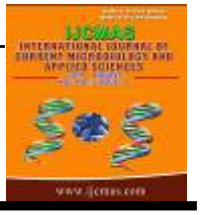
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 977-987**
http://www.ijcmas.com



Original Research Article

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

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Keywords

Grapevine, Esca-BDA, Symptoms, Diplodia seriata, Phomopsis viticola, necrosis.

A B S T R A C T

Esca and Black Dead Arm (BDA) are two major actors of grapevine trunk decline diseases that affect worldwide. The knowledge about the symptoms of these diseases and the microflora associated with these wood pathologies is still incomplete in Tunisia, although they begin to cause considerable damage in our vineyards. In order to better characterize the microflora colonizing the trunk of vine, samples were collected from eight vineyards in the north of Tunisia. Symptoms were described and isolates taken at the level of sick and healthy vines. A halophilic bacterium J9 was used as antagonistic agent and has been tested against fungi isolated. Two symptoms were observed: rapid total or partial drying resulting in the death of the cep (apoplectic form of esca) and the presence, under the bark of the trunk of an orange to brown band, characteristic of the Black Dead Arm Disease (BDA). For the varieties studied, cultivated and diverse microflora are colonizing the vines. Many saprophytic fungi such as *Aspergillus spp* and *Alternaria alternata* were detected in the healthy wood and at the level of necrosis. The identification of fungi by molecular methods including by PCR and sequencing showed the presence of *Phomopsis viticola* and *Diplodia seriata*, two pathogens described in the literature as involved in diseases of wood. Next to these pathogens, the molecular analysis confirmed the presence of *Alternaria alternata* as majority saprophyte in healthy and diseased wood. The use of bacterial antagonist gave pretty promising results especially with *Diplodia seriata*. This is the first report of *Phomopsis viticola* and *Diplodia seriata* infections of grapevines in Tunisia.

Introduction

Grapevine (*Vitis vinifera* L.) is a perennial crop widely cultivated throughout the world for raisin, table grape and wine grape production. Most varieties are grafted on rootstock cultivars, predominantly to counter phylloxera, but also to prevent soil

problems such as chlorosis (Delas, 1992). Among fungal diseases affecting yield, trunk diseases can severely damage crops in most vine-growing areas (Mungnai et al., 1999). Esca, Eutypa dieback and Botryosphaeria cankers are the main wood diseases of grapevine common in adult vineyards (Liminana et al., 2009). These grapevine trunk diseases are very harmful to wine-growing heritage durability because the fungi responsible, by attacking perennial organs, cause at a more or less long-term the death of the vine stock (Larignon et al., 2009). Esca and BDA are two main pathogens inducing such decaying diseases. The infection can be diagnosed by the presence in the wood forming tissues of sectorial and/or central necrosis, which revealed itself by brown stripes or canker and at the foliar level by discoloration and withering (Larignon and Dubois, 1997; Pascoe and Edwards, 2002). Since 2001, there has been a disturbing progression of these diseases. Several comments suggest that this phenomenon is probably only in the beginning of cycle: (i) the prohibition of sodium arsenite, only way currently known to combat esca and Black Dead Arm (BDA) or 'dead black arms' from (Dubos, 1999), (ii) the annual increase of the mortality rate of 4-5% from the fifth year plots where treatment with sodium arsenite was arrested and (iii) the rate high of ceps asymptomatiques contaminated in the vineyard (Dubos, 1999).

The ban sodium arsenite in all wine producing countries for its toxicity not only to the environment but also to human (Spinosi and Fevotte, 2008) increased worry between growers since any satisfactory control method was proposed to them. This puts at risk the maintenance of the production tool and its longevity and that globally. Therefore, these wood diseases will cause either impairment of the wine's quality after parcels rejuvenation or a loss of the wine's typicality from a wine-growing region upon non replanting of the most sensitive varieties.

Despite the damage caused by these diseases, they are often neglected for their slower growth rate Despite the considerable damage noted in vineyards, very few research has been carried out in Tunisia with regard to wood pathologies and associated microflora.

