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The Effect of Environmental pH on the Antifungal Activity of *Pseudomonas protegens* CHA0 against *Verticillium dahliae*

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SUMMARY *Pseudomonas protegens* CHA0 is a potential biocontrol agent for root rot diseases such as Verticillium wilt, caused by several species of the *Verticillium* genus of fungi. However, to optimize *P. protegens* CHA0 for biocontrol, it is important to determine how its antifungal properties are affected by environmental conditions in agricultural settings. Soil pH, for instance, has been shown to affect both bacterial and fungal activity *in vitro* and *in vivo*. Since soil pH can be controlled in agricultural practices, it is important to understand the effect of pH on the fungal inhibition of *P. protegens* CHA0 before it can be widely used as a biocontrol agent. In this study, we used co-culturing and inhibition assays to test the inhibition of *V. dahliae* by *P. protegens* CHA0 at three different pH values: 5.3, 6.3, and 6.9. We measured the resulting inhibition diameters over a 7 day period and observed smaller inhibition rings in lower pH conditions. We found a significant difference between inhibition at different pH conditions, and evidence that lower pH values may negatively affect the antifungal activities of *P. protegens* CHA0.

INTRODUCTION

Verticillium dahliae is a fungal pathogen and major cause of Verticillium wilt, a fungal disease with a broad host range of over 400 plant species, including strawberry, potato, tomato, olive, lettuce, and sunflowers (1). This disease can lead to severe yield losses of crops (1,2). *V. dahliae* is a major concern for disease management because it can persist in the soil for many years, even in the absence of a plant host, by forming black seed-like structures called mycosclerotia formed by masses of melanized hyphae (2).

P. protegens strains are part of the plant rhizosphere microbiome and have been studied as possible biocontrol agents for plant diseases (1). In terms of disease management, biocontrol agents are a promising alternative to chemical pesticides, which harm the environment by polluting the soil and atmosphere (3). One major mechanism of *P. protegens* biocontrol activity is the production of antimicrobial compounds including 2,4-diacetylphloroglucinol (DAPG), phenazines, pyoluteorin, hydrogen cyanide, and cyclic lipodepsipeptides such as syringomycin and syringopeptin (1). The inhibitory activity of *P. protegens* has been demonstrated *in vitro*. For example, Nesemann *et al*. have demonstrated that fluorescent pseudomonads inhibit *Verticillium* growth in a media-dependent fashion (4).

P. protegens CHA0, a well-characterized model biocontrol strain, has been extensively studied in crop-pathogen systems for its production of disease-suppressive antimicrobial compounds (5). The potential of CHA0 as a biocontrol strain is highlighted by its possession of one of the broadest biocontrol repertoires and has the strongest inhibitory potential of known *P. protegens* strains (1, 4).

Because the biocontrol activity of biocontrol strains can be unpredictable, they have not been widely adopted and relatively few biocontrol strains are registered for agricultural use (5). One major reason for this unpredictable activity is the changes in environmental conditions. Thus, understanding environmental factors and their influence on biocontrol agents is important for improving the reliability of biocontrol approaches to pest control (5).

Soil pH is important because it can affect the activity of soil microbes and cause predictable changes in bacterial community behaviour (6). In addition, soil acidification due to the overuse of nitrogen fertilizers has become problematic for sustainable agriculture, and poses another challenge to biocontrol (6). For instance, acidic conditions were found to favor growth of the pathogen *Ralstonia solanacearum,* which causes bacterial wilt, but suppress

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the growth and antagonistic activity of *P. protegens* in an *in vivo* study in Southwest China (6).

Previous studies have shown that pH inhibits antifungal activity of fluorescent pseudomonads. For example, Ownley et al (2003) showed different impacts of *P. protegens* 2-79 against wheat take-all disease caused by the fungus *Gaeumannomyces tritici* (7). In their study, the authors highlight an optimal pH to be 6.0-6.6 in vitro on KMPE media, and pH 4.9 in vivo (7). Trivedi et al. (2006), on the other hand, demonstrated that the antifungal activity of Pseudomonas corrugata against the phytopathogenic fungi *Alternaria alternata* and *Fusarium oxysporum* was greater at acidic pH than at alkaline pH, with the greatest inhibition seen at pH 5.5 and no antagonistic activity seen at pH 8.5 or above (8).

