Eur J Plant Pathol
https://doi.org/10.1007/s10658-018-1458-z

Study of *Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune*, three pathogenic fungi associated with the Grapevine Trunk Diseases in the North of Tunisia

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Accepted: 6 March 2018

Abstract Re-emergence of Grapevine Trunk Diseases (GTDs) in many world vineyards within the span of a mere two decades has become a subject of major concern for the viticulture sector. In Tunisia, knowledge about the symptoms of GTDs and the associated microflora is hitherto incomplete. In order to investigate this point, ten vineyards served as model for the analysis and monitoring of the symptoms of GTDs on cv. Italia (Muscat d'Italie) used to produce table grapes, in the Northern region of Tunisia. Depending on the vineyards, typical GTD-foliar symptoms with incidence ranging from 0.03% to 6% were observed. Fungal strains were isolated from necrotic wood tissues of the trunk of grapevines showing decline, small and distorted leaves and chloroses. Sequencing of the rDNA-Internal Transcribed Spacer region of the fungal strains allowed us to identify for the first time in Tunisia three fungi described in the literature as involved in GTDs: *Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune.* The ability of these fungal strains to grow in Mediterranean climate was investigated by studying mycelial growth at various temperatures. As these fungal strains were isolated from the same wood samples, their ability to compete in vitro and *in planta* was tested, as well as their pathogenicity. For that purpose, strains were inoculated singly or in combination on cv. Italia young vines. Pathogenicity of the isolated fungi was evident but positive interaction between fungal strains was not observed.

Keywords GTDs · Microbial competition · Pathogenic fungi · Pathogenicity · Table grape cultivar · Temperature

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Introduction

Viticulture has a long history in Tunisia dating back to the Carthaginian civilization (Zoghlami et al. 2001, 2009). This longstanding viticulture tradition has persisted until now and numerous international as well as autochthonous cultivars are planted in the Tunisian vineyards. Grapevines are used to produce either wine or table grapes, accordingly grape is amongst the most important fruit crops in Tunisia (Ben Salem et al. 2000; Zoghlami et al. 2001). Table- and wine-grape productions are of great economic importance in Tunisia and

Published online: 10 March 2018

worldwide. Grapevine, i.e. *Vitis vinifera* L., is however susceptible to many fungal pathogens that can alter to a considerable extent the yield and/or quality of the harvest (Bertsch et al. 2013; Compant et al. 2013; Steel et al. 2013). Amongst the fungal diseases that can severely damage crops, Grapevine Trunk Diseases (GTDs), i.e. Esca, Eutypa and Botryosphaeria diebacks, are among the most severe diseases that affect grapevines worldwide. Although GTDs, mainly Esca, have been recorded for centuries, they have become within the span of a mere two decades, a subject of major concern for the wine industry. GTDs decrease harvest, increase costs, limit grape and wine qualities, and reduce the lifespan of plants in many vine-growing areas (Mugnai et al. 1999; Crous and Gams 2000; Armengol et al. 2001; Larignon et al. 2009; Liminana et al. 2009; Lorrain et al. 2012; Bertsch et al. 2013; Bruez et al. 2013, De la Fuente et al. 2016). This epidemic is often associated with the ban on the use of sodium arsenate in 2003 in Europe but GTDs have also progressed in countries where this pesticide has never been used, e.g. Germany, Switzerland, or in a very limited manner, e.g. Italy. Other factors are therefore involved. Abiotic factors such as climatic conditions (Sosnowski et al. 2007), soil types (Mugnai et al. 1999) and/or pruning practices (Armengol et al. 2001; Valtaud et al. 2009; Agustí-Brisach et al. 2015) may trigger or aggravate GTDs foliar expression. As regards the biotic factors, several fungi are associated with GTDs worldwide (Armengol et al. 2001), including various Botryosphaeriaceae species. Those are important destructive pathogens that cause canker, dieback and fruit rot in grapevine and in other woody host plants; and that can result in plant mortality (Johnson et al. 1991, 1993; Ramos et al. 1991; Brown-Rytlewski and McManus 2000; Smith et al. 2001; Slippers et al. 2005; Damm et al. 2007; Amponsah et al. 2011; Thomidis et al. 2011; Ni et al. 2012; Bruez et al. 2014). The infection can be diagnosed by the presence in the wood forming tissues of sectorial and/or central necrosis, and at the foliar level by discoloration and withering (Ben Ghnaya-Chakroun et al. 2014). The predisposition of plants to Botryosphaeriaceae attacks can result from various elements such as mechanical injuries, mineral deficiencies and environmental factors (Slippers and Wingfield 2007; Ismail et al. 2012). Amongst the Botryosphaeriaceae family, many species are linked to GTDs, e.g. *Lasiodiplodia theobromae* (*Botryodiplodia theobromae*) cause dead-arm and dieback disease in major crops with up to 500 plant hosts including grapevine (El-Goorani and El Meleigi 1972; Punithalingam 1980; Bester et al. 2007; Ismail et al. 2012; Abo rehab et al. 2013; Tovar Pedraza et al. 2013; Che et al. 2015); and *Neofusicoccum parvum* (*Botryosphaeria parva*) is one of the most virulent fungal species associated with GTDs (van Niekerk et al. 2004; Laveau et al. 2009; Úrbez-Torres and Gubler 2009). Several other fungi have been associated with GTDs, in particular three basidiomycetes, i.e. *Fomitiporia punctata* (Armengol et al. 2001), *Fomitiporia mediterranea* (Fischer 2002) and *Stereum hirsutum* (Larignon and Dubos 1997); and two ascomycetes, i.e. *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* (Crous and Gams 2000).

In Tunisia, GTDs attacks on table grape cultivars have been recently reported by Ben Ghnaya-Chakroun et al. (2014) and Chebil et al. (2014), they isolated some pathogenic fungi associated with GTDs, i.e. *Botryosphaeria dothidea*, *Diplodia seriata*, *Neofusicoccum luteum* and *Phomopsis viticola*. However, the situation in the vineyards and the knowledge on the pathogenic fungal species complex involved are still relatively incomplete in Tunisia. In that context, a survey was firstly made to investigate whether GTDs were observed on the cultivar most frequently planted in Tunisia, cv. Italia (also known as Muscat d'Italie in Tunisia). Secondly, pathogenic fungal strains were looked for and isolated from necrotic wood tissues of diseased grapevines and investigations were carried out so as: (i) to determine their ability to grow at various temperatures, (ii) to compete in vitro and *in planta* and (iii) to cause necrosis in the wood of cv. Italia young vines after strains were inoculated singly or in combination.

Materials and methods

Field survey

Ten vineyards (10 to 17 year-old) planted with a table grape cultivar, i.e. Italia, were studied in different regions of Tunisia: four vineyards in the region of Mornag, two in Medjez El Bab, two in Borj el Amri and two in the Cité El Mehrine (Table 1). These ten vineyards served as model for the analysis and monitoring of the symptoms of GTDs in the Northern region of Tunisia.

Table 1 Italia cultivar (Muscat d'Italie) vineyards surveyed for GTDs in the northern Tunisian vine growing regions

Vine growing regions	Vineyards	Age of the cultivars in 2013	Rootstocks	Areas/ number of vines	Disease incidence
Mornag	Mornag	15 years	P1103	2 ha 2500 vines	6.00%
	Sidi Sâad	10 years	P1103	3 ha 4500 vines	0.33%
	Kheledia	17 years	P1103	1 ha 1200 vines	1.66%
	Zawiyet Mornag	15 years	P1103	5 ha 6000 vines	3.00%
Medjez El Bab	V1	14 years	P1103	1 ha 1275 vines	0.00%
	V2	13 years	P1103	2 ha 1700 vines	0.06%
Cité El Mehrine	V3	12 years	P1103	1 ha 1275 vines	0.00%
	V4	14 years	P1103	0.5 ha 637 vines	0.00%
Borj El Amri	V5	15 years	P1103	2 ha 2550 vines	0.03%
	V6	13 years	P1103	1 ha 1275 vines	0.30%

Information about disease incidence and symptoms mentioned in Table 1 were given by the owners of the prospected vineyards. All the vineyards visited were trellised as single vines on a pergola system. They were irrigated with a drip irrigation system and protected under hail nets.

Fungal isolation and characterisation

In order to maximize the potential presence of pathogenic strains involved in GTDs, cordons were randomly collected from five mature vines exhibiting external GTDs-foliar symptoms in vineyards showing the most important disease incidence according to the survey: the Mornag vineyard (6%) of the Mornag region and the vineyard V6 (0.3%) of the Borj El Amri region (Table 1). From the inner part of each cordon, wood samples were taken from necrotic and/or from non-necrotic (apparently healthy) tissues (around 5 mm in length). In total, 75 wood pieces (five cordons × 15 chips) were collected and analysed.

In order to eliminate the epiphytic fungi, the wood fragments were surface-sterilized by immersion in 2.5% calcium hypochlorite solution for 30 s, and then rinsed in sterile distilled water. The disinfected chips were dried on a sterile filter paper, plated onto Malt Agar medium (MA) (Biokar Diagnostics, France), and then incubated at 25 °C in the dark for 4 weeks. Plates were monitored daily and when fungal colonies emerged from wood tissues, mycelial fragments were subcultured onto fresh MA plates.

In order to verify the efficacy of the surface disinfection, imprints of sample surfaces were made on MA plates and monitored for one week to confirm that no epiphytic fungi had grown (Hyde and Soytong 2008).

