Talaromyces variabilis interferes with Pythium aphanidermatum growth and suppresses Pythium-induced damping-off of cucumbers and tomatoes

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Pythium-induced damping-off disease is a major disease limiting cucumber and tomato production in different parts of the world. The current study investigated the efficiency of Talaromyces variabilis and its bioactive metabolites in suppressing Pythium-induced damping-off of cucumbers and tomatoes. T. variabilis inhibited the in vitro growth of P. aphanidermatum in solid and liquid media. In addition, abnormalities in P. aphanidermatum hyphae were observed as a result of T. variabilis. Extracts from T. variabilis induced cellular leakage and suppressed oospore production of P. aphanidermatum. Biochemical analyses of T. variabilis metabolites showed that T. variabilis produces glucanase, cellulase and siderophores, suggesting the contribution of these metabolites in the inhibition of P. aphanidermatum growth and in hyphal abnormalities. Treating cucumber seeds with spore and mycelial suspension of T. variabilis isolates led to a significant improvement in the seedling survival of P. aphanidermatum-inoculated seedlings from 18 to 52% (improvement by 34%) for isolate 48 P and from 30–66% (improvement by 36%) for isolate 28 R. Similarly, treating tomato seeds with spore and mycelial suspension of T. variabilis isolates led to a significant improvement in the seedling survival of P. aphanidermatum-inoculated seedlings from 7 to 36% (improvement by 29%) for isolate 28 R and from 20 to 64% (improvement by 44%) for isolate 48 P. Differences in the percent improvement in seedling survival between experiments may be related to difference in the efficacy of the two different isolates or their interaction with the hosts and pathogen. The use of T. variabilis in the biocontrol of Pythium-induced diseases may offer alternatives to the currently used chemical control.

Soil-borne pathogens represent a major challenge to crops worldwide. Diseases and loses caused by soil-borne pathogens vary from one place and crop to another depending on the pathogen, environmental conditions and management strategies. Pythium species are a major problem worldwide especially in vegetable crops. Pythium-induced damping-off and root diseases of cucurbits and tomatoes can result in losses of up to 100%1,2. Diseases in these crops are caused by various Pythium species, the most common of which is P. aphanidermatum3,4.

Damping-off and root diseases of vegetable crops can be managed using chemical treatments (e.g. Mefenoxam, Hymexazol and Captan)5,6. Since chemical control has several negative effects on the environment and humans, other environmentally safe methods have been used or developed, including the use of solarization7. Biological control, which depends on microorganisms, including endophytes, is better than using synthetic chemical fungicides, because of their hazards to the environment as well as the potential development of resistance to fungicides8.
Endophytic microorganisms reside inside plant tissues and have multiple benefits to their hosts and environments including mineral solubilization\textsuperscript{9,10}, phytohormones production\textsuperscript{11}, phytoremediation of heavy metals\textsuperscript{12,13} and disease suppression\textsuperscript{14,15}.

Several endophytic fungi are used as biocontrol agents against plant disease such as \textit{Botryosphaeria ribis}, \textit{Trichoderma} sp. and \textit{Aspergillus terreus}\textsuperscript{14,16}. \textit{Talaromyces} species have multiple benefits for plants; they have been used as biocontrol agents against several plant pathogen\textsuperscript{17,18}. In Oman, little attention has been given to finding biocontrol agents against soil borne diseases.

There are several mechanisms that microbes use to promote disease stress tolerance in plants including hydrolytic enzymes production\textsuperscript{19}, siderophores production\textsuperscript{20} and hydrogen cyanide synthesis\textsuperscript{21}. Generally, endophytes reduce pathogen effects in plants immediately after infection by promoting plant stress response systems\textsuperscript{22,23}. Elucidating the ways by which biocontrol agents affect other pathogens is helpful in coming up with effective management strategies for pathogens.

During a recent study in Oman, two \textit{Talaromyces} isolates with potential biocontrol activities were isolated. This study aimed at investigating the suppressive effects of these isolates against growth, and spore production by \textit{Pythium aphanidermatum}, the potential metabolites involved in the inhibition, and the potential biocontrol activities of the isolates against Pythium damping-off of cucumbers and tomatoes.

Knowledge in these areas could help come up with effective biocontrol agents against soil borne disease affecting crops in Oman.

