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Diversity of Fungal Trunk Pathogens Associated with Grapevine Dieback of Grapevine in Algeria

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Abstract

A significant reduction in vine production has recently been recorded in several vine regions in Algeria due to the death (total or partial) of many vines and to the pulling of many vineyards that had showed too many symptoms of decline. This study was conducted during spring 2010 - 2012 to detect the responsible agents for this disease. Samples of grapevine wood were collected in five grapevine fields in two regions (Medea and Tipaza). A cross section of infected trunks revealed many types of necrosis: sectorial brown colored, central necrosis sectorial gray and light-brown central necrosis. Several fungi were isolated from the margin between healthy and diseased tissues. *Eutypa lata* and *Fomitiporia mediterranae* were identified fungi based on the morphological characteristics of the culture and confirmed by partial sequences analysis of the nuclear ribosomal internal transcribed Spacer (ITS). The sequences submitted in GenBank under accession number revealed 94-100% homology. *F.mediterranae* was the dominant species, followed by *E. lata*. Other fungal species (*Alternaria, Fusarium, Pestolozzia, Botrytis, Rhizopus, and Penicillium*) were also isolated with high frequency. Two kinds of fruiting structures were found where one type showed the presence of perithecia (sexual form) of *Eutypa lata*.

Keywords: Grapevine, Dieback, Eutypiosis, Esca, Algeria.

1. Introduction

Grapevine growing occupied important places in the agricultural sectors of many countries. In Algeria, grapevine was cultivated principally to produce table grapes, in addition to some extent products such as fresh juice and raisins. The soil and climatic conditions in Algeria are favorable to the development and extension of this culture. Grapevines are grown in the north regions (South Mediterranean Sea) on an area of 100.200 ha according to the Ministry of Agriculture statistics done in 2012. The decline of the vine was due to fatal diseases. The most destructive diseases were esca and eutypiosis, respectively developed in vineyards (Larignon and Dubos, 1997; Mugnai *et al.,* 1999). *Eutypa* dieback, caused by the fungus *Eutypa lata*, threatened the sustainability of vineyards, especially those of eight years or older; it is becoming a serious problem in most cool climate growing regions. The fungus infects vines through pruning wounds, colonized wood tissue and caused a characteristic wedge of dead tissue. It was thought that the fungus produce toxic metabolites which were transported through vascular tissue to the foliage, causing stunting of the shoots, distortion and necrosis of leaves (Moller and Kasimatis, 1978; Molyneux *et al.,* 2002). The second disease, called esca, was a complex disease, more complicated than that of *Eutypa* dieback, including a vascular symptoms and an internal white rot in the trunk, which gradually changed the hard wood to a soft one (Mugnai *et al.,* 1999). It was attributed to a group of systematically diverse fungi that were considered to be latent pathogens. The principal pathogenic taxa associated with esca were *Eutypa lata*, *Phaeomoniella chlamydospora,* and various species of the genera *Botryosphaeria,Cylindrocarpon, Fomitiporia*, and *Phaeoacremonium* (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Surico *et al*., 2006).

As a result of the gravity of this phytosanitary problem of this decline in the vineyards in Algeria, this study was conducted in five vineyards located in two

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regions known by their vine vocation. This study aims at identifying and characterizing the causal agents of grapevine dieback in Algerian vineyards on morphological characteristics of culture; besides, a partial sequences analysis of the nuclear ribosomal internal transcribed Spacer (ITS) was investigated. Detection of fruiting bodies in wood samples was also investigated.

2. Materials and methods

2.1. Sampling and Isolation

A field survey was conducted on some vineyards which showed dieback symptoms on local cultivars, namely Dattier de beyrouth, Muscat, Cinsault, in the north region of Algeria (Tipaza, Medea) during the spring period of 2010 and 2012 (Table 1). After carrying out the descriptive symptomatology and localization the vines with dieback symptoms, samples were collected randomly from each vineyard vines (10 among the 500 observed). Some vines showed symptoms of decline on herbaceous parts for each cultivar were cut at the base of the trunk, wood slices of 0,5 mm of large were sectioned from the margin of all necrosis categories at the frontier of necrotic tissues and apparently healthy. Slices surface were disinfected by immersion in sodium hypochlorite (NaOCl) (2%) for 4 min, after that they were rinsed and dried with sterilized filter paper. Then, they were placed on potato dextrose agar (PDA) plates and stored at 25°C. Observations of fungal development were recorded weekly. Morphological and microscopic analyses of mycelia culture of *Fomitiporia* and *Eutypa* were done according to Fischer (2002), Moller and Kasimatis (1978), respectively.

