Pathogenicity of *Ilyonectria pseudodestructans* propagules to grapevine rootstocks

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Abstract Black foot disease of grapevines is a major economic issue for the viticulture industry, with several *Dactylonectria* and *Ilyonectria* species identified as causal agents worldwide. This study aimed to confirm the pathogenicity of an *Ilyonectria pseudodestructans* isolate recovered from a symptomatic grapevine in a nationwide survey. An initial pot experiment inoculated callused and root-wounded grapevine propagation material of varieties '101-14' and '5C' with *I. pseudodestructans* conidia. The second pot experiment compared the pathogenicity of *I. pseudodestructans* conidial, chlamydospore and mycelial inocula. Disease incidence, severity and root and shoot dry weights were determined after 4–5 months of growth. *Ilyonectria pseudodestructans* was recovered from inoculated plants resulting in higher disease incidence and severity compared with the uninoculated control. Disease severity and incidence was higher for callused compared to rooted propagation material, but did not differ between grapevine varieties. Conidial inoculum caused greater disease incidence and severity compared with chlamydospore and mycelial inocula. *Ilyonectria pseudodestructans* propagules infected grapevine plant material via the callused basal ends or wounded roots, indicating this species is a potentially important pathogen of grapevines both in nurseries and vineyards.

Keywords *Cylindrocarpon* spp., *Cylindrocarpon destructans, Ilyonectria radicicola,* blackfoot disease, *Vitis* spp., propagation material, propagules

INTRODUCTION

Black foot disease of grapevines (*Vitis vinifera*) causes the decline and death of vines especially in young vineyards and nurseries worldwide, and is a major economic issue for the viticulture industry due to the loss of productive vines and the costs associated with replanting. A wide range of soil-borne pathogens have been identified as being associated with the disease worldwide, including several *Campylocarpon, Cylindrocladiella, Cylindrocarpon, Dactylonectria, Ilyonectria, Neonectria* and *Thelonectria* species (Cabral et al. 2012a, b; Brown et al. 2013; Agustí-Brisach & Armengol 2013; Lombard et al. 2014; Úrbez-Torres et al. 2014), with *Cylindrocarpon destructans, C. macrodidyma* and *C. liriodendri* reported the most widely associated species. However, based on recent taxonomic revision,

many *Cylindrocarpon* species have been reclassified as either *Dactylonectria* or *Ilyonectria* species, with the description of several new species within each genus (Cabral et al. 2012 a, b; Lombard et al. 2014). For example, *C. destructans* has been reclassified as an *I. radicicola* species complex consisting of 12 species, including *I. radiciola, I. pseudodestructans* and *I. europaea*. Therefore, for research reported prior to the taxonomic revision of *C. destructans*, it is now unclear as to which of the new species within the *I. radicicola* species complex the research was reporting on.

A New Zealand wide survey of symptomatic vines identified a number of species within the new *I. radicicola* complex including *I. pseudodestructans* (Ridgway & Jones unpublished results). Although *I. pseudodestructans* has been isolated from grapevines with black foot decline symptoms (Cabral et al., 2012a), its pathogenicity has not been determined. Due to the uncertainty of the exact species referred to in previous studies as a result of the nomenclature changes, there is a need to determine whether or not this species is a pathogen of grapevines.

Dactylonectria or *Ilyonectria* species associated with black foot disease are known to produce several different propagules including mycelium, macro- and micro-conidia and chlamydospores as survival propagules (Agustí-Brisach & Armengol 2013). However, the role of these different propagules in the disease cycle of is unclear. A recent study by Probst et al. (2019a) showed that all propagules of *I. liriodendri* and *D. macrodidyma* resulted in infection of grapevine rootstocks, but with higher disease rates resulting from the conidial inoculum than from other propagule types. However, it is not known what roles propagules of other pathogenic species, such as *I. pseudodestructans* play in the disease cycle. Further, Probst et al. (2019a) reported no difference in susceptibility of the two commonly grown grape rootstock varieties, '101-14' and '5C', to *I. liriodendri* and *D. macrodidyma* in a pot experiment using potting mix. However, the relative susceptibility of these rootstock varieties was reported to differ when planted in soil artificially inoculated with a mixture of *Dactylonectria* and *Ilyonectria* spp. isolates, with '101-14' reported to be one of the most susceptible rootstocks whilst '5C' the least (Bleach 2012). This difference in the relative susceptibility of the varieties in the two studies is potentially related to the pathogen species used in the experimental set up.