The aims of the present work were therefore to characterize the fungi associated with esca and BDA disease in grapevine from different regions of the Northern Tunisia.

Materials and Methods

Sampling locations

Sampling was made between mid-May and August 2013. Eight vineyards were studied in different regions, three vineyards in the region of Mornag (Governorate of Ben Arous), two in Medjez El Bab (Governorate of Beja), two in Borj el Amri and one in the city of Mehrine (Manouba governorate). These eight vineyards served as model for the analysis and monitoring of the symptoms of Esca-BDA in the Northern region of Tunisia. Samples were taken either from necrosis or vascular streaking for the following table grape cultivars: Red globe, Mikkeli Palieri and Muska d'Italie.

Fungal isolations

Fungal isolates were obtained from grapevines showing decline, small and distorted leaves and Chlorosis. 15 small and thin pieces of wood (< 1 cm^2) were disinfected in 70% alcohol, rinsed with sterile distilled water (SDW), dried and plated onto potato dextrose agar (PDA)

plates. The plates were incubated at 25°C for at least 5 days or until fungi were observed growing from the symptomatic wood. The fungi isolated are subsequently purified on PDA.

Morphological characterization

After the purification step, the fungal isolates were identified based on morphological characters.

Molecular Characterization

Extraction of total DNA

The total DNA was extracted according to the method of Liu et al. (2000) with some modifications. For each pure fungus, a quantity of mycelium was collected with a sterile toothpick and then put in an eppendorf tube containing 500 µl of solution 1 (400mM Tris, 60mM EDTA, 150mM NaCl and 1% SDS). The mixture was then left at room temperature. Then 150µl of solution 2 (60ml Acetate ammonium (5 M), 11.5 ml of cold acetic acid, 28.5 ml sterile water) were added and thoroughly mixed with a Vortex before centrifugation for 2 min at 12000 rpm.

The supernatant was thereafter retrieved carefully avoiding the pellet and then centrifuged again for 2 min at 12000 rpm. The supernatant was then recovered and added to 600µl of isopropanol for DNA precipitation, shaked 10 times by inversion and centrifuged once more for 2 min at 12000 rpm at 4°C, the supernatant will be next disposed and 300 µl ethanol 70% are added to the Pellet, centrifuge again 2 min has 12000 rpm at 4 ° C, are then disposed of supernatant and left to dry the pellet under hood (Speed Vac) for 40 min, we finally add 50µl of TE, then it stores the DNA at-20 ° C for later use. The concentration and purity of the extracted DNA was checked by a spectrophotometer (ND-1000, Nanodrop, pays).

The ITS region amplification

The ITS (Internal Transcribed spacer) region, described by different studies (White et al., 1990) was chosen as a target region for the identification of fungal species. The ITS1 sequence-5.8 S-ITS2 presents a conserved region of DNA in most fungal species and variable regions used in the studies of populations, ITS1 and ITS4 described by White and al. (1990) are used in this study (table 1).

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')
ITS4 (5'- TCCTCCGCTTATTGATATGC)

Antagonism test

An antagonism test was carried out to assess the effectiveness of bacteria J9 *in vitro*. Indeed, this test was performed on the PDA medium. The growth inhibition percentage of the pathogen was measured according to the formula of Sadfi et al. (2008 a,b).

Effect of volatile compounds on the biomass and growth of pathogens

The J9 bacterium was grown on agar TSA with 5% NaCl for 24 hours at 30 ° C, a second box containing the agar covered MEA of a cellophane paper disk on which is inoculated pathogen is placed above the bacterial culture the two boxes are then sealed by the parafilm and incubated at 27 ° C for 5 days measurement of biomass is determined by calculating the difference between the weight of cellophane containing the fungal culture and the paper weight empty before the test.

