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In vitro screening of *Trichoderma* isolates for biocontrol of black foot disease pathogens

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Summary. Management of black foot disease (BFD) of grapevines is difficult due to limited control options. Biological control fungi, in particular *Trichoderma* spp., hold potential as part of integrated management of BFD. *Trichoderma atroviride*, *T. fertile*, *T. harzianum* and *T. virens* were evaluated *in vitro* against four common BFD pathogens in South Africa, including *Campylocarpon fasciculare*, *C. pseudofasciculare*, *Dactylonectria macrodidyma* and *Ilyonectria liriodendri*. Effects of *Trichoderma* volatile organic and diffusible antifungal compounds (VOCs and DACs) and direct antagonistic effects were determined in Petri dish assays. Pathogen growth inhibition was determined in the VOC and DAC assays. Macro- and microscopic observations of fungus interaction zones were made in dual culture plate assays. Greater BFD pathogen growth inhibition occurred with the DACs than the VOCs. For both classes of compounds, *D. macrodidyma* was the most sensitive pathogen (100% inhibition by DACs and 65% by VOCs). In some cases, depending on the *Trichoderma* spp. isolate, growth stimulation occurred for *I. liriodendri*, *C. fasciculare* and *C. pseudofasciculare*. Macroscopically observed *Trichoderma* and BFD pathogen interactions included total or partial overgrowth, often associated with sporulation of the *Trichoderma* spp., and arrested growth or the formation of inhibition zones. Microscopic interactions included adhesion of the *Trichoderma* to pathogen hyphae, pathogen hyphae swelling, malformation and disintegration. In general, *in vitro* efficacy was isolate-dependent, both for *Trichoderma* spp. and the BFD pathogen.

Keywords. Volatile organic compounds, diffusible antifungal compounds, *Campylocarpon*, *Dactylonectria*, *Ilyonectria*.

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INTRODUCTION

Black foot disease (BFD) is a major fungus disease associated with young grapevine decline in nurseries and young vineyards (Halleen *et al*., 2004, 2006a, 2006b; Gramaje *et al*., 2010). Incidence and severity of BFD has increased and is now one of the major grapevine trunk diseases (Petit and Gubler, 2005; Rego *et al*., 2009; Compant *et al*., 2013; Úrbez-Torres *et al*.,

2014). The causal pathogens of this disease include species of *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew., *Dactylonectri*a L. Lombard and Crous, *Ilyonectria* P. Chaverri and C. Salgado, *Pleiocarpon* L. Lombard and D. Aiello and *Thelonectria* P. Chaverri and C. Salgado (Agustí-Brisach *et al*., 2012; Agustí-Brisach and Armengol, 2013; Dos Santos *et al*., 2016; Carlucci *et al*., 2017, Aigoun-Mouhous *et al*., 2019). Fungi in these genera are common soil inhabitants, occurring as saprophytes, root colonizers or weak plant pathogens that can survive in soil for extended periods due to the formation of chlamydospores (Brayford, 1993; Halleen *et al*., 2004).

Apart from hot water treatments (HWT) of dormant nursery plants, no treatment is available to prevent nursery plants from becoming infected with BFD pathogens after planting. Hot water treatment (50°C for 30 min) is considered effective against BFD (Halleen *et al*., 2006a; Alaniz *et al*., 2011; Agustí-Brisach and Armengol, 2013), but may result in a short-term effect, and also vine failure if not correctly applied to low quality propagation material (Rego *et al*., 2009). The application of *Trichoderma* spp. as biological control agents (BCAs) offers sustainable and lasting protection against grapevine trunk diseases when applied as pruning wound protectants (Mondello *et al*., 2018), so BCAs offer potential as part of integrated disease management of BFD. However, *Trichoderma harzianum* was evaluated on nursery vines in South Africa, and although '*Cylindrocarpon*' re-isolation was reduced, this was not significantly less than untreated controls (Fourie *et al*., 2001). Other studies investigating effects of *Trichoderma* isolates on nursery vines and BFD did not consistently find lower BFD pathogen infections (Dos Santos *et al*., 2016; Berlanas *et al*., 2018; Berbegal *et al*., 2019).

