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# Occurrence of Botryosphaeriaceae species associated with grapevine dieback in Algeria

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**Abstract:** Grapevine dieback is an increasing problem in grape vineyards in Algeria. We conducted field surveys each spring between 2006 and 2012 to determine the fungal pathogens associated with grapevine decline in various grape-growing regions. We isolated and identified 3 species of *Botryosphaeriaceae* (*Botryosphaeria dothidea*, *Diplodia seriata*, and *Neofusicoccum parvum)* from infected wood using a combination of morphological and cultural characters, as well as phylogenetic analyses of ribosomal DNA internal transcribed spacer (ITS) regions and fruiting body. We identified 20 isolates of *Diplodia seriata*, 13 isolates of *Botryosphaeria dothidea*, and 7 isolates of *Neofusicoccum parvum*. Inoculation of grapevine plantlets with the 3 *Botryosphaeriaceae* species produced necrosis and vascular lesions in the wood after a 6-week incubation; *Neofusicoccum parvum* isolates were the most virulent, followed by *D. seriata* and *B. dothidea*. All 3 species were re-isolated from lesions on infected plantlets. Our results represent the first report of a canker disease of grapevine associated with species of the *Botryosphaeriaceae* in Algeria.

**Key words:** Grapevine trunk diseases, pathogenicity test, phylogenetic analysis

## 1. Introduction

Many farmers cultivating grapevine (*Vitis vinifera* L.) around the world suffer serious dieback problems associated with the development of a range of grapevine trunk disease pathogens. Among them, *Eutypa* dieback, caused by the Diatrypaceae *Eutypa lata* (Pers.: Fr.) Tul. & C.Tul. (Syn.: *Eutypa armeniacae* Hansf. & Carter), is one of the most important canker diseases (Moller, 1978). Another disease, called esca, is one of the most destructive diseases affecting woody grapevine tissues, causing decline and loss of vine productivity (Duboset al., 1988). Several species in the *Botryosphaeriaceae* are also known to infect vines (Van Niekerk et al., 2004). These species have been reported in the last decade as pathogens of grapevine cankers in the United States, South Africa, Australia, France, Italy, Portugal, Egypt, India, Mexico, Chile, and Brazil (Úrbez-Torres, 2011) and in China (Xie et al., 2010). The disease associated with these fungal pathogens is referred to as *Botryosphaeria* dieback (Úrbez-Torres, 2011). This disease, formerly named black dead arm (BDA), was identified for the first time in a field in Tokaj, Hungary (Lehoczky, 1974), where it was observed on the basis of wood lesions only. Later, this disease was associated with foliar symptoms in Italy (Rovesti and Montermini, 1987) and in France in 1999 (Larignon et al., 2001). A recent survey however, provided evidence that foliar symptoms attributed to BDA in fact belonged to esca syndrome (Lecomte et al., 2012). The *Botryosphaeriaceous* fungi, which can be localized in healthy tissue on any part of the whole plant, mostly cause disease after a tree or fruit experiences stress after harvest and higher rainfall (Johnson, 1992). The fungus develops in the wood and causes slow decline and death. Symptoms generally associated with *Botryosphaeria* dieback on grapevine include mortality, dieback, cankers and sector-sharped lesions inside the wood, bud mortality, reduced growth, and in some cases branch rot (Van Niekerk et al., 2004).

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According to statistics from the Algerian Ministry of Agriculture, in 2012 the total vineyard area in Algeria was 100,200 ha, for grape production of 334,021 t. In spite of significant funding from the Ministry of Agriculture in recent years to improve the wine sector, a noticeable fall in vineyard area accompanied by a decrease in production was recorded due to the death (total or partial) of many vines, and the pulling of many vineyards that had shown too many symptoms of decline. In response to this, a preliminary survey (Ammad, 2006) reported the presence of dieback in some grapevine regions in Algeria. However, research into this serious problem has been very limited. The aim of this study was therefore to identify and characterize the causative agents of grapevine dieback in Algerian vineyards using morphological, cultural characteristic, and molecular tests. In addition to these means used to identify the agents involved in this decline, we conducted a search on the presence of the sexual stage on dead wood (fruiting body) and in vitro pathogenicity tests on grapevine vitro-plantlets.

## 2. Materials and methods

The study was conducted each spring between 2006 and 2012 (Figure 1), in 4 viticulture areas: Tipaza, Mostaganem, Blida, and Medea, located in the west and center of northern Algeria. Each of these sites was planted with different cultivars: Dattier de Beyrouth, Gros noir, Cabernet Sauvignon, Muscat, Cardinal, Carignan, Cinsault, Merlot, and Syrah. These areas are situated in a bioclimatic zone with an average annual precipitation of 600 mm. Vines were 5–45 years old and were not treated with sodium arsenite or any other fungicide (Table 1). The survey was based on the detection of typical dieback symptoms (specifically foliar symptoms), such as reduced growth in small leaves compared to their normal state; distorted, sometimes chlorotic and necrotic leaves; and shoots that often had very short internodes. These symptoms were mostly attributed to *Eutypa* dieback (Dubos, 2002) but some may also have corresponded to *Botryosphaeria* dieback. Wood samples were collected from a total of 17 vines; the samples comprised 10 vines from each vineyard expressing dieback symptoms including dead shoots, cankers, and discolored tissues. Isolations were made from each type of necrotic tissue.