Table 1. Characteristic vine yards studied in (Médea and Tipaza) provinces during 2010 growing season.

Location	Medea		Tipaza		
site	Benchicao		Hamrelain		
Vine yards	1	2	1	2	3
Cultivar	Dattier	Muscat	Dattier	Muscat	Cinsault
Age (an)	26	45	40	10	10
Sup (Ha)	12	06	05	04	03
Mode of pruning	Guyot simple	Guyot double	Cordon double	Cordon double	Guyot
Rootstock	41B	41B	41B	41B	41B

2.2. Body Fruiting

Dead branches and wood were inspected in the vineyard for the presence or absence of the sexual forms (Perithecia and Pycnida) of the fungus implicated in the decline of vines such as the genus *Eutypa*. Infected samples, collected from vineyards, were transported to the laboratory where they were left to dry for examination. The preparation of ascosporic was conducted from fruiting in sterilized water and was placed on PDA plates. After 24 h at 25 ± 2°C, individual germinated spores were observed under a microscopic observation.

2.3. DNA Extraction and PCR Amplification

Total genomic DNA of all isolates, identified morphologically as *Eutypa* and *Fomitiporia*, were extracted from pure culture mycelia as reported by Liu *et al.* (2000). Oligonucleotide primers ITS1 (5'-TCCGTAGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC-3') were used to amplify the ITS regions of the nuclear ribosomal DNA (including the 5.8s gene) according to (White *et al.*, 1990). The amplification reactions were performed in a 25µl volume of reaction mixture containing (1mM of each primer, 0.2 Mm of dNTP, 15 ml $MgCL_2$ and 2.5 U of Taq polymerase adjusted with purified distilled water to a final volume of 25 µl). The PCR program for ITS genes was run according to Guizhen and Mitchell (2002) included an initial denaturation 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 final elongation step at 72 C° for 10 min. The PCR amplification products were separated by electrophoresis in 1.5% agarose gels prepared in tampon TBE 0,5 X (Tris-Borate 100 Mm ;pH 8,3; EDTA 2 mM) added 50 µg ethidium bromide (BET), and visualized under UV light (Sambrook *et al*., 1989). The PCR products were purified with QIAquick Wizard PCR purification Kit (Promega) according to the manufacturer's instructions. The sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, HTDS, Tunisia).

2.4. Molecular Identification

The nucleotide sequences were read and edited with Chromas1.7.5(http://www.technelysium.com.au/ chromac. html) . All sequences were checked manually. They were initially analyzed by searching the National Center for Biotechnology Information (NCBI) database using the BLAST (Basic local alignment search tool) (Altschul *et al*., 1997). Reference sequences for the ITS regions for the *Eutypa* and *Fomitiporia* spp. were obtained from GenBank.

3. Results

3.1. Fungal Isolation

Based on their appearance in culture, the isolates obtained in this study were assigned to main fungal groups. The first group of isolates, occurs in the first days (2-3 days) of incubation; this later became of white color and of a cottony texture. All isolates were typical of *Fomitiporia* genus and produced high density and aerial hyphae; after 10 days of incubation they developed white-yellowish mycelia, which became yellow-brown over time.

The second group was characterized by having white to white-cream cottony and slow-growing an intense aerial mycelium of the developed hyphae on PDA. With age, some cultures of *Eutypa* change color, turning from white to yellow with no fruiting structures. After four weeks, the accumulation of a brown color which became darker blackish determining melanisation. The higher frequency was attributed to the genus *Eutypa* and

Fomitiporia, the fungal agents associated with *Eutypa* dieback and esca, respectively. Their genus were isolated from all vines showing disease symptoms and frequently isolated from central and sectorial necrosis of soft and hard texture (Table 2) and (Table 3). Other fungi were isolated from grapevine cankers, such as *Alternaria, Fusarium; Pestolozzia, Botrytis, Rhizopus* and *Penicillium.*

Table 2. Frequency of isolation of the fungus depending on the variety in (Médea and Tipasa) provinces during 2010.

