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Grapevine dieback caused by *Botryosphaeriaceae* species, *Paraconiothyrium brasiliense, Seimatosporium vitis-viniferae* and *Truncatella angustata* in Piedmont: characterization and pathogenicity

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Summary. Grapevine Trunk Diseases (GTDs) are major threats in Mediterranean countries, causing economic losses due to reduced grape yields and long-term vine productivity, as well as death of grapevines. A survey was conducted in Piedmont (Northern Italy) during 2021-2022 to investigate the species diversity and distribution of GTD pathogens in this important Italian wine region. Morphological and multilocus phylogenetic analyses (based on ITS, *tef1*, *tub2*, *act* and *rpb2*) identified species of *Botryosphaeriaceae* at high frequency, including *Botryosphaeria dothidea*, *Diplodia mutila, Diplodia seriata* and *Neofusicoccum parvum*. Other pathogens commonly associated with GTDs, including *Eutypa lata*, *Fomitiporia mediterranea* and *Phaeomoniella chlamydospora*, were also isolated. Less commonly isolated species included *Neocucurbitaria juglandicola, Paraconiothyrium brasiliense*, *Seimatosporium vitis-viniferae* and *Truncatella angustata*. Pathogenicity tests with two representative isolates of each species were carried out using one-year-old potted grapevine cuttings ('Barbera'). All isolates (except *N. juglandicola*) caused brown wood necrotic vascular discolourations on inoculated plants and were successfully re-isolated. Effects of temperature on colony growth were also assessed. For all tested isolates there was no growth at 5°C, only four isolates (*Botryosphaeriaceae*) grew at 35°C, and optimum growth temperatures were between 20 and 25°C. This is the first record of *Paraconiothyrium brasiliense* and *Neocucurbitaria juglandicola* associated with symptomatic grapevines in Italy.

Keywords. Botryosphaeria dieback, *Neofusicoccum, Neocucurbitaria juglandicola,* pathogenicity, Grapevine Trunk Diseases.

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important cultivated crop, with a worldwide vineyard area of 6.73 million ha (FAOSTAT, 2021), mainly grown for wine and table grape production. Reports have increased of diseases caused by grapevine trunk diseases (GTDs) associated fungi causing severe economic and yield losses as a result of reduced grape quality and early plant death. Grapevine trunk diseases are severely destructive in Europe and Mediterranean countries (Guerin-Dubrana *et al*., 2019), representing major threats to vineyard productivity.

Several factors, including climate change and rapid expansion and industrialization of viticulture, are related to increased incidence and severity of GTDs (Graniti *et al*., 2000; Surico *et al*., 2004). Up to 133 pathogens belonging to nine families have been associated with GTDs (Luque *et al.,* 2009; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Mondello *et al*., 2018). These xylem-colonizing fungi are predominantly found in the grapevine framework (spurs, cordons and trunk), but also in root (Gramaje *et al*., 2018).

Symptoms of GTDs vary among the different diseases, and include sectorial wood necroses (cankers), black-brown streaking (discolourations or blackening of xylem vessels), central brown-red discolouration, and white rot, while external symptoms include leaf necrosis and chlorosis with typical tiger-stripes, reduced vine vigour and stunted growth, dieback of shoots and leaf drop (apoplexy). These symptoms generally lead to death of affected vines, caused by the Esca complex of diseases, decline and dieback or stunted shoots growth, including witch's broom symptoms, as in Eutypa dieback (Mugnai *et al*., 1999; Luque *et al.,* 2009; Úrbez-Torres, 2011; Bertsch *et al*., 2013; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Mondello *et al*., 2018; Billones-Baaijens and Savocchia, 2019; Guerin-Dubrana *et al*., 2019; Reis *et al*., 2019; Claverie *et al*., 2020).

Grapevine trunk diseases are associated with different vascular xylem-colonizing pathogenic fungi. *Phaeomoniella chlamydospora* and several *Phaeoacremonium* spp. are responsible for Petri disease and Esca complex, the major GTDs reported in all European and Mediterranean countries. *Fomitiporia mediterranea*, in the same areas, is the most common lignin-degrading Basidiomycete fungus responsible for white rot (Surico *et al*., 2004). Different dieback diseases such as Botryosphaeria dieback, Diaporthe dieback and Eutypa dieback are associated with different species of related fungal families (Claverie *et al*., 2020).

Fungi in the Botryosphaeriaceae have cosmopolitan distribution, and have been associated with many host plants (Úrbez-Torres, 2011; Carlucci *et al*., 2015; Guarnaccia *et al*., 2022). Several species are reported to be plant pathogens while others are endophytes or saprophytes of annual or perennial plants. Over the last decade, interest in species in Botryosphaeriaceae has increased, due to importance of Botryosphaeria dieback of grapevines, where at least 26 species have been associated with this disease (Úrbez-Torres, 2011; Carlucci *et al*., 2015; Gramaje *et al*., 2018; Arkam *et al*., 2021). Botryosphaeria dieback, has been associated with different fungal genera, including *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinsia* (Yang *et al*., 2017; Gramaje *et al*., 2018). Typical symptoms occur on grapevine trunk and shoot wood, and include wedge-shaped cankers, dark V-shaped wood necroses, and elongated black lesions in vessels. Vascular streaking, foliar discolouration, bud necrosis and severe decline can cause death of plants (Úrbez-Torres, 2011; Billones-Baaijens and Savocchia, 2019; Úrbez-Torres *et al*., 2020). Botryosphaeria dieback symptoms can discontinue but are commonly observed on mature vineyards (> 8 years), but have also been reported on 3- to 5-years-old vines (Úrbez-Torres *et al*., 2008). All GTDs pathogens can cause asymptomatic infections as latent pathogens (González and Tello, 2011; Bruez *et al*., 2014).

Among Fungi commonly associated with GTDs, several recent studies have highlighted the association of other wood-degrading fungi with symptomatic plants (Raimondo *et al*., 2019; Bekris *et al*., 2021). Numerous fungi belonging to *Neopestalotipsis*, *Truncatella*, *Seimatosporium* and *Sporocadus* have recently been reported as part of the grapevine microbiome, while their roles as causes of symptoms need to be clarified (Maharachchikumbura *et al*., 2017; Geiger *et al*., 2022; Vanga *et al*., 2022).

With more than 718,000 ha of wine and table grapevines (O.I.V, data 2022, https://www.oiv.int/it/what-we-do/country-report?oiv), Italy is leading grapevine production worldwide and represent the fourth largest vineyard acreage after Spain, France and China. Among all Italian regions, Piedmont (Northern Italy, with the Langhe area included in UNESCO's World Heritage list), is a renowned wine production region. In the last 30 years, incidence of GTDs has increased in all Italian regions, and several fungi have been reported associated with grapevines showing various symptoms (Surico *et al*., 2000; Guerin-Dubrana *et al*., 2019). Since the 1990s, studies have reported high disease incidence and mortality of plants in the first year of planting. The Esca complex, including apoplexy, is frequent and widespread in all grape growing Italian regions (Guerin-Dubrana *et al*., 2019), and reaches high incidence in climatical-

ly favourable seasons, up to 80% in mature vineyards (Romanazzi *et al*., 2009).

Botryosphaeria dieback associated with different pathogens, including *Diplodia seriata*, *Neofusicoccum parvum* and *Lasiodiplodia theobromae,* was reported in Apulia, Marche, Molise, Tuscany, Sardinia and Sicily (Burruano *et al*., 2008; Romanazzi *et al*., 2009; Spagnolo *et al*., 2014; Carlucci *et al*., 2015; Linaldeddu *et al*., 2015; Mondello *et al*., 2020), but no investigations have been carried out in Piedmont. Due to limited information on distribution of GTD related pathogens in Piedmont, the present research aims were: 1) to investigate the species diversity and distribution of GTD pathogens in Piedmont, focusing on canker agents and wood-degrading fungi associated with dead cordons, independently from detected foliar symptoms; 2) to characterize obtained isolates; and 3) to test representative isolates for pathogenicity to healthy grapevine plants.

MATERIALS AND METHODS

Field sampling and isolation of fungi

Surveys were carried out from July 2021 to November 2022, in five vineyards in the Alba and Alessandria areas of Piedmont, Northern Italy. Wood samples were collected from necrotic cordons, and from trunk portions of declining vines of 12 different grapevine cultivars (Table 1). Sampled vines were aged between 10 and 25 years, and showed dieback symptoms, including foliar discolourations, dieback and internal dark V-shaped wood necroses. The sampling method was destructive; vines were cut and transverse sections of the affected trunk and branches of each plant were examined to check for wood necroses. Each sample was reduced into small fragments, including the necrotic zone, and was sterilized in a sodium hypochlorite solution (1%) for 1 min and then rinsed in sterilized distilled water for 30 sec. Excess water was removed using sterilized filter paper. The wood samples were cut into small pieces from the margins of the necrotic zones. Five wood fragments from each sample were plated onto the surface of Potato Dextrose Agar (PDA, Merck) in a Petri plate, supplemented with streptomycin sulphate (25 ppm L^{-1}, PDA-S), and incubated at 25 ± 1°C. After 5 d the plates were examined. From the margins of resulting fungal colonies, single hyphal tips were cut and placed on new PDA plates, to obtain pure cultures.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

For recurring fungal colonies, mycelium from each 10-d-old pure culture on PDA was scraped and collected into a 2 mL capacity centrifuge tube. Total DNA was extracted directly from fresh mycelium using the

Table 1. Information on the vineyards surveyed and sampled in Piedmont, with respective fungi from different grapevine cultivars.

