**Screening *Trichoderma* spp. as a Biocontrol Agent of Impatiens Downy Mildew (*Plasmopara obducens*)**

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## ABSTRACT

A relatively recent epidemic of *Plasmopara obducens*, the oomycete responsible for Impatiens downy mildew, has spread throughout several countries including the United States. If *P. obducens* oospore behavior is similar to other, better studied downy mildews, oospores may be able to remain in the soil for long periods of time, making it extremely difficult to eliminate the disease. The objective of this experiment was to determine if *Trichoderma asperellum* isolate 04-22 and a second unidentified *Trichoderma* species (isolate Tri-4) has the potential to remediate soils infested with *P. obducens* oospores. The experiment showed the effects of *T. asperellum* 04-22 and *Trichoderma* sp. isolate Tri-4 on *P. obducens* oospores was negligible, and no mycoparasitism was observed. Only one replicate showed a lower number of oospore plasmolysis in the treatment plate compared to the control. The *Trichoderma* spp. treatment plates showed greater than 50% plasmolysis in all cases. The experiment demonstrated that the two *Trichoderma* spp. are unlikely to be an effective biological control agent of *P. obducens* oospores, particularly for soil sanitation purposes.

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## INTRODUCTION

Impatiens downy mildew is caused by the oomycete *Plasmopara obducens.* Oomycetes are a group of organisms also known as "water molds." They have previously been classified as lower fungi, but recent morphological and molecular studies have shown that they are more closely related to algae (Fry and Grünwald 2010). Today, most taxonomists classify them in the Kingdom Chromista. However, oomycetes share many ecological and epidemiological characteristics with fungal plant pathogens (Ash 2000). Oomycete organisms are responsible for a number of devastating plant disease epidemics, such as the Irish potato famine, caused by *Phytophthora infestans*, sudden oak death caused by *Phytophthora ramorum*, and grape downy mildew caused by *Plasmopara viticola* (Agrios 2005). Many fungicides do not inhibit oomycetes due to biochemical differences between oomycetes and fungi (Hardham 2007).

An epidemic of *P. obducens* has recently affected commercial species of impatiens, *Impatiens balsamina*, and *I. walleriana* (Farr and Rossman 2018). The disease became a concern for the US in 2011 when it hit the landscape; prior to that it had been mainly reported in greenhouses (Wegulo *et al.* 2004; Palmateer *et al.* 2013; Crouch *et al.* 2014). It was first reported in California in 2004 and in 2011 severe outbreaks of *P. obducens* on *I. walleriana* occurred in Florida (Wegulo *et al.* 2004; Palmateer *et al.* 2013). The disease was reported in the United Kingdom in 2003 and has spread throughout Europe and into Australia (Lane *et al.* 2005; Cunnington *et al.* 2008; Toppe *et al.* 2010; Bulajić *et al.* 2011; Vajna 2011 ). *P. obducens* can cause huge losses in sales for commercial growers given the extreme popularity of *I. walleriana* as bedding plant for shade and partial shade areas. Unfortunately, little is known about this plant pathogen.

Grape downy mildew, *P. viticola,* is a well-studied oomycete that is in the same genus as impatiens downy mildew (*Plasmopara*), making it a model organism for the basis of this experiment. Like *P. obducens* and other downy mildews, *P. viticola* is an obligate biotroph, meaning it is completely reliant on nutrients from host tissues (Fawke *et al.* 2015). The life cycle of *P. viticola* has been documented. The oospores mature, over-wintering in decaying plant material. In the spring, the oospores

produce sporangium, which release zoospores. The zoospores infect the plant via penetration of the stomata. Sporangia form on the undersides of leaves and fruits of infected plants. The zoospores cause secondary infection when dispersed to another host by rain or wind (Ash 2000).

Oospores' ability to survive for long periods of time in the soil and in decaying plant material make downy mildews extremely difficult to control. Oospores are the primary inoculum for downy mildew (Pertot and Zulini 2003), and it was found that oospore-derived primary infections were responsible for the early epidemic stages of *P. viticola* (Pertot *et al.* 2003). Germination of *P. viticola* oospores is highly variable as Vercesi *et al.* (1999) showed; germination of oospores occurred at varying times depending on the year and sampling month. Maturation of the oospore is a prerequisite of germination, although environmental conditions may be the limiting factor in oospore germination (Vercesi *et al.* 1999).