\textbf{Results}

\textbf{Identification of \textit{Talaromyces} isolates.} The combined ITS, TUB and CMD dataset comprises 18 isolates of \textit{Talaromyces}. Phylogenetic analysis showed that isolates 48 P and 28 R belong to \textit{T. variabilis} (Fig. 1).

\textbf{Antagonistic effect of \textit{T. variabilis} isolates against \textit{P. aphanidermatum}.} Both \textit{T. variabilis} isolates showed antagonistic activity against \textit{P. aphanidermatum} in PDA medium (Table 1). \textit{T. variabilis} isolates 48 P and 28 R produced inhibition zones of 8.5 mm and 6.25 mm, respectively.

The second experiment was conducted in PDA plates to observe the antagonistic effect of \textit{T. variabilis} isolates over time. \textit{P. aphanidermatum} ceased its growth. However, \textit{T. variabilis} isolates 48 P and 28 R continued to grow towards \textit{P. aphanidermatum} and filled the plate after 13.5 days and 15.5 days, respectively (Table 1).

\textbf{Effect of culture filtrates of \textit{T. variabilis} on \textit{P. aphanidermatum} growth and oospore production.} Treating \textit{P. aphanidermatum} with culture filtrates of \textit{T. variabilis} isolates led to considerable inhibition in mycelial growth in all the tested concentrations (Fig. 2). \textit{T. variabilis} isolates fully suppressed the growth of \textit{P. aphanidermatum} at 75% concentration. However, the growth was reduced at 50% and 25% concentrations (Fig. 2). Furthermore, oospore production by \textit{P. aphanidermatum} decreased significantly when it was exposed to 20% culture filtrate of \textit{T. variabilis} (48 P: 8 oospores; 28 R: 9 oospores) compared to the control (56 oospores) (Table 1).
Extracellular conductivity. Addition of 48 P and 28 R culture filtrates to *P. aphanidermatum* mycelium resulted in an increase in the extracellular conductivity values to 21.56 mV and 13.66 mV respectively, compared to PDB control (0.86 mV) (Table 1).

Glucanase activity of *T. variabilis* culture filtrates. The concentration of glucanase enzyme produced by both *T. variabilis* isolates were similar: 8.09 for 48 P and 8.21 for 28 R (Table 1). The enzyme activity is expressed in nmoles substrate consumed h\(^{-1}\) ml\(^{-1}\).

Determination of cellulase activity using filter paper assay (FPA). Both isolates of *T. variabilis* strains had cellulase enzyme in their culture filtrates. Isolate 48 P had significantly higher concentration of cellulase activity, 2.12 \(\mu\)mol/min/ml compared to 28 R isolate, 0.43 \(\mu\)mol/min/ml (Table 1).

Siderophore production by *T. variabilis* isolates. Both isolates of *T. variabilis* produced siderophore in both media. However, King B medium contains the highest amount of siderophore, 24.25 \(\mu\)M for 48 P and 21.85 \(\mu\)M for 28 R compared to Glucose medium, 15.86 \(\mu\)M for 48 P and 13.56 \(\mu\)M for 28 R (Fig. 3).

Effect of *T. variabilis* on *P. aphanidermatum* morphology. Both isolates of *T. variabilis* induced significant abnormalities in general shape, internal content and the tips of main hyphae and hyphal branches of *P. aphanidermatum*. Isolate 48 P had a greater impact on hyphal morphology compared to isolate 28 R (Fig. 4).

Furthermore, scanning electron microscope showed similar observations such as shrunken and wavy hyphae, hyphal content exits and hyphae have protrusions and narrowings, as compared to the control which had straight, smooth surface and full hyphae (Fig. 6).

Biocontrol potential of *T. variabilis* isolates against damping-off diseases of cucumber and tomato. *T. variabilis* isolates (48 P and 28 R) did not cause any significantly harmful effects on the length, fresh weight and dry weight of cucumber and tomato seedlings (Tables 2, 3). Both *T. variabilis* isolates 48 P and 28 R had considerable biocontrol efficacy against damping-off disease in cucumber and tomato (Table 3). Treating cucumber seeds with spores and mycelial suspension of *T. variabilis* isolate 48 P and *P. aphanidermatum* led to significant improvement in seedlings survival (51.78%) compared to the control (17.86%). Similar results were observed by *T. variabilis* isolate 28 R with significant improvement in cucumber survival (66.07%) compared to the control (30.36%) (Table 3).

