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RESEARCH PAPER

Fungi from the *Diaporthaceae* and *Botryosphaeriaceae* families associated with grapevine decline in Tunisia

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Abstract

S. Chebil, R. Fersi, M. Bouzid, F. Quaglino, S. Chenenaoui, I. Melki, G. Durante, E. Zacchi, B.A. Bahri, P.A. Bianco, and A. Rhouma. 2017. Fungi from the *Diaporthaceae* and *Botryosphaeriaceae* families associated with grapevine decline in Tunisia. Cien. Inv. Agr. 44(2): 127-138. Severe decline of mature grapevines has recently been observed in several vineyards in grape regions in northern Tunisia. Between August 2011 and June 2013, wood samples from diseased vines showing dead spur and cordons, shoot dieback associated with sunken necrotic bark lesions and brown to black vascular streaking were collected from numerous diseased vineyards. Several fungal species were isolated from the margin between healthy and symptomatic tissue. Three species of *Botryosphaeriaceae*, namely, *Diplodia seriata*, *Neofusicoccum australe,* and *N. vitifusiforme* and one species of *Diaporthaceae*, namely, *Diaporthe neotheicola*, were observed to be associated with the decline of old vines. Other fungal species were recovered from diseased wood, namely, *Alternaria alternata*, *Botryotinia fuckeliana* (anamorph of *Botrytis cinerea*), *Acremonium* spp., *Aspergillus* spp., and *Fusarium* spp. In addition, *Penicillium* spp. inter- and intra-species diversity were assessed based on virtual RFLP gel analyses and identification of restriction enzymes able to distinguish fungi strains within species based on determination of single nucleotide polymorphism (SNP) lineages within cluster members based on the sequencing of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the rDNA.

Keywords: *Botryosphaeriaceae*, *Diaporthaceae*, trunk disease, virtual RFLP, *Vitis vinifera*

Introduction

In Tunisia, grapevine (*Vitis vinifera* L.) is cultivated in two grape-growing regions; the Northern Coastal Region for late cultivars and the Southern Region for earlier cultivars to maintain a long period of table grape production extending from June to December.

Over the past several decades, 'Grapevine Trunk Diseases' (GTD) have been the most destructive

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diseases of grapevines overall in grape-growing countries (Bertsch *et al.,*2009). Generally, the term 'Grapevine Trunk Diseases' refers to a set of complex diseases, primarily including Esca, Eutypa dieback and Botryosphaeria dieback (Larignonand Dubos, 1997; Mugnai *et al.,*1999; Larignon *et al.,*2009; Bertsch *et al.,*2013). Several fungi are associated with trunk diseases throughout the world, including the following 18 genera: *Botryosphaeria*, *Diplodia*, *Lasiodiplodia*, *Fusicoccum*, *Neofusicoccum*, *Dothiorella*, *Phomopsis*, *Diaporthe*, *Eutypa*, *Eutypella*, *Diatrypella*, *Diatrype*, *Cryptovalsa*, *Cylindrocarpon*, *Phaeomoniella*, *Fomitiporia*, *Phaeoacremonium*, and *Greeneria* (Armengol *et al.,*2001; Kaliterna *et al.,*2012; Mostert *et al.,*2004). Other genera that affect young vineyards are *Campylocarpon, Cylindrocladiella, Dactylonectria, Ilyonectria* and *Neonectria spp.* (Gramaje and Armengol, 2011).

In Tunisia, common grape fungal diseases are powdery and downy mildews (Zouba, 2001). However, due to the ban of sodium arsenate, several vine dieback cases have been reported, often with unidentified causes. However, recent studies conducted on vineyards showing declining grapevines reported the occurrence of three *Botryosphaeriaceae* species to be associated with grapevine dieback in Tunisian vineyards (Chebil *et al.,* 2014). Symptoms included the decline or death of plant parts and, eventually, of whole vines due to a variety of necrosis phenomena, such as cankers, browning, vascular streaking, longitudinal lesions, and cane bleaching, which affect the woody parts of the plant (Luque *et al.,* 2009).

