Folia Forestalia Polonica, Series A – Forestry, 2021, Vol. 63 (1), 10–20

ORIGINAL ARTICLE

DOI: 10.2478/ffp-2021-0002

First report of *Apiognomonia errabunda* on *Quercus ilex* in Algeria

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Abstract

Anthracnose of holm oak (*Quercus ilex* L.) was studied in several sites located in Belezma National Park (Eastern Algeria). Disease severity varied according to the site, but symptoms were particularly frequent on leaves supported by south-facing twigs. Leaves inserted on the distal part of twigs were more affected by anthracnose regardless of twigs' position on trees. The fungal isolates obtained from damaged tissues were sorted to five morphotypes and identified as *Apiognomonia errabunda* based on morphological characteristics and ITS sequencing. This is the first record of the occurrence of the fungus *Apiognomonia errabunda* on *Quercus ilex* in Algeria. Particular climatic conditions marked by late spring rains followed by high temperatures may play a key role in the increased leaf vulnerability of *Q. ilex* to infection.

Key words

anthracnose, leaf position, *Quercus ilex*, severity, twigs orientation

Introduction

The holm oak (*Quercus ilex*) is a typical Mediterranean sclerophyllous species, dominating woody vegetation and occupying large areas, especially in the western part of the Mediterranean basin. This Fagaceae species is noted for its remarkable adaptive potentialities (Barbero and Loisel 1980; Barbero et al. 1992). Variability of *Q. ilex* populations was the subject of several investigations; in fact, Albert and Jahandiez (1908) described 31 varieties, but currently two morphotypes are recognized: *Q. ilex* and *Q. rotundifolia* Lam., which differ morphologically at the foliage level (veins number) (Saenz De Rivas 1967, 1970) vigour and shape (Barbero et al. 1992).

In Algeria in the 1950s, holm oak occupied an estimated area of approximately 700,000 ha (Boudy 1955) but had declined by the early 1980s to 354,000 ha (Dahmani-Megrerouche 2002). In the Belezma and the Aures mountains of eastern Algeria, *Q. ilex* is considered the main tree in the forest landscape, often associated with *Pinus halepensis* Mill., *Juniperus oxycedrus* L. and *Fraxinus xanthoxyloides* (G.Don) Wall. ex A.DC. in the semi-arid zones, with *Cedrus atlantica* (Endl.) Manetti ex Carrière in the subhumid zones situated on middle and high altitudes

Received 10 April 2019 / Accepted 9 November 2020

and with *Juniperus phoenicea* L. and *J. oxycedrus* on degraded lands influenced by the Sahara bioclimatic regime.

In recent years, natural events such as drought and irregular precipitation, anthropogenic actions (fires, overgrazing, and over-exploitation) have negatively affected *Q. ilex* forests in these regions. In addition, unusual development of the vegetation cover dominated by this species has been reported in Belezma, particularly in sites formerly occupied by *C. atlantica*, which had declined rapidly (Bensaci et al. 2015). In this situation, certain diseases and pests have increased greatly including anthracnose caused by *Apiognomonia errabunda* (Roberge ex Desm.) Höhn. (anamorph = *Discula quercina* (Westend.) Arx), known on *Quercus* species in the Mediterranean region (Morelet 1989; Ragazzi et al. 1999; Ragazzi et al. 2007; Linaldeddu et al. 2009).

Several studies highlighted the links between the aetiology of this disease and the trophic behaviour of the causal fungus, switching between the endophyte and pathogenic phase, as well as the phytosanitary state of the oak forests (Ragazzi et al. 2007; Moricca et al. 2012). Based on recent observations (2010 to 2016) in Belezma National Park, anthracnose in *Q. ilex* spread in parallel with recent outbreaks of the gall-forming mite *Aceria ilicis* Canestrini, which causes deformation, overgrowth, and browning of leaves, shoots, and inflorescences (Stork and Wüest 1996; Karioti et al. 2011).

The objectives of this work were to evaluate the extent of anthracnose on *Q. ilex* in the Belezma massif through the quantification of foliar symptoms, to determine the geographical variability of disease symptoms, and the influence of twig orientation and leaf position on twigs on the intensity of symptoms. To date, no studies have been conducted on the aetiology of this disease in North Africa.

