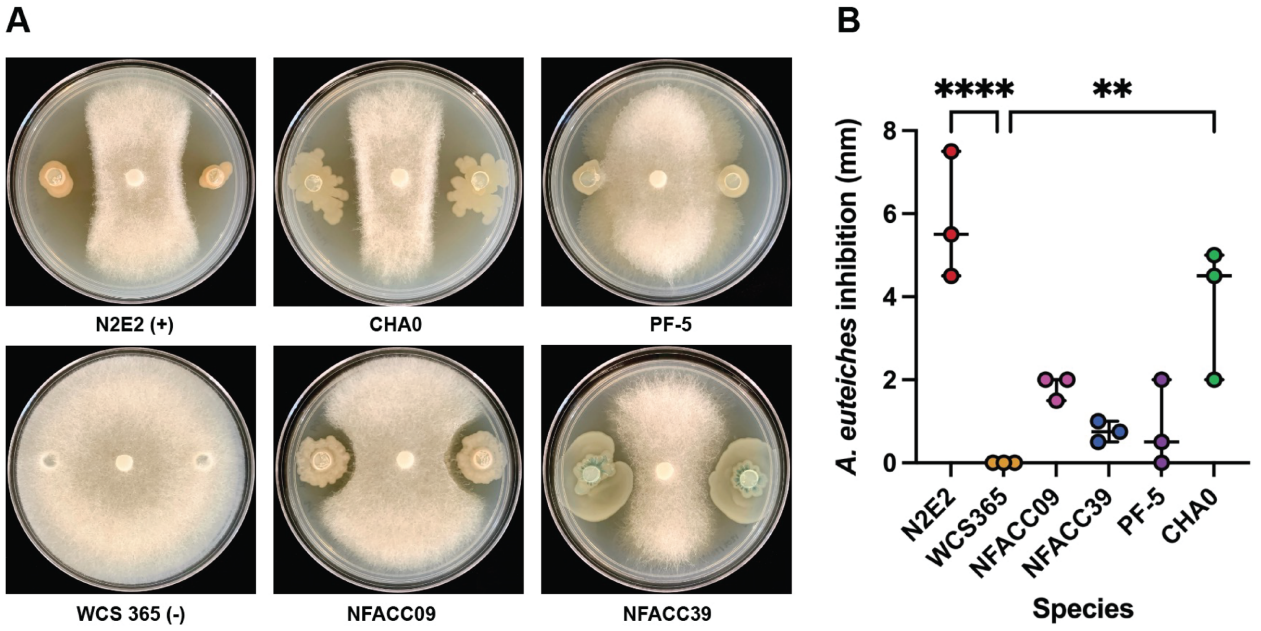

FIG. 2 *P. protegens* CHA0 lawn sections significantly inhibited *A. euteiches* mycelial growth *in vitro*. A) Representative images of co-culture Assay 3. B) Inhibition zones (mm) of each bacterial strain. Ordinary one-way ANOVA and Dunnet's multiple comparisons test, **** $p < 0.0001$, ** $p < 0.01$. Error bars represent 95% confidence intervals.

Mycelial growth inhibition assay 4. A co-culture of *A. euteiches* and one experimental bacterial strain were performed in triplicate. A 0.75cm diameter plug of *A. euteiches* was excised from the edge of the growing source plate and placed in the centre of the petri plate. Then, a loop was dragged through a confluent bacterial lawn, and dotted onto each side of the *A. euteiches* plug (Fig. 3A). These steps were repeated with each experimental and control bacterial strain. All plates were incubated in the dark for 5 days at 23°C. Following incubation, the zones of inhibition were measured between the closest edge of *A. euteiches* mycelial growth and bacterial colony using a ruler.

