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Characterization of *Trichoderma* isolates from southern Italy, and their potential biocontrol activity against grapevine trunk disease fungi

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Summary. Grapevine trunk diseases (GTDs) are one of the most economically important diseases of grapevines, causing yield reductions and limiting vineyard lifespans. Fungal pathogens responsible for GTDs primarily infect grapevines through pruning wounds. The lack of systemic fungicides to stop the advance of fungi in vine vascular systems makes the use of pruning wound protection with either synthetic chemicals or biological control agents (BCAs) the main available GTD control strategy. Demands for sustainable grape production and increasing limitations on pesticide usage have raised interest in potential use of BCAs as pruning wound protectants against GTDs. The objectives of this study were to characterize 16 *Trichoderma* isolates from southern Italy using molecular methods, and to evaluate their potential activity as BCAs *in vitro* against the GTD canker-causing fungi *Diplodia seriata*, *Eutypa lata* and *Neofusicoccum parvum*. Molecular studies along with phylogenetic analyses identified eight species, including *Trichoderma atroviride*, *T. guizhouense*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and one unidentified *Trichoderma* sp. All these species had optimum mycelium growth between 25 and 35°C. *In vitro* dual culture experiments assessed antagonistic capabilities of all the *Trichoderma* isolates against *D. seriata*, *E. lata* and *N. parvum*, and showed that the isolates inhibited mycelial growth of the pathogens by up to 70%. The most inhibitory isolates were tested *in planta* for capability to protect pruning wounds against *D. seriata* and *N. parvum* in a detached cane assay. All the selected *Trichoderma* isolates gave >80% control of *D. seriata* and 60% control of *N. parvum*, and protected pruning wounds for up to 21 d after treatment.

Keywords. Biological control, *Botryosphaeriaceae*, canker, *Diatrypaceae*, *Vitis vinifera*.

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INTRODUCTION

Vitis vinifera L., with 7.15 million ha cultivated and 79.2 million tonnes of grapes produced in 2018, is one of the largest and most profitable perennial crops (FAO, 2020). However, grapes host the widest variety of pathogens of

any woody agricultural plant (Martelli, 1997), with fungi being the most prevalent (Wilcox *et al*., 2015). Among all fungal diseases affecting grapes, grapevine trunk diseases (GTDs) are considered the most destructive, causing significant economic losses to grape and wine industries (Bertsch *et al*., 2013; Gramaje *et al*., 2018). GTDs cause a wide range of foliar and vascular symptoms, and include black foot and Petri disease in young vineyards and Botryosphaeria dieback, esca, Eutypa dieback, and Phomopsis dieback causing overall decline and eventual vine death in mature vineyards (Gramaje *et al*., 2018).

With the exception of Black foot disease, caused by soil-borne fungi, most GTD fungal pathogens are air-borne, and these infect grapevines through pruning wounds (Gramaje *et al*., 2018). Under optimum environmental conditions, fruiting bodies (pycnidia or perithecia) embedded in the bark of cordons and/or trunks of infected grapevines, and/or from other woody hosts surrounding vineyards, discharge spores (conidia or ascospores). These are primarily spread short and/or long distances by rain droplets and wind (Kuntzmann *et al*., 2009; Petzoldt *et al*., 1983; Úrbez-Torres *et al*., 2010). Spores land on susceptible pruning wounds of nearby vines and infection commences. No systemic products are currently available to detain colonization by GTD fungi in vine vascular systems once infections move beyond points of entry. Preventive control measures such as pruning wound protection have been shown to be the most effective (Gramaje *et al*., 2018). Many different products, including synthetic, biological and organic fungicides are available and registered as pruning wound protectants against GTD fungi. Much has been published on this subject, and several reviews on specific products and their use for GTD control have been recently published (Gramaje *et al*., 2018; Mondello *et al*., 2018; Úrbez-Torres, 2011). Increasing consumer demands for more sustainable grapevine production and restrictions on synthetic pesticide usage have resulted in further interest by grape and wine industries to develop integrated pest management (IPM) programmes to control GTDs. These will include organic products, biological control agents (BCAs), cultural practices and responsible use of synthetic chemicals (Bertsch *et al*. 2013).

BCAs are organisms selected for their intrinsic antagonism towards a particular pathogen or pathogen groups, and that can function as effective alternatives to chemical pesticides (Wilson, 2003). BCAs are diverse and include either generalist species of *Bacillus*, *Pseudomonas*, *Streptomyces*, *Trichoderma*, *Clonostachys* or *Beauvaria*, or specialists in *Agrobacterium*, *Ampelomyces*, *Fusarium* or *Aspergillus* (Woo *et al*., 2014). Among them, *Trichoderma* species are the most studied as bio-pesticides, with over 60% of registered fungal BCAs products based on *Trichoderma* spp. (Verma *et al*., 2007). This is also true for grapevines, in which *Trichoderma*-based products are becoming more prevalent as alternatives for reducing pesticide use to control economically important diseases, including GTDs (Pertot *et al*., 2017; Mondello *et al*., 2018; Berbegal *et al*., 2020). Several *Trichoderma*-based commercial products using different *Trichoderma* species or strains are available as pruning wound protectants against GTDs, but there are conflicting reports on their effectiveness depending on the GTD fungal pathogen targeted and/or the grape cultivar and region (Mutawila *et al*., 2011; Gramaje *et al*., 2018; Mondello *et al*., 2018). The success of *Trichoderma* spp. as BCAs depends on their ability to survive under unfavourable conditions, their high reproductive capacity, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against plant pathogenic fungi, and efficiency in promoting plant growth and defense mechanisms (Verma *et al.,* 2007). Disease control efficacy by *Trichoderma* spp. may depend to their adaptability to host plants and regional environmental conditions.

The main objectives of the present study were; i) to characterize, using molecular methods, several *Trichoderma* isolates obtained from different substrates in southern Italy; and ii) to assess their potential biocontrol activity against commonly found canker-causing GTD fungi.

MATERIALS AND METHODS

Trichoderma *isolates and grapevine trunk disease fungal pathogens*

Sixteen hyphal-tipped *Trichoderma* isolates were selected for this study (Table 1). The isolates were stored in the fungal collection of the Department of Soil, Plant and Food Sciences at the University of Bari Aldo Moro, Bari, Italy. *Trichoderma* isolates were retrieved from the collection, revived on potato dextrose agar (PDA) and submitted to the Summerland Research and Development Centre (SuRDC), Summerland, British Columbia, Canada for characterization. The GTD pathogens *Diplodia seriata* (PARC91), *Eutypa lata* (PARC381) and *Neofusicoccum parvum* (PARC15) were selected for this study. Isolates of these pathogens were kept dormant as mycelium plugs in glass vials in autoclaved distilled water at 2°C in the Plant Pathology Fungal Collection at the SuRDC. The isolates were revived by placing three to four mycelium plugs on PDA and incubating at 22°C in the dark. When colonies were observed, they were transferred to fresh PDA and incubated until needed.

