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Identification and pathogenicity of Fungi associated with Decline of cork oak in the North West of Tunisia

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Abstract - Decline events have recently been observed in several cork oak (*Quercus suber*) forests in northwest Tunisia (Kroumirie). The affected trees show crown thinning, leaf yellowing, branch dieback, cankers, epicormic shoots and exudates on branches and trunk. Six species: *Biscogniauxia mediterranea*, *Botryosphaeria corticola*, *Cytospora* sp., *Discula quercina*, *Fusicoccum* sp. and *Pleurophoma cava* were constantly isolated from cankered and dieback branches of affected plants. Pathogenicity tests on 3-years-old cork oak seedlings confirmed the aggressiveness of all species. The obtained results emphasize the need to develop further research into the real incidence and distribution of these pathogens involved in the etiology of decline phenomena in the Tunisian cork oak forest.

Keywords: pathogens, decline, cork oak

1. Introduction

During the recent years many works were carried out in the south of Europe (Italy, Spain, Portugal and France) to study the decline of *Quercus* spp., and to characterize the fungal endophytic associations of oak trees, as well as the opportunistic involved pathogens **(**Franceschini et al., 2005**)**.

In Tunisia, cork oak (*Quercus suber*) forests are widespread mainly in the Northwest regions (Kroumirie-Mogods). Over the last three decades, recurring environmental stresses and high anthropogenic pressure (firewood harvesting, grazing) have contributed to causing progressive degradation of the Tunisian oak woods (Hasnaoui et al., 2005).

Furthermore, recently it has been reported an increase in decline phenomenon and tree mortality in different cork oak stands (Ben Jamâa et al., 2005). In the etiology of this complex disease lethal attacks of plant pathogenic fungi play a crucial role. However, little information is available about the pathogens occurring in Tunisia.

This study reports the results of research carried out in some declining cork oak forests of North-Western Tunisia in order to study the fungal pathogens associated with canker and branch dieback.

2. Materials and methods

In autumn 2006, samples of cankered and wilted branches of declining cork oak trees were collected in different stands located in Aïn Draham (Aïn Draham I, III and V), Tabarka (figure 1) (Mekna II, III and IV) and Nefza (figure 2) (Bellif).

Endophytes were isolated from 2 declining plants at each site. These samples were disinfected by immersion for 15 min in oxygenated water at 10 %, then dipped 5 times in sterile distilled water, and finally dried with sterile filter paper. Three fragments for each symptomatic part of declining plants were taken. A total of hundred samples were used for isolation.

Fungal isolations were made in Petri dishes containing malt extract agar (MEA) from the disinfected bark of symptomatic branches. All the dishes were incubated at 25°C in a darkened thermostat. The fungal colonies which developed in each dish after 3 to 5 days, were transplanted in potato dextrose agar (PDA) and kept in the same conditions for 20 days, or until sufficient characteristics for identification had developed. The identification was confirmed by experts from the University of Agronomy Sassari (Italy).

Isolation Frequency (IF) of each endophyte was defined by the formula: $IF = N_i/N_t * 100$, where N_i and N_t are the number of segments colonized by the endophyte and the total number of examined segments, respectively.

Pathogenicity tests of all fungi isolated were carried out on 2 years old cork oak seedlings maintained in a greenhouse for two months at 16-37°C. Groups of 7 seedlings were inoculated at the basis of the trunk with a mycelial plug (3–4 mm^2) of each fungus, taken from the margin of an actively growing fungal colony on MEA.As control,7 seedlings were inoculated with a sterile MEA plug. At the end of the experimental period, the length of stem lesions caused by fungal pathogens was measured after removal of the outer bark. Re-isolation of the pathogens was performed on five disinfected stem segments (1 cm^2) of bark removed from each seedling.

Data of stem lesion lengths (only one independent variable) were analysed by one-way ANOVA, and mean values were compared by LSD test using Statgraphics Plus software test at $p = 0.05$. This analysis for each parameter was performed to evaluate the fungi effects on plant health.

