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Fungal pathogens associated with grapevine trunk diseases in young vineyards in Sicily

Vincenzo MONDELLO[1], Selene GIAMBRA[2], Gaetano CONIGLIARO[2], Nicola FRANCESCA[2], Santella BURRUANO[2,*]

[1] *SFR Condorcet FR CNRS 3417, Université de Reims Champagne-Ardenne, Résistance Induite et Bioprotection des Plantes – RIBP, EA 4707, BP 1039, Reims, Cedex 2 51687, France*
[2] *Università degli Studi di Palermo, Dipartimento di Scienze Agrarie, Alimentari e Forestali, viale delle Scienze, 4, 90128 Palermo, Italy*
*Corresponding author. E-mail: santella.burruano@unipa.it

Summary. After the first report of grapevine decline caused by *Botryosphaeriaceae* in Sicily in 2007, epidemiological studies carried out in mature vineyards until 2011 confirmed the widespread occurrence of "Botryosphaeria dieback" and the "Esca complex" disease. Dieback symptoms were also recently observed in two young vineyards in Partanna and Castellammare del Golfo in western Sicily (Trapani province). Declining vines were inspected for grapevine trunk disease (GTD) symptoms, and were uprooted and submitted for analyses. Fungal isolates were collected and identified using culturing and molecular analyses. One isolate per identified species was inoculated to three grapevine shoots to evaluate pathogenicity and fulfil Koch's postulates. Several GTD *Botryosphaeriaceae* pathogens in the genera *Cadophora, Ilyonectria, Neonectria*, *Phaeoacremonium* and *Phaeomoniella* were isolated from the symptomatic young vines. Artificial inoculation confirmed the pathogenicity of these fungi. In addition, virulence variability was observed among the isolates, with *P. chlamydospora* causing the largest lesions. The different species were associated with specific symptoms and/or host vine parts, especially in the roots and around the grafting areas. Several fungi associated with Petri disease and black foot were shown to be responsible of young vine decline.

Keywords. Young vine decline, grapevine trunk diseases, Petri disease, black foot.

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INTRODUCTION

Within grapevine trunk diseases (GTDs), the term "young vine decline" (YVD) refers to a complex of diseases (Petri disease, black foot and young Esca) observed in vines less than 6 years old, and caused by different fungal pathogens (Agustı-Brisach and Armengol, 2013; Gramaje and Armengol, 2011a). The main external symptoms of YVD are progressive dieback characterized by reduced vigour, shortened internodes, leaf chlorosis and eventual plant death. Internally, infected vines show dark-brown streaking along the trunks and/or necrotic wood mainly at the bases of the rootstocks or scions in self-rooted vines. Decreased survival of young plants in nurseries and

newly established vineyards affected by YVD have been reported from many wine-producing areas (Halleen *et al.,* 2003; 2004; Gimenez-Jaime *et al.,* 2006; Gramaje *et al.*, 2009, 2018; Rego *et al.,* 2009; Agustì-Brisach *et al.,* 2011; Gramaje and Armengol 2011a; Cabral *et al.,* 2012; Pintos *et al.,* 2018). At least 51 GTD pathogens are now considered associated to the YVD (Úrbez-Torres *et al.,* 2015). Economic impacts of YVD are significant in nurseries and vineyards (due to required replacement of dead vines). In nurseries, the lack of effective prophylaxis techniques along the plant production process has increased GTD infections, causing grafting failures, poor plant vigour and, in general, the production of poor-quality vines with latent infections (Smart *et al.,* 2012; Gramaje and Di Marco, 2015; Waite *et al.,* 2015).

In Sicily, the first report of YVD was in 1912, when Lionello Petri found two isolates of *Cephalosporium* and one of *Acremonium* associated with symptomatic wood of young declining vines. Sixty years later, *Cylindrocarpon obtusisporum* (Grasso and Magnano di San Lio, 1975), *Phoma glomerata* (Granata and Refatti, 1981), and *Cylindrocarpon destructans* and *Fusarium solani* (Grasso, 1984) were identified as causal agents of YVD in different areas of Sicily. Sidoti *et al.* (2000) reported *Phaeomoniella chlamydospora* associated with YVD in a young vineyard of cv. Victoria vines in the southeast of Sicily.

More recently, vineyard surveys in the western part of Sicily (Agrigento, Caltanissetta, Palermo and Trapani provinces) have highlighted the presence and spread of Botryosphaeria dieback and Esca pathogens, but only in mature vines (Burruano *et al.*, 2008; 2010; Mondello *et al.,* 2013). In 2015 and 2018, contrary to previous studies, dieback symptoms were also observed in two young vineyards (2- and 1-year-old), located in the Trapani province. Different abiotic and biotic factors can be associated with the decline of young vineyards, including nutritional deficiencies, fungal, bacterial and viral diseases, and insect and nematode pests (Halleen *et al.,* 2003). Accordingly, the aim of this research was to investigate the observed decline of young vines, to identify the associated fungi and assess their virulence towards *Vitis vinifera*.

MATERIALS AND METHODS

Sampling and fungus isolation

Three surveys were carried out in a 2-year-old vineyard (cv. Grecanico) in Castellammare del Golfo in 2015. In addition, two surveys were carried out in a 1-year-old vineyard (cv. Muller-Thurgau) in Partanna in 2018. Both vineyards were in the province of Trapani, Sicily, Italy. Plants in both vineyards showed dieback and general reduced vigour, with foliar discolouration frequently observed in the Partanna vineyard. In both vineyards, vines with canes with stunted growth, including short internodes, uneven lignification, leaf zig-zag lesions and chlorosis, were collected in spring. In total, 20 samples were collected for analysis from the Castellammare del Golfo vineyard, and nine samples were from Partanna. After preliminary inspections, each vine trunk was superficially sterilized, the bark removed and the trunk divided into three different sections, including the base of the rootstock, the rootstock and the grafting point. Small wood portions (approx. 2 mm^2) from each trunk position were placed into Petri plates containing potato dextrose agar (PDA) supplemented with 500 mg L^{-1} streptomycin sulphate, and these were incubated at 24± 1°C in darkness. After 7 to 10 d incubation, fungal colonies similar to known GTD fungi were individually transferred to PDA in Petri plates, to obtain pure cultures. Isolation frequencies for each fungus per sample and per sample wood part were calculated. The obtained isolates are maintained in the fungal culture collection of the Dipartimento di Scienze Agrarie Alimentari e Forestali (University of Palermo).