DNA extraction, PCR amplification, sequencing and phylogeny of Trichoderma *isolates*

Trichoderma isolates were grown on PDA for up to 3 d at 22°C in the dark. Total genomic DNA was extracted from actively growing colonies of each isolate using the Power Soil DNA Isolation Kit (MO BIO Laboratories Inc.) following the manufacturer's instructions. Oligonucleotides primers ITS1/ITS4 (White *et al.*, 1990), tef71f /tef997R (Braithewaite *et al.*, 2017) and fRBP2-7cR/fRBP2-5F (Liu *et al.*, 1999) were used to amplify, respectively, the ITS1-5.8S-ITS2 gene (ITS), a portion of the translation elongation factor 1-α (*EF-1α*) gene, and the RNA polymerase II second largest subunit (*RPB2*). ITS PCR mixtures each contained 4 µL of 5× Phusion HF Buffer, 11.9 µL of SD-H_2O, 2 µL of 2.0 mM dNTPs, 0.4 µL of Blotto 10%, 0.25 µL of 20 mM of each primer, 0.2 µL of *Phusion*, 1 µL of DNA, and ultrapure water up to 20 µL. EF1-α and RPB2 polymerase chain reaction (PCR) mixtures each contained 2 µL of 10× PCR buffer, 13.85 µL of SD-H_2O, 2 µL of 2.0 mM dNTPs, 0.4 µL of Blotto 10%, 0.25 µL of 20 mM of each primer, 0.1 µL of *DreamTaq* DNA polymerase, 1 µL of DNA, and ultrapure water up to 20 µL. Amplifications were performed in a GeneAmp 2700 thermal cycler (Applied Biosystems) using the following PCR conditions. ITS PCR conditions were one cycle at 98°C for 30 s followed by 35 cycles at 98°C for 10 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. EF-1α PCR conditions were 95°C for 6 min followed by four cycles at 95°C for 60 sec, 70°C for 90 sec, 72°C for 90 sec; 26 cycles with the annealing temperature decreasing from 68°C to 55°C (reduction gradient 0.5°C per cycle), and 12 cycles with the annealing temperature at 55°C; with a final extension at 72°C for 7 min. RBP2 PCR conditions were of hot start at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 55°C for 2 min, an increase of 1°C per 5 s to 72°C, and 72°C for 2 min; with a final extension at 72°C for 10 min. All PCR products were purified using a QIAquick PCR purification Kit (QIAGEN Inc.), and both strands of the ITS, *EF1-α* and *RPB2* were sequenced using a eight-capillary AB 3500 genetic Analyzer Sanger Sequencer at the SuRDC.

Forward and reverse sequences from each *Trichoderma* isolate were edited and assembled using Lasergene SeqMan Pro version 9.1.1.4 (DNASTAR Inc.). Consensus sequences were then compared with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST), and closest related sequences were selected to conduct phylogenetic analyses (Table 1). *Trichoderma* sequences from southern Italy were aligned with published GenBank sequences, including ex-type specimens when available for comparison, using the computer software BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999) (Table 1). Alignments were corrected visually and manually edited. Single phylogenetic analyses for each ITS, *EF-1α* and *RPB2* dataset were first conducted using Maximum Parsimony (MP) with the bootstrap test (1,000 random additional sequence replicates) and the Tree-Bisection-Regrafting (TBR) algorithm in MEGA-X (Kumar *et al.*, 2018). In addition, each aligned locus was further analyzed by Neighbor-Joining (NJ) using the Maximum Composite Likelihood method with 1,000 replicates to assess the robustness and the Tamura-Nei model. Tree topologies from individual trees were compared. ITS, *EF-1α* and *RPB2* trees with the greatest log likelihood values were selected. *Trichoderma* sequences from this study were deposited into GenBank, and representative isolates are maintained in both University of Bari Aldo Moro and SuRDC fungal collections.

Optimum temperatures for mycelium growth of Trichoderma *isolates*

Mycelium plugs (4 mm diam.) from the edges of 3-d-old actively growing colonies on PDA from 12 *Trichoderma* isolates (Table 1), were used to inoculate 90 mm diam. Petri dishes each containing 20 mL of PDA. Three replicates of each isolate were incubated separately at eight different temperatures ranging from 5 to 40°C, at 5°C intervals, in the dark. The orthogonal diameters of developing colonies were measured after 3 d incubation. The experiment was conducted twice. Paired T-test analyses were conducted to test for statistical differences between the first and the second experiment, to determine if data from separate experiments could be pooled for analyses. The mean radial mycelial growth and standard error for each temperature/species combination was calculated. Statistical analyses were performed using R.

Dual culture antagonism assay

Antagonistic capability of the selected *Trichoderma* isolates was evaluated *in vitro* against the GTD pathogens *D. seriata* (PARC 91), *E. lata* (PARC 381) and *N. parvum* (PARC 15) using dual culture assays (Rahman *et al.*, 2009). Mycelium plugs (6 mm diam.) of each *Trichoderma* and pathogen were obtained from the edges of 3-d-old actively growing colonies on PDA, and were placed opposite to each other at the edge of 90 mm diam. Petri dishes containing PDA. Plates were incubated at 22°C in the dark for 5 d. *Diplodia seriata*, *E. lata* and *N. parvum* were individually grown without the

Table 1. *Trichoderma* isolates used in this study and their GenBank accession numbers.

Species	Isolate[a]	Host	Geographic origin	ITS[d]	EF-1α[e]	RPB2[f]
Trichoderma atroviride	CBS 142.95	*Quercus* sp.	Slovenia	MH862505	-	-
T. atroviride	CBS 693.94	Mushroom compost	Northern Ireland	MH862501	-	-
T. atroviride	CBS 119499	*Prunus padus*	Austria	FJ860726	-	FJ860518
T. atroviride	DAOM 222144	n/a	n/a	AF456916	-	FJ442754
T. atroviride	DAOM 230838	n/a	Japan	-	KJ871107	-
T. atroviride	DAOM 238037	n/a	Thailand	-	KJ871093	-
T. atroviride	DAOM 242935	n/a	n/a	-	KX463440	-
T. atroviride	DAOM 242940	n/a	n/a	-	KX463436	-
T. atroviride	**PARC1011[b] / FV146**	n/a	Italy	MT448957	MT454114	MT454130
T. atroviride	**PARC1014[b] / FV54**	n/a	Italy	MT448960	MT454117	MT454133
T. atroviride	**PARC1017[b] / FV178**	n/a	Italy	MT448963	MT454120	MT454136
T. atroviride	**PARC1018[c] / FV271**	n/a	Italy	MT448964	MT454121	MT454137
Trichoderma guizhouense	BG8	*Ceriops tagal*	China	MH712265	-	-
T. guizhouense	BG9	*Ceriops tagal*	China	MH712266	-	-
T. guizhouense	DAOM 231412	n/a	n/a	-	AY605764	-
T. guizhouense	DAOM 231435	n/a	n/a	-	EF191321	-
T. guizhouense	NJAU 4742	Plant tissue	China	-	KP292611	-
T. guizhouense	**PARC1022[b] / Tch_4**	*Prunus persica*	Italy	MT448968	MT454125	MT454141
T. guizhouense	**PARC1023[b] / 5B**	*Prunus persica*	Italy	MT448969	MT454126	MT454142
T. guizhouense	**PARC1025[b,c] / Tch_6**	*Prunus persica*	Italy	MT448971	MT454128	MT454144
T. guizhouense	**PARC1026[c] / Tch_7**	*Prunus persica*	Italy	MT448972	MT454129	MT454145
T. guizhouense	S628	n/a	Greece	-	-	KJ665273
Trichoderma harzianum	*CBS 226.95*	Soil	England	AF057606	-	AF545549
T. harzianum	CBS 354.33	Soil	U.S.A.	MH855457	-	-
T. harzianum	DAOM 233986	n/a	n/a	EF392757	EF392749	KJ842171
T. harzianum	DAOM 242937	n/a	n/a	-	KX463434	-
T. harzianum	PAN12-34	Soil	U.S.A.	MK32267	-	-
T. harzianum	**PARC1013[b] / FV185**	n/a	Italy	MT448959	MT454126	MT454132
T. harzianum	**PARC1019[b] / Tch_1**	*Prunus persica*	Italy	MT448965	MT454122	MT454138
Trichoderma koningiopsis	Arak-96	Soil	Iran	-	KP985652	-
T. koningiopsis	*GJS 93-20*	Wood	Cuba	DQ313140	-	-
T. koningiopsis	ITCC 7291	Soil	India	-	LN897322	-
T. koningiopsis	**PARC1024[c] / Tch_5**	*Prunus persica*	Italy	MT448970	MT454127	MT454143
T. koningiopsis	S359	n/a	France	-	-	KJ665285
Trichoderma longibrachiatum	*ATCC 18648*	Mud	U.S.A.	NR_120298	AY865640	HQ260615
T. longibrachiatum	CIB T13	n/a	Colombia	-	EU280033	-
T. longibrachiatum	DAOM 167674	n/a	n/a	EU280099	-	-
T. longibrachiatum	DAOM 234103	n/a	n/a	-	DQ125467	-
T. longibrachiatum	**PARC1015[b] / FV144**	n/a	Italy	MT448961	MT454118	MT454134
T. longibrachiatum	S328	n/a	n/a	JQ685875	-	KJ665291
Trichoderma nigricans	NBRC 31285	n/a	n/a	JN943368	-	-
Trichoderma paratroviride	*CBS 136489*	n/a	Spain	-	KJ665627	KJ665321
T. paratroviride	**PARC1012[b] / FV145**	n/a	Italy	MT448958	MT454115	MT454131
T. paratroviride	S489	n/a	Spain	-	KJ665628	KJ665322
T. paratroviride	TE11	n/a	n/a	MH549109	-	-
T. paratroviride	TES15	n/a	n/a	MH549108	-	-
Trichoderma paraviridescens	*Hypo 372*	n/a	Austria	MT187973	-	KC285763
T. paraviridescens	S122	n/a	Italy	-	-	KC285764

