

## entomology &amp; pathology

# Phytophthora cinnamomi Involved in the Decline of Holm Oak (*Quercus ilex*) Stands in Southern Italy

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During a survey of forest stands of holm oak (*Quercus ilex*) in the Salento peninsula, Apulia region (southern Italy), the oomycete *Phytophthora cinnamomi* was found to be consistently associated with tree decline and mortality in 7 municipalities of the province of Lecce. The pathogen was recovered directly from roots using a selective medium and from rhizosphere soil samples with leaf baits and subsequent isolation on the same selective medium used for direct isolation from roots. It was identified on the basis of morphological characters and by sequencing of the internal transcribed spacer (ITS) regions of the rDNA after amplification with conventional PCR. All *P. cinnamomi* isolates were A2 mating type and proved to be highly aggressive on seedlings of evergreen Mediterranean oak species, including holm oak, cork oak (*Q. suber*) and kermes oak (*Q. coccifera*). *P. cinnamomi* is a well known pathogen of several forest trees worldwide and on the basis of its widespread and consistent occurrence in forest stands of the Lecce province it was assumed to be the primary causal agent of holm oak decline in this area. Options for the management of this phytosanitary environmental emergence are discussed.

**Keywords:** Cork oak, Kermes oak, ITS rDNA, invasive pathogen, Mediterranean evergreen oaks

Apulia (southeastern Italy) is the least forested region in Italy and the one with the lowest ratio of woodland area/inhabitant. It is subdivided into diverse climatic sub-regions, and the sub-region of the Salento Peninsula (latitude 40° 30' 00"; longitude 18° 00' 00"), which includes the whole province of Lecce and part of the provinces of Brindisi and Taranto (Figure 1), is characterized by a mild climate with January and February isotherms of 19°C and an average yearly rainfall ranging from 500 up to 900 mm. Apulia's forests historically have been dominated by oaks.

Ten different evergreen, semi-deciduous, and deciduous oak species are present in this region (Bianco et al. 1989). Two evergreen oaks, holm oak (*Quercus ilex* L.) and kermes oak (*Q. coccinea* s.l.), are the most common native species in the southern part of the Salento Peninsula, where their distribution areas partly overlap (Sabato

1972, Biondi et al. 2004). Holm oak is widespread in the midwestern Mediterranean basin and forms evergreen climax forests (climax of *Quercion ilicis* or holm oak forest) that are regarded as typical of the Mediterranean region (Sabato 1972). Indeed, the native range of this sclerophyllous oak is even larger than the climax of *Quercion ilicis* and includes all coastal circum-Mediterranean areas.

In Italy, holm oak can be found in dense or sparse, pure or mixed, usually not very extensive stands, reaching even 1500 m a.s.l. in the southern Apennines. According to a phylogeographic study (Lumaret et al. 2002), the holm oak population of the Salento Peninsula, supposed to be a relic of more extensive forests, is closely related to the Greek population of this Mediterranean evergreen oak. In the province of Lecce, dense sclerophyllous native forest stands with holm oak as the dominant overstory tree species

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escaped excessive anthropogenic pressure and deforestation for agricultural land uses because they were private parks or game reserves for throstle hunting. These reserved areas were delimited by stone walls (Figure 2A), which have protected them from wildfires, cutting for lumber production, and overgrazing, thus favoring natural regeneration. These stands generally range from 3 to 40 ha. Nowadays, many of them are public amenity parks.

Since 2012, a severe and widespread dieback in these holm oak stands has been observed (Figure 2B–C). Symptoms included yellowing and wilting of leaves, small-sized and sparse foliage, thinning and dieback of the crown, decrease in production of acorns, and damping off of young seedlings, all adversely affecting natural regeneration. These symptoms were accompanied by rot of fine and coarse roots of trees, necrosis of taproot, and eventually tree mortality. Occasionally, in a few sites the presence of bleeding cankers incited by *Botriosphaeriaceae* or associated with typical zigzag-shaped galleries of *Agrilus* oak borer (oak Buprestidae beetle) and charcoal cankers of *Biscogniauxia mediterranea* were observed on the trunk and branches of mature trees. In all stands, dead trees showed a clumped distribution pattern and mortality incidence in a single stand varied from 10 to 60%, while almost all the trees were affected by decline symptoms.