Material and methods

Study areas

Four sites were examined: Bordjem, Tougurt, Ain Ali and Chelaâlaâ (Fig. 1). Leaves of *Q. ilex* showing typical symptoms of anthracnose (Morelet 1989; Moricca and Ragazzi 2011) were sampled at each site (Fig. 2). Each twig was subdivided into apical or distal (AP) and non-apical (NAP) parts. Twigs were sampled from four cardinal directions (North, South, East and West). Leaves were stored in paper bags for transfer to the laboratory.

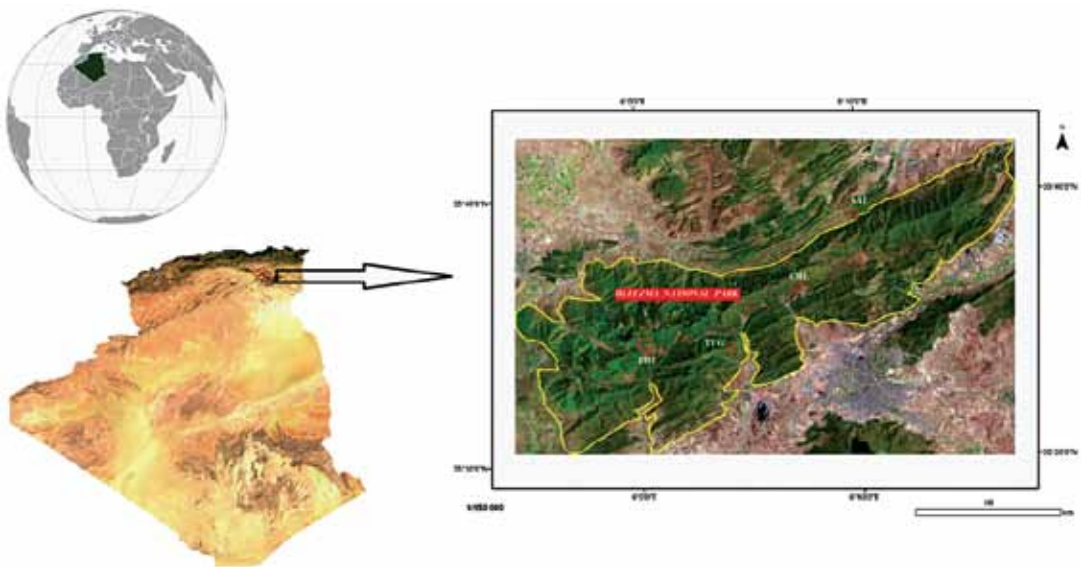

Figure 1. Map depicting the study sites situated in Belezma National Park: BRG: Bordjem, TUG: Tougurt, CHL: Chelaâlaâ, AAL: Ain Ali