(Continued)

Table 1. (Continued).

Species	Isolate[a]	Host	Geographic origin	ITS[d]	EF-1α[e]	RPB2[f]
T. paraviridescens	ATCC 20898	Soil	U.S.A.	-	-	EU252009
T. paraviridescens	BMCC:LU786	n/a	New Zealand	-	KJ871271	-
T. paraviridescens	KX098484	n/a	New Zealand	-	KX098484	-
T. paraviridescens	**PARC1016[b] / FV154**	n/a	Italy	MT448962	MT454119	MT454135
Trichoderma simonsii	CBS 130431	Decaying bark	U.S.A.	NR_137297	-	-
T. simonsii	GJS 91-138	Wood	U.S.A.	-	-	FJ442757
T. simonsii	GJS 92-100	Wood	U.S.A.	-	-	FJ442710
T. simonsii	S7	n/a	Italy	-	-	KJ665337
Trichoderma sp.	**PARC1020[c] / Tch_2**	*Prunus persica*	Italy	MT448966	MT454123	MT454139
Trichoderma spirale	*DAOM 183974*	Soil	Thailand	DQ083014	-	AF545553
T. spirale	DIS 311D	*Irvingia gabonensis*	Cameroon	FJ442232	-	-
T. spirale	**PARC1021[b] / Tch_3**	*Prunus persica*	Italy	MT448967	MT454124	MT454140
T. spirale	UNISS 3b-11	n/a	n/a	-	EF596973	-
T. spirale	UNISS 23-9	n/a	n/a	-	EF596975	-

[a] Isolate numbers in bold represent *Trichoderma* isolates from Italy used in this study. Isolate numbers in italics and underlined are ex-type specimens. CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), DAOM (Department of Agriculture Ottawa Mycology, Ottawa, ON, Canada), PARC (Pacific Agri-Food Research Centre Fungal Collection, Summerland, BC, Canada). ATCC (American Type Culture Collection, Manassas, VA, USA).
[b] *Trichoderma* isolates used in the temperature study.
[c] *Trichoderma* isolates used in the detached cane assay experiment.
[d] ITS: Internal Transcribed Spacer.
[e] EF1-α: Translation elongation factor 1-α.
[f] RNA polymerase II second largest subunit.
n/a: Not available.

presence of *Trichoderma* under the same conditions as experimental controls. Each *Trichoderma*/pathogen combination was replicated four times, and the experiment was conducted twice. The percent of mycelium inhibition was calculated using the formula:

Percent Inhibition (%) = [(B – A) / B] * 100

where A is the radius of pathogen mycelium growth co-inoculated with *Trichoderma* and B is radius of the pathogen mycelium growth alone in the control plate.

Statistical data analyses were adapted from Haidar *et al.* (2016). The experimental data from the dual culture antagonism assays were subjected to analysis of variance (ANOVA) followed by Tukey-Kramer's Honest Significant Difference post-hoc comparison tests (Tukey HSD) ($P = 0.05$). The standard errors of the means were calculated for all mean values.

Detached grapevine cane assay

The antagonistic activity of most inhibitory *Trichoderma* isolates from the dual culture experiments was evaluated *in planta* against the GTD pathogens *D. seriata* (PARC 91) and *N. parvum* (PARC 15) using a detached cane assay (DCA) (Ayres *et al.*, 2014) (Table 1). *Trichoderma* inoculum was obtained by scraping the surfaces of 4-d-old *Trichoderma* colonies grown on PDA at 22°C with a UV light /darkness regime of 12 h:12 h, after flooding each plate with sterile water containing 0.05% Tween 20 and filtering through autoclaved 25 µm pore diam. Miracloth (Sigma-Aldrich) to remove mycelium fragments. Conidium suspensions of each *Trichoderma* isolate were adjusted to 10^6 conidia mL^{-1} with a haemocytometer. Pycnidiospores of *D. seriata* and *N. parvum* were obtained from pycindia formed on colonies growing on PDA in the dark at 25°C with a UV light/darkness regime of 12 h:12 h, with the UV light source(Phillips UVB TL 20W/12RS bulb) at 80 cm distance from the Petri plates. Pycnidia and pycniospores formed after 4-5 weeks were harvested by adding 1-2 mL of sterile distilled water previously amended with a drop of Tween 20, then gently scraping the upper layer of the PDA with a sterile thin spatula to expose the pycnidia. Suspensions containing pycnidia was transferred into a sterile pestle and ground with a mortar to release spores from pycnidia. Suspensions were filtered through Miracloth into test tubes, which were vortexed, and spore concentrations were adjusted

to 10^5 spores mL^{-1} for both *D. seriata* and *N. parvum* using a haemocytometer.

Dormant grapevine canes (cv. Chardonnay) were collected from an experimental vineyard block located at the SuRDC. The canes were cut into two node sections (≈ 20 cm length), and were placed vertically through holes in Styrofoam trays floating in water, in a contained plant growth chamber (CONVIRON) set at 25°C, 70% RH, and 12 h light:12 h dark regime. The canes were then each pruned to 4 cm above the upper bud to simulate a fresh pruning wound. Soon after pruning, the wounds were individually treated with 50 µL of 10^6 conidia mL^{-1} (50,000 conidia per wound) of each *Trichoderma* spp. Different wounds were then challenged 24 h, 7 d or 21 d after treatment with 50 µL of 10^5 conidia mL^{-1}(5,000 spores per wound) of *D. seriata* or *N. parvum* to determine how long any *Trichoderma* activity lasted on each pruning wound. Positive controls included non-treated but inoculated wounds with *D. seriata* or *N. parvum* at 24 h, 7 d, or 21 d after pruning. Negative controls included non-treated and non-inoculated canes to determine if natural infections occurred in the collected canes from the experimental vineyard. A total of 30 canes per treatment were used, and canes were placed in randomized blocks of ten canes each. The cane cuttings were maintained under controlled conditions in the plant growth chamber for 5 weeks. After this time, the canes were collected, roots and leaves were removed, and the canes were prepared for re-isolation of the inoculated pathogens. The bark around the pruning wounds was shaved off, then the surface of each cane was flame sterilized with 95% ethanol. A tissue piece (≈ 1 mm) from the surface of each cane was discarded and then ten pieces of tissue (≈ 0.5 cm^2) were plated onto PDA amended with 1 mg mL^{-1} tetracycline (Sigma-Aldrich) (PDA-tet). Plates were the incubated for up to 10 d at 22°C in the dark. If a plate yielded either *D. seriata* or *N. parvum*, the corresponding cane was rated as colonized by the respective pathogen. The mean percent infection (MPI) by each pathogen was determined from all positive controls and treatments. *Trichoderma* spp. inhibition effectiveness was calculated as the mean percent disease control (MPDC) using the formula:

MPDC = 100 × [1-(MPI treated canes / MPI control canes)]

Statistical data analyses were adapted from the method used by John *et al.* (2005). The binary (infected or not-infected) data produced from the detached cane assays were analyzed using a logistic regression to determine the significance of timing of inoculation, and were subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests to determine if there were statistically significant differences between the means (P = 0.05). All statistical analyses were performed using R (R Core Team, 2019).

RESULTS

Molecular characterization of Trichoderma *isolates from southern Italy*

PCR amplifications of ITS, *EF-1α* and *RPB2* regions gave, respectively, products of approx. 500, 700, and 800 bp. To identify the different isolates and study the phylogenetic relationships among *Trichoderma* from southern Italy, ITS, *EF-1α* and *RPB2* sequences were BLAST analysed to select closely related sequences for the phylogenetic analyses (Table 1). Thirty seven taxa were included in the phylogenetic analysis of the ITS dataset, 36 taxa were included in the *EF-1α* dataset, and 33 taxa were included in the *RPB2* dataset. There were a total of 608 (ITS), 819 (*EF-1α*) and 849 (*RPB2*) positions in each final dataset, including gaps. The ITS and *RPB2* MP evolutionary histories both generated ten equally parsimonious trees. The *EF-1α* MP evolutionary history generated nine equally parsimonious trees (trees not shown). Lengths, consistency indices (CI), retention indices (RI), and rescaled consistency indices (RC) were, respectively, 98, 0.956989, 0.995056, and 0.954441 for ITS; 659, 0.835385, 0.968994, and 0.811662 for EF-1α; and 423, 0.808290, 0.959496, and 0.791641 for RPB2. The NJ analyses of each ITS, *EF-1α* and *RPB2* dataset resulted in trees with similar topology to the MP tree. The ITS, *EF-1α* and *RPB2* trees with the greatest log likelihoods are shown, respectively, in Figures 1, 2 and 3.

Based on individual phylogenetic analyses, *Trichoderma* isolates from southern Italy were identified as *Trichoderma atroviride* (PARC1011, PARC1014, PARC1017 and PARC1018), *T. guizhouense* (PARC1022, PARC1023, PARC1025, and PARC1026), *T. harzianum* (PARC1013 and PARC1019), *T. koningiopsis* (PARC1014), *T. longibrachiatum* (PARC1015), *T. paratroviride* (PARC1012), *T. paraviridescens* (PARC1016), *T. spirale* (PARC1021) and *Trichodrma* sp. (PARC1020).

The ITS phylogenetic analyses resulted in eight clades, each corresponding to a well-supported species. However, ITS analyses were not informative enough to discriminate among *T. harzianum* and *T. guizhouense* isolates, and included isolates of both species in a single clade along with *T. nigricans*, another species within the *T. harzianum* species complex (Figure 1). In addition, a second well-supported clade (82% NJ) containing *T. har-*

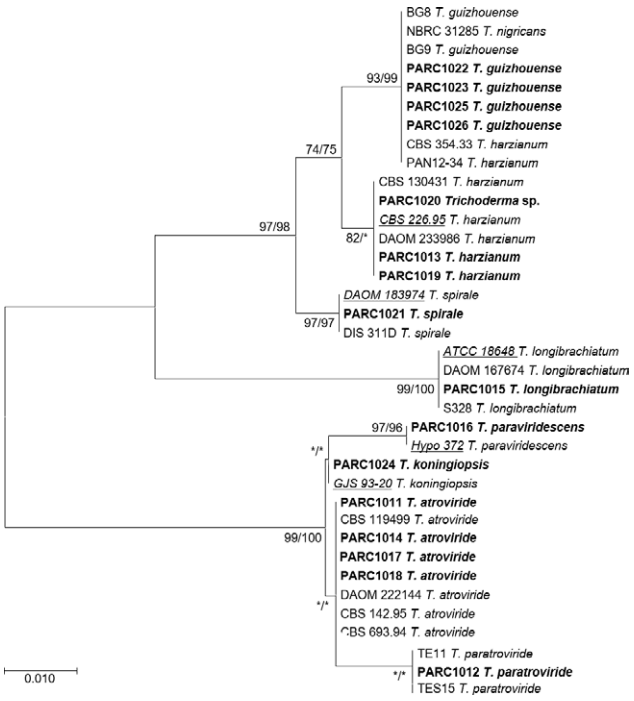

Figure 1. Greatest log likelihood ITS Neighbor-Join phylogenetic tree generated with 37 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values represented by asterisks were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

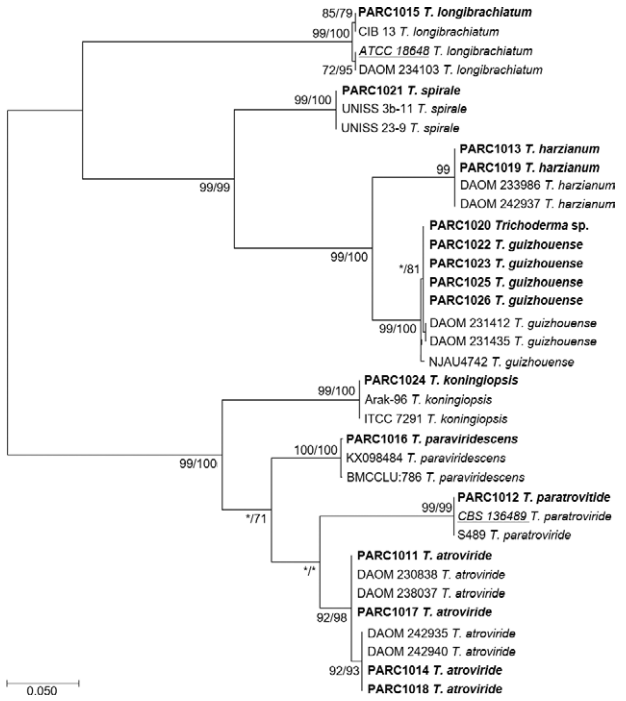

Figure 2. Greatest log likelihood EF-1α Neighbor-Join phylogenetic tree generated with 36 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values accompanied by asterisks were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

zianum isolates, including the ex-type CBS 226.95, was formed in the ITS analyses (Figure 1).

The *EF-1α* phylogenetic analyses placed *Trichoderma* isolates from southern Italy in eight different well-supported clades corresponding to each of the species identified, with the exception of isolate PARC1020, which grouped within one of the *T. harzianum* clades in the ITS analyses but fell within the *T. guizhouense* clade in the *EF-1α* analyses (Figure 2). Intraspecific variation was observed in three clades in the *EF1-α* analyses, including the *T. atroviride*, *T. guizhouense*, and *T. longibrachiatum* (Figure 2). Bootstrap values in the *EF-1α* analyses were greater than in the ITS analyses.

