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Mario Riolo

Emerging fungal and oomycete pathogens of olive with a wide host range: characterization, monitoring and management

PH.D. THESIS

Tutor
Prof. Gaetano Magnano di San Lio

Co-Tutor
Prof.ssa Santa Olga Cacciola

Supervisor
Prof. Giuseppe Meca de Caro

Supervisor CREA
Dott.ssa Elena Santilli

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Ph.D. Coordinator
Prof. Leonardo Schena

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Abstract

This Ph.D. thesis deals with emerging and re-emerging diseases of olive (*Olea europeaea* L.) caused by fungi (*Colletotrichum* species) and oomycetes (*Phytophthora* species) in southern Italy. It is aimed at clarifying some unexplored aspects of the etiology and epidemiology of these diseases, determine the host range and monitoring the distribution of pathogens responsible for them in several olive-growing areas, on other host plants (most of these pathogens are polyphagous) or different ecosystems that may be inoculum reservoires, including agricultural and less anthropized ecosystems such as nature reserves, and seek for sustainable management strategies based on use of varietal genetic resistance and application of biological control agents (BCAs). Major specific objectives include: i. To test the susceptibility of italian olive cultivars to the infections of diverse *Colletotrichum* species associated with olive anthracnose (OA). ii. To characterize the secondary metabolites produced by diverse *Colletotrichum* species on olive cultivars differing in susceptibility to OA. iii. To characterize and describe a new *Phytophthora* species emerging in nurseries and new commercial plantings of olive in southern and insular Italy. iv. To investigate the distribution and ecology of *Phytophthora oleae* (a recently described species) and other *Phytophthora* species in agricultural and natural ecosystems with particular emphasis on those infecting olive. v. To study the effectiveness of selected lactobacilli sourced from olives as candidate BCAs of fungal plant diseases in alternative to chemical pesticides.

The studies carried out to fulfill these objectives are described more in detail as it follows. Most of the results obtained have been already published or have been submitted to scientific journals. Published scientific articles form an integral part of this thesis.

i. Susceptibility of italian olive cultivars to various *Colletotrichum* species associated with fruit anthracnose.

   In this study, eight among the most popular olive cultivars from central Italy and one cultivar originating from Spain were tested for their susceptibility to five different species of *Colletotrichum*, including *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae* and C. *karsti.* In the first set of experiment four fungal isolates (one isolate per *Colletotrichum* species) and nine olive cultivars were tested by wounding. The results obtained, based on rAUDPC, shown a significant variability among the nine olive cultivars towards *C. acutatum* was detected, with a greater susceptibility of Ottobratica and Coratina cultivars and an intermediate susceptibility of all other cvs, except for Leccino and Frantoio that showed a lower susceptibility to *C. acutatum*. No variability was recorded with *C. karsti* for all olive varieties tested. While a medium-high variability was registered for all nine

olive cultivars inoculated with *C. gloeosporioides* and *C. godetiae*. On the contrary, with these latter species, Leccino and Frantoio cultivars showed to be more resistant. In the second set of experiment the *Colletotrichum* species × olive cultivar interaction was evaluated using different isolates of the *Colletotrichum* species tested, and also including *C. nymphaeae*. Ten isolates (two for each of the five *Colletotrichum* species tested, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. karsti* and *C. nymphaeae*) and four olive cultivars (Coratina, Frantoio, Leccino and Ottobratica) were included. The results of the second set of experiments confirmed the olive cultivars tested differed in susceptibility to *Colletotrichum* species. Coratina and Ottobratica were shown to be susceptible while Frantoio and Leccino were relatively resistant to all the *Colletotrichum* species tested. *C. acutatum* was confirmed to be the most virulent among the *Colletotrichum* species tested, followed by *C. nymphaeae*. Conversely, *C. karsti* was again the least aggressive. No significant difference in virulence was observed between isolates of the same *Colletotrichum* species. The third set of experiments evaluated the effect of both wounding and maturity stage on the response of drupes to inoculation with isolates of diverse *Colletotrichum* species. Both mature and green drupes of Coratina were inoculated singly with four *Colletotrichum* species (two diverse isolates of each species). In the third set of experiments, only the isolates of *C. acutatum* and *C. nymphaeae* induced symptoms on unwounded drupes, and exclusively on mature ones, although in the parallel test on wounded drupes, Coratina was confirmed to be very susceptible to infections by these two *Colletotrichum* species. Conversely, isolates of *C. godetiae* were the least aggressive and *C. gloeosporioides* were more aggressive than isolates of *C. godetiae,* but less aggressive than isolates of both *C. acutatum* and *C. nymphaeae.* In general, symptoms were more severe on mature than on green drupes.

Results of this study were published in Riolo et al., (2022), *Plant Pathology*, 00, 1– 13 https://doi.org/10.1111/ppa.13652

ii. Characterization of the secondary metabolites produced by diverse *Colletotrichum* species on olive cultivars differing in susceptibility to OA

This study was aimed to characterize the secondary metabolites produced by four *Colletotrichum* species, *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. karsti*, both *in vitro*, on potato desxtrose agar (PDA) and oatmeal agar (OA), and during the infection process of fruits of four olive cultivars differing in susceptibility to anthracnose, 'Coratina' and 'Ottobratica', both susceptible, 'Frantoio' and 'Leccino', both resistant. The metabolites were extracted from axenic cultures after seven days incubation and from olives at three

different times, 1, 3 and 7 days post inoculation (dpi). They were identified using the HPLC-QTOF analysis method. In total, 45 diverse metabolites were identified; of these 32 were detected on infected fruits, 24 in axenic cultures and 11 on both fruits and axenic cultures. The 45 identified metabolites comprised compounds of metabolite class, including fatty acid, miscellaneous compounds, phenolics, pyrones, sterols, terpenes. Each *Colletotrichum* species produced a different spectrum of metabolites and the metabolite profile of each species varied depending of the type of matrices. On artificially inoculated olives the severity of symptoms, the amount of fungal secondary metabolites and their number peaked 7 dpi irrespective of the cultivar susceptibility and the virulence of the *Colletotrichum* species. The metabolite profiles as represented by heat maps were the result of the interaction olive cultivar x *Colletotrichum* species.

Results of this study have been submitted as an article to the Fungal Biology Journal (Elsevier).

iii. Identification and characterization *Phytophthora* species in olive orchards in southern Italy.

This study comprises two different lines of research:

- First report of root rot caused by *Phytophthora bilorbang* on *Olea europaea* in Italy. The main aim of the present study was to identify and characterize the causative agent of root and crown rot in olive tree of the cv. Nera di Gonnos in Calabria. Based on morphological characters and sequencing of Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) the oomycete was identified as *Phytophthora bilorbang.* Koch's postulates were fulfilled by reproducing the symptoms on potted *Olea europaea* var. Nera di Gonnos saplings transplanted into infested soil. At the end of the experiment *P. bilorbang* was re-isolated from roots of symptomatic trees. This is the first report of *P. bilorbang* on *O. europaea*. Results of this study were published in Santilli et al., (2020), *Plants*, 2020, 9, 826. https://doi.org/10.3390/plants9070826.

- *Phytophthora heterospora* sp. nov., a new conidia-producing sister species of *P. palmivora.*

  In this study, morphology, breeding system and growth characteristics of an unusual *Phytophthora* associated with stem lesions, root and collar rot on young olive trees in Southern Italy were examined, and multi loci phylogenetic analyses were performed. Morphologically the *Phytophthora* isolates, identified as

Phytophthora heterospora, were characterized by the abundant production of caducous, non-papillate conidia-like sporangia compared to resembling *P. palmivora.* Phylogenetically, these isolates grouped in a distinct well-supported clade sister to *P. palmivora*, thus they constitute a separate species. The new species, described here as *Phytophthora heterospora* sp. nov., proved to be highly pathogenic to both olive and durian plants in artificial stem inoculations. Results of this study were published in Scanu et al., (2021), *Journal of Fungi*, 2021, 7(10), 870; https://doi.org/10.3390/jof7100870.

iv. Diversity of *Phytophthora* communities across different types of Mediterranean vegetation in a nature reserve area.

The Objective of this study was to investigate and correlate the diversity and distribution of *Phytophthora* species with the vegetation in aquatic, riparian and terrestrial habitats within a protected area in Eastern Sicily, Southern Italy. Water and soil samples were collected from two streams running through the reserve and six different types of vegetation, including *Platano-Salicetum pedicellatae*, the *Sarcopoterium spinosum* community, *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis*, *Oleo-Quercetum virgilianae* and a gallery forest dominated by *Nerium oleander*. *Phytophthora* species isolations were performed using leaf baiting technique and the species obtained were classified on the basis of morphological and molecular characteristics. Overall, 11 *Phytophthora* species, were identified, including *P. asparagi*, *P. bilorbang*, *P. cryptogea*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. nicotianae*, *P. oleae*, *P. parvispora*, *P. plurivora* and *P. syringae*. No *Phytophthora* species were found in the S*arcopoterium spinosum* comm. *Phytophthora asparagi*, *P. lacustris* and *P. plurivora* were the prevalent species in the plant communities, but only *P. plurivora* was present in all of them. Overall aquatic species from clade 6 were the most common; they were recovered from all five types of vegetation, streams and riparian habitats. *Phytophthora* populations found in the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* show the highest diversity, while no correlation was found with the physicochemical characteristics of the soil. The vegetation type and the aquatic or terrestrial habitat were identified as major environmental factors correlated with the diversity of *Phytophthora* communities in this reserve. Results of this study were published in Riolo et al., (2020), *Forests*, 11(8), 1–21. https://doi.org/10.3390/F11080853.

v. Antifungal activity of selected lactic acid bacteria from olive drupes.