Earlier studies on grapevine trunk disease have largely considered *Botryosphaeriaceae* species to be saprophytes, secondary colonizers or weak pathogens in grapevine wood (Phillips, 2002) until recent studies identified 21 different *Botryosphaeriaceae* species occurring in grapevines worldwide (Úrbez-Torres, 2011). In addition, canker symptoms caused by *Botryosphaeriaceae* in grapevines have probably been unnoticed due to the difficulty to differentiate them from those caused by other grapevine trunk disease pathogens, such as *Eutypa lata* (Leavitt, 1990), *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Phillips, 2002; Surico *et al.,*2006). Moreover, grapevine trunk diseases, such as Eutypa dieback and Esca, have been the primary and, occasionally, sole focus of study for most researchers throughout the past century, which has not helped to clarify the role that *Botryosphaeriaceae* played in grapevine diseases (Úrbez-Torres, 2011). Moreover, fungal species from the genera *Phomopsis* and *Diaporthe,* belonging to the family *Diaporthacea,e* are known as grapevine pathogens and/or endophytes, from which the most commonly reported are *Phomopsisviticola*, *Diaporthe neotheicola*, *D. ambigua* and *D. eres* (Mostert *et al.,* 2000; Schilder *et al.,* 2005). Recent studies suggested the addition of *P. viticola* to the fungi involved in the grapevine trunk disease complex and *Diaporthe* species to be saprophytes or weak pathogens on grapevine wood (Úrbez-Torres *et al.,* 2013).

Therefore, the aim of this study was the identification of fungal species associated with grapevine trunk disease in young and mature vines in Tunisian vineyards.

Material and Methods

Field survey and sampling

A field survey was conducted between the 2011 and 2013 seasons in several vineyards (n=48) in which diseased grapevines surpassed 5% of planted vines. The vineyards were located in the North-East (MORNAG and ZAGHOUAN) and North-West (TAKELSA) regions of Tunisia, known to be the main grape producing areas, where vineyards are planted mainly with cv. Muscat Italia. Wood samples were collected (n=250), from diseased mature vines (8–12-years-old) showing dead spur and cordons, shoot dieback, sunken necrotic bark lesions and brown to black vascular streaking. Wood segments were cut from the affected

canes and cordons and washed under running tap water, and the surface was disinfected for 1 min in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water. Small pieces from the margin between healthy and discolored or decayed wood tissue were plated on PDA (Potato Dextrose Agar). Plates were incubated at 25 °C in the dark for 14 to 21 d, and in order to cover all present fungal species, all colonies were transferred and sub-cultured on PDA medium. Single spore colonies were derived prior to morphological and molecular identification using the serial dilution method and stored in 20% glycerol solution at -80 °C.

DNA extraction

Fungal mycelium and conidia from pure cultures grown on PDA for 2 wk at 25 °C in the dark were scraped and homogenized at room temperature in plastic mesh bags (Mesh Bags REF: SAC-Acc provided by Sediag: http://www.sediag.com) with a ball-bearing device in 10 ml of CTAB buffer (3% CTAB,1 M Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl) with the addition of 0.2% 2-mercaptoethanol; 1 ml was transferred to an Eppendorf tube and incubated in a water bath at 65 °C for 20 min. After extraction with 1 ml of chloroform, total genomic DNA was extracted and precipitated from the aqueous phase with an equal volume of isopropanol, collected by centrifugation, washed with 70% ethanol, dried, dissolved in 100 µl of water and stored at -20 °C.

PCR analysis

Identity of the different taxa was assessed by sequence analyses of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the rDNA using the primers ITS1 and ITS4 (White *et al.,* 1990). Each PCR reaction was carried out in a reaction volume of 25 µl containing 1X buffer, 3 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM primers ITS1/ITS4, 5% DMSO, 0.02 U/µl DNA polymerase (Go Taq Flexi DNA polymerase, PROMEGA) and 5 µl of template DNA. PCR experiments were performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems). The amplification was started with an initial denaturation step at 94 °C for 1 min followed by 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Finally, the reaction was completed with an extension step at 72 °C for 5 min. Samples were analyzed after electrophoretic separation in ethidium-bromide stained 2.5% (w/v) agarose gels (10 µl of 1% ethidium bromide solution in 100 ml of 2.5% agarose). The PCR products were visualized under UV light (302 nm). Considering the PCR inhibitors possibly present in each DNA sample, amplification with a diluted DNA sample was performed if there were no PCR products from the starting template.

Sequencing and nucleotide accession numbers

Amplicons were sequenced in both directions by the PTP Genomic Platform service (Parco Tecnologico Padano, Lodi, Italy). Sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Nucleotide sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/) under accession numbers reported directly on the phylogenetic tree (Figure 1) and listed in Supplementary Material.