*Trichoderma* spp. are fungi that can be found in agricultural soils of all types, as well as in decaying materials (Naher *et al.* 2014). *Trichoderma* spp. are also found as fungal endophytes (Widmer 2014). *Trichoderma* spp. have been used as biocontrol agents for managing phytopathogens (Bell *et al.* 1982; Lorito *et al.* 1994; Naher *et al.* 2014). Each *Trichoderma*-pathogen pairing may exhibit its own unique interaction (Harman and Kubicek 1998; Verma *et al.* 2007). Anti-phytopathogen activities exhibited by *Trichoderma* spp. include induced plant defenses, the production of various antimicrobial compounds, mycoparastism, and niche exclusion (Hanhong 2011). *Trichoderma* spp. produce a large number of different secondary metabolites that have biocontrol activities; some of these chemicals include alamethicin, trichotoxins, trichopolins, paracelsin, trichorzins, harzianins, and trichokonins (Zeilinger *et al.* 2016). *Trichoderma* spp.-produced chemicals have shown to degrade, as well as inhibit the reconstruction of, fungal and bacteria cell walls and to induce apoptotic programmed cell death in microbes (Hanhong 2011; Zeilinger *et al.* 2016). A number of genes associated with biocontrol properties from *Trichoderma* species have been isolated and characterized, with variable mechanisms and antagonistic activity (Munir *et al.* 2014; Zeilinger *et al.* 2016). *Trichoderma* species have been commercialized as biological control agents to manage plant pathogens, for example Root Shield®

(*Trichoderma harzianum* Rifai) (Raj *et al.* 2005; Peng *et al.* 2014; Vos and Kazan 2016; Widmer and Samuels 2016).

A study by Widmer (2014) found two *T. asperellum* isolates (04-22 and 02-64) that exhibited mycoparasitic activity against *Phytophthora ramorum* chlamydospores and sporangium. The two isolates also mediated *P. ramorum* infested soil (Widmer 2014). *P. ramorum* is a member of the oomycetes and is the pathogen responsible for sudden oak death and ramorum blight (Widmer and Samuels 2010; Widmer 2014; Widmer and Samuels 2016). A dual culture bioassay screened *Trichoderma* isolates for mycoparasitism of *P. ramorum*; nine *Trichoderma* isolates demonstrated a lack of *P. ramorum* mycelial growth in the dual culture assay (indicating antagonistic activities). Examining the interactions microscopically showed mycoparasitism of *P. ramorum* chlamydospores and sporangium. A soil bioassay was then performed and showed *T. asperellum* isolate 04-22 and 02-64 significantly reduced *P. ramorum* in the soil over time compared to the controls (Widmer 2014).

***Objective and Hypotheses***

The objective of this study was to determine if *Trichoderma asperellum* isolate 04-22 and *Trichoderma* sp. isolate Tri-4 have the potential to remediate soils infested with *Plasmopara obducens* oospores. Given the antagonistic activities displayed by *Trichoderma* sp. and *T. asperellum* isolate's 04-22 ability to effectively mycoparasitize *P. ramorum*, we hypothesized that (i) a significant reduction in the viability of *P. obducens* oospores would occur when treated with the *Trichoderma* isolates and (ii) the *Trichoderma* isolates would mycoparasitize *P. obducens* oospores.

## MATERIALS AND METHODS

The two *Trichoderma* isolates (04-22 and Tri-4) were cultured on half strength potato dextrose agar (½ PDA). *T. asperellum* 04-22 isolate was used because it was collected from the United States (Maryland) and it previously showed mycoparasitism of the oomycetes' *Phytophthora ramorum* (Widmer 2014). The second, unidentified *Trichoderma* sp. isolate Tri-4 was selected because it had previously shown some antagonistic activities on oospores of the oomycetes' *Phytophthora kernoviae* (T. Widmer, personal communication, June 2017). We used ½ PDA media to culture the *Trichoderma* isolates because it is more limiting in nutrients, which allowed for sporulation and less mycelia growth of the fungus (T. Widmer, personal communication, October 2016). Once the *Trichoderma* isolates were transferred to ½ PDA plates, the cultures were incubated at 20$^{o}$C to allow the fungus to fully colonize the plate.

### *Plasmopara obducens isolate*

Leaves and stems from *Impatiens balsamina* grown from seed (Eden Brothers, Arden, NC) were used for maintenance of *P. obducens* and for production of oospores. *P. obducens* isolate OW, collected in 2015 from Mt. Airy, Maryland, was used for inoculation in this study. Single sporangium culture of the OW isolate was prepared prior to the study by Dr. Nina Shishkoff.