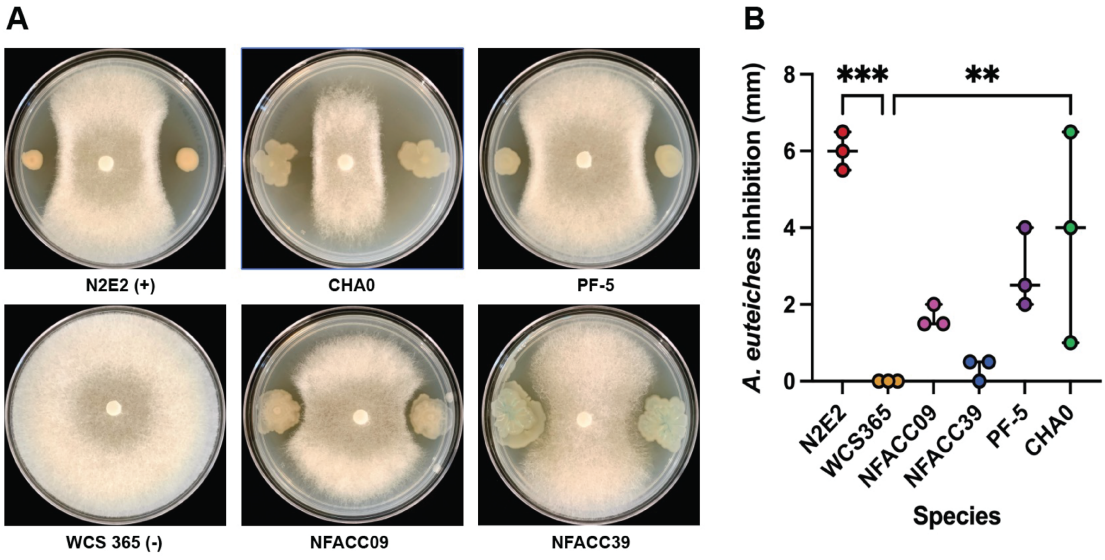

FIG. 3 *P. protegens* CHA0 lawn streaks significantly inhibited *A. euteiches* mycelial growth *in vitro*. A) Representative images of co-culture Assay 4. B) Inhibition zone (mm) of each bacterial strain. Ordinary one-way ANOVA and Dunnet's multiple comparisons test , *** $p < 0.001$, ** $p < 0.01$. Error bars represent 95% confidence intervals.

Imaging and statistical tests. Photos of co-culture assays were taken from the top down on a black background using a phone. Data analysis was performed using a one-way ANOVA and Dunnet's multiple comparisons tests (Fig. 1B, 2B, 3B, and Supplemental Fig. 1B) as well as a two-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test (Fig. 4). All statistical analyses were done in GraphPad Prism.

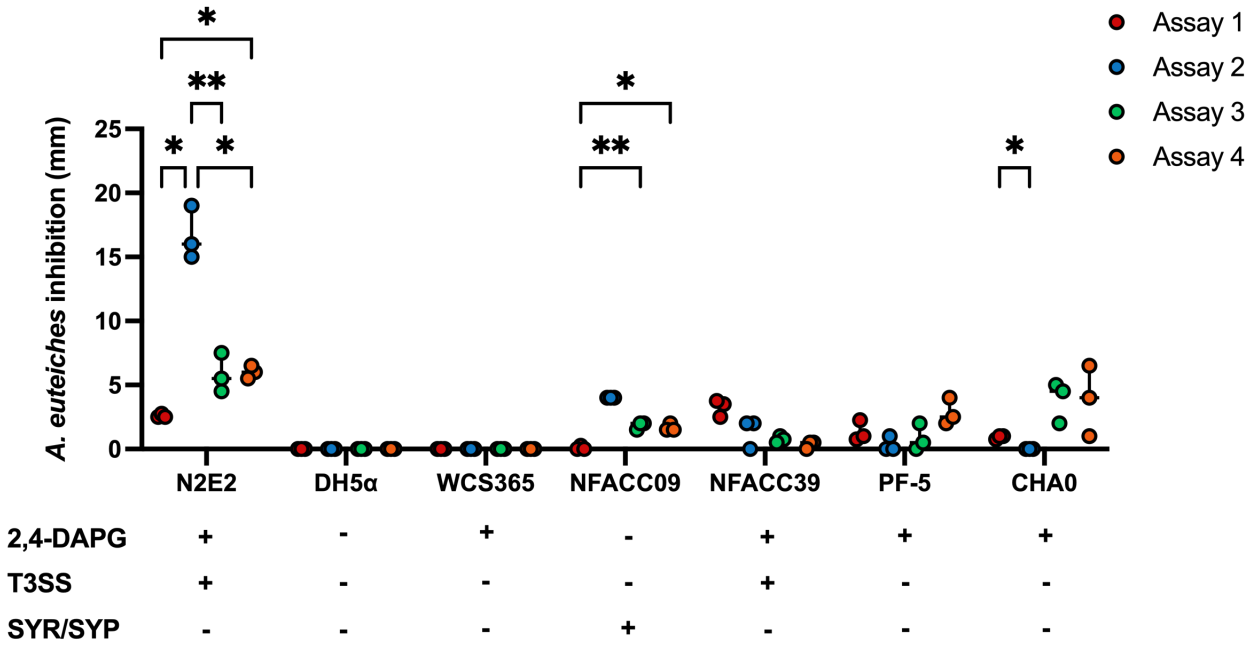

FIG. 4 Inhibition varies upon assay type and is not strongly correlated to genes of interest. Inhibition zone (mm) of each bacterial species and each assay. Two-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test, ** $p < 0.01$, * $p < 0.05$. Error bars represent 95% confidence intervals.