The taxonomic identification of the recovered fungal strains was based on morphological and cultural features. Fifty-eight fungal strains were recovered from the 75 wood pieces collected: 16 strains from the necrotic tissues, 26 from the non-necrotic tissues and 16 strains from the cordon ends. Fifty-four strains were saprophytes and the four others, isolated from the necrotic tissues, belonged to three pathogenic species involved in GTDs according to the literature, i.e. *Neofusicoccum parvum*, *Lasidiodiplodia pseudotheobromae* and *Schizophyllum commune* (two strains). The colour and growth of those pathogenic isolates were recorded on MA medium after an incubation of three and seven days at 25 °C.

Identification of fungi by rDNA-ITS sequencing

The 16 strains isolated from the necrotic tissues were selected for molecular characterization. The strains were cultured onto MA plates at 25 °C for 7–10 days and the fresh mycelia were scraped at the surface of each plate

with a sterile tip and then freeze-dried overnight (Alpha 1–4 LOplus, Bioblock Scientific). The dried mycelia were ground with a little glass ball in a TissueLyserII (Qiagen) before the DNA extraction with a classical CTAB procedure (Bruez et al. 2014). The DNA extracts were then quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 50 ng/μL.

DNA samples were sent to Beckman Coulter Genomics (Takeley, United Kingdom) for sequencing the internal transcribed spacer region (ITS) of the rDNA with the primers ITS1 (5'-TCCGTAGGTGAACC TGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTG ATATGC-3′) (White et al. 1990). For species level identification (>97% sequence similarity threshold), sequences were compared with the Genebank database by using the Blastn program (Altschul et al. 1997).

Effect of the temperature on the in vitro mycelial growth of *L. pseudotheobromae*, *N. parvum* and *S. commune*

The effect of the temperature on the in vitro mycelial growth was assessed by placing a mycelium plug from the margin of a five-day-old culture of *L. pseudotheobromae*, *N. parvum* and *S. commune* in the centre of a new MA plate. Plates were then incubated in the dark at 5–40 °C in 5 °C intervals with 14 plates per isolate and per temperature. The radial growth of each fungus was measured (in mm) after 24, 48, 72 and 96 h (Valtaud et al. 2009; Ismail et al. 2012).

Fitting thermal-response model

Theory and data concur that biological rates essentially show unimodal responses to temperature (Johnson et al. 1974). Metabolic reaction rates tend to increase up to an optimal temperature, then decline due to protein degradation and other processes (Johnson et al. 1974; Dell et al. 2011). As the mycelial growth rate is expected to be unimodal with respect to temperature, we fit Brière function (Brière et al. 1999) to the mycelial growth of each strain.

The Brière function is define as $[cT(T-T0)(Tm-T)^{1/2}]$, where T is temperature of fungal development in degrees Celsius and the three parameters c, T0 and Tm represent a rate constant, the minimum and maximum temperatures respectively. We fit the model using non-linear least squares, with the *nls* function in R.

In vitro competition between *L. pseudotheobromae*, *N. parvum* and *S. commune*

The in vitro competition between the three fungi was determined by placing mycelium plugs of each fungal strain (from the margin of five-day-old cultures) at equidistance (40 mm) onto MA plates. Seven treatments each containing 14 plates were tested at 25 °C and 30 °C for 72 h: (i) *N.P:* plates inoculated with *N. parvum,* (ii) *L.P*: plates inoculated with *L. pseudotheobromae,* (iii) *S.C*: plates inoculated with *S. commune*, (iv) *N.P* + *L.P:* dual combination of *N. parvum* with *L. pseudotheobromae,* (v) *N.P* + *S.C*: dual combination of *N. parvum* with *S. commune,* (vi) *L.P* + *S.C*: dual combination of *L. pseudotheobromae* with *S. commune*, and (vii) *N.P* + *L.P* + *S.C*: triple combination of *N. parvum* with *L. pseudotheobromae* and *S. commune.* The competition ability of each fungus was evaluated through the calculation of the space colonization (C%) by the method of Camporota (1985): C = (DT*100)/DE where DT is the distance travelled by a fungus on the axis connecting two mycelial discs after 72 h; and DE, the distance between two mycelial discs.

In planta pathogenicity assay

Grapevine material and experimental design

A total of 225 cuttings of cv. Italia were grown in a greenhouse in INRA precincts (Villenave d'Ornon, Bordeaux, France) where the air temperature (the average was 21 °C) and the air relative humidity (the average was 66%) were measured during the period of incubation using Vaisala HMP 35C probes (Campbell Sci., Logan, UT). Nine treatments each applied on 25 plants were tested: i) C: not inoculated with the pathogenic fungi tested, ii) CP: plants inoculated with sterile malt agar plugs, iii) *N.P*: plants inoculated with *N. parvum,* iv) *L.P*: plants inoculated with *L. pseudotheobromae*, v) *S.C*: plants inoculated with *S. commune*, vi) *N.P* + *L.P*: plants inoculated with *N. parvum* and *L. pseudotheobromae*, vii) *N.P* + *S.C*: plants inoculated with *N. parvum* and *S. commune,* viii) *L.P* + *S.C*: plants inoculated with *L. pseudotheobromae* and *S. commune*, and ix) *N.P* + *L.P* + *S.C*: plants inoculated with *N. parvum, L. pseudotheobromae* and *S. commune.*

Inoculation of plants with pathogenic fungi

Before the inoculations (in June 2015), the woody stem of each plant was surface-disinfected with 95% ethanol, then, the centre of each stem cutting was artificially wounded by drilling a hole up to the pith (4 mm diameter). The wound was then immediately filled with a mycelium plug excised from the margin of a fresh fungal MA culture, with the mycelium facing the cambium. In the case of the co-inoculations with two or three fungal strains, for each cutting the mycelium plugs were randomized into the same hole to avoid an inoculation effect. The treated wounds were then wrapped with parafilm for being protected during all the incubation period.

Wood necrosis measurement

After an incubation period of 120 ± 5 days (October 2015), the bark was removed and the stem of each plant was cut longitudinally, and the percentage of the internal vascular lesions or necrosis in the cutting was recorded as described by Rezgui et al. (2016).

GTD-Koch's postulate verification

In order to test the GTD-Koch's postulate, five wood chips were sampled in two different zones for each cutting: A = necrotic tissues, B = non-necrotic wood (Fig. 1). For control cuttings, i.e. C: not inoculated with the pathogenic fungi tested and CP: inoculated with sterile malt agar plugs, ten wood chips were sampled in zone B as no necrotic tissues were found. Thus, a total of 450 wood chips (10 chips per cutting × five cuttings × nine treatments) were collected, plated after disinfection onto MA medium and incubated at 25 °C in the dark for seven days. Based on morphological features, 58 fungal strains corresponding to the three pathogenic species inoculated were recovered. Their DNA was extracted and sequenced according to the protocol previously described.

Statistical analyses

Data from the in vitro competition test and *in planta* pathogenicity assay were analyzed using the statistical software R, version 3.2.2. The data were first subjected to the Shapiro-Wilks and Levene's tests to check the normality and equality of variances before being subsequently subjected to the non-parametric Kruskal-Wallis test or Student's t-test and the relative contrast effects analyzed by the nparcomp package (version 2.0).

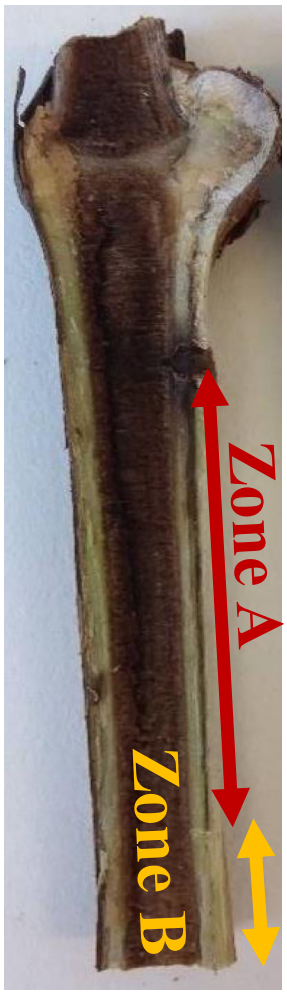

Fig. 1 Zones of isolation of fungi to test the Koch's postulates: zone A = necrotic tissues, zone B = non-necrotic woody tissues

Results

GTDs symptoms on leaves in the vineyards

In the current study 22,912 vines of the cultivar Italia covering 18.5 ha were surveyed. GTDs foliar symptoms (Table 1) were observed, namely chlorotic, irregular spots between the veins or along the leaf margins, wilting on shoots and fruits, and necrosis of the xylem vessels (Mugnai et al. 1999). Table 1 describes the characteristics of each vineyard. Great variations in the incidence of GTDs were recorded amongst vineyards

(0.03% to 6%). The vineyards of the Mornag region showed the greatest disease incidence with percentage of symptomatic vines up to 6%.