Vineyard		No. plants sampled	Isolated fungi				
Grapevine cultivar	Location		*Botryosphaeriaceae* spp.	*N. juglandicola*	*P. brasiliense*	*S. vitis*	*T. angustata*
Alba rossa	Carpeneto (AL)	2	+	-	-	+	-
Barbera	Dogliani (CN)	2	+	-	+	+	+
	Castiglione Falletto (CN)	3	+	-	-	-	-
	Fubine (AL)	3	+	-	-	+	-
Cabernet	Fubine (AL)	2	+	-	-	-	-
Cortese	Carpeneto (AL)	2	+	-	-	-	-
Erbaluce	Carpeneto (AL)	2	-	-	-	-	-
Grignolino	Carpeneto (AL)	1	+	-	-	-	-
Merlot	Fubine (AL)	2	+	-	-	-	-
Moscato	Carpeneto (AL)	2	+	-	-	-	-
Nebbiolo	Dogliani (CN)	3	+	-	-	-	+
	Monforte d'Alba (CN)	2	+	+	+	-	-
Rossese bianco	Monforte d'Alba (CN)	1	+	-	-	-	-
	Fubine (AL)	1	+	-	-	-	-
Sauvignon blanc	Carpeneto (AL)	2	-	-	-	-	-
Timorasso	Carpeneto (AL)	2	-	-	-	-	-

E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. DNA amplification and sequencing of different loci were carried out to achieve species identification. *Botryosphaeriaceae*-like isolates were characterized through DNA amplification and sequencing of the partial translation elongation factor-1α (*tef1*) gene, using the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). For the remaining isolates, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified using universal primers ITS1 and ITS4 (White *et al*., 1990), while the primers T1 and Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), fRPB2-5f and fRPB2-7cr (Liu *et al*., 1999), and ACT512f and ACT783r (Carbone and Kohn, 1999), were used to amplify, respectively, genes for partial beta-tubulin (*tub2)*, the fragment of the RNA polymerase II subunit 2 (*rpb2*), and γ-actin (*act*). The PCR reactions and conditions adopted for all the loci were described in the above respective cited studies. Polymerase chain reaction assays were carried out in a final 25 µL volume, using a Taq DNA polymerase kit (Qiagen) and 25 ng of DNA. Five microliters of each PCR reaction product were analyzed by electrophoresis at 100V in a 1% agarose (VWR Life Science AMRESCO® biochemicals) gel stained with GelRed™ in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA; pH 8.0). Amplified PCR fragments were sequenced by Eurofins Genomics Service (Cologne). Obtained sequences were trimmed in Geneious v. 11.1.5 (Auckland, New Zealand), and the blast function of NCBI's GenBank nucleotide database was used to determine the closest relatives of the studied isolates.

Phylogenetic analyses

To give an overview of isolated genera, an initial phylogenetic analysis was conducted with sequences of the partial translation elongation factor-1α (*tef1*) gene for *Botryosphaeriaceae*-like isolates, and with the nuclear ribosomal internal transcribed spacer (ITS) gene for other isolates. Subsequently, a subset of representative isolates was then selected based on the previous results, to distinguish the isolates at species level. A multilocus phylogenetic analysis was conducted using the following locus combinations: ITS and *tef1* for members of *Botryosphaeriaceae* (Pintos *et al*., 2018; Guarnaccia *et al*., 2020); ITS, *tub2* and *act* were amplified for *Paraconiothyrium* (Verkley *et al*., 2004); ITS, *tub2* and *rpb2* for *Neocucurbitaria* (Jaklitsch *et al*., 2018); and ITS, *tub2* and *tef1* for isolates related to the family *Sporocadaceae* including the genera *Truncatella* and *Seimatosporium* (Raimondo *et al*., 2019). Isolate sequences, including references downloaded from GenBank, were aligned with the software MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley, 2013), and were then manually adjusted in MEGA v.7 (Kumar *et al*., 2016). Multi-locus analyses, based on Maximum Parsimony (MP) were performed using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Cummings, 2004), while MrModeltest v. 2.3 (Nylander, 2004) and MrBayes v. 3.2.5 (Ronquist and Huelsenbeck, 2003) were used for the Bayesian Inference (BI) analyses. The best nucleotide substitution model for each gene was estimated using MrModeltest. Based on obtained results for optimal setting criteria for each locus, BIs were performed using the Markov Chain Monte Carlo (MCMC) method. Four simultaneous Markov Chains were run for 1,000,000 generations starting from a random tree topology. Trees were saved each 1000 generation, while preburn and heating parameters were set, respectively, to 0.25 and 0.2. Based on burn-in fraction, the remaining trees were used to calculate the majority rule consensus tree and posterior probability (PP). The analyses stopped once the average standard deviation of split frequencies fell below 0.01. For Maximum Parsimony, phylogenetic relationships were estimated using the heuristic search option with 100 random addition sequences. Tree bisection reconnection (TBR) was used with branch swapping option as "best trees"; characters were treated as equally weighted, and gaps as fifth base. Parsimony and the bootstrap analyses were based on 1,000 replications, and tree lengths (TL), consistency indices (CI), retention indices (RI) and rescaled consistence indices (RC) were calculated. Resulting trees were visualized with FigTree version 1.4.4 (Page, 1996). Sequences generated in this study were deposited in GenBank (Table 2).

Morphological analyses

Two isolates of each species identified using molecular analyses were selected for the morphological observation. Mycelium plugs (each 6 mm diam.) were taken from each 10-d-old fungal colony growing on PDA and were transferred to Petri dishes containing different media. To enhance sexual sporulation or conidium production, for *Botryosphaeriaceae*-like isolates, 2% water agar supplemented with sterile pine needles (Pine Needle Agar or PNA, Smith *et al*., 1996) was used, with incubation at 25°C under near-UV light (Crous *et al*., 2006). For *Neocucurbitaria*, malt extract agar (MEA) and PDA were used, with incubation at 20°C and alternating light-dark periods (Jaklitsch *et al*., 2018). Corn meal agar (CMA), oat agar (OA) and MEA were used for *Paraconiothyrium*, incubated at 25°C under UV light. PDA, MEA

Table 2. List of isolates used for phylogenetic analyses, and their GenBank accession numbers.

Species	Isolate code	Country	Host	GenBank accession number [a]				
				ITS	tef1	tub2	act	RPB2
Alloconiothyrium aptrootii	CBS 980.95	Papua Nuova Guinea	Soil	JX496121	-	JX496460	JX496347	-
Allocucurbitaria botulispora	CBS 142452	USA	Superficial tissue	LT592932	-	LT593001	-	LT593070
Bartalinia robillardoides	CBS 122705	Italy	*Leptoglossus occidentalis*	LT853104	LT853202	LT853252	-	-
Botryosphaeria corticis	CBS 119047	USA: New Jersey	*Vaccinium corymbosum*	DQ299245	EU017539	EU673107	-	-
Botryosphaeria dothidea	CBS 110302	Portugal	*Vitis vinifera*	AY259092	AY573218	EU673106	-	-
Botryosphaeria dothidea	CBS 115476	Switzerland	*Prunus* sp.	AY236949	AY236898	AY236927	-	-
Botryosphaeria dothidea	CBS 145971	Australia	*Grevillea* sp.	MT587332	MT592034	MT592470	-	-
Botryosphaeria dothidea	CVG 1582*	Italy	*Vitis vinifera*	*OQ612689*	*OQ680488*	*OQ680508*	-	-
Botryosphaeria dothidea	CVG 1615	Italy	*Vitis vinifera*	*OQ612691*	*OQ680490*	*OQ680509*	-	-
Botryosphaeria fabicerciana	CGMCC 3.20320	Unknown	Unknown	MW642163	MW651965	MW651966	-	-
Botryosphaeria fusispora	MFLUCC 10-0098	Thailand	*Entada* sp.	JX646789	JX646854	JX646839	-	-
Botryosphaeria kuwatsukai	KUMCC 20-0106	Unknown	Unknown	OP714405	-	-	-	-
Botryosphaeria pseudoramosa	CERC 2001	China	*Eucalyptus* hybrid	KX277989	KX278094	KX278198	-	-
Botryosphaeria ramosa	CBS 122069	Australia	*Eucalyptus camaldulensis*	EU144055	EU144070	KF766132	-	-
Botryosphaeria wangensis	CERC 2298	China	*Cunninghamina deodara*	KX278002	KX278107	KX278211	-	-
Cucurbitaria berberidis	CBS 142401	Austria	*Berberis vulgaris*	MF795756	-	MF795886	-	MF795798
Diplodia africana	CBS 120835	South Africa	*Prunus persica*	MH863094	KF766397	KF766129	-	-
Diplodia agrifolia	CBS 132778	California, U.S.A.	*Quercus agrifolia*	MH866051	MT592036	MT592472	-	-
Diplodia corticola	CBS 112548	Portugal	*Quercus suber*	AY259099	KX464559	KX464789	-	-
Diplodia fraxini	CBS 136012	Portugal	*Fraxinus angustifolia*	MT587345	MT592050	MT592492	-	-
Diplodia intermedia	CBS 124462	Portugal	*Malus sylvestris*	MH863374	GQ923826	MT592503	-	-
Diplodia mutila	CBS 136014	Portugal	*Populus alba*	KJ361837	KJ361829	MZ073932	-	-
Diplodia mutila	CBS 112553	Portugal	*Vitis vinifera*	MW810279	MZ073947	MZ073931	-	-
Diplodia mutila	CVG 1739	Italy	*Vitis vinifera*	*OQ612693*	*OQ680492*	*OQ680512*	-	-
Diplodia mutila	CVG 1741*	Italy	*Vitis vinifera*	*OQ612695*	*OQ680494*	*OQ680513*	-	-
Diplodia pseudoseriata	CBS 124906	Uruguay	*Blepharocalyx salicifolius*	EU080927	EU863181	-	-	-
Diplodia pyri	CBS 121862	Netherlands	*Pyrus* sp.	KX464093	KX464567	KX464799	-	-
Diplodia rosulata	CBS 116470	Ethiopia	*Prunus africana*	EU430265	EU430267	EU673132	-	-
Diplodia sapinea	CBS 114864	Unknown	Unknown	KX464096	KX464570	KX464803	-	-
Diplodia scrobiculata	CBS 118110	Wisconsin, U.S.A.	*Pinus banksiana*	KF766160	KF766399	-	-	-
Diplodia seriata	CBS 112555	Italy	*Vitis vinifera*	AY259094	AY573220	DQ458856	-	-
Diplodia seriata	CBS 119049	Italy	*Vitis vinifera*	DQ458889	DQ458874	DQ458857	-	-
Diplodia seriata	CVG 1577*	Italy	*Vitis vinifera*	*OQ612688*	*OQ680487*	*OQ680515*	-	-
Diplodia seriata	CVG 1753	Italy	*Vitis vinifera*	*OQ612694*	*OQ680493*	*OQ680514*	-	-
Diplodia subglobosa	CBS 124133	Spain	*Lonicera nigra*	GQ923856	GQ923824	MT592576	-	-

(Continued)

Table 2. (Continued).