Beneficial activity of *Trichoderma* that have been studied extensively *in vitro* includes production of secondary metabolites and antibiosis, and direct mycoparasitism of plant pathogens (Harman, 2006; Samuels and Hebbar, 2015). Secondary metabolite composition is specific to the *Trichoderma* strain (Vinale *et al*., 2008a, 2008b), and includes a variety of compounds that can be categorized as volatile organic compounds (VOCs) or diffusible antifungal compounds (DACs). However, factors contributing to overall plant health and productivity cannot be evaluated *in vitro*. For example, improved plant health can be attributed to the induction of host plant resistance (Harman *et al*., 2004; Harman 2006; Vinale *et al*., 2008a) and growth stimulation (Vinale *et al*., 2008a, 2009; Samuels and Hebbar, 2015).

Despite various commercial *Trichoderma*-based products being available in South Africa, none has yet been registered for control of BFD of grapevine. The aim of the present study was, therefore, to investigate the efficacy of *Trichoderma* spp. isolates, derived from commercial products or potential products, against BFD pathogens *in vitro*. The tests conducted included screening for VOCs and DACs and examination of competitive growth between *Trichoderma* isolates and BFD pathogens.

MATERIALS AND METHODS

Fungal isolates

Ten *Trichoderma* spp. isolates, of which eight were from commercial products (Table 1), were tested *in vitro* for abilities to inhibit mycelium growth of BFD pathogens, using the assay methods by Dennis and Webster (1971a; 1971b) with modifications. The BFD pathogens used were three isolates each of *C. fasciculare*, *C. pseudofasciculare*, *D. macrodidyma* and *I. liriodendri* (Supplementary Table 1). Pure *Trichoderma* cultures were made from the products TrichoPlus™ and Awegenic Tri-cure™, by making a spore suspension from each product, plating it onto potato dextrose agar (PDA) (Biolab), and hyphal tip subculturing of developing fungi. The other *Trichoderma* isolates were obtained as pure cultures from the respective companies or providers. Fungus cultures were grown on PDA plates at 25°C for approx. 10 d, cut into small blocks and stored in sterile 14 mL capacity McCartney bottles with 10 mL sterile distilled water at room temperature until required.

Screening Trichoderma *isolates for production of volatile organic compounds*

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 7 d. Mycelium plugs (5 mm diam.) cut from the growing margins of colonies were placed face down in the centres of 90 mm PDA Petri dishes. Petri dishes containing the pathogens were then inverted over Petri dishes containing *Trichoderma* isolates in all combinations, and were sealed with Parafilm® M (Bemis). The Petri dishes were then incubated in the dark at 25°C for 7 d. Control plates were set up in the same manner, except that BFD pathogen-inoculated dishes were combined with sterile PDA Petri dishes. Following incubation, two colony diameters (perpendicular and horizontal) of each BFD pathogen were measured.

Table 1. *Trichoderma* products and isolates used in this study.

Isolate code[a]	Species (commercial isolate)	Product name	Institution/Company	Recommended or registered use
T1	*Trichoderma atroviride* (USPP-MT1)		Stellenbosch University	Grapevine pruning wounds
T2	*Trichoderma atroviride* (USPP-T1)		Stellenbosch University	Grapevine pruning wounds
T3	*Trichoderma atroviride*	Eco 77®	Plant Health Products	Grapevine pruning wounds – *Eutypa*; Tomato and cucumber – *Botrytis cinerea*
T4 T5	*Trichoderma atroviride* (Vitic 2) *Trichoderma harzianum* (Sp)	Bio-Tricho	Agro-Organics	Multiple crops and diseases
T6	*Trichoderma fertile*	TrichoPlus™	BASF	Seed treatment of tobacco
T7 T8 T9	*Trichoderma harzianum* (K2) *Trichoderma atroviride* (K4) *Trichoderma virens* (K1)	Excalibur Gold™	Advanced Biological Marketing®	General seed treatment
T10	*Trichoderma harzianum* (MIT04)	Awegenic Tri-cure™ MBFi		Beans, maize – *Rhizoctonia* and *Fusarium*; Potatoes – stem canker, black scurf, *Rhizoctonia*; Wheat – Fusarium; Tomato, curcurbit, lettuce – *Fusarium*, *Pythium* spp.

[a] Isolate codes used in study.