Similar declines of holm oak and cork oak (*Q. suber*) caused by the oomycete *Phytophthora cinnamomi* were previously reported across the natural range of these evergreen oak species in several Mediterranean countries, including France, Portugal, and Spain (Brasier et al. 1993, Robin et al. 1998, Corcobado et al. 2013, Jung et al. 2013), as well as in the Sardinia region and central Italy (Scanu et al. 2013, Linaldeddu et al. 2014). *P. cinnamomi* also was reported to be a major contributing factor of extensive diebacks of white oak (*Q. alba*) in the eastern United States (Nagle et al. 2010, McConnel and Balci 2014). Several other soil-borne *Phytophthora* species were found to be associated with oak decline all over the world (Jung et al. 1996, Balci and Halmschlager 2003a, Balci et al. 2007, Perez-Sierra et al. 2013). *P. quercina*, for example, alone or with other species, is regarded as a major pathogen of oaks in central Europe, Sweden, Italy, Spain, and Turkey (Jung et al. 2000, Balci and Halmschlager 2003b, Jönsson et al. 2005, Jung et al. 2013). Perhaps the most relevant case is sudden oak death (SOD) incited by *P. ramorum* on native oaks in California and Oregon coastal forests (Garbelotto and Hayden 2012, Rizzo et al. 2002).

The threat posed by this invasive and polyphagous pathogen for agricultural and natural ecosystems has raised biosecurity issues and stimulated the application of strict quarantine measures for forest pathogens and nursery stocks in the United States, Europe, and many other parts of the world (Frankel 2008, Grünwald et al. 2008). More recently another exotic invasive species, *P. kernoviae*, with a wide host range including potentially holm oak and pedunculated oak (*Q. robur*), was identified in the UK (Giltrap et al. 2013). Both *P. ramorum* and *P. kernoviae* have been added to the EPPO A2 list of quarantine pathogens.

The objective of this study was to investigate a possible involvement of *Phytophthora* spp. in the outbreak of holm oak decline observed in the Salento Peninsula.

## Materials and Methods

### Sampling, Visual Inspection of Trees, and Isolation

In 2014 and 2015 between April and May, soil samples containing fine roots were collected from around symptomatic holm

oak trees in seven forest stands with holm oak as the dominant tree species, in the municipalities of Castro, Maglie, Ortelle, Poggiardo, Scorrano, Tiggiano, and Uggiano la Chiesa, all located in the province of Lecce (Figure 1). Each sample was obtained by mixing together four subsamples collected beneath the same tree. Five composite soil samples (each of approximately 2 L) were collected in each stand from five scattered, distinct symptomatic trees and processed within 24 to 72 hours after collection. The crown status of the sampled trees was assigned to class 4 (crown transparency more than 55%), following the method of Balci and Halmschlager (2003a).

To isolate *Phytophthora* directly from roots, fine roots were separated from the soil, washed free of soil with tap water, cut into small segments (3–5 mm), blotted dry, and plated in Petri dishes (9 cm diameter) onto a slightly modified BNPAH selective medium (Masago et al. 1977), with V8 Agar (V8A) (V8° juice, Campbell Soup Company, USA) instead of Potato-Dextrose-Agar (PDA, Oxoid Ltd, UK) (PDA) as basal nutritive medium. V8A was amended ( $\mu\text{g mL}^{-1}$ ) with benomyl (10), pentachloronitrobenzene (25), nystatin (25), ampicillin (500), rifampicin (10), and