Results and Discussion

Symptoms, incidence and Fungal isolations

Four vine cultivation stations were visited in the North of Tunisia to evaluate attacks by wood diseases (the esca, the BDA and the eutypa) on harvests in the region. The rate of diseased vines was variable between and within the four regions explored: Mornag (three vineyards), Medjez El Bab (three vineyards), Borj el Amri (three vineyards) and Marshall city (one vineyard). Hence for Morneg, it varied between 3% in the vineyard n°1 and 0.8% in the vineyard n°3. The rate was less than 0.1% for the other vineyard.

The identification of diseases through the study of the symptoms for each cep. For the first station visited, the following symptoms were noted: in the vineyard n°1, there was typical symptoms of the apoplectic form of esca characterized by a dry, fast and total causing the death of the cep (Fig. 1A). In the vineyard n°2, found the existence of Central necrosis of light colour at the level of the cross section of the wood (Fig. 1B), but also mottling at the level of the typical leaves of esca. On other feet examined in the vineyard n°2, there is appearance of light brown to orange under the bark and bands which is a symptom of essential and typical disease of Black Dead Arm (BDA) (Fig. 1 C). The different symptoms were observed in the vineyard n°3, but could essentially save the existence of sectoral necrosis of darkish brown color at the level of the vine wood cross-section, which is a key symptom of eutypa (Fig. 1 D).

For the second station, Medjez El Beb, no symptom of wood diseases was found in the vineyard n°4 and only one foot was attacked by the BDA in the vineyard n°5. In the third station (Mehrine city), no sick foot was identified in the vineyard n°6. For the Station 4 (Borj el Amri), the same symptoms of eutypa, previously observed, were detected in the vineyard n°7, and 4 feet with BDA symptoms was noted in the vineyard n°8. At the whole, symptoms of Esca-BDA were observed only on old vines of 10, 15 and 17 years but not on young ones aged 3, 4 or 5 years.

Morphological characterization

In this study, a total of 630 touchwoods were analyzed. At least a fungus was isolated from half of them, or 315 shives (50%). When testing, fungi known for their non-pathogenicity on vine or indeterminate were grouped in a class called "saprophytes" while other fungi were subsumed under the term "pathogens". Isolation made from healthy wood and at the level of necrosis (Brown band) in the sick vine. After a minimum of 3 weeks of incubation at 25°C the fungal species colonizing the Middle were identified on the basis of phenotypic characters. Cep level asymptomatic (witness), the isolation revealed the existence only of belonging saprophytic fungi in the genus *Penicillium, Aspergillus,* and especially *Alternaria* (70%) (Fig. 2 A, B and C).

For the sick vine, isolation also showed the presence of a saprophytic community and another group of fungi, possibly the suspected pathogens. They were selected based on phenotypic traits and on litterature (Larignon et al. 2009).

After several purifications and monosporous cultures, 12 mushrooms were selected: F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, F12. A relatively diverse and similar fungal microflora were isolated from necrotic and healthy wood (Brown band) of symptomatic

plants. The isolated strains had different aspects and colors as well as different growth rate. The appearance of a few isolated fungi and their macroscopic patterns are presented in Fig. 3.

Isolation from diseased vines has also shown the presence of several bacterial colonies and whose appearance corresponds to the genus *Bacillus* (Fig. 4).

Molecular Characterization

ITS1 and ITS4 primers were used for the amplification of a region of the rDNA including a conserved region 5.8 S and two noncoding ITS1 and ITS2 regions where resides the interspecific and intraspecific, variability obtained after PCR amplicons are sized 600 pb (Fig. 5), the numbers of each isolate are presented in table 2.

Sequence analysis bands amplified and their alignment with the fungal species identified in the NCBI gene bank has identified five fungal species: *Quambalaria cyanescens*, *Alternaria alternata*, *Emericella nidulans*, *Diplodia seriata*, *Diaporthe ampelina*, other isolates are being sequenced, each mushroom data are presented in table 3.