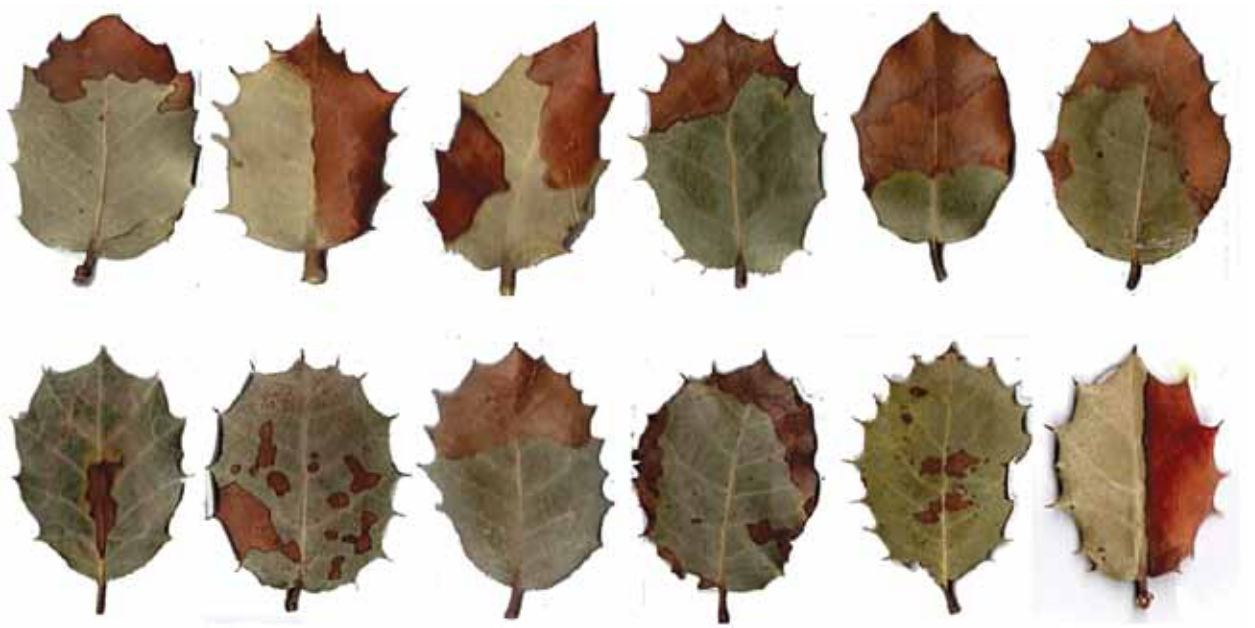

Figure 2. Leaves of *Q. ilex* showing characteristic brown-necrosis caused by *D. quercina*

Evaluation of anthracnose symptoms on Quercus ilex

The severity of the symptoms on the leaves was determined by direct quantification of the necrotic areas. The leaves were glued on to A4 white paper and scanned at 700 dpi resolution (Epson Perfection V370, Seiko Epson Co.). The scan was performed against a background of white light to maximize the contrast of the object before processing images with ImageJ software (Abràmoff et al. 2004; Ferreira and Rasband 2012). The resulting images were calibrated in mm against a ruler placed in the background. Image files were saved in TIFF format and analysed in the RGB (Red-Green-Blue) scale. A reference line (20 mm) was drawn to standardize the dimensions to the ruler and the object, and the set scale menu activated to set the measurement unit. Using the colour thresholds extraction method, selected necrotic areas of the leaf blade were extracted and measured (mm^2).

Disease severity index (*DSI*) was determined as the ratio between the percentage of necrotic leaf area (%) and the corresponding severity scale (Horsfall and Barratt 1945; Nutter et Esker 2006), as follows:

DSI = (Σ Xi.ni / R Nt) x 100, here *Xi*: disease severity (scale score), *ni*: leaf number with *i* severity, *R*: the highest score of the scale and *Nt*: the total number of examined leaves.

Since the sampling of symptomatic leaves was based on the orientation of the twigs and the position of the leaf (insertion point) on, severity was also considered in relation to these two parameters.

Pathogen isolation and identification

To isolate and identify the causative agent, leaves were surface-sterilized in 10% H_2O_2 for 15 min followed by threefold rinsing in sterile distilled water (Ragazzi et al. 2001). After sterilization, the leaves were dried on sterile filter paper and aseptically cut into small square pieces (5 mm^2) containing for each both necrotic and non-necrotic areas. Leaf pieces were placed on malt extract agar (MEA), as 5 pieces per Petri dish incubated at 24°C for one week, with daily observations to characterize the appearance of hyphae giving the fungal colonies. Mycelium was identified on morphological traits (Hughes 1953; Monod 1983; Ellis and Ellis 1997; Sogonov et al. 2008). Characterization of morphotypes was based on macroscopic features including colony shape and colour (Ragazzi et al. 2000; Lacap et al. 2003). Once identified, the fungal cultures are subcultured by transferring to water agar (WA).

For molecular characterization, discs taken from three-week colonies were transferred to glass tubes of 15 ml WA. Tubes were incubated at 24°C. After 15 days,

the purity of emerging colonies was tested according to Smith and Onion (1994). Subsequently, the mycelium was scraped from the agar surface and crushed on a sterilized stainless steel support. The crushed materials were placed in previously autoclaved glass tubes, alternating with layers of microgranules of autoclaved silica gel (Smith and Onion 1994). Tubes were sealed and stored at 4°C. Three days after, the crushed mycelia were recovered and resuspended in glass Petri dishes containing potato dextrose agar (PDA), then incubated at 26°C for 15 days, before subculturing to Erlenmeyer flasks containing 100 ml of potato dextrose broth. The flasks were incubated with shaking at 250 rpm at 25°C for one week prior to DNA extraction.

DNA extraction was performed according to Lee and Taylor (1990) using Tris-HCl (50 mM), EDTA (50 mM), 3% SDS, 1% 2-mercaptoethanol, chloroform-TE-saturated phenol 1:1 (v/v), NaOAc (3M)/pH: 8.0; isopropanol, and 70% ethanol as lysis buffer. PCR was carried out according to White et al. (1990), using ITS1, 5.8S and ITS2 regions (White et al. 1990; O'Donnell et al. 2000).