Figure 1. Declining cork oak in Tabarka.

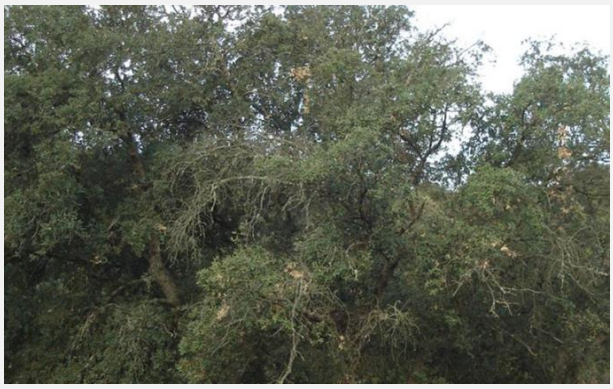

Figure 2. Cork oak trees with symptoms of branch dieback.

3. Results

In all the examined cork oak forests, a high occurrence of both symptomatic and dead trees was observed. The affected trees showed crown thinning, leaf yellowing, branch dieback, cankers, epicormic shoots and blackish exudates on branches and trunk. Six fungal pathogens: *Biscogniauxia mediterranea*, *Botryosphaeria corticola*, *Cytospora* sp., *Discula quercina*, *Fusicoccum* sp. and *Pleurophoma cava* were constantly isolated from the symptomatic samples.The majority of these fungi are identified on cork oak trees in Tunisia for the first time.

Pathogenicity tests confirmed the aggressiveness of all the six species. *B. corticola* produced the longest stem lesions on inoculated seedlings (Tab. 1).It caused sunken lesions associated with dark brown discolorations and vascular necrosis, and after 4 weeks all the seedlings died from girdling. The other fungi caused cankers and necrotic lesions around the inoculation point. All pathogens inoculated were recovered from the symptomatic seedlings, thus fulfilling Koch's postulates. No symptoms were visible in the control seedlings.

Biscogniauxia mediterranea and *Botryosphaeria corticola* have always been the predominant species (with IF values varying from 33 % to 76 %).

The fungal endophytic community at the various sites was composed by a variable number of species (six fungal pathogens)(table 1). Three species *Biscogniauxia mediterranea*, *Botryosphaeria corticola* and *Discula quercina* were isolated from all the sites and are the principal pathogens responsible for the decline of *Quercus* sp.

The main morphological parameters were used to identify these species are summarized in table 2 and figure4.

These results contribute to improve the knowledge on the fungal pathogens involved in cork oak decline in Tunisia and emphasize the need to carry out further research to evaluate their effective incidence and distribution in the Maghreb areas.

Table 1. Isolation frequency (IF), lesion lengths, wilted plant number, and positive re-isolations of the fungi inoculated on cork oak seedlings.

Inoculated fungal species	IF	lesion length (cm)	Wilted plants number	Positive re-isolations number
Biscogniauxia mediterranea	48 %	2.1cd*	0	7
Botryosphaeria corticola	56 %	9.1f	7	7
Cytospora sp.	13 %	1.4b	0	7
Discula quercina	37 %	3.9e	0	7
Fusicoccum sp.	19 %	2.8d	0	7
Pleurophoma cava	14 %	1.6bc	0	7
Control	-------	0.1a	0	-

*Values in column followed with different letters are significantly different according to LSD test ($P \leq 0.05$).

Table 2. The main morphological parameters use to identify these pathogens.