Antagonism test

The bacterial strain J9, a halophilic bacterium isolated by Essghaier et al. (2009), was tested *in vitro* on Potato Dextrose Agar (PDA) against fungal isolates. To this effect, the dual culture technique was applied, and the percentage of inhibition calculated as described in material and methods. Cultivation of the antagonist (J9) with the mushrooms for 5 days at 27°C showed the appearance of an inhibition zone around isolates F9, F11, and F4= *Diplodia seriata* (Fig. 6). For each percentage of inhibition (I) isolate the following F11: I (%) = 50% F9: I (%) = 30% *Diplodia seriata*: I (%) = 12%.

Test of volatile compounds

To study the effect of volatile compounds produced by the bacterial strain on biomass, weight net of each mushroom was calculated and then compared to that of the control (biomass of the fungus in the absence of the bacteria), the results are presented in table 4. The fungus F11 was the most inhibited with a percentage of inhibition of 67.9% followed by F7, F4, and F10. The fungus F4 identified by sequencing as *Diplodia seriata* belonged to the family of Botryosphaeriaceae and is involved in BDA disease, showed a growth reduction of 22.4%. Hence the bacteria J9 which could be considerd as effective and promising agent for the biocontrol of the Black Dead Arm (BDA).

Very few knowledge on micro-organisms colonizing the vine wood attacked by the Esca-BDA is currently avalaible elucidated (Gerbore, 2009). In order to provide new evidence on this crucial topic for the understanding of these pathologies, the first part of this work was devoted to the study of symptoms of vine wood diseases in the northern region of Tunisia and isolate the fungi responsible of these diseases. The second part was carried out to identify these pathogens using molecular biology tools., while in the third part, assays of culture with antagonist bacteria was realized to investigate the potential biological control against Esca-BDA.

Indeed, the surveys made in Tunisian vineyards throughout the summer 2013 demonstrate the existence of these diseases of dieback of Grapevine, namely: esca, the BDA and the eutypa and whose symptoms are similar to those described by Valtaud et al. (2009), Larignon and Dubos (1997);

Larignon and al. (2001), or Larignon et al.(2009) as predominant mostly french vineyards than in other countries. Isolation revealed that the Tunisian vine wood diseases were not due to single pathogen but to several groups of pathogens since various fungi were isolated and purified. Similar finding was made by other recent studies in France and in the United States of America (Larignon et al, 2009).

Macroscopically and from the morphological point of view, three mushrooms among the isolates have the same color and the same growth rate compared to some pathogenic fungal species associated with vine wood diseases Armengol et al. (2001), Fisher and Kassmeyer (2003). These isolates (F3, F7, and F4) were similar macroscopically and morphologically and were identified as *Phaeoacremonium aleophilum* (causal agent of esca), Eutypalata (the eutypa agent) and Botryosphaeria obtusa (BDA agent).

Identification by sequencing the ITS regions allowed to assign the fungus F4 isolate to *Diplodia seriata* species described by Morales et al. (2012) and Phillips et al. (2007). This species is the Anamorph form (asexual reproductive form) of *Botryosphaeria obtusa* (telomorphe), a pathogen involved in wood diseases, and belonging to the family of Botryosphaeriacea. As it has also shown the presence of *Diaporthe ampelina* (also called *Phomopsis viticola*) which is a pathogen endophyte of plants but also to other hosts and which was found to be associated with *vitis vinifera* in Italy, Turkey and the United States (Gomes et al., 2013). This work constitutes the first report which allowed the identification of fungi involved in wood diseases in Tunisia: *Diplodia seriata* and *Phomopsis viticola* in association with *Vitis vinifera*.

Other species were identified by sequencing as opportunistic phytopathogen. These were *Emericella nidulans*, *Quambalaria cyanescen,* and *Alternaria alternata*. These results provide important and valuable information on the existence and the association of these species with dieback disease in Tunisian vineyards.