However, the impact of pH on the antifungal activity of *P. protegens* CHA0 against *V. dahliae* has not yet been studied. The goal of our investigation is to determine the impact of pH on the biocontrol activity of *P. protegens* CHA0 against the fungal pathogen *V. dahliae* in vitro using an inhibition assay. Given the trends from previous literature, we hypothesize that the biocontrol activity of *P. protegens* CHA0 against *V. dahliae* is greatest at a neutral pH of approximately 7.

METHODS AND MATERIALS

Preparation of Bacterial Culture. To prepare bacterial cultures for the co-culture and inhibition assays, *P. protegens* CHA0 was grown on LB agar at 30℃ for 24 hours. A single colony was inoculated in 5mL of LB broth and incubated at 30℃ for 24 hours, shaking at 220rpm. For long-term storage, the liquid overnight *P. protegens* CHA0 was preserved with 20% glycerol at -80℃.

Preparation of Fungal Culture. Fungal spores were prepared by growing *V. dahliae* on simulated xylem medium (SXM) at 30℃ for a minimum of one week. Modified SXM agar [2% (w/v) pectin, 4% (w/v) tryptone, 1x AspA solution (70 mM $NaNO_3$, 7 mM KCl, 11 mM KH_2PO_4, pH 5.5 with KOH), 1x (v/v) trace element solution (18 µM $FeSO_4$, 134 µM EDTA, 76.5 µM $ZnSO_4$, 178 µM H_3BO_3, 25.3 µM MnC_{l2}, 6.7 µM $CoCl_2$, 6.4 µM $CuSO_4$, 4.1 µM $(NH_4)_6Mo_7O_{24}$, pH 6.5 with KOH), 2mM $MgSO_4$, 2% agar] was used in this experiment under the direction of our instructor Dr. Cara Haney. A section of the *V. dahliae* (approximately 1cm x 1cm) agar culture was inoculated in liquid SXM medium at 30℃ , shaking at 220rpm for 120 hours.

Co-cultivation and Inhibition Assay. In order to simulate the nutritional composition of soil, potato dextrose agar (PDA) medium was used to co-culture *P. protegens* CHA0 and *V. dahliae* to study bacteria-fungi interactions. PDA plates were made at pH 5.3, 6.3, and 6.9 to examine the effect of pH on the biocontrol activity of *P. protegens* CHA0 against *V. dahliae*. The optimal pH for pea plants is pH 6 - 7 (9) and *V. dahliae*, which has the optimal growth at pH 5.2, and *P. protegens* CHA0, which has the optimal growth at pH 7, were expected to show inversely proportional growth efficiency within the range of pH 5.3 - 6.9 based on effects seen in previous studies (10, 11). The PDA plates were aseptically adjusted to each pH value by adding the estimated volume of sterile 1M KOH. Afterwards, the pH of the remaining media after pouring plates were measured to determine the specific pH of the final PDA plates.

The co-culturing and inhibition assays were adapted from previous bacterial and fungal co-cultivation experiments and antifungal sensitivity tests (1, 12). This involved measuring the size of the circular zones of inhibition at the center of the PDA plates, indicating inhibition activity *P. protegens* CHA0 against *V. dahliae*. For the co-culturing and inhibition assays, we spread-plated 100 µL of the *V. dahliae* spore solution (1×10^8 spores mL^{-1}) onto each PDA plate. At the center of each plate, a 1.2 cm diameter hole was made, and inoculated with 60µL of CHA0 overnight culture ($OD_{600nm} = 1$, 1×10^8 CFU mL^{-1}). The zones of inhibition were observed for 7 days, and we measured the diameter of the distinct zones of inhibition from three replicates of each pH group. Fungal solution without liquid bacterial culture was used as a negative control for each pH PDA value.

Analysis and Data Visualization. The inhibition diameter measurements taken over 7 days were collected and imported to GraphPad (GraphPad Software, USA) to visualize changes in the size of the inhibition region at different pH values over time. Data was visualized by scatter plot and bar graph analysis and standard deviation, and means were calculated for the diameter measurements of each replicate. The data was also examined for statistical significance by a two-tailed unpaired Student's T-test using the t Test Calculator in GraphPad to determine the p-value.