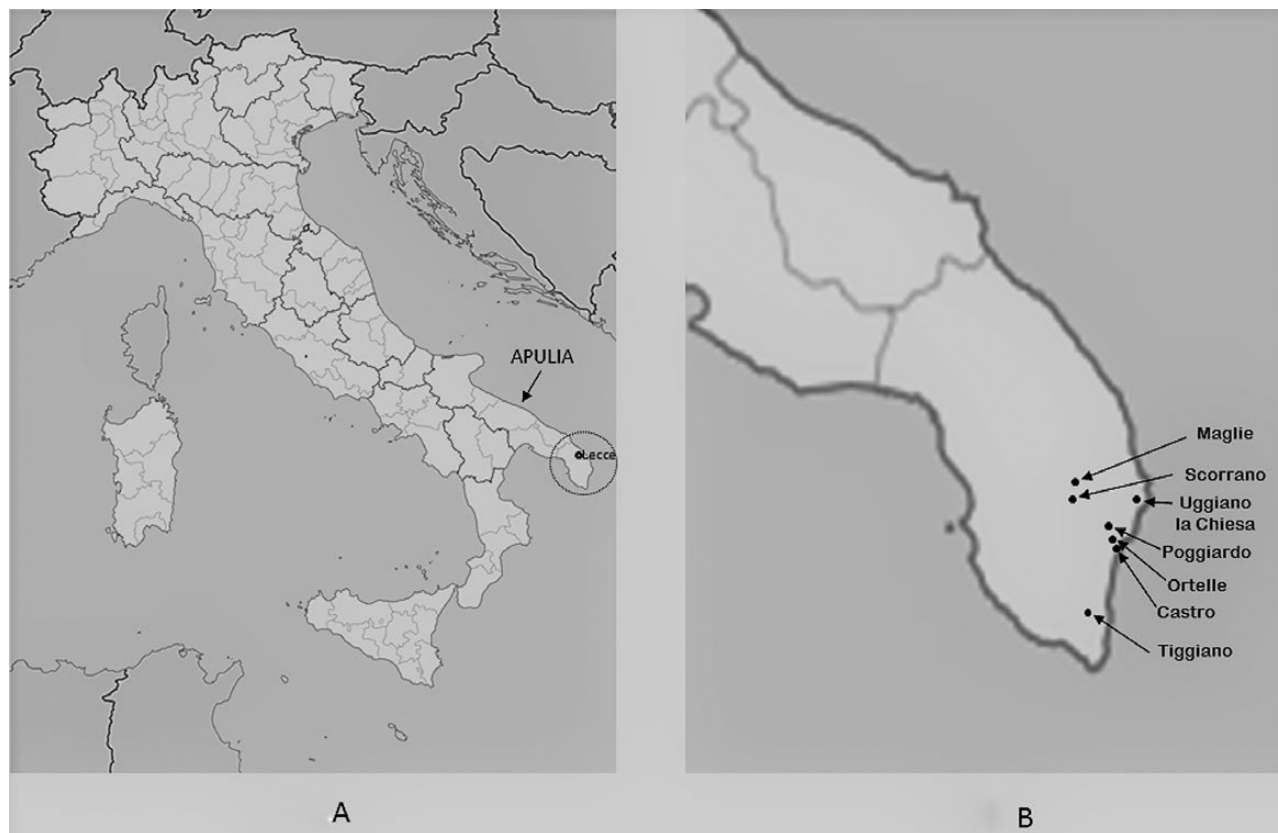
## Management and policy implications

The identification of the causal agent associated with the mortality of holm oak trees in the Salento Peninsula as *Phytophthora cinnamomi* indicates that the decline of holm oak stands in this area of southern Italy is primarily caused by *Phytophthora* root rot disease and has practical implications for land management. Although *P. cinnamomi* is no longer included in the EPPO (European and Mediterranean Plant Protection Organization) lists of quarantine pathogens, it is regarded as one of the worst invasive alien species on a global scale and appropriate management is required in order to prevent its further spread in horticulture as well as in natural ecosystems.

*Phytophthora* root rot, like other infectious diseases, can spread epidemically. As a consequence, the intervention measures that can be adopted by the Regional Forest Service include the use of certified seedlings for reforestation and replanting, avoiding the dispersal of the pathogen inoculum through infested soil adhering to visitors' shoes, car wheels, boots, tools and equipment of forest laborers, and avoiding soil waterlogging by drainage and thinning of the forest stands in order to favor ventilation and reduce shading of the underwood.

The identification of the primary causal agent of the decline in the forest stands in the Salento Peninsula also has implications for regional policymakers as they provide financial and human resources to contain the epidemics and prevent spoiling the typical landscape of this area, which is endangered by another destructive disease of olive caused by the bacterium *Xylella fastidiosa*.

Efforts to restrict these two diseases could be coordinated. There is, however, the risk that the destructive potential of *P. cinnamomi* is underestimated, as the attention of public opinion is presently focused on the damage of *X. fastidiosa*, which is spreading more rapidly because it is transmitted by winged insects. Eradication and replanting strategies like those applied in Australia to manage jarrah disease (a disease of native *Eucalyptus* species) caused by *P. cinnamomi* are not feasible in Apulia because of the different economic, social, and natural context. However, a public information campaign to make people aware of the risk posed by the epidemic spread of *P. cinnamomi* in both forest and agricultural ecosystems could be successful in limiting the impact of this exotic, invasive pathogen.



**Figure 1.** Map of the Salento Peninsula (A) showing the surveyed area and the location of the municipalities (B) where oak stands were assessed for the presence of *Phytophthora cinnamomi*.