Pathogens	Keys of identification
Biscogniauxia mediterranea	The carbonaceous stroma are present on the branches and trunk all year, and emit a large amount of spores (asci). ascospores are dark brown and ovoid and are grouped.
Botryosphaeria corticola	Rot caused by *Botryosphaeria corticola* produces small black spots (pycnidia) embedded inside the bark of trunks and branches. These stains are difficult to remove.
Discula quercina	The pathogen causes staining on leaf ends and ribs. The necrotic areas grow and end up covering the entire leaf. The pathogen product a large quantities of elongate and transparent conidiums.
Pleurophoma cava	Pycnidia spherical, brown; ostioles lined by compacted periphyses; cells of the pycnidium wall separated by dark, encrusted material. Conidiogenous cells integrated in filiform, septate conidiophores. Small conidia, cylindrical
Cytospora sp.	Asci were free and cylindrical. Ascospores regrouped, elongate, hyaline and aseptate. Perithecia arranged at different depths in the stroma.
Fusicoccum sp.	*Fusicoccum* sp. caused canker, leaf blight and twig dieback of cork oak. Conidia were hyaline, oval and elliptic in form and aseptate.

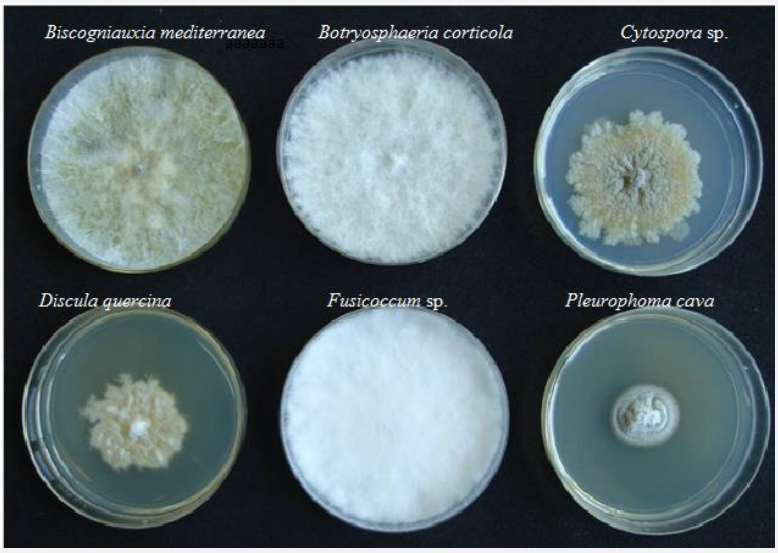

Figure 3. Aspect of the colonies, on MEA after 6 days, of the fungi isolated in this study.

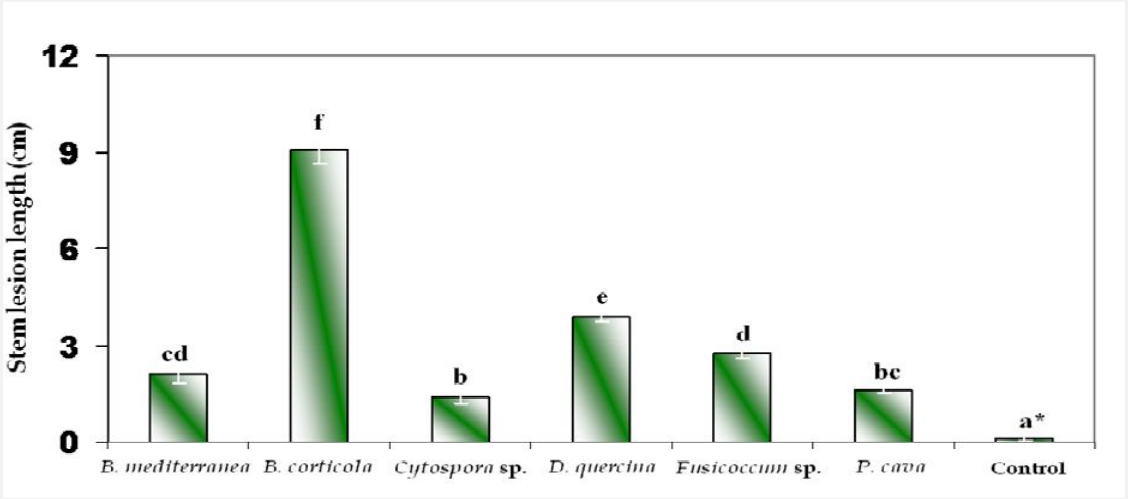

Figure 4. Stem lesion length measured at the end of the experiment in inoculated cork oak seedlings. Data presented as mean plus-minus standard error. *Histograms marked with different letters are significantly different according to LSD test ($P \leq 0.05$).