Characterization of the fungal strains isolated from necrotic wood samples

A total of 16 endophytic fungal strains were recovered from the necrotic woody tissues sampled in Tunisian vine cordons. Table 2 illustrates the general features of the isolated fungi. Based on partial ITS genes sequence similarity, the fungal strains belonged to: *Alternaria* spp. (8 strains), i.e. *Alternaria tenuissima (3), Alternaria alternata* (4) and *Alternaria mali (1); Acremonium strictum* (3); *Fusarium lateritium* (1); *Lasidiodiplodia pseudotheobromae* (1); *Neofusicoccum parvum* (1) and *Schizophyllum commune* (2). The last three species, known to be involved in GTDs, were isolated from the Mornag vineyard whereas only saprophytic fungi were isolated from the Borj El Amri one.

Morphological characterization of *L. pseudotheobromae*, *N. parvum* and *S. commune*

Observations were made after three and seven days of incubation on MA at 25 °C. The three pathogenic fungi tested had different aspects and colors, and mycelial growth rates. Indeed, *N. parvum* isolates were initially white, grew rapidly and formed a dense aerial mycelium that became dark grey. The reverse side of the colonies became an olive colour, and finally passed to dark grey. *L. pseudotheobromae* colonies grew rapidly, were initially grey-white and became dark grey-black. *S. commune* colonies grew slowly, were initially white and cottony; the reverse side of the colonies became yellow.

Effect of the temperature on the in vitro mycelial growth of *L. pseudotheobromae*, *N. parvum* and *S. commune*

The results obtained showed that the temperature and the incubation period affected the in vitro mycelial growth of the three pathogenic fungi. Whatever the incubation period, the optimal temperature for mycelial growth was 26.884 ± 0.639 °C for *N. parvum*; 32.325 ± 0.442 °C for *L. pseudotheobromae* and 33.704 ± 1.028 °C for *S. commune* (Table 3).

The extreme temperatures tested, i.e. 5 °C and 40 °C, inhibited the mycelial growth excepted for *S. commune* that were still able to develop at 40 °C (Fig. 2). That strain had a more limited mycelial growth in each condition tested compared to *N. parvum* and *L. pseudotheobromae*

Table 2 Origin and molecular identification of the fungal strains isolated from necrotic tissues of Italia cultivar

Strains	Sampling Site	Molecular Identification	GenBank reference accession number (ITS)
F15	Borj El Amri	*Alternaria tenuissima*	(JN542519.1, 100%)
F17	Borj El Amri	*Alternaria alternata*	(FJ228163.1, 99%)
F33	Borj El Amri	*Alternaria tenuissima*	(JN542519.1, 100%)
F34	Borj El Amri	*Alternaria. Alternata*	(KC692221.1, 99%)
F35	Borj El Amri	*Alternaria alternata*	(AY154682.1, 100%)
F36	Borj El Amri	*Alternaria. Alternata*	(FJ228163.1, 99%)
F39	Borj El Amri	*Acremonium strictum*	(EU497953.1, 99%)
F41	Mornag	***Schizophyllum commune***	(KJ093499.1, 99%)
F42	Mornag	***Schizophyllum commune***	(FJ478109.1, 99%)
F46	Mornag	*Fusarium lateritium*	(GU480949.1, 99%)
F47	Mornag	***Lasidiodiplodia pseudotheobromae***	(GQ469969.1, 100%)
F48	Mornag	***Neofusicoccum parvum***	(JQ647911, 99%)
F50	Mornag	*Alternaria mali*	(AY154683.1, 100%)
F51	Mornag	*Acremonium strictum*	(EU497953.1, 99%)
F53	Mornag	*Acremonium strictum*	(EU497953.1, 99%)
F58	Mornag	*Alternaria tenuissima*	(KP942908.1, 99%)

The 4 fungi are in bold because they are used for other experiments in the publication

Table 3 Mycelial growths of *L. pseudotheobromae* (LP), *N. parvum* (NP) and *S. commune* (SC) after 24, 48, 72 and 96 h of incubation at various temperatures

Strain	Time (H)	c ± (SE)	T0 ± (SE)	Tm ± (SE)	Temp_Op±(SE)	Temp_OpMean
SC	24	0.001 ± 0.000	6.862 ± 2.217	43.696 ± 1.029	35.716 ± 0.504	33.704 ± 1.028
SC	48	0.003 ± 0.000	3.518 ± 1.235	41.329 ± 0.188	33.435 ± 0.119	
SC	72	0.007 ± 0.000	2.727 ± 1.300	40.703 ± 0.121	32.847 ± 0.099	
SC	96	0.011 ± 0.000	3.379 ± 1.028	40.577 ± 0.088	32.818 ± 0.078	
LP	24	0.011 ± 0.001	9.108 ± 1.129	40.006 ± 0.013	33.061 ± 0.088	32.325 ± 0.442
LP	48	0.024 ± 0.002	4.629 ± 1.743	40.001 ± 0.006	32.499 ± 0.118	
LP	72	0.029 ± 0.002	0.393 ± 1.638	40.001 ± 0.003	32.040 ± 0.097	
LP	96	0.027 ± 0.002	3.162 ± 1.903	40.001 ± 0.003	31.699 ± 0.101	
NP	24	0.006 ± 0.001	0.045 ± 3.860	35.104 ± 0.102	28.088 ± 0.231	26.884 ± 0.639
NP	48	0.052 ± 0.002	9.095 ± 0.355	31.304 ± 0.122	26.144 ± 0.064	
NP	72	0.071 ± 0.003	7.541 ± 0.401	32.459 ± 0.199	26.844 ± 0.097	
NP	96	0.065 ± 0.004	4.794 ± 0.702	32.417 ± 0.250	26.460 ± 0.126	

Calculations were made using Brière $[cT(T-T0)(Tm-T)^{1/2}]$ function, in which T is temperature of fungal development in degrees Celsius and the three parameters c, T0 and Tm represent a rate constant, the minimum and maximum temperatures respectively. SE is set for standard deviation

For each strain (LP, NP and SC) Temp_OP and Temp_OpMean represent the optimal temperature at the different times of experiments (24, 48, 72, 96 h) and the mean optimal temperature during the four times of experiments respectively

for which the measured mycelial diameters were at least two times bigger (Fig. 2).

It should also be noted that *N. parvum* and *L. pseudotheobromae* had a faster growth rate than *S. commune*. Both fungal strains reached their maximal growth after 3 days of incubation whether *S. commune* reached its maximum after 4 days (Fig. 2).

In vitro competition between the three pathogenic fungi *L. pseudotheobromae*, *N. parvum* and *S. commune*

The results obtained in Fig. 3 showed variable colonization percentages for the three fungal pathogens tested, i.e. *L. pseudotheobromae* ($P < 0.05$), *N. parvum* ($P < 0.05$), and *S. commune* ($P < 0.05$) on dual and triple combination. Regarding *N. parvum* (Fig. 3a), its mycelial growth was reduced by *L. pseudotheobromae* and *S. commune* when they were co-cultured in dual and/or triple combination after 72 h incubation at 25 °C and/or 30 °C. The inhibitory effect of *L. pseudotheobromae* (60%) and *S. commune* (20%) was not enhanced when both were co-cultured with *N. parvum* in the triple combination assay. The greatest inhibition was observed with *L. pseudotheobromae* in the dual competition test (*N. parvum* growth reduced by 60% compared to the control).

In the case of *L. pseudotheobromae* (Fig. 3b), its growth was significantly affected ($P < 0.05$) in presence of *N. parvum* (42%) and/or *S. commune* (22%) at 30 °C. At 25 °C, the growth of this fungus was inhibited only in presence of *N. parvum* in dual (42%) and/or triple combination (46%), whereas *S. commune* had an inhibitory effect only in the triple combination (22%).

For *S. commune* colonization, the results obtained showed that *L. pseudotheobromae* had an inhibitory effect in the double (14%, 17%) and/or triple (20%, 14%) combination assays at 25 °C and 30 °C (Fig. 3c). *N. parvum* inhibited the growth of *S. commune* in the triple combination assays at 25 °C and/or 30 °C ($P < 0.05$), whereas it had no effect ($P > 0.05$) at 25 °C and 30 °C in the dual culture combinations.

L. pseudotheobromae, N. parvum and *S. commune* pathogenicity on young vines of cv. Italia

All the stem cutting cv. Italia artificially inoculated with the three pathogenic fungi, i.e. *L. pseudotheobromae* (*L.P*), *N. parvum* (*N.P*) and *S. commune* (*S.C*), singly or in combination, exhibited internal necrotic lesions, namely brown stripe with hard texture, developed downward from the inoculation point at the end of the