Therefore, the aim of the study was to determine the relative pathogenicity of different propagules of *I. pseudodestructans* to two commonly grown rootstock varieties '101-14' and '5C'. Two grapevine propagation materials, callused and rooted cuttings, were assessed for relative susceptibility as these are used by the viticulture industry.

MATERIALS AND METHODS

Ilyonectria pseudodestructans inoculum preparation

Ilyonectria pseudodestructans isolate Mar13a (ICMP 16792), originally isolated from a black foot symptomatic grapevine plant from a Marlborough vineyard in 2005, was obtained from the Lincoln University Plant Pathology Culture collection. The isolate was stored as mycelium colonised agar discs in glycerol at -80°C, and routinely cultured from the stock cultures onto potato dextrose agar (PDA, Oxoid Ltd Basingstoke, UK) and incubated at 20°C for 2–3 weeks in the dark.

Conidial suspension inocula were produced by flooding 3-week-old PDA cultures with 5 mL of sterile water plus 3 drops/L of Tween 80 (polyoxylethylene (20) sorbitan mono-oleate; BDH Chemicals Ltd, Poole, UK) and scraping the surface with a sterile glass microscope slide. This process was repeated twice per culture plate. The resulting suspension was then sieved through a sterile 150 µm mesh sieve to remove mycelial fragments. The final conidial suspension was adjusted to 10^6 conidia/mL using sterile water based on haemocytometer counts.

Grapevine plant material

Two different grapevine propagation methods, rooted and callused grapevine cuttings, of

two grapevine varieties '101-14' and '5C' were used. For the rooted planting material, two-node dormant shoot cuttings collected in late winter from visually healthy vines from the Lincoln University vineyard (Canterbury) that had shown no latent infection by black foot pathogens in previous experiments (data not shown). Cuttings were placed in trays containing pumice then placed on a heat pad at 25°C for 6 weeks, with an average air temperature of 5.4°C in a shade house. The standard New Zealand nursery protocol was used to callus a separate set of cuttings, whereby the cuttings were placed in 20-cm deep trays of vermiculite at 27°C for 4 weeks and then hardened off in a greenhouse (14–28°C) for 1–2 weeks before inoculation.

Pathogenicity of *I. pseudodestructans*

Inoculation

Rooted cuttings were carefully removed from the trays and the roots trimmed to 10 cm then inoculated by soaking for 30 minutes in a suspension of the *I. pseudodestructans* conidia prepared above (10^6 conidia/mL). The bases of callused cuttings were treated in the same way. Control cuttings were soaked in sterile water. The cuttings were then planted into 1.5 L pots containing potting mixture (80% horticulture bark: 20% pumice, amended with 2 kg of Osmocote Exact (Scotts Australia, NSW; N:P:K (16:5:9.2), 1 kg agricultural lime and 1 kg Hydralow (Scotts Australia) per m^3). Seven replicates for each plant propagation type and rootstock variety combination treatment and controls were set up and the plants laid out in a randomised block design on mesh tables in a greenhouse. The plants were grown for 4 months and to ensure plants had adequate light for 16 h per day, high pressure sodium lamps (Son-T Agro 400, Philips) were switched on from 4 am to 12 pm and 4 pm to 8 pm for the duration of the experiment. The plants were watered daily.

Assessments

Plants were harvested by removal from the pots and the roots of each plant were washed under running tap water. The roots were then removed from the stem base, placed into a paper bag and dried at 70°C to constant weight before being weighed. The main stems were washed thoroughly with running tap water and re-isolation of the pathogen from the infected plant was carried out as described by Halleen et al. (2003). The lower 20 cm stem section was removed and surface sterilised by submerging the stem in 70% ethanol for 30 s, followed by 5 min in 0.35% sodium hypochlorite and finally 30 s in 70% ethanol. After air drying the stem in a laminar flow cabinet for 10 min, the root crown comprising the lowest 1–2-cm of the stem base was removed and discarded.

To determine pathogen infection, a 1–3 mm piece of tissue was sliced from the basal end of the stem (0 cm) and cut into four pieces of approx. 3 mm^2 with these pieces placed equidistant around the edge of a PDA amended with 250 mg/L chloramphenicol plate. A 1–2 mm tissue slice was also cut from the stem at 5 cm above the base, cut into two pieces and one piece placed in the centre of the same PDA plate. After the plates were incubated at 20°C for 7 days in the dark, *I. pseudodestructans* like colonies growing from the wood pieces were identified by comparing the colony morphology with the *I. pseudodestructans* isolate used for inoculation. The presence of the pathogen in the grapevine at 0 cm and 5 cm (disease incidence) and the proportion of wood pieces at 0 cm colonised by the pathogen (disease severity) were recorded (Probst et al. 2012).