Screening Trichoderma *isolates for production of diffusible antifungal compounds*

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 8 d. Mycelium plugs (5 mm diam.) cut from the growing margins of the *Trichoderma* colonies were placed face down on autoclaved 50 µm thick cellophane membranes (85 mm in diam.; Sigma) covering PDA in Petri dishes. The Petri dishes were then incubated in the dark at 25°C until colony diameters of 50 mm were reached (after 42 to 48 h incubation). Following the incubation period, the cellophane membranes were removed ensuring that the Petri dishes were completely free of *Trichoderma* conidia and mycelia. The Petri dishes were then re-inoculated with mycelium plugs (5 mm diam.) cut from the growing margins of BFD pathogen colonies, and were incubated in the dark at 25°C for 6 d. Control plates were set up in the same manner, with the exception that the cellophane membranes were not inoculated with the *Trichoderma* isolates. Following the incubation period, two colony diameters (perpendicular and horizontal) of each BFD pathogen were measured.

Competitive growth assessments

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for, respectively, 3 or 7 d. Mycelium plugs (5 mm diam.) of the pathogens were cut from the growing margins of the colonies and placed face down on one side of 90 mm diam. Petri dishes. The Petri dishes were then incubated in the dark at 25°C for 4 d. Mycelium plugs (5 mm diam.) cut from the growing margins of the *Trichoderma* colonies were placed face down opposing the BFD pathogen colonies and incubated for a further 6 d. These dual-inoculated plates were then used for macroscopic observations of the colony interaction zones. Hyphae interactions, in particular *Trichoderma* hyphal adhesion or coiling and pathogen hyphal swelling, malformation or disintegration, were observed by mounting agar blocks cut from the interaction zones between *Trichoderma* isolates and one isolate of each BFD pathogen on a microscope slide and viewing it with a Nikon Eclipse E600 compound microscope at different magnifications (×200, ×400 or ×1000 with oil immersion).

Statistical analyses

Six replicates were used for the VOC and DAC experiments. The percentage inhibition from each experimental treatment was calculated, using the formula: Percentage inhibition of pathogen colony = [(Colony radius of control – Colony radius of treatment)/Colony radius of control] × 100. Normality of standardized residuals of the data was confirmed by the Shapiro-Wilk test (Shapiro and Wilk, 1965). Levene's test was used to verify the homogeneity of factor (treatment) variances (Levene, 1960). The data were subjected to analysis of

variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS Version 9.2 (SAS Institute Inc.). Fisher's least significant difference (LSD) was calculated at $P = 0.05$ to compare factor means (Ott and Longnecker, 2001), and this probability level was considered statistically significant for all tests.

RESULTS AND DISCUSSION

The *Trichoderma* spp. showed variable levels of growth inhibition towards the four BFD pathogen species tested, and, in a few cases, caused growth promotion of the pathogens. The biocontrol activity varied according to *Trichoderma* isolate, pathogen isolate, and according to the VOC or DAC tests.

Analyses of variance revealed significant pathogen × *Trichoderma* interactions ($P < 0.0001$), so the data are presented for each isolate (Tables 2 and 3). Results from the VOC assay differed to a large extent from those obtained in the DAC assay. When taking both classes of antifungal compounds into account, two isolates of *T. atroviride* (T1 and T8) gave the greatest overall mycelium growth inhibition, although growth inhibitioni-

Table 2. Mean percentage of mycelium growth inhibition of four species of black foot disease pathogens (three isolates each) from volatile organic compounds (VOCs) produced by different *Trichoderma* spp. Isolates (T1 to T10).