The culture was maintained on detached *I. balsamina* leaves. The detached leaves were first surface-sterilized for 60 seconds in 10% bleach (0.6% sodium hypochlorite), rinsed three times with water, and then placed in a moist-chamber. The moist chambers were created by lining nine-centimeter diameter Petri dishes with moist filter paper. Sporangia, suspended in water, were poured over the leaves in the moist-chamber (approx. five mL). The moist chambers were then placed in the dark, overnight at 20 $^{o}$C. The following day, the inoculum suspension was decanted from the moist-chambers and they were then stacked in one gallon self-sealing plastic bags. They were incubated in a growth chamber with a 14-hr photoperiod for one to two weeks, when inoculum was ready to be collected for the next transfer (N. Shishkoff, personal communication, October 2016).

***Production of Oospores***

*I. balsamina* stem segments (five-six cm long) were surface-sterilized for 60 seconds in 10% bleach (0.6% sodium hypochlorite). The stem segments were put in moist-chambers that were created as previously described, and a sporangia in water suspension was poured over the stems until they were 1/4-1/2 submerged. The moist-chambers were then placed in the dark, overnight at 20 °C. The following day, the inoculum suspension was decanted from the moist-chambers and they were then stacked in one gallon self-sealing plastic bags. The bags of moist-chambers with stem segments were incubated in a growth chamber at 20°C with a 14-hr photoperiod for a minimum one month to allow oospores to develop and mature. A total of 14 moist-chambers (per replicate) were prepared to ensure a sufficient amount of oospores for the experiment.

***Screening bioassay***

Oospores of the downy mildew were exposed to conidia of the biocontrol agents in order to screen for antagonistic activity. To collect oospores from stem tissue, the infected stems were macerated using sand, then passed through several sieves with different size screens. The oospores were collected from the smaller screens (<45 $\mu$m) and then rinsed from the sieve with water into a beaker. The water containing the oospores was filtered under vacuum (-58 psi), where the oospores were collected on a 0.45-$\mu$m Millipore™ filter (Fig. 1). Each filter was placed in the center of a 9-cm diameter Petri dish containing two percent water agar (WA).

**Figure 1.** Millipore™ vacuum filter set-up to fix oospores on filters.

To inoculate oospores with *Trichoderma,* inoculum of candidate isolates were added to the plates containing oospores. Three experiments were done, the first exposing oospores to conidia of *T. asperellum* (04-22) conidia suspended in water, the second exposing oospores to conidia of *T. asperellum* (04-22) conidia suspended in three percent dextrose water, and the third exposing oospores to conidia of *T. asperellum* (04-22) conidia grown in wheat bran. This third method was also used to screen the second *Trichoderma* (Tri-4) isolate.

*Experiment 1:* Conidia of *T. asperellum* were collected from the ½ PDA plates by placing a small amount of water in the plate and lightly scraping the surface until conidia were released. With the use of a hemocytometer, the concentration of conidia in solution was adjusted to a concentration of 1 x $10^7$ conidia/ml. The conidial solution was sprayed on the treatment plates using an atomizer, and the control plates were sprayed with sterile water. The plates were considered sufficiently sprayed once droplets were visible on the water agar plates. Three *Trichoderma*-treated Petri plates and three control plates were used for each time interval. The time intervals were every seven days for 21 days, including a time 0 counted right after performing the inoculation. There were a total of 24 Petri plates per replicate.

*Experiment 2* Conidia of *T. asperellum* were collected from the ½ PDA plates and used to inoculate the plates containing oospores, in the same manner as Experiment 1, with the exception of using sterile three-percent dextrose water rather than sterile water. The time intervals and number of plates, treatment and control, were the same as Experiment 1.

*Experiment 3:* The two *Trichoderma* isolates (04-22 and Tri-4) were grown in a wheat bran formulation as described by Widmer *et al.* (2017) to a concentration of 1 x $10^7$ colony forming units (CFU)/g. The *Trichoderma* isolates in the wheat bran formulation were used to inoculate the two percent WA plates with oospores. The wheat bran formulation was sprinkled over the top of the filter paper centered on the Petri plate as shown in Figure 3a and b (Widmer and Samuels 2016). The oospores were counted using the same method as described in Experiment 2. Three replicates for *T. asperellum* (04-22) were performed with this inoculation method. Two replicates for *Trichoderma* sp. (Tri-4) were performed with this inoculation method. For second replicate for isolate Tri-4, four plates were inoculated for each time interval.