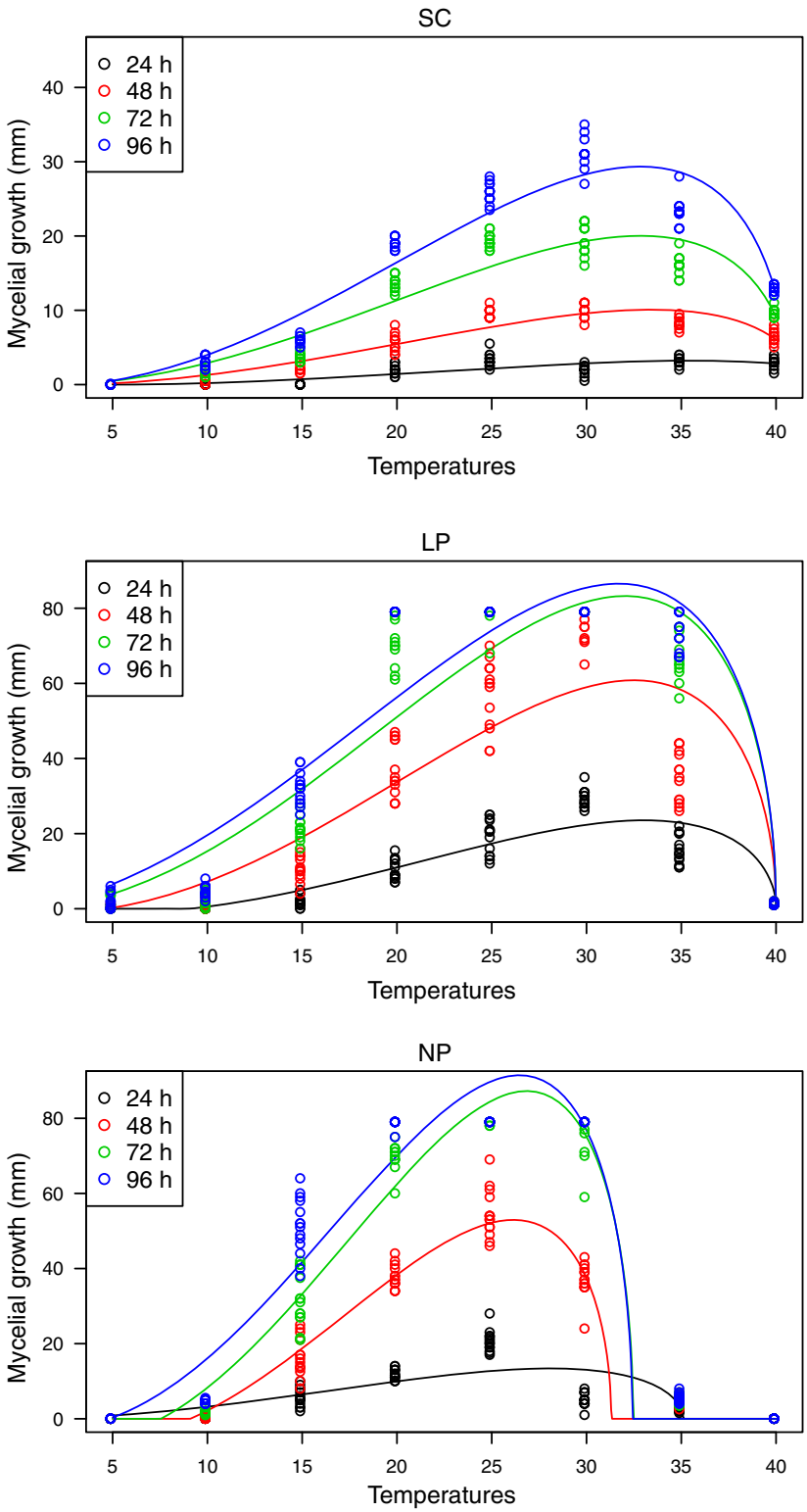

Fig. 2 Effect of the temperature on the mycelial growth (in mm) of *L. pseudotheobromae* (L.P), *N. parvum* (N.P) and *S. commune* (S.C) after 24, 48, 72 and 96 h of incubation. Each point is one replicate made per strain and per condition tested

incubation period of 120 ± 5 dpi (data not shown). In the control cuttings (non inoculated with the pathogen), no lesion was observed.

Evaluation of the pathogenic potential of the fungal strains on cv. Italia showed significant variations depending on the isolates used ($P < 0.05$). *N. parvum* caused the greatest damages as the necrosis rates were in the range of 80%, whereas those due to *L. pseudotheobromae* or *S. commune* were 2 to 2.5 times lower; they were

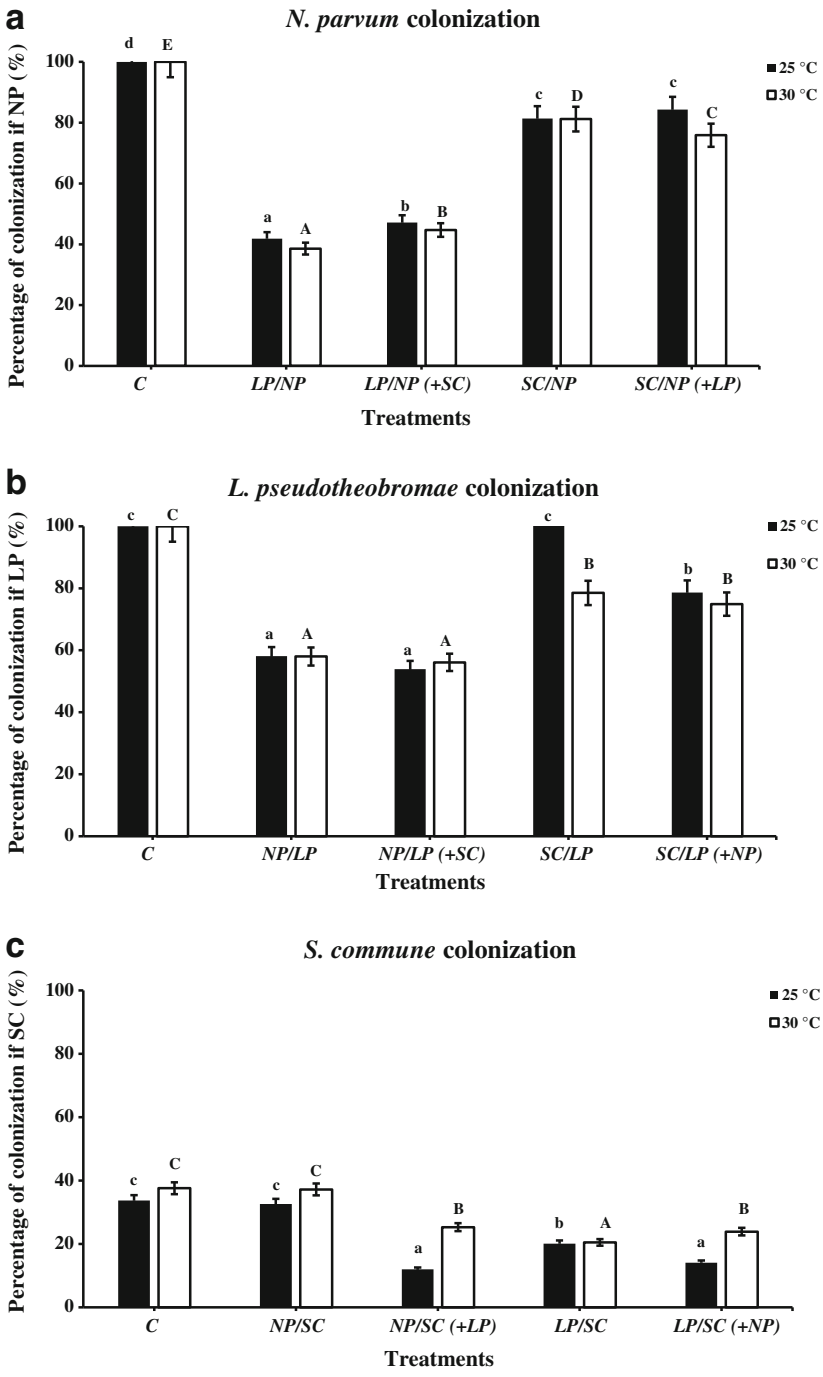

Fig. 3 In vitro competition (% colonization) between (**a**) *N. parvum* (NP), **b** *L. pseudotheobromae* (LP), and **c** *S. commune* (SC) after 72 h of incubation on MA. C: control, i.e. *N. parvum*, *L. pseudotheobromae* and *S. commune* alone, LP/NP: effect of LP on NP in double combination, SC/NP: effect of SC on NP in double combination, LP/NP (+SC): effect of LP on NP in triple combination, SC/NP (+LP): effect of SC on NP in triple combination, NP/LP: effect of NP on LP in double combination, SC/LP: effect of SC on LP in double combination, NP/LP (+SC): effect of NP on LP in triple combination, SC/LP (+NP): effect of SC on LP in triple combination, NP/SC: effect of NP on SC in double combination, LP/SC: effect of LP on SC in double combination, NP/SC (+LP): effect of NP on SC in triple combination, and LP/SC (+NP): effect of LP on SC in triple combination. Percentage values are the mean of 14 measures per treatment. Mean values sharing the same uppercase or lowercase letters are not significantly different according to the Kruskal-Wallis' non-parametric relative contrast effects post-hoc test at $P < 0.05$. Error bars show the 95% confidence intervals

respectively 32% and 35% (Fig. 4). It should also be noted that the length of the stem necrosis also differed depending on the treatment ($P < 0.05$), i.e. if the pathogenic strains were inoculated alone or in combination. Indeed, *N. parvum* attack rates were reduced when the fungus was co-inoculated with *L. pseudotheobromae* (N.P + L.P - 32%) or *S. commune* (N.P + S.C - 31%) or both (N.P + L.P + S.C - 45%) compared to control cuttings inoculated only with *N. parvum* (80%). *N. parvum* did not however reduce the size of the necrosis induced by *L. pseudotheobromae* and/or *S. commune*.