hymexazol (50). Plates were incubated at  $20 \pm 2^\circ\text{C}$  for 2 to 3 days in the dark. Growing colonies were purified by subculturing hyphal tips onto the same selective medium and subsequently onto V8A. Soil samples were baited as described by Jung (2009). They were flooded with distilled water in a plastic tray to 3 cm depth, and any organic matter floating on the surface of the water was removed to prevent organic debris from coming into contact with the baits. Juvenile leaves of holm oak, carob (*Ceratonia siliqua*), sweet orange (*Citrus sinensis* “Lane Late”), and brush cherry (*Eugenia myrtifolia*) were floated on the water, acting as baits for *Phytophthora*. Trays were incubated in a growth chamber at  $20 \pm 2^\circ\text{C}$ , under artificial light with a 12 h photoperiod. After 2–5 days, leaves showing brown to dark brown spots (on *E. myrtifolia* leaves, symptoms appeared as irregular, edematous patches with a dark-brown irregular margin) were examined under the microscope (250 x magnification) for presence of sporangia. Leaves with spots were rinsed with distilled water and blotted dry. Discolored leaf lamina was cut into small pieces and plated onto BNPRAH selective medium. The plates were checked daily under the stereomicroscope, and developing colonies were purified by subculturing hyphal tips onto selective medium and subsequently onto V8A. Purified isolates were grown on V8A for morphological identification and DNA extraction.

### Morphological and Molecular Identification of *Phytophthora* Isolates

Isolates recovered from roots and soil (a total of 305 isolates) were identified by examining the colony growth pattern, cardinal

temperatures of growth, and microscopic morphological characteristics on both V8A and PDA, according to the keys of Stamps et al. (1990) and Erwin and Ribeiro (1996) as well as the diagnostic protocol PM7/26 of OEPP/EPP (Cacciola et al. 2004). Sporangia were observed on 15 mm squares cut from the growing edge of a 5- to 7-day-old culture grown on V8A at  $20^\circ\text{C}$  in the dark, and flooded for 24–36 h in 90 mm Petri dishes with non-sterile soil extract (Jung et al. 1996). Measures of 20 sporangia were recorded for each isolate.

Pure cultures of *Phytophthora* isolates were identified through amplification of the internal transcribed spacer (ITS) of the rDNA with the ITS6/ITS4 primer pair (Cooke et al. 2000). The PCR was performed in a 25  $\mu\text{l}$  final volume containing 1x PCR buffer with 1.5 mM  $\text{MgCl}_2$  (Invitrogen), 0.5  $\mu\text{M}$  of each primer, 0.1 mM dNTPs (Invitrogen), 1.25 U of Taq DNA polymerase (Invitrogen), and 1  $\mu\text{l}$  of DNA template. The reaction was incubated at  $95^\circ\text{C}$  for 2 min followed by 35 cycles each consisting of  $94^\circ\text{C}$  for 1 min,  $55^\circ\text{C}$  for 1 min,  $72^\circ\text{C}$  for 1 min, with a final cycle of  $72^\circ\text{C}$  for 10 min.

Amplification products were analyzed by electrophoresis on 1.5% agarose gel. The approximate length (800 bp) of the amplification products was determined using the Low DNA mass ladder (Invitrogen). Amplification products were purified using the ExoSAP-IT kit for PCR Product Cleanup (Affymetrix, UK) and sequenced using an external sequencing service (BMR-Genomics, Italy). ITS sequences were analyzed using BLASTN (NCBI database) to identify the most similar available sequences.



**Figure 2.** A native, walled holm oak forest stand in the Salento Peninsula used in the past as private park and game reserve (Maglie, Lecce; A); the picture was taken before the epidemic of the dieback; B–C); dead holm oak trees in native, almost pure stands in the Salento Peninsula with natural regeneration and overgrowth of understory plants which are favored by the sunlight (Tiggiano, Lecce); D); clear cut of dead holm oak trees and replanting with nursery grown holm oak seedlings (protected with a plastic screen) in an almost pure holm oak stand (Scorrano, Lecce).