In this study, different Lactobacilli (LABs) were isolated from the drupes of olive (*Olea europaea*) oil varieties. LABs were identified as *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecium* and *Streptococcus salivarius* by MALDI-TOF and sequencing of the 16S rRNA. To evaluate *in vitro* the antifungal activity of LABs and their cell-free fermentates (CFSs) against several plant pathogenic, including *Alternaria*, *Aspergillus Colletotrichum*, *Penicillium*, *Plenodomus* and *Phytophthora*, the culture overlaying and the agar diffusion tests were used. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined. The results obtained revealed that LABs showed antifungal activity against the fungi sensu lato tested. *Lactobacillus plantarum* and *P. pentosaceus* against *Fusarium oxysporum*, *Colletotrichum* species and *Penicillium nordicum* shown the most noticeable inhibitory activity. Chemical analysis revealed CFSs contained acid lactic and variable quantities of 14 diverse phenolic acids and 26 volatile organic compounds (VOCs). No obvious correlation was found between the metabolic profile of LABs and their antifungal efficacy. However, it is the first time that the potential of fermentates of LABs, recovered from drupes of olive oil varieties, as natural fungicides, was demonstrated.

Results of this study have been submitted as an article to the LWT Journal (Elsevier).

Riassunto

Questa tesi di dottorato ha riguardato malattie emergenti e riemergenti dell'olivo (*Olea europeaea* L.) causate da funghi (specie *Colletotrichum*) e oomiceti (specie *Phytophthora*) nell'Italia meridionale. L'obiettivo è quello di chiarire alcuni aspetti inesplorati dell'eziologia e dell'epidemiologia di queste malattie, determinare l'areale d'ospite e monitorare la distribuzione dei patogeni responsabili in diverse aree olivicole, su altre piante ospiti (la maggior parte di questi patogeni sono polifagi) o su diversi ecosistemi che possono essere serbatoi di inoculo, compresi gli ecosistemi agricoli e quelli meno antropizzati come le riserve naturali, e cercare strategie di gestione sostenibile basate sull'uso della resistenza genetica varietale e sull'applicazione di agenti di controllo biologico (BCA). I principali obiettivi specifici includono: i. Testare la suscettibilità delle cultivar di olivo italiane alle infezioni di diverse specie di *Colletotrichum* associate all'antracnosi dell'olivo (OA). ii. Caratterizzare i metaboliti secondari prodotti da diverse specie di *Colletotrichum* su cultivar di olivo con diversa suscettibilità all'OA. iii. Caratterizzare e descrivere una nuova specie di *Phytophthora* emergente nei vivai e nei nuovi impianti commerciali di olivo nell'Italia meridionale e insulare. iv. Indagare la distribuzione e l'ecologia di *Phytophthora oleae* (una specie recentemente descritta) e di altre specie di *Phytophthora* in ecosistemi agricoli e

naturali, con particolare attenzione a quelle che infettano l'olivo. v. Studiare l'efficacia di alcuni BCA candidati e di composti ecologici come alternativa ai pesticidi chimici.

Gli studi condotti per raggiungere questi obiettivi sono descritti più dettagliatamente di seguito. La maggior parte dei risultati ottenuti è già stata pubblicata o è stata presentata a riviste scientifiche. Gli articoli scientifici pubblicati costituiscono parte integrante di questa tesi.

i. Studio della suscettibilità di varietà olivicole italiane a diverse specie di *Colletotrichum* associate all' antracnosi dell'olivo. In questo studio, otto tra le più popolari cultivar di olivo dell'Italia centrale e una cultivar originaria della Spagna sono state testate per la loro suscettibilità a cinque diverse specie di *Colletotrichum*, tra cui *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae* e *C. karsti*. Nella prima serie di esperimenti sono stati testati per ferita quattro isolati fungini (un isolato per ogni specie di *Colletotrichum*) e nove cultivar di olivo. I risultati ottenuti, basati sulla rAUDPC, hanno evidenziato una significativa variabilità tra le nove cultivar di olivo nei confronti di *C. acutatum*, con una maggiore suscettibilità delle cultivar Ottobratica e Coratina e una suscettibilità intermedia di tutte le altre, ad eccezione di Leccino e Frantoio che hanno mostrato una minore suscettibilità a *C. acutatum*. Non è stata registrata alcuna variabilità con *C. karsti* per tutte le varietà di olivo testate. Mentre una variabilità medio-alta è stata registrata per tutte le nove cultivar di olivo inoculate con *C. gloeosporioides* e *C. godetiae*. Al contrario, con queste ultime specie, le cultivar Leccino e Frantoio si sono dimostrate più resistenti. Nella seconda serie di esperimenti l'interazione *Colletotrichum* specie × cultivar di olivo è stata valutata utilizzando diversi isolati delle specie di *Colletotrichum* testate e includendo anche *C. nymphaeae*. Dieci isolati (due per ciascuna delle cinque specie di *Colletotrichum* testate, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. karsti* e *C. nymphaeae*) e quattro cultivar di olivo (Coratina, Frantoio, Leccino e Ottobratica) sono stati inclusi. I risultati della seconda serie di esperimenti hanno confermato che le cultivar di olivo testate differiscono nella suscettibilità alle diverse specie di *Colletotrichum*. Coratina e Ottobratica si sono dimostrate suscettibili, mentre Frantoio e Leccino sono risultate relativamente resistenti a tutte le specie di *Colletotrichum* testate. *C. acutatum* si è confermato il più virulento tra le specie di *Colletotrichum* analizzate, seguito da *C. nymphaeae*. Al contrario, *C. karsti* è risultato ancora una volta il meno aggressivo. Non sono state osservate differenze significative nella virulenza tra isolati della stessa specie di *Colletotrichum*. La terza serie di esperimenti ha valutato l'effetto della ferita e dello stadio di maturazione sulla risposta delle drupe all'inoculazione con isolati di diverse specie di *Colletotrichum*. Sia le drupe mature che quelle verdi di Coratina sono state

inoculate singolarmente con quattro specie di *Colletotrichum* (due diversi isolati di ciascuna specie). Nella terza serie di esperimenti, solo gli isolati di *C. acutatum* e *C. nymphaeae* hanno indotto sintomi su drupe non ferite, ed esclusivamente su quelle mature, anche se nel test parallelo su drupe ferite, la Coratina si è confermata molto sensibile alle infezioni di queste due specie di *Colletotrichum*. Al contrario, gli isolati di *C. godetiae* sono stati i meno aggressivi e *C. gloeosporioides* è stato più aggressivo degli isolati di *C. godetiae*, ma meno aggressivo degli isolati di *C. acutatum* e *C. nymphaeae*. In generale, i sintomi erano più gravi sulle drupe mature che su quelle verdi.
I risultati di questo studio sono stati illustrati in Riolo et al., (2022), Plant Pathology 2022, 00, 1- 13 https://doi.org/10.1111/ppa.13652

ii. Caratterizzazione dei metaboliti secondari prodotti da diverse specie di *Colletotrichum* su cultivar di olivo che differiscono per la suscettibilità all'OA.

Questo studio ha avuto come obiettivo la caratterizzazione dei metaboliti secondari prodotti da quattro specie di *Colletotrichum*, *C. acutatum*, *C. gloeosporioides*, *C. godetiae* e *C. karsti*, sia in vitro, su potato desxtrose agar (PDA) e oatmeal agar (OA), sia durante il processo di infezione dei frutti di quattro cultivar di olivo diverse per suscettibilità all'antracnosi, 'Coratina' e 'Ottobratica', entrambe suscettibili, 'Frantoio' e 'Leccino', entrambe resistenti. I metaboliti sono stati estratti da colture axeniche dopo sette giorni di incubazione e da olive in tre momenti diversi, 1, 3 e 7 giorni dopo l'inoculazione (dpi). Sono stati identificati con il metodo di analisi HPLC-QTOF. In totale, sono stati identificati 45 metaboliti diversi; di questi, 32 sono stati rilevati sui frutti infetti, 24 nelle colture axeniche e 11 sia sui frutti che sulle colture axeniche. I 45 metaboliti identificati comprendevano composti della classe dei metaboliti, tra cui acidi grassi, composti vari, fenoli, pironi, steroli e terpeni. Ogni specie di *Colletotrichum* ha prodotto un diverso spettro di metaboliti e il profilo metabolico di ciascuna specie variava a seconda del tipo di matrice. Su olive inoculate artificialmente, la gravità dei sintomi, la quantità di metaboliti secondari fungini e il loro numero hanno raggiunto il picco a 7 dpi, indipendentemente dalla suscettibilità della cultivar e dalla virulenza della specie di *Colletotrichum*. I profili dei metaboliti, rappresentati dalle mappe di calore, erano il risultato dell'interazione cultivar di olivo x specie di *Colletotrichum*.

I risultati di questo studio sono stati presentati come articolo alla rivista Fungal biology (Elsevier).

iii. Identificazione e caratterizzazione delle specie di *Phytophthora* negli oliveti dell'Italia meridionale. Questo studio è stato sviluppato da due diverse linee di ricerca:

- Prima segnalazione di marciume radicale causato da *Phytophthora bilorbang* su *Olea europaea* in Italia.

  Lo scopo principale del presente studio è stato quello di identificare e caratterizzare l'agente causale del marciume radicale e della chioma in un olivo della cv. Nera di Gonnos in Calabria. Sulla base dei caratteri morfologici e del sequenziamento delle regioni ITS (Internal Transcribed Spacer) del DNA ribosomiale (rDNA), l'oomicete è stato identificato come *Phytophthora bilorbang*. I postulati di Koch sono stati soddisfatti riproducendo i sintomi su alberelli di Olea europaea var. Nera di Gonnos trapiantati in vaso nel terreno infestato. Alla fine dell'esperimento *P. bilorbang* è stato nuovamente isolato dalle radici di piante sintomatiche. Si tratta della prima segnalazione di *P. bilorbang* su *O. europaea*. I risultati di questo studio sono stati illustrati in Santilli et al., (2020), Plants, 2020, 9, 826. https://doi.org/10.3390/plants9070826.

- *Phytophthora heterospora* sp. nov., una nuova specie affine a *P. palmivora* che produce conidi.

  In questo studio sono stati esaminati la morfologia, il sistema di riproduzione e le caratteristiche di crescita di un'insolita *Phytophthora* associata a lesioni del fusto, marciume radicale e del colletto su giovani olivi dell'Italia meridionale e sono state eseguite analisi filogenetiche multi-loci. Morfologicamente gli isolati di *Phytophthora*, identificati come *Phytophthora heterospora*, si sono caratterizzati per l'abbondante produzione di sporangi caduchi, simili a conidi non papillati, rispetto a quelli simili a *P. palmivora*. Dal punto di vista filogenetico, questi isolati si sono raggruppati in un clade distinto e ben supportato, gemello di *P. palmivora*, costituendo così una specie separata. La nuova specie, qui descritta come *Phytophthora heterospora* sp. nov., si è dimostrata altamente patogena per le piante di olivo e di durian in inoculi artificiali di fusto.