Sequence analyses and SNP detection

Nucleotide sequences of the fragment ITS1/ITS4, amplified from the 70 fungal strains associated with grapevines in the present study, and internal transcribed spacer (ITS1-5.8S-ITS2) region sequences of representative strains of fungi previously identified in diseased grapevines, available at NCBI GenBank, were trimmed to the annealing sites of the primers ITS1/ITS4 and used for the creation of a database through the software BioEdit (Hall, 1999). GenBank accession

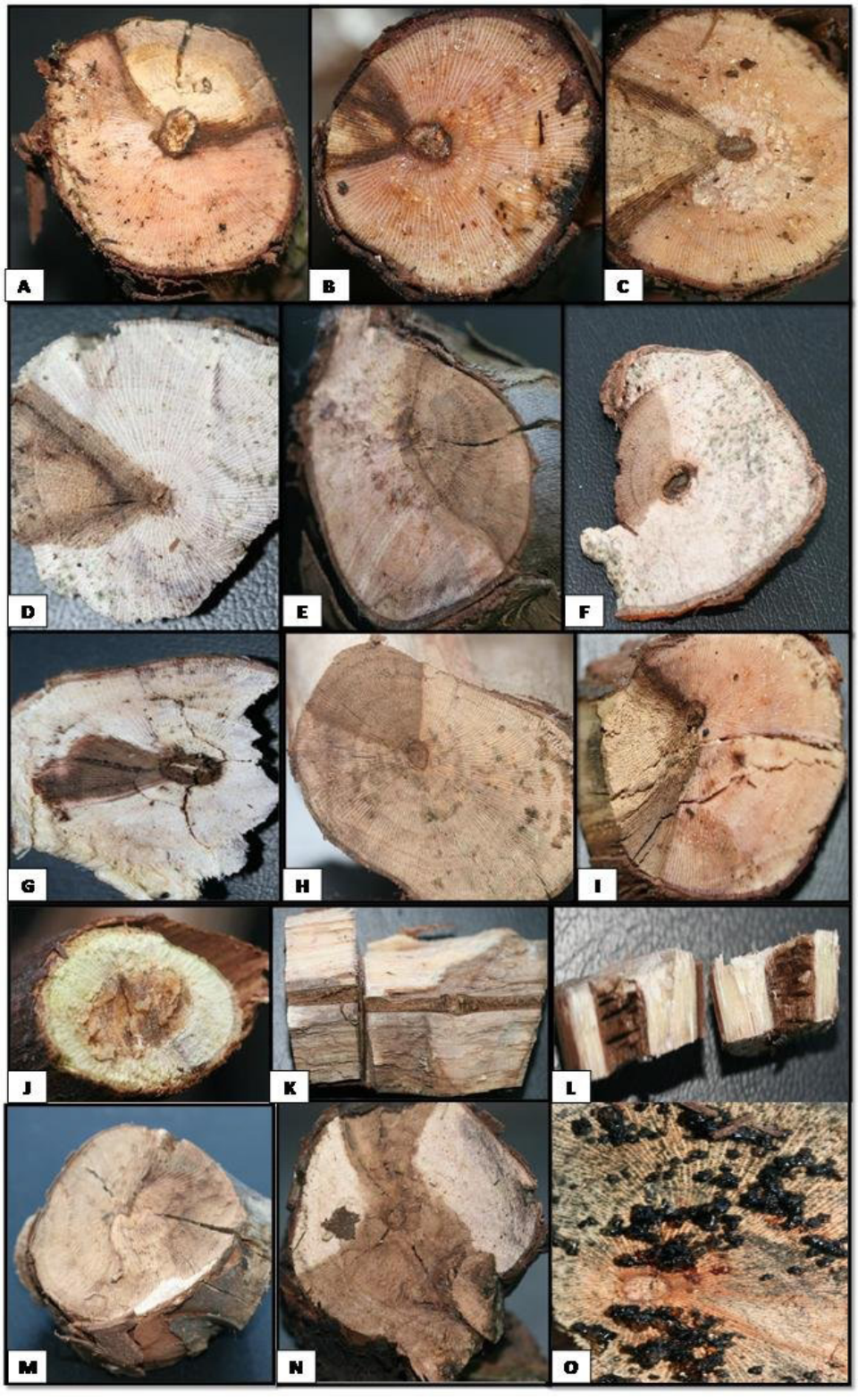

Figure 1. Longitudinal (K, L) and transversal (A, B, C, D, E, F, G, H, I, J, M, N) sections of cordons and spurs from symptomatic vines. Fungal fruiting structures on the surface of diseased wood (O).