The *RPB2* phylogenetic analyses were the most informative, and these grouped *Trichoderma* isolates from Italy in eight well-supported clades, including the ex-types (Figure 3). Similar to ITS and *EF-1α* analyses, *RPB2* was did not group isolate PARC1020 with any identified clade, and it was closely related to *T. simonsii*, another species identified within the *T. harzianum* species complex. Therefore, isolate PARC1020 was identified as *Trichoderma* sp. (Figure 3). *RPB2* phylogenetic analyses resulted in the greatest bootstrap support for each clade.

Optimum temperatures for mycelium growth of Trichoderma *isolates*

Radial colony growth values for each *Trichoderma* isolate were plotted versus temperature (Figure. 4A). All isolates grew over the temperature range of 10 to 30°C, except for PARC1016, which did not grow at 30°C. Isolates PARC1013, PARC1015, PARC1019, PARC1022, PARC1023, PARC1025 grew at 35°C. None of the isolates used in the study grew at 5°C or 40°C (Figure 4A). Optimum temperatures for mycelium growth varied among isolates. The temperatures at which each *Trichoderma* isolate reached the maximum radial growth were 25°C for PARC1011, PARC1012, PARC1014, PARC1016, and PARC1017; 30°C for PARC13, PARC19, PARC1021, PARC1022, PARC1023, and PARC1025; and 35°C for PARC1015 (Figure 4A). Among all the isolates, PARC1017 had the least radial colony growth. Colony colours were cream for iso-

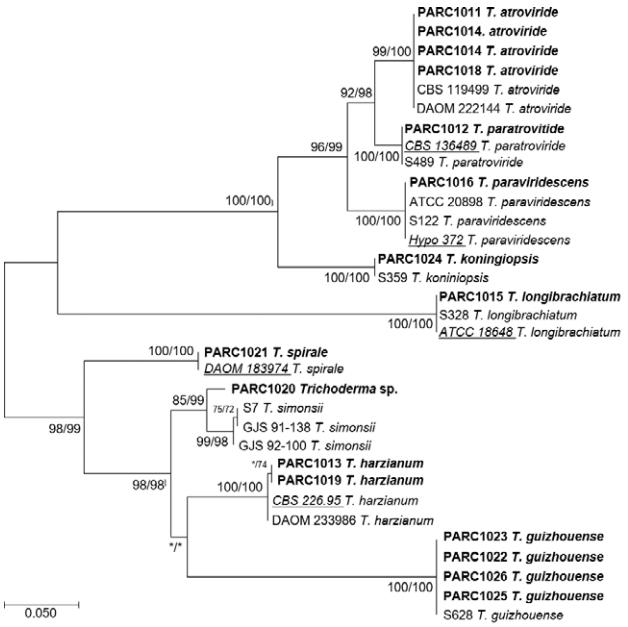

Figure 3. Greatest log likelihood RPB2 Neighbor-Join phylogenetic tree generated with 33 *Trichoderma* nucleotide sequences. Numbers in before and after each slash (/) represent, respectively, likelihood and parsimony bootstrap values from 1,000 replicates. Values accompanied by an asterisk were less than 70% bootstrap. *Trichoderma* isolates from southern Italy included in this study are indicated in bold. Ex-type isolates are indicated in underlined italics.

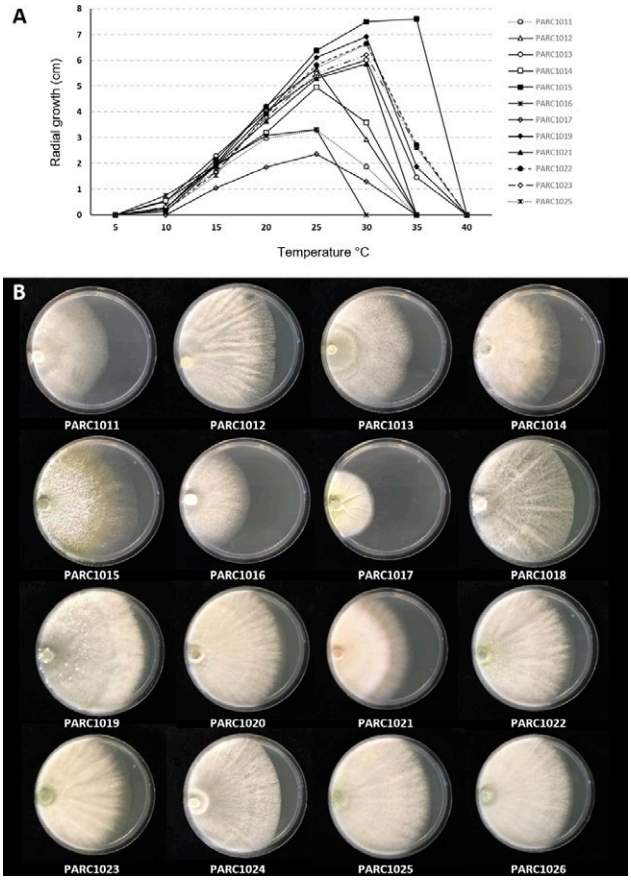

Figure 4. Phenotypical features of *Trichoderma* isolates from southern Italy. A. Mean radial mycelium growth at different temperatures after 72 h. B. Colony growth and colour after 72 h on potato dextrose agar for the different *Trichoderma* isolates from southern Italy used in this study.

lates PARC1014, PARC1018, and PARC1021, green for PARC1015, and white for the other *Trichoderma* isolates used in this study (Figure 4B).

Dual culture antagonism assays

The levels of antagonism of *Trichoderma* isolates used in this experiment against the GTD pathogens *D. seriata*, *N. parvum* and *E. lata* is shown in Figures 5 and 6. The mean percentage of inhibition of radial mycelial growth (PIRG) of *D. seriata*, *N. parvum* and *E. lata* by *Trichoderma* isolates used in this experiment ranged from 24.7% to 74.3% (Figure 6). Mean PIRG ranged from 51.4% to 69.6% for *D. seriata*, from 44.5% to 74.3% for *N. parvum* and from 24.7% to 68.2 for *E. lata* (Figure 6). The greatest PIRG for *D. seriata* (69.6%) and *E. lata* (68.2%) were recorded from *T. atroviride* PARC1018, and for *N. parvum* (74.3%) for *T. koningiopsis* PARC1024 (Figure 6). Overall, antagonism of *Trichoderma* isolates against *E. lata* mycelium growth was less than for both *D. seriata* and *N. parvum*, with seven out of 16 *Trichoderma* isolates giving mean PIRG less than 50% (Figure 6).

Detached grapevine cane assays

Results of the DCA trial are shown in Table 2. MPI of *D. seriata* and *N. parvum* from positive controls inoculated with 5,000 spores per wound were, respectively, 60% and 80% or greater, even when wounds were inoculated 21 d after pruning. *Diplodia seriata* and *N. parvum* were not isolated from non-treated and non-inoculated negative controls (natural infection, data not shown). All isolates tested in the DCA gave MPDC of 84 to 100% against *D. seriata* and 23 to 100% against *N. parvum* (Table 2). *Trichoderma atroviride* (PARC1018) and *T. harzianum* (PARC1020) gave the greatest MPDC (100%) against *D. seriata* from day one to day 21 after treatment. The other *Trichoderma* isolates gave high MPDCs (89 to 94%) when pruning wounds were challenged with *D. seriata* one day after treatment, and 100% MPDC when wounds were challenged 21 d after treatment

