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Development of a high throughput optical density assay to determine fungicide sensitivity of oomycetes

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ABSTRACT

A high-throughput assay was developed to screen *Phytophthora* species for fungicide sensitivity using optical density measurements for unbiased, automated measurement of mycelial growth. The efficacy of the optical density assay (OD) to measure phosphite sensitivity in *Phytophthora* species was compared to two widely used methods, radial growth (RG) and dry weight (DW) assays. Three isolates of each of *Phytophthora cinnamomi*, *P. multivora* and *P. pluvialis*, with known phosphite exposure and three isolates of each species with no prior phosphite exposure, were screened for phosphite sensitivity using the three assays. Mycelial growth measurements were taken after culturing for 6, 14 and 15 days for the OD, DW and RG assays respectively. Mycelial growth inhibition at 15, 80, 200 and 500 µg/mL phosphite relative to growth on control media was used to determine effective concentration values for 50% growth reduction (EC50). The species varied in their tolerance to phosphite with *P. cinnamomi* being the least sensitive followed by *P. multivora* and *P. pluvialis*. No significant differences in tolerance were found between isolates within the same species using any method. The OD assay produced comparable EC50 values to the RG and DW assays. The growth of the three species was more sensitive to phosphite in the DW than the RG and OD assays, however limited sample throughput and greater variation in measuring small amounts of mycelia in the dry weight assessment increase variability and limits throughput. The OD assay offers a fast method to enable an inventory of chemical resistance and is particularly advantageous for slow growing species as it requires less time and offers greater throughput than existing RG and DW methods.

1. Introduction

Phytophthora species are a genus of plant pathogens which cause disease on horticultural, ornamental, and forest plants worldwide (Erwin and Ribeiro, 1996). They include some of the most successful plant pathogens globally, and there are currently no methods to eradicate them from an area once they are established practically. However, there are several types of chemicals that are effective at controlling Phytophthora diseases, including phenylamide fungicides and phosphite. Chemical control is widely used to protect crops from Phytophthora diseases, resulting in frequent reapplication of chemicals. Management of chemical control is very important to prevent *Phytophthora* species isolates becoming tolerant to fungicides. Tolerance to the phenylamide fungicide metalaxyl (Dowley and O'Sullivan, 1981) has been found in *Phytophthora infestans*, the causal agent of potato (*Solanum tuberosum*) late blight. Isolates of *P. cinnamomi* have been shown to have decreased sensitivity to phosphite after prolonged exposure (Dobrowolski et al., 2008; Duvenhage, 1994; Ma and McLeod, 2014).

Phosphite is a widely used fungicide for controlling many Phytophthora diseases which affect horticultural crops, planted forests, culturally and ecologically important species (Hardy et al., 2001). Phosphite is used extensively in nurseries and botanical gardens to manage diseases caused by oomycete pathogens, including establishment and damping off caused by a range of oomycetes genera and foliar, soil and water-borne species within the genus *Phytophthora*.

On-going monitoring of resistance to fungicides in true fungi and oomycete plant pathogens is vital to ensure that treatments which reduce sporulation, growth and spread of plant pathogens continue to be effective in the future. This is most pertinent to the agricultural industry, particularly horticulture, in the face of increasing food production demands worldwide, population growth and climate change. Fungicide resistance is increasingly important for plant production nurseries globally and within natural ecosystems where continual

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Table 1
Phytophthora isolates used to compare the radial growth (RG), dry weight (DW) and optical density (OD) methods for measuring dose response.

Species	NZFS number	Date Collected	Host	Location	Location type	Historic phosphite application
Phytophthora cinnamomi	4446	27/02/2017	*Persea americana* (Avocado)	Bay of Plenty	Avocado orchard	Yes
	4447	27/02/2017	*P. americana*	Bay of Plenty	Avocado orchard	Yes
	4449	27/02/2017	*P. americana*	Bay of Plenty	Avocado orchard	Yes
	3030	1/10/2008	*Agathis australis* (Kauri)	Auckland	Park	No
	3953	21/07/2014	*Vitex lucens* (Puriri)	Auckland	Beachfront park	No
	2960	13/12/2007	*Araucaria heterophylla* (Norfolk Island pine)	Auckland	Reserve	No
Phytophthora multivora	3732	24/10/2012	*Magnolia* sp.	Wellington	Botanical gardens	Yes
	3740	24/10/2012	*Magnolia* sp.	Wellington	Botanical gardens	Yes
	3796	30/09/2013	*A. australis*	Auckland	Nursery	Yes
	3766	11/06/2013	*Araucaria bidwillii*	Napier	Reserve	No
	3842	31/01/2014	*Pinus radiata* (Monterey pine)	Gisborne	Pine plantation	No
	3797	9/07/2013	*A. heterophylla*	Mount Maunganui	Beachfront park	No
Phytophthora pluvialis	4057	10/10/2014	*P. radiata*	Tokoroa	Nursery	Yes
	4058	9/10/2014	*P. radiata*	Tokoroa	Nursery	Yes
	4059	10/10/2014	*P. radiata*	Tokoroa	Nursery	Yes
	4317	18/07/2016	*P. radiata*	Bay of Plenty	Pine plantation	No
	4340	19/10/2016	*P. radiata*	Gisborne	Pine plantation	No
	4368	26/08/2016	*P. radiata*	Nelson	Pine plantation	No

fungicide use is vital for maintaining ecosystems processes and species assemblages (Barrett and Rathbone, 2018).