Species	Isolate code	Country	Host	GenBank accession number [a]				
				ITS	tef1	tub2	act	RPB2
Discosia artocreas	CBS 124848	Germany	*Fagus sylvatica*	MH553994	MH554420	MH554662	-	-
Heterotruncatella proteicola	CBS 144020	South Africa	*Protea acaulos*	MH554077	MH554512	MH554751	-	-
Heterotruncatella spartii	CBS 143894	USA	*Pinus edulis*	MH554134	MH554569	MH554807	-	-
Lecanosticta acicola	LNPV252	France	*Pinus attenuata* x *Pinus radiata*	JX901755	JX901639	JX902213	-	-
Massarina eburnea	CBS 473.64	Switzerland	*Fagus sylvatica*	AF383959	-	-	-	GU371732
Neocucurbitaria acanthocladae	CBS 142398	Greece	*Genista acanthoclada*	MF795766	-	MF795894	-	MF795808
Neocucurbitaria acerina	CBS 142403	Austria	*Acer pseudoplatanus*	MF795768	-	MF795896	-	MF795810
Neocucurbitaria cava	CBS 257.68	Germany	Wheat-field soil	JF740260	-	KT389844	-	LT717681
Neocucurbitaria hakeae	CBS 142109	Australia	*Hakea* sp.	KY173436	-	KY173613	-	KY173593
Neocucurbitaria irregularis	CBS 142791	USA	Subcutaneous tissue	LT592916	-	LT592985	-	LT593054
Neocucurbitaria juglandicola	CBS 142390	Austria	*Juglans regia*	MF795773	-	MF795901	-	MF795815
Neocucurbitaria juglandicola	CVG 1779	Italy	*Vitis vinifera*	*OQ612696*	-	*OQ680503*	-	*OQ884601*
Neocucurbitaria quercina	CBS 115095	Italy	*Quercus robur*	LT623220	-	LT623237	-	LT623277
Neocucurbitaria rhamni	CBS 142391	Austria	*Rhamnus frangula*	MF795775	-	-	-	MF795817
Neocucurbitaria rhamnicola	CBS 142396	Spain	*Rhamnus lycioides*	MF795780	-	MF795906	-	MF795822
Neocucurbitaria ribicola	CBS 142394	Austria	*Ribes rubrum*	MF795785	-	MF795911	-	MF795827
Neofusicoccum algeriense	CBS 113072	Unknown	Unknown	KX464150	KX464645	KX464920	-	-
Neofusicoccum arbuti	CBS 116131	Washington, U.S.A.	*Arbutus menziesii*	AY819720	KF531792	KF531793	-	-
Neofusicoccum batangarum	CBS 127348	USA: Florida	*Schinus terebinthifolius*	MH864533	KX464674	KX464952	-	-
Neofusicoccum eucalypticola	CBS 115679	Australia	*Eucalyptus grandis*	KF766201	-	-	-	-
Neofusicoccum italicum / parvum	MFLUCC 15-0900	Italy	*Vitis vinifera*	KY856755	KY856754	MT592684	-	-
Neofusicoccum kwambonambiense	CBS 123639	South Africa	*Syzygium cordatum*	MH863317	-	-	-	-
Neofusicoccum occulatum	CBS 128008	Australia	*Eucalyptus grandis*	EU301030	EU339509	EU339472	-	-
Neofusicoccum parvum	CBS 123650	South Africa	*Syzygium cordatum*	KX464182	KX464708	KX464994	-	-
Neofusicoccum parvum	CBS 145623	Italy	*Ficus carica*	MN611180	MN623347	MN623344	-	-
Neofusicoccum parvum	CVG 1588*	Italy	*Vitis vinifera*	*OQ612690*	*OQ680489*	*OQ680510*	-	-
Neofusicoccum parvum	CVG 1731	Italy	*Vitis vinifera*	*OQ612692*	*OQ680491*	*OQ680511*	-	-
Neofusicoccum protearum	CBS 114176	South Africa	*Leucadendron laureolum*	AF452539	KX464720	KX465006	-	-
Neofusicoccum ribis	CBS 122553	Panama	*Theobroma cacao*	EU683673	EU683654	MT592742	-	-
Neofusicoccum umdonicola	CBS 123645	South Africa	*Syzygium cordatum*	MH863318	-	KF766145	-	-
Neofusicoccum vitifusiforme	CBS 110887	South Africa	*Vitis vinifera*	AY343383	AY343343	KX465061	-	-
Paraconiothyrium africanum	CBS 121166	South Africa	*Prunus persica*	EU295650	-	JX496142	JX496255	-
Paraconiothyrium brasiliense	CBS 100299	Brazil	*Coffea arabica*	AY642531	-	JX496350	JX496237	-
Paraconiothyrium brasiliense	CBS 159.60	Unknown	Unknown	JX496044	-	JX496383	JX496270	-
Paraconiothyrium brasiliense	CBS 587.84	Italy	*Vitis vinifera*	JX496099	-	JX496438	JX496325	-

(Continued)

Table 2. (Continued).

Species	Isolate code	Country	Host	GenBank accession number [a]				
				ITS	tef1	tub2	act	RPB2
Paraconiothyrium brasiliense	CVG 1579*	Italy	*Vitis vinifera*	*OQ612697*	-	*OQ680500*	*OQ680507*	-
Paraconiothyrium brasiliense	CVG 1736	Italy	*Vitis vinifera*	*OQ612698*	-	*OQ680499*	*OQ680506*	-
Paraconiothyrium cyclothyrioides	CBS 432.75	Sri Lanka	*Hevea brasiliensis*	JX496088	-	JX496427	JX496314	-
Paraconiothyrium fuckelii	CBS 508.94	Italy	*Rosa* sp.	JX496096	-	JX496435	JX496322	-
Paraconiothyrium hawaiiense	CBS 120025	Hawaii	*Sophora chrysophylla*	JX496027	-	JX496366	JX496253	-
Paraconiothyrium hawake	CBS 142521	Australia	*Hakea* sp.	KY979754	-	KY979920	-	-
Paraconiothyrium minitans	CBS 286.81	Venezuela	*Solanum tuberosum*	JX496063	-	JX496402	JX496289	-
Paraconiothyrium sporulosa	CBS 105.76	Norway	*Picea abies*	JX496014	-	JX496353	-	-
Paraconiothyrium variabile	CBS 112.72	Italy	*Dianthus* sp.	JX496019	-	JX496358	JX496245	-
Parafenestella pseudoplatani	CBS 142392	Austria	*Acer pseudoplatanus*	MF795788	-	MF795914	-	MF795830
Paraphaeosphaeria neglecta	CBS 124076	Italy	*Actinidia chinensis* var. Hort16A	JX496037	-	JX496376	JX496263	-
Phlogicylindrium eucalypti	CBS 120080	Australia	*Eucalyptus globulus*	NR_132813	MH704607	MH704633	-	-
Pseudopyrenochaeta lycopersici	CBS 306.65	Germany	*Lycopersicon esculentum*	NR103581	-	LT717674	-	LT717680
Robillarda africana	CBS 122.75	South Africa	Unknown	KR873253	MH554414	MH554656	-	-
Robillarda roystoneae	CBS 115445	Hong Kong	*Roystonea regia*	KR873254	KR873310	KR873317	-	-
Seimatosporium botan	NBRC 104200	Japan	*Paeonia suffruticosa*	AB594799	-	LC047770	-	-
Seimatosporium luteosporum	CBS 142599	USA	*Vitis vinifera*	KY706284	KY706334	KY706259	-	-
Seimatosporium physocarpi	CBS 139968	Russia	*Physocarpus opulifolius*	KT198722	MH554434	MH554676	-	-
Seimatosporium vitifusiforme	CBS 142600	USA	*Vitis vinifera*	KY706296	KY706346	KY706271	-	-
Seimatosporium vitis	MFLUCC 14-0051	Italy	*Vitis vinifera*	KR920363	-	-	-	-
Seimatosporium vitis	Napa774	Napa County, U.S.A.	*Vitis vinifera*	-	KY706326	KY706251	-	-
Seimatosporium vitis-viniferae	CVG 1681	Italy	*Vitis vinifera*	*OQ612700*	*OQ680496*	*OQ680502*	-	-
Seimatosporium vitis-viniferae	CVG 1682*	Italy	*Vitis vinifera*	*OQ612699*	*OQ680495*	*OQ680501*	-	-
Seimatosporium vitis-viniferae	CBS 123004	Spain	*Vitis vinifera*	MH553992	MH554418	MH554660	-	-
Seimatosporium vitis-viniferae	CBS 116499	Iran	*Vitis vinifera*	MH553984	MH554402	MH554643	-	-
Sporocadus biseptatus	CBS 110324	Unknown	Unknown	MH553956	MH554374	MH554615	-	-
Sporocadus incanus	CBS 123003	Spain	*Prunus dulcis*	MH553991	MH554417	MH554659	-	-
Truncatella angustata	CBS 144025	France	*Vitis vinifera* Prunelard	MH554112	MH554546	MH554785	-	-
Truncatella angustata	CBS 113.11	Germany	*Picea abies*	MH553966	MH554384	MH554625	-	-
Truncatella angustata	CBS 165.25	Unknown	*Prunus armeniaca*	MH554010	MH554444	MH554686	-	-
Truncatella angustata	CVG 1601	Italy	*Vitis vinifera*	*OQ612701*	*OQ680497*	*OQ680504*	-	-
Truncatella angustata	CVG 1631*	Italy	*Vitis vinifera*	*OQ612702*	*OQ680498*	*OQ680505*	-	-
Truncatella restionacearum	CBS 118150	South Africa	*Restio filiformis*	DQ278914	MH554407	MH554649	-	-

[a] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; act: actin; tef1: translation elongation factor 1-α gene; rpb2: RNA polymerase second largest subunit; tub2: beta-tubulin. Sequences generated in this study indicated in italics. Ex-type and ex-epitype isolates are indicated in bold font; Genbank accession numbers generated in this study are indicated in italic font. CVG strains marked with * were selected to investigate effects of temperature on fungal radial growth.

Figure 1. Grapevine trunk disease symptoms observed in Piedmont. A. Symptoms attributed to Botryosphaeria dieback on a grapevine shoots, with complete branches dissection, drying, and fall of affected leaves. B. Cross section of a cordon with internal necrotic wood cankers (wedge-shaped) characteristic of Botryosphaeria dieback.

and OA, with incubation at 25°C in dark, were used for *Seimatosporium* isolates (Kanetis *et al.*, 2022). For *Truncatella* isolates, CMA and MEA were used, and colonies were incubated at 21°C with alternating light-dark periods (Liu *et al.*, 2019).

Effects of temperature on fungal colony radial growth

To investigate the effect of temperature on the colony radial growth of selected fungal isolates (Table 2), each isolate was grown on PDA amended with streptomycin sulphate (25 ppm L^{-1}) for 7 d in the dark at 25°C. Mycelium plugs were taken from the margins of 10-d-old colonies using a cork borer (0.6 cm diam.) and were placed upside down at the centers of 9 cm diam. Petri dishes, each containing 10 mL of PDA-S medium. All plates were then incubated for 7 d at 5, 10, 15, 20, 25, 30 or 35°C, and each isolate was tested using seven replicate plates per temperature. Following incubation, the Petri plates were examined without being opened, and the mean colony diameters (minus the diameter of the initial inoculation plugs) of each growing mycelial colony were measured in two perpendicular directions at the end of the 4th and 7th day. Radial growth rates (mm d^{-1}) were calculated for each temperature. The variations in mycelium growth rates at different temperatures were analyzed using the generalized Analytics Beta model (López-Moral *et al.*, 2017). Based on this analysis, optimum growth temperature and the corresponding maximum growth rate were calculated for each isolate. Box-Cox transformation was applied to optimum growth temperature data. To satisfy ANOVA assumptions, normality and homogeneity of variance were evaluated with, respectively, Shapiro-Wilk and Levene's tests. One-way ANOVA was carried out, followed by Tukey's test for evaluation of statistically significance differences between means (at $P < 0.05$), as both ANOVA assumptions were satisfied for the growth rate data. Welch's ANOVA was performed on optimum growth temperature data because only the normality assumption was satisfied. Statistical differences ($P < 0.05$) were analyzed with the Games-Howell *post hoc* test. All statistical analyses were carried out using R (https://www.R-project.org/).