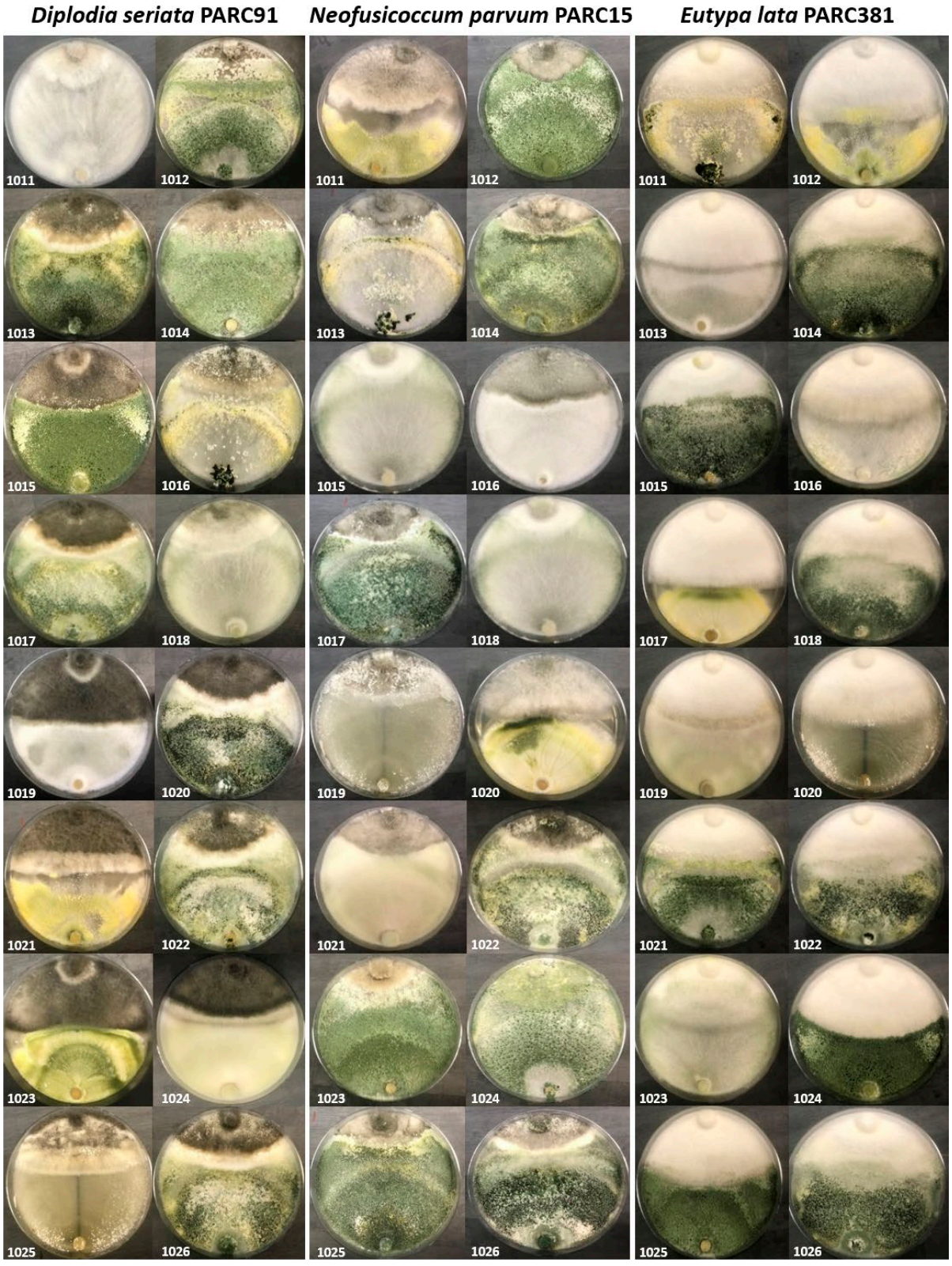

Figure 5. Dual culture antagonism experiment. Petri plates show antagonistic activity of each *Trichoderma* isolate used in this study (bottom side of Petri dish) against the GTD pathogens *Diplodia seriata* PARC91, *Neofusicoccum parvum* PARC15 and *Eutypa lata* PARC 381 (top of Petri plate) five days after culturing on PDA at 23°C.

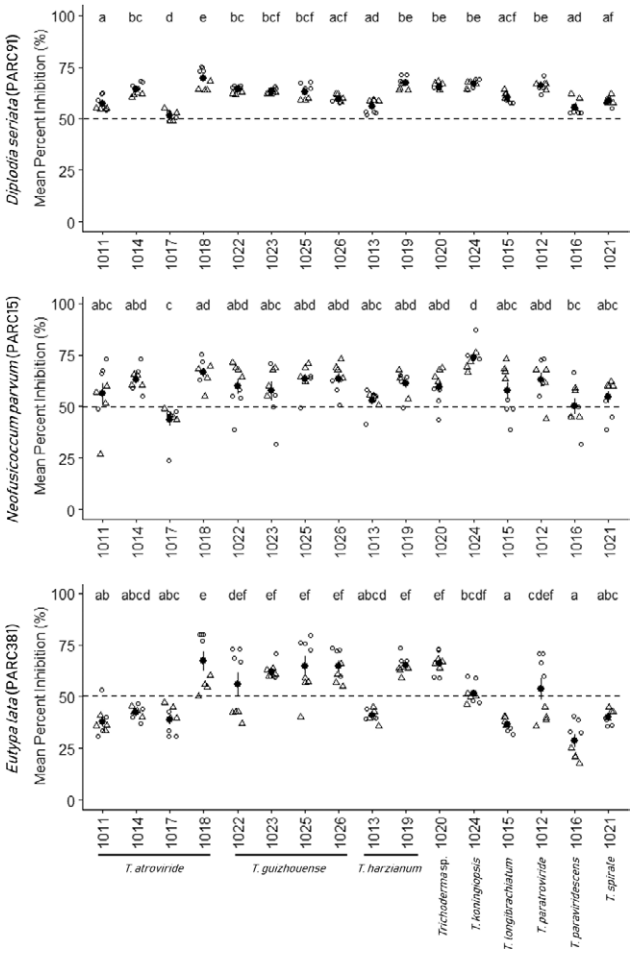

Figure 6. Mean percent inhibition of radial growths (PIRG) for *Trichoderma* isolates from southern Italy against GTD pathogens *Diplodia seriata* PARC91, *Neofusicoccum parvum* PARC15 and *Eutypa lata* PARC 381, measured after 5 d. White dots and triangles represent, respectively, data from experiment 1 and repeated experiment 2. Solid black dots represent the mean percent inhibition for each isolate calculated from eight replicates, and bars represent standard errors of the means. Dash line represents 50% inhibition threshold. Columns accompanied by the same letter were not statistically different ($P = 0.05$), Tukey-Kramer Honest Significant Difference post-hoc tests.

(Table 2). *Trichoderma atroviride* (PARC1018) and *T. paratroviride* (PARC1012) gave the greatest MPDC (88-100%) against *N. parvum* from day one to day 21 after treatment. The remaining isolates provided much lower MPDCs (23 to 70%) against *N. parvum*, except for *T. guizhouense* (PARC1026) that gave MPDCs of 69% at 1 d after treatment, 75% at 7 d, and 83% at 21 d after treatment. All the isolates tested in the DCA experiment gave MPDCs greater than 50% when pruning wounds were challenged with 5,000 *N. parvum* spores per wound 7 d after treatment (Table 2).

DISCUSSION

This study has identified nine different *Trichoderma* spp. from southern Italy and characterized their *in vitro* potential as biocontrol agents against the GTD fungal pathogens *D. seriata*, *N. parvum* and *E. lata*. *Trichoderma* isolates in this study were identified to species level based on molecular and phylogenetic analyses of the ITS, *EF1-α* and *RPB2* genes, and included *T. atroviride*, *T. guizhouense*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and an unidentified *Trichodrma* sp.