### 2.1. Isolation

We isolated a total of 1560 wood slices, originating from mature and young vines. The slices showed disease symptoms including longitudinal lesions of canes, wedge-shaped and central necrosis symptoms in cross sections, and dead cordons. We sectioned wood slices, 0.5-mm thick, from the margins of necrotic and apparently healthy tissues. Slices were surface disinfected in sodium hypochlorite (NaOCl, 2%) for 4 min, rinsed, and dried on sterilized filter paper. The slices were then placed on potato-dextrose agar (PDA) plates, and incubated at 25 °C. We recorded weekly observations of fungal development, and on the basis of morphological characteristics identified the isolates as *Botryosphaeria* according to Phillips et al. (2002) and Slippers et al. (2004).

### 2.2. Fruiting body surveys

Dead branches and wood were inspected in the vineyard for the presence or absence of perithecia and pycnidia (sexual and asexual fruiting bodies of the fungus). Infected samples that showed pycnidia forms were transported to the laboratory, where they were left to dry for examination. The preparation of ascospores was conducted from fruiting bodies in sterilized water and placed on PDA plates. After 24 h at 25 ± 2 °C, individual germinated spores were removed under a microscope for observation (Van Niekerk et al., 2010).

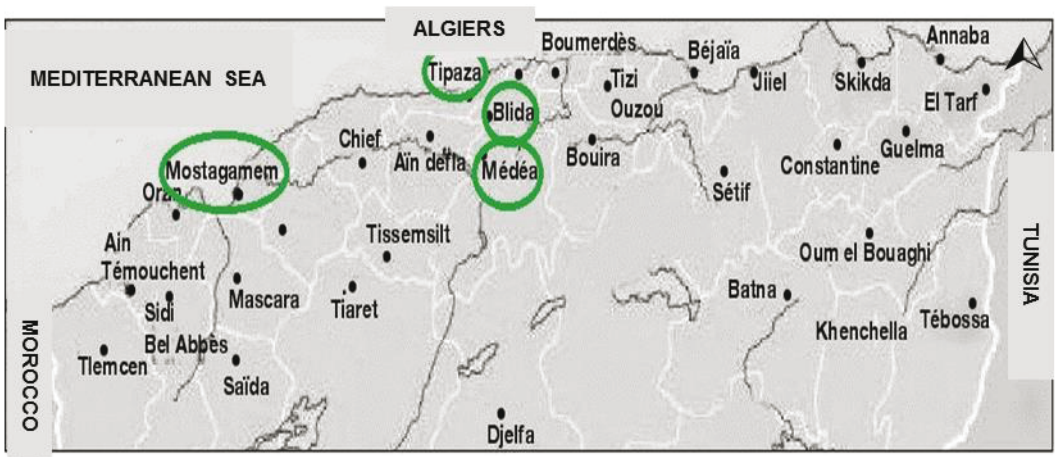


**Figure 1.** The 4 regions in Algeria under study.

**Table 1.** Characteristics of grapevines and grape regions surveyed in Algeria.

| Locality | Médéa | | Blida | | | | | | | | Tipaza | | | | | Mostaganem | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | Benchicao | | Mouzaia | | | | | | | | Hamr El Ain | | | | | ONCV | |
| Vineyards | 1 | 2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 1 | 2 |
| Cultivar | Dattier de Beyrouth | Carignan | Cinsault | Dattier de Beyrouth | Cardinal | Carignan | Syrah | Cabernet sauvignon | Merlot | Cinsault | Dattier | Gros noir | Cinsault | Syrah | Cabernet sauvignon | Muscat | Muscat |
| Age (year) | 26 | 45 | 11 | 10 | 12 | 12 | 05 | 10 | 09 | 12 | 40 | 10 | 12 | 11 | 11 | 6 | 30 |
| Area (ha) | 12 | 06 | 10 | 6.5 | 05 | 05 | 04.5 | 04 | 03 | 06.5 | 05 | 04 | 03 | 04.5 | 03 | 10 | 11 |
| Type of pruning | Guyot simple | Guyot double | Guyot | Cordon double | Cordon double | Guyot | Guyot | Guyot | Guyot | Guyot | Cordon double | Cordon double | Guyot | Guyot | Guyot | Goblet | Goblet |
| rootstock | 41B | 41B | 41B | 41B | 41B | 41B | SO4 | SO4 | 41B | 99R | 41B | 41B | SO4 | 99R | 41B | 41B | 41B |
| Time of pruning | December | | | | | | | | | | | | | | | | |

ONCVNational (Algerian) Office of Marketing of Wine Products

**2.3. DNA extraction, PCR, and multigene phylogenic analysis**

The total genomic DNA of all isolates identified morphologically as *Botryosphaeriaceae* was extracted from pure culture mycelia as reported by Liu et al. (2000). Oligonucleotide primers ITS1 (5'-TCC GTA GT GAA CCT GCG G-) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the ITS region of the nuclear ribosomal DNA (including the 5.8S gene) (White et al., 1990). The amplification reactions were performed in a 25-µL volume of reaction mixture and contained 1 mM of each primer, 0.2 mM of dNTP, 15 mM of $MgCl_2$, and 2.5 U of Taq polymerase adjusted with purified distilled water to a final volume of 25 µL. The PCR program for ITS region amplification included an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 1 min denaturation at 94 °C, annealing for 40 s at 53 °C, and 1 min elongation at 72 °C, with a final elongation step at 72 °C for 10 min. The PCR amplification products were separated by electrophoresis on 1.5% agarose gels prepared in 0.5X TBE (Tris-Borate 100 mM; pH 8.3; EDTA 2 mM), stained with 0.5 mg/L solution of ethidium bromide and visualized under UV light (Sambrook et al., 1989). The sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing kit and carried out with an ABI Biosystem 3130 (Applied Biosystems, HTDS, Tunisia).