The sequences of the used primers were 5'-TCCGTAGGTGAACCTGCGG-3' (ITS1) and 5'-TCCTCCGCTTATTGATATGC-3' (ITS4) (White et al. 1990; O'Donnell et al. 2000).

Amplification cycles were carried out in a ProFlex^{T-M}PCR System (Applied Biosystems), with initial denaturation at 94°C (15 min) followed by three repeated phases (35 times): denaturation at 94°C for 2 min; annealing at 55°C for 1 min and elongation at 72°C for 3 min. A final elongation step of 72°C for 5 min was included. PCR products were examined on a 1% agarose gel in Tris-borate-EDTA buffer. DNA purification was carried out using the QiaQuick Gel Extraction kit (Qiagen, Germany). The product was stored at –20°C before sequencing.

Sequencing of the amplified fragments was performed in an ABI-Prism 373A DNA sequencer (Applied Biosystems) with enzymatic lysis and neo-amplification using the Big Dye Xterminator purification Kit (Applied Biosystems). Sequences were aligned using Bioedit Sequence Alignment Editor 7.0.0. (Hall 1999) and compared with those available in GenBank using BLAST search (http://blast.ncbi.nih.gov).

Statistical analyses

For phylogenetic analyses, multiple sequence alignment was performed including the genomic sequences of fungal isolates from the present study, compared with related mycotaxa in the Gnomoniaceae and Diaporthales using the ClustalW algorithm. The phylogenetic dendrogram was elaborated using MEGA 7.0 (Kumar et al. 2016) implying a Neighbour-joining statistical method. Evolutionary distances were computed using the Jukes-Cantor model (Yarza et al. 2017). The test adopted for phylogenetic analysis was the Bootstrap method with 1000 replicates. Statistical analyses were performed using XLSTAT 2014.5.03 (Addinsoft-Microsoft).

Results

Disease symptoms on leaves of Quercus ilex

Digital image analyses of the symptomatic leaves of *Q. ilex* allowed characterizing the severity of anthracnose (Tab. 1).

Anthracnose severity varied according to the site. The most intense severity was recorded in the leaves sampled from Tougurt (34.52%), followed by Chelaâlaâ

Table 1. Mean severity (%) calculated for each sites according to digital image analyses of *Q. ilex* leaves affected by anthracnose

Sites	Number of sampled trees	Number of sampled leaves	Severity (%)	Severity index	Horsfall-Barratt Scale
Bordjem	10	374	21.85 ± 0.18 ^{b}	0.10	3.99 ± 0.18
Tougurt	10	350	34.52 ± 0.15^{a}	0.13	4.76 ± 0.15
Chelaâlaâ	10	396	21.96 ± 0.17^{b}	0.11	4.01 ± 0.17
Ain Ali	10	319	13.66 ± 0.22^{c}	0.03	3.03 ± 0.22

Values represent means ± SE. Means followed by the same letter(s) within the same column are not significantly different ($\alpha = 0.05$) according to LSD Fisher test.

and Bordjem, with 21.96% and 21.85', respectively, while the leaves sampled from Ain Ali were recorded with severity of 13.66% ($P < 0.0001$) (Tab. 1). Severity index and the Horsfall-Barratt (HB) scale also differed according to the site. The most important severity index was recorded for Tougurt (0.13), unlike Ain Ali with a minimum scale of 0.03 (Tab. 1).

Severity was greater in the south-facing twigs for all the sites (Tab. 2), while minimum values were recorded for the west-facing twigs (Tab. 2).

Regarding leaf position on a twig, we have found that anthracnose severity was more prominent apical leaves compared to non-apical leaves except for those sampled from Ain Ali (Tab. 3).

Fungal isolation

The causal agent of anthracnose was isolated in all leaf samples in the anamorphic form and identified under species level as *Apiognomonia errabunda*. 35 isolates distributed over five morphotypes were characterized from 330 leaf pieces (Tab. 4, Fig 3).

Morphological characterization

All the fungal colonies have a circular shape. After 15 days of growth at 25°C on MEA, colony diameters in Tougurt isolates averaged 43.57 mm; those of Bordjem have an average diameter of 40.95 mm. For Chelaâlaâ isolates, mean colony diameter was 42.28 mm and 43.19 mm for Ain Ali isolates (Tab. 4).