  I risultati di questo studio sono stati illustrati in Scanu et al., (2021), Journal of Fungi, 2021, 7(10), 870; https://doi.org/10.3390/jof7100870.

iv. Diversità delle comunità di *Phytophthora* in sei comunità vegetazionali mediterranee presenti in una riserva naturale protetta del sud Italia.
L'obiettivo di questo studio è stato quello di indagare e correlare la diversità e la distribuzione delle specie di *Phytophthora* con la vegetazione in habitat acquatici, ripariali

e terrestri all'interno di un'area protetta della Sicilia orientale, nell'Italia meridionale. Sono stati raccolti campioni di acqua e suolo da due corsi d'acqua che attraversano la riserva e da sei diversi tipi di vegetazione, tra cui *Platano-Salicetum pedicellatae*, la comunità *Sarcopoterium spinosum*, *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis*, *Oleo-Quercetum virgilianae* e una foresta a galleria dominata da *Nerium oleander*. Gli isolamenti delle specie di *Phytophthora* sono stati eseguiti con la tecnica dell'esca fogliare e le specie ottenute sono state classificate sulla base delle caratteristiche morfologiche e molecolari. Complessivamente sono state identificate 11 specie di *Phytophthora*, tra cui *P. asparagi, P. bilorbang, P. cryptogea, P. gonapodyides, P. lacustris, P. multivora, P. nicotianae, P. oleae, P. parvispora, P. plurivora* e *P. syringae*. Non sono state trovate specie di *Phytophthora* nella comunità a *Sarcopoterium spinosum Phytophthora asparagi*, *P. lacustris* e *P. plurivora* erano le specie prevalenti nelle comunità vegetali, ma solo P. plurivora era presente in tutte. Nel complesso, le specie acquatiche del clade 6 erano le più comuni; sono state recuperate da tutti e cinque i tipi di vegetazione, dai corsi d'acqua e dagli habitat ripariali. Le popolazioni di *Phytophthora* trovate nel *Platano-Salicetum pedicellatae* e nell'*Oleo-Quercetum virgilianae* mostrano la più alta diversità, mentre non è stata trovata alcuna correlazione con le caratteristiche fisico-chimiche del suolo. Il tipo di vegetazione e l'habitat acquatico o terrestre sono stati identificati come i principali fattori ambientali correlati alla diversità delle comunità di *Phytophthora* in questa riserva. I risultati di questo studio sono stati illustrati in Riolo et al., (2020), Forests, 11(8), 1-21. ttps://doi.org/10.3390/F11080853.

v. Attività antifungina di batteri lattici selezionati da drupe di olivo.
In questo studio sono stati isolati diversi lattobacilli (LAB) dalle drupe delle varietà di olio di oliva (*Olea europaea*). I LAB sono stati identificati come *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecium* e *Streptococcus salivarius* mediante MALDI-TOF e sequenziamento del 16S rRNA. Per valutare in vitro l'attività antifungina dei LAB e dei loro fermentati senza cellule (CFS) nei confronti di diversi patogeni vegetali, tra cui *Alternaria*, *Aspergillus, Colletotrichum*, *Penicillium*, *Plenodomus* e *Phytophthora*, sono stati utilizzati i test di sovrapposizione delle colture e di diffusione su agar. Sono state determinate la concentrazione minima inibitoria (MIC) e la concentrazione minima fungicida (MFC). I risultati ottenuti hanno rivelato che i LAB hanno mostrato attività antifungina contro i funghi sensu lato testati. *L. plantarum* e *P. pentosaceus* hanno mostrato l'attività inibitoria più evidente contro *Fusarium oxysporum*, *Colletotrichum* species e *Penicillium nordicum*. L'analisi chimica ha rivelato che i CFS contenevano acido lattico e quantità variabili di 14 diversi acidi fenolici e

26 composti organici volatili (VOC). Non è stata trovata alcuna correlazione evidente tra il profilo metabolico dei LAB e la loro efficacia antifungina. Tuttavia, è la prima volta che viene dimostrato il potenziale dei fermentati di LAB, recuperati da drupe di varietà di olio d'oliva, come fungicidi naturali.

I risultati di questo studio sono stati presentati come articolo alla rivista LWT (Elsevier).

1. General introduction

1.1. Olive anthracnose

Olive anthracnose caused by *Colletotrichum* species is the most damaging fungal disease of olive fruits worldwide. It occurs in humid olive-growing areas of several countries (in the Mediterranean macroregion, Asia, Australia, New Zealand, Northern and Southern America and South Africa) and causes severe yield losses and detrimental effects on oil quality.

It was first reported from Portugal by J.V. d'Almeida [1] and named gaffa. In Italy, it is known as lebbra (leprosy) and in Spain as aceituna jabonosa (soapy fruit) or momificado (mummification) [2,3]. The most typical symptoms of olive anthracnose (OA) are fruit rot and mummification. In moist conditions, infected fruits show a soft to dark brown rot with an abundant production of an orange gelatinous matrix embedding conidia emerging from acervuli, while, in dry conditions, the fruits mummify and lose weight due to dehydration. Affected fruits fall prematurely to the ground and only a few mummies remain attached to the tree. Fruit drop may also be the consequence of infections in the peduncle [4]. Mature drupes are mostly affected, but in favorable environmental conditions green fruits of susceptible cultivars may also be severely affected. In cultivars with oblong drupes, rot often starts from the apical end but, in cultivars with large, sub-spherical drupes, the first symptoms appear as circular sunken lesions and acervuli form in concentric rings starting from the centre of the lesion (as reviewed by Cacciola et al. [5]). Trees affected by anthracnose show also chlorosis and necrosis of the leaves, defoliation, and dieback of twigs and branches [2]. OA is also a post-harvest disease with decay developing from quiescent infections that took place in the field.

OA infections affect both fruit yield and commercial quality of the oil. In fact, oil produced from infect-ed olives has off-flavor, a reddish color and shows chemical alterations such as high acidity, a considerablereduction of β-sitosterol, polyphenols and α-tocopherol [6,7].

The fungus causing OA was originally described as a distinct species, *Gloeosporium olivarum*, by de Almeida [1] in Portugal. Subsequently, *G. olivarum* was found to be indistinguishable from *G. fructigenum* Berk., the agent of bitter rot of apples. As a consequence of the revision of the genus *Gloeosporium* (vonArx, 1957), both species were transferred to *Colletotrichum gloeosporioides* (Penz.) Penz. et Sacc., the anamorph of *Glomerella cingulata* (Stonem.) Spauldinget v. Schrenk. Subsequently, two anamorphic species of *Colletotrichum*, *C. gloeosporioides* (Penz.) Penz. et Sacc. and *C. acutatum* J.H. Simmonds ex J. H. Simmonds, were reported to be associated with OA in Spain by Martín and García-Figueres [8] and Moral et al. [3], according to whom *C. acutatum* prevails in areas where the disease occurs epidemically.

Colletotrichum acutatum sensu lato (s.l.) was first introduced by Johnston and Jones [9] to accommodate isolates that clustered with *C. acutatum sensu* Simmonds and others that showed a wide range of morphological and genetic diversity. Like *C. gloeosporioides*, *C. acutatum s. l.* is a species complex showing high phenotypic and genotypic diversity. After the introduction of multiloci phylogenetic analysis as a taxonomic criterion numeorus species have been segregated from the *Colletotrichum* species complexes and now it is recognized that around 18 different *Colletotrichum* species are associated with OA worldwide [10]. The progress of research on OA in the last 30 years and state of the art on this disease has been updated and summarized in four successive exhaustive reviews [2,5,10].

The specific objectives of this thesis are listed below.

i. To test the susceptibility of Italian olive cultivars to the infections of diverse *Colletotrichum* species associated with OA.

ii. To characterize the secondary metabolites produced by diverse *Colletotrichum* species on olive cultivars differing in susceptibility to OA.

1.2. Root and crown rot of olive caused by *Phytophthora* spp.

As a typical Mediterrranean plant olive has been traditionally cultivated in arid lands. However, during the last decades in many olives growing countries, including Italy, the olive cultivation has been extended to different types of soil and irrigation has become a common practice in olive orchards. An emerging phytopathological problem of olive trees growing in wet or waterlogged soils is root rot caused by *Phytophthora* spp. The genus *Phytophthora* (Pythiaceae, Peronosporales, Oomycota, Chromista) comprises more than 200 described taxa [11–13]. The discovery of new taxa is constantly evolving, due both to the discovery of new molecular methods, combined with classical molecular techniques, and to sampling in forest and natural ecosystems in regions with limited accessibility [12,14–16]. Several *Phytophthora* species of different phylogenetic clades have been reported as causative agents of leaf chlorosis, wilting, defoliation, and twig dieback, associated with root rot and basal stem cankers on olive worldwide, including *P. acerina*, *P. cactorum*, *P. cinnamomi*, *P citricola sensu lato, P. cryptogea, P. drechsleri, P. inundata, P. megasperma, P. nicotianae, P. oleae, P. palmivora, P. pini and P. plurivora*. [17–29]. They differ in aggressiveness, temperature-growth requirements, geographical distribution and ecology. *P. palmivora*, alone or in association with *Verticillium dahliae*, was reported as causal agent of rot of fine roots and wilt of young olive trees in nurseries and new plantings in Italy [18,30]. *Phytophthora oleae* was recovered from soil and roots of wild olive trees in protected natural areas in Spain and Sicily (southern Italy) and is widespread in soil of olive orchards in Calabria (southern Italy)

[19,21,31,32]. *Phytophthora inundata* is responsible for root rot and wilt of olive trees in clay soils after flooding acting as an opportunistic albeit aggressive root pathogen [25,29]. In moist environments some soil-inhabitant species, like *P. nicotianae* and *P. oleae* have occasionally adapted to an aerial life style and may infect aboveground parts of olive trees such drupes, leaves and twigs causing fruit rot, leaf drying, filloptosis and twig dieback [33–35].