### Mating Type Determination

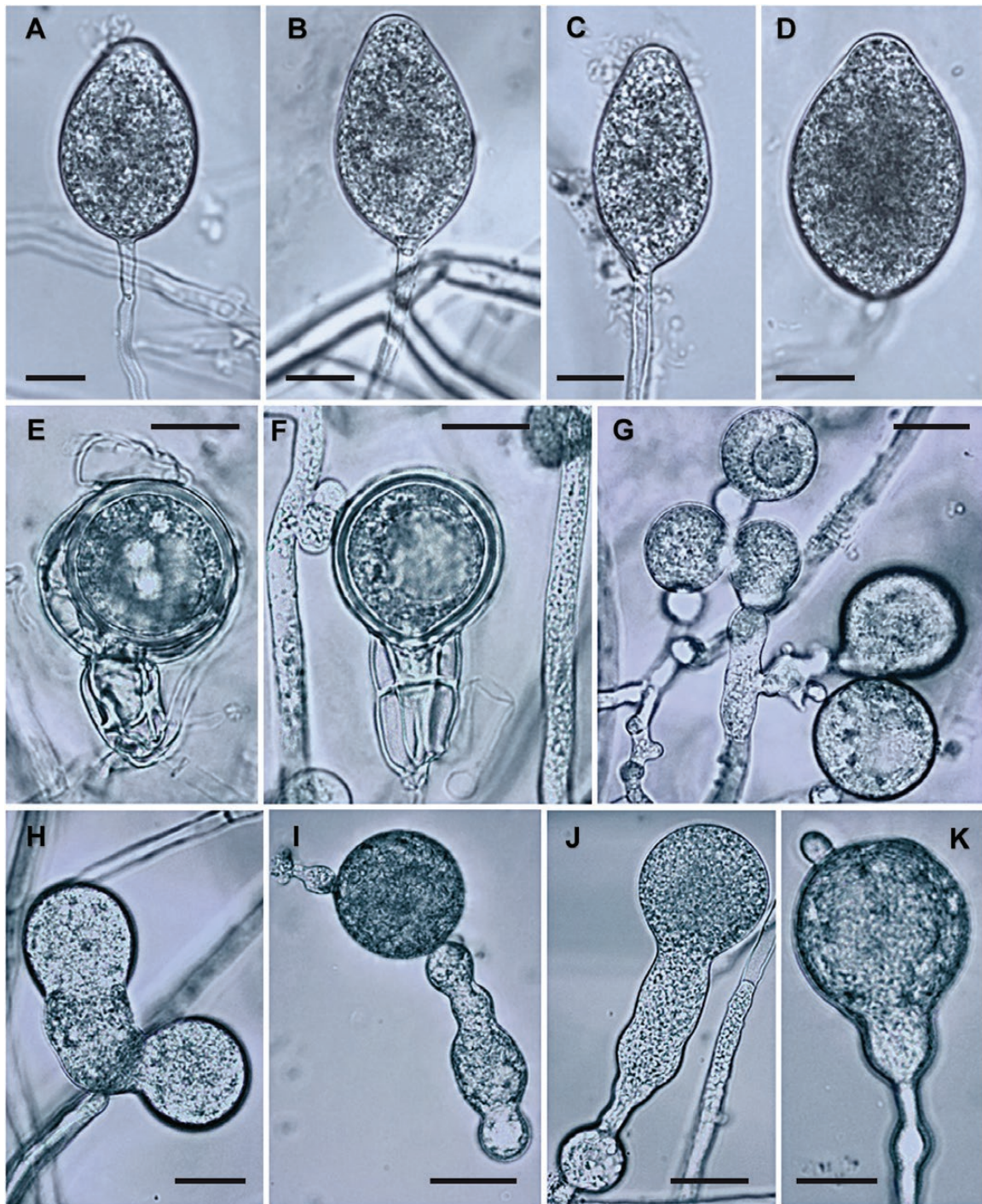
Isolates were paired with A1 and A2 mating type of *P. cinnamomi* and *P. cambivora* tester strains to stimulate production of oogonia. Isolates of *P. cinnamomi* used as tester were PRC-102 (A1, Mycological Collection of Reggio Calabria, Italy) and CBS 144.22 (A2, ex-type isolate, Scanu et al. 2014) isolates; for *P. xcambivora* IMI 229178 (A1) and CBS 141.218, (A2) (Jung et al. 2017b) isolates were used. A 5 mm plug was taken from each isolate and transferred to the edge of four 90 mm Petri dishes on V8A. A 5 mm plug of each A1 and A2 isolate of the two species was placed opposite each isolate, and binary cultures were incubated at 22°C in the dark for two weeks. Measures of 20 oospores were recorded for each isolate.

### Pathogenicity Tests

Inoculations were carried out using 6-month-old seedlings of holm oak, cork oak, and kermes oak. Inoculum was produced in 500 ml Erlenmeyer flasks on an autoclaved mixture of 250 cm<sup>3</sup> of vermiculite, 20 cm<sup>3</sup> of whole oat grains, and 175 ml of V8 juice (200 ml V8 juice, 800 ml deionized water, 3 g CaCO<sub>3</sub>) (Jung et al. 1996). Each flask was inoculated with 4 plugs from actively

growing cultures of *P. cinnamomi* on V8A. A representative *P. cinnamomi* isolate obtained from Scorrano holm oak rhizosphere soil was used for inoculations. Flasks were incubated at 20°C for 6 weeks. Inoculum was mixed with a twice-autoclaved mixture of peat, vermiculite, and volcanic sand (1:1:1, v/v/v) at 20 cm<sup>3</sup> inoculum per 1000 cm<sup>3</sup> potting medium. The control medium consisted of peat, vermiculite, and sand mixed with a non-infested mixture of vermiculite, whole oat grains, and V8 juice. Twenty seedlings of each oak species were transplanted into 14 cm diameter pots (a single seedling per pot) containing the infested potting medium. The same number of seedlings of each oak species was transplanted into pots containing non-infested substrate (control). Potted seedlings were grown in a growth chamber at 20 ± 1°C, 14/10 h light/dark photoperiod and a relative humidity of 65% maintained throughout the experiment. Pots were flooded for 48 h soon after the seedlings were transplanted and subsequently every 2 weeks.