Table 2. Variability of anthracnose severity (%) on leaves of *Q. ilex* according to the orientation of twigs

Twig orientation	Bordjem	Tougurt	Chelaâlaâ	Ain Ali
East	20.61 ± 0.39^{ab}	31.56 ± 0.32^{b}	21.51 ± 0.35^{ab}	12.63 ± 0.38^{b}
West	18.35 ± 0.40^{b}	26.80 ± 0.34^{b}	17.53 ± 0.38^{b}	9.79 ± 0.38^{b}
North	20.53 ± 0.34^{ab}	26.64 ± 0.34^{b}	19.36 ± 0.64^{b}	13.87 ± 0.51^{ab}
South	25.79 ± 0.31^{a}	43.87 ± 0.24^{a}	26.08 ± 0.28^{a}	21.60 ± 0.48^{a}
P	0.0773	<0.0001	0.0075	0.0093

Values represent means ± SE. Means followed by the same letter(s) within the same column are not significantly different (α = 0.05) according to LSD Fisher test.

Table 3. Severity (%) variation of *Q. ilex* anthracnose according to leaf position on twigs

	Bordjem	Tougurt	Chelaâlaâ	Ain Ali
NAP	20.02 ± 0.22^{b}	32.93 ± 0.21^{a}	21.55 ± 0.23^{a}	14.08 ± 0.28^{a}
AP	25.43 ± 0.63^{a}	36.34 ± 0.22^{a}	22.43 ± 0.25^{a}	13.07 ± 0.33^{a}
P	0.0193	0.1347	0.6597	0.6916

Values represent means ± SE. Means followed by the same letter(s) within the same column are not significantly different (α = 0.05) according to LSD Fisher test. NAP: non-apical leaves, AP: apical leaves.

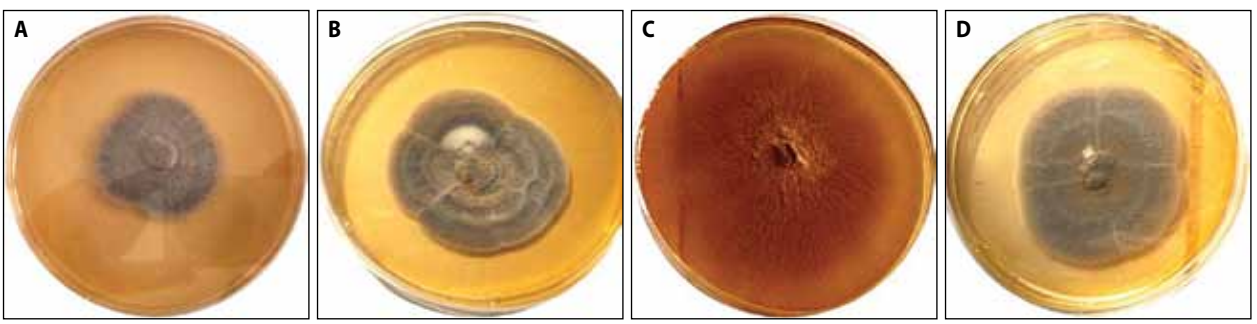

Figure 3. *Apiognomonia errabunda* cultures on MEA medium. (A) AECS3 isolated from Chelaâlaâ. (B) AETS2 isolated from Tougurt. (C) AEBS5 isolated from Brdjem. (D) AEAN6 isolated from Ain Ali