RESULTS

Fungal growth and inhibition diameters were similar in all three pH conditions. To confirm inhibition of *V. dahliae* by *P. protegens* CHA0, a co-culture of *P. protegens* and *V. dahliae* was plated on PDA at pH 6.9 in triplicate resulting in a clear inhibition ring in all three replicates. To control for the effect of pH on the growth of *V. dahliae*, the same co-culture was performed in pH 5.3 and 6.3 (Figure 2). We monitored the plates over six days and did not see any inhibition. A similar density of fungal growth for all nine plates was observed for all replicates at each pH value tested. The PDA plates started off as a cloudy-white and translucent shade on day zero, and changed to a dark green opaque color by day 6. This is likely due to microsclerotia, which are black seed-like structures formed by masses of melanized hyphae (2). This green, opaque appearance covered the entire PDA plate surface for all three pH conditions (Figure 1). The plates looked identical in all conditions and replicates.

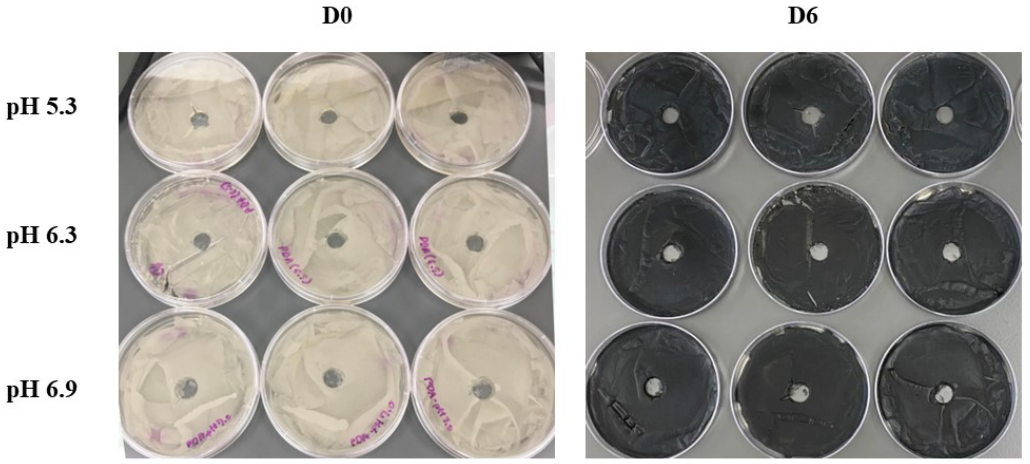


FIG. 1 *V. dahliae* grown in various pH. Photographed image of PDA plates containing *V. dahliae* at three different pH conditions (5.3. 6.3, 6.9) with three replicates each, at day one (left) and day six (right).

Smaller inhibition rings were observed in lower pH conditions. To compare fungal inhibition between different pH conditions, we plated a co-culture of *P. protegens* CHA0 and *V. dahliae* on PDA plates with pH values of 5.3, 6.3 and 6.9. We observed clear inhibition rings (Figure 2; the circles inside the white translucent areas) for all replicates at each pH value tested. We also observed cloudy, irregularly shaped inhibition rings surrounding the smaller clear inhibition rings on all plates. Through days 1-7 we observed the largest clear inhibition rings at pH 6.9 condition and the smallest at pH 5.3 for all 6 days (Figure 2). On day 6, the average inhibition radius was the highest at pH 6.9 condition and measured 2.13 cm. The average inhibition radius at pH 6.3was 1.97 cm, and at pH 5.3, the inhibition radius was 1.6 cm (Figure 3).

DISCUSSION

This study examined fungal inhibition by *P. protegens* CHA0 against *V. dahliae* in vitro at three distinct pH values: 5.3, 6.3, and 6.9. The results of the co-culturing and inhibition assays indicate that pH may affect fungal inhibition of *V. dahliae* by *P. protegens* CHA0,