RESULTS

Single colonies of *Pseudomonas* sp. NFACC39 and *Pseudomonas protegens* PF-5 significantly inhibited *Aphanomyces euteiches* mycelial growth *in vitro*. In order to test the ability of *Pseudomonas* to inhibit *A. euteiches*, we developed a single colony-based assay (Assay 1, Methods). This assay found that the positive control *P. fluorescens* N2E2, as well as experimental strains *P.* sp. NFACC39 and *P. protegens* PF-5 were capable of significantly inhibiting mycelial spread of *A. euteiches*, with mean inhibition zones of 2.5mm, 3.5mm and 1.0mm respectively (Fig. 1A, B). The experimental strains *P.* sp. NFACC09 and *P. protegens* CHA0 appeared visually to inhibit *A. euteiches* mycelia (Fig. 1A), however, one-way ANOVA tests indicated that this inhibition was statistically insignificant when compared to the inhibition zone measurements of the negative control, *E. coli* DH5α (Fig. 1B). As expected, the negative control *E. coli* DH5α showed no inhibition of *A. euteiches* (Fig. 1A, B). However, *E. coli* DH5α exhibited very poor growth on PDA (Supplemental Fig. 2), giving uncertainty to the results in Assay 1, as it is difficult to rule out whether poor growth restricted its ability to inhibit *A. euteiches*. Additionally, we observed that *P.* sp. NFACC39 discolours surrounding media neon-yellow, and bears a deep blue morphology. In conclusion, several single-individual colonies of *P.* sp. NFACC39 and *P. protegens* PF-5 were found capable of *A. euteiches* mycelial growth inhibition in Assay 1.

***Pseudomonas protegens* CHA0 lawn sections significantly inhibited mycelial *Aphanomyces euteiches* growth *in vitro*.** While Assay 1 worked well and demonstrated significant *A. euteiches* mycelial growth inhibition of *Pseudomonas*, we wanted to test the *A.*

euteiches inhibition potential of many individuals of a species (as opposed to that of colonies grown from one individual). For these reasons, we chose to use sections of bacterial lawns instead of single-individual colonies (Assay 2 and 3, Methods). Unfortunately, strains exhibited poor growth in Assay 2, most likely due to the placement of the lawn section in an excised well on the plate. The lawn section inserted dried out and shrank, leaving a gap between the lawn section and the plate that the bacteria were not able to bridge well (Supplemental Fig. 1A). Thus, we developed Assay 3, which differed from Assay 2 in that lawn sections were placed upside down directly on the co-culture plate, allowing bacteria to spread easily. Assay 3 demonstrated that lawn sections of *P. protegens* CHA0 were the only experimental strain capable of significantly inhibiting *A. euteiches* mycelial growth, with a mean inhibition zone of 4.5mm (Fig. 2A, B). As expected, the positive control *P. fluorescens* N2E2 significantly inhibited *A. euteiches* mycelial growth, while the negative control *P. fluorescens* WCS365 did not display any inhibition. As with Assay 1, *P.* sp. NFACC09, *P.* sp. NFACC39, and *P. protegens* PF-5 displayed visual inhibition of *A. euteiches* compared to the negative control *P. fluorescens* WCS365, however, inhibition zone measurements were not statistically significant (Fig. 2A, B). Similarly to Assay 1 results, *P.* sp. NFACC39 produced blue pigment, though the intensity was noticeably reduced. As well, *P. protegens* PF-5 displayed interesting *A. euteiches* mycelial morphology (Fig. 2A). Unlike other co-cultures where the edges of the mycelial growth appeared healthy and white, the mycelia closest to the *P. protegens* PF-5 lawn section appeared dead as it had collapsed and was yellow, wilted, and gel-like. In conclusion, lawn sections of *P. protegens* CHA0 were found capable of *A. euteiches* mycelial growth inhibition in Assay 3.