Pathogenicity of different *I. pseudodestructans* propagules

Production of I. pseudodestructans *propagules*

Conidial suspension for *I. pseudodestructans* isolate Mar13a was produced as previously described. Chlamydospores were produced in liquid cultures using the method of Yoo et al. (1996), whereby 1 L flasks containing 500 mL of 1/3 strength Czapex dox broth (CDB; Sigma Chemicals, St. Louis, USA) were inoculated with five mycelium plugs (5.5-mm diameter) cut from the margin of a 14-day-old *I. pseudodestructans* colony growing on PDA. Three replicate flasks were set up and incubated on a shaker at 100

rpm at room temperature (approx. 20°C) for 30 days. The conidia were removed by washing the contents of the flask with sterile water in a 150 µm mesh sieve three times. The washed mycelium was then homogenising for 2 min at high speed in a Sunbeam Multiblender to separate the chlamydospores from the mycelium and sieved again to remove the mycelium. The concentration of chlamydospores in the filtrate was adjusted 10^6 chlamydospores/mL with sterile water based on haemocytometer counts.

Wheat grains colonised with mycelia were used for mycelium inoculum. Wheat grain (Champion Flourmills, Christchurch, New Zealand; 400 g) along with 500 mL of water and 250 mg of chloramphenicol were placed in 1 L conical flasks and heated to boiling. After leaving the grains to cool for 10 min they were rinsed three times with water with the excess water drained off. The grains were then autoclaved at 121°C at 15 psi for 15 min on two consecutive days, and once cooled inoculated with five mycelial discs (7-mm diameter) cut from the margin of a 14-day-old *I. pseudodestructans* PDA colony. The flasks were incubated for 14 days at 20°C in the dark, with the flasks shaken by hands daily for 3–5 sec to facilitate even colonisation of the grains.

Inoculation

Rooted and callused grapevine cuttings of rootstock varieties '101-14' and '5C' were inoculated by soaking them in either conidial or chlamydospore spore suspensions for 30 min and then potted in 1.5-L pots containing potting mixture as previously described. Control plants were treated with sterile water.

For mycelial inoculation, 1.5-L pots were half filled with potting mixture and 5 g of colonised wheat grains placed in the bottom of a 5-6 cm deep hollow made in the potting mixture. Rooted or callused grapevine plants were then placed on top of the wheat grains and the pots filled up with potting mixture. For control plants, uncolonised sterile wheat grains were used. The 12 replicates for each treatment were arranged in a split plot design on a mesh table in a greenhouse. The plants were grown for 5 months, after which they were harvested and assessed as previously described. In addition, the dry weight of new shoots that grew within the 5 months were also assessed by removing the leaves and drying the shoots in paper bags at 70°C for 2–3 days to constant weight and weighed.

Statistical analysis

Disease incidence at 0 cm and 5 cm above the stem base was analysed by general linear model with bionomial distribution and logit link. For significant main effects or interactions, treatment means were compared using standard error of difference (SED). Disease severity percentage data were arcsine transformed prior to analysis. Root and shoot dry weights, and disease severity were analysed using a general linear model with terms appropriate to the design and the interaction amongst the factors of interest. The treatment means for significant main effect of interactions were compared using Fisher's Protected least significant difference (LSD) test at P=0.05. All analysis were conducted using Genstat version 16 (VSN International Ltd, Hemel Hempstead, UK).

RESULTS

Pathogenicity of *I. pseudodestructans*

Disease incidence at 0 cm above stem base for all plants was significantly higher (P=0.010) for *I. pseudodestructans* (51.9%) compared with the uninoculated control (21.4%). Disease incidence at 0 cm was higher for callused plants than rooted plants (P=0.023), with means of 51.8% and 18.7%, respectively but did not differ significantly (P=0.536) between rootstock varieties (means of 37.8% for '101-14' and 28.5% for '5C').

The disease incidence at 5 cm above the stem base was significantly higher (P=0.043) for *I. pseudodestructans* (33.3%) compared with the control (10.7%). Disease incidence at 5 cm showed significant differences between propagation methods, with callused plants having greater incidence than rooted plants (P<0.001), means being 40.7 and 3.9%, respectively, but did not differ significantly (P=0.111) between

varieties with mean disease incidences of 28.8% and 21.4% for '101-14' and '5C', respectively.