numbers of the fungi reference strains retrieved from NCBI GenBank are listed in Table 1 and reported in Figure 1 and in Supplementary Material. Sequences from the database were aligned by ClustalW Multiple Alignment application, and the obtained alignment was analyzed by a Sequence Identity Matrix application of the software BioEdit to define the species affiliation of the isolated fungal strains based on sequence identity percentage in comparison with reference fungi (Table 1 and Supplementary Material). Moreover, alignments were analyzed by eye in order to identify single nucleotide polymorphisms (SNPs), describing the inter- and intra-species variability of the examined fungi.

Virtual restriction fragment length polymorphism (RFLP) analyses

Positions of identified SNPs within restriction sites of enzymes (endonucleases) were verified using the software pDRAW32 (www.acaclone.com), selecting the option 'all enzymes', as previously described for other plant pathogens (Wei *et al.,* 2007; Quaglino *et al.,* 2009). Virtual RFLP profiles using enzymes able to distinguish strains of diverse species (analysis carried out on reference strains) and of the same species (analysis carried out on isolated strains) were generated. Briefly, each DNA sequence was analyzed through an automated *in silico* restriction assay, and digestion results were plotted on virtual gels as described by Wei *et al.* (2007).

Phylogenetic analysis

Nucleotide sequence alignments of ITS1/ITS4 trimmed fragments, amplified from fungal strains isolated in the present study and GenBank reference fungi, were used to construct phylogenetic trees. A Minimum-Evolution method was employed using the Jukes-Cantor model and bootstrap replicated 1000 times with the software MEGA7 to obtain an unrooted phylogenetic tree (Kumar *et al.,* 2016).

Table 1. rDNA sequence identity and prevalence of fungal isolates identified in grapevine.

No. isolates	Seq. Id. Range (%)	*Closest relative species*	GenBank Accession Number
30	99.5 - 100	*Diplodia seriata*	AY259094
20	99.5 - 99.7	*Alternaria alternata*	EU530004
6	99.7	*Neofusicoccum australe*	AY339262
2	99.7	*Diaporthe neotheicola*	JQ663436
4†	96.1 - 97.8	*Diaporthe neotheicola*	JQ663436
3	98 - 100	*Penicillium spinulosum*	KF646101
1	98.2	*Fusarium solani*	KC771504
1†	89.5	*Fusarium solani*	KC771504
2	98.2	*Neofusicoccum vitifusiforme*	AY343382
2	99.1 - 100	*Aspergillus insuetus*	KC473933
1†	96.9	*Chaetomium globosum*	JX241646
1†	93	*Peyronellaea prosopidis*	KF777181
1†	94.1	*Fusarium oxysporum*	AY462580
1	100	*Botryotinia fuckeliana*	HM849615
1†	96.9	Acremonium sp.	HQ649807

†Nine isolates showed best sequence identity <98% and were not assigned to a species.

Results

Field survey and disease symptoms

Over the two years (2011–2013) since the first report of grapevine decline in northern Tunisia was recorded, declining plants were gradually detected, and a portion of these were collected in different grape-growing areas (Northern Coastal and Southern Regions). During the pruning process, common symptoms of grapevine dieback were frequently observed on mature table grapes with ages of 8 to 12 yr in the same region but extended to several vineyards growing the cultivar Muscat Italia. The symptoms included dead spur and cordons, shoot dieback, and sunken necrotic bark lesions, which progressed into the trunk, resulting in the death of large sections of the vine. Indeed, longitudinal and transversal sections of cordons and spurs from symptomatic vines revealed brown wedge-shaped "V" cankers of hard consistency and more or less regular shape (Figure 1). This necrosis may evolve to occupy almost all of the wood. Moreover, a soft yellowish necrosis delimited by a blackish brown lucent line was observed while transversally cutting the stem. A longitudinal section through the wood having a central necrosis shows an altered or even completely decayed marrow tissue, and for other samples, fungal fruiting structures were observed (Figure 1).