Morphological characterization of fungus isolates

One isolate of each colony type was selected for identification to genus on the basis of macroscopic (colony colour, morphology, texture and type of growing margin), microscopic and biometric characteristics (Domsch *et al.,* 1980; Barnett and Hunter 1998; Pitt and Hocking 1999). Microscopic characteristics, including conidium length, width, shape and number of septa, were examined using a light microscope (Axioskop; Zeiss) coupled to an Axio-Cam MRc5 (Zeiss) digital camera, and images were captured using the AxioVision 4.6 software (Zeiss). Fifty conidia were measured per isolate, to calculate length and width means, standard deviations and 95% confidence intervals for the conidium dimension data.

Biometric analyses were used to evaluate effects of temperature on mycelium growth in the same isolates used for microscope measurements. Agar plugs (6 mm diam.) from 7-day-old colonies were inoculated onto PDA plates which were incubated at different temperatures of 5 to 40°C (5°C intervals) in the dark. Three replicates for each isolate and temperature were prepared, and the experiment was performed twice. Colony diameters were each measured along two perpendicular axes when the colony reached at least two-thirds of the plate diameter, and these data were converted to daily radial

growth (mm d^{-1}). For each isolate, average data of radial growth were adjusted to a regression curve using Statgraphics Plus 5.1 software (Manugistics Inc.), and the best polynomial model was chosen based on parameter significance ($P < 0.05$) and coefficient of determination (R^2) to estimate the optimum growth temperature for each isolate. Data were subjected to analysis of variance (one-way ANOVA) and significant differences between mean values were determined by Fisher's least significant difference (LSD) multiple range test at $P = 0.05$, using SAS version 9.0 (SAS Institute).

After 15 days, culture plates incubated at temperatures in which there was no growth were placed at 25°C to determine if these temperatures were fungistatic or fungicidal.

DNA extraction, PCR amplification and sequencing

Isolates grouped according their morphology were also processed for molecular identification. Total genomic DNA was extracted from each isolate using a standard cetyltrimethylammonium bromide (CTAB)-based protocol (O'Donnell *et al.,* 1998). The internal transcribed spacer region (ITS) of the ribosomal DNA operon, including the ITS1, 5.8S and ITS2, were amplified by PCR using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et *al.,* 1990). Each 40 µL reaction volume contained 50–100 ng of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM of each primer, 0.5 U of Taq DNA polymerase (Dream Taq, Fermentas) and 1 × Dream Taq buffer (Fermentas). PCR products were sequenced in both directions, and the nucleotide sequences were compared to those present in GenBank using the BLAST program (Altschul *et al.,* 1997). Sequences obtained in this study were deposited in GenBank (Table 1).

Pathogenicity tests

Following previous studies (Úrbez-Torres and O'Gorman, 2014; Carlucci *et al*, 2017; Aigoun-Mouhous *et al*., 2019), the identified isolates were tested for pathogenicity in 1-year-old dormant canes cut from 3-year-old grapevine plants (cv. Grecanico; five replicates per isolate). Before artificial inoculation, the canes were subjected a hot water treatment (53°C for 30 min: Carlucci *et al.,* 2017), to obtain shoots with low presence of pathogens.

A mycelium plug (6 mm diam.) from a 10-day-old PDA colony of each selected isolate was inserted into a wound made in the wood of each inoculated cane after removing the bark. Inoculated wounds were wrapped with wet sterile cotton wool for approx. 2 d, and the canes were then placed vertically in a plastic box sealed with cellophane film for another 13 d. Negative controls were inoculated with non-colonized PDA plugs. Each experiment included six replicates per isolate. After incubation at 23 ± 2°C for 15d, inoculated shoots were examined for necrosis by removing the bark from each shoot, and the length of brown streaking was measured. To fulfill Koch's postulates, all the inoculated canes were subjected to re-isolation of the inoculated pathogen. Data of lesion lengths were checked for normality using the Shapiro-Wilk test, and were subjected to analysis of variance (one-way ANOVA). Significant differences between mean values were determined by LSD tests at $P = 0.05$, using SAS version 9.0 (SAS Institute).

RESULTS

Sampling and fungal isolations

Sampled plants from the two different vineyards showed stunted/reduced vegetative vigour, interveinal leaf chlorosis, thin canes with shortened internodes and reduced root growth (Figure 1a, b, c and d). Longitudinal sections of symptomatic plants showed brown/black discolouration of the wood along almost the entire trunk lengths. In cross section, discolourations were often associated with dark-coloured exudates or with annular browning (Figure 1e and f). Most of the cv. Grecanico samples showed larger discolouration around the grafting-points than discolourations observed in basal ends and collars of the rootstocks (Figure 1g, h and i). In some of the observed rooting areas, severe blackening of the wood was also present. Muller-Thurgau vines all showed prominent vascular discolouration (Figure 1j).

Morphological and molecular identification of isolated fungi

A total of 570 fungal isolates were obtained from the symptomatic plants. Of these, 264 were classified as fungi belonging to the genera *Acremonium, Aspergillus, Cladosporium, Lophiostoma*, and *Penicillium*, which are known as endophytes and/or saprophytes in plants. These isolates were not further considered in the present study (Halleen *et al.,* 2007). The other 306 isolates, grouped according similarity for colony and conidium morphology, were classified as fungi associated with GTDs. Sixty-two isolates were associated with biometric features of Botryosphaeria dieback (BD) pathogens, 118 with features related to Black foot disease (BFD) patho-

Figure 1. Symptoms of YVD observed in western Sicilian vineyards. a-d, whole plants with stunted development in cv. Muller-Thurgau (a-b) and cv. Grecanico (c-d). Vines showed delayed growth, chlorosis, canes with short internodes and poor root system development. e-j, YVD internal symptoms: dark-coloured exudates in cv. Grecanico (e), and annular browning in cv. Muller-Thurgau (f). Declining vines in longitudinal section showed wood discolouration at the grafting points (g), along the trunk (h) and at the base of the rootstock (h, cv. Grecanico). j, longitudinal sections of vines cv. Muller-Thurgau.

gens, and 126 isolates had features of Petri disease (PD) pathogens.