Pathogenicity tests

Fifteen representative isolates from seven identified fungal species were used to inoculate one-year-old potted 'Barbera' grapevine cuttings grafted on K5BB rootstock (Table 2). Ten plants were inoculated with each isolate. The inoculations were carried out in May 2022. Cuttings were inoculated above the grafting point by forming a slit (1.0–1.5 cm long) using a sterile scalpel as described by Carlucci *et al.* (2015) and Bezerra *et al.* (2021). Agar plugs (6 mm diam.) were taken from 10-d-old fungal cultures grown on PDA and plugs were placed with mycelium in contact with plant tissues, under the stem bark. Each inoculated wound was wrapped with wet sterile cotton wool soaked in sterilized distilled water and was then sealed firmly with Parafilm® (American National Can) to maintain high humidity at the inoculation point. Control plants were inoculated with sterile agar plugs. Inoculated plants were the placed in a greenhouse at 25 ± 3°C, from May to November 2022. After 180 d. from inoculation, the plants were examined after bark removal and lengths of any visible necrotic wood lesions were measured from the inoculation points. Small tissue pieces (0.5 cm) from the necrotic area were placed on PDA supplemented with streptomycin sulphate (25 ppm L^{-1}), and incubated at 25 ± 1°C. To fulfil Koch's postulates, resulting colonies were identified based on their morphological characteristics. Data of necrotic lesion lengths were subjected to statistical analysis. Shapiro-Wilk (W) tests were used to determine if the data followed normal distributions. Levene's tests were carried out to assess the homogeneity of the variances of the dataset. A Welch's ANOVA was performed because the dataset was normally distributed, but data were not homoscedastic. The Games-Howell *post hoc* test was used to evaluate statistically significant differences among mean lesion lengths caused by the different fungal isolates (at $P \leq 0.05$). All statistical analyses were carried out using R (https://www.R-project.org/).

RESULTS

Sampling, isolation and morphological identification of isolates

In sampled vineyards, more than 30% of the plants showed Botryosphaeria dieback related symptoms. Approximately 5–10% of plants showed decline with severe dieback and death. Sampled grapevines showed typical dieback symptoms, primarily associated with Botryosphaeria dieback, such as defoliation and wedge-shaped cankers of internal wood tissues and dark streaking of wood.

A total of 248 fungal isolates were obtained from a total of 32 symptomatic vines of 12 cultivars. The first screen and identification of isolates was based on their morphological and cultural characteristics. A group of isolates identified as *Botryosphaeriaceae*-like showed high isolation frequency (80 isolates, 37% of total isolates obtained) compared with other common GTDs fungi, such as *P. chlamydospora* (38 isolates), *E. lata* (5 isolates) and *F. mediterranea* (12 isolates) that were occasionally present.

Other less frequently isolated genera were also detected using morphological characteristics, including *Diaporthe* (5 isolates), *Kalmusia* (3 isolates), *Neocucurbitaria* (2 isolates), *Paraconiothyrium* (3 isolates), *Seimatosporium* (7 isolates), and *Truncatella* (4 isolates) species. Some common saprophytes, not considered to be associated with observed symptoms, were also isolated, including *Alternaria*, *Epicoccum, Cladosporium*, and *Dydimella* spp. Table 1 shows details of the fungal species isolated from each vineyard surveyed in Piedmont.

Phylogenetic analyses

The multi-locus analyses conducted on all isolates confirmed the genera obtained with the initial phylogenetic analysis of the *tef1* and ITS regions. The combined locus analysis of *Botryosphaeriaceae*-like isolates consisted of 35 sequences and *Lecanosticta acicola*, which was chosen as the outgroup. A total of 995 characters (ITS: 1–660 and *tef1*: 666-995) were included in the phylogenetic analyses of *Botryosphaeriaceae*-like isolates. A total of 1405 characters (ITS, 1–628; *tub2*, 633–1123; *tef1*, 1128–1405) were included in the *Paraconiothyrium* analyses where, for a total of 15 sequences, *Alloconiothyrium aptrootii* was chosen as the outgroup. For *Neocucurbitaria*, *Pseudopyrenochaeta lycopersici* was chosen as outgroup, and a total of 1810 characters (ITS, 1–507; *tub2*, 511–896; *rpb2*, 901-1810) were included in the phylogenetic analyses performed with 16 sequences. The combined phylogenetic session for *Seimatosporium* and *Truncatella* had a total of 1823 characters (ITS, 1–579; *tef1*, 583–1071; *tub2*, 1076–1823) with 25 sequences. *Discosia artocreas* was chosen as the outgroup. For each session, a tree was created based on a maximum of 1000 equally most parsimonious trees. Bootstrap support values for all MP trees obtained are shown in Figures 2, 3, 4 and 5.

In the *Botryosphaeriaceae*-like analyses, two isolates (CVG15777 and CVG1753) clustered with *D. seriata* reference strains, and isolates CVG1582 and CVG1615 clustered with *B. dothidea* strains. Isolates CVG1588 and CVG1731 were grouped with *Neof. parvum*, and isolates CVG1739 and CVG1714 clustered with *D. mutila* reference strains. For *Paraconiothyrium*, both strains clustered with reference strains of *P. brasiliense*. In the phylogenetic tree from the *Truncatella* and *Seimatosporium* analysis, two isolates (CVG1601, CVG1631) were identified as *T. angustata*, and two isolates (CVG1681, CVG1682) were grouped with *S. vitis-viniferae* reference strains. Isolate CVG1779 of *Neocucurbitaria* clustered in the *Neoc. juglandicola* clade. The recommended evolutionary model, unique site patterns, number of generations, and tree produced and sampled for each partition of the Bayesian analyses are reported in Table 3, as well as other parameters produced by MB analyses, including tree lengths, consistency, retention, and rescaled consistency indices. Data obtained from the multi-locus analyses carried out on the 15 selected representative isolates gave four *Botryosphaeriaceae* species, including *B. dothidea, D. mutila, D. seriata* and *Neof. parvum*. Among other less frequently isolated taxa, *Neocucurbitaria juglandicola*, *P. brasiliense*, *S. vitis-viniferae* and *T. angustata* were identified.

Morphology

Morphological observations were performed for all the selected species. Colonies characteristic, including edges shape, colony front and reverse color, mycelia appearance and conidia morphology of *B. dothidea*, *D. mutila, D. seriata* and *Neof. parvum* were congruent with previous descriptions of species belonging to *Botryosphaeriaceae* family (Phillips *et al.* 2013). Different conidia were observed (cylindrical to fusiform, hyaline to dark brown) and all isolate showed fast growth mycelia, becoming dark with age starting from the center, spreading to the whole colony.

Colonies of *Neoc. juglandicola* on PDA and MEA showed slow growth with uneven margins. Colony upper surfaces were brown to dark brown with dense zonate mycelium. Pycnidia appeared as dots, which were numerous and centrally located. Reverse colony sides

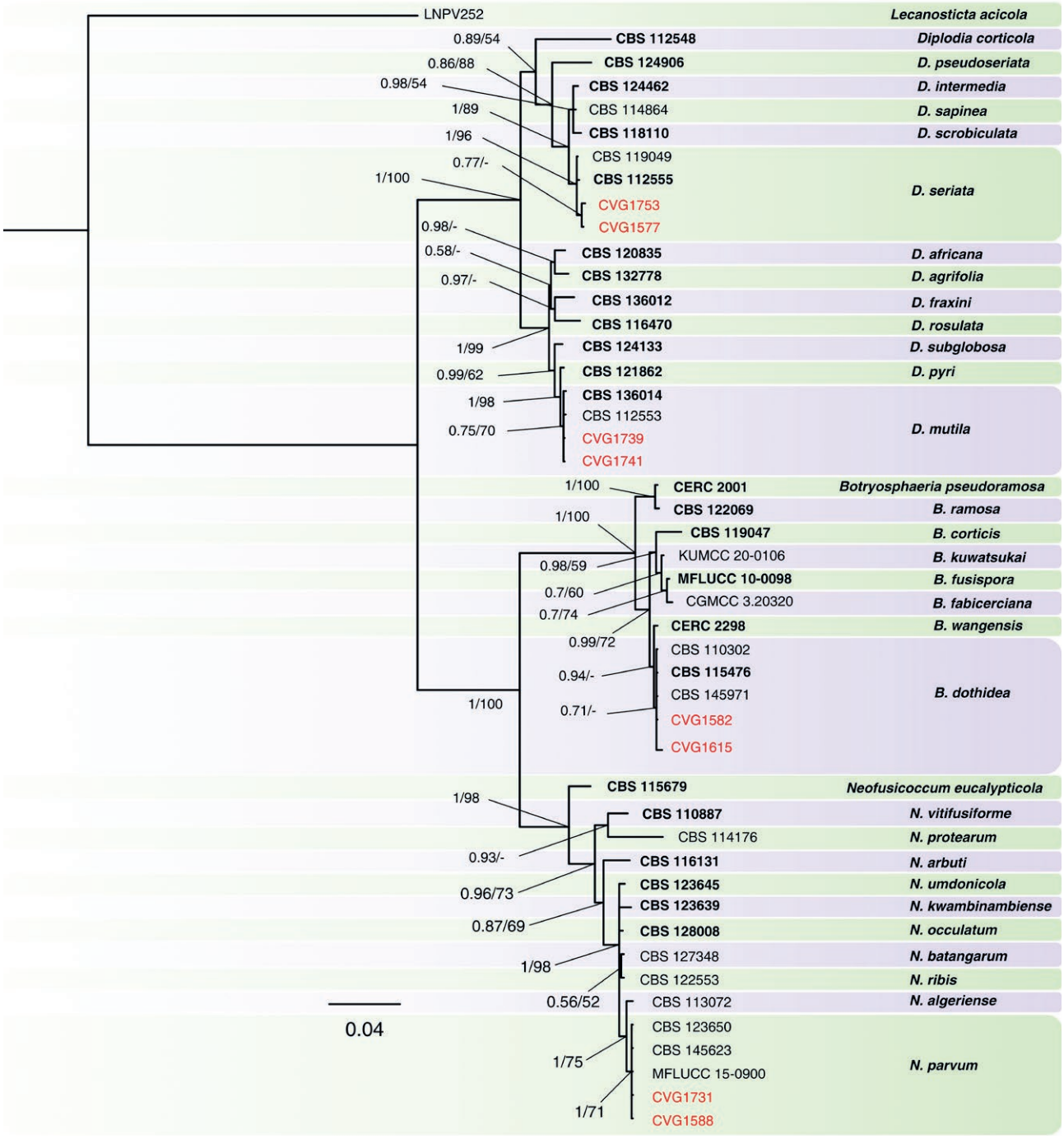

Figure 2. Phylogenetic tree for *Botryosphaeriaceae*, resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font, and species are delimited with coloured blocks. Isolates collected in the present study are indicated in red font. The tree was rooted to *Lecanosticta acicola* (LNPV252).

were dark brown. Conidia, produced on PDA measured 2.0–3.1 × 1.3–1.5 µm, mean (± S.D) = 2.5 ± 0.5 × 1.4 ± 0.1 µm, and were unicellular with smooth surfaces, hyaline, and ellipsoid with rounded apices. Based on morphological features, colonies had similar characteristic to those reported by Jaklitsch *et al.* (2018).