Fungal collection and morphological characterization

A total of 76 isolates were recovered from samples of diseased wood collected from infected vineyards and regrouped in 12 groups based on morphological characteristics including six taxa known to be associated with trunk diseases in grapevine, primarily from *Botryosphaeriaceae* and *Diaporthaceae* families. *Botryosphaeriaceae* spp. were the most common fungi isolated with 38 isolates covering 3 taxa, namely, *Diplodia seriata*, *Noefussicoccum australe* and *N. vitifusiforme*, in which *Diplodia seriata* (n=30) was the main species of *Botryosphaeriaceae* spp. Six isolates belonging to *Diaporthaceae* family were identified as *Diaporthe neotheicola*. Other fungal genera (*Alternaria*, *Fusarium*, *Aspergillus*, and *Penicillium*) were also recovered from diseased wood with variable frequency. The rest of the collection cannot be identified based on morphological characterization and still require further molecular identification.

Based on colony and conidia morphological characteristics of *Botryosphaeriaceae* spp., isolates were divided in three species; *Diplodia seriata*, *N. australe* and *N. vitifusiforme*. *D. seriata* colonies were gray-brown with dense aerial mycelium producing brown cylindric to ellipsoid conidia rounded at both ends and averaged 22.4×11.7 µm (n=50). *N. australe* colonies produced globose, black pycnidia with unicellular, hyaline, ellipsoidal, densely granulate, externally smooth, and thin-walled conidia of 17. 7×6.4 µm (n=50). A yellow pigmentation was observed at the center of 48 h colonies on PDA. *N. vitifusiforme* colonies produced aerial mycelium that became gray with age. Conidia were aseptate hyaline, fusoid to ellipsoid, widest in upper third, apex obtuse, base flattened and sub-truncate with average dimensions of 21.4×5.6 µm (n=50). Colonies of *Diaporthaceae* species, identified as *Diaporthe neotheicola*, produced alpha conidia that were unicellular, fusoid, hyaline, and biguttulate with average dimensions of 7.8×2.2 µm (n=50) and beta conidia were unicellular, filiform, curved, hyaline, and eguttulate with average dimensions of 24.5×1.1 µm (n=50).

Molecular identification and phylogenetic analysis

Sequence analyses of the ITS region of the rDNA confirmed the identity of the *Botryosphaeriaceae*. *Diaporthaceae* species were identified using morphological characterization, as well as the identity of saprophytic fungi among them *Botryotinia fuckeliana*, *Alternaria alternata*, *Aspergillus insuetus*, and *Fusarium solani* were observed. According

to the sequence identity shared with the GenBank fungal reference strains (Table 1 and Supplementary Material) and phylogenetic clustering (Figure 2), among 76 isolates, 40 isolates could be associated with trunk disease in which 30 isolates were identified as *Diplodia seriata*, 6 isolates as *N. australe*, 2 isolates as *Diaporthe neotheicola*, and 2 isolates as *N. vitifusifurme*. In the remaining isolates, considered as saprophytes, *Alternaria alternata* was the predominant species with 20 isolates. Due to the low sequence identity value (<98%), 9 isolates were not assigned to a fungal species.