Morphological features, biometric and molecular analyses of the selected isolates of GTD fungi identified two genera and four species (Table 1). Optimum temperatures for growth in culture varied among the tested isolates, ranging from 20.7°C of *Ilyonectria liriodendri* to 28.7°C of *Phaeoacremonium* sp. All optimum growth temperatures were statistically different among isolates, except between *Phaeomoniella chlamydospora* and *Cadophora lutea-olivacea*, which had the same optimum growth temperature (22.7°C). All tested isolates grew at 5°C except for *N. parvum* which did not grow at 5°C but did at 10°C. Different values were recorded for the upper temperature limits for growth, which were 25°C for *C. luteo-olivacea* and *I. liriodendri*, 30°C for *Neonectria* sp., and 35°C for *N. parvum*, *Phaeoacremonium* sp. and *P. chlamydospora*. When incubated at lower temperatures, mycelial plugs of *I. liriodendri* isolates maintained at 35°C did not show any growth, while 40°C killed all the other tested isolates, except *Phaeoacremonium* sp.

Comparisons between nucleotide sequences of the isolates and those present in Genbank confirmed the specific morphological identifications for *C. luteo-olivacea*, *I. liriodendri*, *N. parvum* and *P. chlamydospora*. For these species, the BLAST searches yielded 100% similarity with ex-type sequences in the GenBank database. For strains T2 and T21, the BLAST results did not allow their identification at species level.

Cadophora lutea-olivacea is reported to be associated with declining *V. vinifera* in Italy, confirming the report by Raimondo *et al.* (2019). *Cadophora lutea-olivacea* colonies on MEA were flat, felty, greenish-olivaceous in colour, with even edges (Figure 2a). All *C. luteo-olivacea* isolates grown on MEA had aerial mycelium consisting of branched and septate hyphae, singly or grouped up to three to five. Hyphal swellings were occasionally observed (Figure 2b). Conidiophores originating from hyphae were not usually branched, but were septate, mostly short, erect (Figure 2c) and sometimes flexuous (Figure 2d). Terminal or lateral phialides were mostly monophialidic (Figure 2e), smooth, hyaline, and with cylindrical collarettes, and were sometimes long with

Table 1. Morphological and molecular details of GTD fungi assessed in this study.

Isolate	Genus and species	*Vitis vinifera* cultivar	GenBank Access No. ITS	Conidium dimensions (µm)*	Mean ± SD (µm) **	L/W (µm) ***	Opt. growth T (°C)	GenBank Ex-type Access No. *(no ex-type)*	Coverage %	Similarity with ex-type *(no ex-type)*
T7	*Cadophora luteo-olivacea*	cv. Muller-Thurgau	MK589330	-	-	-	-	**NR_165945**	99.81	100
T23	*C. luteo-olivacea*	cv. Muller-Thurgau	MK589331	(4.6-)4.7 - 7.2(-7.9) x (1.6-)1.8 - 3.1(-3.4)	5.9 ± 0.8 x 2.5 ± 0.4	2.5 ± 0.5	22.7 b	**NR_165945**	**99.81**	**100**
T12	*Ilyonectria liriodendri*	cv. Muller-Thurgau	MK589332	microconidia (8.2-)8.6 - 19.0(-20.3) x (2.2-)2.5 - 6.5(-7.0) macroconidia (23.3-)23.6 - 38.5(-39.0) x (2.9-) 3.1 - 4.6(-5.7)	13.1 ± 3.4 x 4.0 ± 1.2	3.4 ± 0.6	20.7 a	**N_R119565.1**	**95**	**100**
T21	*Neonectria* sp.	cv. Muller-Thurgau	MK589335	microconidia (2.6-)5.2 - 7.3(-8.0) x (1.6-)2.0 x 3.7(-3.8) macroconidia (12.2-)12.3 - 35.5(-37.5) x (2.1-) 2.4 - 5.3(-6.0)	6.3 ± 0.9 x 2.8 ± 0.5	2.3 ± 0.4	24.7 c	***(HQ843921)***	**99**	***(100)***
T1	*Neofusicoccum parvum*	cv. Muller-Thurgau	MK589318	(11.7-)12.9 - 19.2(-21.1) x (5.2-)5.8 – 8.1(-9.0)	16.3 ± 1.9 x 7.1 ± 0.8	2.3 ±0.4	26.2 d	**FJ545228**	**99.63**	**100**
T9	*N. parvum*	cv. Muller-Thurgau	MK589319	-	-	-	-	**FJ545228**	**99.63**	**100**
T24	*N. parvum*	cv. Muller-Thurgau	MK589320	-	-	-	-	**FJ545228**	**99.63**	**100**
T2	*Phaeacremonium* sp.	cv. Muller-Thurgau	MK589321	(2.8-)3.6 – 4.8(-6.0) x (1.1-)1.2 - 2.1(-2.3)	4.2 ± 0.5 x 1.6 ± 0.3	2.6 ± 0.5	28.7 e	***(AF19783.1)***	**100**	***(99.73)***
PC1	*Phaeomoniella chlamydospora*	cv. Grecanico	MN597999	(2.7-)2.8 – 3.7(-3.8) x (0.7-)0.9 – 1.4(-1.5)	3.2 ± 0.3 x 1.1 ± 0.2	2.9 ± 0.5	22.7 b	**MH862510**	**99.82**	**100**
PC2	*P. chlamydospora*	cv. Grecanico	MN598000	-	-	-	-	**MH862510**	**99.82**	**100**
PC3	*P. chlamydospora*	cv. Grecanico	MN598001	-	-	-	-	**MH862510**	**99.82**	**100**

* Data presented as the lower and upper 95% confidence limits from 50 conidia, with minimum and maximum dimensions in parenthesis.
** Mean and S.D., standard deviation of 50 conidia.
*** L/W, length/width ratio, mean and standard deviation from 50 conidia.
Means accompanied by the same letters are not statistically different (Fisher's LSD tests at $P < 0.05$).
Data in bold are for ex-type specimens, in brackets to non-ex-type.