Following identification of these pathogens a test of antagonism *in vitro* and a test of the volatile compounds were made using a halophilic bacteria (J9) and *Bacillus* specie. These two tests helped to assess the effectiveness of the studied bacteria to reduce Esca/BDA. In particular an inhibitory effect exerted by J9 on *Diplodia seriata* was revealed. Moreover, the growth of *Diplodia seriata* was reduced by 22.4% by the volatile compounds produced by J9. It is a fairly large percentage, proving that J9 bacterium can be an effective and promising antagonist agent for biological control against the vine wood diseases. This study confirms the ability of the halophilic bacteria for biological control which was shown for the first time by the team of Sadfi et al. (2000), and then by another more recent work in 2008 by the same team (Sadfi et al., 2008 a and b).

Table.1 Composition of the reaction mixture of PCR

Reagents	Final concentration	Initial concentration	Volume per tube (25µl)
H_2O stérile			11.25
Tampon buffer		10	5
$MgCl_2$		25 mM	1.5
dNTP	25mM	20 mM	1
ITS1	100pmoles/µL	20 pmoles/µl	2
ITS4	100pmoles/µl	20 pmoles/µl	2
Taq Polymerase	5U/µl	1U/µl	0.25
ADN			2

Table.2 Numbers of isolates on agarose gel

Numbers	**Fungal strains**
1	F11
2	F9
3	F12
4	F5
5	F10
6	F4
7	F3
8	F2
9	F7
10	F8
11	F6
12	F1

Table.3 Identity of the species identified by sequencing

Numbers	Fungal strains	Identified species	vineyards	diseases	Percentage of Homology (NCBI)	Accessions
12	F1	*Alternaria alternata*	n°8	Any cep studied	99%	KF293887.1
6	**F4**	***Diplodia seriata***	n° 1,2,5 and 8	**Esca**	**98%**	JF934890.1
8	F2	*Emericella nidulans*	n° 1,2,5 and 8	**Esca**	97%	KF381094.1
4	F5	*Quambalaria cyanescens*	n° 1,2 and 8	**Esca**	96%	DQ823421.1
3	**F12**	***Diaporthe ampelina (Phomopsis viticola)***	n°5 and 8	**BDA**	**96%**	KF017926.1

Table.4.Effect of volatile compounds on fungal biomass

Isolate	Biomass (g)	Biomass control (g)	Percentage inhibition (%)
F3	0.533	0.600	11,1%
F7	0.414	0.812	49%
F11	0.340	1.060	67,9%
F10	0.398	0.500	20 ,4%
F4	**0.500**	**0.612**	**22 ,4%**
F5	0.300	0.312	3 ,8%
F12	0.420	0.450	6 ,6%