The specific objectives of this thesis are listed below.

iii. Identification and characterization *Phytophthora* species in olive orchards in southern Italy;

iv. Investigation of the distribution, epidemiology and ecology of *Phytophthora oleae* (a recently described species) and other *Phytophthora* species in agricultural and natural ecosystems with particular emphasis on those infecting olive.

2. Susceptibility of italian olive cultivars to various *Colletotrichum* species associated with fruit anthracnose

Mario Riolo[1,2,3], Antonella Pane[2], Elena Santilli[1†], Salvatore Moricca[4†], Santa Olga Cacciola[2†*]

[1] Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus crops (CREA- OFA), 87036 Rende, Cosenza, Italy;

[2] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[3] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[4] Department of Agricultural, Food, Environmental and Forestry Science and Technology, University of Florence, 50144 Firenze, Italy;

*Corresponding author: Santa Olga Cacciola, E-mail olga.cacciola@unict.it

† Co-senior authors: Santa O. Cacciola, Elena Santilli and Salvatore Moricca

2.1. Abstract

Fruit anthracnose caused by *Colletotrichum* species is a major disease of olive (Olea europaea) worldwide. In this study, we tested in vitro the susceptibility of eight widely grown Italian olive cultivars ('Carolea', 'Cassanese', 'Coratina', 'Dolce Agogia', 'Frantoio', 'Leccino', Ottobratica' and 'Sant'Agostino') and one Spanish cultivar ('Picual'), included as a reference, to five *Colletotrichum* species (*C. acutatum*, *C. gloeosporioides*, *C. godetiae C. karsti* and *C. nymphaeae*). Olive drupes at comparable ripening stage were wound-inoculated with a conidial suspension and the severity of infections was rated at various time intervals after inoculation using a scale of 0 to 6. Results were expressed in terms of relative Area Under Disease Progress Curve (rAUDPC). *Colletotrichum acutatum* was the most aggressive while *C. karsti* was the least aggressive among the *Colletotrichum* species tested. 'Frantoio' and 'Leccino' were the least susceptible cultivars while 'Ottobratica', 'Coratina' and 'Carolea' were the most susceptible to all *Colletotrichum* species. In separate experiments aimed at evaluating the effect of both inoculation method and drupe ripening stage on the interaction between *Colletotrichum* species and olive cultivars, only *C. acutatum* and *C. nymphaeae* induced symptoms in non-wounded drupes, while in general, the disease severity in green drupes was significantly lower than in mature drupes. However, the rankings of olive cultivars for their susceptibility to *Colletotrichum* species on both green and mature drupes showed similar trends.

Keywords: *Olea europaea*, *Colletotrichum acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. karsti*, *C. nymphaeae*, AUDPC, Phylogenetic analysis, ITS and TUB2

2.2. Introduction

Olive anthracnose (OA), caused by *Colletotrichum* species, is the most damaging disease of olive fruit worldwide and together with Mediterranean olive fruit fly (*Bactrocera oleae*) and olive leaf spot (*Venturia oleaginea*) is a major phytopathological concern of olive crops in the Mediterranean region [1–6]. It causes a substantial deterioration of oil quality, the severity of which depends on the proportion of infected fruits, the *Colletotrichum* species inciting the disease and the olive cultivar [3,7,8]. In some areas of southern Italy, where environmental conditions, such as warm, rainy, and humid climate during fruit ripening, are disease conducive, OA is endemic, with severe epidemic outbreaks occurring frequently. Beside environmental conditions, the susceptibility of olive cultivars, the ripening stage of drupes and the differences in virulence of *Colletotrichum* species responsible for the disease are major driving factors triggering the onset and conditioning the severity of epidemics. A direct correlation was observed between the severity of OA outbreaks and attacks of the Mediterranean olive fly or the Queensland fruit fly (*B. tryoni*) as wounds caused by these insect pests on olive drupes facilitate pathogen entry [9–11]. So far, around 18 *Colletotrichum* species, most of the acutatum, gloeosporioides and boninense species complexes, were reported to be associated with OA worldwide, but not all species are equally aggressive or are responsible for disease outbreaks [1,6,11–17]. The *Colletotrichum* species associated most frequently with olive anthracnose outbreaks in the Mediterranean macroregion are *C. acutatum*, *C. godetiae* (syn. *C. clavatum*) and *C. nymphaeae* all in the acutatum species complex. Species in the gloeosporioides complex, such as *C. gloeosporioides* sensu stricto (hereinafter referred to as *C. gloeosporioides*), and those in the boninense complex, such as *C. karsti*, occur sporadically and in a lower proportion. Although the role of *C. gloeosporioides* in OA outbreaks is controversial some isolates of this species were virulent in tests on detached olive drupes [17]. By contrast, in these tests isolates of *C. karsti* were only weakly pathogenic [17]. Yet, both species are common pathogens of citrus in the Mediterranean microregion [18,19]. The distribution and prevalence of *Colletotrichum* species may vary in different olive-growing countries or areas within the same country and even on different organs of the tree [1,2,8,11,16,17,20–22]. Seasonal fluctuations of *Colletotrichum* populations and relative presence of different species have also been observed [23]. Until recently, *C. godetiae*, originally described as *C. clavatum*, was the prevalent species associated to OA outbreaks in Calabria (southern Italy) [14,15] . However, in the last decade *C. acutatum* has prevailed over *C. godetiae* and presently is the most frequent species associated to the disease in this region [23,24]. Interestingly, *C. acutatum* has been recently reported for the first time as causal agent of olive anthracnose in Albania and Greece [25,26], the same countries from which very probably *C. godetiae*, the species responsible for the first epidemic

outbreaks of OA in the 1950s, was inadvertently introduced into southern regions of Italy [1]. Moreover, *C. acutatum*, which as a species is supposed to be native to the southern hemisphere, is already widespread in some olive growing regions of Portugal and Tunisia [13,20]. A similar shift in *Colletotrichum* population associated with OA is probably occurring in olive orchards in Portugal. *Colletotrichum godetiae*, which presently is the prevalent species in northeastern part of the country, is being replaced by the more aggressive *C. nymphaeae*, which has been already dominant in olive orchards of the southwestern part of the country for many years [5,6,11,21]. Also, in several olive-growing countries where OA is present, differences in susceptibility among olive cultivars were observed and genetic resistance to the disease was considered as part of an integrated strategy to manage OA [1,3,11,27–29]. Differences in in-field susceptibility were reported among different olive cultivars in Italy, Spain and Portugal [1,11,29]. Talhinhas et al. [6] evaluated the susceptibility of a set of eight olive cultivars commonly grown in Portugal to the infections of diverse *Colletotrichum* species in the acutatum and gloeosporioides species complexes, including *C. acutatum*, *C. godetiae*, *C. nymphaeae*, *C. fioriniae*, *C. rhombiforme* and *C. gloeosporioides*. They found a high variability in virulence among *Colletotrichum* species as well as a significant cultivar x *Colletotrichum* isolate interaction. Moral et al. [29] screened a large collection of olive cultivars of worldwide origin for the susceptibility to natural OA infections in a major olive-growing area in Spain where the largely prevalent *Colletotrichum* species was recently demonstrated to be *C. godetiae* [15]. These authors selected a representative cultivar for each susceptibility class (highly susceptible, susceptible, moderately susceptible, resistant and highly resistant) and indicated 'Picual' as representative of the group of resistant cultivars and 'Frantoio' as representative of the group of highly resistant cultivars. Inoculation of detached olive drupes under controlled environmental conditions has been used by many researchers as a rapid test to evaluate the pathogenicity of *Colletotrichum* isolates and the susceptibility of olive cultivars to anthracnose [6,17,27,30]. With a few exceptions, results of artificial inoculations on detached drupes were consistent with the rating of olive cultivars for the susceptibility to OA infections as inferred from field trials [30]. As in the case of OA natural infections, the susceptibility of olive drupes artificially inoculated with *Colletotrichum* increases with maturity. No standard protocol has been universally accepted for the in vitro artificial inoculations of olive drupes to test their susceptibility to *Colletotrichum* infections. Moral et al. [27] evaluated the effect of wounding on the susceptibility of detached drupes to artificial inoculation with *Colletotrichum* and concluded that although wounding was not necessary for fruit infection it enhanced the severity of symptom expression. This is consistent with the observation that in field susceptibility to OA increases when fruit are wounded by olive fly [9–11,31]. According to Moral et al. [27], in

assays on detached olive drupes, differences in resistance between olive cultivars were more easily detected when high concentration of inoculum (10^5 to 10^6 conidia ml^{-1}) and less mature (green) drupes were used. Talhinhas et al. [6] inoculated unwounded mature olive drupes with a drop of conidium suspension (10^6 condia ml^{-1}) to test the virulence of isolates of diverse species of *Colletotrichum* on a set of olive cultivars commonly grown in Portugal. Conversely, Schena et al. [17] used wounded mature drupes and a mycelium plug as inoculum to evaluate the differences in pathogenicity of *Colletotrichum* species of the gloeosporioides and boninese complexes.

In this study, eight of the most popular Italian olive cultivars and the cultivar Picual originating from Spain were tested for their susceptibility to five different species of *Colletotrichum*, (*C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. karsti* and *C. nymphaeae*) with the aim of investigating the interaction *Colletotrichum* species and olive cultivar,'Picual' was included as reference due to its resistance to OA in field [6,7,29,30]. In addition, the effect of the inoculation method (by wounding and without wounding) and the ripening stage (green and mature) of olive fruits on the *Colletotrichum* species x olive cultivar interaction was evaluated.

2.3 Materials and methods

2.3.1. Fungal isolates and production of inoculum

This study included 10 fungal isolates of five different *Colletotrichum* species, *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. karsti* and *C. nymphaeae*. All isolates, except C9D2C, RD9B and C12D1A, were identified in previous studies based on morphological characteristics and phylogenetic sequence analysis of the internal transcribed spacer regions of rDNA (ITS), intervening 5.8S nrDNA, as well as part of the β-tubulin gene (TUB2) regions as barcode markers [32,33]. The following isolates were used in pathogenicity tests: isolate C9D2C and UWS149 of *C. acutatum s.s.*; isolates RD9B and AC24 of *C. gloeosporioides*; isolates OLP12 and OLP16 of *C. godetiae*; isolates RB012 and RB428 of *C. nymphaeae*; isolates CAM, C12D1A and ALL2I of *C. karsti* (Table 1). The isolates C12D1A and ALL21 of *C. karsti* were selected in the preliminary screening on olive drupes, they were the most virulent in a set of 10 *C. karsti* isolates, including five isolates from olive collected in southern Italy. All isolates included in this study were sourced from the culture collection of the Molecular Plant Pathology Laboratory of the Department of Agriculture, Food and Environment of the University of Catania, Italy.