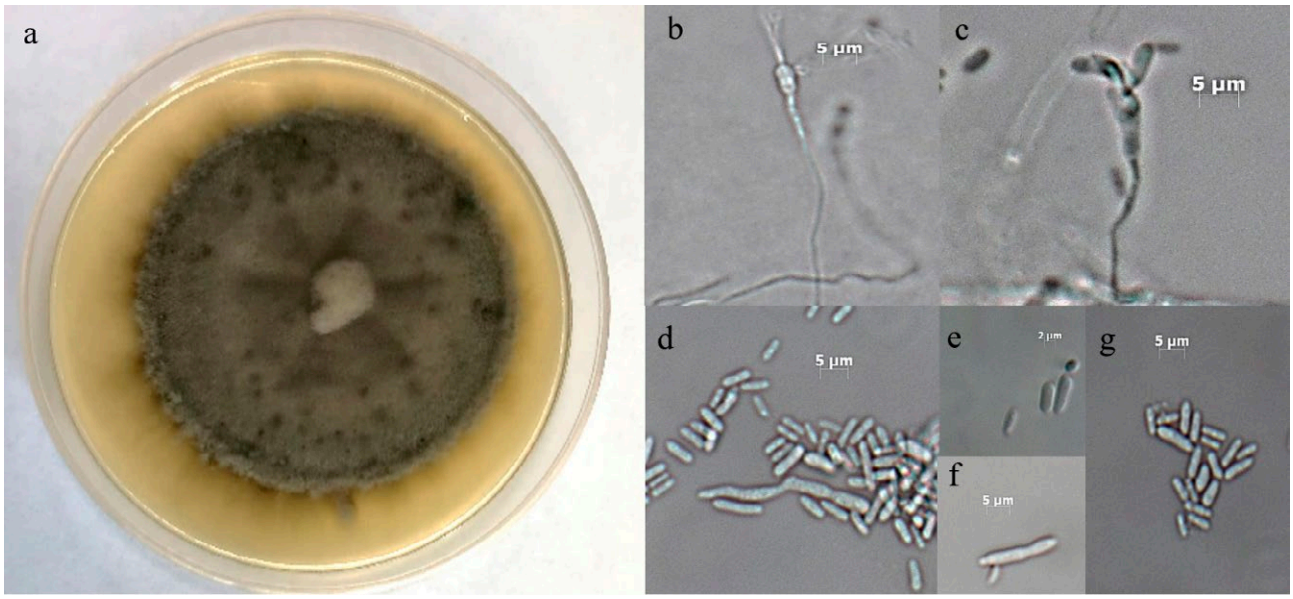

Figure 2. Macroscopic and microscopic features of *Cadophora luteo-olivacea* isolated from declining young grapevines cv. Muller-Thurgau in Sicily. a, colony growing on MEA. b, c and d, conidiophores of *C. luteo-olivacea*. d and e, short terminal or lateral phialides. g, hyaline conidia.

ampulliform bases (Figure 2f). Conidia were hyaline, with 2-3 guttules, ovoid or oblong ellipsoidal (Figure 2g). These microscopic features were similar to those reported for *C. luteo-olivacea* by Gramaje *et al.* (2011b).

Incidence of Botryosphaeria dieback, black foot and Petri disease fungi in sampled vines

Eighty-three percent of the analysed plants were infected by at least one GTD pathogen. Two GTD fungi were isolated from the same plant in 41% of the collected vines and 17% of these vines yielded three GTD fungi. Incidence of the different GTD pathogens, as indicated by the fungi isolated from young grapevines, is shown in Table 2. Overall, the proportion of vines infected by GTD pathogens was less for cv. Grecanico plants than for cv. Muller-Thurgau plants. BD associated fungi were the less isolated in both the cultivars. In cv. Grecanico vines, PD associated pathogens were dominant, while both BFD and PD associated pathogens prevailed in cv. Muller-Thurgau vines.

Distribution and relative presence of grapevine trunk disease pathogens in declining vines

The isolation frequencies (IFs) of fungi associated with GTDs are shown in Table 3. These were different, both in number and type, depending on grapevine cultivar and on trunk portion. Among the fungi identified, *Neonectria* sp. and *P. chlamydospora* were isolated from all cv. Grecanico trunk portions, and similarly for *I. liriodendri*, *C. luteo-olivacea* and *Phaeoacremonium* from cv. Muller-Thurgau trunk portions.

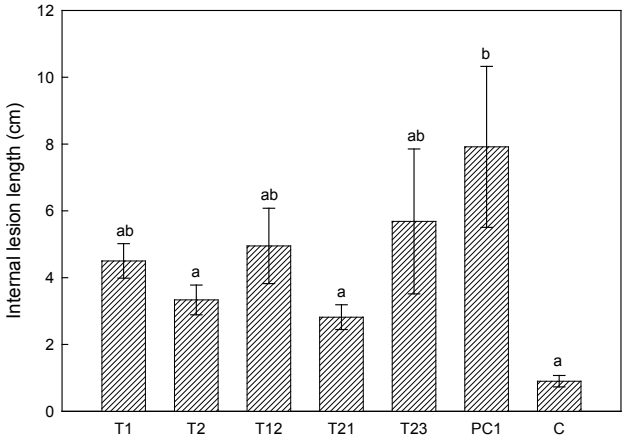

Figure 3. Mean internal lesion lengths (cm) on excised grapevine canes (cv. Grecanico) 15 d after inoculation with isolates of *Neofusicoccum parvum* (T1), *Phaeoacremonium* sp. (T2), *Ilyonectria liriodendri* (T12), *Neonectria* sp. (T21), *Cadophora luteo-olivacea* (T23), or *Phaeomoniella chlamydospora* (PC1), compared to non-inoculated controls (C). The vertical lines indicate standard errors. Bars accompanied by the same letter are not significantly different ($P < 0.05$, Fisher's LSD test).