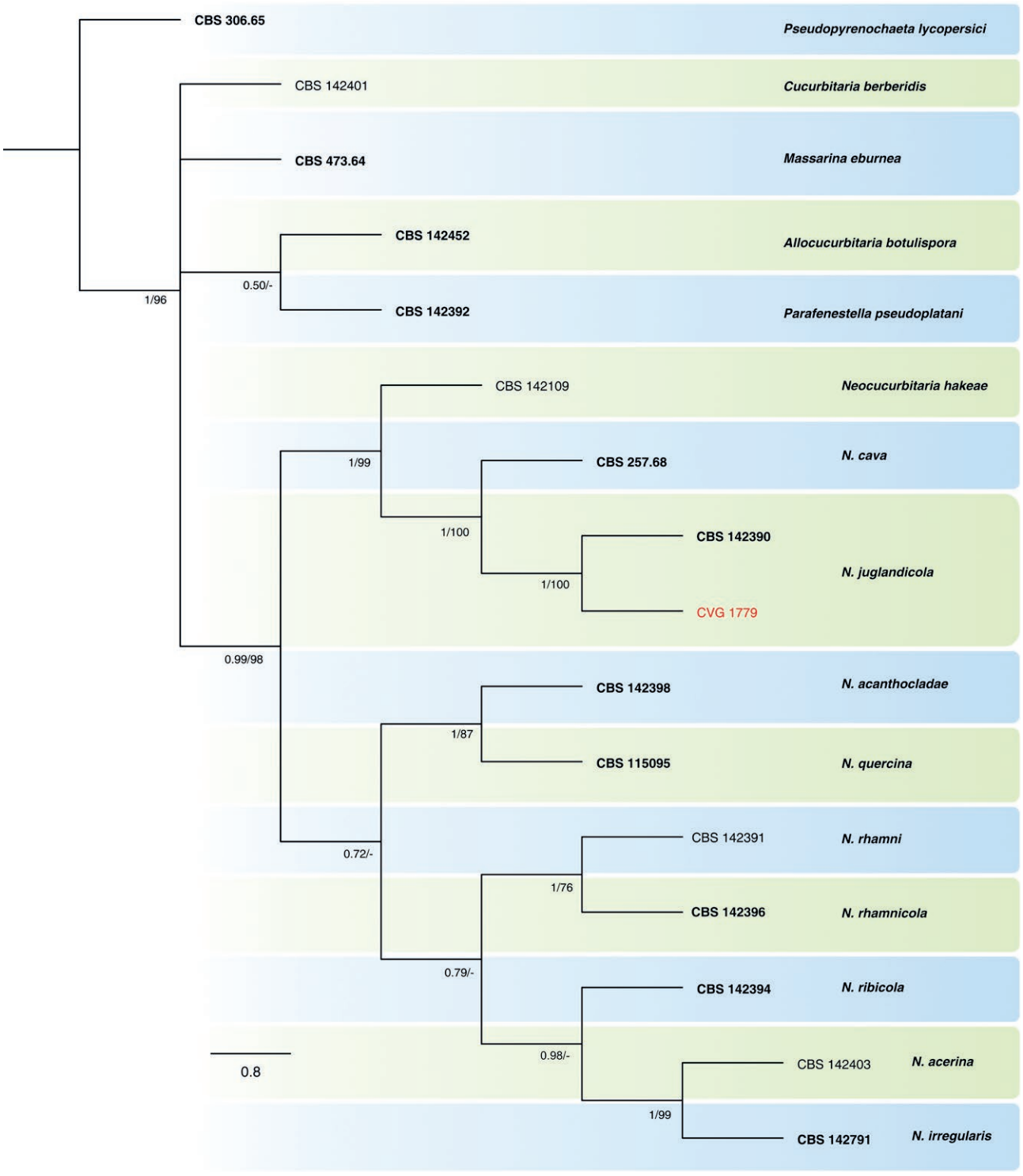

Figure 3. Phylogenetic tree of *Neocucurbitaria* sp., resulting from a Bayesian analysis of the combined ITS, *tub2* and *rpb2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font, and species are delimited with coloured blocks. The isolate collected in the present study is indicated in red font. The tree was rooted to *Pseudopyrenochaeta lycopersici* (CBS 306.65).

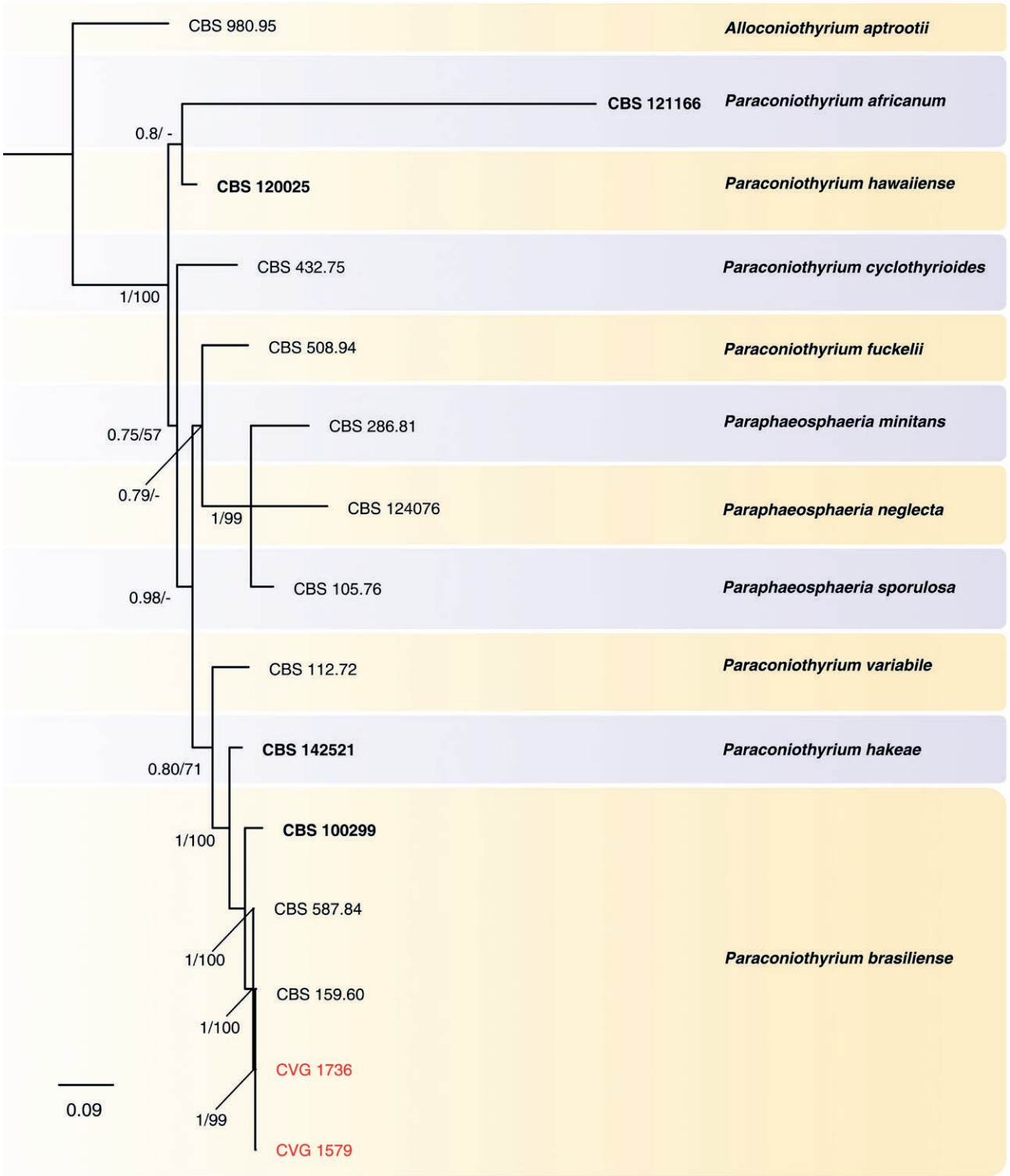

Figure 4. Phylogenetic tree of *Paraconiothyrium* sp. resulting from a Bayesian analysis of the combined ITS, *tef1* and *act* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated in bold font and species are delimited with coloured blocks. Strains collected in this study are indicated in red. The tree was rooted to *Alloconiothyrium aptrootii* (CBS 980.95).