A conidial suspension of each isolate at a concentration of 10^6 conidia ml^{-1} in sterile distilled water (SDW) was prepared from 10-day-old cultures grown on Potato Dextrose Agar (PDA; Oxoid Ltd., Basingstoke, UK) at 25 °C and used as inoculum. To produce the inoculum single-conidium

isolates were cultured in Petri dishes containing PDA. Dishes were incubated at 23 ±2 °C with a 12-h photoperiod of fluorescent light (40 μmol m^{-2} s^{-1}). After one week incubation, dishes were flooded with sterile distilled water and mycelium was scraped with a spatula. The suspension was filtered through four layers of sterile gauze and the concentration of inoculum was adjusted with a hemocytometer.

Table 1. GenBank and source of isolates of *Colletotrichum* used in this study.

Isolate code	Species	Host (species and cultivar)	Organ	Geographical origin	GenBank Accession	
					ITS-rDNA	ß-tubulin 2
UWS149	*C. acutatum*	*Olea europaea*	Fruit	Australia	MT997785	MW001517
C9D2C	*C. acutatum*	*O. europaea*	Fruit	Calabria	MZ502315	MZ508448
AC24	*C. gloeosporioides*	*Citrus sinensis*	Leaf	Sicily	MT997808	MW001540
RD9B	*C. gloeosporioides*	*O. europaea*	Fruit	Calabria	MZ502314	MZ508447
OLP 12	*C. godetiae*	*O. europaea*	Fruit	Calabria	JN121131	JN121218
OLP 16	*C. godetiae*	*O. europaea*	Fruit	Calabria	JN121137	JN121224
ALL2I	*C. karsti*	*C. sinensis*	Twig	Sicily	MT997861	MW001546
CAM	*C. karsti*	*Camellia* sp.	Leaf	Sicily	KC425664	KC425716
C12D1A	*C. karsti*	*O. europaea*	Fruit	Sicily	ON231821	ON246203
RB012	*C. nymphaeae*	*O. europaea*	Fruit	Spain	JQ948201	JQ949852
RB428	*C. nymphaeae*	*Walnut* sp.	Bud	France	MG58986	MG666308

2.3.2. Molecular characterization of fungal isolates

The RD9B, C9D2C and C12D1A isolates, which had not been previously identified, were grown on PDA for 7 days at 25 ± 1° C. Mycelium of each isolate was harvested with a sterile scalpel, and the genomic DNA was extracted using a PowerPlant® Pro DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. The DNA was preserved at -20 C°. The ITS1–5.8S–ITS2 region and the fragment of the β-tubulin 2 gene (TUB2) between exons 2 and 6 were amplified with primers ITS5 and ITS4 and primers βt2a- βt2b [34,35], respectively, and sequenced to confirm the isolate species. PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Monza-Brianza, Italy). All PCR reactions were carried out by using Taq DNA polymerase recombinant (Invitrogen™) in a total volume of 25 μL containing PCR Buffer (1 X), dNTP mix (0.2 mM), MgCl2 (1.5 mM), forward and reverse primers 0.5 μM each), Taq DNA Polymerase (1 U) and 1 μL of genomic DNA. Reaction conditions were 94°C for 3 min followed by 35 cycles of 94° C for 30 s, 58°C (ITS region)/ 60°C (TUB2) for 30 s, and 72°C for 30 s, followed by an additional 10 min extension at 72°C.

Amplified products were analyzed by agarose gel electrophoresis and single bands of the expected size were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with both forward and reverse primers by Macrogen Europe (Amsterdam, The Netherlands). The ChromasPro v. 1.5 software was used to evaluate the reliability of sequences and to create consensus sequences. Unreliable sequences in which both forward and reverse

sequences, or one or the other, were not successful or contained doubtful bases were re-sequenced. The ITS and TUB2 sequences obtained in the present study were deposited in GenBank and the relative accession numbers are presented in Table 1. Validated sequences representative of all species identified within the acutatum, boninense and gloeosporioides species complexes were phylogenetically analysed to determine the relationship between different isolates and define their taxonomic status. Sequences from ex-type or authentic culture were included in the analysis as reference (Table 2).

Table 2. GenBank accession numbers of sequences of the isolates of worldwide origin used as references in phylogenetic analyses

Species	Isolate	Clade	Origin	Host	Source	GenBank accession number	
						ITS-rDNA	ß-tubulin 2
C. acutatum	IMI 117620	acutatum	Australia	*Carica papaya*	[36]	FJ788417	FJ788419
C.acutatum	CBS 127598	acutatum	South Africa	*Olea europaea*	[37]	JQ948363	JQ950014
C. acutatum	CBS 144.29	acutatum	Sri Lanka	*Capsicum annuum*	[37]	JQ948401	JQ950052
C. aenigma	ICMP 18608	gloeosporioides	USA	*Persea americana*	[38]	JX010244	JX010389
C. aeschynomenes	ICMP 17673	gloeosporioides	USA	*Aeschynomene virginica*	[38]	JX010176	JX010392
C. alatae	CBS 304.67	gloeosporioides	India	*Dioscorea alata*	[38]	JX010190	JX010383
C. alienum	ICMP 12071	gloeosporioides	New Zealand	*Malus domestica*	[38]	JX010251	JX010411
C. annellatum	CBS 129826	boninense	Colombia	*Hevea brasiliensis*	[39]	JQ005222	JQ005656
C. aotearoa	ICMP 18537	gloeosporioides	New Zealand	*Coprosma* sp	[38]	JX010205	JX010420
C. beeveri	CBS 128527	boninense	New Zealand	*Brachyglottis repanda*	[39]	JQ005171	JQ005605
C. boninense	CBS 123755	boninense	Japan	*Crinum asiaticum* var. *sinicum*	[39]	JQ005153	JQ005588
C. brasiliense	CBS 128501	boninense	Brazil	*Passiflora edulis*	[39]	JQ005235	JQ005669
C. brisbaniense	CBS 292.67	acutatum	Australia	*C. annuum*	[37]	JQ948291	JQ949942
C. carthami	SAPA100011	acutatum	Japan	*Carthamus tinctorius*	[37]	AB696998	AB696992
C. clidemiae	ICMP 18658	gloeosporioides	USA	*Clidemia hirta*	[38]	JX010265	JX010438
C. colombiense	CBS 129818	boninense	Colombia	*Passiflora edulis*	[39]	JQ005174	JQ005608
C. fioriniae	CBS 128517	acutatum	USA	*Fiorinia externa*	[37]	JQ948292	JQ949943
C. gloeosporioides	CBS 112999	gloeosporioides	Italy	*Citrus sinensis*	[38]	JQ005152	JQ005587
C. gloeosporioides	STE-U4295	gloeosporioides	Italy	*Citrus* sp.	[40]	AY376532	AY376580
C. godetiae	CBS 133.44	gloeosporioides	Denmark	*Clarkia hybrida*	[38]	JQ948402	JQ950053
C. godetiae	CBS 160.50	gloeosporioides	/	*Citrus aurantium*	[38]	JQ948406	JQ950057
C. godetiae	CBS 796.72	gloeosporioides	USA	*Aeschynomene virginica*	[38]	JQ948407	JQ950058
C. karstii	CBS 132134/ CORCG6	boninense	China	*Vanda* sp.	[41]	HM585409	HM585428
C. karstii	CBS 106.91	boninense	Brazil	*Carica papaya*	[17]	JQ005220	JQ005654
C. nymphaeae	CBS 515.78	acutatum	/	*Nymphaea alba*	[29]	JQ948197	JQ949848
C. nymphaeae	CBS 231.49	acutatum	/	*Olea europaea*	[29]	JQ948202	JQ949853
C. paspali	MAFF 305403	graminicola	Japan	*Paspalum notatum*	[42]	EU554100	JX519244;
Monilochaetes infuscans	CBS 869.96	/	South Africa	*Ipomoea batatas*	[42]	JQ005780	JQ949805

2.3.3. Morphological characteristics and optimum growth temperature of fungal isolates

Agar plugs (5mm) were taken from the edge of actively growing cultures on PDA and transferred onto the center of 9-cm diameter Petri dishes containing PDA. Dishes were incubated at 25 °C either in the dark for 10 days to determine both the colony morphology and radial growth rate or with continuous fluorescent light to observe microscopic morphological traits. Conidial and mycelial suspensions were prepared in SDW from 10-day-old cultures and examined microscopically.

Optimum temperature for radial growth was determined for all isolates used in this study. The following isolates of *C. acutatum* (UWS149), *C. gloeosporioides* (AC 24) and *C. karsti* (ALL 2I) characterized in previous studies were used as reference [19]. Agar plugs (5-mm) were taken from the edge of actively growing cultures on PDA and transferred onto the center of 9-cm diameter Petri dishes containing PDA media. Dishes were incubated at 5, 7, 10, 12, 15, 20, 25, 30 and 35°C in the dark with three replicates per temperature and per isolate. Two orthogonal diameters were measured per each colony after 3-, 5- and 7-days incubation. The experiment was conducted twice with similar results, so results of only one experiment are reported.

2.3.4. Plant material for pathogenicity tests

Nine olive (*Olea europaea* L.) cultivars were included in this study to evaluate their susceptibility to the infections by diverse *Colletotrichum* species: 'Carolea', 'Cassanese', 'Coratina', 'Dolce Agogia' 'Frantoio', 'Leccino', Ottobratica', 'Picual' and 'Sant'Agostino'. All the cultivars were of Italian origin, with the only exception of 'Picual, which was included as a reference.