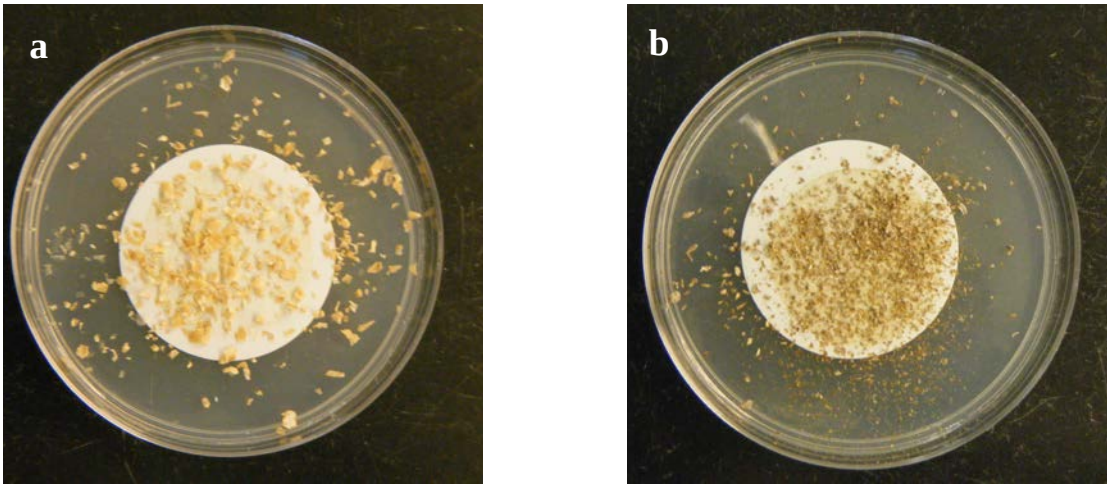


**Figure 2**. Two percent water agar plates with *Plasmopara obducens* oospores adhered to a 0.45-$\mu$m Millipore™ filter **(a)** control plate inoculated with sterile wheat bran and **(b)** treatment plate inoculated with wheat bran formulation of *Trichoderma* sp. isolate Tri-4.

For all three experiments, at seven-day intervals, including time 0 right after inoculation, some of the oospores were removed from the filter paper and put on a slide. A four mol/L sodium solution was added to the slides. The oospores were recorded as either plasmolyzed, where the cytoplasm either contracts to form a ball-like structure or cytoplasm can remain in contact with the cell wall, causing the spore to collapse and the wall to buckle both being microscopically visible, or not plasmolyzed (Fig. 3a

and b). With a compound microscope, 50 oospores were counted randomly from each plate. A key assumption is that plasmolysis infers oospore viability. The plasmolysis method has been shown to be superior for determining oospore viability compared to the conventional method of tetrazolium bromide staining (Etxeberria *et al.* 2011).

***Statistical analysis***

The arithmetic mean of the three control plates and the plates treated with the *Trichoderma* isolate, at each time interval, was calculated for each replicate. We analyzed the data from the three replicates inoculated with the wheat bran method. Individual replicates were analyzed separately; a Kaplan-Meier analysis was conducted to visualize survival over time and a log-rank test was performed to determine whether or not survival (number of oospores plasmolyzed) between the control and treatment groups was significant different. Replicates were also individually analyzed with Cox's regression in order to model the hazard function with a log minus log plot to assess the proportional hazard assumption (Berwick *et al.* 2004). The data analysis was done with the statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

## RESULTS

We did not observe *T. asperellum* (04-22) colonization of the treatment plates in experiments 1 and 2 (data not shown). As a result, we did not run a second trial for the first two experiments. We hypothesized that the *T. asperellum* (04-22) was not establishing, so we added a food base in the form of wheat bran for Experiment 3.

The wheat bran formulation of the *Trichoderma* spp. allowed the fungus to successfully establish on the filter paper containing oospores (Fig. 2b). Plasmolysis was clearly visible when present (Fig. 3a and b). The average percent plasmolyzed at each time interval for all replicates individually is summarized in Figure 4. No mycoparasitism was observed on any of the plates. The number of plasmolyzed oospores varied between intervals as well as replicates, with the first replicate (wheat bran 1 *T. asperellum* (04-22)) showing the greatest difference in oospore survival on the treatment plates compared to the control plates; with the treatment plates having less oospores plasmolyzed compared to the control plates. The lowest number of plasmolyzed oospores observed was at the 14 day time interval for the first wheat bran replicate (with *T. asperellum* (04-22)). The variability was highest in the first replicate of wheat bran; all three wheat bran replicates inoculated with *T. asperellum* (04-22) had greater than or equal to 55% of oospores (greater than or equal to 25 oospores) plasmolyzed at any given time interval. The second replicate of *Trichoderma* sp. (Tri-4) had the least amount of variability between time intervals (Fig. 4e).