Disease severity was significantly higher (P<0.001) for *I. pseudodestructans* (41.2%) compared with the control (12.5%). Disease severities differed for propagation methods (P=0.004), with callused plants (29.1%) having greater disease severity than rooted plants (1.9%). There was no significant difference (P=0.655) between varieties, which had means of 12.3% for '101-14' and 8.1% for '5C'.

Root dry weights did not significantly differ (P=0.230) between *I. pseudodestructans* inoculated (6.2 g) compared with the control (6.8 g). Similarly, there was no significant differences between propagation method (P=0.705) being 6.6 g and 6.4 g for rooted and callused cuttings, respectively, and cultivar (P=0.305) being 6.8 g for '101-14' and 6.4 g for '5C'.

The pathogenicity of the different pathogen propagules.

Disease incidences at 0 cm above the stem base showed significant differences between propagule types (P=0.004) with conidia having a greater mean disease incidence (76.9%) than chlamydospores (59.1%) and mycelium (43.8%), compared with the two controls (33.3% and 31.3% for water and wheat, respectively) (Figure 1). Callused plants had a greater disease incidence than rooted plants (P<0.001), with means of 66.4 and 37.2%, respectively, while disease incidences at 0 cm were not significantly different (P=0.394) between plants from the different rootstock varieties (means of 50.3% for '101-14' and 53.2% for '5C').

There was a significant difference (P=0.014) in disease incidences at 5 cm above the stem base between propagule types, with *I. pseudodestructans* conidia and chlamydospores (36.5% and 27.3%, respectively) having significantly greater mean disease incidence compared with the water control (6.3%) and the wheat control (16.7%), with the level in the *I. pseudodestructans* mycelium (27.1%) not being significantly different to the wheat control (Fig. 1). Callused plants had greater disease incidences at 5 cm than rooted plants (P<0.003), with means of 33.3 and 16.0%, respectively, but varieties did not differ, with '101-14' and '5C' having means of 22.8 and 26.6%, respectively (P=0.429).

Disease severity showed significant differences between propagule types (P=0.002), with conidia causing significantly greater mean disease severity (52.0%) than chlamydospores (26.1%) and mycelium (17.9%; Fig. 1). All plants

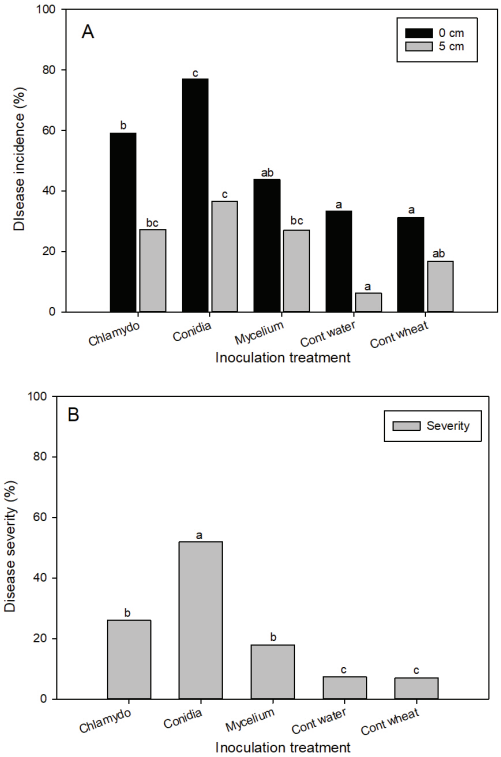

Figure 1 A) Mean disease incidences at 0 and 5 cm above stem bases; and B) disease severity at 0 cm of grapevine plants inoculated with three inoculum types (chlamydospores, conidia and mycelium) of *Ilyonectria pseudodestructans*. For 0-cm and 5-cm data individually, bars with different letters are significantly different (P<0.05) based on the SED for disease incidence and LSD for disease severity data generated from GLM analysis.

inoculated with propagules showed significantly greater mean disease severities than their respective control.

Disease severity differed between propagation methods (P<0.001), with callused plants having greater disease severity than rooted plants (39.1 and 10.0%, respectively). Disease severity did not differ between rootstock varieties (P=0.508), with mean severities being 21.7% for '101-14' and 23.7% for '5C'.