The ripening stage of olive drupes was determined according with the maturation index (MI) of Guzmán et al. [43], with value 0 corresponding to >50% bright green and value 4 to 100% blackish-purple or black color of drupes. In tests aimed at evaluating only the *Colletotrichum* species x olive cultivar interaction, drupes with MI values between 3 and 4 were used. While in comparative tests aimed at evaluating the effect of ripening stage and wounding on the *Colletotrichum* species x olive cultivar interaction, both mature (MI 4) and green (MI 2) drupes of 'Coratina' collected from the same trees during the veraison were used. Olive fruits were collected from 15-year-old olive trees from two distinct experimental orchards in Calabria (southern Italy): one in the municipality of Mirto-Crosia, province of Cosenza (Geographic Coordinates from DATUM WGS 84, 39°36'54.5"N 16°46'11.7"E) and another in the municipality of Rende, province of Cosenza, (Geographic Coordinates from DATUM WGS 84, 39°21'59.4"N 16°13'44.4"E). Drupes of the same cultivar from the two orchards, and from three distinct trees per orchard, were pooled together. Drupes were stored for 3 hours inside a refrigerated bag, before being transported to the laboratory and were inoculated the day after collection. Before the inoculation, they were surface

disinfected by immersion in a 0.5 % NaClO solution for 30 s, rinsed in sterile distilled water, blotted dry and placed in incubation trays.

2.3.5. Inoculation and rating of disease severity

Drupes were punctured with a sterile needle in an equatorial position and a 20 µl droplet of the conidial suspension (10^6 conidia ml^{-1}), prepared as described in a previous paragraph, was pipetted on the surface of the wound. Control drupes received 20 µl of sterile water. In the first set of experiments, four fungal isolates (an isolate per *Colletotrichum* species) and nine olive cultivars were tested. The experimental design was a complete randomized block with four replications. In each block 25 drupes were inoculated per each isolate × cultivar combination, i.e. in total 500 drupes per cultivar were used in each experiment, including the non-inoculated control. After inoculation, drupes were incubated in a humid chamber at 23±1 °C, with 80% relative humidity, and a photoperiod of 16 h of light and 8 h of dark. Health conditions of inoculated drupes were assessed at 3, 5 and 7 days post inoculation (dpi). In the second set of experiments, the *Colletotrichum* species x olive cultivar interaction was evaluated using different isolates of the Colletotrichum species tested, and also including *C. nymphaeae*, which was the species responsible for severe epidemics in southwestern part of Portugal. Ten isolates (two for each of the five *Colletotrichum* species tested, *C. acutatum*, *C, gloeosporioides*, *C. godetiae*, *C. karsti* and *C. nymphaeae*) and four olive cultivars ('Coratina', 'Frantoio', 'Leccino' and 'Ottobratica') were included. All isolates were from olive except for the isolate CAM and ALL2I of C. karsti and isolate AC24 of *C. gloeosporioides*. Olive drupes were inoculated by wounding, as described previously. After inoculation, drupes were incubated in plastic boxes at 23±1 °C, with 80% relative humidity and a photoperiod of 16 h of light and 8 h of dark. The severity of symptoms on inoculated drupes was rated at 3, 5 and 7 dpi. The experimental design was a complete randomized block with four replicates and six drupes per replicate. The third set of experiments it was evaluated the effect of both wounding and maturity stage on the response of drupes to the inoculation with isolates of diverse *Colletotrichum* species. Both mature (MI 4) and green (MI 2) drupes of 'Coratina' were inoculated singularly with four *Colletotrichum* species (two diverse isolates of each species). The following fungal isolates were included: isolates C9D2C and UWS149 of *C. acutatum*, isolates RB012 and RB428 of *C. nymphaeae*, isolates OLP12 and OLP16 of *C. godetiae*, isolates RD9B and AC24 of *C. gloeosporioides*. Both mature and green drupes were split into two subgroups encompassing drupes inoculated by wounding and drupes inoculated without wounding, respectively. A 20 µl drop of a conidial suspension (10^6 conidia ml^{-1}) in 0.5% water-agar was pipetted on unwounded drupes in equatorial position. Wounded drupes received the same amount of inoculum and were inoculated with the method described

previously. After inoculation, both wounded and unwounded drupes were incubated in plastic boxes at 23±1 °C, with 80% relative humidity and a photoperiod of 16 h of light and 8 h of dark. The experimental design was a complete randomized block with four replicates and six drupes per replicate. The severity of symptoms on wounded and unwounded drupes was monitored up to 7 and 14 dpi, respectively.

The disease severity index (DSI) on olive fruits was scored based on an empirical scale in accordance with Talhinhas et al. [6] where: 0, no symptoms; 1, mycelium only; 2, small necrosis (<5 mm diameter) and absence of sporulation; 3, large necrosis (>5 mm diameter) and absence of sporulation; 4, few spore masses on the inoculation point; 5, abundant spore masses expanding away from the inoculation point; 6, spore masses entirely covering the fruit (Figure 1).

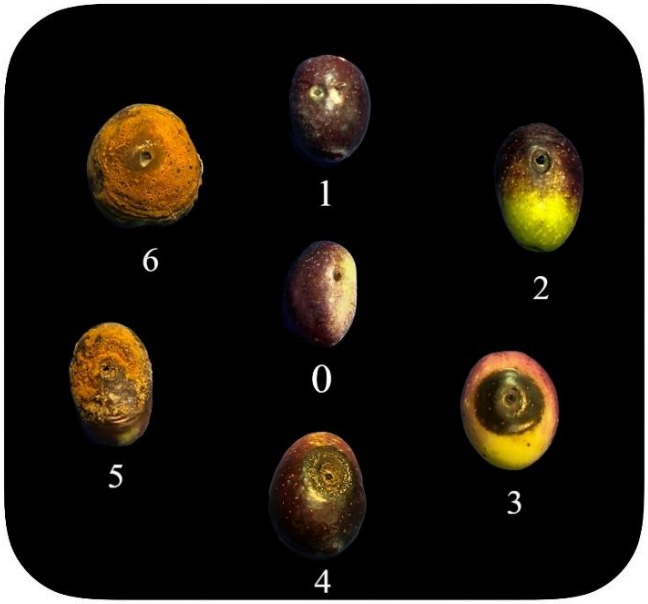

Figure 1. Rating scale used in this study to assess the OA severity index on olive drupes. 0, no symptoms; 1, mycelium only; 2, small necrosis (<5 mm diameter) and absence of sporulation; 3, large necrosis (>5 mm diameter) and absence of sporulation; 4, few spore masses on the inoculation point; 5, abundant spore masses expanding around the inoculation point; 6, spore masses entirely covering the fruit.

Symptoms were recorded daily after inoculation and the absolute Area Under the Disease Progress Curve (AUDPC) was calculated conforming with Talhinhas et al. [6]. Then, the relative Area Under Disease Progress Curve (rAUDPC) was estimated and reported in the figures. Each set of experiments was performed for two consecutive years, with similar results, so results of only the second year are reported (2020 for the first set and 2021 for the second and third sets).

2.3.6. Statistical analysis

All the data were normalized by square root transformation and then subjected to ANOVA followed by Tukey's Honestly Significant Difference (HSD) test as a post hoc test (R software). Differences at $P \leq 0.05$ were considered significant.

2.4. Results

The phylogenetic analysis of the combined data set of sequences from ITS and TUB2 regions of all single-conidium *Colletotrichum* isolates tested in this study (Table 1), along with sequences of the reference isolates of *C. acutatum* (UWS 149), *C. gloeosporioides* (AC24) and *C. karsti* (CAM) and the reference sequences of *Colletotrichum* species separated within the gloeosporioides, boninense and acutatum species complexes, produced a phylogenetic tree (Figure 2) with a similar topology and high concordance with those reported by the authors who revised the systematics of these species complexes using multigene sequence analysis [37–39].

In the interval between 12 and 35°C, the *C. gloeosporioides* isolates grew faster than the isolates of the acutatum complex, as expected. All tested isolates of both *C. gloeosporioides* and *C. karsti* as well as the isolates of *C. acutatum* and *C. godetiae* showed an optimum temperature for radial growth at 25°C and grew faster at 20 than at 30 and 35 °C (Figure 3). No one of the isolates grew at 5°C and growth was not resumed when the Petri dishes were transferred to 25°C after 7 days of incubation at 5°C. However, at 30 and 35° C, *C. gloeosporioides* isolates were less inhibited than isolates of the other species. In particular, the radial growth of the two tested *C. gloeosporioides* isolates at 30° C was reduced only by 8.7 and 4.5%, respectively, compared to the growth at 25°C, and by 25 and 18%, respectively, at 35°C. The growth of the two *C. karsti* isolates at 30 °C was reduced by 30 and 37.5%, while at 35 °C it was inhibited by 55 and 42.5 %, respectively. Also, the growth of the two isolates of *C. acutatum* was dramatically reduced at 30°C, by 59 and 61.5%, respectively. The radial growth of *C. godetiae* isolates at 30°C was reduced by 60.5 % and 62%, respectively, compared to the growth at 25°C, and by 60% for both isolates, compared to their growth at 20°C. Conversely, at 10°C the *C. godetiae* and *C. karsti* isolates grew significantly faster than *C. gloeosporioides* and *C. acutatum* isolates.