**2.4. Phylogenetic analysis**

The nucleotide sequences were read and edited with Chromas 1.7.5 (http://www.technelysium.com.au/chromas.html). We checked all sequences manually and initially analyzed them by searching the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). We obtained reference sequences for the ITS regions for the *Botryosphaeriaceae* spp. from GenBank and deposited all new sequences in GenBank under the accession numbers listed in Table 2. The trees were rooted to *Mycosphaerella konae* (GenBank AY260085).

The ITS sequences were aligned with Clustal X v.1. using pairwise alignment parameters (gap opening = 10, gap extension = 0.1), multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 30%), and manual adjustments made where necessary. The phylogenetic tree was constructed by maximum parsimony (MP) analysis, using MEGA 5.2.2 software. For the MP analysis, we used the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping options. All characters were unordered and of equal weight. Statistics included tree length (TL), consistency index (CI), retention index (RI), and composite index (IC) values. The robustness of the tree was evaluated by bootstrap analysis based on 1000 replications.

**2.5. Pathogenicity test**

Pathogenicity tests were performed in vitro on grapevine vitro-plantlets using 2 Algerian local cultivars, as already described by Conceição Santos et al. (2005, 2006). They used calluses of grapevine that were cultured in the presence of strains as *Phaeoacremonium* and *Phaeomoniella.* The same study was conducted with *Botryospheria* species by Montserrat (2010). Cuttings from 2 internodes of Valenci and Hmar Bouamar were maintained in culture in vitro micropropagation. After sterilization, micro-cuttings (fragments of stem, leaf, and bud) were placed on the middle ML-vitis. This medium was supplemented with agar (7 g/L), sucrose (30 g/L), acetic naphthalene acid (0.6 g/L) and 6-benzylaminopurine (0.01 g/L), and activated carbon (0.05 g/L). After 2 months of culture under a cycle of 16 h light and 8 h dark at 23 °C and 75% humidity during the day and at 18 °C and 85% humidity at night, we obtained each seedling or micro-cutting and transplanted them under aseptic conditions in a test tube for in vitro infection with our range of fungi. We tested 30 isolates belonging to the 3 fungal species isolated in this study: *B. dothidea*, *D. seriata*, and *N. parvum*, with 3 isolates per species. A total of 90 in vitro plantlets for each cultivar were inoculated with isolates of the 3 tested species (10 plantlets per isolate and 3 isolates per species). The mycelium suspension was prepared in $MgSO_4$. Using fine sterile needles, we injected 0.1 mL of each fungal suspension into superficial wounds at different positions without touching the cambium on the vines. The inoculation sites were covered by cotton swabs moistened with sterile water to avoid desiccation. We used 9 in vitro plantlets as negative controls for the 3 isolates of each cultivar; they were inoculated with sterile distilled water. Vitro plantlets were observed after 6 weeks and inspected for development and disease symptoms. The intensity of infection was evaluated via area of infection and apparition of necrosis from the inoculation point; it was classed as low, medium, or high by visual observations. We gave the rating 1/1 when a net necrosis appeared a few millimeters below the contact area between the in vitro plantlets and the mycelium (symptoms away from the point of infection are visible yellowing and necrosis of leaves). The rating 1/0 corresponded with the appearance of a net necrosis a few millimeters below the contact area associated with the fungus without foliar symptoms, and a score of 0/0 was given in the case where there was no visible necrosis or foliar symptoms. Small fragments (0.5 to 1 cm) of necrotic tissue were placed on PDA plates to determine the cause of the lesions. After 5 days, fungi were identified based on cultural and morphological characters.