Table 4. Cultural characteristics of the 35 *D. quercina* isolates on MEA medium

Site	Isolates	Morphotype	Sporulation	Acervuli	Diam (mm)	CUps	CLws	Shape	ConidiaL (µm)	ConidiaW (µm)
Bordjem	DQBS1	M1	S	–	37.00	LB	LB	Circular	9.00	5.00
	DQBS2	M1	S	–	39.33	LB	LB	Circular	11.00	3.00
	DQBS3	M1	S	–	47.00	LB	LB	Circular	9.00	3.00
	DQBS4	M1	S	–	41.67	LB	LB	Circular	10.00	4.00
	DQBS5	M3	S	–	41.00	LB	LB	Circular	9.00	3.00
	DQBS6	M5	S	–	40.00	LB	LB	Circular	6.00	4.00
	DQBN1	M5	NS	–	40.67	LB	LB	Circular	n/a	n/a
Tougurt	DQTS1	M1	S	–	45.67	LB	LB	Circular	7.00	3.00
	DQTS2	M1	S	–	43.33	BG	LG	Circular	8.00	3.00
	DQTS3	M1	S	–	41.67	BG	LG	Circular	6.00	3.00
	DQTN1	M1	NS	–	39.67	LB	LB	Circular	n/a	n/a
	DQTS4	M2	S	–	45.33	LB	LB	Circular	8.00	5.00
	DQTN2	M2	S	–	44.00	LB	LB	Circular	7.00	3.00
	DQTS5	M2	S	–	46.67	LB	LB	Circular	8.00	2.00
	DQTN3	M3	S	–	40.00	LB	LB	Circular	8.00	4.00
	DQTN4	M3	NS	–	41.67	LB	LB	Circular	n/a	n/a
	DQTS6	M5	S	–	47.67	LB	LBCC	Circular	9.00	3.00
Chelaâlaâ	DQCN1	M1	NS	–	41.33	BG	LG	Circular	n/a	n/a
	DQCS1	M3	S	+	43.67	LB	LB	Circular	7.00	4.00
	DQCS2	M4	S	–	43.67	LB	LB	Circular	9.00	3.00
	DQCS3	M5	S	–	43.33	BG	LG	Circular	8.00	4.00
	DQCS4	M5	S	–	42.67	LB	LB	Circular	9.00	3.00
	DQCS5	M5	S	–	39.00	LB	LB	Circular	9.00	4.00
Ain Ali	DQAN1	M1	S	–	33.00	LB	LB	Circular	11.00	3.00
	DQAN2	M1	S	–	42.33	LB	LB	Circular	11.00	4.00
	DQAN3	M4	S	+	43.33	LB	LB	Circular	9.00	3.00
	DQAS1	M1	S	–	35.33	LB	LB	Circular	8.00	4.00
	DQAS2	M1	S	–	45.67	LB	LBCC	Circular	8.00	3.00
	DQAS3	M1	S	–	41.00	LB	LB	Circular	9.00	4.00
	DQAN4	M1	NS	–	41.33	LB	LB	Circular	n/a	n/a
	DQAN5	M1	S	+	49.67	BG	LG	Circular	6.00	3.00
	DQAS4	M2	S	+	50.00	BG	LG	Circular	7.00	3.00
	DQAN6	M2	NS	–	46.00	LB	LB	Circular	n/a	n/a
	DQAS5	M3	S	–	40.00	LB	LB	Circular	7.00	3.00
	DQAS6	M3	S	–	50.67	LB	LB	Circular	7.00	4.00

CSup: colony color in the upper side of Petri dish. CLws: colony color in the lower side of Petri dish. ConidiaL: conidia length. ConidiaW: conidia width. n/a: not-applicable. NS: non-sporulating isolate. S: sporulating isolate. (+) presence. (-) absence. LB: light brown. BG: brownish-gray. LBCC: light brown with gray concentric circles. LG: light gray.

Colonies' colour was usually light brown on both sides of the Petri dish, brownish-grey on the upper side and light grey on the underside, or light brown on the upper side and light-brown on the upper side with grey concentric circles on the lower side (Tab. 4). Conidia were ellipsoid, one-celled, 3.30–3.58 µm x 7.50–8.86 µm. Acervuli could not be detected, except in some isolates where they were spherical and grouped in clusters of 2 to 4.

Phylogenetic analyses

The identification of *A. errabunda* species by molecular characterization was confirmed by comparing ITS sequences against GenBank accessions (Tab. 4). The database search yielded two strains: MH111403 with approximately 613 bp and MG594496 with approximately 595 bp. The two sequences were deposited at GenBank. The MH111403 had ITS similarity of 99% with sequences of *A. errabunda* isolated from *Quercus pubescens* in Italy (GQ452265, GQ452259). The phylogenetic tree showed isolates MH111403 and MG594496 together in a single clad (Fig. 4).