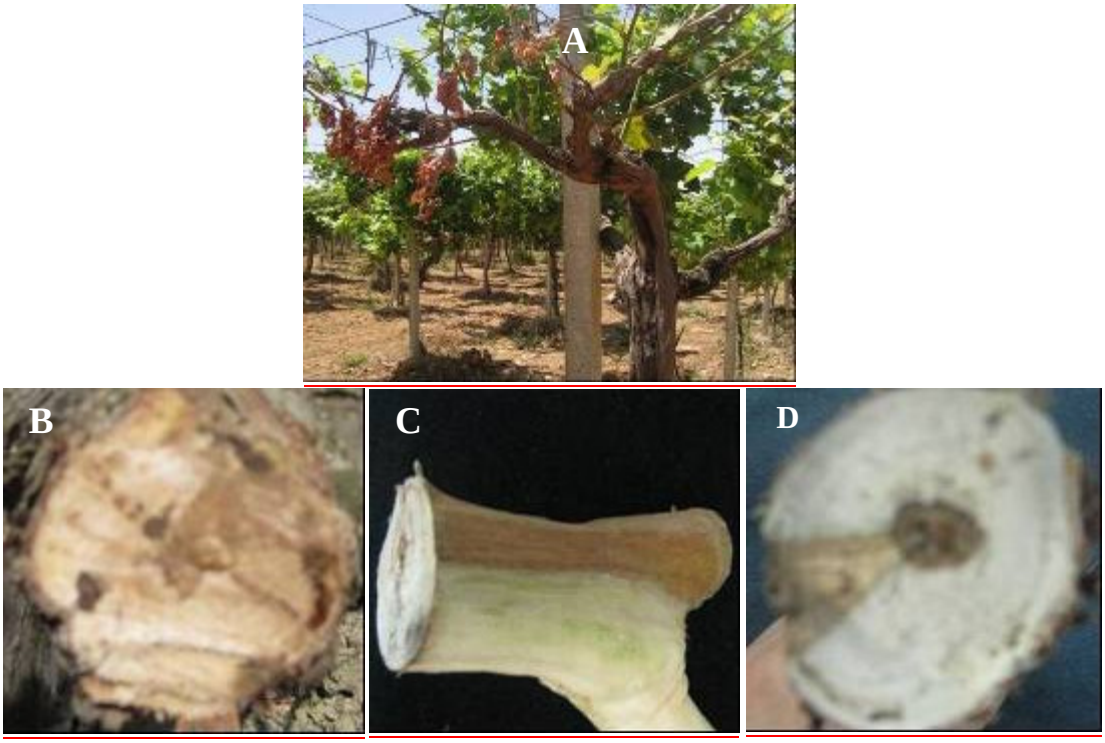

Figure.1 The different wood disease symptoms in Morneg station. **A-** The apoplectic form of Esca. **B-** Characteristic Central necrosis of the esca. C- The brown orange stripe under bark attributed to the BDA. **D-** The eutypa characteristic brown sectoral necrosis.

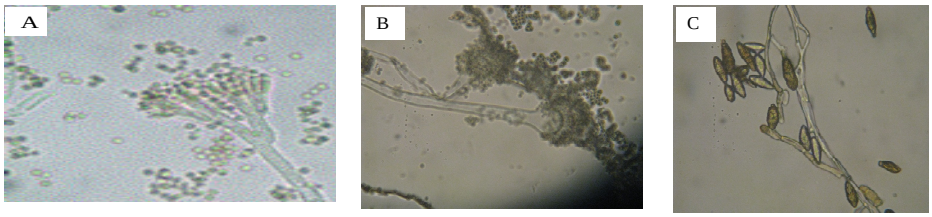

Figure.2 Microscopic observation of a few saprophytic fungi. **A-** *Penicillium* **B-** *Aspergillus.* C- *Alternaria*

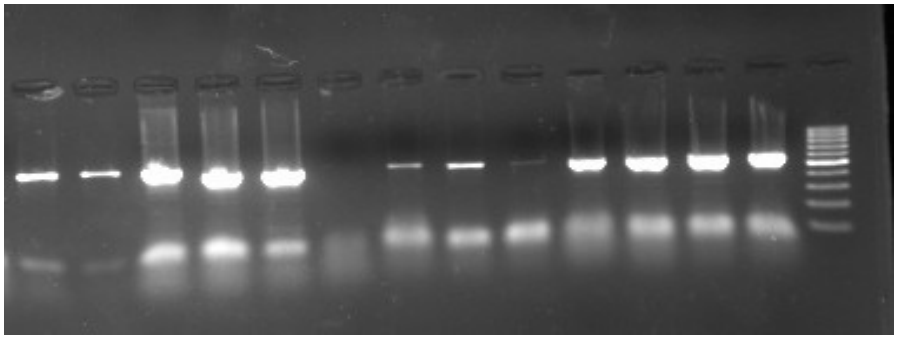

Figure.3 Agarose gel amplification products of the ITS1 region-5, 8s - ITS2 of isolates obtained by primers ITS1 and ITS4.

References

Armengol,J.,Vicent,A., Torné,L., Guarcia, F., Figueres,Guarcia-Jimenez,J.2001. Fungi associated with esca and grapevine declines in Spain:A three-year survey.Phytopath.Medit.40: S325-S329.

Delas, J. 1992. Criteria used for rootstock selection in France. Rootstock Seminaar: a Worldwide Perspective. American Society for Enology and Viticulture, (S. A. Fike, ed.), Reno NV, USA, 1-14.