**Table 2.** Fungal isolates included in phylogenetic analyses.

| Isolate number | Identity | Host | Country | Collector, year | ITS GenBank code |
|---|---|---|---|---|---|
| CBS 121485 | *Diplodia seriata* | *Vitis vinifera* | Spain | Martos et al., 2011 | EU650671 |
| CBS 200.49 | *Diplodia seriata* | *Alnus* sp. | Portugal | Phillips, 2006 | EF127892 |
| GA-422 | *Diplodia seriata* | *Prunus persica* | China | Wang et al., 2012 | HQ660463 |
| CCTU370b | *Diplodia seriata* | *Morus nigra* | Iran | Arzanlou, 2012 | KC020171 |
| CCTU370 | *Diplodia seriata* | *Morus nigra* | Iran | Arzanlou, 2012 | KC020170 |
| CXY1571 | *Diplodia seriata* | *Carya cathayensis* | China | Quan, 2012 | KC527828 |
| BB9 | *Diplodia seriata* | *Vitis vinifera* | Australia | Wunderlich, 2011 | HQ392699. |
| B2 | *Diplodia seriata* | – | Italy | Carlucci et al., 2009 | FJ481588 |
| B39 | *Diplodia seriata* | – | Italy | Carlucci et al., 2009 | FJ481599 |
| B24 | *Diplodia seriata* | – | Italy | Carlucci et al., 2009 | FJ481596 |
| IMI 395176 | *Botryosphaeria dothidea* | *Populus tremula* | Italy | Grasso and Granata | GU119957 |
| CBS 121484 | *Botryosphaeria dothidea* | *Vitis vinifera* | Spain | Luque | EU650670 |
| CAP248 | *Botryosphaeria dothidea* | *Olea europaea* | Italy | Lazzizera et al., 2009 | EF638767 |
| CAP246 | *Botryosphaeria dothidea* | *Olea europaea* | Italy | Lazzizera et al., 2009 | EF638766 |
| CAP244 | *Botryosphaeria dothidea* | *Olea europaea* | Italy | Lazzizera et al., 2009 | EF638765 |
| CAP243 | *Botryosphaeria dothidea* | *Olea europaea* | Italy | Lazzizera et al., 2009 | EF638764 |
| *Lijiang09* | *Botryosphaeria dothidea* | *Vaccinium corymbosum* | China | Yu et al., 2012 | JX096631 |
| ZY-713 | *Botryosphaeria dothidea* | *Prunus persica* | China | Wang et al., 2012 | HQ660462 |
| V473 | *Botryosphaeria dothidea* | *Vitis vinifera* | Uruguay | Abreo et al., 2012 | JX271827 |
| V122 | *Botryosphaeria dothidea* | *Vitis vinifera* | Uruguay | Abreo et al., 2012 | JK217826 |
| *CIAD 02111* | *Neofusicoccum parvum* | *Avocado fruit* | Mexico | Molina-Gayosso et al., 2012 | JN203129 |
| UY1050 | *Neofusicoccum parvum* | – | Uruguay | Perez et al., 2008 | EU080928 |
| UY754 | *Neofusicoccum parvum* | – | Uruguay | Perez et al., 2008 | EU080926 |
| UY1720 | *Neofusicoccum parvum* | – | Uruguay | Perez et al., 2008 | EU860399 |
| UY1366 | *Neofusicoccum parvum* | – | Uruguay | Perez et al., 2008 | EU080935 |
| UCR1166 | *Neofusicoccum parvum* | *Citrus* | California | Adesmoye and Eskalen, 2011 | JF271762 |
| PVFi-Np8 | *Neofusicoccum parvum* | *Vitis vinifera* | Italy | Spagnolo et al., 2011 | GU187987 |
| PVFi-Np28 | *Neofusicoccum parvum* | *Vitis vinifera* | Italy | Spagnolo et al., 2011 | GU188007 |
| PVFi-Np27 | *Neofusicoccum parvum* | *Vitis vinifera* | Italy | Spagnolo et al., 2011 | GU188006 |
| UY1267 | *Neofusicoccum parvum* | *Vitis vinifera* | Uruguay | Perez et al., 2008 | EU860385 |
| STE-U 2125 | *Mycosphaerella konae* | Proteaceae | South Africa | Taylor et al., 2003 | AY260085 |
| AF9F | *Neofusicoccum parvum* | *Vitis vinifera* | Algeria | Ammad, 2012 | KF465683 |
| AF10F | *Neofusicoccum parvum* | *Vitis vinifera* | Algeria | Ammad, 2010 | KF465684 |
| AF29F | *Neofusicoccum parvum* | *Vitis vinifera* | Algeria | Ammad, 2006 | KF465685 |
| AF30F | *Neofusicoccum parvum* | *Vitis vinifera* | Algeria | Ammad, 2010 | KF465686 |
| AF9F1 | *Neofusicoccum parvum* | *Vitis vinifera* | Algeria | Ammad, 2010 | KF465687 |
| AF52 | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465689 |
| AF5F | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2009 | KF465693 |
| AF5 | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465694 |
| AF27F | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465695 |
| AF44F | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465696 |
| AF27 | *Botryosphaeria dothidea* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465688 |
| AF45F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465697 |
| AF46F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465698 |
| AF3F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465691 |
| AF3 | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2006 | KF465692 |
| AF331F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465700 |
| AF332F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2009 | KF465702 |
| AF8 | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2010 | KF465703 |
| AF9 | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2010 | KF465704 |
| AF7 | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2009 | KF465705 |
| AF8F | *Diplodia seriata* | *Vitis vinifera* | Algeria | Ammad, 2011 | KF465706 |

### 2.6. Data analysis

The presence of necroses was used as an indicator of pathogenicity, and was analyzed using the general linear model (GLM) with the aid of the Systat software (version 6.0) with "grapevine variety" and "isolate" as independent factors. Tukey's test was used to detect differences among isolates within a given species.

## 3. Results

In this study, *Botryosphaeriaceous* fungi were the most commonly isolated species, followed by the fungal agents associated with *Eutypa* dieback and esca, namely *Eutypa lata* and *Fomitiporia mediterranea*, respectively. *Botryosphaeriaceous* fungi were detected in 85% of infected wood samples. These fungi were isolated from all vines showing disease symptoms, and were frequently isolated from central and sectorial gray and brown necrosis of soft and hard textured wood. There were 2 species that were frequently isolated from grapevine cankers in all regions; 40% of isolates conformed to the *Diplodia seriata* anamorph of the genus *Botryosphaeria obtusa* and 30% of isolates were identified as *Botryosphaeria dothidea.* They were found in all vineyards surveyed in Algeria. This was followed by *Neofusicoccum parvum,* represented by 10% of isolates and found only on syrah in Tipaza. Among the other fungi isolated from grapevine cankers, 10% of 4 species were isolated in all regions: *Coniolariella gamsii*, *Coniolariella hispanica*, *Biscogniauxia mediterranea*, and *Xylaria hypoxylon.*

### 3.1. Morphological and cultural characterization

The *Botryosphaeriaceae* colonies that occurred on the PDA media in the first 2–3 days of incubation were white and had a cottony texture; all isolates were typical of *Botryosphaeriaceae* species producing high density and aerial hyphae after 10 days of incubation (Figure 2). These isolates developed gray pigment that turned dark green