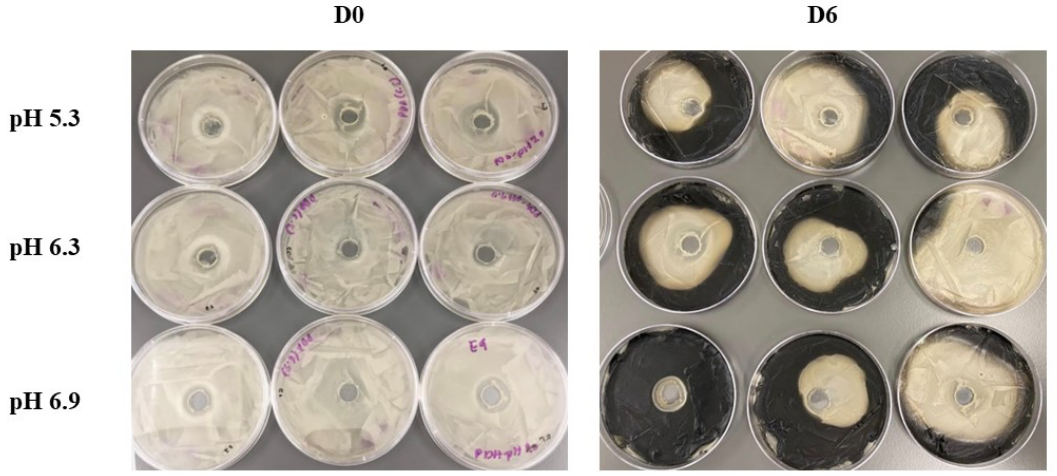
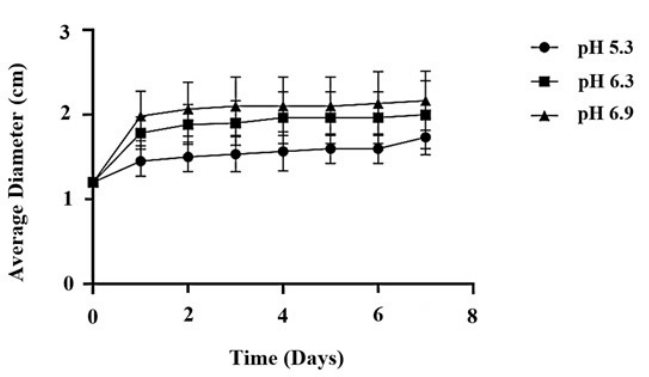


FIG. 2 The effects of pH on the growth of *P. protegens* CHA0 in the presence of *V. dahliae*. Photographed image of PDA plates containing co-culture of *V. dahliae* and *P. protegens* CHA0 at three different pH conditions (5.3. 6.3, 6.9) with three replicates each, at day one (left) and day six (right).

A

Day	Average Diameter (cm)		
	pH 5.3	pH 6.3	pH 6.9
0	1.2	1.2	1.2
1	1.45	1.78	1.98
2	1.5	1.88	2.07
3	1.53	1.9	2.1
4	1.57	1.97	2.1
5	1.6	1.97	2.1
6	1.6	1.97	2.13



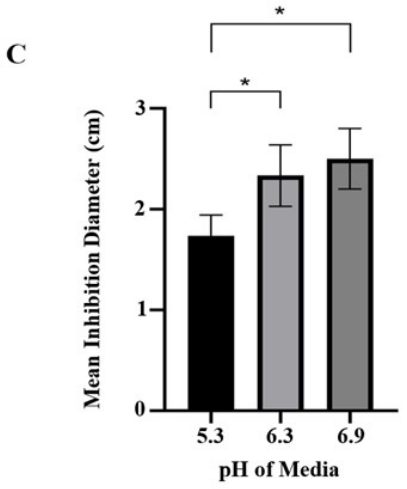


FIG. 3 Average inhibition diameter of clear inhibition rings (not including surrounding cloudy rings) is greater at higher pH. A) Table of mean inhibition diameters of clear inhibition rings at pH 5.3, 6.3, 6.9 obtained from daily measurements over 7 days. **B)** Scatter plot analysis of the average inhibition diameters of clear inhibition rings over time at pH 5.3, 6.3, and 6.9, with error bars indicating SD. **C)** Mean inhibition diameter of clear inhibition rings on Day 7 for pH 5.3, 6.3, and 6.9. Statistical differences were calculated with two-tailed unpaired Student's T-test, where * indicates $p < 0.05$, and a statistically significant difference between two experimental groups.

with increased inhibition of *V. dahliae* at a higher pH value. The mean inhibition diameter at pH 6.3 and 6.9 were significantly greater than the mean inhibition diameter at pH 5.3 over the 7 day observation period, with pH 6.9 showing the greatest mean inhibition diameter (Figure 3). However, the difference between the mean inhibition diameters in pH 6.3 and pH 6.9 was not significant at any point over the observation period. These relationships are more evident in the mean inhibition diameters measurements on Day 7, after which no change in inhibition diameters was observed (Figure 3).