### Table 1. Antagonistic effects of *T. variabilis* isolates against *P. aphanidermatum*. Values with the same letters in the same column are not significantly different from each other (Duncan test, P > 0.05 for 3 treatments, ANOVA Test, P > 0.05 for 2 treatments), the values represent the mean ± SD. NA (not applicable).

| Treatments | Inhibition zone (mm) | Time taken to fill plate (days) | Number of produced spores | Extracellular conductivity: 24 h-0 min (mV) | Glucanase activity (nmoles hr\(^{-1}\) ml\(^{-1}\)) | Cellulase activity: \(\mu\)mol/min/ml |
|------------|----------------------|---------------------------------|----------------------------|------------------------------------------|------------------------------------------|-----------------------------|
| Control    | 0 ± 0c               | NA                              | 56.33 ± 7.5a               | 0.86 ± 0.95b                            | NA                                       | NA                          |
| 48 P       | 8.5 ± 1.29a          | 13.5 ± 0.58a                    | 8.66 ± 3.78b               | 21.56 ± 1.67a                           | 8.09 ± 0.15a                            | 2.12 ± 1.09a               |
| 28 R       | 6.25 ± 0.96b         | 15.5 ± 2.08a                    | 13.66 ± 5.16a              | 8.21 ± 0.04a                            | 8.21 ± 0.04a                            | 0.43 ± 0.09b               |

### Figure 2. Influence of three different concentrations of *T. variabilis* culture filtrates (75%, 50% and 25%) on *P. aphanidermatum* growth. Columns and bars represent means ± SD.
Similarly, treating tomato seeds with spore and mycelial suspension of 48 P and *P. aphanidermatum* led to significant improvement in seedling survival (64.28%) compared to the control (19.64%). Also, 28 R significantly improved tomato survival (35.71%) compared to the control (7.14%).

**Discussion**

Two strains of *T. variabilis* 48 P and 28 R were isolated from *Rhazya stricta* and *Zygophyllum coccineum*, respectively. The two plants are native to Oman. Species of *Talaromyces* are known endophytes and found on a wide range of plants such as *Potentilla fulgens* 24, *Dactylis glomerata* 25 and *Aloe vera* 26. The current study proved that *T. variabilis* isolates were not pathogenic to cucumbers and tomatoes plants. This result was compatible with the definition of endophytes as fungi which colonize the stems and leaves of plants without causing any symptoms of disease 27.

Our study clearly demonstrated that *T. variabilis* isolates produced inhibition zones against *P. aphanidermatum* on PDA media. Many other fungi such as *Trichoderma* spp. 28 and bacteria such as *Pseudomonas fluorescens* 29 are known to be antagonistic against harmful pathogens such as *Aspergillus flavus*, *Fusarium moniliforme* and *Rhizoctonia solani*. The inhibition activity is mainly due to their ability to secrete bioactive compounds that inhibit plant pathogens 30.

The culture filtrates of *T. variabilis* isolates were effective against *P. aphanidermatum* growth in liquid media. They significantly decreased *P. aphanidermatum* dry weight at 25% and 50% concentrations, and fully suppressed its growth at 75% concentration. Previous studies showed that *Streptomyces hydrogenans* culture filtrates inhibited the growth of *Alternaria brassicicola* 31. Oospore production was greatly decreased in the presence of culture filtrates of 48 P and 28 R. Similar observation was made by 16, where *Aspergillus terreus* affected spore production by *P. aphanidermatum*.

Our data showed that both *Talaromyces* isolates produce cellulase enzyme. Cellulase enzyme can be produced by several fungal genera such as *Trichoderma* 32, *Aspergillus* 33,34 and *Talaromyces* 35. Fungi and bacteria that secrete hydrolytic enzyme have biocontrol ability against plant pathogen. For example chitinase, glucanase and protease enzymes produced by *Trichoderma harzianum* are antagonistic against some fungi 36. Chitinase and β-1,3-glucanase enzymes produced by *Clonostachys rosea* f. *catenulata* were responsible for efficient biocontrol against fungal plant pathogens 37. About 18% of the *P. aphanidermatum* cell wall consists of cellulose 38. The
efficacy of Talaromyces isolates as biocontrol agents against damping-off disease may be in-part due to cellulose production by Talaromyces isolates.

Loss in integrity of P. aphanidermatum cells was observed due to T. variabilis isolates 48 P and 28 R culture filtrates. Consistently, the antifungal metabolites produced by Sporothrix flocculosa led to cellular leakage in several phytopathogens. Another study by Zhao, et al. showed that Streptomyces bikiniiensis causes cellular leakage in Fusarium oxysporum.