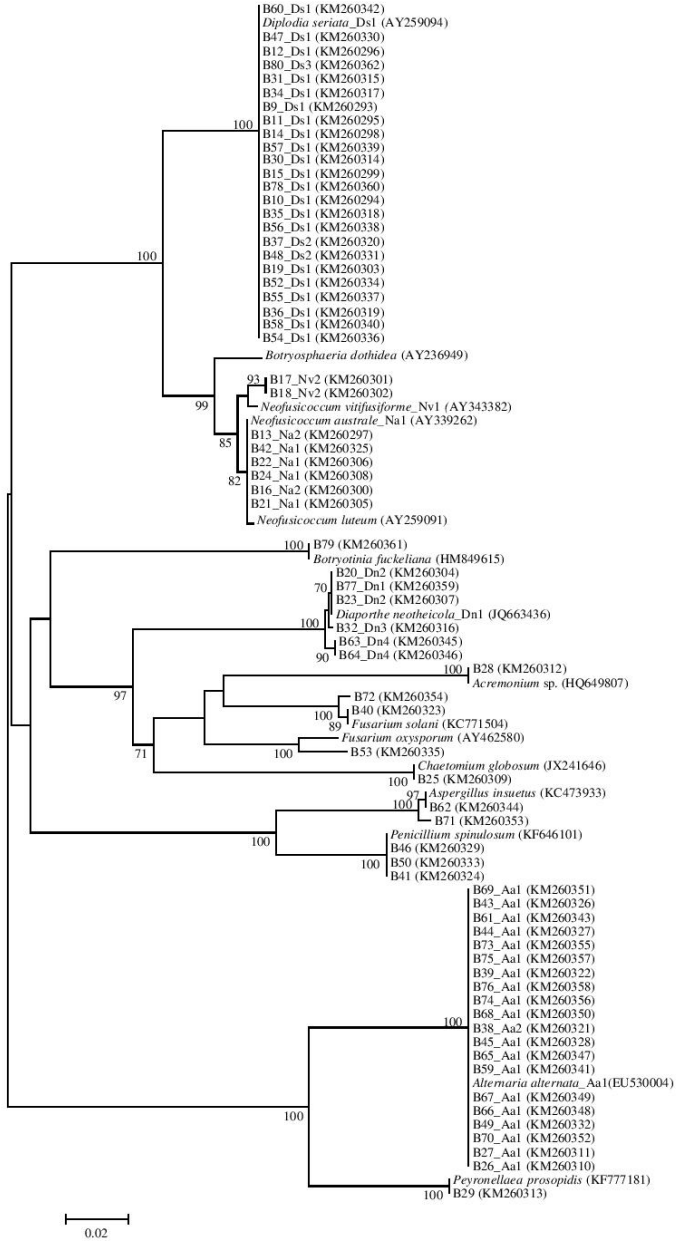

Figure 2. Unrooted phylogenetic tree inferred from nucleotide sequences of rDNA ITS1/ITS4 trimmed fragments of fungi strains isolated in the present study and GenBank reference fungi. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1000 times. List of isolates inserted in phylogenetic analyses is reported in Table 1 and Supplementary Matherial. GenBank accession number of each sequence is given in parenthesis.

SNP detection and RFLP profiling

Virtual-RFLP analyses carried out on nucleotide sequences of fungal reference strains enabled identification of three key enzymes (*Aci*I, *Bcl*I, *Set*I) able to resolve the genetic diversity among species more frequently associated with grapevines in the present study (Figure 3). Moreover, sequence alignments carried out on more representative fungal strains, found in association with diseased plant trunks (*D. seriata*, *N. australe*, *N. vitifusiforme*, and *D. neotheicola*) or as grapevine saprophytes (*A. alternata*), revealed the presence of SNPs distinguishing genetic lineages within each species (Table 2). Restriction analyses using enzymes able to distinguish restriction sites carrying the SNPs produced lineage-specific patterns (Figure 4). In detail, distinct genetic lineages were highlighted using (i) the enzyme *Bsl*I within the species *A. alternata* (Figure 4a), (ii) the enzyme *Eci*I within the species *D. seriata* (Figure 4b), (iii) the enzyme *Bce*AI within the species *N. australe* (Figure 4c), (iv) the enzyme *Ava*I within the species *N. vitifusiforme* (Figure 4d) and (v) the enzyme *Hpy*CH4III within the species *D. neotheicola* (Figure 4e).

Discussion

Vineyards in Northern Tunisia are known to produce seasonal and late cultivars, primarily Muscat Italia, where the symptoms of Botryosphaeria-related dieback were observed.

In this study, fungi recovered from diseased mature vines belong mainly to the *Botryosphaeriaceae* and *Diaporthaceae* families. Several studies showed that *D. seriata* is the most frequently recovered species from diseased vines and found to be associated with dieback of grapevines worldwide and have been identified as causal agents of Black Dead Arm (BDA) (Larignon and Dubos, 1997; Armengol*et al.,* 2001; Bertsch*et al.,* 2013).

Table 2. Single nucleotide polymorphisms distinguishing fungal intra-species lineages associated with grapevine.