The significant difference in the mean inhibition diameters between pH 5.3 and 6.9 and between pH 5.3 and pH 6.3 indicate that higher pH values are associated with increased inhibition of *V. dahliae* by *P. protegens* CHA0. Notably, these trends were only observed in the measurements taken for the clear inhibition rings (Figure 2). In contrast, the cloudy irregularly shaped outer rings varied greatly from each other within and between pH values. This effect may be explained by uneven fungal growth rather than inhibitory action by *P. protegens* CHA0, since these cloudy regions indicate partial inhibition of *V. dahliae* (13). The clear inhibition rings, however, were uniformly shaped and relatively similar in size between replicates, likely indicating areas of complete inhibition via production of antifungal compounds by CHA0 (13). While the significance of the cloudy regions was not explored in this study, future studies could focus on the partial inhibition seen in these regions.

The effect of pH on the antifungal activity of *P. protegens* CHA0 may be attributed to the optimal pH for *P. protegens* CHA0 and *V. dahliae* growth. Cell culturability and optimal growth of *P. protegens* CHA0 is supported at a neutral pH of approximately 7.0 (9). Consequently, the greater inhibition seen at pH 6.9 could have resulted from improved growth of the bacterial strain at this pH, supporting higher rates of production of antifungal compounds such as pyoluteorin, 2,4-DAPG, and HCN. It is also important to note that the optimal pH for fungal growth is more acidic than that of *P. protegens* CHA0 (11,12,13), which could account for the greater fungal inhibition observed in higher pH values. Though a similar density of fungal growth was observed on control plates grown at all three pH values (Figure 2), it is possible that *V. dahliae* cannot sustain this growth in the presence of an antagonistic organism, such as *P. protegens* CHA0. This competition could require more resources to overcome the inhibitory effects of the bacterial strain.

Another possible explanation for increased fungal inhibition at more neutral pH values is that the antifungal compounds produced by *P. protegens* CHA0 have lower activity in acidic conditions. However, this does not agree with existing literature on the three main antifungal compounds produced by this strain: pyoluteorin, 2,4-DAPG, and HCN. Pyoluteorin appears to be stable from pH 5.8-7.8 in the absence of light, and lowers in stability as pH increases or when exposed to light (17). Lower pH is also associated with higher activity of 2,4-DAPG (18) and HCN (19). As a result, the stability of these three main antifungal compounds responsible for the inhibitory effect of *P. protegens* CHA0 on *V. dahliae*, is likely not altered in less acidic conditions. Rather, a more neutral pH may support increased production of antifungal compounds, which could explain the increased fungal inhibition in pH 6.9.

Conclusions Our study aimed to investigate the effect of pH on the antifungal activity of *P. protegens* CHA0 against *V. dahliae* by comparing the diameters of clear fungal inhibition rings at pH 5.3, 6.3, and 6.9. Overall, our results suggest that pH affects the antifungal activity of *P. protegens* CHA0 against *V. dahliae*, and that a more neutral pH value is associated with higher antifungal activity. Due to the limitations of our study, these findings should be confirmed with a similar study with more replicates, which would increase confidence in the trends observed. Nonetheless, our results support our hypothesis that a neutral pH results in greater inhibition of *V. dahliae* by *P. protegens* CHA0. This relationship could be further investigated to confirm the results and understand the underlying mechanism.

Future Directions As this experiment only tested pH 5.3, 6.3 and 6.9, a greater range of pH values could be tested in future experiments. Furthermore, our co-culturing and inhibition assays were performed *in vitro*, which may change in natural environments as *P. protegens* growth is affected by soil acidity and could further confound its antifungal properties (9). Thus, another next step could be an *in vivo* assay testing soil pH to determine if a similar effect on antifungal activity is observed in semi-agricultural conditions.

Future studies could also explore the underlying mechanisms behind the effect of pH on fungal inhibition by *P. protegens* CHA0. For instance, the effect of pH on the production of key antifungal molecules produced by CHA0 could be tested. This could be investigated by repeating co-culturing and inhibition assays with knockouts for genes encoding the production of antifungal compounds and observing if there is still a decrease in the level of fungal inhibition in lower pH.

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CONTRIBUTIONS

P.B., K.H., H.K., and J.C. all contributed equally to the design and implementation of this study, preparation of chemical reagents and materials, execution of each experiment, the interpretation of the results, and the writing and editing of the final manuscript.

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