Mortality rate of seedlings was assessed at 60 days after transplanting. Roots were washed free of soil with tap water, cut into small segments (3–5 mm), blotted dry, and plated onto selective medium to re-isolate the pathogen in order to complete Koch's postulates.



**Figure 3.** Morphological structures of *Phytophthora cinnamomi* from holm oak tree of the Salento Peninsula. Non papillate, persistent sporangia (A–D); gametangia and oospores (E–F) with amphigynous unicellular (E) and bicellular (F) antheridium; hyphal swellings (G–K) and a thin-walled chlamydospore (G). Scale bar: 20  $\mu\text{m}$ .

## Results

A sole *Phytophthora* species was consistently recovered from roots and rhizosphere soil of declining holm oak trees in all seven surveyed sites. It was isolated from fine roots of 30 out of 35 examined holm oak trees and from all 35 soil samples collected beneath these trees. The isolation frequency from the fine roots of the soil samples collected in Castro, Maglie, Ortelle, Poggiardo, Tiggiano, and Uggiano la Chiesa municipalities was 73, 80, 27, 24, 72, and 56%, respectively, whereas the highest isolation frequency (96%)

was recorded in Scorrano. More than 35% of isolates obtained from roots and soil (107 out of 305) were from soil samples collected at Scorrano.

Isolates showed a woolly colony pattern on selective medium and a distinct rosette colony pattern on PDA. They produced coral-loid hyphae and botryose swellings on V8A, often in clusters, and formed globose thin-walled, mainly terminal chlamydospores, often in characteristically grape-like clusters of 3–10 chlamydospores (Figure 3G–K). The mean diameter size of chlamydospores was

45 µm, with a range of 32 to 80 µm. Cardinal temperatures for radial growth were determined and minimum, optimum, and maximum temperatures were 5, 25, and 35°C. Sporangia were large, ovoid, obpyriform, or ellipsoid, non-papillate, persistent (Figure 3A–D). Their mean size was 74 (35–82) x 40 (35–46) µm with a length-breadth ratio of about 1.5. New sporangia were produced by internal or external proliferation or by sympodial development of the sporangiophore immediately below empty sporangia. Pairing tests revealed all isolates were A2 mating type. Antheridia (mean size 19 x 17 µm) were amphigynous, occasionally bicellular. Oogonia, whose size ranged from 36 to 55 µm in diameter (mean 40 µm), were round, with a funnel-shaped stalk (Figure 3E–F), hyaline to yellow brown and smooth walled. Oospores ranged from 33 to 41 µm in diameter (mean 36 µm).

Nucleotide sequences of the isolates from holm oak showed 100% and 99.9% homology with reference isolates of *P. cinnamomi* in GenBank, KU723593 (Jung et al. 2017a) and KC478663 (ex-type isolate CBS 144.22, Cacciola et al. 2004), respectively. Isolates from holm oak were also compared with the ex-type isolate of *P. parvispora* (CBS 132.772, Scanu et al. 2014) with 97.94% identity. The ITS sequence of only one representative isolate from Scorrano was deposited in GenBank (Acc. No. KT366917) because all the isolates have identical sequences.

The *Phytophthora* species associated with holm oak decline in the Salento Peninsula was unambiguously identified as *P. cinnamomi* Rands on the basis of both morphological characteristics and the DNA analysis. Pathogenicity of *P. cinnamomi* isolates associated with oak decline in the Salento Peninsula was demonstrated. Holm oak, cork oak, and kermes oak seedlings artificially inoculated with the suspected pathogen showed wilt symptoms within 15 days, and all died within 60 days after transplanting, while control seedlings showed no symptoms and produced new sprouts. *P. cinnamomi* was re-isolated only from roots of inoculated dying seedlings. The test was repeated once with similar results. No differences in susceptibility were observed between holm oak, cork oak, and kermes oak in pathogenicity tests.

## Discussion

The *Phytophthora* species isolated from rhizosphere of declining holm oak trees in the Salento Peninsula was clearly identified as *P. cinnamomi* on the basis of both DNA analysis and morphological characteristics, which matched those reported in the EPPO diagnostic protocol PM7/26 (Cacciola et al. 2004) and the recent re-definition of this species, which has been separated from *P. parvispora* (formerly *P. cinnamomi* var. *parvispora*) (Scanu et al. 2014).