Species	SNP lineage	No. of strains†	Acc. No.	Single nucleotide polymorphism (SNP) position‡																			Enzyme
				2	3	13	15	16	17	24	32	42	80	82	83	99	129	130	375	403	437	442	
A. alternata	Aa1	20 (T65A)	EU530004							-													
	Aa2	1 (B38)								G													*Bsl*I
D. seriata	Ds1	30 (CBS 112555)	AY259094	C	T	A	C	T	C	A	A					T							
	Ds2	1 (B80)		G	C	-	T	C	T	-	G					C							*Eci*I
D. neotheicola	Dn1	2 (CRO_PV1053)	JQ663436									A		C									
	Dn2	1 (B23)										G		C									*Hpy*CH4III
N. australe	Na1	5 (CMW6837)	AY339262																		T		
	Na2	2 (B13)																			C		*Bce*AI
N. vitifusiforme	Nv1	1 (STE-U 5050)	AY343382										C	T	C		T	G	A	A		C	
	Nv2	2 (B17)											T	C	G		C	A	G	G		T	*Ava*I

†Representative strain of each SNP lineage is given between parentheses

‡Position within ITS1-5.8S rRNA-ITS2 fragment trimmed for alignment and phylogenetic analyses

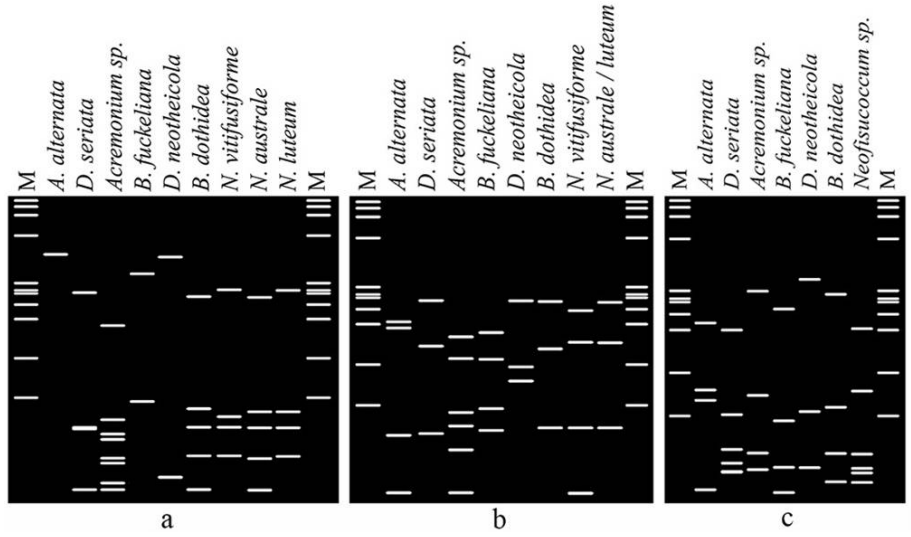

Figure 3. Virtual-RFLP analyses carried out on nucleotide sequences of fungi reference strains using the key enzymes *Aci*I (a), *Bcl*I (b), and *Set*I (c). Obtained profiles allowed to resolve the genetic diversity among species associated with grapevines.

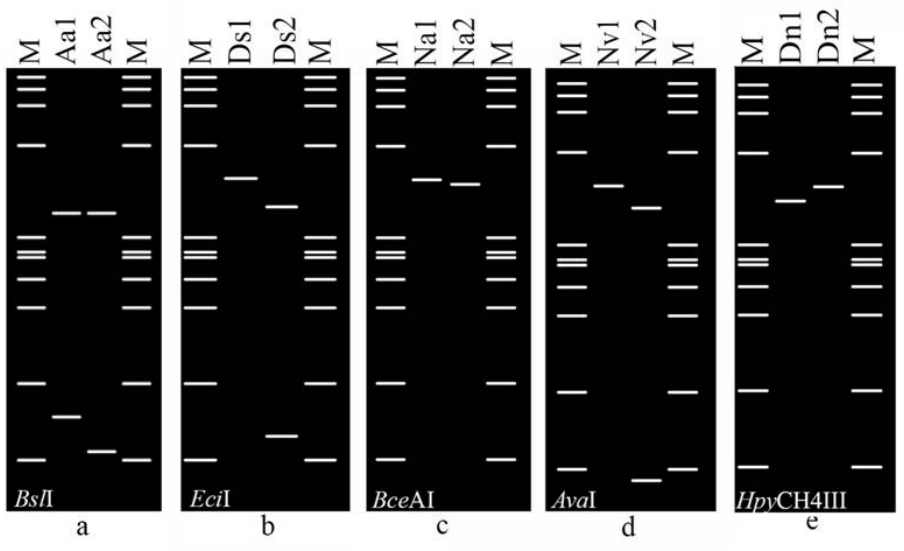

Figure 4. Virtual-RFLP analyses carried out using enzymes able to distinguish restriction sites carrying the lineage-specific SNPs within the species *Alternaria alternata* (a), *Diplodia seriata* (b), *Neofusicoccum australe* (c), *N. vitifusiforme* (d), and *Diaporthe neotheicola* (e).