Table 2. Incidence (%) of the different grapevine trunk diseases (GTDs) in whole grapevine plants and in plant parts of cvs Grecanico and Muller-Thurgau from two localities in Sicily. BD = Botryosphaeria dieback, BFD = black foot disease, PD = Petri disease. Percentages were calculated from the number of plants showing symptoms (in brackets) and the total number of samples per vineyard.

Locality (cultivar)	Number of plants	Disease	% of GTD-affected plants (N° of plants)	% of GTD affected plant parts (N° of plants)		
				apical	medium	basal
Castellammare (cv. Grecanico)	20	BD	25 (5)	25.0 (5)	5.0 (1)	0.0 (-)
		BFD	25 (5)	5.0 (1)	10.0 (2)	20.0 (4)
		PD	45 (9)	30.0 (6)	25.0 (5)	30.0 (6)
		Total GTD	***75 (15)***	***55.0 (11)***	***35.0 (7)***	***45.0 (9)***
Partanna (cv. Muller-Thurgau)	9	BD	66.7 (6)	66.7 (6)	0.0 (-)	0.0 (-)
		BFD	88.9 (8)	33.3 (3)	55.5 (5)	88.9 (8)
		PD	88.9 (8)	22.2 (2)	66.7 (6)	33.3 (3)
		Total GTD	*100.0 (9)*	*88.9 (8)*	*88.9 (8)*	*100.0 (9)*

Table 3. Isolation frequency (IF) of grapevine trunk disease (GTD) fungi from symptomatic cv. Grecanico and Muller-Thurgau grapevine samples from two localities in Sicily.

Locality (cultivar)	Disease	GTD fungi isolated	Total IF (%)	Isolation frequency of GTD fungi from trunk portions (%)		
				apical	medium	basal
Castellammare (cv. Grecanico)	BD	*Botryosphaeriaceae*	2.0	1.1	0.9	0.0
		Lasiodiplodia sp.	0.4	0.4	0.0	0.0
	BFD	*Neonectria* sp.	1.3	0.1	0.2	1.0
	PD	*P. chlamydospora*	4.7	1.0	1.7	1.9
Partanna (cv. Muller-Thurgau)	BD	*N. parvum*	4.1	4.1	0.0	0.0
	BFD	*I. liriodendri* sp.	10.4	1.7	3.2	5.4
		Neonectria sp.	2.2	0.0	2.0	0.2
	PD	*C. luteo-olivacea*	5.1	0.5	2.8	2.0
		Phaeoacremonium sp.	3.6	0.4	2.3	0.9

Pathogenicity tests

At 15 d after inoculation, control canes developed only slight subcortical discolouration upward and downward from the wound sites (mean total length = 0.9 cm). Canes inoculated with GTD pathogen isolates had bark lesions and subcortical longitudinal discolouration extending upward and downward from the inoculation points. Mean discolouration lengths varied among the fungal isolates. The greatest discoloration length (mean = 7.9 cm) resulted from inoculation with *P. chlamydospora*. The least discolouration length (2.8 cm) was caused by *Neonectria* sp., while *C. luteo-olivacea* (5.7 cm), *I. liriodendri* (5.0 cm) and *N. parvum* (4.5 cm) each gave similar amounts of discolouration. Only lesions from *P. chlamydospora* inoculations were significantly longer than the experimental controls (Figure 3). All the GTD fungi tested were re-isolated from inoculated plants, fulfilling Koch's postulates, and no fungal pathogens were isolated from the control plants.

DISCUSSION

This study has confirmed the presence of YVD in western Sicilian vineyards and has shown that different GTD pathogens were associated with the disease. All fungi identified in this study are known to be pathogens causing YVD in different wine growing regions (Rego *et al.,* 2006; Gramaje *et al.*, 2009; Agusti-Brisach and Armengol, 2013, Urbez-Torres and O'Gorman, 2014; Armengol, 2014, Carlucci *et al.*, 2015, 2017; Baranek *et al.*, 2018).

Morphological and molecular analyses identified six genera, including *Cadophora*, *Ilyonectria*, *Neonectria*, *Neofusicoccum*, *Phaeoacremonium* and *Phaeomoniella*

associated with YVD in cv. Grecanico and cv. Muller-Thurgau vines. According to their low IF and sporadic presence in the sub-samples, *Lasiodiplodia* and the other *Botryosphaeriaceae* were not considered to be associated with YVD in the two vineyards sampled in this study. Overall, a large proportion of vines was infected by at least one GTD fungus. This incidence was greater than previously recorded in Spain (minimum 9.6%, maximum 76.4%: Aroca *et al.,* 2010; Gramaje *et al.,* 2009; Gimenez–Jaime *et al.,* 2006), but similar to the values reported in 2018 by Pintos *et al.* (81-100%). Other recent studies carried out in Italy (Puglia and Molise regions) showed lower incidence of YVD fungi, both in nurseries and young plantations (4.4 to 24.4%), (Carlucci *et al.,* 2017).

In the present study, the incidence of the different GTDs (BD, BFD and PD) linked to the observed YVD was different for the two grapevine cultivars. In cv. Muller-Thurgau vines, all the samples were infected with GTD fungi with greater incidences of PD and BFD fungi than BD pathogens. For cv. Grecanico, lower incidence was recorded, with greater prevalence of PD than BD and BFD pathogens. Among fungi known to be associated with PD, *Phaeoacremonium* spp. have been the most frequently isolated (Raimondo *et al.,* 2014) along with *P. chlamydospora* (Mugnai *et al.,* 1999; Raimondo *et al.,* 2014), and *Pleurostoma richardsiae* (Carlucci *et al.,* 2013; Réblová *et al.,* in 2015). In the present study, the PD pathogens were *P. chlamydospora* from cv. Grecanico, and *P. chlamydospora*, *C. luteo-olivacea* and *Phaeoacremonium* sp. from cv. Muller-Thurgau vines.