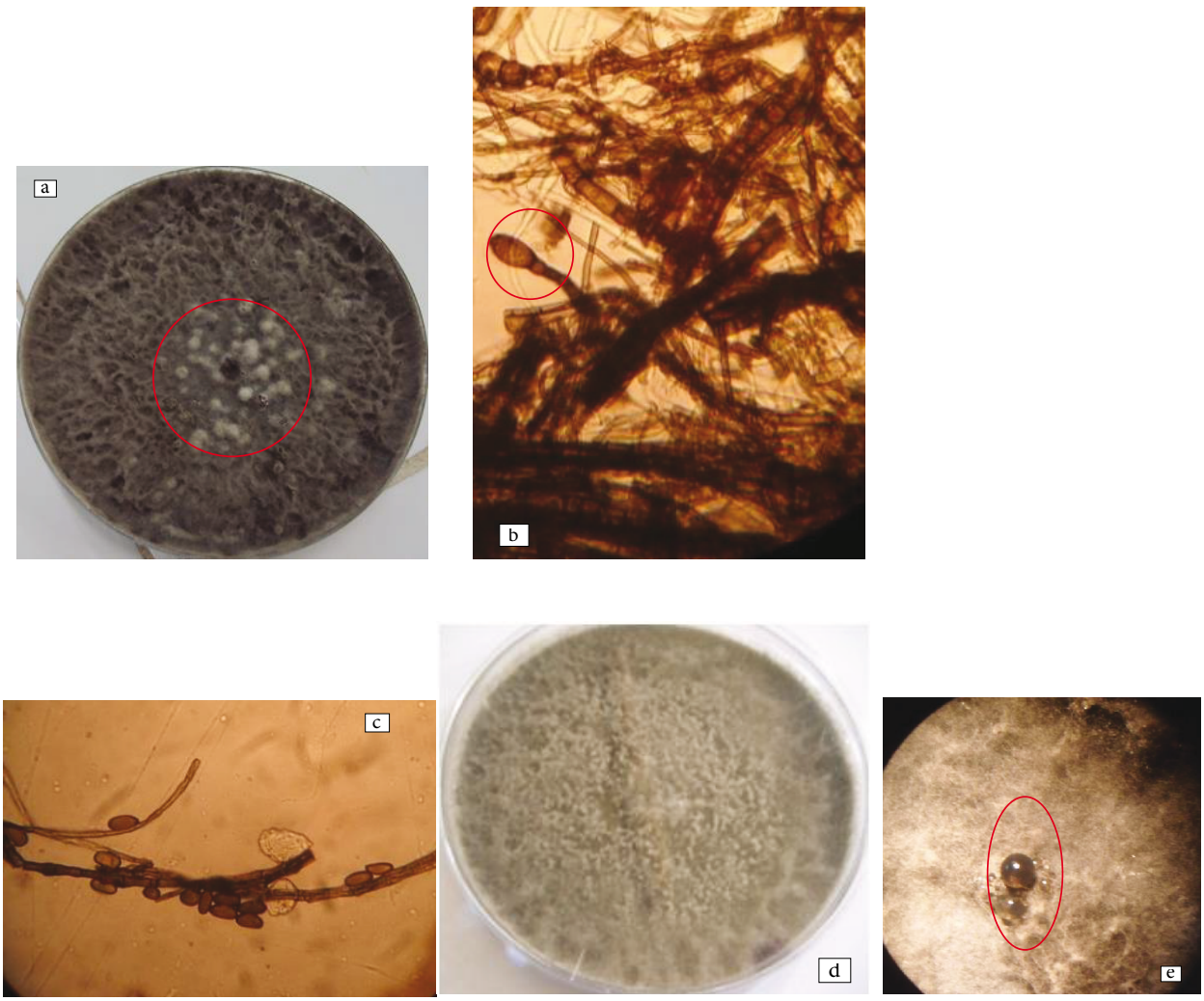


**Figure 2.** *Botryosphaeria* colonies: (a) *Diplodia seriata* on PDA medium aged 1 month, dark gray; (b) ramified mycelium (G: 40 × 10); (c) conidia (G: 10 × 10 × 40); (d) *Botryosphaeria dothidae* on PDA medium, brown; (e) pycnidia on colonies.

and black. Many colonies developed fruiting structures (blackish and globular, grouped or single) with pycnidia on the surface after 3 months of incubation. Microscopic observation showed many kinds of conidia. The conidia from *Diplodia* isolates were hyaline when immature and dark brown at maturity, occasionally aseptate, but mostly 1-septate. The conidia from *Botryospheria dothidea* were hyaline at maturity. The *N. parvum* may be hyaline, with 2-septate or nonseptate. The tree species were present in each of the 4 regions surveyed; however, not all species were present in each individual vineyard. The origins of isolates used in pathogenicity tests are presented in Table 3. We selected 21 isolates for molecular analysis, and the strains of *Botryosphaeriaceae* used for comparison are shown in Table 2.

### 3.2. Fruiting body

We found many fruiting structures of various trunk disease pathogens or saprobes on the surface of the dead branches and debris collected in some prospected vineyards in the 4 regions visited, especially on the Sauvignon Cabernet, Muscat, and Dattier de Beyrouth varietals. In particular, we observed an abundance of mature pycnidia. Microscopic observation of these pycnidia showed the presence of conidiogenous cells and conidia typical of 2 species, *Diplodia seriata* (Figure 3) and *Botryosphaeria dothidea* (Figure 4).

### 3.3. Molecular identification and phylogenetic analysis

To confirm identification based on morphology, BLAST searches in GenBank showed that ITS sequences of *Botryosphaeriaceae* isolates had 99%–100% homology with isolates of *D. seriata*, *B. dothidea*, and *N. parvum*. The ITS sequences of a second group of isolates were 95% identical to those of *Conilariella hispanicaa* and *C. gamsii*. A third group of isolates presented 98% homology with *Biscogniauxia mediterranea* and *Xylaria hypoxylon*.