Our study showed the production of glucanase enzyme by 48 P and 28 R isolates. Several previous studies also documented a role of extracellular enzymes in biocontrol of pathogens. Examples include cellulases produced by Lysinibacillus sphaericus and chitinases and glucanases produced by Trichoderma species. Our results showed that siderophores were produced by 48 P and 28 R isolates in King B and glucose media. Numerous fungi and bacteria could produce siderophores that are effective against pathogens. Siderophores produced by Rhizobium meliloti led to inhibition of Macrophomina phaseolina. Moreover, cucumbers damping-off disease was controlled by Aspergillus terreus which was able to produce siderophores. These siderophores may deprive the pathogen of iron, thus limiting essential nutrient.
The morphology of *P. aphanidermatum* hyphae were significantly affected by 48 P and 28 R isolates, showing several abnormalities. Getha and Vikineswary\(^44\) showed the following abnormalities in *Fusarium oxysporum* hypha: swelling, distortion and excessive branching of hyphae, thickened with bulbous-like formation along the ends. Another study by Halo, et al.\(^16\) showed abnormalities in hyphae of *P. aphanidermatum* such as shrunken, semi empty and empty content and wrapped up ends under the effect of *Aspergillus terreus*.

Our study confirmed the efficient role of 48 P and 28 R isolates against cucumbers damping-off and tomatoes damping-off diseases. A previous study by Sivan, et al.\(^45\) showed the suppression *P. aphanidermatum* by *Trichoderma harzianum*. Similarly, *Gliocladium catenulatum* inhibited cucumbers damping-off and root rot diseases caused by *P. aphanidermatum*\(^46\). Also, damping-off of tomatoes disease caused by *P. aphanidermatum* was inhibited by *Trichoderma viride* and *Pseudomonas fluorescens* biocontrol agents\(^47\). Furthermore, endophytic

**Table 2.** Effects of *T. variabilis* isolates on shoot length, shoot fresh weight and shoot dry weight of cucumbers and tomatoes. Values with the same letters in the same row for each fungal treatment and its control are not significantly different from each other (ANOVA Test, *P* > 0.05). Values represent the mean ± SD.

|                         | Isolate 48 P                      | Isolate 28 R                      |
|-------------------------|----------------------------------|----------------------------------|
|                         | Control                          | Treatment                        | Control                          | Treatment                        |
| Cucumber shoot length (cm) | 29.34 ± 1.77a                     | 28.96 ± 2.05a                     | 28.57 ± 2.6a                     | 29.62 ± 2.74a                     |
| Tomato shoot length (cm)  | 19.33 ± 2.05a                     | 21.86 ± 1.12a                     | 19.33 ± 2.05a                    | 20.71 ± 1.41a                     |
| Cucumber fresh shoot weight (g) | 7.84 ± 0.87a                     | 8.1 ± 0.94a                      | 5.9 ± 0.85a                      | 6.41 ± 1.01a                      |
| Tomato fresh shoot weight (g) | 1.54 ± 0.43a                     | 2.18 ± 0.38a                     | 1.54 ± 0.43a                     | 1.58 ± 0.31a                      |
| Cucumber dry shoot weight (mg) | 0.572 ± 0.09a                    | 0.573 ± 0.08a                    | 0.358 ± 0.05a                    | 0.362 ± 0.06a                     |
| Tomato dry shoot weight (mg) | 0.114 ± 0.03a                    | 0.193 ± 0.04a                    | 0.11 ± 0.03a                     | 0.13 ± 0.03a                      |

The morphology of *P. aphanidermatum* hyphae were significantly affected by 48 P and 28 R isolates, showing several abnormalities. Getha and Vikineswary\(^44\) showed the following abnormalities in *Fusarium oxysporum* hypha due to antagonistic influence of *Streptomyces violaceusniger*: swelling, distortion and excessive branching of hyphae, thickened with bulbous-like formation along the ends. Another study by Halo, et al.\(^16\) showed abnormalities in hyphae of *P. aphanidermatum* such as shrunken, semi empty and empty content and wrapped up ends under the effect of *Aspergillus terreus*.