The presence/persistence of the inoculated pathogenic fungi (Koch's Postulates) was assessed: 145 fungal strains were recovered from the 450 wood chips sampled in the nine treatments tested, i.e. 87 saprophytic fungi and 58 pathogenic strains (29 *N. parvum*,

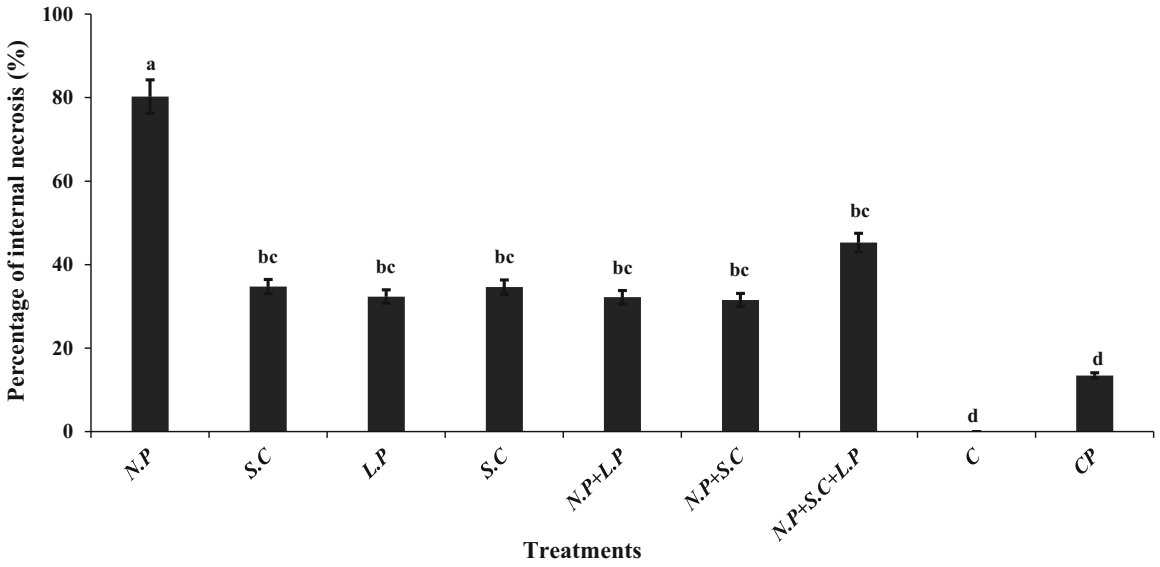

Fig. 4 Internal necrosis induction by *L. pseudotheobromae* (*L.P*), *N. parvum* (*N.P*) and *S. commune* (*S.C*) on young vines cv. Italia under greenhouse conditions. Percentage values are the mean of 25 measures per treatment. Mean values sharing the same letters are not significantly different according to the Kruskal-Wallis' non-parametric relative contrast effects post-hoc test at $P < 0.05$. C: control cuttings not inoculated with the fungus, CP: control cuttings inoculated with sterile malt agar plugs. Error bars show the 95% confidence intervals

13 *L. pseudotheobromae* and 16 *S. commune*) (Table 4). Strains of the three pathogenic fungi, e.g. *L. pseudotheobromae, N. parvum* and *S. commune*, were all isolated from the necrotic tissues (zone A), whereas only saprophytic fungi, e.g. *Alternaria* sp., *Penicillium* sp., *Aspergillus* sp., were isolated from the non-necrotic ones (zone B) (Fig. 1, Table 4). The molecular characterization of the 58 pathogenic strains based on the rDNA-ITS sequencing confirmed their identification (Genebank >99% sequence similarity) and that they corresponded to the strains inoculated at the beginning of the assay (data not shown).

Discussion

The aim of this study was first to gain a more comprehensive understanding of the extent of GTDs in 10 Tunisian vineyards selected by chance, and secondly to look for and isolate some pathogenic fungal species

Table 4 Number of fungal strains per treatment and zone of isolation

Treatments	Zone A				Zone B			
	N.P	*L.P*	*S.C*	Others	*N.P*	*L.P*	*S.C*	Others
N.P	25	0	0	0	0	0	0	4
L.P	0	10	0	2	0	0	0	7
S.C	0	0	4	1	0	0	0	1
N.P + *L.P*	1	1	0	5	0	0	0	3
N.P + *S.C*	3	0	3	3	0	0	0	1
L.P + *S.C*	0	2	5	3	0	0	0	3
N.P + *L.P* + *S.C*	0	0	4	4	0	0	0	4
C	–	–	–	–	0	0	0	23
CP	–	–	–	–	0	0	0	23
Total	29	13	16	18	0	0	0	69

Zone A = necrotic woody tissues, zone B = non-necrotic wood, *L.P* = *Lasidiodiplodia pseudotheobromae*, *N.P*=*Neofusicoccum parvum*, *S.C*=*Schizophyllum commune*

associated with cv. Italia symptomatic grapevines. Investigations on the isolated pathogenic fungi were carried out to (i) determine their ability to grow at various temperatures, (ii) to compete in vitro and *in planta* and (iii) to cause necrosis in the wood of cv. Italia young vines after strains were inoculated singly or in combination.

In the survey made in ten table-grape vineyards, plants from the cultivar Italia showed leaf symptoms of GTDs, e.g. Esca-like foliar symptoms as described by Mugnai et al. 1999, but depending on the vineyards, frequency of symptomatic vines ranged from 0.03% to 6%. The highest percentages were obtained in the Mornag vine-growing region, suggesting thus that GTDs could, in the future, seriously impact vineyards longevity in that region. In the three other regions, GTDs frequency ranged from 0% in Cité El Mehrine to 0.3% in Borj El Amri. This result is in agreement with Bruez et al. (2013) who reported that for a given variety, different levels of GTDs can be shown depending on the regions it is planted.

Most research on GTDs has been focused on cultivars used for wine grape production (Bruez et al. 2013; Haidar et al. 2016; Yacoub et al. 2016) but some studies indicate that table grape-cultivars are attacked as well, e.g. Red Globe and Victoria cultivars in Chile and South Africa (Auger et al. 2004; Mutawila et al. 2011; Morales et al. 2012). *To our knowledge,* in the literature with regards to the cultivar Italia, few studies have investigated its susceptibility to GTDs (Sparapano et al. 2001b).

In the present study, three fungal species, known to be involved in GTDs according to the literature, i.e. *Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune* (Armengol et al. 2001; Fischer 2002), were isolated from the necrotic tissues of vines cv. Italia and their identification was confirmed by the rDNA-ITS sequencing.

Many reports showed that *L. pseudotheobromae* belongs to the species of the Botryosphaeriaceae family linked to decline, canker and dieback of many crops, i.e. maize (Diab et al. 1984), citrus (Abo-El-Dahab et al. 1992), sugar beet (Abd-El Ghani and Fatouh 2005), mango (Abdalla et al. 2003; Ismail et al. 2012) and grapevine worldwide (El-Goorani and El Meleigi 1972; Yan et al. 2013; Dissanayake et al. 2015). According to Burgess et al. (2003), *L. theobromae* has to be regarded as a tropical and subtropical pathogen and cooler climates might prevent its establishment in such regions. This might explain why *L. pseudotheobromae* has been isolated in Tunisian vineyards characterized by semi-arid to arid climatic conditions with generally dry and hot summers and mild winters (Donat et al. 2014). This phenomenon is of key importance and should also be linked to the incidence, virulence and symptoms on grapevines caused by Botryosphaeriaceae, including *L. pseudotheobromae*, that may differ from one cultivar and country to another (Alves et al. 2008; Cai et al. 2011; Ko-Ko et al. 2011a, b; Liu et al. 2012).

N. parvum is one of the most virulent species associated with GTDs (Laveau et al. 2009; Úrbez-Torres and Gubler 2009). This species has been frequently associated with internal and external wood necrosis in grapevine wood (Phillips 2002; Laveau et al. 2009; Amponsah et al. 2011). This pathogenic fungus is found in grapevine worldwide; i.e. Australia, Chile, China, Egypt, Hungary, Italy, Lebanon, New Zealand, Portugal, South Africa, Spain, and USA (Baskarathevan et al. 2011; Yan et al. 2011a, b, 2012, 2013). In France it was detected on mature grapevines of *Vitis vinifera* (Larignon et al. 2001, 2009; Kuntzmann et al. 2010; Bruez et al. 2013); but also in young vines (3–6 years old) (Larignon et al. 2015). So, the wide adaptation of *N. parvum* to various environmental conditions suggests that this pathogen may occur and attack grapevines under different climatic conditions, including in Tunisia.

S. commune is also among the fungi known to be involved in GTDs (Larignon and Dubos 1997; Úrbez-Torres et al. 2012); it causes wood disease of many other crops, i.e. pistachio (Michailides et al. 1995), olive trees (Úrbez-Torres et al. 2013) and peach trees (Chen et al. 2015).

The results of the present study support these earlier reports by showing that *L. pseudotheobromae, N. parvum* and *S. commune* also inhabit necrotic woody tissues of mature grapevines (10–17 years old) in Tunisia.

Numerous other pathogenic fungi not isolated in our experiment are also involved in GTDs such as *Botryosphaeria* spp., *Diplodia seriata, Eutypa lata, Phaeomoniella chlamydospora, Togninia minima* (Taylor et al. 2005; Savocchia et al. 2007; Billones et al. 2010; Úrbez-Torres et al. 2012). Such result may be due to the sampling method (i.e. 5 mm wood pieces) that selected for, or provided an advantage to faster growing fungi to be isolated. For instance, *Phaeoacremonium* spp. and *P. chlamydospora* are known as slow-fungal growers and it take them longer time to grow from the wood pieces than other fast-growing fungi such as

L. pseudotheobromae and *N. parvum*. In such a situation, they likely outgrow and overgrow *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora*. This could be one of the reasons why these fungi were not isolated in our experiment. A larger sampling size and the inclusion of various cultivars from different growing regions within Tunisia might shed more light onto the microbiota of Tunisian grapevines.