We performed phylogenetic analysis with genera of *Botryosphaeriaceae* species, using maximum composite likelihood to construct the maximum parsimony tree, as shown in Figure 5.

The ITS dataset comprised sequences from 21 isolates collected in this study and 31 reference sequences retrieved from GenBank, representing 3 in-group and 1 out-group taxa. The alignment contained 465 characters including coded alignment gaps. After a heuristic search, 10 parsimonious trees with the same topology were retained (TL = 129 steps, CI = 0.82, RI = 0.98, composite index = 0.87) for all sites.

### 3.4. Pathogenicity test

*Diplodia seriata*, *B. dothidea*, and *N. parvum* isolates were pathogenic on grapevine plantlets of the Hmar Bouamar and Valenci cultivars, which showed necroses and lesions upward and downward from the point of inoculation.

**Table 3**. Length of necrosis obtained 6 weeks after inoculation with each of the fungal species (in 2011) Valenci (Algerian cultivar).

| Species | Mean necrosis length (mm) | Test GLM | Isolates | Mean necrosis length (mm) | Test GLM |
|---|---|---|---|---|---|
| Valenci | 3.3 ± 0.9[a] | | *N. parvum* | 4.8 ± 0.1[a] | |
| | | | *D. seriata* | 3.2 ± 0.2[b] | |
| | | | *B. dothidea* | 2.4 ± 0.2[c] | |
| | | F = 6.63 ; P = 0.013, P < 5% | Control | 1.1 ± 0.0[d] | F = 51.88; P = 0.000, P < 0.01% |
| Hmar Bouamar | 2.4 ± 0.7[b] | | *N. arvum* | 4.0 ± 0.4[a] | |
| | | | *D. seriata* | 3.2 ± 0.1[b] | |
| | | | *B. dothidea* | 1.9 ± 0.2[c] | |
| | | | Control | 0.7 ± 0.2[d] | |

Data are means of 3 replicates per treatment ± standard deviation.
Cankers not measured, observed after inoculation.
Rating
Significant at P < 0.05; significant at P < 0.01; significant at P < 0.001; ns: nonsignificant.


**Figure 3.** *Botryosphaeria dothidae* fruiting body found on the infected grapevine: (a) Pycnidia; (b) Conidia (G: 40 × 10).

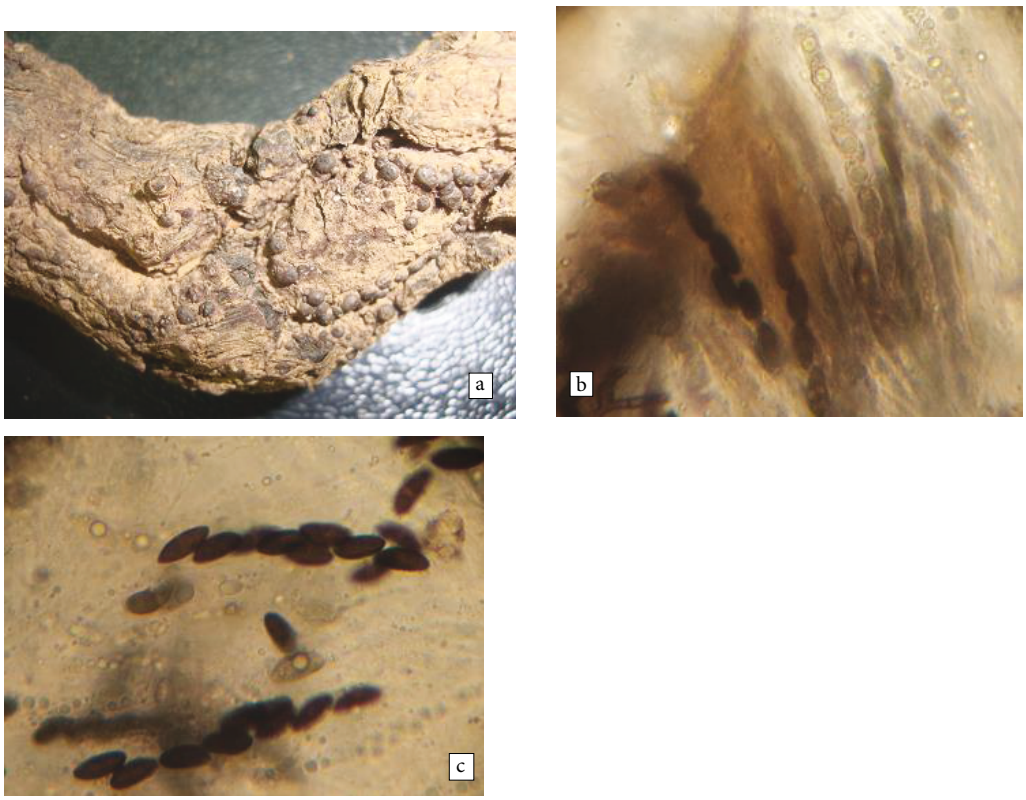


**Figure 4.** *Diplodia seriata* fruiting body found on the infected grapevine: (a) pycnidia; (b) conidigenous (G: 40 × 10); (c) conidia (G: 40 × 10).