***Pseudomonas protegens* CHA0 lawn streaks significantly inhibited mycelial *Aphanomyces euteiches* growth *in vitro*.** Following Assay 3, we wanted to see whether co-culturing bacteria at a later growth stage with *A. euteiches* gave them an advantage in inhibiting mycelial growth compared to starting the co-culture with few individuals. Thus, we chose to streak the assay plate using a loop that had been passed through a lawn for Assay 4. Similarly to Assay 3, Assay 4 showed that *P. protegens* CHA0 was the only experimental strain capable of significantly inhibiting mycelial *A. euteiches* growth, with a mean inhibition zone of 4.2mm (Fig. 3A, B). These results are congruent with Assay 3, in which *P. protegens* CHA0 had a mean inhibition zone of 4.5mm. *P. protegens* PF-5, *P.* sp. NFACC09, and *P.* sp. NFACC39 once again showed visual inhibition of *A. euteiches* mycelial growth compared to the negative control *P. fluorescens* WCS365, however, one-way ANOVA tests yielded statistically insignificant results (Fig. 3B). Again, the positive control *P. fluorescens* N2E2 significantly inhibited *A. euteiches* mycelial growth while the negative control *P. fluorescens* WCS365 did not display any inhibition. Once more, yellow discolouration of the media and blue pigmentation of *P. sp.* NFACC39 was present, but the discolouration was noticeably reduced in comparison to Assay 1. The conclusions from Assay 3 and 4 indicate that *P. protegens* CHA0 lawns at early and later growth stages are capable of significantly inhibiting *A. euteiches* mycelial growth *in vitro*.

DISCUSSION

In this study, we investigated the *A. euteiches* inhibition ability of several *Pseudomonas* strains. Overall, we found that *P. protegens* CHA0, *P.* sp. NFACC39, and *P. protegens* PF-5 significantly inhibited *A. euteiches* mycelial growth *in vitro*.

While Assay 1 (Fig. 1) worked well and provided significant results, we determined that it would be more scientifically relevant to analyse the *A. euteiches* inhibition capacity of *Pseudomonas* at an advanced growth stage. This would more closely resemble *in vivo* conditions and could potentially induce stronger anti-oomycete activity due to the population dynamics between individual bacteria, which may be dependent or independent of QS. Such phenomena include the production of biofilms, siderophores, and secondary metabolites with antibiotic capabilities (20). Consequently, single colonies were replaced with bacterial lawn sections embedded in the plate for Assays 2, 3, and 4. Assay 2 showed that *P.* sp. NFACC09 significantly inhibited *A. euteiches* mycelial growth, however, we did not feel that these

results were representative due to poor bacterial growth caused by the PDA of lawn sections drying out, inhibiting bacterial growth (Supplemental Fig. 1A). Thus, we flipped the excised lawn section upside down on the assay plate or dotted a loop dragged through a lawn for Assays 3 and 4 (Fig. 2, 3).

The results obtained from Assay 1 conflicted with Assays 3 and 4. Assay 1 showed significant *A. euteiches* mycelial growth inhibition by *P.* sp. NFACC09 and *P. protegens* PF-5, and no significant inhibition by *P. protegens* CHA0, while Assays 3 and 4 only showed significant inhibition by *P. protegens* CHA0. Because we compared the inhibitory effects of lawns to single colonies, we hypothesised that the QS dependent or independent population dynamics present in lawns resulted in a differential regulation of factors that control anti-oomycete function (20). In support of this hypothesis, we observed that the positive control *P. fluorescens* N2E2, and the experimental strain *P. protegens* CHA0 were significantly more inhibitory in Assays 3 and 4 (Fig. 4) compared to Assay 1. Population-based differential gene expression has been a documented phenomenon that can drive *Pseudomonas*-based biocontrol (25). Therefore, it is possible that bacteria such as *P. protegens* CHA0 and *P. fluorescens* N2E2 heavily utilise population-dependent factors to drive anti-oomycete abilities, resulting in increased *A. euteiches* inhibition in the latter two assays utilising bacterial lawns.