The three pathogenic fungi isolated in this study were then studied for their in vitro mycelial growth depending on the temperature. The optimal temperature was 26.88 ± 0.639 °C for *N. parvum* and 33.70 ± 1.028 °C for *S. commune* after 96 h of incubation, almost equal to previous results reported by Espinoza et al. (2009), Thomidis et al. (2011) and Teoh and Mat Don (2015). In the case of van Niekerk et al. (2004) and Ploetz et al. (2009), the *N. parvum* temperature optimum recorded was higher; respectively 30 °C and 27.8 °C. The reasons for these discrepancies are unknown, but may reflect genetic differences between the strains used, geographical differences (Baskarathevan et al. 2011), and/or a possible adaptation of the *N. parvum* strain to the Tunisian Mediterranean climate.

As regards *L. pseudotheobromae*, the maximum radial growth was obtained at 32.325 ± 0.442 °C. This optimum temperature was a bit higher to the one obtained, i.e. 30.8 °C, by Úrbez-Torres et al. (2006). The mycelial growth was reduced at 10 °C, 15 °C and 40 °C but not stopped. At 5 °C, in contrast with *N. parvum* and *S. commune, L. pseudotheobromae* showed a slight growth.

L. pseudotheobromae, N. parvum and *S. commune* showed therefore their ability to grow at high temperatures (>30 °C), which may suggest their adaptation to the Mediterranean climate in the north of Tunisia.

The pathogenicity of *L. pseudotheobromae*, *N. parvum* and *S. commune* was confirmed when these fungi were inoculated singly or in combination in the trunk of young vines of cv. Italia. All these fungi infected the vines through wounds, colonized and degraded the woody tissues, and caused internal disease symptoms. The symptoms severity, i.e. the length of internal necrosis, differed depending on the fungal inocula applied ($P < 0.05$): *N. parvum* caused the longest internal necrosis. This result is in agreement with Úrbez-Torres and Gubler (2009) who reported that *L. theobromae, Neofusicoccum luteum, N. parvum,* and *N. australe* were more highly virulent than *D. seriata, D. mutila, Dothiorella iberica* and *D. viticola* in Californian vineyards.

It is also worth noting that in this *in planta* assay, a competitive effect between the three pathogens *L. pseudotheobromae*, *N. parvum* and *S. commune* was observed. It subsequently reduced the length of the internal necrosis caused by *N. parvum* when this fungus was inoculated together with *L. pseudotheobromae* and/or *S. commune*. This may result from antagonism between the pathogenic fungi when they defend themselves and try to exclude *N. parvum*. Likewise Sparapano et al. (2000, 2001a) reported a competitive association of *Phaeoacremonium minimum* and *P. chlamydospora*, and a marked antagonistic effect of *P. minimum* against *Fomitiporia punctata* during wood colonization.

In vitro confrontation assays between *L. pseudotheobromae, N. parvum* and *S. commune* on synthetic medium supported this result. Greater or lesser antagonistic effects were observed depending on the fungal combination applied ($P < 0.05$) and/or the temperature of incubation ($P < 0.05$). These discrepancies are unknown, but may be explained by the ability of fungi to produce antifungal compounds (Teoh and Mat Don 2015) and the temperature effect on their growth that would influence their antagonism capacity.

In conclusion, this study reported that the table grape cultivar Italia, which is the most planted in Tunisia, is susceptible to GTDs attacks. Three pathogenic fungi *L. pseudotheobromae*, *N. parvum* and *S. commune* were isolated for the first time from necrotic woody tissues of this cultivar. These pathogens reproduced typical internal symptoms of GTDs, namely brown stripe with hard texture as described by Mugnai et al. (1999), on stem cuttings of cv. Italia artificially inoculated. However, no foliar symptoms of GTDs had been reproduced on young plants. Although pathogenicity of the isolated fungi was evident, positive cooperation between fungal strains was not observed. On the contrary, in vivo and *in planta* competitions between strains were noticed. The results also suggest that temperatures, and certainly those of Tunisia, can influence the growth of the pathogenic fungi inhabiting the wood of vineyards and therefore the GTDs attacks.

Acknowledgements The authors are grateful to the program PHC-Utique France-Tunisie managed by CMCU (project 13G0924), the French Ministry of Agriculture, Food-processing industry and Forest (Casdar program V1302), the Poupelain Foundation and the International Organisation of Vine and Wine (OIV). We are grateful to Mokhtar Dridi (Laboratoire de Protection des végétaux, Institut National de la Recherche Agronomique de Tunisie (INRAT) 2049, Ariana, Tunisia) for his help on sampling

vines. We are also indebted to the INRA UMR SAVE technical staff, Gilles Taris and Sébastien Gambier, for their valuable help for the greenhouse and laboratory experiments.

References

Abdalla, M. A., Safie, M. H., El-Boghdady, M. M., & Soltan, H. H. M. (2003). Fruit coating with certain plant oils for controlling post-harvest diseases of mangoes with special reference to stem end rot. *Egyptian Journal of Applied Science, 18*, 116–136.

Abd-El Ghani, H. S., & Fatouh, H. M. (2005). First record of sugar beet root rot disease caused by *Botryodiplodia theobromae* in Egypt. *Egyptian Journal of Phytopathology, 33*(1), 107–108.

Abo rehab, M. E. A., Korra, A. K. M., Kamhawy, M. A. M., & Youssef, K. Y. A. (2013). Fungal species associated with graft union on grapevine, its impact on graft failure process and attempted solutions in Egypt. *International Journal of Agriculture Research, 3*(2), 52–59.

Abo-El-Dahab, M. K., El-Kazazz, S. A., Shoeib, A. A., & El-Sheikh, M. A. (1992). Biochemical changes in citrus fruits infected with *Botryodiplodia theobromae*. *Journal of Agriculture Science Mansoura University, 17*, 3525–3532.

Agustí-Brisach, C., León, M., García-Jiménez, J., & Armengol, J. (2015). Detection of grapevine fungal trunk pathogens on pruning shears and evaluation of their potential for spread of infection. *Plant Disease, 99*(7), 976–981.

Altschul, S. F., Madden, T. L., Schäffer, 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.

Alves, A., Crous, P. W., Correia, A., & Phillips, A. J. L. (2008). Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity, 28*, 1–13.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., & Jaspers, M. V. (2011). Identification, potential inoculum sources and pathogenicity of Botryosphaeriaceous species associated with grapevine dieback disease in New Zealand. *European Journal of Plant Pathology, 131*, 467–482.

Armengol, J., Vicent, A., Torné, L., García-Figueres, F., & García-Jiménez, J. (2001). Fungi associated with Esca and grapevine declines in Spain: A three-year survey. *Phytopathologia Mediterranea, 40*, 325–329.

Auger, J., Esterio, M., Ricke, G., & Pérez, I. (2004). Black dead arm and basal canker of *Vitis vinifera* cv. Red globe caused by *Botryosphaeria obtusa* in Chile. *Plant Disease, 88*, 1286.

Baskarathevan, J., Marlene, V., Jaspers, E., Jones, E., & Ridgway, H. J. (2011). Incidence and distribution of Botryosphaeriaceous species in New Zealand vineyards. *European Journal of Plant Pathology, 132*(4), 549–560.

Ben Ghnaya-Chakroun, A., Rezgui, A., Vallance, J., Kharoubi, I., Dridi, M., Hajlaoui, M. R., Rey, P., & Sadfi-Zouaoui, N. (2014). First molecular and biochemical characterization of *Phomopsis viticola* and *Diploidia seriata* two pathogens of Esca and black dead arm diseases of grapevine in the northern region of the Tunisia. *International Journal of Current Microbiology and Applied Sciences, 3*(8), 977–987.

Ben Salem, A., Jemâa, R., Gugerli, P., & Ghorbel, A. (2000). Introduction and rehabilitation of vine in Tunisian Sahara. *Bulletin de L'Organisation International du Vigne et du Vin* (pp. 574–580).

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clément, C., & Fontaine, F. (2013). Grapevine trunk diseases: Complex and still poorly understood. *Plant Pathology, 62*, 243–265.

Bester, W., Crous, P. W., & Fourie, P. H. (2007). Evaluation of fungicides as potential grapevine pruning wound protectants against Botryosphaeria species. *Australasian Plant Pathology, 36*, 73–77.

Billones, R. G., Ridgway, H. J., Jones, E. E., & Jaspers, M. V. (2010). First report of *Neofusicoccum macroclavatum* as a canker pathogen of grapevine in New Zealand. *Plant Disease, 94*(12), 1504.

Brière, J. F., Pracros, P., Le Roux, A. Y., & Pierre, J. S. (1999). A novel rate model of temperature-dependent development for arthropods. *Environmental Entomology, 28*(1), 22–29.

Brown-Rytlewski, D. E., & McManus, P. S. (2000). Virulence of *Botryosphaeria dothidea* and *Botryosphaeria obtusa* on apple and Management of Stem Cankers with fungicides. *Plant Disease, 84*, 1031–1037.