The presence of BFD is now confirmed in many wine-growing areas, including Portugal, Spain, South Africa and the United States of America (Rego *et al.,* 2000; Halleen *et al.,* 2004; Petit and Gubler, 2005; Alaniz *et al.,* 2007; Gramaje *et al.,* 2010; Agusti-Brisach and Armengol, 2013). In the 1980s, BFD in Italy was associated with *C. obtusisporum*, *C. destructans* and *P. glomerata* (Grasso and Magnano di S. Lio, 1975; Granata e Refatti, 1981; Grasso, 1984), and more recently also with *Dactylonectria torresensis*, *I. liriodendri* and *Thelonectria* sp. (Carlucci *et al*., 2017). In the present study, the BFD-associated fungal genera were *Ilyonectria* and *Neonectria*, both present in cv. Muller-Thurgau vines, with *Neonectria* only found in cv. Grecanico samples.

Regarding the status of BD in Italy, the role of *Botryosphaeriaceae* damaging vineyards has been recognised since the late 1970s (Cristinzio, 1978; Rovesti and Montermini, 1987; Burruano *et al.,* 2008, Mondello *et al.,* 2013; Carlucci *et al.,* 2015). Results from the present study confirm the presence of *Botryosphaeriaceae* spp. in young vineyards, indicating a potential secondary role for these pathogens in YVD.

Among the four fungi identified to species level, the presence of *C. luteo-olivacea* isolated from symptomatic wood of cv. Muller-Thurgau is significant. This fungus is now considered a pathogen and part of the Petri disease causal agents in young vines (Halleen, *et al.,* 2007; Gramaje *et al.,* 2011b; Raimondo *et al*., 2019). The present study confirms *C. luteo-olivace*a to be associated with declining *V. vinifera* in Italy.

Despite most YVD-affected vines yielding multiple fungi associated with BD, BFD and PD, this study revealed different localization and abundance of specific GTD pathogens from different parts of the collected vines. In cv. Grecanico samples, *P. chlamydospora* was the most isolated fungus from along the rootstocks and grafting areas, and was always associated with brown wood streaking. In cv. Muller-Thurgau, the black foot pathogens *Ilyonectria* and *Neonectria* were the most abundant, and were specifically found at the bases of the rootstocks, associated with necrotic wood. These results indicate that PD could be the main GTD responsible for the vine decline observed in Castellammare vineyards, and BFD was important in Partanna vineyards.

High pathogen incidence in such young vines, and their abundance in the distal parts (grafting points and rootstock bases) indicate the pre-existence of the observed GTD infections before young vines are planted in the field. This has also been suggested by several authors, where *P. chlamydospora*, *Phaeoacremonium* spp., *Botryosphaeriaceae*, *Cylindrocarpon* spp., *Ilyonectria* and other pathogens have been commonly found during the vine propagation processes in nurseries, frequently resulting as mixed infections (Waite *et al.,* 2015). The frequency of mixed infections was also indicated in our assays, where the different genera associated with the observed YVD did not show significant differences in growth at low temperatures. This suggests high probability of multiple infections, in the field. Variability in the optimum and maximum growth temperature values would also influence the evolution of infections. Host colonization could vary, if environmental temperatures diverge from pathogen thermal needs, so affecting the incidence of the diseases they cause.

There is no single and simple control method for vine trunk diseases (Mondello *et al.,* 2018a), and the present results further confirm the urgency in adopting a "global strategy" for management of GTDs along all stages of grapevine vegetative and productive life (Armengol, 2014). Strategies using different methods can be suggested to nurserymen and winegrowers (Halleen and Fourie, 2016; Gramaje *et al.,* 2018, Mondello *et al*., 2018b) to limit the economic impacts of YVD and GTDs.

It is important to emphasize that the pathogenicity tests on detached canes did not show statistically significant differences among several isolates, except for *P. chlamydospora*. This result could be explained by the generally accepted complexity of interactions between *V. vinifera* and GTD pathogens. Disease symptoms are linked to the diversity of fungi, to the metabolites they produce (toxins and exopolysaccharides), the environmental conditions, and to the phenological stages of host grapevine plants (Spagnolo *et al*., 2017; Fisher and Peighami Ashnaei, 2019; Mondello *et al*., 2019; Reis *et al*., 2019). Models used to study GTD pathogen virulence are unlikely to represent all these variables.

Further studies are required to evaluate the spread of YVD in other young vineyards in Sicily, and to better characterize the roles of the different pathogens in YVD in this part of Italy.

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LITERATURE CITED

Agustí-Brisach C., Gramaje D., León M., García-Jiménez J., Armengol J., 2011. Evaluation of vineyard weeds as potential hosts of Black-foot and Petri Disease pathogens. *Plant Disease* 95: 803–810. DOI: 10.1094/PDIS-12-10-0888

Agustí-Brisach C., Armengol J., 2013. Black-foot disease of grapevine: an update on taxonomy, epidemiology and management strategies. *Phytopathologia Mediterranea* 52(2): 245–261. DOI: 10.14601/Phytopathol_Mediterr-12662

Aigoun-Mouhous W., Elena G., Cabral A. Lèon M., Sabaou N., …, Berraf-Tebbal A., 2019. Characterization and pathogenicity of *Cylindrocarpon*-like asexual morphs associated with black foot disease in Algerian grapevine nurseries, with the description of *Pleiocarpon algeriense* sp. nov. *European Journal of Plant Pathology* 154: 887–901.

Alaniz S., León M., Vicent A., García-Jiménez J., Abad-Campos P., Armengol J., 2007. Characterization of *Cylindrocarpon* species associated with black foot disease of grapevine in Spain. *Plant Disease* 91: 1187–1193.

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389–3402

Armengol J., 2014. Fungal trunk pathogens in grapevine nurseries: current status and future. In: COST Action FA1303 Sustainable control of Grapevine trunk diseases Workshop. Warsaw. Retrieved from http://managtd.eu/images/uploads/content/125/Armengol%20J_Grapevine%20nurseries.pdf

Aroca Á., Gramaje D., Armengol J., García-Jiménez, J., Raposo, R., 2010. Evaluation of the grapevine nursery propagation process as a source of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* and occurrence of trunk disease pathogens in rootstock mother vines in Spain. *European Journal of Plant Pathology* 126(2): 165–174. DOI: 10.1007/s10658-009-9530-3

Baránek M., Armengol J., Holleinová V., Pečenka J., Calzarano F., …, Eichmeier A., 2018. Incidence of symptoms and fungal pathogens associated with grapevine trunk diseases in Czech vineyards: fist example from a north-eastern European grape-growing region. *Phytopathologia Mediterranea* 57(3): 49–458.