The genus *Trichoderma* was first described by Christiaan Hendrik Persoon in 1794 and later linked to the sexual state *Hypocrea*, which was described by Elias Fries in 1825. The type species of the *Hypocrea* is *Hypocrea rufa* (Pers. : Fr.) Fr. (anamorph *T. viride*) (Jaklitsch *et al.*, 2006). Species of *Trichoderma* and their *Hypocrea* teleomorphs can colonize different substrates; however, they are primarily isolated from soil. *Trichoderma* spp. have cosmopolitan distribution and have been isolated from many different ecosystems, including from the most extreme northern and southern latitudes (Chaverri and Samuels, 2003). Many studies made taxonomic reassessments of *Hypocrea* and *Trichoderma* anamorphs, and the current status and species delimitation of these fungi has been outlined by Braithwaite *et al.* (2017), Chaverri and Samuels (2003), Chaverri *et al.* (2015), Jaklitsch *et al.* (2006) and Jaklitsch and Voglmayr (2015). Most of the *Trichoderma* spp. identified in the present study have been previously described to commonly occur in many different regions, including Italy (Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015; Lorenzini *et al.*, 2016; Innocenti *et al.*, 2019). However, the present study describes for the first time the presence of *T. koningiopsis*, *T. paratroviride*, and *T. spirale* in Italy.

Trichoderma koningiopsis was recently described as a new species within the *T. koningii* species complex, based on multi-locus sequence analyses, and this species has been reported from Brazil, Canada, Cuba, Ecuador, France, Germany, New Zealand, Peru, Puerto Rico, and the United States of America (Samuels *et al.*, 2006; Braithwaite *et al.*, 2017). *Trichoderma koningii* has long been known to occur in Italy and was described from citrus in Sicily in the early 1990s (Greuter *et al.*, 1991). It is likely that *T. koningiopsis* could also have been long present in Italy as part of the *T. koningii* species complex, but only now has this species been confirmed using DNA analyses.

Trichoderma paratroviride is a recently described new species, and it has been only reported to occur in China (Zhang *et al.*, 2015), Hungary (Chen *et al.*, 2019),

Table 2. Detached cane assay results under controlled conditions. Values represent the mean percent recovery (MPR) of *Diplodia seriata* (PARC91) and *Neofusicoccum parvum* (PARC15) from pruning wounds treated with selected *Trichoderma* isolates from southern Italy and inoculated 1, 7 or 21 d after treatment. Values in each row followed by same letters are not statistically different (P = 0.05), Tukey-Kramer multiple comparison test.

Pathogen / Inoculation day[a]		I-Controlc	*T. paratroviride*	*T. atroviride*	*Trichoderma* sp.	*T. koningiopsis*	*T. guizhouense*	
			PARC1012	PARC1018	PARC1020	PARC1024	PARC1025	PARC1026
Diplodia seriata								
1 d	MPR	60a	0b	3b	0b	7b	3b	7b
	MPDCb		100	94	100	89	94	89
7 d	MPR	63a	0b	3b	0b	10b	0b	0b
	MPDC		100	95	100	84	100	100
21 d	MPR	60a	0b	0b	0b	3b	0b	0b
	MPDC		100	100	100	94	100	100
Neofusicoccum parvum								
1 d	MPR	87a	7b	10b	47c	37cd	67c	27bd
	MPDC		92	88	46	58	23	69
7 d	MPR	80a	3b	0b	40c	33cd	40c	20bd
	MPDC		96	100	50	58	50	75
21 d	MPR	80a	0b	0b	27c	23cd	33c	13bd
	MPDC		100	100	67	70	58	83

[a] Number of days after the *Trichoderma* treatment when pruning wounds were inoculated with *D. seriata* or *N. parvum*.
[b] MPDC: mean percent disease control was calculated as 100 x [1-(MPR treatment/MPR control)].
[c] Inoculated positive control.

and Spain (Jaklitsch and Voglmayr, 2015). The present study expands the geographical distribution of *T. paratroviride* into Italy. Whether the geographical distribution of this species is restricted to Europe, or more precisely, to southern European countries, cannot yet be confirmed. However, based on the cosmopolitan nature of *Trichoderma* spp., it is likely that future studies and surveys could expand the geographical distribution of *T. paratroviride* to more countries in Europe and/or other continents.

Trichoderma spirale was first described by Bissett (1991) from samples collected in Canada, Thailand, and the United States of America. This species has since been reported from several countries in Africa and Central America, from Iran and Turkey in the middle east and from New Zealand in Oceania (Kindermann *et al.*, 1998; Jaklitsch and Voglmayr, 2015; Braithwaite *et al.*, 2017). To date, *T. spirale* has only been found once in Europe, in the Canary Islands of Spain (Jaklitsch and Voglmayr, 2015). The present study also expands the distribution of *T. spirale* into another European country.

Hypocrea teleomorphs possess highly conserved structures, so they are of limited use for species identification (Chaverri and Samuels, 2003; Samuels *et al.*, 1998). Consequently, phenotypical and molecular characters of *Trichoderma* anamorphs have traditionally been used for species delineation (Chaverri and Samuels 2003). However, with over 250 *Trichoderma* spp. currently accepted (Bissett *et al.*, 2015) and many of them having overlapping morphological features, species differentiation based only on anamorph characters it is not definitive. Implementation of DNA sequencing and multi-locus sequence analyses has facilitated resolution of taxonomic uncertainty and species delineation in *Trichoderma* (Jaklitsch *et al.*, 2006; Chaverri and Samuels, 2003; Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015; Samuels *et al.*, 2006).

Different genetic markers, including a-actin (*ACT*), calmodulin (*CAL*), *EF-1α*, ITS, the large subunit of ATP citrate lyase (*acl1*), and *RPB2* have been commonly used in phylogenetic analyses of the genus (Chaverri *et al.*, 2015; Jaklitsch and Voglmayr, 2015). We used ITS, *EF-1α* and *RPB2* sequences in individual phylogenetic analyses to determine the phylogenetic relationships among *Trichoderma* isolates from southern Italy. All three markers supported species delineation, with *EF-1α* and *RPB2* giving the strongest bootstrap supports, as reported elsewhere (Chaverri and Samuels, 2003; Jaklitsch and Voglmayr, 2015). However, *Trichoderma* isolate PARC1020 could not be assigned to a specific species in the current study. ITS analyses grouped this isolate in the *T. harzianum* clade containing the Ex-type speci-

men and thus, it could have been named *T. harzianum*. However, the *EF-1α* analyses grouped PARC1020 in the *T. guizhouense* clade, while the *RPB2* analyses grouped it in a single clade closely related to *T. simonsii*. These species all belong to the *T. harzianum* species complex, in which a total of 14 cryptic species have been identified (Chaverri *et al*., 2015). We are confident, based on current information, that PARC1020 is not *T. harzianum* but belongs to the *T. harzianum* species complex. Further multi-locus sequence analyses, including other loci such as *ACT* and *CAL*, are required to identify this isolate to species, so we have named PARC1020 as *Trichoderma* sp. until further studies provide more precise nomenclature.