There was a significant effect (P<0.001) of propagule on the root dry weight, with mean root dry weights of plants inoculated with mycelium being significantly higher (9.5 g) than those inoculated with chlamydospores (8.8 g) and conidia (8.4 g; Table 1). No significant differences were observed between the root dry weight of the inoculated plants and their respective controls, apart from for plants inoculated with chlamydospores, which were significantly higher than for the water control plants.

Mean root dry weights were significantly higher (P<0.001) for rooted plants (9.5 g) than callused plants (8.3 g) and showed significant interaction (P<0.001) between propagation method and propagules. For chlamydospores and conidia the root dry weights were significantly higher for rooted compared with callused, but for mycelium the root dry weight was significantly higher in callused plants, with there being no significant difference between propagation method for the wheat and water controls (Table 1). The root dry weight of rootstock variety '5C' (9.2 g) was significantly (P=0.008) higher than for '101-14' (8.4 g), and there was significant interaction (P<0.001) between rootstock variety and propagation type. The root dry weight of rooted '101-14' cuttings (9.6 g) was significantly higher than for callused '101-14' cuttings (7.6 g), whereas there was no significant difference between the dry root weight of callused and rooted '5C' cuttings (9.4 g and 9.0 g, respectively).

Shoot dry weights showed significant differences between propagules (P<0.001), with plants inoculated with mycelium having a significantly higher mean shoot dry weight (8.8 g) than those inoculated with chlamydospores (7.1 g) and conidia (6.6 g), Table 1. No significant differences were observed between the mean shoot dry weights of the inoculated plants and their respective controls, except that the shoot dry weight of plants inoculated with chlamydospores were significantly higher that for the water control plants.

The shoot dry weights differed significantly with respect to propagation methods (P<0.001), being 8.5 g for rooted plants and 6.6 g for callused plants, and the interaction between propagation methods and propagule types (P<0.001). For chlamydospores, conidia and water inoculations, the shoot dry weight was significantly higher for rooted compared with

Table 1 Mean shoot and root dry weights of callused and wounded rooted grapevine plants for rootstock varieties 101–14' and '5C' 5 months after inoculation with three propagule types of *Ilyonectria pseudodestructans.*

Treatment	Propagules	Shoot dry weight (g)			Root dry weight (g)		
		Rooted[1]	Callused[1]	Mean[2]	Rooted[2]	Callused[2]	Mean[2]
I. pseudodestructans	Chlamydospores	8.4	5.7	7.1 b	9.9	7.7	8.8 b
	Conidia	8.6	4.6	6.6 ab	10.1	6.6	8.4 bc
	Mycelium	8.1	9.5	8.8 c	9.1	10.0	9.5 a
Control	Wheat	8.8	8.5	8.6 c	9.6	10.2	8.0 c
	Water	6.6	5.0	5.8 a	8.4	7.5	9.9 a
	LSD	1.22		0.86	0.90		0.63

[1]Mean values for shoot and root dry weight across rooted and callused and [2] across both propagation method, followed by different letters are significantly different based on LSD at P<0.05.

callused plants. In contrast, for mycelium the shoot dry weight was higher for callused plants, with there being no significant difference in the shoot dry weight between the propagation method for the wheat control (Table 1). There was no significant difference (P=0.367) in dry shoot weight for the different rootstock varieties being 7.5 g for '101-14' and 7.2 g for '5C'.

DISCUSSION

This is the first report of the pathogenicity of *I. pseudodestructans* on grapevine. Although *I. pseudodestructans* has been isolated from grapevines with black foot symptoms (Cabral et al. 2012a), its pathogenicity has not been verified. As well as being isolated from grapevines in Portugal, it has also been reported from *Poa pratensis* from Canada and *Quercus* sp. from Austria (Cabral et al. 2012a); however information on the pathogenicity on these hosts has not been reported. The species has also been isolated from roots of symptomatic strawberry (*Fragaria* × *ananassa*) and raspberry (*Rubus idaeus*) plants although again their pathogenicity was not verified (Weber et al., 2017). It is important to determine the host range of these pathogens, as previous crops or those planted as cover crops could provide a source of pathogen inoculum for infection of subsequently planted grapevines. Additionally, since nurseries often produce propagation material for a wide range of horticultural crops including grapevine, this information would inform nurseries of the potential carry-over of inoculum from other host plants to infect grapevine propagation material.