Barnett H.L., Hunter B., 1998. *Illustrated Genera of Imperfect Fungi*. St Paul, MN, USA, APS Press.

Burruano S., Mondello V., Conigliaro G., Alfonzo A., Spagnolo A., Mugnai L., 2008. Grapevine decline in Italy caused by *Lasiodiplodia theobromae*. *Phytopathologia Mediterranea* 47(2): 132–136.

Burruano S., Alfonzo A., Conigliaro G., Mondello V., Torta L., 2010. Il mal dell'esca della vite in Sicilia. In: *Il Mal dell'Esca della Vite Interventi di ricerca e sperimentazione per il contenimento della malattia-Progetto MESVIT*. Ed. ARSIA Regione Toscana: 171-177.

Cabral A.C.G.P., 2012 - New insights in *Ilyonectria* black foot disease of grapevine. PhD Thesis, Lisbon: ISA, 2012 http://hdl.handle.net/20.500.11755/cf66c9a9-46e1-4bef-881f-467a9fc08d42.

Carlucci A., Raimondo M. L., Cibelli F., Phillips A. J. L., Lops F., 2013. *Pleurostomophora richardsiae*, *Neofusicoccum parvum* and *Phaeoacremonium aleophilum* associated with a decline of olives in southern Italy. *Phytopathologia Mediterranea* 52(3): 517–527.

Carlucci A., Cibelli F., Lops F., Raimondo, M. L., 2015. Characterization of Botryosphaeriaceae Species as Causal Agents of Trunk Diseases on Grapevines. *Plant Disease* 99: 1678–1688. DOI: 10.1094/PDIS-03-15-0286-RE

Carlucci A., Lops F., Mostert L., Halleen F., Raimondo M., 2017. Occurrence fungi causing black foot on young grapevines and nursery rootstock plants in Italy. *Phytopathologia Mediterranea* 56(1): 10-39.

Cristinzio G., 1978. Gravi attacchi di *Botryosphaeria obtusa* su vite in provincia di Insernia. *Informatore Fitopatologico* 6: 21–23.

Domsch KH, Gams W, Anderson TH., 1980. *Compendium of Soil Fungi*. London, UK, Academic Press.

Fischer M., Peighami Ashnaei S., 2019. Grapevine, esca complex, and environment: the disease triangle. *Phytopathologia Mediterranea* 58(1): 17–37. DOI: 10.14601/Phytopathol_Mediterr-25086

Gardes M., Bruns T.D., 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118. DOI: 10.1111/j.1365-294X.1993.tb00005.x

Giménez-Jaime A., Aroca A., Raposo R., García-Jiménez J., Armengol J., 2006. Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. *Journal of Phytopathology* 154: 598–602.

Gramaje D., Muñoz, R. M., Lerma M. L., García-Jiménez, J., Armengol, J., 2009. Fungal grapevine trunk pathogens associated with Syrah decline in Spain. *Phytopathologia Mediterranea* 48 (3): 396–402. DOI: 10.14601/Phytopathol_Mediterr-2934

Gramaje D., Alaniz S., Abad-Campos P., García-Jiménez J., Armengol, J., 2010. Effect of hot-water treatments in vitro on conidial germination and mycelial growth of grapevine trunk pathogens. *Annals of Applied Biology* 156(2): 231–241. DOI: 10.1111/j.1744-7348.2009.00382.x

Gramaje D., Armengol, J. 2011a. Fungal trunk pathogens in the grapevine propagation process: potential inoculum sources, detection, identification, and management strategies. *Plant Disease* 95: 1040–1055. DOI: 10.1094/PDIS-01-11-0025

Gramaje D., Mostert L., Armengol J., 2011b. Characterization of *Cadophora luteo-olivacea* and *C. melinii* isolates obtained from grapevines and environmental samples from grapevine nurseries in Spain. *Phytopathologia Mediterranea* 50 (S): 112–126.

Gramaje D., Di Marco S., 2015. Identifying practices likely to have impacts on grapevine trunk disease infections: a European nursery survey. *Phytopathologia Mediterranea* 54 (2), 313–324. DOI: 10.14601/Phytopathol_Mediterr-16317

Gramaje D., Urbez-Torres J. R., Sosnowski M. R., 2018. Managing grapevine trunk diseases with respect to etiology and epidemiology: current strategies and future prospects. *Plant Disease* 102: 12–39. DOI: 10.1094/PDIS-04-17-0512-FE

Granata, G., Refatti E., 1981. Decline and death of young grapevines by infection of *Phoma glomerata* on the rootstock. *Vitis* 20(8): 341–346.

Grasso S., 1984. Infezioni di *Fusarium oxysporum* e di *Cylindrocarpon destructans* associate a una moria di giovani piante di vite in Sicilia. *Informatore Fitopatologico* 1: 59–63.

Grasso S., Magnano Di San Lio G., 1975. Infezioni di *Cylindrocarpon obtusisporum* su piante di vite in Sicilia. *Vitis* 14: 38–39.

Halleen F., P. W. Crous, Petrini O., 2003. Fungi associated with healthy grapevine cuttings in nurseries, with special reference to pathogens involved in the decline of young vines. *Australasian Plant Pathology* 32: 47–52 DOI: 10.1071/AP02062

Halleen F., Schroers H. J., Groenewald J. Z., Crous P. W., 2004. Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). *Studies in Mycology* 50(2): 431–455.

Halleen F., Mostert L., Crous P.W., 2007. Pathogenicity testing of lesser-known vascular fungi of grapevines. *Australasian Plant Pathology* 36: 277–285.