Moreover, *D. seriata* was predominantly isolated from diseased wood among the *Botryosphaeriacae* species in comparison to other fungal species with low frequencies. However, other studies conducted by Larrignon (2010) in French vineyards especially revealed the presence of *N. parvum* as a causal agent of grapevine decline associated with other fungi but in low frequencies such as *Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Botryosphaeria dothidea*, *Chaetomium* sp., *Clonostachysrosea*, *Diplodia seriata*, *Eutypalata*, *Fusarium* sp., *Phaeoacremoniumaleophilum*, *Phaeomoniellachlamydospora*, *Phomopsis* sp. Contradictorily, other authors (Pollastro *et al.,*

2000; Van Niekerk *et al.,* 2006; Úrbez-Torres *et al.,* 2006; Pitt *et al.,* 2010), indicate other causal agents of grapevine decline such as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp. and *Eutypalata* which were not isolated in this study.

To investigate the etiology and the epidemiology of diseases associated with multiple pathogens occupying complex ecological niches, methods allowing to identify specifically and easily the involved pathogens are required. Previous studies employed molecular methods to identify the fungal pathogens associated with grapevine trunk diseases (Martin *et al.,* 2013; Bruez *et al.,* 2014; Urbez-Torres *et al.,* 2014). In the present work, we developed a highly reproducible SNP-based system for discriminating the more representative fungal strains found in association with diseased plant trunks (*Diplodia seriata*, *Neofusicoccum australe*, *N. vitifusiforme*, *Diaporthe neotheicola*) or as grapevine saprophytes (*Alternaria alternata*). This molecular approach was previously applied to specifically detect closely related phytoplasmas infecting diverse plant hosts and to investigate their epidemiological cycle (Lee *et al.,* 1998; Wei *et al.,* 2007; Casati *et al.,* 2011; Molino Lova *et al.,* 2011). Our method can be used for large surveys on fungi associated with grapevine trunk diseases. Moreover, based on nucleotide sequence alignments, additional PCR-RFLP assays can be accurately designed for detecting fungi rarely associated with diseased grapevines in Tunisia but were found as extremely pathogenic in this study.

Acknowledgments

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Resumen

S. Chebil, R. Fersi, M. Bouzid, F. Quaglino, S. Chenenaoui, I. Melki, G. Durante, E. Zacchi, B.A. Bahri, P.A. Bianco, y A. Rhouma. 2017. Declinación de la vid en Túnez asociada a hongos de las familias Diaporthaceae y Botryosphaeriaceae. Cien. Inv. Agr. 44(2): 127-138. Recientemente se ha observado un fuerte deterioro de las vides maduras en varios viñedos de las regiones de uva del norte de Túnez. Entre agosto de 2011 y junio de 2013, se recolectaron muestras de madera de vid enfermas que mostraban espolones y cordones muertos, desvirones de tallos asociados con lesiones de corteza necrótica hundidas y rayas vasculares de color café a negro de numerosos viñedos enfermos. Se aislaron varias especies de hongos del margen entre tejido sano y sintomático. Tres especies de *Botryosphaeriaceae*, a saber, *Diplodia seriata*, *Neofusicoccum australe*, y *N. vitifusiforme* y una especie de *Diaporthaceae*, a saber, *Diaporthe neotheicola*, se observaron a ser asociado con la disminución de viñas viejas. Otras especies fúngicas se recuperaron de la madera enferma, a saber, *Alternaria alternata*, *Botryotinia fuckeliana* (anamorfo de *Botrytis cinerea*), *Acremonium* spp., *Aspergillus* spp. Y *Fusarium* spp. Además, *Penicillium* spp. inter e intra-diversidad especies fueron evaluados en base del gel RFLP virtual análisis y la identificación de las enzimas de restricción capaz de distinguir cepas de hongos dentro de las especies basado en la determinación del polimorfismo de un solo nucleótido (SNP) linajes dentro de los miembros de clúster en base a la secuenciación del espaciador transcrito interno (ITS1-5.8S-ITS2) región del rDNA.

Palabras clave: *Botryosphaeriaceae*, *Diaporthaceae*, enfermedad del tronco, RFLP virtual, *Vitis vinífera.*

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