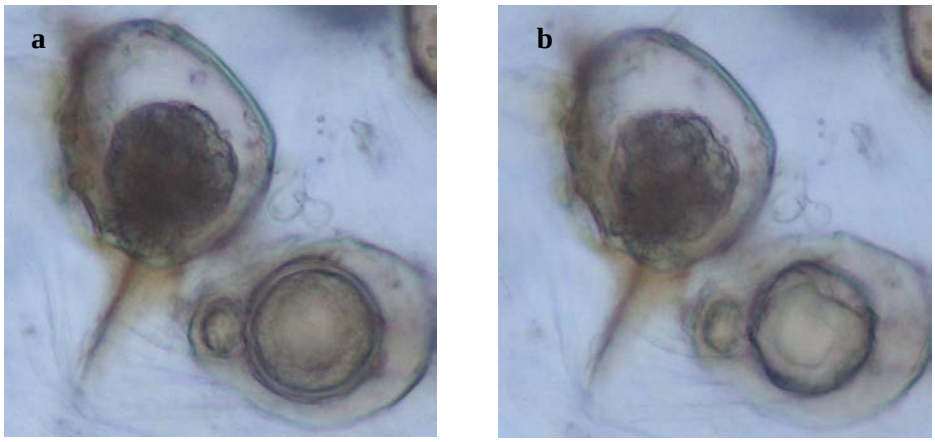


**Figure 3**. The images from plates **(a)** non-plasmolyzed/ nonviable *Plasmopara obducens* oospore on top and viable oospore on bottom right before adding 4M NaCl (x200), **(b)** non-plasmolyzed/ nonviable oospore on top and plasmolyzed oospore on bottom right after 4M NaCl (x200). Photos by Emily Smallwood.

Wheat bran 1 replicate had a statistically significant chi-square value from the log-rank test (df = 1 *p*-value = 0.008). The number plasmolyzed was significantly higher in the control plates compare to the treatment plates. Therefore, we can reject the null hypothesis that there is no difference in survival curves between the *T. asperellum* (04-22) treated and control plates for wheat bran 1 replicate. The other two *T. asperellum* (04-22) replicates, wheat bran 2 and wheat bran 3, did not have a statistically significant chi-square value (df = 1, *p*-value = 0.739 and df = 1, *p*-value = 0.909), from the log-rank test. The replicates with *Trichoderma* sp. isolate Tri-4 also showed not to be statistically significant (Table 1). In the case of *T. asperellum* (04-22) replicates, wheat bran 2 and wheat bran 3, as well as the two replicates with *Trichoderma* sp. isolate Tri-4, we fail to reject the null hypothesis that survival differs between the treated and control plates (df = 1, *p*-value = 0.275 and df = 1, *p*-value = 0.798).

The log minus log plot produced in our Cox's regression model for all replicates showed the proportional hazard assumption was met. The results of the regression showed the hazard ratio for wheat bran 1 replicate was statistically significant (df = 1, *p*-value= 0.015). The hazard ratio indicates whether or not the treatment has a significant effect on survival. Given significant hazard ratio value, we look at the 95% confidence intervals (CI) for that hazard ratio, with a lower limit of 0.441 and an upper limit of

0.915. The value hazard ratio value for wheat bran 1 replicate suggests that the survival rate between treatment groups is approximately 0.635 times higher for the *T. asperellum* (04-22), and at least 0.44 times higher than the control group. However, wheat bran replicates 2 and 3 *T. asperellum* (04-22) did not have a statistically significant hazard value (df = 1, *p*-value = 0.753 and df = 1, *p*-value = 0.914). The replicates with the *Trichoderma* sp. isolate Tri-4 also did not have statistically significant hazard ratio values (df = 1, *p*-value = 0.308; df = 1, *p*-value = 0.807; Table 2).

**Table 1**. The results of the Log Rank (Mantel-Cox) test. $H_0$: There is no difference between survival in treatment groups; $H_A$: The survival differs between treatment groups. * denotes statistical significance.

| Log Rank (Mantel-Cox) | | |
|---|---|---|
| Replicate | Chi-Square | Sig. (*p*-value) |
| Wheat Bran 1 *T. asperellum* (04-22) | 7.149* | 0.008 |
| Wheat Bran 2 *T. asperellum* (04-22) | 0.111 | 0.739 |
| Wheat Bran 3 *T. asperellum* (04-22) | 0.013 | 0.909 |
| Wheat Bran 1 *Trichoderma* sp. isolate Tri-4 | 1.190 | 0.275 |
| Wheat Bran 2 *Trichoderma* sp. isolate Tri-4 | 0.066 | 0.798 |