The variable inhibition observed from our experimental strains did not appear to be correlated to the genes of interest we identified in the literature (Fig. 4). While *P. protegens* CHA0 significantly inhibited *A. euteiches* growth, *P. protegens* PF-5 was incapable of significant inhibition. Both strains have genes to produce 2,4-DAPG, which has been shown to be effective against root rot (26). Furthermore, while our negative control *P. fluorescens* WCS365 also contains genes that produce 2, 4-DAPG, it was incapable of inhibiting mycelial growth. This observed incongruence may be due to the fact that the level of 2,4-DAPG expression in *Pseudomonas* strains may be regulated by different environmental triggers, and are not ubiquitously expressed (27, 28). Thus, if 2,4-DAPG was necessary for the mycelial inhibition of *A. euteiches*, one could assume that *P. protegens* PF-5 had not sufficiently expressed 2,4-DAPG. Additionally, the positive control *P. fluorescens* N2E2 encodes 2,4-DAPG and a T3SS, as does the experimental strain *P. sp.* NFACC39, both of which were capable of significantly inhibiting mycelial growth in Assay 1 (Fig. 1). In Assays 3 and 4, however, they appeared to have very different measurable zones of inhibition (Fig. 2A, 3A). Previous findings suggest that while 2,4-DAPG production by *P. fluorescens* CHA0 may not be under the control of QS, it may still be synthesised in a cell population dependent manner (29). Therefore, the differences observed may once again circle back to the difference in inhibition capability of single colonies vs. confluent lawns.

The morphology of *A. euteiches* in co-culture assays containing *P. protegens* PF-5 was particularly interesting as it appeared to have a dead outer ring of mycelia, as evidenced by the wilted, gel-like texture and yellow colour (Fig 2A). To compare, no mycelial growth could be seen in the vicinity of *P. fluorescens* N2E2, and the edges of the mycelial growth were healthy and white (Fig. 2A). This indicates that *P. protegens* PF-5 was not able to inhibit mycelial spread, however, it was capable of causing mycelial collapse and presumed death upon contact. Thus, we propose that there may be several mechanisms of *A. euteiches* inhibition by *Pseudomonas* strains. The first is what we would term classical inhibition, wherein the PDA surrounding the bacteria is unable to support mycelial growth. This is presumably due to the secretion and spread of anti-oomycete gene products into the media. The second would be latent inhibition, wherein oomycetes are able to sustain growth on the media, but are unable to survive after making physical contact with the inhibitory bacterium. While these are interesting possibilities, it is important to note that this phenomenon was only significantly observable in Assay 3. Thus, further exploration and repetition is required to gain more insight into the exact mechanisms of mycelial inhibition.

P. sp. NFACC39 was another experimental strain with unexpected morphology. In solo culture, *P.* sp. NFACC39 appeared bright blue and discoloured the PDA yellow (Supplemental Fig. 2A). These effects were significantly more pronounced in Assay 1, with deep blue bacterial colonies and neon yellow PDA discoloration (Fig. 1A). In this assay, *P.* sp. NFACC39 also appeared to inhibit the *A. euteiches* the most, with an average inhibition zone 1mm larger than that of the positive control *P. fluorescens* N2E2 (Fig. 1B). Furthermore,

two days after the inhibition zones were measured, blue pigment was found seeping into the middle of the mycelial growth (Supplemental. Fig. 2B). On the other hand, Assays 3 and 4 did not show as significant of a blue pigmentation or PDA discoloration of *P.* sp. NFACC39. As well, the parent lawn used for these assays only had a light blue tinge, with no significant media discolouration. It is possible that the production of blue pigment and compounds that discolour PDA by *P.* sp. NFACC39 is suppressed by the population dynamics in bacterial lawns, which may be dependent or independent of QS. Interestingly, the assays with low levels of blue pigment and discoloration also were observed to have reduced anti-oomycete activity, which may also be linked to the population dynamics of lawns versus single colonies. The blue pigmentation observed in *P.* sp. NFACC39 has not been previously documented in the literature, and thus serves as an interesting avenue for future investigation.

As aforementioned, the incongruence in the results between the assays is likely due to the use of lawns in Assay 3 and 4, wherein the population dynamics were different in comparison to the single colonies of Assay 1. As a result, we determined that Assays 1, 3, and 4 were all conclusive for selecting potential *A. euteiches* biocontrol agents as the three assays produced significant results, depending on the growth stage that is being tested. With this in mind, we found that *P.* sp. NFACC39, *P. protegens* PF-5, and *P. protegens* CHA0 inhibit *A. euteiches* mycelial growth in their respective growth conditions.