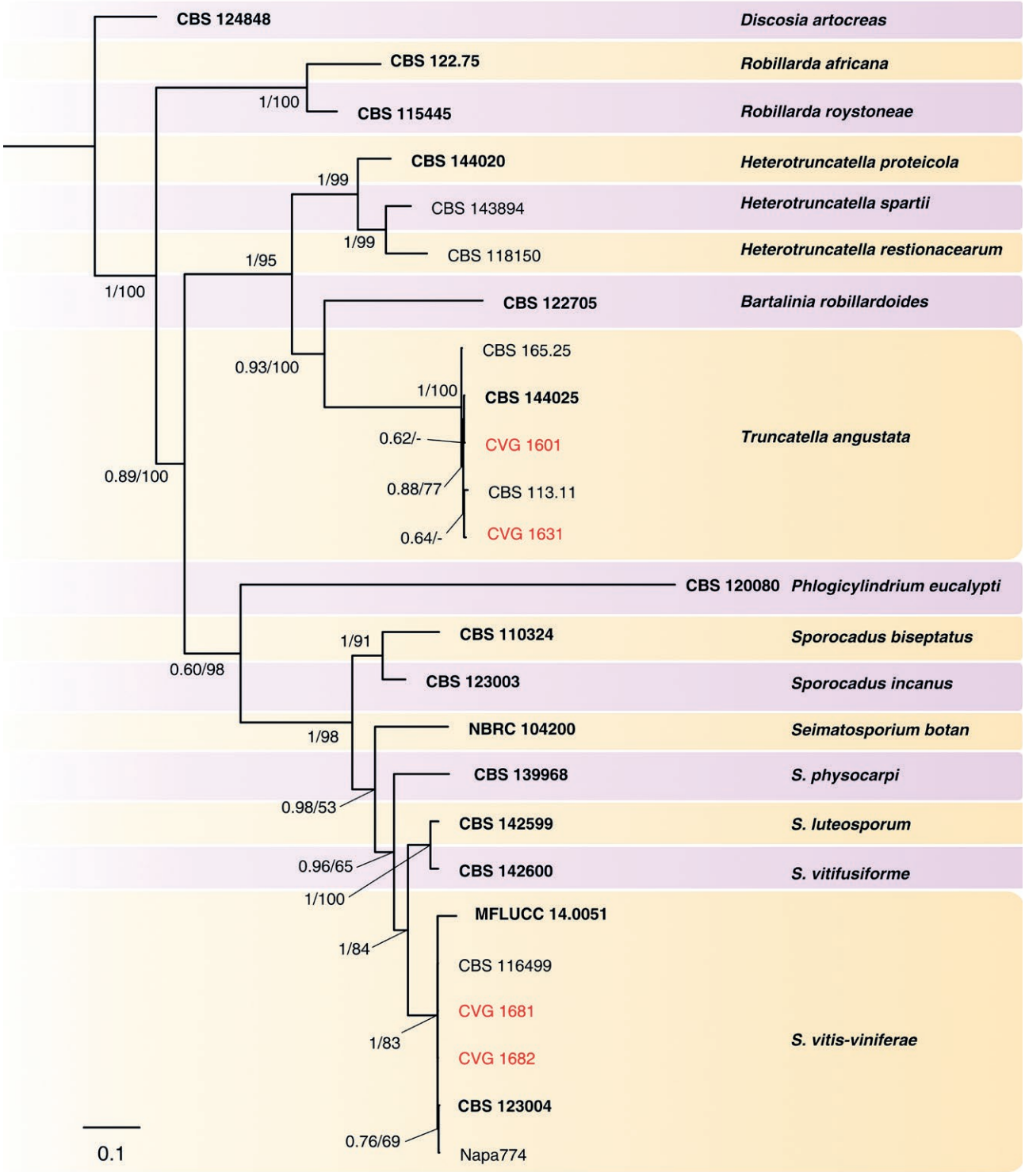

Figure 5. Phylogenetic tree of *Seimatosporium* sp. and *Truncatella* sp. resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequence alignment. Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type isolates are indicated in bold font and species are delimited with coloured blocks. Isolates collected in the present study are indicated in red font. The tree was rooted to *Discosia artocreas* (CBS 124848).

Table 3. Parsimony and Bayesian parameters for each phylogenetic analysis to identify different fungi.

Species	Locus(i)	Bayesian analysis				Parsimony analysis						
		Evolutionary model	Unique site pattern	Generation ran	Generated trees	Sampled trees	Total sites	Constant sites	Variable sites	Parsimony Informative sites	Tree length	Consistency index	Retention index	Rescaled consistence index
Botryosphaeraceae	ITS	SYM+G	242	1140000	2282	1712	1395	763	263	369	/	0.787	0.946	0.744
	tef1	HKY+I+G	242											
	tub2	GTR+G	121											
Neocucurbitaria sp.	ITS	GTR+I+G	145	805000	1612	206	1803	1063	324	416	1666	0.628	0.495	0.311
	tef1	SYM+G	148											
	RPB2	HKY+G	334											
Paraconiothyrium sp.	ITS	GTR+I+G	167	305000	612	93	1397	734	372	291	1273	0.742	0.612	0.454
	tub2	SYM+G	238											
	act	HKY+G	132											
Seimatosporium sp. and *Truncatella* sp.	ITS	HKY+G	178	705000	1412	711	1929	762	261	906	3376	0.68	0.803	0.546
	tef1	GTR+I+G	404											
	tub2	HKY+I+G	512											

Colonies of *P. brasiliense* on MEA were white-gray, had regular margins with rapid growth and darker aerial mycelium in the centre. Reverse colony sides were light brown-honey to light yellow amber. On OA the colonies were light gray, each with a darker area. On CMA the colonies were white, with mycelium development in the centres showing concentric and radiating patterns. Conidia were cylindrical to ellipsoid with rounded apices, and measured 2.5-4.4 × 1.3-2.8 µm, mean (± S.D) = 3.3 ± 0.9 × 1.9 ± 0.7 µm. They were hyaline and unicellular, with smooth walls, and granular contents. Based on morphological features, colonies had similar characteristic to those reported by Kanetis *et al.* (2022).

Colonies of *S. vitis-viniferae* on PDA and MEA had entire edges and were light brown to reddish with wooly aerial mycelium with smooth whitish margins. On OA the colonies were slightly to light brown with off-white wooly margins. Conidia were fusiform, each with three septa, and were constricted at each septum, measuring 15.8-22.7 × 4.2-6.1 µm, mean (± S.D) = 18.9 ± 3.5 × 5.1 ± 1.0 µm. The conidia were pale to dark brown, and each basal cell had an appendage while the apical cell had a rounded apex.

Colonies of *T. angustata* on PDA had entire edges, had pale gray to white fuzzy mycelium from above and grayish to white on the reverse sides, and were fast-growing. Black pycnidia were observed at the centre of each colony after 7 d. On MEA the colonies had entire edges, and grew slowly, with cottony white to light brown mycelia. Conidia were fusiform (17.5–19.7 × 6.1-7.3 µm, mean (± S.D) = 18.6 ± 1.1 × 6.7 ± 0.6 µm), mostly with three cells and were transversally septate without septal constrictions, and with truncate bases and several appendices. Based on morphological features of colonies and conidia, isolates studied have similar characteristic to those reported by Raimondo *et al.* (2019).

Effects of temperature on fungal growth

None of the tested isolates grew at 5°C, growth was slow between 10 to 15°C, and was optimum at 20 to 25°C. Four isolates, CVG1577 (*D. seriata*), CVG1582 (*B. dothidea*), CVG1588 (*Neof. parvum*) and CVG1741 (*D. mutila*), grew at 35°C. A generalized Analytics Beta model was used to describe the relationship between mycelial growth and selected temperatures (Figure 7) and optimum growth temperature, and the corresponding maximum growth rates were calculated. Coefficients of determination (R^2) for the Analytics Beta model ranged between 0.88 and 0.99. Analysis of variance (ANOVA) was carried out on data of mycelial growth rates and optimum growth temperatures.

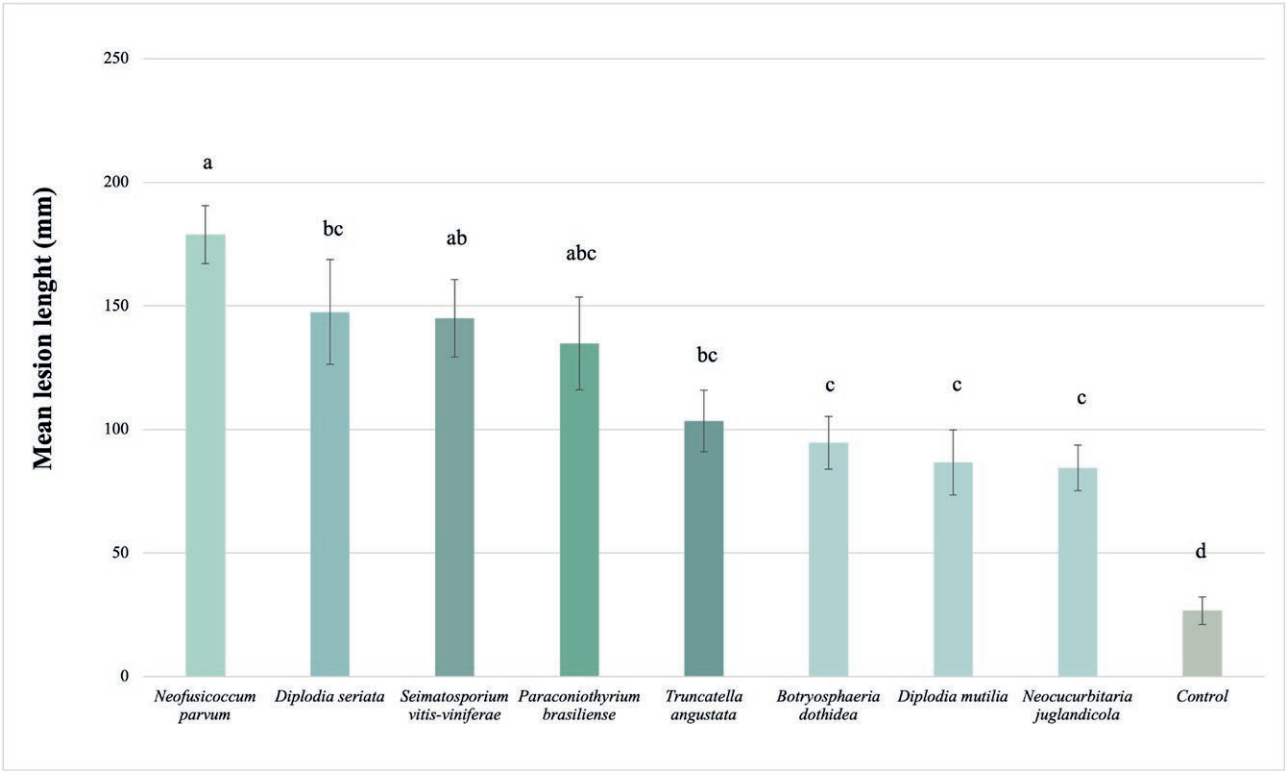

Figure 6. Mean necrosis lengths (mm) in grapevine stems resulting from inoculations with different fungi. A Games-Howell *post hoc* test was performed and means accompanied by different letters are significantly different ($P < 0.05$).