**Table 2**. The results of the Cox's regression analysis, displaying hazard ratio value as Exp (β), and the lower and upper 95% confidence intervals for the hazard ratio. * denotes statistical significance.

| Cox's Regression Variables in the Equation | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 95.0% CI for Exp(B) | |
| Replicate | Coefficient B | Standard Error | Exp(B) | Sig. (*p*-value) | Lower | Upper |
| Wheat Bran 1 *T. asperellum* (04-22) | -0.454 | 0.187 | 0.635* | 0.015 | 0.441 | 0.915 |
| Wheat Bran 2 *T. asperellum* (04-22) | -0.066 | 0.21 | 0.936 | 0.753 | 0.621 | 1.412 |
| Wheat Bran 3 *T. asperellum* (04-22) | 0.024 | 0.217 | 1.024 | 0.914 | 0.669 | 1.566 |
| Wheat Bran 1 *Trichoderma* sp. isolate Tri-4 | -0.209 | 0.205 | 0.811 | 0.308 | 0.534 | 1.213 |
| Wheat Bran 2 *Trichoderma* sp. isolate Tri-4 | -0.058 | 0.239 | 0.943 | 0.807 | 0.590 | 1.507 |

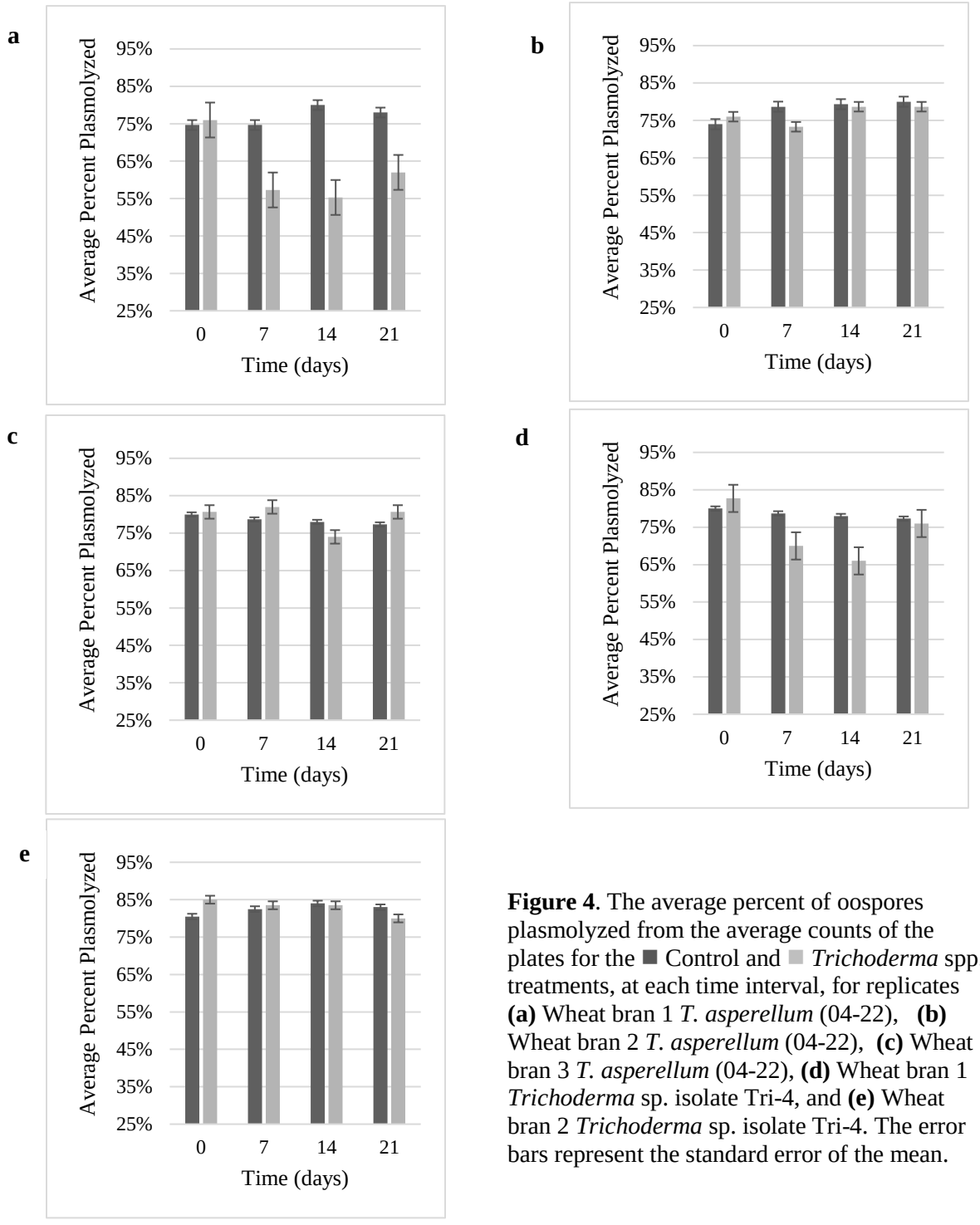


**Figure 4**. The average percent of oospores plasmolyzed from the average counts of the plates for the ■ Control and ■ *Trichoderma* spp. treatments, at each time interval, for replicates **(a)** Wheat bran 1 *T. asperellum* (04-22), **(b)** Wheat bran 2 *T. asperellum* (04-22), **(c)** Wheat bran 3 *T. asperellum* (04-22), **(d)** Wheat bran 1 *Trichoderma* sp. isolate Tri-4, and **(e)** Wheat bran 2 *Trichoderma* sp. isolate Tri-4. The error bars represent the standard error of the mean.

## DISCUSSION

The methods of inoculation in Experiments 1 and 2 may have proved insufficient for the establishment and initial survival of *T. asperellum* (04-22) conidia. Altering the inoculation method with three-percent dextrose water in Experiment 2 showed a slight increase in the visibility of the fungus on the experimental plates, but the method may still not have allowed for sufficient growth of the fungus. The lack of *T. asperellum* (04-22) growth on the experimental plates in the first two experiments was likely due to limiting sugars and/or other nutrients, essentially starving the fungus. The wheat bran formulation of the *Trichoderma* isolates allowed for the fungus to colonize the treated plates. The wheat bran likely provided the *Trichoderma* spp. with an exogenous food source, which allowed the fungus to become established.

A significantly lower number of oospore plasmolyzed in the treatment plates compared to the control plates and the *T. asperellum* (04-22) treatments was only apparent in the first replicate. The wheat bran formulation added depth to the slides, making it more difficult to detect the oospores, which may have resulted in non-random and/ or bias counting. The second and third replicates were also counted under a higher magnification (x200 and x400) on the compound microscope compared to the first replicate (x100), making plasmolysis more discernable.