Dubos, B. 1999. Maladies cryptogamiques de la vigne. Editions Féret, Bordeaux. 176p.

Essghaier, B., Fardeau, M.L., Cayol, J.L., Hajlaoui, M.R., Boudabous, A., Jijakli, H., et Sadfi-Zouaoui, N.2009. Biological control of grey mould in strawberry fruits by halophilic bacteria. *Journal of Applied Microbiology* 106 (3):833-46.

Fisher,M.,Kassemeyer,H.H.2003.Fungi associated with esca disease of grapevine in Germany.Vitis42 : 109-116.

Gerbore, J. 2009. Caractérisation de la microflore colonisant les tissus ligneux sains et nécrosés chez la vigne atteinte d'une maladie du bois, l'Esca-BDA, mémoire de fin d'études pour l'obtention du titre d'Ingénieur des Techniques Agricoles, à l'Ecole Nationale s'Ingénieurs des Travaux Agricoles de Bordeaux. p45.

Gomes, R.R., Glienke, C., Videira, S.I.R., Lombard, L., Groenewald, J.Z., Crous, P.W.2013.Diaporthe : a genus of endophyticsaprobic and plant pathogenic fungi. Personia 31: 1-41.

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.Observations of Black Dead Arm in French vineyards. Phytopathologia Mediterranea. 40: 36-42.

Larignon, P., Fontaine, F., Farine, A., Clément, C., Bertsch, C. 2009.Esca et Black Dead Arm : deux acteurs majeurs des maladies du bois de la vigne. R. biologies, 332.

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: 00-00.

Liu, D., Coloe, S.,Baird, R., Pederson, J.2000. Rapid Mini-Preparation of Fungal DNA for PCR. J clin Microbial vol 38.n °3: p 471.

Morales, A., Latorre, B.A., Piontelli, E., Besoain, X. 2012. Botryosphaeriaceae species affecting table grape vineyards in Chile and cultivar susceptibility. Cien. Inv. Agr. 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

Pascoe, I., Edwards, J. 2002. Esca or not? The Australian Grapegrower and winemaker 439:14.

Phillips, A.J.L., Crous, P.W., Alves, A.2008. *Diplodia seriata* the anamorphe of *Botryosphaeriaobtusa*.FungalDiversit y 25: 141-155.

Sadfi N, Hajaij M, Cherif M, Boudabous A. 2000.Selection of halophilic *Bacillus sp* isolates antagonistic against *Fusarium roseumvar*.

sambucinum. *Annales de l'association Française de la protection des plantes* (AFPP) 2: 615-622.

Sadfi–Zouaoui, N., Essghaier, B., Hajlaoui, M.R., Fardeau, M.L., Cayaol, J.L., Olivier, B., Boudabous, A.2008a. Ability of Moderately Halophilic Bacteria to Control Grey Mould Diseases on Tomato Fruits .*Journal* .*Phytopathology* 156: 42-52.

Sadfi–Zouaoui, N., Hannachi, I., Andurand, D., Essghaier, B., Boudabous, A., Nicot, P.2008b. Biological control of *Botrytis cinerea* on stem wounds with moderately halophilic Bacteria .*World Journal Microbiology and Biotechnology* 24:2871-2877.

Spinosi, J., Fevotte, J. 2008. Eléments techniques sur l'exposition professionnelle aux pesticides arsenicaux. Matrice cultures-ex-positions aux pesticides arsenicaux. Institut de veille sanitaire.

Valtaud,C.,Larignon,P.,Roblin,G.,Fleurat-Lessard, P. 2009. Developmental and ultrastructural features of *Phaeomoniella chlamydospora* and *Phaeacremonium aleophylum*in relationship with the capacity with xylem degradation in esca disease of grapevine.Journal of Plant Pathology 91:37-51

White, T.J., Bruns, T., Lee, S., Taylor, J.1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *Genetics and evolution Part three* 38: 315-322.