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	27.8ab[a]	33.8ab	23.2b-e	28.6a	15.8a	15.7a	55.3b	54.3a	66.9a	14.6a	15.8ab	21.9a
T2	23.9a-c	32.9ab	26.9bc	29.4a	11.8ab	10.4ab	37.7c	54.4a	58.0ab	-4.2b-d	10.6bc	14.5ab
T3	28.5a	33.9ab	30.8b	27.1a	11.3ab	12.1a	60.2ab	49.7a	41.0cd	5.7a-c	15.1ab	23.3a
T4	19.0b-d	23.5a-d	18.7c-e	21.3ab	3.6bc	2.6bc	60.7ab	53.6a	51.4bc	-11.0d	8.0bc	4.8bc
T5	24.7a-c	24.1a-c	22.1c-e	21.3ab	6.1bc	2.7bc	64.2a	58.3a	61.4ab	-10.7cd	7.9bc	3.6c
T6	6.3e	7.0e	9.0f	8.2c	-1.6c	-1.5c	8.3e	8.8c	21.8ef	-15.3d	4.4cd	-0.6c
T7	17.6cd	22.3b-d	24.7b-d	16.4bc	7.5a-c	9.7ab	33.1c	33.3b	34.2de	-7.3b-d	6.7c	17.3a
T8	28.6a	36.1a	39.6a	13.3bc	10.9ab	-2.5c	36.6c	37.6b	57.2ab	6.3ab	21.8a	23.9a
T9	12.6de	11.9c-e	18.6de	-2.6d	-1.2c	2.9bc	21.5d	14.1c	18.5f	-18.6d	-1.6d	-4.1c
T10	11.7de	10.6de	15.8ef	9.9c	6.4bc	3.9bc	18.4d	31.4b	30.5d-f	-5.2b-d	3.8cd	3.0c
LSD[b]	9.38	13.30	8.31	9.43	9.23	8.05	8.14	9.81	14.06	16.54	8.03	9.93

[a] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Least significant differences of each column.

Table 3. Mean percentage of mycelium growth inhibition of four species of black foot disease pathogens (three isolates each) from diffusible antifungal compounds (DACs) produced by *Trichoderma* spp. isolates (T1 to T10).

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	48.0a[a]	53.8a	52.8a	46.1c	30.7b	36.4a	100.0a	100.0a	100.0a	35.9a	70.7a	38.3b
T2	16.4c	22.3bc	19.6b	35.7d	17.4c	18.7bc	46.1b	43.9b	47.1c	19.1bc	25.6b	16.4de
T3	30.5b	20.6b-d	18.2bc	16.4f	17.4c	13.6cd	29.0cd	35.7bc	82.6b	9.5d	28.7b	16.4de
T4	20.0c	19.9cd	13.0bc	7.9fg	10.5d	3.7d	25.0c-e	12.4ef	26.8d	10.0cd	15.3bc	8.4ef
T5	20.6c	14.6c-e	18.6bc	26.1e	14.4cd	13.2cd	34.5bc	21.9de	23.7de	24.2b	24.9b	13.8d-f
T6	0.9e	-6.3f	-14.1e	2.6g	8.2d	3.6d	15.1e	13.0ef	16.8ef	2.5d	-1.1c	4.7f
T7	3.6de	5.0ef	-2.8de	2.7g	9.0d	4.0d	18.2de	9.9f	10.1f	6.6d	3.1c	8.2ef
T8	35.4b	48.4a	39.7a	100.0a	33.1b	29.9ab	96.4a	100.0a	94.5a	38.0a	57.5a	65.5a
T9	39.6ab	33.8b	52.0a	76.7b	97.8a	25.2ab	99.1a	29.9cd	53.2c	19.6b	23.0b	22.5cd
T10	11.5cd	8.7de	4.3cd	14.5f	13.7cd	13.9cd	21.1de	39.6bc	20.1de	19.2bc	14.7bc	26.0c
LSD[b]	9.37	13.33	14.69	8.68	6.34	11.30	11.67	11.27	9.74	9.50	16.81	9.08

[a] Values within each column followed by the same letter do not differ significantly ($P = 0.05$).
[b] Least significant differences of each column.

Table 4. Macroscopic interactions observed between *Trichoderma* isolates (T1 to T10), and the respective black foot disease pathogens (three isolates of each) in dual culture assays.

Isolate	*Campylocarpon fasciculare*			*Campylocarpon pseudofasciculare*			*Dactylonectria macrodidyma*			*Ilyonectria liriodendri*		
	8691	8692	8693	8694	8280	8279	8264	8265	8702	8267	8266	8699
T1	OGS[a]	OGS	OGS	OGS	SG	IZ	OGS	OGS	OGS	SG	SG	IZ
T2	OG	OG	OG	OG	PO	SG	PO	OG	OG	SG	PO	PO
T3	OGS	OGS	OGS	OGS	IZ	IZ	OGS	OGS	OGS	SG	IZ	SG
T4	OGS	OGS	OGS	OGS	IZ	SG	OGS	OGS	OGS	SG	IZ	SG
T5	OGS	OGS	OGS	OGS	SG	SG	OGS	OG	OGS	SG	IZ	SG
T6	POS	POS	POS	POS	IZ	IZ	OGS	POS	OGS	SG	IZ	SG
T7	OGS	OGS	OGS	OGS	OGS	OGS	OGS	POS	OGS	POS	OGS	POS
T8	OGS	OGS	OGS	OGS	SG	SG	OGS	OGS	OGS	SG	SG	SG
T9	OG	OG	OG	SG	SG	SG	OGS	OG	OGS	SG	IZ	SG
T10	OGS	OGS	OGS	OGS	IZ	IZ	OGS	OGS	OGS	SG	SG	SG