The two replicates with *Trichoderma* sp. isolate Tri-4 did not exhibit a difference between the control and treatment plates. The addition of a fourth plate to each time interval in the second replicate of *Trichoderma* sp. (Tri-4) reduced the variability between sampling times.

We conducted the experiment in this manner to give the *Trichoderma* optimal conditions to mycoparasitize *P. obducens* oospores. However, neither *T. asperellum* 04-22 nor *Trichoderma* sp. isolate Tri-4 exhibited the expected direct antagonistic activity against the *P. obducens* oospores. No mycoparasitic activity was observed on any of the experimental plates. We hypothesize that the *Trichoderma* isolates used in this experiment did not produce the secondary metabolites required to degrade the oogonial and/or oospore wall, which is required for mycoparasitism.

The lack of statistical significance in the second and third replicates using the wheat bran inoculation method suggests that *T. asperellum* isolate 04-22 would not make an effective biological control agent (BCA) of *P. obducens*, particularly in terms of sanitation of oospore infested soil. This is also true for *Trichoderma* sp. isolate Tri-4 (Figure 4).

The *Trichoderma* isolates may still help prevent downy mildew infection by inducing plant defenses, which was not tested in this study (Zeilinger *et al.* 2016; Szczech *et al.* 2017). *Trichoderma harzianum* T39 (T39) has demonstrated this ability to reduce downy mildew (*P. viticola*) in grapevines (Perazzolli *et al.* 2008; Palmieri *et al.* 2013; Banani *et al.* 2014). T39 inoculated grapevines were shown to have increased production of proteins associated with photosynthesis and energy metabolism in response to the grape downy mildew pathogen. The T39-induced resistance was also found to be correlated with callose at infection sites and the accumulation of reactive oxygen species, which suggests an active defense response to the pathogen (Palmieri *et al.* 2013). T39-induced resistance had different levels of efficiency on different grape cultivars indicating plant genotype plays a role in resistance activation. Activation of plant defense is a low-impact method for control of crop diseases, but complete disease control rarely occurs with resistance inducers and their effects are usually not consistent in field conditions (Banani *et al.* 2014).

Triggering stimuli are required for the production of certain secondary metabolites in *Trichoderma* spp. (Zeilinger *et al.* 2016). It is possible that the currently unknown triggering stimuli for the *Trichoderma* spp. to produce antagonistic metabolites may have not been present in our experiment.