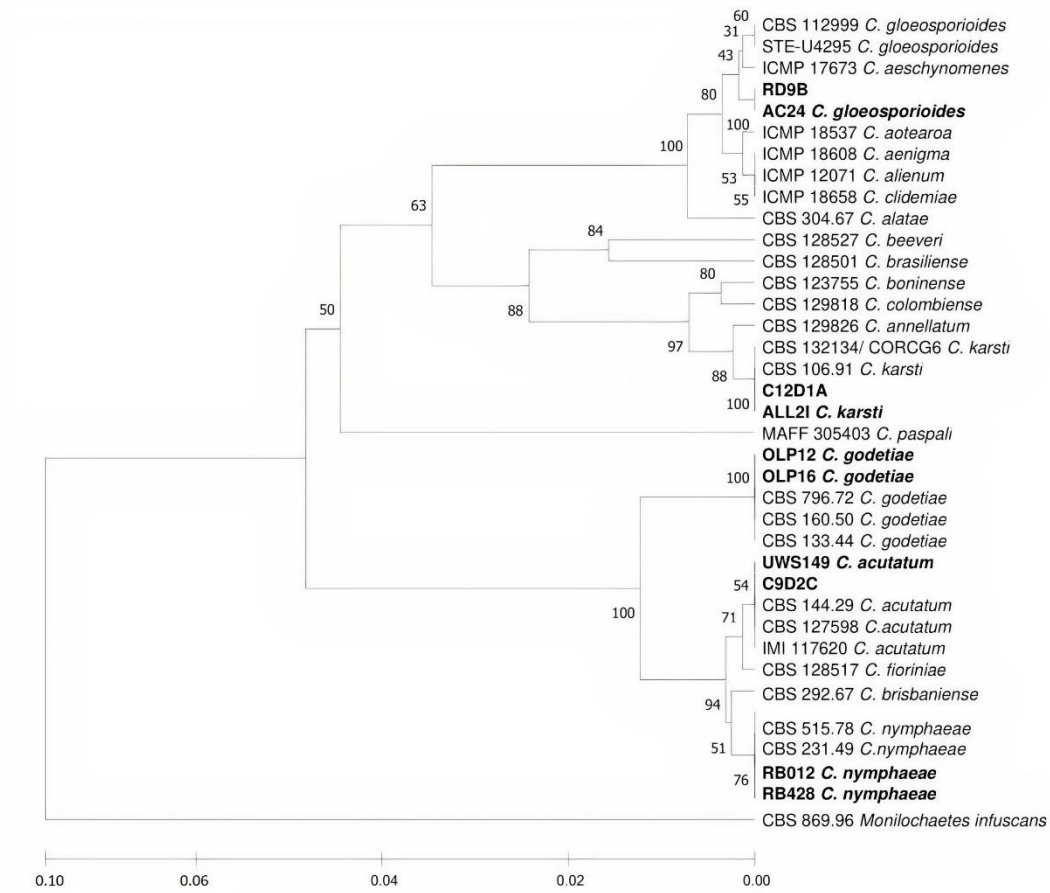

Figure 2. Phylogenetic tree obtained using combined internal transcribed spacers (ITS) and β-tubulin (TUB2) sequences of isolates of *Colletotrichum* species used in the present study (in bold) along with reference isolates of *C. karsti*, *C. gloeospoiroides*, and other representative species in the boninense, gloeospoirioides and acutatum species complex. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

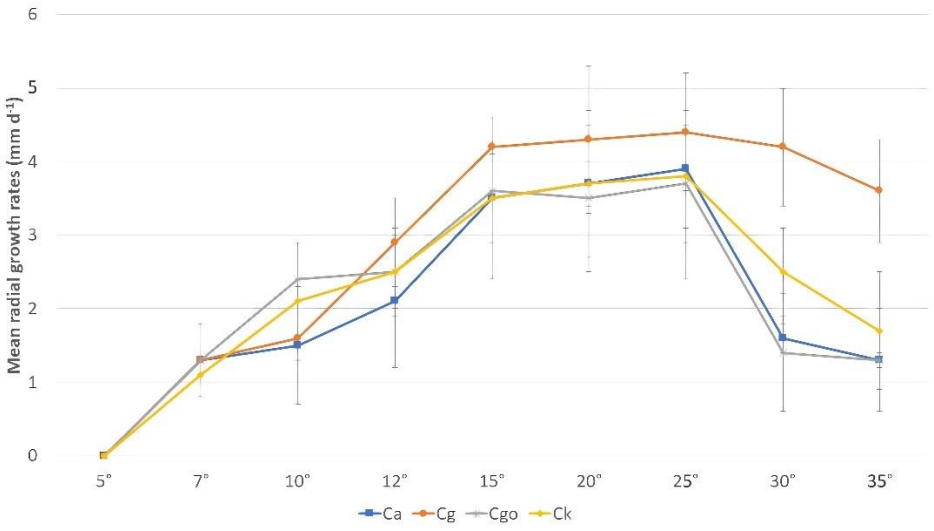

Figure 3. Mean radial growth rates of four isolates of *Colletotrichum acutatum* (Ca), *C. gloeosporioides* (Cg), *C. godetiae* (Cgo), *C. karsti* (Ck) on PDA at different temperatures.

In the first set of pathogenicity assays, isolates of all *Colletotrichum* species were pathogenic to wound-inoculated drupes of olive cultivars while no disease symptoms was recorded in control fruits. However, isolates differed markedly in virulence. Also, olive cultivars showed substantial differences in susceptibility to the infection by diverse *Colletotrichum* species. Statistical analysis of values of disease intensity over time (rAUDPC) revealed significant differences in both virulence among *Colletotrichum* isolates and susceptibility among olive cultivars and showed a significant isolate x cultivar interaction ($p<0.05$) (Figure 4).

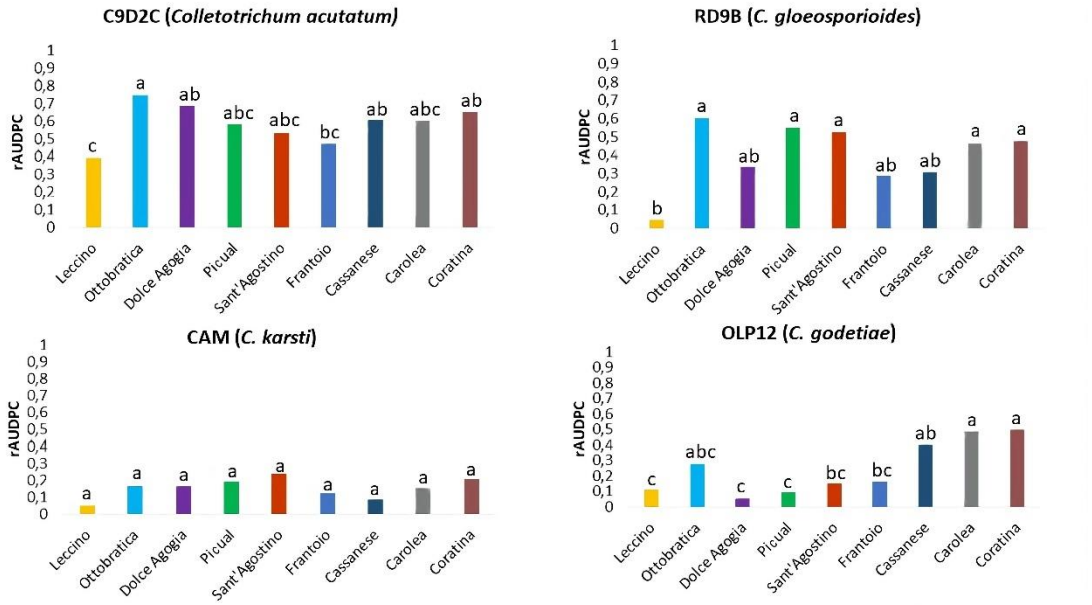

Figure 4. Olive anthracnose intensity over time (rAUDPC) on drupes of nine olive cultivars inoculated with four isolates of *Colletotrichum acutatum*., *C. gloeosporioides*; *C. godetiae* and *C. karsti*. Severity of symptoms on inoculated olive drupes was assessed daily for 7 days and was rated on a 0 to 6 scale according to the size of necrotic lesion and the abundance of fungus sporulation. Values sharing a common letter are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$). ANOVA F (8,81) value of C9D2C: 9.6, $P \leq 0.05$; RD9B: 9.4, $P \leq 0.05$; CAM: $P > 0.05$; OLP 12: 5.3, $P \leq 0.05$.

The most virulent among the isolates tested was the *C. acutatum* isolate, while the most resistant to this aggressive *Colletotrichum* species among the cultivars tested was 'Leccino', although the response of this cultivar did not differ significantly from that of 'Frantoio'. Moreover, 'Leccino' was the cultivar that segregated most clearly from the others for its low susceptibility to *C. gloeosporioides* infections. The mean rAUDPC values in this cultivar were 0.38 for *C. acutatum*, 0.04 for *C. gloeosporioides*, 0.11 for *C. godetiae* and 0.05 for *C. karsti*. Only 'Frantoio' showed resistance to artificial infections of *C. acutatum*, comparable to that of 'Leccino'. The susceptibility of 'Picual' to this *Colletotrichum* species did not differ significantly ($p>0.05$), from the susceptibility of cultivars commonly regarded as very susceptible to OA infections in field, such as 'Carolea',

'Coratina' and 'Ottobratica'. The least virulent among the isolates tested was the *C. karsti* isolate, which was weakly pathogenic on all olive cultivars. No significant difference ($p<0.05$) in susceptibility to this *Colletotrichum* species was detectable among the olive cultivars tested, probably due to a low level of virulence (Figure 4). Conversely, more clear differences in susceptibility were observed among olive cultivars inoculated with the *C. godetiae* isolate. 'Dolce Agogia', 'Frantoio', 'Leccino' and 'Picual' were the least susceptible while 'Carolea' and 'Coratina' were the most susceptible to this *Colletotrichum* species. The last two cultivars together with 'Ottobratica' were also amongst the most susceptible to *C. acutatum* and *C. gloeosporioides* with mean rAUDPC values of 0.6 and 0.46, respectively, for 'Carolea', 0.65 and 0.47, respectively for 'Coratina', and 0.74 and 0.6, respectively, for 'Ottobratica'. Both the Picual and Dolce Agogia cultivars that were relatively resistant to *C. godetiae* (mean rAUDPC values 0.09 and 0.05, respectively) proved to be highly susceptible to *C. acutatum* (mean rAUDPC values 0.57 and 0.68, respectively).

The results of the second set of experiments confirmed the olive cultivars tested differed in susceptibility to *Colletotrichum* species (Figure 5). 'Coratina' and 'Ottobratica' were shown to be susceptible while 'Frantoio' and 'Leccino' were relatively resistant to all the *Colletotrichum* species tested. *Colletotrichum acutatum* was confirmed to be the most virulent among the *Colletotrichum* species tested, followed by *C. nymphaeae*. Conversely, *C. karsti* was again the least aggressive. No significant difference in virulence were observed between isolates of the same *Colletotrichum* species.