The sensitivity of *Phytophthora* species and other fungal plant pathogens to fungicides has been studied *in vitro* using mycelial growth tests. These techniques have been used to quantify chemical suppression of mycelial growth *in vitro* by measuring radial growth (RG) and dry weight (DW). RG involves measuring linear growth of cultures on agar amended with fungicide and has been used for true fungi (Chapman et al., 2011; Chen et al., 2008; LaMondia, 2014; Patón et al., 2017) and *Phytophthora* species (Bashan et al., 1990; Coffey and Bower, 1984; Duvenhage, 1994; Garbelotto et al., 2009; Horner and Hough, 2013; Keinath and Kousik, 2011; Qi et al., 2012; Wilkinson et al., 2001). DW involves weighing dried mycelium of cultures grown in liquid media amended with fungicide and has been used for true fungi (Datta et al., 2016; Özer et al., 2010; Tremblay et al., 2003) and *Phytophthora* species (Fenn and Coffey, 1984; Ma and McLeod, 2014).

There are advantages and disadvantages to using each of these different techniques. RG assays can be used to obtain time series measurements and provide an opportunity to assess the dose-response of culture morphology. However, linear growth measurements do not reflect variation in mycelial density and the aerial growth habit of some species (Guest and Grant, 1991). DW assays provide a more accurate measure of growth inhibition; however, as mass can only be determined on the dried material, it is not possible to measure growth variation in the same culture over time or determine when growth is limited by resource availability. DW assays can, therefore, provide a more accurate measurement of growth inhibition by assessing the impact of treatment on mycelial density which can be greatly reduced (Guest and Grant, 1991; Ma and McLeod, 2014). Ma and McLeod (2014) found *P. cinnamomi* isolates were more sensitive to phosphite in liquid medium than a solid medium. They concluded that the relative sensitivity of the isolates was influenced by both the phosphite concentration and media density due to a higher surface area of mycelia being exposed to the phosphite in the liquid media. While the DW assay may be more accurate and informative than the RG assay for determining growth inhibition, it is also more time consuming with limited throughput. Furthermore, dry weights of mycelia are very small which increases the chance of error, especially when grown in the presence of inhibitory chemicals.

Optical density (OD) measurements are a commonly used measure in bacteriology for quantification of bacterial suspensions. Recently OD has been used to quantify the growth of fungal isolates within multi-well plates containing fungicide amended liquid media (Akhavan et al., 2017; Cox et al., 2009; Frac et al., 2016; Rampersad, 2011; Seyran et al., 2010; Wedge et al., 2013) and to assess *Phytophthora* zoospore germination (Elliott et al., 2015; Kuhajek et al., 2003). While optical density has been used previously with zoospore suspensions (Kuhajek et al., 2003), here we demonstrated a similar assay for oomycete mycelial growth which enables the screening of larger numbers of isolates without the need to produce zoospores which is incredibly challenging to synchronise across isolates and species. Furthermore, it is difficult to get a truly homogenous solution of zoospore inoculum because zoospores often aggregate at the surface of a liquid (Cameron and Carlile, 1977), resulting in a heterogeneous suspension with high standard deviations in optical density values. Zoospores may be more sensitive to fungicides than mycelium (Garbelotto et al., 2009), as a result, isolate sensitivity to fungicides may be underestimated if zoospores are solely used in inhibition experiments.

Mycelium is the most relevant propagule to use when testing the impacts of systemic fungicides, such as phosphite, on *Phytophthora* species because mycelium grows through host plants and will be exposed to systemic fungicides. Phosphite is also used to manage disease development and symptoms within infected plants. *Phytophthora* mycelium is aseptate therefore it is not possible to use a homogenised mycelial solution as an inoculum source because hyphal fragments may not survive excessive maceration (Kuhajek et al., 2003). Therefore, it is necessary to inoculate with a standardised amount of mycelial mat from a *Phytophthora* culture when inoculating with mycelia. The RG and DW assays have been used widely for testing tolerance to phosphite in *Phytophthora* species, but the OD assay has never been used to test hyphal growth inhibition in *Phytophthora* species as a response to phosphite treatment.

The specific objectives of this study were: 1) to determine if the OD method can detect the same levels of variation in sensitivity as the RG and DW assays between species; and 2) to analyse the effects of phosphite on hyphal growth of three *Phytophthora* species, including *P. cinnamomi*, *P. multivora* and *P. pluvialis*.

2. Materials and methods

2.1. Phytophthora isolates and media used

Phytophthora isolates from the New Zealand Forest Research Institute Culture Collection (NZFS) (Table 1) were maintained in water vials at 4 °C on carrot agar containing blended frozen carrots 100 g/L and agar 15 g/L (Erwin and Ribeiro, 1996). Isolates were cultured onto a modified Ribeiros' Minimal Medium (RMM) (Ribeiro et al., 1975), modified as outlined below. The glucose concentration was 9.0 g/L, and β-sitosterol was omitted. MES hydrate buffer (2-(N-morpholino) ethanesulfonic acid) was added at a final concentration of 0.03 M and the