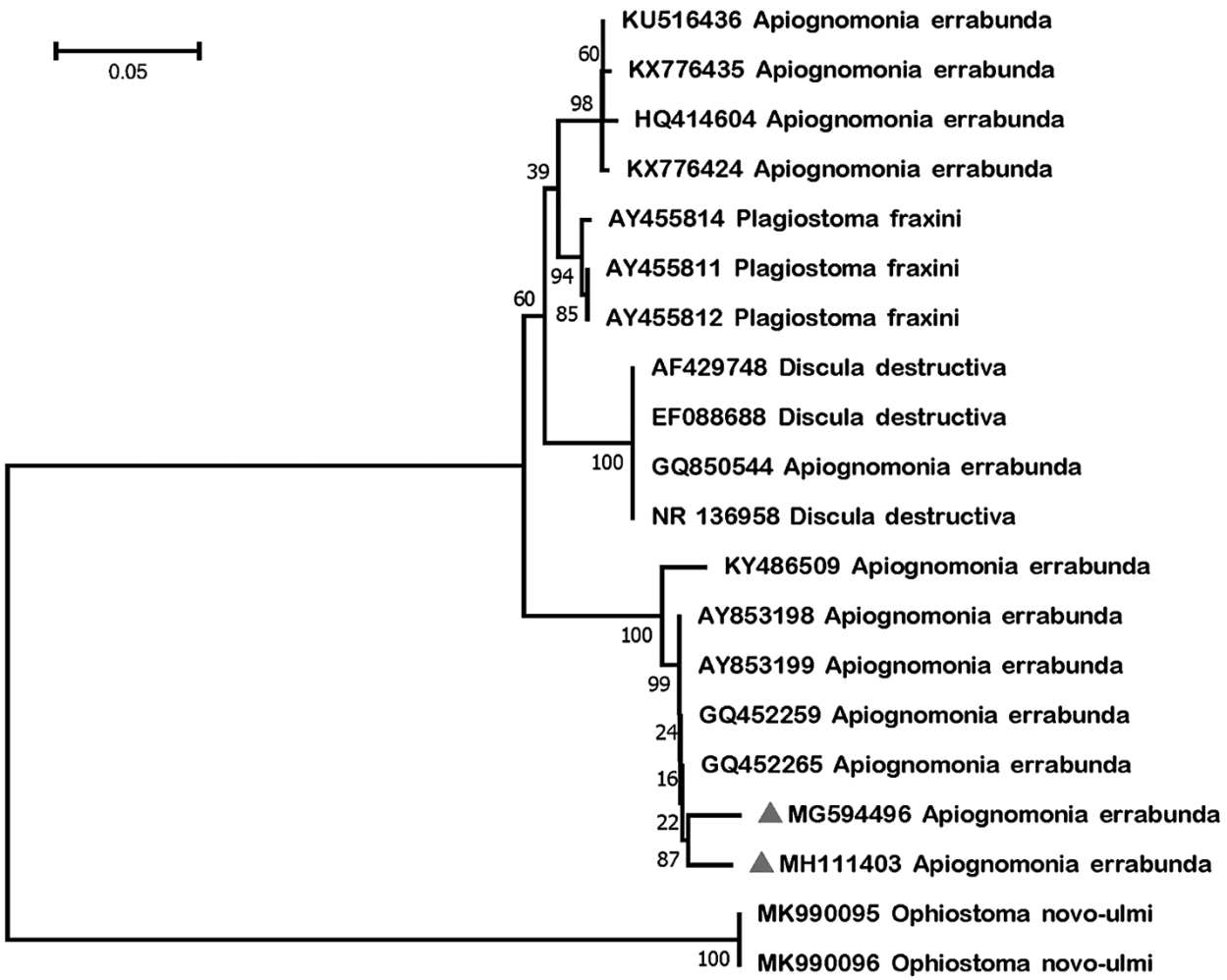

Figure 4. Phylogenetic tree (rDNA ITS) of *A. errabunda* including the two isolates from *Q. ilex* in Belezma National Park. Dendrogram was elaborated implying the Neighbor-Joining method based on the Jukes-Cantor model. The bootstrap tree inferred from 1000 replicates (The optimal tree with the sum of branch length = 0.62132144). Two accessions of *Ophiostoma novo-ulmi* were considered in this tree as out-group taxa

Discussion

Prospection of the five sites in the Belezma massif has shown a considerable incidence of anthracnose in *Q. ilex*. The incidence of *A. errabunda* was determined for several *Quercus* spp. In Tuscany region (Italy), the incidence of anthracnose *Q. pubesens* Willd. was a recorded level of 15% to 20% (Moricca and Ragazzi 2011). In Italian oaks, the incidence of anthracnose was characterized by interannual variability: during the 1990s, it was particularly high among Fagaceae in mixed oak sites: (*Quercus*/*Fagus*; *Quercus*/*Castanea* and *Quercus*/*Ostrya*) to a lesser degree in sites with monospecies or scattered oak trees (Ragazzi et al. 2007). While pathogenicity of this fungus was determined experimentally on several *Quercus* species (Hecht-Poinar and Parmeter 1986; Hanifeh et al. 2018).