The majority of the inoculum responsible for the reoccurrence of downy mildew year after year comes from wild hosts, seed, and soil borne oospores. Oospores are easily, and in large numbers, deposited into soil from infected plant material, which can remain dormant and cause infection the next year. Elimination of oospore populations is needed to prevent primary infection and also lower the risk of secondary infection by decreasing sporangia and zoospores present (Lynch 2002). Further investigations into other organisms that may act as potential BCAs of oospores is important for management of *P. obducens*.

Nine other isolates of *Trichoderma* spp., from the study done by Widmer (2014), displayed the ability to eliminate *P. ramorum* chlamydospores from infested soil and could potentially be tested for antagonistic activities against *P. obducens* oospores, in the same manner this experiment was performed. To reduce variability, more plates and sampling oospores at more, shorter time intervals for each replicate, is advisable. However given that both *Trichoderma* isolates did not sufficiently reduce oospore viability, other antagonistic microorganisms should be examined.

Other organisms with the potential to destroy oospores of *P. obducens* are species belonging to hyphomycetes, particularly the genus *Dactyella* (Drechsler 1938; Sneh *et al.* 1977). *Dactyella spermatophaga* was isolated from oospores of *Phytophthora megasperma* var. *sojae* in Michigan, USA, and attacked the oospores of several *Pythium* spp. In dual culture, *D. spermatophaga* demonstrated the ability to parasitize oospores from a number of species, which included *Pythium graminicolum, P. myriotylum, P. oedochilum, Phytophthora cactorum, P. megasperma,* and *Aphanomyces euteiches* (Drechsler 1938).

Abiotic factors, such as soil moisture, influence interactions between organisms (Sneh *et al.* 1977; Zeilinger *et al.* 2016). *Phytophthora megasperma* var. *sojae* and *P. cactorum* incubated in different soil conditions resulted in oospore mycoparasitism by a number of different species. In flooded soils, oospores became infected with epibiotic and endobiotic chytrids, such as *Rhizidiomycopsis japonicus, Canteriomyces stigeoclonii*, and *Hyphochytrium catenoides*. In flooded soils, the oomycetes *Pythium* sp., and *Leptolegnia* sp. also attacked *P. megasperma* var. *sojae* and *P. cactorum* oospores. In soils with moisture below saturation, the hyphomycetes *Humicola fuscoatra* Traaen., *Diheterospora chlamydosporia, Fusarium oxysporum* (Schlecht.), and *Dactyella spermatophaga* attacked oospores (Sneh *et al.* 1977).

Biotic factors also impact the effectiveness of a BCA like *Trichoderma* for soil dwelling plant pathogens due to interactions with other microorganisms in the soil and/or plant rhizosphere. This could increase or decrease the effectiveness of the BCA and is also why follow-up tests in the field are

important. Due to biotic and abiotic factors, looking for naturally occurring, endemic organisms is the best approach to finding an effective BCA (Sneh *et al.* 1977; Zeilinger *et al.* 2016; Szczech *et al.* 2017).

Other than the commercial impatiens bedding industry, downy mildew species have impacted production of pearl millet, hops, currants, geranium sp., cucurbits, and grapes (Farr and Rossman 2018). Fungicides are heavily relied on to control downy mildew in commercial agricultural settings, such as grape production. In areas where grape downy mildew occurs frequently or is well-established, pre-infection spray programs are commonly used. It is difficult to kill and control the downy mildew organisms once they become visible on the grapevine. Post-infection fungicide sprays are not usually effective and require follow-up applications, increasing the risk of fungicide resistance developing (Taylor and Fisher 2013).

Fungicides, such as metalaxyl, used to control downy-mildew are expensive, environmental pollutants, may have limited effectiveness, and pose the risk for resistance developing (Lynch 2002). Therefore there is a need for alternative strategies of pathogen control. For example, *Plasmodiophora brassica*e, responsible for clubroot of canola, resting spore populations in soil were tested and even after a two-year period, the spore remained too high to produce commercially acceptable yields. Crop rotation with non-host plants for a greater than two-year intervals along with planting resistant cultivars proved to increase canola yields in fields infested with *P. brassica*e resting spores (Peng *et al.* 2014). Developing integrated management strategies and resistant cultivars are important areas to investigate and may be necessary to effectively manage downy mildew infestations.

Downy mildews are a problem for numerous species, and given the destructive nature of this disease, along with the long survival period of resting spores, an easy, relatively inexpensive, and effective way to sanitize infested soil is the ideal method for controlling *P. obducens* and other oomycete pathogens alike (Drechsler 1938; Farr and Rossman 2018).

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