Results indicate that *P. cinnamomi* is widespread in holm oak stands of the Salento Peninsula, as it was isolated in all surveyed forest stands. By contrast, bleeding cankers incited by *Botryosphaeriaceae*, galleries of insect borers, and charcoal cankers caused by *B. mediterranea*, reported to be major contributing factors of the decline of oak forests in other parts of the Mediterranean basin (Linaldeddu et al. 2014, Moricca et al. 2016), were found in only three out of the seven sites involved, i.e., Maglie, Scorrano, and Tiggiano (data not reported).

All tested *P. cinnamomi* isolates from forest stands of the Salento Peninsula proved to be virulent on Mediterranean evergreen oak species. These results provide circumstantial evidence of the role of *P. cinnamomi* as primary causal agent of the decline of holm

oak in the Salento Peninsula, but at some sites additional biotic factors, including infections by *Botryosphaeriaceae* such as *Diplodia corticola*, and *Xylariaceae* such as *B. mediterranea*, may synergistically contribute to the more rapid decline of these oak ecosystems (Linaldeddu et al. 2014, Moricca et al. 2016).

A strong association between the presence of *P. cinnamomi* and decline of Mediterranean evergreen oaks *Q. ilex* and *Q. suber* was first reported in the Iberian Peninsula (Brasier et al. 1993). *P. cinnamomi* was also reported to cause cankers and mortality on native oak species in California and Mexico (Tainter et al. 2000, Wood and Tainter 2002, Garbelotto et al. 2006, Alvarado-Rosales et al. 2008) and in the eastern United States as a major contributing factor of extensive dieback of white oak (*Q. alba*) (Balci et al. 2007, Nagle et al. 2010, McConnel and Balci 2014).

Holm oak is extremely susceptible to *P. cinnamomi* (Robin et al. 1998, Serrano et al. 2012, Seddaiu et al. 2014) and, in this study, forest ecosystems dominated by this evergreen Mediterranean oak were confirmed to be extremely vulnerable to such an invasive pathogen, which has been introduced into 15 of the 25 global biodiversity hotspots and is threatening rare plant species and degrading unique plant communities (Dunstan et al. 2010). Dunstan et al. (2010) reported that, when assessing forest ecosystems, a site should be regarded at risk whenever *P. cinnamomi* is detectable in the soil. Serrano et al. (2015) also drew the same conclusion for oak forests in the Mediterranean region.

In the last decade, numerous studies have dealt with the role of *Phytophthora* species in forest ecosystems worldwide and the impact that alien, invasive species of this Oomycetes genus have on native vegetation in areas where they have been introduced and become established.

In Italy, the first sporadic reports of *P. cinnamomi* in chestnut (*Castanea sativa*) stands and as causal agent of root rot and dieback of avocado (*Persea americana*) trees in commercial plantings, respectively, date back to around twenty years ago (Cristinzio 1986, Cacciola et al. 1998). Subsequently, this *Phytophthora* species was reported as causal agent of root and crown rot of English walnut trees and ink disease of chestnut in northern and central Italy, respectively (Belisario et al. 2001, Vettraino et al. 2001). Its presence was also reported in stands of Mediterranean oaks, including *Q. cerris*, *Q. frainetto*, and *Q. ilex*, but was not associated with any visible symptom on trees (Vettraino et al. 2002). By contrast, recently it was identified as primary causal agent of decline and mortality of cork oak forests in Sardinia (Scanu et al. 2013, Linaldeddu et al. 2014).

The decline of holm oak stands associated with *P. cinnamomi* is a serious concern for the maintenance of the typical Mediterranean landscape in the Salento Peninsula, which is facing another dramatic phytosanitary and environmental emergency due to the rapid spread and the destructive effects of *Xylella fastidiosa* subsp. *paucispora* epidemics in olive plantings, which are a historic and culturally significant aspect of the landscape in the Salento Peninsula (Martelli et al. 2016). Moreover, the presence of *P. cinnamomi* in oak forests in this area poses a serious threat to both agricultural crops and biodiversity of natural ecosystems because of the considerable polyphagy of this pathogen, whose host range includes over 1,000 plant species (Erwin and Ribeiro 1996).