The incidence of oak anthracnose is largely dependent on climatic conditions during the vegetative and reproductive growth seasons, including rainfall, spores-carrying winds, high temperatures, and humidity. In Italy, anthracnose was almost present in the southern parts; in Sicily, to regions with temperate Mediterranean conditions as for Tuscany (Moricca and Ragazzi 2008). While causal fungus was repeatedly isolated as an endophyte from several Fagaceae and more specifically *Quercus* spp. (Moricca and Ragazzi 2008), *A. errabunda* is one of the associated pathogens with recent decline phenomenon of oaks, as infection levels in declining trees far exceeded those recorded in healthy asymptomatic trees (Ragazzi et al 2003; Moricca and Ragazzi 2008). The causal fungus of anthracnose shares the same etiological characteristics compared to some endophytic fungi in that it can shift from asymptomatic (mutualistic) to the pathogenic symptomatic behaviour (La Porta et al. 2008).

Anthracnose severity was varied according to the site but more importantly in Tougurt, indicating that disease occurrence is probably dependent on certain site-related factors, which may include *Q. ilex* occurrence among tree species composition, age and its density. The calculated severity indexes indicate that holm oak was moderately sensitive for all studied sites, more particularly in Tougurt.

In terms of twigs direction, the severity of foliar symptoms was considerable for those exposed to the south. No work has addressed this parameter and its effect on the magnitude of anthracnose on *Q. ilex*. Infection occurs over a wide temperature range, but the leaves must be moistened for an extended period (Morelet 1989). We consider that a combination of leaf wetness during delayed spring rains and high temperatures, resulting in a prolonged time of twigs exposure to "energizing" effect of solar radiation leading to an amplified oak infection. The exceptional climatic conditions may influence not only the extent of oak infection by *A. errabunda*, but also the large-scale dissemination of the disease.

Our results prove that the most important severity was recorded in leaves inserted on the apical parts of twigs. It has been shown in several studies that foliar symptoms of anthracnose caused by fungi belonging to the genus *Apiognomonia* Höhn, *Gloeosporium* Desm. & Mont., and *Gnomonia* Ces. & De Not. were particularly concentrated in the distal leaves (Gregory and Redfern 1998). According to Morelet (1989), young organs are particularly susceptible to *Apiognomonia quercina*, especially on *Q. bicolor* Willd. and *Q. macrocarpa* Michx.

The asexual form of the causal agent of oak anthracnose (*D. quercina*) was described for the first time by Saccardo in 1884. This mycotaxon is commonly considered as a typical endophyte of the Mediterranean Fagaceae (Moricca and Ragazzi 2011). Colony growth on MEA suggests that *A. errabunda* respond by the same magnitude to the nutrient quality of the medium. According to the present work, the *A. errabunda* isolates from *Q. ilex* are less vigorous on cultures than other isolates characterized from *Q. frainetto* and *Q. cerris* by Ragazzi et al. (2000). Thus, host taxa may probably affect the growth potential of the fungus on synthetic medium (Toti et al. 1992).

The shape and colour of colonies are generally similar to that described by Moricca and Ragazzi (2011) on PDA as "very light-brown," while conidia sizes are variable according to the geographical origin of the isolates.

In combination with the morphological data, the molecular characterization confirmed the identity of the causal agent as *A. errabunda*. The resulting ITS sequences of Belezma strains seem to be more related phylogenetically to those isolated from *Q. cerris* in Italy (Turco et al., unpublished data). We suggest expanding the geographical area of the sampled sites to check whether the populations of this fungus in North Africa

are divergent or not compared to those of South European regions.

A. errabunda offers a typical example of the trophic behaviour shift of endophytes from mutualism or asymptomatic to the symptomatic pathogenetic aspect. If *A. errabunda* is considered as an element involved in *Quercus* decline (Bendixsen et al. 2015), it is necessary and essential to apply the best control strategies for anthracnose through reasonable silvicultural practices and preventive treatments if necessary, while giving importance to the climatic data that must be exploited by the forest manager in order to choose the appropriate timing of the intervention.

Acknowledgments

Authors thank Mr. Said Abderrahmani, the director of Belezma National Park for providing logistic support during the period of study.

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