Bruez, E., Lecomte, P., Grosman, J., Doublet, B., Bertsch, C., Fontaine, F., Da Costa, J. P., Ugaglia, A., Teissedre, P. L., Guerin-Dubrana, L., & Rey, P. (2013). Overview of grapevine trunk diseases in France in the 2000s. *Phytopathologia Mediterranea, 52*, 262–275.

Bruez, E., Vallance, J., Gerbore, J., Lecomte, P., Da Costa, J. P., Guerin-Dubrana, L., & Rey, P. (2014). Analyses of the temporal dynamics of fungal communities colonizing the healthy wood tissues of Esca leaf-symptomatic and asymptomatic vines. *PLoS One, 9*, e95928.

Burgess, T., Wingfield, M. J., & Wingfield, B. D. (2003). Development and characterization of microsatellite loci for the tropical tree pathogen *Botryosphaeria rhodina*. *Molocular Ecology Notes, 3*, 91–94.

Cai, L., Giraud, T., Zhang, N., Begerow, D., Cai, G. H., & Shivas, R. G. (2011). The evolution of species concepts and species recognition criteria in plant pathogenic fungi. *Fungal Diversity, 50*, 121–123.

Camporota, P. (1985). Antagonisme *in vitro* de *Trichoderma spp*. vis-à vis de *Rhizoctonia solani Kühn*. *Agronomy Journal, 5*(7), 613–620.

Che, J., Liu, B., Ruan, C., Tang, J., & Huang, D. (2015). Biocontrol of *Lasiodiplodia theobromae*, which causes black spot disease of harvested wax apple fruit, using a strain of *Brevibacillus brevis* FJAT-0809-GLX. *Crop Protection Journal, 67*, 178–183.

Chebil, S., Fersi, R., Yacoub, A., Chenenaoui, S., Melki, I., Zemni, Z., Rhouma, A., Durante, G., & Mliki, A. (2014). First report of *Botryosphaeria dothidea*, *Diplodia seriata* and *Neofusicoccum luteum* associated with canker and dieback of grapevines in Tunisia. *Plant Disease, 98*(3), 420.

Chen, C., Bock, C. H., Hotchkiss, M. H., Garbeletto, M. M., & Cottrell, T. E. (2015). Observation and identification of wood decay fungi from the heartwood of peach tree limbs in Central Georgia, USA. *European Journal of Plant Pathology, 143*, 11–23.

Compant, S., Brader, G., Muzammil, S., Sessitsch, A., Lebrihi, A., & Mathieu, F. (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *Biological Control, 58*, 435–455.

Crous, P. W., & Gams, W. (2000). *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri Grapevine decline and Esca. *Phytopathologia Mediterranea, 39*, 112–118.

Damm, U., Crous, P. W., & Fourie, P. H. (2007). Botryosphaeriaceae as potential pathogens of Prunus species in South Africa, with descriptions of *Diplodia africana* and *Lasiodiplodia plurivora sp. nov. Mycologia, 99*(5), 664–680.

De la Fuente, M., Fontaine, F., Gramaje, D., Armengol, J., Smart, R., Nagy, Z.A., Borgo, M., Rego, C., & Corio-Costet, M.F. (2016). Grapevine trunk diseases. A review. *OIV publications, 1st Edition Paris, France,* (pp. 24). ISBN: 979-10-91799-60-7.

Dell, A. I., Pawar, S., & Savage, V. M. (2011). Systematic variation in the temperature dependence of physiological and ecological traits. *Proceedings of the National Academy of Sciences USA, 108*, 10591–10596.

Diab, M. M., Kahlil, I., Dawood, N. A., & El-Assiuty, E. M. (1984). Ear and grain rot of maize caused by *Botryodiplodia theobromae* pathogens in Egypt. *Minufiya Journal of Agricultural Research, 9*, 129–138.

Dissanayake, A. J., Zhang, W., Mei, L., Chukeatirote, E., Yan, J. Y., Li, X. H., & Hyde, K. D. (2015). *Lasiodiplodia pseudotheobromae* causes pedicel and peduncle discoloration of grapes in China. *Australasian Plant Disease Notes, 10*, 21.

Donat, M. G., Peterson, T. C., Brunet, M., King, A. D., Almazroui, M., Kolli, R. K., Boucherf, D., Al-Mulla, A. Y., Nour, A. Y., Aly, A. A., Ali Nada, T. A., Semawi, M. M., Al Dashti, H. A., Salhab, T. G., El Fadli, K. I., Muftah, M. K., Dah Eida, S., Badi, W., Driouech, F., El Rhaz, K., Abubaker, M. J. Y., Ghulam, A. S., Sanhouri Erayah, A., Ben Mansour, M., Alabdouli, W. O., Al Dhanhaniw, J. S., & Al Shekailiw, M. N. (2014). Changes in extreme temperature and precipitation in the Arab region: Long-term trends and variability related to ENSO and NAO. *International Journal of Climatology, 34*, 581–592.

El-Goorani, M. A., & El Meleigi, M. A. (1972). Dieback of grapevine by *Botryodiplodia theobromae Pat.* in Egypt. *Phytopathologia Mediterranea, 11*, 210–211.

Espinoza, J. G., Briceño, E. X., Chávez, E. R., Úrbez-Torres, J. R., & Latorre, B. A. (2009). *Neofusicoccum spp.* associated with stem canker and dieback of blueberry in Chile. *Plant Disease, 93*(11), 1187–1194.

Fischer, M. (2002). A new wood-decaying basidiomycete species associated with Esca of grapevine : *Fomitiporia mediterranea (Hymenochaetales)*. *Mycological Progress, 1*(3), 315–324. https://doi.org/10.1007/s11557-006-0029-4.

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P., & Fermaud, M. (2016). Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control, 92*, 55–65.

Hyde, K. D., & Soytong, K. (2008). The fungal endophyte dilemma. *Fungal Diversity, 33*, 163–173.

Ismail, A., Cirvilleri, M. G., Polizzi, G., Crous, P. W., Groenewald, J. Z., & Lombard, L. (2012). *Lasiodiplodia* species associated with dieback disease of mango (*Mangifera indica*) in Egypt. *Australasian Plant Pathology, 41*, 649–660.

Johnson, F. M., Eyring, H., & Stover, B. J. (1974). *The theory of rate processes in biology and medicine* (p. 704). New York: John Wiley.

Johnson, G. I., Cooke, A. W., Mead, A. J., & Wells, I. A. (1991). Stem end-rot of mango in Australia: Causes and control. *Acta Horticulturae, 219*, 288–295.

Johnson, G. I., Cooke, T., & Mead, A. (1993). Infection and quiescent of mango stem-end rot pathogens. *Acta Horticulturae*, (341), 329–336.

Ko-Ko, T. W., Stephenson, S. L., Bahkali, A. H., & Hyde, K. D. (2011a). From morphology to molecular biology: Can we use sequence data to identify fungal endophytes? *Fungal Diversity, 50*, 113–120.

Ko-Ko, T. W., Stephenson, S. L., Hyde, K. D., & Lumyong, S. (2011b). Influence of seasonality on the occurrence of Myxomycetes. *Chiang Mai Journal of Sciences, 38*, 71–84.

Kuntzmann, P., Villaume, S., Larignon, P., & Bertsch, C. (2010). Esca, BDA and Eutypiosis: Foliar symptoms, trunk lesions and fungi observed in diseased vine stocks in two vineyards in Alsace. *Vitis, 49*, 71–76.

Larignon, P., & Dubos, B. (1997). Fungi associated with Esca disease in grapevine. *European Journal of Plant Pathology, 103*, 147–157.

Larignon, P., Fulchic, R., Cere, L., & Dubos, B. (2001). Observation on black dead arm in French vineyards. *Phytopathologia Mediterranea, 40*, S336–S342.

Larignon, P., Fontaine, F., Farine, S., Clément, C., & Bertsch, C. (2009). Esca et Black Dead Arm : deux acteurs majeurs des maladies du bois chez la Vigne. *Comptes Rendus Biologies, 332*, 765–783.

Larignon, P., Spagnolo, A., Bertsch, C., & Fontaine, F. (2015). First report of young grapevine decline caused by *Neofusicoccum parvum* in France. *Plant Disease, 99*(12), 1859.

Laveau, C., Letouze, A., Louvet, G., Bastien, S., & Guerin-Dubrana, L. (2009). Differential aggressiveness of fungi implicated in Esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea, 48*, 32–46.

Liminana, J. M., Pacreau, G., Boureau, F., Menard, E., David, S., Himonnet, C., Fermaud, M., Goutouly, J. P., Lecomte, P., & Dumot, V. (2009). Inner necrosis in a grapevine rootstock vineyard in the Cognac area (Charentes, France). *Phytopathologia Mediterranea, 48*, 92–100.

Liu, J. K., Phookamsak, R., Doilom, M., Wikee, S., Li, Y. M., Ariyawansha, H., Boonmee, S., Chomnunti, P., Dai, D. Q., Bhat, J. D., Romero, A. I., Zhuang, W. Y., Monkai, J., Jones, E. B. G., Chukeatirote, E., Ko Ko, T. W., Zhao, Y. C., Wang, Y., & Hyde, K. D. (2012). Towards a natural classification of Botryosphaeriales. *Fungal Diversity, 57*, 149–210.