Cultivar	*Eutypa lata spp*	Frequency of isolation	*Fomitiporia mediterrania*	Frequency of isolation
Muscat	$8^{a}/10^{ь}$	80%	$6^{a}/10^{ь}$	60%
Cinsault	4/10	40%	5/10	50%
Dattier de Beyrouth	12/30	40%	15/30	50%
Total	24/50	48%	26/50	52%

* The report is (a) number of isolated fungus, (ь) number of vines analyzed.

Table 3. Frequency of isolation of the fungus depending in (Médea and Tipasa) provinces during 2010.

Region	Location	*Eutypa lata*	Frequency of isolation	*Fomitiporia mediterania*	Frequency of isolation
Médéa	Ben chicao	$6^{a}/20^{ь}$	30%	$11^{a}/20^{ь}$	55%
Tipasa	Hamrelain	18/30	60%	15/20	75%
Total	2	24/50	48%	26/50	52%

* The report is : (^{a}) number of isolated fungus, (ь) number of vines analyzed.

3.2. Fruiting Bodies

On the surface of dead branches and debris collected in the field, many fruiting structures of various trunks diseases pathogens were found (Fig 1). Microscopic observation of these fruiting bodies showed the presence of many asci, rounded with a pedicel (Fig. 2). Each ascus contained ascospores arched liberated from an ostiole (Fig. 3). The perithecia were the infectious form of *Eutypa lata*. None of the fruiting bodies was recorded for the second genus: *Fomitiporia*. In the present study, other forms of fruiting bodies on dead wood were found (Fig. 4) and presence of asci contained ascospore (Fig.5), the line identification helped distinguish isolates genre *Hysterium* (www.lenaturaliste.net).

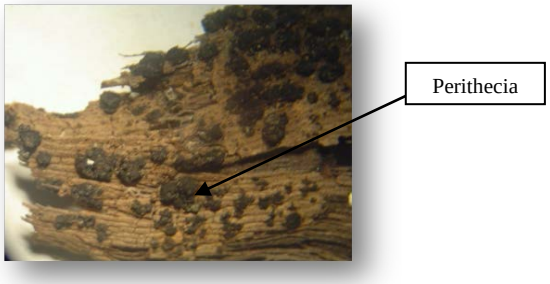

Figure 1. Body fruiting of Eutypa lata (Dattier de Beyrouth cultivar, Tipaza,2010).

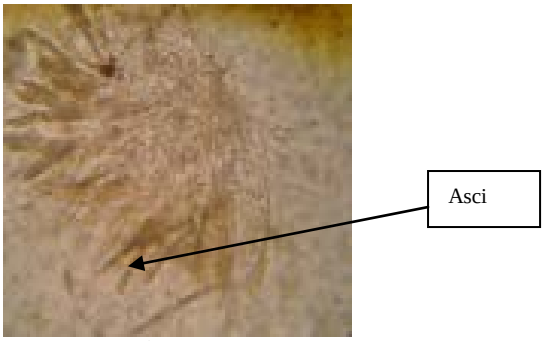

Figure 2. Asci of Eutypa lata contain ascospores (G : 40× 10). (Dattier de Beyrouth cultivar, Tipaza,2010).

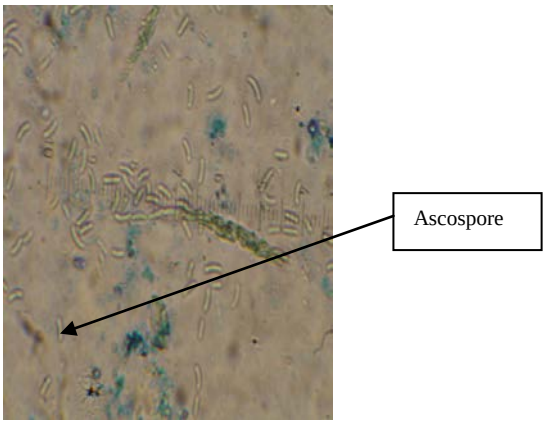

Figure 3. Liberation of ascospores of Eutypa lata (G: 40 × 10)(Dattier de Beyrouth cultivar, Tipaza,2010).

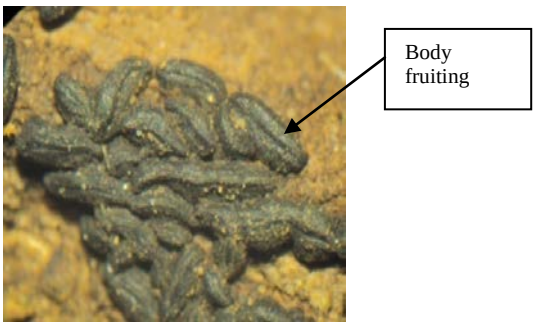

Figure 4 . Body fruiting of Hysterium sp (Cinsault cultivar, Tipaza,2010).