Trichoderma spp. are well-known for their antifungal properties, and they have long been used as BCAs against a wide range of pathogens in many different crops (Schuster and Schmoll, 2010). Species in *Trichoderma* are now the most studied organisms for use in products employed as BCAs, bio-pesticides, and/or bio-fertilizers. Over 60% of registered fungal BCA products are based on *Trichoderma* spp. (Verma *et al*., 2007). *Trichoderma* spp. are also being studied as potential BCAs against GTD fungi. Mondello *et al*. (2018) reviewed trials conducted and compounds used to control GTDs in the last 15 years, including BCAs and *Trichoderma* spp. To date, *T. asperellum*, *T. atroviride*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, and *T. polysporum* have been tested in different countries against GTD pathogens causing Botryosphaeria dieback, Esca and Eutypa dieback (Mondello *et al*., 2018). These *Trichoderma* spp. were shown to be highly efficient against GTD fungi in the different studies. However, most of these data were from experiments conducted *in vitro* and only very few studies completed trials *in planta* and/or under natural field conditions (Mondello *et al*., 2018). The present study adds six new taxa to the list of effective *Trichoderma* spp. against GTD fungi, including *T. guizhouense*, *T. koningiopsis*, *T. paratroviride*, *T. paraviridescens*, *T. spirale*, and a yet unidentified *Trichoderma* sp. In addition, we report results from *in vitro* and *in planta* experiments.

The *in vitro* dual culture antagonism assays conducted showed at least one isolate from each *Trichoderma* spp. from southern Italy to be highly effective on reducing mycelium growth (60 to 75%) of *D. seriata* and *N. parvum*, two of the most common grapevine canker-causing fungi (Úrbez-Torres, 2011). These results agree with most *in vitro* experiments conducted against these two botryosphaeriaceous fungi (Mondello *et al*., 2018). However, only isolates of *T. guizhouense* and *Trichoderma* sp. and one isolate each of *T. atroviride* and *T. harzianum* gave similar reductions of mycelium growth of reduction against *E. lata* in the present study. This indicates less efficacy of *Trichoderma* spp. against this second canker-causing pathogen. *Trichoderma atroviride* and *T. harzianum* have routinely been shown to be effective against *E. lata*, although mostly used in trials as commercial products and not as pure isolates (Halleen *et al*., 2010; Kotze *et al*., 2011). These studies along with the present results indicate that *T. atroviride* and *T. harzianum* are the most promising species to be used as BCAs against *E. lata*. However, we also showed that isolates of *T. guizhouense* and one isolate of *Trichoderma* sp. were very effective against *E. lata*, but further research is required to expand the number of *Trichoderma* spp. that could be used as BCAs against *E. lata*.

No field trials to assess the efficacy of the different *Trichoderma* spp. under natural conditions were conducted in this study, due to the foreign origins of all the *Trichoderma* isolates. However, the selected isolates were screened against *D. seriata* and *N. parvum in planta* under controlled conditions in a growth chamber using a modified DCA similar to that described by Ayres *et al.* (2014). Due to the lack of a native source of *E. lata* ascospores at the time of conducting this study, we did not include this pathogen in the DCA. The DCA showed all species tested, including *T. atroviride*, *T. guizhouense*, *T. koningiopsis*, *T. paratroviride*, and *Trichoderma* sp., effectively protected pruning wounds against *D. seriata* for at least 21 d after treatment. In contrast, only *T. atroviride*, *T. paratroviride* and *T. guizhouense* isolate PARC1026 showed similar control against *N. parvum*. The greater control of *D. seriata* may correspond to the well-known lower virulence compare to *N. parvum* isolates (Úrbez-Torres, 2011). However, inconsistent efficacy has been shown for *Trichoderma* against GTD pathogens, including botryosphaeriaceous fungi (Halleen *et al*., 2010; Kotze *et al*., 2011; Mondello *et al*., 2018).

The antifungal activity of *Trichoderma* has been extensively documented, and is based on different factors, including antibiosis, mycoparasitism, and nutrient and/or space competition (Schuster and Schmoll, 2010). Though still not well-understood, it is hypothesized that nutrient and/or space competition in grapevine pruning wounds could be an important antifungal mode of action of *Trichoderma* spp. against GTD fungi. Therefore, effective disease control as pruning wound protectants requires time for *Trichoderma* to become established, so most commercial products report greatest antifungal activity several days after application. High levels of control activity were shown for *Trichoderma* spp. in the present study only 1 d after treatment. MPDCs greater than 90% 1 d after treatment were

recorded from four of six *Trichoderma* isolates against *D. seriata*, and from isolate PARC1012 against *N. parvum*. This could have resulted from conducting the DCA experiment under controlled conditions with optimum temperature and humidity for *Trichoderma* conidium germination and wound colonization, conditions that would not occur in field situations. However, grapevine pruning is conducted during the dormant plant period (late fall to late winter), in which temperatures are usually below optima for *Trichoderma* conidium germination and mycelium growth. Therefore, it is important to test the survival and effectiveness of these *Trichoderma* spp. under natural conditions during the time when pruning wound protection is normally carried out. Nevertheless, the results obtained in this study are promising and also showed the possibility of *Trichoderma* spp. to immediately protect pruning wounds if applied under optimum growing conditions.

The species identified in the present study showed optimum mycelium growth between 25 and 35°C. The differences in growth at different temperatures can be important in the selection of BCAs for aspects related to the "industrial production" of biomass, and for fitness in different environmental conditions in different habitats and for multiple pathogens with different biological requirements (Kredics *et al*., 2003). *Trichoderma* spp. from the present study are likely to be well-adapted for use in grape-growing regions with mild winters (main pruning season), as in southern Italy. Further studies are needed to determine the optimum application time in the field to give greatest pruning wound protection from GTD pathogens. In order to secure sufficient infection in the positive experimental controls, we used high inoculum pressure (5,000 spores per wound) of *D. seriata* and *N. parvum* to challenge the *Trichoderma* treatments tested. It is very unlikely that pruning wounds are exposed to such high levels of inoculum under natural field conditions. It is therefore possible that we have underestimated the efficacy of *Trichoderma* spp. against *N. parvum* or other GTD pathogens (Mondello *et al*., 2018). Field evaluations under natural conditions using lower inoculum pressures in southern Italy (origin of the isolates) should be carried out to better determine the capacity the *Trichoderma* spp. as BCAs against GTDs.

Trichoderma spp. have also been widely used to minimize the impacts of GTDs during grapevine nursery propagation processes, with some promising results primarily against fungi in the Esca complex (Gramaje *et al*., 2018; Mondello *et al*., 2018). Similar to studies conducted on the use of *Trichoderma* spp. for pruning wound protection, the number of *Trichoderma* spp. tested in propagation processes is limited to *T. atroviride*, *T. harzianum* and *T. longibrachiatum* (Gramaje *et al*., 2018; Mondello *et al*., 2018). The present study provides efficacy data for several different *Trichoderma* spp. against GTD fungi in the *Botryosphaeriaceae* and the *Diatrypaceae*. Further screening of *Trichoderma* spp. identified in this study should be conducted to assess their efficacy against other GTD fungi, including species of *Ilyonectria* and *Phaeoacremonium*, and *Cadophora luteo-olivacea* and *Phaeomoniella chlamydospora*, all of which are commonly found in the nursery propagated material, and are responsible for decline and death of young grapevines. These studies could provide more alternatives to the current BCAs available during nursery propagation processes.

Chemical pesticides have become increasingly relied upon for control of grapevine pathogens and those causing other crop diseases. The deleterious effects of many of these chemicals are due to overuse, toxic residues, applicator hazards, development of pathogen resistance, and adverse environmental impacts (Nicolopoulou-Stamati *et al*., 2016). Therefore, integrated pest management (IPM) approaches, which include the use of cultural practices, BCAs and responsible use of chemical pesticides, have been suggested as strategies for management of many crop diseases, including GTDs (Bertsch *et al*., 2013; Gramaje *et al*., 2018). The present study has demonstrated the potential of several different *Trichoderma* spp. as alternatives to chemicals to reduce the important economic impacts that GTD have for grape production. However, further research is required to determine: i) the antifungal modes of action these *Trichoderma* spp. have against GTD pathogens, ii) the adaptability of the potential BCAs under natural conditions, and iii) their potential to be used as effective commercial products.

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