Statistically significant differences ($P < 0.05$) obtained with Games-Howell *post hoc* tests for optimum growth temperatures and Tukey's test for mycelial growth rates are shown in Figure 8. Among the *Botryosphaeriaceae* species, optimum mean growth temperatures ranged from 23.2°C for *D. seriata* and 23.9°C for *D. mutila*, to 25.5°C for *Neof. parvum* and 27.3°C for *B. dothidea*. *Neocucurbitaria juglandicola* did not grow 35°C. At the respective optimum temperatures, *D. mutila* grew the most rapidly at 10.9 mm d^{-1}, followed by *D. seriata* (10.5 mm d^{-1}), *B. dothidea* (10.0 mm d^{-1}) and *Neof. parvum* (8.7 mm d^{-1}). Mean optimum temperatures for mycelial growth were 24.8°C for *S. vitis-viniferae* and 24.6°C for *Neoc. juglandicola*. Both the species showed slow growth at, respectively, 0.83- and 0.54-mm d^{-1}. Maximum growth for *T. angustata* (3.8 mm d^{-1}) was obtained at 20.8°C and for *P brasiliense* (1.69 mm d^{-1}) was at 22.8°C. Based on maximum growth rates, *Botryosphaeriaceae* isolates had the fastest growth rate (>8 mm d^{-1}), followed by *T. angustata* and *P. brasiliense* (< 5 mm d^{-1}). *Seimatosporium vitis-viniferae* and *Neoc. juglandicola* had the slowest growth rates (< 1 mm d^{-1}).

Pathogenicity tests

The fungal isolates used for pathogenicity tests caused brown necroses and vascular discolourations in the wood of inoculated grapevines, 180 d after inoculation. No lesions were observed on inoculation control plants. *Neocucurbitaria juglandicola* (CVG 1779) was not re-isolated from necrotic areas in the wood, while all the other respective inoculated fungi were successfully re-isolated from the grapevine plants, fulfilling Koch's postulates. Re-isolated identifications were confirmed through morphological and molecular analyses (partial *tub* gene sequencing) while frequencies of re-isolations of inoculated species ranged from 80% and 90%. A Shapiro-Wilk (W) test was used for data of necrotic lesion lengths on the inoculated plants to determine if they followed normal distributions with W = 0.9807 (P-value = 0.07556). Levene's test showed that the homogeneity of variance was not significant for the dataset (P = 0.001825). Because data were normally distributed but not homoscedastic, a Welch's ANOVA was performed. This showed that statistically significant differences occurred among the inoculated fungi (P= 3.167e-15). Results of the Games-Howell *post hoc* test to evalu-

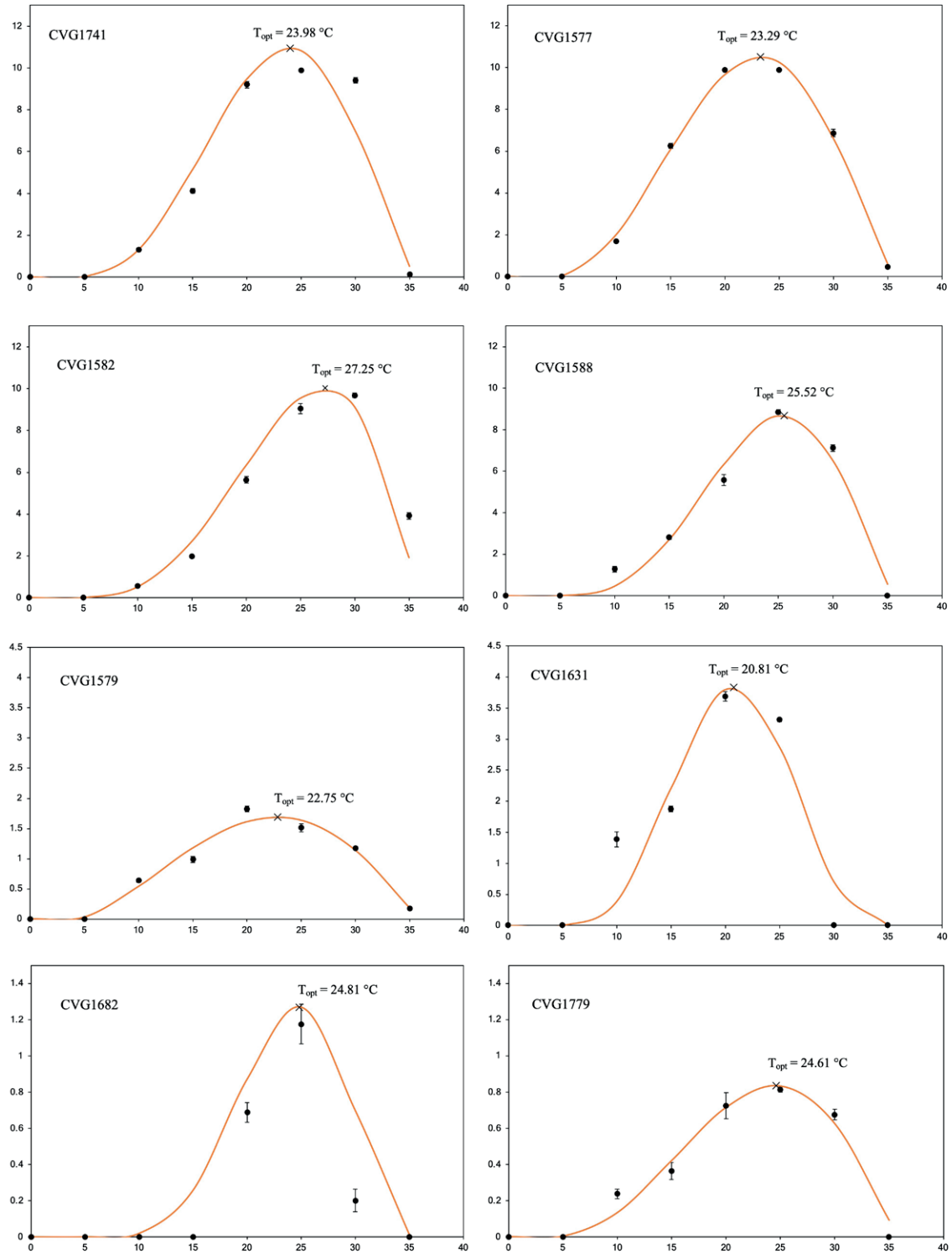

Figure 7. Mean mycelium growth rates (mm d-1) at different temperatures for *D. mutila* (CVG1741), *D. seriata* (CVG1577), *B. dothidea* (CVG1582), *N. parvum* (CVG1588), *P. brasiliense* (CVG1579), *T. angustata* (CVG1631), *S. vitis-viniferae* (CVG1682) and *N. juglandicola* (CVG1779). Strains were grown on PDA at 0, 5, 10, 15, 20, 25, 30, and 35°C for 4 days. A nonlinear regression curve based on Analytis Beta model was used to plot average growth rates over temperature. Data points are the means of seven independent replicated and standard error of the means is showed as vertical bars.

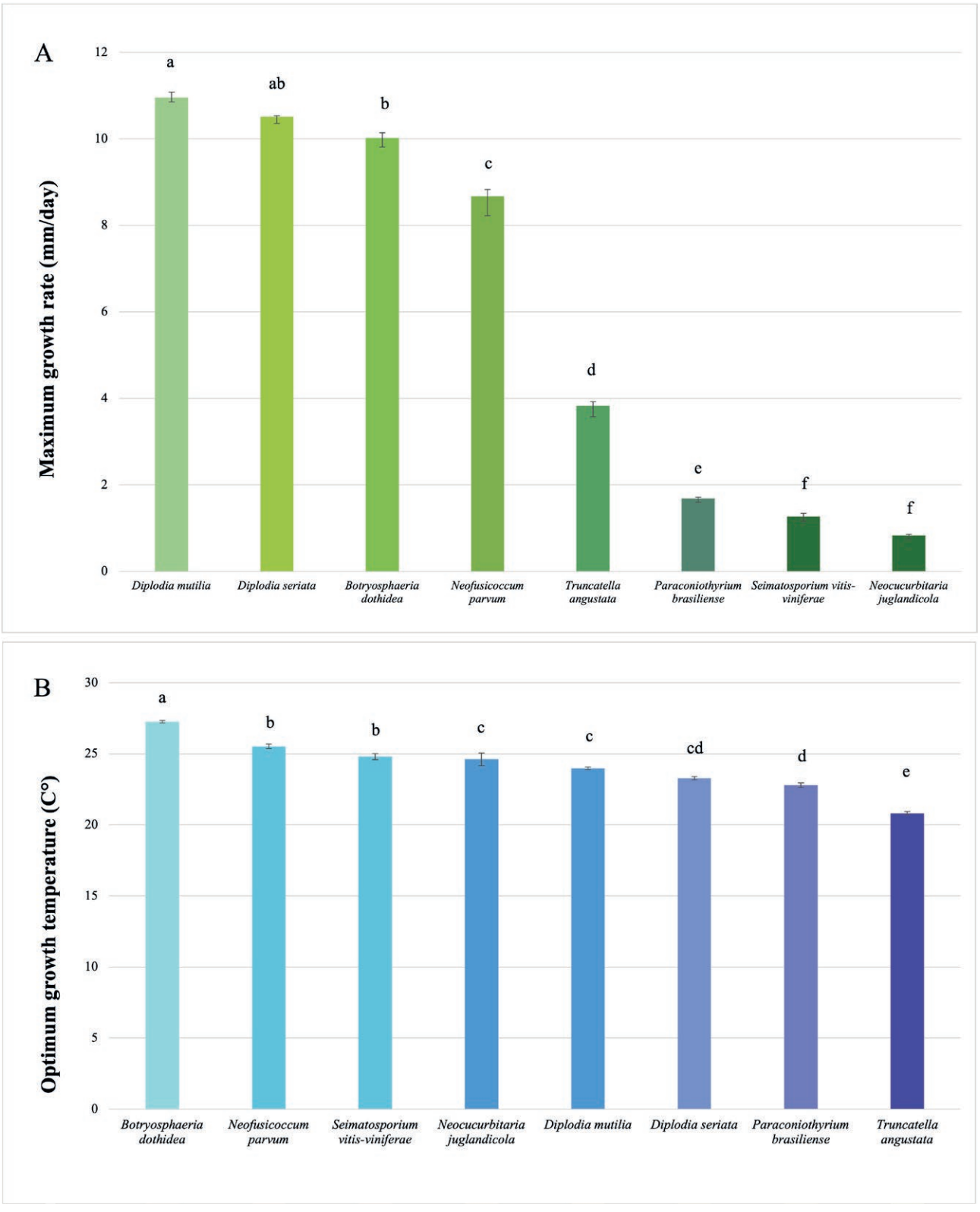

Figure 8. Mean maximum growth rates (A) at respective optimum growth temperature (B) for eight fungi, Vertical bars indicate standard errors. Means in each histogram accompanied by different letters are significantly different ($P = 0.05$).

ate differences among mean lesion lengths are shown in Figure 6. The longest necrotic lesions were produced by *Neof. parvum* (mean length = 178.8 mm). Aggressiveness of the other *Botryosphaeriaceae* was also confirmed: these strains produced variable vessel discolouration, with mean lesion lengths of 147.5 mm from *D. seriata*, 94.6 mm from *B. dothidea,* and 86.6 mm from *D. mutila*. *Paraconiothyrium brasiliense* and *S. vitis-viniferae* each showed similar aggressiveness compared to the *Botryosphaeriaceae* isolates, with, respectively, mean lesion lengths of 134.8 mm and 145.0 mm. *Neocucurbitaria juglandicola* (mean lesion length = 84.4 mm) and *T. angustata* (mean lesion length = 103.4 mm) were the least aggressive among the non-reported GTD pathogens.