Limitations The scope of our study contains limitations such as the representation of the zones of inhibition in our assays and the applicability of our findings *in vivo.* Firstly, mycelial growth inhibition Assays 1, 3, and 4 showed clear visual differences between the inhibition capacity of the negative control and that of strains deemed statistically insignificant. In our study, the inhibitory capabilities of each bacterial strain were quantified by measuring the closest distance between bacterial growth and mycelial growth. Although some of these measurements were not statistically significant, clear inhibitory effects were seen from all experimental strains (Fig. 1A, 2A, 3A). This was especially obvious when compared directly to the negative control, which displayed complete overgrowth of healthy, white *A. euteiches* mycelia on top of the bacterial colonies (Supplemental Fig. 3B). Furthermore, we did not distinguish between healthy and dying mycelia, though co-cultures with the experimental strains showed wilted, yellowed mycelia in proximity to bacterial colonies (Fig. 2A). For these reasons, utilising a different technique of measurement such as measuring the *A. euteiches* growth instead of the zones of inhibition which takes into account these differences, would better represent the inhibitory effects of the bacterial strains.

Our study was conducted in a laboratory setting with very strict growth conditions. Thus, our findings *in vitro* may not be applicable *in vivo,* as the growth of the experimental strains of bacteria in soil and in other media was not tested and consequently may result in differential outcomes. Furthermore, the ability for the bacteria to withstand varying temperatures and climates may change the rate of growth of the bacteria and could inhibit their anti-oomycete capabilities altogether. Therefore, future studies should test the ability of these bacteria to grow and inhibit mycelial growth in a setting that more closely mimics this variability in the environment to confirm the robustness of their *A. euteiches* growth inhibition *in vivo*.

Conclusions In this study, we utilised mycelial growth inhibition assays to determine the anti-oomycete capability of *P. protegens* CHA0*, P. protegens* PF-5, *P.* sp. NFACC09, and *P.* sp. NFACC39 against *A. euteiches in vitro.* We found that single colonies of *P.* sp. NFACC39 and *P. protegens* PF-5, as well as lawn sections and lawn streaks of *P. protegens* CHA0, significantly inhibited *A. euteiches* mycelial growth *in vitro*. These results suggest a role for bacterial population dynamics in *A. euteiches* inhibition. Furthermore, mycelial growth inhibition does not seem correlated to specific genes of interest, and investigation into gene expression can be further explored. Our findings suggest that *P. protegens* CHA0, *P.* sp. NFACC39, and *P. protegens* PF-5 could serve as potential *A. euteiches* biocontrol agents, however, further research is necessary to explore their *in vivo* viability, as well as their exact mechanisms of inhibition.

Future Directions As mentioned in Limitations, the method we used to quantify inhibition was not precise enough to detect lower levels, or different types of *A. euteiches* mycelial

inhibition by *Pseudomonas* strains. This warrants a change in the method used to quantify inhibition. When viewing negative controls (*E. coli* DH5α and *P. fluorescens* WCS365, Fig. 1-3A) and 5-day-old *A. euteiches* growth plates (Supplemental Fig. 3A), mycelia reach from the centre *A. euteiches* plug to the edge of the plate in each direction. Thus, we can assume that zero inhibition means a growth radius equal to the distance between the centre and the edge of the plate, in addition to healthy, white mycelia (Supplemental Fig. 4A). For future *in vitro* growth inhibition assays, we would recommend comparing the radius of mycelial growth from the centre *A. euteiches* plug to the closest healthy mycelia near the bacterial colony (Supplemental Fig. 4B).

Not only do we believe that it is worthwhile to repeat these growth inhibition assays using the radius method to quantify inhibition of mycelia, we believe it is worthwhile to optimise and perform *in vitro* growth inhibition assays on *A. euteiches* oospores, considering that *A. euteiches* can infect pea roots and cause root rot at any stage in its life cycle (4). Once these assays are complete, the *in vivo* potential of promising *Pseudomonas* strains to inhibit *A. euteiches* growth and prevent root rot could be explored. To this end, pea plant roots grown in soil enriched with *A. euteiches* and *Pseudomonas* could be compared to pea plant roots grown in normal and singly enriched soils.

Additionally, we chose our four experimental *Pseudomonas* strains based on their expression of one or more known antifungal and/or anti-oomycete genes. Studying how the expression of these genes relates to their ability to inhibit *A. euteiches* could elucidate the pathways and molecular mechanisms necessary for inhibition. To confirm the necessity of specific genes in the inhibitory capacity of bacterial strains, we could create bacterial strains with deactivated genes of interest, and compare their *in vitro A. euteiches* inhibition ability to wild-type bacteria. Using qPCR, we could also study how the expression of bacterial genes of interest correlates to their level of *A. euteiches* inhibition, as well as the baseline expression of these genes in the absence of *A. euteiches*.

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CONTRIBUTIONS

All authors contributed equally to the final experiment design, performance of experiments, and manuscript writing and editing. T.L. and V.P. conceptualised the study, performed statistical analyses, and created all figures.

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