The two grapevine propagation methods used here were designed to investigate both the infection situation in the nursery, where callused cuttings are placed in the soil, and in vineyards where vines with trimmed roots are planted. Wounds on roots or below-ground rootstock tissue can also be entry points for pathogens due to the activity of soil nematodes or insects, emergence of secondary roots (Horsfeld & Dimond 1960) or damage to planting material during transplanting. *Ilyonectria pseudodestructans* was isolated from stems after 4 to 6 months, indicating that the pathogens can infect both through the callused basal ends and wounded roots. However, disease incidence and severity were higher for callused compared with rooted vines, especially disease incidence at 5 cm above the stem base. Halleen et al. (2003) reported that incompletely callused grapevines cuttings were predisposed to infection by soil borne black foot pathogens. Further, entry of the pathogen into the stem through the incomplete callused stem base would likely result in faster colonisation of the stem, than for the pathogen infecting and colonising from root entry points. Disease incidences were lower for infected root wounded plants than for callused plants but the pathogen could possibly move from roots to the stems after a longer period. The results obtained here showed the importance of early detection of the pathogen in nursery and in vineyard soils to enable appropriate control strategies to be implemented to prevent infection.

No difference was seen in the susceptibility of the two rootstocks, which is similar to that reported by Probst et al. (2019a) in a previous study conducted using plant material inoculated and grown in potting mix. However, Bleach (2012) found that isolate '5C' was the least susceptible whilst '101-14' was one of the most susceptible when different rootstock varieties were planted in soil artificially inoculated with a mixture of *Dactylonectria* and *Ilyonectria* spp. This difference was probably related to soil factors including biotic and physicochemical properties which may differentially affect the susceptibility of the rootstocks, and the pathogenicity of these species in soil and these factors need to be investigated further.

All three *I. pseudodestructans* propagule types tested here were able to infect grapevine rootstocks resulting in disease. Conidial inoculum resulted in higher rates of infection than mycelium, which is similar to that reported for *D. macrodidyma* and *I. liriodendri* by Probst et al. (2019a). In soil in the absence of a host, mycelium propagules have been shown to disintegrate or rapidly convert into conidia or chlamydospores, resulting in lower inoculum

levels (Probst et al. 2019b). The fate of the different inoculum propagules in potting mix and in the presence of a host is not known but these results indicate that conidial and chlamydospore inocula may germinate and infect the grapevine rootstocks directly. However, it is unclear whether the lower disease levels observed with mycelial inoculum indicates that the mycelium converts into conidia/chlamydospores prior to these propagules germinating and infecting the grapevine, or whether the inoculum infects the rootstocks directly.

Inoculation with *I. pseudodestructans* conidia and chlamydospores reduced the root dry weights but not those of shoots, and may indicate that the higher disease levels observed in the *I. pseudodestructans* conidial and chlamydospore inoculated callused rootstocks resulted in a decrease in root biomass. Similar results were observed by Halleen et al. (2004), whereby inoculation of rooted vines with *I. radicicola* complex (as *Cylindrocarpon destructans*) and *D. macrodidyma* complex (as *Cylindrocarpon macrodidymum*) reduced root mass after 4.5 months growth in potting mix. However, overall, the shoot dry weight for plants soaked in water or spore suspension was lower than for plants inoculated with wheat grains, irrespective of the presence of *I. pseudodestructans*. This result suggests that the autoclaved wheat grains provided nutrients available to stimulate plant shoot growth.

Infection by *I. pseudodestructans* was seen in the control treatment, which was probably as a result of water splash contamination of propagules from the treated pots into the nearby untreated control pots due to overhead irrigation. This observation highlights the capacity of inoculum to spread in a vineyard by rainfall or overhead irrigation. Similar spread of fungal inoculum by water splash was observed for the biocontrol agent *Coniothyrium minitans* (Williams et al. 1998). Further, these results indicate that even relatively low inoculum concentrations can result in infection of grapevine rootstocks. To limit cross-contamination in future experiments, pots should be more widely spaced to avoid water splash contamination. Further, cross-contamination was prevented where each plant was irrigated using independent drippers (Probst et al. 2019a).

The results of this study have confirmed the ability of *I. pseudodestructans* to infect grapevine rootstocks and adds to the information on the species shown to be involved in the black foot disease complex in New Zealand. All three propagule types were shown to infect the grapevine rootstocks either through callused basal ends or wounded roots indicating their ability to infect grapevines either in the nursery or in vineyards. Management of soil-borne inoculum in nursery and vineyard soils to reduce risk of infection is important, with further work required to identify soil factors which influence survival and infection of grapevines by *I. pseudodestructans* to inform the development of management practices.

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