[a] PO = Partial overgrowth of the pathogen by *Trichoderma*; POS = Partial overgrowth of the pathogen and sporulation by *Trichoderma*; OG = Overgrowth of the pathogen by *Trichoderma*; OGS = Overgrowth of the pathogen and sporulation by *Trichoderma*; IZ = Inhibition zone formed between the pathogen and *Trichoderma*; two cultures; SG = Growth of the pathogen and *Trichoderma* ceased when in contact.

was greatest in the DAC assay. In both assays, and for most of the pathogen isolates, isolates T1 and T8 gave the greatest growth inhibition. For example, with *C. fasciculare*, T1 DACs gave the greatest growth inhibition against all three pathogen isolates (mean = 48.0 to 53.8%), and T8 VOCs gave the greatest growth inhibition against all three pathogen isolates (mean = 28.6 to 39.6%).

Overall, *D. macrodidyma* was the most sensitive pathogen species, for which all three isolates were completely inhibited by T1 DACs, and inhibition from T8 not being significantly different from T1 (mean = 94.5 to 100.0%). Greater inhibition of *D. macrodidyma* isolates (maximum mean = 66.9%) was also measured from the VOCs in comparison to the other species (maximum mean = 39.6% for *C. fasciculare*, 29.4% for *C. pseudofasciculare* and 23.9% for *I. liriodendri*).

In some cases, *Trichoderma* metabolites stimulated growth of the BFD pathogens. This was more often observed for *I. liriodendri* (mean inhibition = -1.6 to -18.6%) and *C. pseudofasciculare* (mean = -1.2 to -2.6%) in the VOCs assay, and also occurred for *C. fasciculare* (mean = -2.8 to -14.2%) and once for *I. liriodendri* (mean = -1.1%) in the DACs assay.

The macroscopic interactions between *Trichoderma* and pathogen isolates are reported in Table 4. For 90% of the *Trichoderma* isolates, the interactions occurred simultaneously and are, therefore, shown for each isolate. The formation of inhibition zones and complete growth inhibition were probably due to the production of secondary metabolites by the BFD pathogens, and this was only observed in a few cases. *Trichoderma* spp. mostly overgrew the pathogens followed by profuse sporulation, which was not observed without direct contact to the pathogens. Numerous factors, including the metabolic rate of hypha cells and the production of antimicrobial metabolites, can cause conidiation (Steyaert *et al*., 2013). Such an example is cell wall degrading enzymes and secondary metabolites that are produced during this process, facilitating entry of *Trichoderma* hyphae into the lumina of the parasitized fungi (Zaidi and Singh, 2013).

These interactions often coincided with mycoparasitic actions at microscopic levels (Supplementary Table 2). Adhesion of the *Trichoderma* hyphae to pathogen hyphae, and disintegration of pathogen hyphae, were often observed, while coiling of the *Trichoderma* hyphae or swelling and malformation of pathogen hyphae were rarely seen. Similar hyphal interactions were observed in isolates of the same species.

This study found two isolates of *T. atroviride* (T1 and T8) gave general antagonistic efficacy, though with varying efficacy against different BFD pathogens. Combining these isolates could offer more effective management of BFD. A matter of concern are the low levels of inhibition observed for the *Campylocarpon* species and *I. liriodendri*. Overall efficacy towards BFD would, therefore, be dependent on the composition of the BFD species present in a particular vineyard, as well as the *Trichoderma* isolates utilized for BFD control. Combining existing knowledge of *Trichoderma* as BCAs with the knowledge obtained from the present study will aid in the screening and combination of *Trichoderma* isolates as BCAs against BFD before field evaluations commence.

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