All 3 species produced necroses and lesions that were significantly different from the control, but the degree of infection and length of lesions varied within and between species. On both cultivars, *N. parvum* isolates were the most virulent, followed by *D. seriata*. These isolates induced internal necrosis and external cankers developing from the point of inoculation. *B. dothidea* isolates caused the smallest lesions. When scoring symptoms for the 540 infected in vitro plantlets, we observed 118 in vitro plantlets without symptoms and 422 in vitro plantlets with symptoms. For the Hmar Bouamar variety, 64 in vitro plantlets had severe symptoms and 152 had mild symptoms. Almost the same results were recorded with the second variety tested, Valenci: 152 in vitro plantlets without severe symptoms and 54 with mild symptoms. The 27 control in vitro plantlets produced light discolorations around the

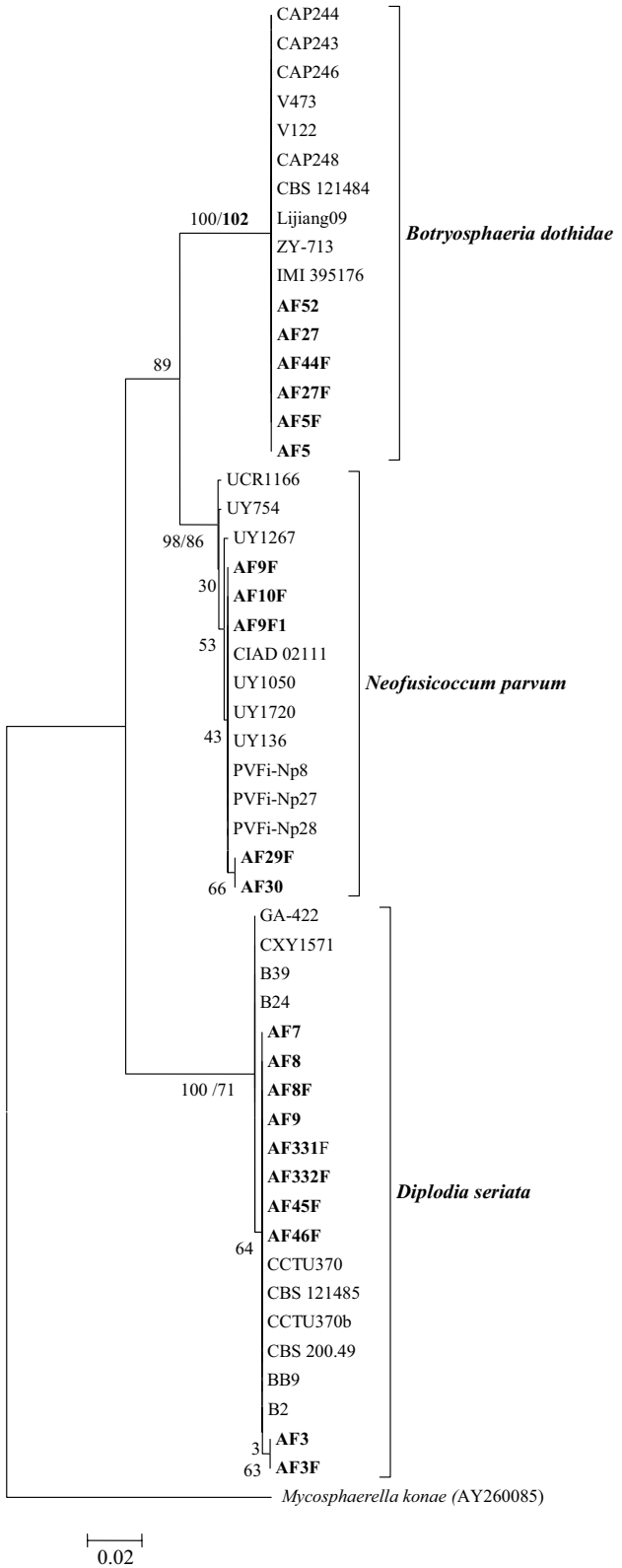


**Figure 5.** One of 10 equally parsimonious trees resulting from the alignment. Bootstrap values from 1000 replications are shown for maximum parsimony (MP); *Mycosphaerellakonae* (GenBank AY260085) were included as out-groups.

point of inoculation. We observed wood necrosis, which showed lesions upward and downward from the point of inoculation. The mean lesion lengths for *N. parvum*, *D. seriata*, *B. dothidea* and the control were 4.80 ± 0.14[a], 3.24 ± 0.22[b], 2.42 ± 0.20[c], and 1.08 ± 0.028[d] mm, respectively on Hmar Bouamar and 3.96 ± 0.35[a], 3.19 ± 0.05, 1.93 ± 0.18[c], and 0.66 ± 0.18[d] mm, respectively on Valenci. A GLM test indicated a significant difference between the cultivars (F = 6.63; P = 0.013, P < 5%). Valenci was more susceptible than Hmar Bouamar to *N. parvum and B. dothidea*. However, significant differences between isolates and their respective controls were more frequent in Valenci (F = 51.88, P = 0.000, P < 0.01%). The results obtained from Tukey's test analyses showed some variability in the length of the lesions caused by various isolates tested for pathogenicity. The most significant lesions were induced by *N. parvum* (maximum mean values), followed by *D. seriata*, whereas reduced variation was observed for *B. dothidea* (Table 3). All 3 species were re-isolated from the margins of necrotic lesions and their identity confirmed on the basis of morphological and cultural characters. No fungi were isolated from the controls.