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**Figure 6.** Abnormalities in hyphae of *P. aphanidermatum* under the effect of *T. variabilis* isolates using scanning electron microscope. Normal hyphae of *P. aphanidermatum* (control; A,B); Effects of 48 P isolate on *P. aphanidermatum* hypha: shrunken (C), wavy (D), loss of hyphal content (E) and hyphae with protrusions and narrowings (F); Effects of 28 R isolate on *P. aphanidermatum* hypha: shrunken (G), wavy (H) and loss of hyphal content and hyphae with protrusions (I).
actinomycetes were able to suppress pathogenic activities of *P. aphanidermatum* because they produce glucanase enzyme.

Our study is the first comprehensive report on the efficacy of *T. variabilis* isolates and their byproducts on *P. aphanidermatum* and *Pythium* damping-off of cucumbers and tomatoes.

The efficacy of these endophytes in suppressing *P. aphanidermatum* in the *in vitro* and *in vivo* tests through multiple mechanisms suggests that they may be effective against other harmful phytopathogens, including *Pythium* species that cause diseases in other plants. Also, using these endophytes provide an efficient alternative to the use of synthetic chemicals because *P. aphanidermatum* is less likely to develop resistance against these antagonistic fungi because they have multiple modes of action.

### Materials and Methods

**Talaromyces isolates.** *Rhaya stricta* and *Zygophyllum coccineum* plants from desert sites in the Sultanate of Oman were selected for the isolation of endophytic fungi. The collections of samples was in May-August 2016 from Adam, 150 km from Muscat, the capital area of Oman. The method of Larran, *et al.* was followed for endophytic fungi isolation, as described by Halo *et al.*

Two fungal isolates (*Talaromyces*) were identified using sequences of three genes: the internal transcribed spacer region of the ribosomal RNA (ITS), beta-tubulin (TUB) and Calmodulin (CMD). The three genes were amplified using ITS1/ITS4, BT2A/BT2B and CMD5/CMD6 primers, respectively, using previously described reaction mixtures and conditions. MEGA V.6 was used for sequence alignment. Sequences were deposited in GenBank under accession numbers: ITS (48 P: MG957181, 28 R: MH006605), TUB (48 P: MH000341, 28 R: MH006606) and CMD: (48 P: MG979054, 28 R: MH006607).

**Antagonistic effect of *T. variabilis* isolates against *P. aphanidermatum.*** The antagonistic activity of *T. variabilis* isolates 48 P and 28 R against *P. aphanidermatum* was investigated using fresh cultures of *P. aphanidermatum* and *T. variabilis* as explained in our previous work, using dual culture assay. There were four replicates in each experiment.

Table 3. Biocontrol effect of *T. variabilis* isolates against damping-off disease caused by *P. aphanidermatum* in cucumbers and tomatoes. Values with the same letters are not significantly different from each other (Duncan test, *P* > 0.05). Values represent the percentage ± 95% confidence limit.

| Treatments | Cucumbers seedlings’ survival percentage | Tomatoes seedlings’ survival percentage |
|------------|----------------------------------------|----------------------------------------|
|            | 48 P | 28 R | 48 P | 28 R |
| Control    | 94.64 ± 2.25a | 83.93 ± 4.89a | 83.93 ± 4.3a | 83.92 ± 4.3a |
| Fungus     | 94.64 ± 2.25a | 87.5 ± 2.78a | 78.57 ± 4.64a | 80.35 ± 3.23a |
| *P. aphanidermatum* | 17.86 ± 5.06c | 30.36 ± 8.51b | 19.64 ± 4.6b | 7.14 ± 3.28c |
| Fungus + *P. aphanidermatum* | 51.78 ± 3.98b | 66.07 ± 5.65a | 64.28 ± 4.64a | 35.71 ± 5.19b |

**Effect of culture filtrates of *T. variabilis* on extracellular conductivity and oospore production by *P. aphanidermatum.*** The leakage of cellular components from mycelium of *P. aphanidermatum* was investigated using 5 mg of dried mycelium obtained from the liquid culture. 10 ml of culture filtrates were obtained by centrifugation at 10,000 g, filtered through 0.2 µm Minisart filters, transferred to conical flasks and stored at 4 °C for further experiments.