Halleen F., Fourie P. H., 2016. An integrated strategy for the proactive management of grapevine trunk disease pathogen infections in grapevine nurseries. *South African Journal of Enology and Viticulture* 37(2): 104–114. DOI: 10.21548/37-2-825

Mondello V., Lo Piccolo S., Conigliaro G., Alfonzo A., Torta, L., Burruano S., 2013. First report of *Neofusiccoccum vitifusiforme* and presence of other Botryosphaeriaceae species associated with Botryosphaeria dieback of grapevine in Sicily (Italy). *Phytopathologia Mediterranea* 52(2): 388–396.

Mondello V., Songy A., Battiston E., Pinto C., Coppin C., … Fontaine F., 2018a. Grapevine trunk diseases: a review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Disease* 102: 1189–1217.

Mondello V., Larignon P., Armengol J., Kortekamp A., Vaczy K., …, Fontaine F., 2018b. Management of grapevine trunk diseases: knowledge transfer, current strategies and innovative strategies adopted in Europe. *Phytopathologia Mediterranea* 57(3): 369-383 DOI: 10.14601/Phytopathol_Mediterr-23942

Mondello V., Spagnolo A., Larignon P., Clément C., Fontaine F., 2019. Phytoprotection potential of *Fusarium proliferatum* for control of Botryosphaeria dieback pathogens in grapevine. *Phytopathologia Mediterranea* 58(2): 293-306. DOI: 10.14601/Phytopathol_Mediter-10617.

Mugnai L., Graniti A., Surico G., 1999. Esca (Black Measles) and Brown Wood-Streaking: Two Old and Elusive Diseases of Grapevines. *Plant Disease* 83(5): 404–418. DOI: 10.1094/PDIS.1999.83.5.404

O'Donnell K., Cigelnik E., Nirenberg H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 90: 465–493.

Petit E., Gubler W. D., 2005. Characterization of *Cylindrocarpon* species, the cause of Black Foot disease of grapevine in California. *Plant Disease* 89(10): 1051–1059. DOI: 10.1094/PD-89-1051

Pintos C., Redondo V., Costas D., Aguín O., Mansilla P., 2018. Fungi associated with grapevine trunk diseases in nursery-produced *Vitis vinifera* plants. *Phytopathologia Mediterranea* 57(3): 407–424.

Pitt J.I, Hocking A.D., 1999. *Fungi and Food Spoilage*, 2nd edn. Gaithersburg, MD, USA, Aspen Publishers, Inc.

Raimondo M. L., Lops F., Carlucci A., 2014. *Phaeoacremonium italicum* sp. nov., associated with esca of grapevine in southern Italy. *Mycologia* 106(6): 1119–1126. DOI: 10.3852/14-080

Raimondo M.L., Carlucci A., Ciccarone C., Sadallah A., Lops F., 2019. Identification and pathogenicity of lignicolous fungi associated with grapevine trunk diseases in southern Italy. *Phytopathologia Mediterranea* 58(3): 639-662. doi: 10.14601/Phyto-10742

Réblová M., Jaklitsch W. M., Réblová K., Štěpánek V., 2015. Phylogenetic Reconstruction of the *Calosphaeriales* and *Togniniales* using five genes and predicted RNA secondary structures of ITS, and *Flabellascus tenuirostris* gen. et sp. nov. *PLOS One* 10(12): e0144616. Retrieved from DOI: 10.1371/journal.pone.0144616

Rego C., Oliveira H., Carvalho A., Phillips A., 2000. Involvement of *Phaeoacremonium* spp. and *Cylindrocarpon destructans* with grapevine decline in Portugal. *Phytopathologia Mediterranea* 39(1): 76–79.

Rego C., Nascimento T., Cabral A., Oliveira H., 2006. Fungi associated with young vine decline in Portugal: results of nine years surveys. *Integrated Protection in Viticulture* IOBC/wprs Bulletin Vol. 29(11), 2006 pp. 123-126 123.

Rego, C., Nascimento, T., Cabral, A., Silva M. J., Oliveira H., 2009. Control of grapevine wood fungi in commercial nurseries. *Phytopathologia Mediterranea* 48(1): 128–135.

Reis P., Pierron R., Larignon P., Lecomte P., Abou-Mansour E., Farine S., … Fontaine F., 2019. *Vitis* methods to understand and develop strategies for diagnosis and sustainable control of Grapevine Trunk Diseases. *Phytopathology* 109(6): 916–931.

Rovesti L., Montermini A., 1978. A grapevine decline caused by *Sphaeropsis malorum* widespread in the province of Reggio-Emilia. *Informatore Fitopatologico* 37: 59–61.

Sidoti A., Buonocore E., Serges T., Mugnai L., 2000. Decline of young grapevines associated with *Phaeoacremonium chlamydosporum* in Sicily (Italy). *Phytopathologia Mediterranea* 39(1): 87–91. DOI: 10.14601/Phytopathol_Mediterr-1533.

Smart R., Mugnai L., Lane C., 2012. International viticultural consultant's view of grapevine trunk diseases and their impact on clients. *Phytopathologia Mediterranea* 51: 433–434.

Spagnolo A., Mondello V., Larignon P., Villaume S., Rabenoelina F., …, Fontaine F. 2017. Defense responses in grapevine (cv. Mourvèdre) after inoculation with the Botryosphaeria dieback pathogens *Neofusicoccum parvum* and *Diplodia seriata* and their relationship with flowering. *International Journal of Molecular Sciences,* 18: 393. DOI: 10.3390/ijms18020393

Úrbez-Torres J. R., O'Gorman D. T., 2014. Trunk Diseases in British Columbia: Incidence and Characterization of the Fungal Pathogens Associated with Black Foot Disease of Grapevine. *Plant Disease* 98: 456–468.

Úrbez-Torres J. R., Haag P., Bowen P., Lowery T., O'Gorman D. T., 2015. Development of a DNA macroarray for the detection and identification of fungal pathogens causing decline of young grapevines. *Phytopathology* 105: 1373–1388.

Waite H., Whitelaw-Weckert M., Torley P., 2015. Grapevine propagation: principles and methods for the production of high-quality grapevine planting material. *New Zealand Journal of Crop and Horticultural Science* 43(2): 144–161. DOI: 10.1080/01140671.2014.978340

White T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In: *PCR protocols: A guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, ed.). Academic Press, San Diego, CA, USA, 315–322.