## 4. Discussion

In Algeria the incidence of grapevine decline has recently significantly increased, and grapevine trunk diseases have become a limiting factor in local vineyards, compromising the Algerian wine industry for long periods. Until now, few studies have been conducted to evaluate the health status of vineyards with symptoms of dieback and showing the presence of the 2 wood diseases *Eutypa* and esca; our study is the first to address the role of the *Botryosphaeriaceae* species in the decline of grapevines in Algeria. We noted the presence of 3 species of *Botryosphaeriaceae* in Algerian vineyards based on cultural and conidial morphology and on fruiting bodies. Úrbez-Torres (2011) recorded the presence of 21 species of *Botryosphaeria* causing multiple grapevine disease symptoms worldwide, and via comparison of ITS sequence data with reference specimens provided the first report in Algeria of *B. dothidea*, *D. seriata*, and *N. parvum* as grapevine pathogens. All 3 species of *Botryosphaeriaceae* were isolated from central and/or sectorial necrosis of grapevine wood. *Neofusicoccum parvum* was only isolated in some Algerian vineyards, while *B. dothidea* and *D. seriata* were isolated from all regions surveyed. *Diplodia seriata* was isolated most frequently, and isolated from V-shaped necroses and central necroses, which is in accordance with previous reports (Dubos et al., 1997; Mugnai et al., 1999).

The presence of pycnidia on dead wood was used to determine and confirm morphological and cultural characteristics. According to a key provided in Phillips et al. (2013), *B. dothidea* can be distinguished from other

members of the genus by conidia that are typically longer than 20 µm. These conidia are narrowly or irregularly fusiform, have thin walls, and are generally transparent or translucent (hyaline) and aseptate but sometimes form up to 2 septa and/or darken when they are older. Pycnidia of the second isolate, *Diplodia seriata* (teleomorph, *Botryosphaeria obtusa*), are brown, ostioles, globular, and contain conidia. The diameter of pycnidia was 500 µm. The conidia were brown, ovoid, and first unicellular and then bicellular, measuring 22–26 µm × 10–12 µm (Dubos, 2002). This species is recognized as an important pathogen in Armengol et al.'s study (2001), which showed a greater incidence of *D. seriata* in Spanish vineyards. Zebib (2011) also reported this species in Lebanese vineyards. *Diplodia seriata* also occurs on olive drupes in Spain (Moral et al., 2008), Tunisia (Chattaoui et al., 2012), and Croatia (Kaliterna et al., 2012). A recent study reported leaf dieback on *Osmanthus* sp. in China (Xie et al., 2010). It has been observed in temperate areas on most continents and described from over 35 different hosts, including *Vitis* spp. (Slippers et al., 2007).

Our results showed that *B. dothidea* has a large distribution in Algeria, and has been found in different stages of climate: temperate and humid in the north near the Mediterranean Sea and in the semiarid western and central regions. This isolate is considered one of the most common species associated with grapevine decline syndrome in Portugal (Phillips, 2002) and Spain (Aroca et al., 2006). *Botryosphaeria dothidea* is also known to infect the fruits of several hosts in Italy (Marinelli et al., 2012), where it was identified as the causal agent of fruit rot for the first time. This species was confirmed to be associated with band canker in almond trees (Inderbitzin et al., 2010). In contrast to our results, where *Neofusicoccum parvum* comprises 10% of the isolates on vines in Algeria, no fruiting was detected on wood and the identity of this species was confirmed by molecular tests. It has been reported as the dominant fungus affecting grapevine in France (Dubos et al., 1997) with 92.6% occurrence in the analyzed plants; it is mostly localized in sectoral necrosis and brown bands. Its ability to cause necrosis in the woods or on the herbaceous part in grapevine has been established by other authors (Larignon et al., 2001; Van Niekerk et al., 2004; Rego et al., 2009), and it was the dominant species reported on almond on Spain (Gramaje et al., 2012). This species has been frequently been reported as an important pathogen of many woody plants in temperate regions around the world (Phillips, 2002; Inderbitzin et al., 2010). Our study revealed the presence of 3 *Botryosphaeriaceous* species threatening the sustainability and longevity of Algerian vineyards. In this work, we conducted pathogenicity studies based on the characterization of plant material infected by different isolates and carried out by scoring symptoms, measuring the length of lesions, and re-isolating fungi. The results of our study showed that *N. parvum*, *D. seriata*, and *B. dothidea* caused lesions and necrosis on wood, that they were pathogenic to local Valenci and Hmar Bouamar grapevine cultivars, and that *N. parvum* was more aggressive than the other *Botryospheaeriacea* studied. Similar results were obtained on the calluses that were in contact with *N. parvum* (Montserrat, 2010). This finding was consistent with those obtained by Phillips (2002), who reported more severe symptoms, such as cane bleaching and wood streaking, with *N. parvum* than with *D. seriata* in grapevines. Similar symptoms have been identified by infecting vine plantlets of different varieties with mycelium (in 6 weeks) or with the culture filtrate (in 4 weeks) of *E. lata* (Mauro et al., 1988; Camps, 2008). Castillo-Pando et al. (2001) reported that the *B. obtusa* anamorph genus of *D. seriata* was responsible for dark streaks in 1-month-old Chardonnay plantlets and potted Chardonnay grapevines. Similar results were recorded by Larignon et al. (2001) when *B. obtusa* was inoculated onto 1-year old Cabernet Sauvignon canes. *Botryosphaeria dothidea* has a wide host range and is a well-known grapevine pathogen all over the world. Toxin production by fungi could explain the phenomenon of the appearance of necrosis. Indeed, Djoukeng et al. (2009) confirmed the production by *Botryosphaeria* isolates of toxins that are transported to the herbaceous parts and cause symptoms away from the point of infection. To our knowledge, this is the first report on the genus *Botryosphaeria* being isolated from grapes in Algeria, and we have also demonstrated the potential pathogenicity of 3 isolates in local grape cultivars. Further research is now required to completely investigate the role of these pathogens in the decline of grapevines and other hosts in Algerian fruit and forestry industries. Further work will be also needed to improve the control of these pathogens, and to ascertain further the role of environmental and cultural factors that may favor their development under local conditions. As reported by Zebib (2012), better knowledge of pathogens could help in the development of potential treatments to limit their impact.

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