Three concentrations of culture filtrates (75%, 50% and 25%) were used to study their effect on *P. aphanidermatum* growth in PDB media while the control included only PDB media. The concentration 75% consisted of 75% filtrate and 25% PDB, and so on for the other concentrations. A 3-mm diameter disk of *P. aphanidermatum* was added to each flask. Flasks were then kept in an incubator shaker at 28 °C and 120 rpm for 7 days. Finally, the liquid was disposed and the mycelium of *P. aphanidermatum* was dried in an oven at 65 °C for 24 h. The dry weights of the treatments and control were measured using three replicates for each isolate.

**Glucanase activity of *T. variabilis* culture filtrates.** Glucanase production by *T. variabilis* isolates was detected using a protocol described by Jackson, *et al.* Samples were analyzed spectrophotometrically at 400 nm using an ELISA spectrophotometer. The final enzyme activity was calculated as per Jackson, *et al.*

**Cellulase activity.** Two *T. variabilis* strains were cultivated in basal medium of Mandels and Weber (1969) supplemented with 1% cellulose. The flasks were incubated in a rotary shaker at 200 rpm at 28 °C for 10 days. Culture filtrates were obtained through centrifugation and used fresh. Total cellulase activities of fungal strains were determined as described by Mandel and Sternberg (1976).

**Siderophore production by *T. variabilis.*** Based on our previous experiment, the highest concentrations of siderophore were obtained using King B medium and glucose medium. The media were inoculated with...
disks from fresh PDA cultures of 48 P and 28 R and kept in an incubator shaker at 120 rpm at 28 °C for 7 days. The supernatants were obtained by centrifugation followed by filtration through 0.2µM Minisart filters. ELISA spectrophotometer at 400 nm was used to detect siderophores concentrations using molar extinction coefficient ε = 20000. The experiment had six replicates.

**Effect of T. variabilis on morphology of P. aphanidermatum.** The antagonistic activity of T. variabilis isolates (48 P and 28 R) was checked against P. aphanidermatum in vitro as detailed in Halo, et al.16. The morphological study focused on main hyphae and their tips. 50 main and hypha tips of P. aphanidermatum near the inhibition zone were examined using a light microscope and scanning electron microscope (SEM, INSTUMENT JSM - 5600). These hyphae were compared with P. aphanidermatum hyphae in PDA control plate. All hyphae were stained with cotton blue.

**Biocontrol potential of T. variabilis isolates against damping-off diseases of cucumber and tomato.** The effect of T. variabilis isolates (48 P and 28 R) on *Pythium* damping-off of cucumber and tomato was tested using three treatments and one control. 48 P and 28 R isolates, used in the experiment, were grown in PDB media in an incubator shaker at 28 °C and 120 rpm for 15 days to obtain fungal suspension (spore/mycelial). Also, this test used fresh cultures of P. aphanidermatum which were grown in PDA for 3 days at 28 °C. Seven sterilised seeds of cucumber or tomato were sown in each pot. Pots (12 cm in diameter) were autoclaved once. However, soil used in the pots was autoclaved twice.

Four replicate pots were kept for each treatment or control. The control pots were irrigated with 25 ml PDB media; the pots in first treatment were inoculated with 25 ml of fungal suspension containing spore and/or mycelia of 48 P or 28 R; the second treatment was treated with full plate of fresh PDA culture of P. aphanidermatum, 2 cm below soil surface; the pots in the third treatment was treated with full plate of fresh PDA culture of P. aphanidermatum and 25 ml of 48 P or 28 R fungal suspension. The experiment was conducted at 28 °C for three weeks at 12–14 hr day length. After that, shoot length, fresh weight and dry weight were determined. Also seedlings survival rate was calculated by dividing the number of surviving seedlings by 7 (total seed sown) and then multiplying them by 100. This experiment was executed twice.

**Statistical analysis.** Data were analysed by IBM SPSS Statistics 24.0 using Chi-Square test for morphological study to compare performance of the isolates to the control. The Poisson test was used to compare oospore production by P. aphanidermatum treated with each of the culture filtrates. Independent sample t-test, One-way ANOVA and Duncan's Multiple Range Test were used to compare means of the different treatments. Each test is explained in the caption of each figure/table in the results section.

**Data Availability.** All data underlying this publication are available in the manuscript.

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Author Contributions

B.A.H., R.A.A. and A.M.A. planned the study, B.A.H. processed samples, B.A.H., R.A.A., S.S.M. and A.M.A. analysed results, B.A.H. and A.M.A. wrote the manuscript and B.A.H., R.A.A., S.S.M. and A.M.A. revised and approved the final version.

Additional Information

Competing Interests: The authors declare no competing interests.

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