Climatic conditions in the warm sub-region of the Salento Peninsula are very favorable to the establishment of *P. cinnamomi*,

which has been included in the group of pathogens and diseases directly affected by climate. This group of pathogens can incite disease in healthy, vigorous hosts if the environmental requirements of the pathogen are met (Sturrock et al. 2011). Floods and droughts are generally assumed to trigger *P. cinnamomi* epidemic outbreaks (Corcobado et al. 2013), and global warming is expected to lead to an expansion of the range of this pathogen in coastal areas of Europe (Brasier and Scott 1994, Brasier 1996, Burgess et al. 2017). Prolonged drought during the last decades in the Mediterranean basin may be a predisposing factor of holm oak decline in the Salento Peninsula. However, the presence of abundant natural regeneration and the fact that similar and such severe decline symptoms have not been reported from more marginal areas of the native range of this typical Mediterranean oak species do not support this hypothesis.

Pathogenicity tests confirmed previous results (Seddaiu et al. 2014), indicating that evergreen Mediterranean oaks such as cork and kermes oak, which are sometimes associated with holm oak in shrub lands and mixed forest stands of the Salento Peninsula, are not only within the host range of *P. cinnamomi*, but are extremely susceptible to infections of this pathogen. In almost all sites of southern part of the peninsula, where the distribution areas of holm and kermes oak overlap, the two oaks compete, and in mixed stands the first species, being more heliophilous than the latter one, often relegates it to the understory or to cleared areas within or at the margin of forests. Theoretically, the presence of *P. cinnamomi* in these forest ecosystems should not directly interfere in this competition, as both oak species are very susceptible. However, it has been observed that in more arid sites with superficial, calcareous soils the clear cut of declining, mature holm oak trees favors regeneration of kermes oak, thus modifying substantially the original vegetation profile. This is a serious concern because some of these relic holm oak stands of the Salento Peninsula, such as the forest of Castro, have been declared Sites of Community Importance for biodiversity conservation (Council Directive 92/43/EEC).

The widespread occurrence of an invasive and destructive pathogen such as *P. cinnamomi* in this part of Apulia has practical implications for the management of forests and natural reserves since eradication is not feasible after the pathogen has already been established in such a vast area.

The only available effective control measure of *P. cinnamomi* dieback is to increase the ecological resilience of ecosystems by mitigating the effects of environmental stressors (e.g., drought, flooding, etc.) that are assumed to be involved in oak decline (Brasier and Scott 1994). The substitution of native oaks with other tolerant oak species conflicts with the conservation guidelines of Council Directive, so this strategy has limited utility. An exclusion strategy aimed at protecting non-infested sites is being applied in Australia to limit the impact of *P. cinnamomi* dieback in eucalyptus forests (Hee et al. 2013). It includes the demarcation of infested areas and the prevention of the spread of inoculum by footwear, tools, vehicles, plants, soil, and water. Accurate monitoring of *P. cinnamomi* inoculum and an information campaign aimed at increasing public awareness on risks associated with the *P. cinnamomi* dispersal are regarded as fundamental for the success of this strategy, which however cannot be practically pursued in stands used as recreational areas such as the forests of Castro and Tiggiano. Similarly, large-scale treatments

with phosphites, which are used in Australia to reduce the impact of *P. cinnamomi* on natural vegetation (Hee et al. 2013), are not feasible in these sites, while phosphite injection into the trunk (Garbelotto et al. 2007) is a possible option to control root rot caused by *P. cinnamomi* on single high-value trees in recreational areas.

Very recently, to face this phytosanitary, environmental emergency, the Regional Government of Apulia has promoted and funded interventions aimed at cutting dead, mature holm oak trees and replanting them with nursery plants of the same species (Figure 2D). Thinning increases ventilation and sunshine, favoring natural regeneration and making the environment less conducive to root infections by *P. cinnamomi*, thus reducing its survival in the soil. However, replanting with container-grown nursery plants increases the risk of introducing new alien, invasive *Phytophthora* species that could exacerbate the impact of *P. cinnamomi*.

Potted nursery plants are the most likely route for these soil-borne pathogens to be accidentally introduced into forest ecosystems (Jung et al. 2016). A recent survey of nursery plants produced in Apulia using new generation molecular diagnostic techniques (Prigigallo et al. 2015) revealed the presence in their rhizosphere of several invasive *Phytophthora* species, including among others *P. cambivora*, *P. cinnamomi*, *P. niederhauserii*, *P. parvispora*, and even the EPPQ quarantine pathogen *P. lateralis*.

As far as the impact of *P. cinnamomi* on natural regeneration is concerned, short-, medium-, and long-term effects should be considered. It was noted that young holm oak seedlings died due to root infections. The highest proportions of dying seedlings were observed at Tiggiano and Scorrano, where mortality reached about 10 and 8%, respectively (data not shown). However, the death of adult trees allowed sunlight to penetrate into the understory, thus favoring regeneration. Very probably, in the long-term the trend will be reversed due to the lack of adult trees producing acorns. Considerable research will be required to limit the impact of these pathogens on forests in southern Italy.

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