Lorrain, B., Ky, I., Pasquier, G., Jourdes, M., Dubrana, L. G., Gény, L., Rey, P., Donèche, B., & Teissedre, P. L. (2012). Effect of Esca disease on the phenolic and sensory attributes of cabernet sauvignon grapes, musts and wines. *Australian Journal of Grape and Wine Research, 18*, 64–72.

Michailides, T.J., Morgan, D.P., & Doster, M.A. (1995). Diseases of pistachio in California and their significance. ISHS Acta Horticulturae 419: I International Symposium on Pistachio. *Acta Horticulturae*, 419, 56.

Morales, A., Latorre, B. A., Piontelli, E., & Besoain, X. (2012). *Botryosphaeriaceae* species affecting table grape vineyards in Chile and cultivar susceptibility. *Ciencia e investigación agraria, 39*(3), 445–458.

Mugnai, L., Graniti, A., & Surico, G. (1999). Esca (black measles) and brown wood streaking two old and elusive diseases of grapevines. *Plant Disease, 83*, 404–418.

Mutawila, C., Fourie, P. H., Halleen, F., & Mostert, L. (2011). Grapevine cultivar variation to pruning wound protection by *Trichoderma* species against trunk pathogens. *Phytopathologia Mediterranea, 50*, S264–S276.

Ni, H. F., Yang, H. R., Chen, R. S., Liou, R. F., & Hung, T. H. (2012). New Botryosphaeriaceae fruit rot of mango in Taiwan: Identification and pathogenicity. *Botanical Studies, 53*, 467–478.

Phillips, A. J. L. (2002). *Botryosphaeria* species associated with diseases of grapevines in Portugal. *Phytopathologia Mediterranea, 41*, 3–18.

Ploetz, R. C., Pérez-Martínez, J. M., Palmateer, A. J., & Tarnowski, T. L. (2009). Influence of temperature, light intensity, and isolate on the development of *Neofusicoccum parvum*–induced dieback of *Eugenia, Syzygium paniculatum*. *Plant Disease, 93*, 804–808.

Punithalingam, E. (1980). Plant diseases attributed to *Botryodiplodia theobromae Pat* (Bibliotheca mycological). J. Carmer. Vaduz. (pp. 123). ISBN 3-7682-1256-4. Record Number: 19801362832.

Ramos, L. J., Lara, S. P., McMillan, R. T., & Narayanan, K. R. (1991). Tip die back of mango (*Mangifera indica*) caused by *Botryosphaeria ribis*. *Plant Disease, 75*, 315–318.

Rezgui, A., Ben Ghnaya-Chakroun, A., Vallance, J., Bruez, E., Hajlaoui, M. R., Sadfi-Zouaoui, N., & Rey, P. (2016). Endophytic bacteria with antagonistic traits inhabit the wood tissues of grapevines from Tunisian vineyards. *Biological Control, 99*, 28–37.

Savocchia, S., Steel, C. C., Stodart, B. J., & Somers, A. (2007). Pathogenicity of *Botryosphaeria* species isolated from declining grapevines in sub tropical regions of Eastern Australia. *Vitis, 46*, 27–32.

Slippers, B., & Wingfield, M. J. (2007). *Botryosphaeriaceae* as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. *Fungal Biology Reviews, Fungal Endophytes, 21*, 90–106.

Slippers, B., Johnson, G. I., Crous, P. W., Coutinho, T. A., Wingfield, B., & Wingfield, M. J. (2005). Phylogenetic and morphological revolution of the *Botryosphaeria* species causing diseases of *Mangifera indica*. *Mycologia, 97*(1), 99–110.

Smith, H., Crous, P. W., Wingfield, M. J., Coutinho, T. A., & Wingfield, B. D. (2001). *Botryosphaeria eucalyptorum sp. nov.*, a new species in the *B. dothidea*-complex on Eucalyptus in South Africa. *Mycologia, 93*(2), 277–285.

Sosnowski, M. R., Shtienberg, D., Creaser, M. L., Wicks, T. J., Lardner, R., & Scott, E. S. (2007). The influence of climate on foliar symptoms of Eutypa dieback in grapevines. *Phytopathology, 97*, 1284–1289.

Sparapano, L., Bruno, G., Ciccarone, C., & Graniti, A. (2000). Infection of grapevines by some fungi associated with Esca. I. *Fomitiporia punctata* as a wood-rot inducer. *Phytopathologia Mediterranea, 39*, 46–52.

Sparapano, L., Bruno, G., & Campanella, A. (2001a). Interactions between associated fungi with Esca of grapevine, and their secondary metabolites. *Phytopathologia Mediterranea, 40*, 417–422.

Sparapano, L., Bruno, G., & Graniti, A. (2001b). Three-year observation of grapevines cross-inoculated with Esca-associated fungi. *Phytopathologia Mediterranea, 40*, S376–S386.

Steel, C. C., Blackman, J. W., & Schmidtke, L. M. (2013). Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. *Journal of Agriculture and Food Chemistry, 61*, 5189–5206.

Taylor, A., Hardy, G. E., Wood, P., & Burgess, T. (2005). Identification and pathogenicity of *Botryosphaeria spp*. associated with grapevine decline in Western Australia. *Australian Plant Pathology, 34*, 187–195.

Teoh, Y. P., & Mat Don, M. (2015). Effect of temperature on *Schizophyllum commune* growth and 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- production using a bubble column bioreactor. *Chiang Mai Journal of Sciences, 42*(3), 539–548.

Thomidis, T., Michailides, T. J., & Exadaktylou, E. (2011). *Neofusicoccum parvum* associated with fruit rot and shoot blight of peaches in Greece. *European Journal of Plant Pathology, 131*, 661–668.

Tovar Pedraza, J. M., Mora Aguilera, J. A., Nava Diaz, C., Teliz Ortiz, D., Villegas Monter, A., & Leyva Mir, S. G. (2013). Control of *Lasiodiplodia theobromae*, the causal agent of dieback of sapote mamey [Pouteria sapota (Jacq.) H. E. Moore and Steam] grafts in Mexico. *Revista Fitotecnia Mexicana, 36*, 233–238.

Úrbez-Torres, J. R., & Gubler, W. D. (2009). Pathogenicity of *Botryosphaeriaceae* species isolated from grapevine cankers in California. *Plant Disease, 93*, 584–592.

Úrbez-Torres, J. R., Leavitt, G. M., Voegel, T. M., & Gubler, W. D. (2006). Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Disease, 90*, 1490–1503.

Úrbez-Torres, J. R., Peduto, F., Striegler, R. K., Urrea-Romero, K. E., Rupe, J. C., Cartwright, R. D., & Gubler, W. D. (2012). Characterization of fungal pathogens associated with grapevine trunk disease in Arkansas and Missouri. *Fungal Diversity, 52*(1), 169–189.

Úrbez-Torres, J. R., Peduto, F., Vossen, P. M., Krueger, W. H., & Gubler, W. D. (2013). Olive twig and branch dieback: Etiology, incidence, and distribution in California. *Plant Disease, 97*(2), 231–244.

Valtaud, C., Larignon, P., Roblin, G., & Fleurat-Lessard, P. (2009). Developmental and ultrastructural features of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in relation to xylem degradation in Esca disease of the grapevine. *Journal of Plant Pathology, 91*(1), 37–51.

Van Niekerk, J. M., Crous, P. W., Groenewald, J. Z. E., Fourie, P. H., & Halleen, F. (2004). DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevines. *Mycologia, 96*, 781–798.

White, T., Birns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *Academic Press,* (pp. 315–322).

Yacoub, A., Gerbore, J., Magnin, N., Chambon, P., Dufour, M. C., Corio-Costet, M. F., Guyoneaud, R., & Rey, P. (2016).

Ability of *Pythium oligandrum* strains to protect *Vitis vinifera L.,* by inducing plant resistance against *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk disease. *Biological Control, 92*, 7–16.

Yan, J. Y., Li, X. H., Kong, F. F., Wang, Z. Y., Gong, L. Z., & He, H. P. (2011a). Occurrence of grapevine trunk disease caused by *Botryosphaeria rhodina* in China. *Plant Disease, 95*, 219.

Yan, J. Y., Peng, Y. L., Xie, Y., Li, X. H., Yao, S. W., Tang, M. L., & Wang, Z. Y. (2011b). First report of grapevine trunk disease caused by *Botryosphaeria obtusa* in China. *Plant Disease, 95*, 616.

Yan, J. Y., Xie, Y., Yao, S. W., Wang, Z. Y., & Li, X. H. (2012). Characterization of *Botryosphaeria dothidea,* the causal agent of grapevine canker in China. *Australian Plant Pathology, 41*(4), 351–357.

Yan, J. Y., Xie, Y., Zhang, W., Wang, Y., Liu, J. K., Hyde, K. D., Seem, R. C., Zhang, G. Z., Wang, Z. Y., Yao, S. W., Bai, X. J., Dissanayake, A. J., Peng, Y. L., & Li, X. H. (2013). Species of *Botryosphaeriaceae* involved in grapevine die-back in China. *Fungal Diversity, 61*, 221–236.

Zoghlami, N., Mliki, A., & Ghorbel, A. (2001). Evaluation of genetic diversity among Tunisian grapevines by RAPD markers. *Vitis, 40*(1), 31–37.

Zoghlami, N., Riahi, L., Laucou, V., Lacombe, T., Mliki, A., Ghorbel, A., & This, P. (2009). Origin and genetic diversity of Tunisian grapes as revealed by microsatellite markers. *Scientia Horticulturae, 120*, 479–486.