4. Discussion

In this study the diversity of endophytic fungal communities associated with cork oak were analyzed and showed that the mycoflore is very varied.

In order to contribute to a better understanding of the factors implied in the decline of oak trees, the prospecting of fungi associated with the oaks was carried out. The identification of certain fungi associated with the observed symptoms showed that *Biscogniauxia mediterranea*, *Discula quercina* and *Diplodia corticola* synonymous with *Diplodia mutila*, represent the main pathogenic fungi that attack the oaks in this area and are directly related to the phenomena of dterioration of this forest.

This mycoflore is very varied if the other different parasites are added, identified of zeen and cork oak: *Penicillium sp*, *Trichoderma sp*, *Nodulisporium sp* (telemorph of *Biscogniauxia mediterranea*), *Alternaria sp*, *Petalotiopsis sp*, *Aureobasidiom pollulans*, *Armillaria mellea*, *Microsphaera alphitoïdes*, *Trabutia quercina* and all the fungi partners of the xylomycetophagous pests *Platypus cylindrus*. (Hasnaoui, 2008).

With regard to pathogenic fungi, the majority of researchers announces attacks by *Armillaria* sp., *Phytophtora cambivora* (Petri) Buism., *Phytophtora cinnamoni* Rands, *Endothiella gyrosa* Sacc., *Biscogniauxia mediterranea* De Not. Kuntze (= *Hypoxylon mediterraneaum*), *Botriosphaeria stevensii* Shoemaker – Anamorphic form *Diplodia mutila* Fr. apaud. Mont., *Corineum nodomium* (Tul.) Griff. and Maube and *Ophiostoma* spp. (De sousa, 2005).

Except *Fusicoccum* sp., the other species are well-known pathogens involved in the etiology of cork oak decline in the Mediterranean countries (Franceschini et al., 2005).

From to the mycocenose, 32 different taxa were isolated, varying from 9 to 21 in the various sites. Four of them were *Aureobasidium pullulans*, *Biscogniauxia mediterranea*, *Discula quercina* and *Penicillium purpurogenum*, and were present in all the sites (Franceschini et al., 2005).

*Biscogniauxia mediterranea*is frequently isolated from declining oak trees, located in the south of Europe and North Africa; it was reported on *Quercus ilex* and *Quercus suber* in Morocco, Spain and Portugal. as well as on *Quercus cerris* in Central Italy **(**Caprettiand Mugnai, 1987; Vannini et al., 1996), on *Quercus suber* in Sardinia (Franceschini et al., 2004), and on *Quercus morisii* in Tuscany (Turco et al., 2004).

Biscogniauxia mediterranea was the mostfrequently isolated fungus. Its isolation frequency was significantly higher in declining covered trees than in control trees (Linaldeddu et al., 2010)

The mycoflore associated with the decline of cork oak in Morocco mainly consists of *Diplodia mutila* synonymous *Diplodia corticola*, *Armillaria mellea*, *Hypoxylon mediterraneum* synonym *Biscogniauxia mediterranea*, *Stuartella formosa*, *Fusarium oxysporum*, *Coryneum sp*., *Alternaria sp*., *Aureobasidium sp*., *Pestalotiopsis sp*., *Funera sp.*, *Pythium sp.*, *Dothiorella sp.*, And *Phoma sp.* (Bakry and Abourouh, 1995).

5. Conclusion

This study focused on the diversity of pathogenic fungi capable of causing damage on the Tunisian oak. These pathogens are added to a series of factors (drought, pathogenic insects, silvicultural operations and aging) and aggravate the state of the Cork oak forests.

This study should be followed by studies aimed at developing the success of stressors that explain the state of our forest ecosystems.

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