DISCUSSION

This study has characterized the different fungal species associated with dieback symptoms observed in representative vineyards in Piedmont, Italy. Some of these fungi are already known to be associated with diseases such as the Esca complex or Botryosphaeria dieback. Among all isolates collected, most were *Botryosphaeriaceae*, with *B. dothidea*, *D. seriata*, *D. mutila* and *Neof. parvum* identified through morphological characterization and confirmed by multi-locus phylogenetic analyses. No *Lasiodiplodia* isolates were found, which may be result because of these fungi is more prevalent in tropical and sub-tropical climatic regions. In Italy, *Lasiodiplodia* was only reported in Sicily (Burruano *et al*., 2008). Other pathogens commonly associated with the Esca complex, were sporadically recovered, including *Fomitiporia mediterranea* and *Phaeomoniella chlamydospora*. No isolates of *Phaeoacremonium* spp. were detected, which is another pathogen commonly associated with the Esca complex (Essakhi *et al*., 2008). *Eutypa lata*, the Eutypa dieback pathogen was also occasionally isolated. The pathogens commonly associated with the various symptoms observed on sampled grapevines were successfully isolated. The wedge-shape cankers, typically caused by *Botryosphaeriaceae*-like fungi or Eutypa infection, were the most common wood symptoms, but Esca-complex associated symptoms were also observed.

Some species less frequently isolated from affected grapevine wood were detected. These included *Neoc. juglandicola, P. brasiliense*, *S. vitis-viniferae* and *T. angustata*. For some of these isolates, association with grapevine woody tissues had been reported previously (Elena *et al*., 2018; Raimondo *et al*., 2019), and their respective pathogenicity related to GTDs was confirmed.

In different countries, including China (Yan *et al*., 2013), Iran (Arzanlou *et al*., 2012), Portugal (Phillips, 2002), Spain (Úrbez-Torres *et al*., 2006), Turkey (Akgul *et al*., 2014), France (Larignon *et al*., 2001), and the United States of America (Úrbez-Torres and Gubler, 2009; Trouillas *et al*., 2010). *Botryosphaeria dothidea* has been described as one of the species associated with typical V-shaped necrotic wood lesions and brown discolouration of the xylem vessels. In Italy, *B. dothidea* was reported on grapevine in the southern and central regions, on which its pathogenicity was confirmed (Carlucci *et al*., 2009). The two *B. dothidea* strains used in the present study produced dark streaks on inoculated cuttings, similar to those previously reported.

Diplodia seriata is known to be widespread in Europe, as it was described associated with symptomatic grapevine in Spain (Martin and Cobos, 2007), Portugal (Rego *et al*., 2009), France (Larignon *et al*., 2001), Turkey (Akgul *et al*., 2014) and Croatia (Kaliterna and Miličević, 2014). Reported as either pathogenic or saprophytic in different hosts, pathogenicity trials conducted by Taylor *et al*. (2005) and Carlucci *et al*. (2015) confirmed its role in causing necrotic wood lesions on *V. vinifera*. Based on the present results, isolates of *D. seriata* produced longer lesions than those caused by *D. mutila* and *B. dothidea*, confirming the variability in virulence among different isolates (Elena *et al*., 2015).

While *D. seriata* has been reported to be associated with grapevine in different Italian regions, including Apulia (Pollastro *et al*., 2000) and Tuscany (Spagnolo *et al*., 2011). However, *D. mutila* has already been reported associated with *Vitis vinifera* in Hungary (Lehoczky, 1974; Kovács *et al*., 2017), Spain, California, and Chile (Morales *et al*., 2012; Díaz *et al*., 2013) and with grapevine canker and dieback in Italy (Carlucci et al. 2015). In the present study, *D. mutila* produced wood discolourations after artificial inoculations, so the association with symptomatic grapevine in Italy was confirmed.

Furthermore, pathogenicity trials carried out in the present study showed *Neof. parvum* to be the most virulent species producing the longest necrotic lesions. This result is similar to those of Billones-Baaijens *et al*. (2013) and Úrbez-Torres and Gubler (2009), who, after pathogenicity trials conducted in, respectively, New Zealand and California, reported *Neof. parvum* as one of the most aggressive species associated with Botryosphaeria dieback. In Italy, Carlucci *et al*. (2015) came to the same conclusion after testing its pathogenicity on two grapevine cultivars. *Neofusicoccum parvum* was usually reported as an aggressive wood pathogen, able to infect many hosts. This fungus was also described in association with Botryosphaeria dieback symptoms on grape-

vine in France (Larignon *et al*., 2015), Algeria (Berraf-Tebbal *et al*., 2014), Spain (Luque *et al*., 2009), Portugal (Phillips, 2002), and Turkey (Akgul *et al*., 2014). Likewise, it was isolated from symptomatic grapevines in the Italian regions of Apulia and Tuscany (Carlucci *et al*., 2009; Spagnolo *et al*., 2011).

The association of *Seimatosporium* with grapevine is known, as well as its wide distribution and ability to colonize many hosts (Raimondo *et al*., 2019). Among all *Seimatosporium* species, recent studies have reported the association of *S. vitis* with GTD symptoms, where it was isolated from necrotic tissues and dead cordons in California (Lawrence *et al*., 2018) and Hungary (Váczy, 2017). In Italy, *S. vitis* was the first *Seimatosporium* species to be described in association with dead stem of *V. vinifera* (Senanayake *et al*., 2015) and Camele and Mang (2019) described it for the first time causing GTDs. In 2022, Kanetis *et al*. reported another species, *S. vitis-viniferae,* associated with lesion and wood discolouration on grapevine. In Italy, Raimondo *et al*. (2019) tested its pathogenicity, confirming its association with GTD symptoms. This agrees with results in the present study, which showed *S. vitis-viniferae* as causing wood necroses after artificial inoculation, with similar severity to necroses caused by *D. seriata*. *Truncatella* genera, which is phylogenetically close to *Seimatosporium*, has been revised by Liu *et al*. (2019) and includes only one species, *T. angustata*, while other *Truncatella* species have been reallocated to other genera. *Truncatella angustata* has been reported in association with grapevine, isolated as an endophyte in Spain (González and Tello, 2011) and Switzerland (Casieri *et al*., 2009). As a pathogen, the involvement of *T. angustata* with GTDs has been demonstrated, isolation from symptomatic grapevines in France (Pintos *et al*., 2018) and Iran (Arzanlou *et al*., 2013), and in pathogenicity tests (Úrbez-Torres *et al*., 2009). This fungus is considered an opportunistic pathogen on grapevine which is not primarily involved in GTDs. In Italy, *T. augustata* was first reported by Raimondo *et al*. (2019), who after isolation from symptomatic grapevines, confirmed its pathogenicity and involvement in GTDs. In accordance with the above studies, the present study confirmed its weak pathogenicity on grapevine, causing necrotic discolourations (mean length = 103.4 mm) after artificial inoculations.

Paraconiothyrium brasiliense was also less frequently isolated from woody tissues, and the role of *Paraconiothyrium* spp. on grapevine requires clarification. Pathogenicity on fruit trees and other woody hosts has been demonstrated for different species (Damm *et al*., 2008), while, *P. brasiliense* has been isolated from symptomatic and non-symptomatic grapevine tissues from Spain (Elena *et al*., 2018) and the United States of America (DeKrey *et al*., 2022). In Italy, *P. brasiliense* was also recently reported associated with dieback of apple trees (Martino *et al*., 2023). Pathogenicity trials conducted in the present study showed that *P. brasiliense* produced wood streaking with similar lesion length (mean = 134.8 mm) to lesions produced by *S. vitis-viniferae* (mean = 145.0 mm), confirming pathogenicity of *P. brasiliense* on grapevine. This study has demonstrated the role of *P. brasiliense* as a weak woody pathogen, and this is the first report of this fungus as a grapevine pathogen in Italy. *Neocucurbitaria juglandicola* has also been identified in this study. *Neocucurbitaria quercina* was reported from grapevine in the United States of America (DeKrey *et al*., 2022), while *Neoc. juglandicola* has been reported in association with *Juglans regia* and *Quercus rubra* (Jaklitsch *et al*., 2018). In the present study, after artificial inoculations, necrotic discolouration was visible, but it was not possible to re-isolate the fungus from necrotic areas. The presence of *Neoc. juglandicola* demonstrates its association with grapevine in Italy, while the pathogenicity tests did not prove its virulence or its association with GTDs. This is the first report of *Neoc. juglandicola* associated with grapevine, however.

Optimum growth temperatures of tested isolates ranged between a maximum of 27°C (for *Neof. parvum*) to a minimum of 21°C (for *T. angustata*). The respective virulences, assessed as lesion lengths, had no relationships with optimum growth temperatures in cultures. Several abiotic factors, including plant drought stress or water excess after climate events, or increases in average temperatures, can play roles in disease development, and may influence pathogen wood colonization and virulence. It is well known that global warming and climate change can increase plant stress and generate favourable conditions for the development of many diseases, including grapevine trunk disease (Guarnaccia *et al*., 2023).

Several fungi, especially *Botryosphaeriaceae*, are known to be able to switch from endophytic to pathogenic behaviors as a result of triggers connected with environmental stresses, such as drought, extreme temperatures and nutrient deficits (Slippers and Wingfield, 2007). These fungi may therefore benefit from the ongoing global warming scenario. The high percentage of isolation of these pathogens from vineyards located in Piedmont suggests a shift of these fungi may also be occurring in northern regions, as has occurred in Mediterranean areas. This expansion may be related to climatic changes. Factors such as prolonged drought, high summer temperatures, and changes in agronomic practices could favor development, spread, and pathogenicity of these fungi.

Results from the present study have demonstrated the presence of well-known GTD pathogens in Piedmont, one of the most important wine-production regions in Italy. The diversity and virulence of these pathogens in Piedmont was previously unexplored. Association of *P. brasiliense* and *N. juglandicola* with grapevine wood in Italy has been reported for the first time.

This first survey in Piedmont aimed to determine the presence and distribution of Botryosphaeria dieback pathogens, and to investigate the occurrence of other fungi associated with symptomatic grapevines. Further research is required to better clarify the distribution of grapevine pathogens in Northern Italy, especially species of *Botryosphaeriaceae*, and to determine which are the GTDs pathogens present in this region and monitor their possible shifts following climate changes.

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