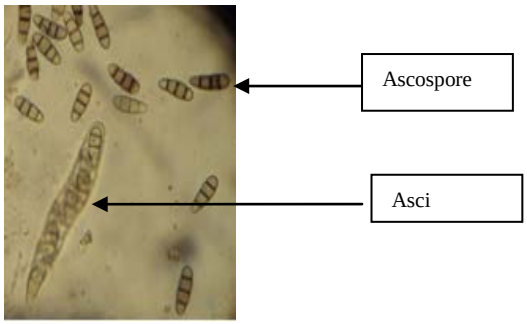

Figure 5 . Asci and ascospore of *Hysterium sp*(G : 40× 10). sp (Cinsault cultivar, Tipaza,2010).

3.3. Molecular Identification

Sequence alignments, used BLASTn in GenBank, showed that all *Fomitiporia* isolates from grapevine, which were tested with ITS sequence analysis, were identified as *F. mediterranea*. Nucleotide identity was 98-100% among sequences of *F. mediterranea* from grapevine. The second group of isolates presented 94-96% of homology with *Eutypa lata*.

4. Discussion

This study shows diversity in fungal trunk pathogens associated with wood decay symptoms on grapevine in Algeria during the period between 2010 and 2012. The result of the isolations shows the complex situation generated by many fungal trunk pathogens on grapevine in Algeria; isolates obtained were classified on clusters based on their appearance in culture and conidial morphology. Morphological and microscopic characters are compared with those reported previously by Moller and Kasimatis (1978) and Fischer (2002).

Including isolates species of *Eutypa lata, Fomitoporia mediterranea* and other detected species, such species could be distinguished based on DNA sequence data and unique morphological characters. Several strains of *Eutypa lata* were isolated during this study. This fungus was a major pathogen of cultivated crops such as apricot and grapevine and has been found all over the world (Carter, 1957). Those species were isolated from grapevine and known as grapevine pathogens in different regions of the world. *Eutypa lata* was the causal agent of *Eutypa* dieback, an important perennial canker disease that occurred in most countries where grapevine is cultivated (Munkvold and Marois, 1994). This species has also been reported from grapevines in Australia, Brazil, South Africa (Mostert *et al*., 2003). In many European countries this specie is considered the primary limiting factor.

The examination of the dead branch revealed the presence of Perthecia, in grapevine the occurrence of a perithecial stroma on the dead stump of a tree was first reported by Carter (1960) in Australia. According to Munkvold and Marois (1994), perithecia is more common on apricot than on grapevine. No perithecia were detected in the vineyards of Merced country. We concluded that infected trees did not develop perthecia but the presence of this sexual structure in some vineyards studied may serve as a source of inoculums (Elmomany, 2002).

In this present investigation, we report the presence of *F. mediterranea* in declining grapevine in Algeria. The importance of *F. mediterranea* as a pathogen has been widely investigated (Mugnai *et al*., 1999; Fischer, 2002; Fischer and Binder, 2004). The association between *F.mediterranea* and *Vitis vinifera* was of particular relevance, since the fungus produces an extensive white rot in the trunks of growing *Vitis* plants, was one symptom of the destructive grapevine disease complex esca, affecting vine cultivation on a global scale (Romanazzi *et al*., 2009). No fructification was detected in all vineyards for *Fomioporia*; we suggest that the conditions were not favorable for the developpement of those structures.

5. Conclusion

The eutypiosis and esca appeared as severe dieback diseases of the vine, because there were no means of control to cure the diseased vines. The majority of vine growers in Algeria have not realized the seriousness and severity of the diseases that affected the vine heritage. The present work has allowed analysis of the grapevines showing symptoms of decline showed the existence of several types of necrosis in the trunks. The analysis of the isolated from necrotic wood revealed the existence of two genus *Eutypa lata* and *Fomitiporia mediterranea* involved in the decline. It is clear that a better understanding of diseases is absolutely necessary to try to find effective solutions. It is advisable to design a diagnostic study involving vine regions to identify the likely origins of decay. Further investigations on *E. lata* and *F. mediterranea* and other wood decay agents should be conducted in order to manage the slow decline phenomenon in Algeria and other regions.

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