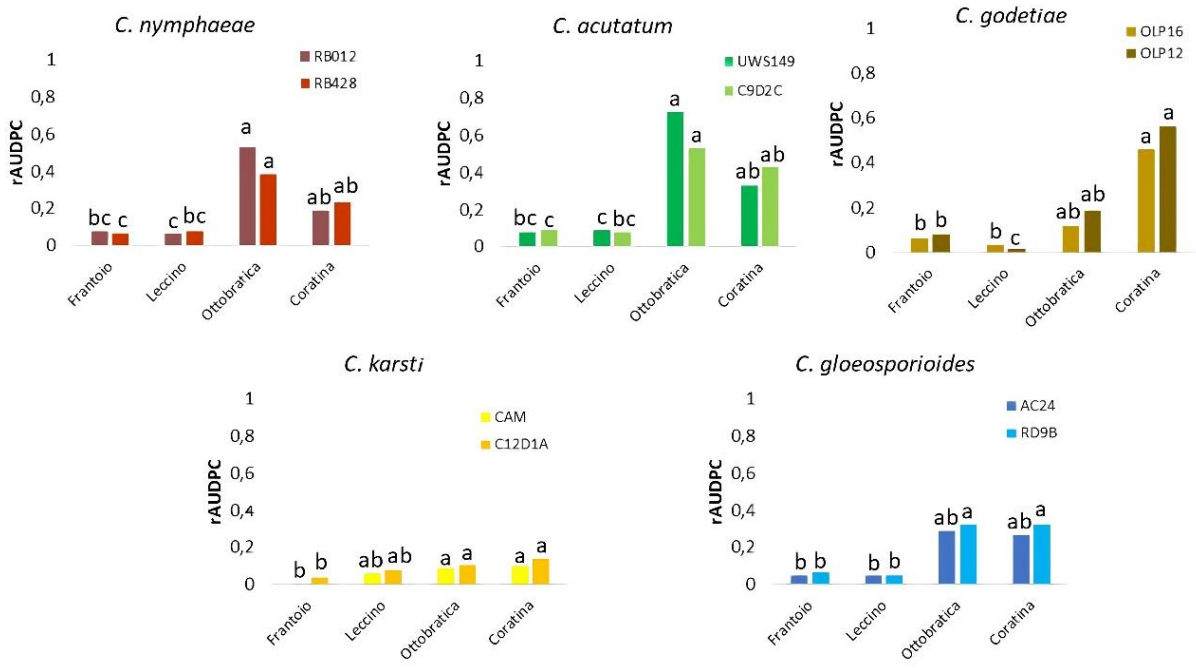

Figure 5. Olive anthracnose intensity over time (rAUDPC) on drupes of four olive cultivars inoculated with five isolates of *Colletotrichum acutatum, C. gloeosporioides*; *C. godetiae, C. karsti* and *C. nymphaeae*. Severity of symptoms on inoculated olive drupes was assessed daily for 7 days and was rated on a 0 to 6 scale according to the size of necrotic lesion and the abundance of fungus sporulation. Values sharing a common letter are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$).

In the third set of experiments, only the isolates of *C. acutatum* and *C. nymphaeae* induced symptoms on unwounded drupes, and exclusively on mature ones, although in the parallel test on wounded drupes, 'Coratina' was confirmed to be very susceptible to the infections of these two *Colletotrichum* species (Figure 6). On wounded drupes, isolates of both *C. acutatum* and *C. nymphaeae* proved to be very aggressive with mean rAUDPC values for UWS149 and C9D2C *C. acutatum* isolates on ripe drupes, 0.72 and 0.6, respectively, and mean rAUDPC values for RB012 and RB428 *C. nymphaeae* isolates, 0.52 and 0.46, respectively. Conversely, isolates of *C. godetiae* were the least aggressive with mean rAUDPC values for OLP12 and OLP16 isolates on ripe olives 0.14 and 0.16, respectively. *C. gloeosporioides* isolates, with mean rAUDPC values for RD9B and AC24 isolates on ripe olives 0.32 and 0.21, respectively, were more aggressive than isolates of *C. godetiae*, but less aggressive than isolates of both *C. acutatum* and *C. nymphaeae*. In general, symptoms were more severe on mature than on green drupes (Figure 6). However, the susceptibility of green drupes to diverse *Colletotrichum* species correlated to the susceptibility of mature drupes. No significant difference in virulence were observed between isolates of the same *Colletotrichum* species (Figure 6).

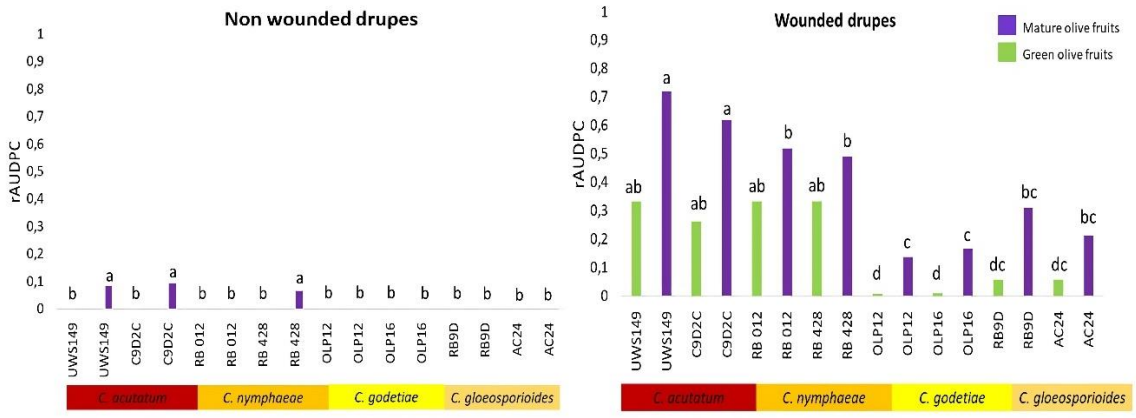

Figure 6. Olive anthracnose intensity over time (rAUDPC) on ripe and green drupes of 'Coratina' (wounded and non wounded) inoculated with four isolates of *Colletotrichum acutatum, C. gloeosporioides*; *C. godetiae* and *C. nymphaeae*. Severity of symptoms on inoculated olive drupes was assessed daily for 7 days for ripe olives and for 14 days for green drupes. Severity of symptoms was rated on a 0 to 6 scale according to the size of necrotic lesion and the abundance of fungus sporulation. Values sharing a common letter are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$).

2.5. Discussion

The introduction of molecular taxonomy contributed greatly to the accurate identification and characterization of cryptic species previously merged in the acutatum, boninense and gloeosporioides species complexes. This, in turn, promoted a notable advancement in both the study and knowledge of the structure of *Colletotrichum* populations associated to OA and the disease epidemiology in different geographical areas [1,2,5,11,15,16,30]. Differences in temperature requirements of diverse *Colletotrichum* species associated to OA, as revealed in this study, by *in vitro* assays of mycelium growth and also evidenced by Moral et al. [44] in experiments aimed at evaluating the effect of temperature on conidial germination of these species, might have epidemiological implications; however, they are not so marked as to explain by themselves why some species prevail over the others in different olive growing areas. Beside climatic conditions, a major driving factors of OA epidemics is the susceptibility of olive cultivars to the disease. Different levels of in-field susceptibility to OA among Italian olive cultivars were previously reported [1,9]. In a field screening for the susceptibility of olive cultivars to OA of worldwide origin, 'Frantoio', a cultivar of Italian origin, was selected as representative of the "highly resistant" susceptibility class, i.e. a higher resistance level than 'Picual', a common cultivar in Portugal and Spain, which was classified as 'resistant' [29]. However, to our knowledge, it is the first time that a set of olive cultivars of Italian origin was screened in comparable conditions for the susceptibility to diverse species of *Colletotrichum* associated to OA. Both 'Frantoio' and 'Picual were included in this study as references.

In the pathogenicity assays on detached olive fruits, 'Frantoio' was confirmed to be resistant to all the species of *Colletotrichum* tested while 'Picual' behaved as resistant to *C. godetiae* but resulted to be susceptible to the more virulent *C. acutatum* and *C. gloeosporioides*. Interestingly, so far *C. godetiae* has been the prevalent *Colletotrichum* species associated to olive anthracnose in Spain. 'Carolea', 'Coratina' and 'Ottobratica' resulted to be very susceptible irrespective of the *Colletotrichum* species tested. 'Coratina', originating from Apulia region, is the most common Italian cultivar with around 90,000 ha, most of which in the province of Bari. 'Ottobratica' is mainly grown in the alluvional plane of Gioia Tauro, province of Reggio Calabria (southern Italy), a vast olive growing area of around 20,000 ha where OA is endemic. Indeed, consistent with the *in vitro* tests, in the Gioia Tauro Plane, 'Ottobratica' is considered one of the local cultivars most susceptible to OA [1,9]. 'Leccino', a popular cultivar originally restricted to Tuscany and Umbria regions and presently grown also in numerous other regions of central and southern Italy, was shown to be as resistant as and in some tests more resistant than 'Frantoio', even to the most aggressive *Colletotrichum* species. This olive cultivar has also been proven to be tolerant of the

quick decline syndrome caused by the bacterium *Xylella fastidiosa* [45,46]. The resistance of 'Leccino' to OA might be interesting not only in view of its widespread diffusion in commercial olive orchards but also as a genetic source of resistance in breeding programs of olive cultivars.

In agreement with previous results of Talhinhas et al. [6] and Moral et al., [15], *C. acutatum* and *C. nymphaeae* were more virulent than *C. godetiae*. A feature of both *C. acutatum* and *C. nymphaeae*, which in this study contributed to attain high scores in pathogenicity tests (DSI values 5 and 6 in the rating scale), was the ability to sporulate on infected drupes of susceptible olive cultivars. The production of a great amount of conidia might increase the fitness of these two species and give them an epidemiological advantage over other *Colletotrichum* species infecting olive drupes. Conversely, in agreement with a previous study by Schena et al. [17], *C. karsti* was scarcely aggressive on olive fruits. Despite its polyphagy and widespread diffusion this *Colletotrichum* species was shown to be a weak pathogen also on other host plants, such as apple and citrus [19]. This would confirm that the presence of *C. karsti* on symptomatic olive fruits is incidental and its role in OA epidemic outbreaks is marginal. Conversely, this study did not contribute to clarify the controverted role of *C. gloeosporioides* as causal agent of OA because on detached olive drupes this *Colletotrichum* species was even more virulent than *C. godetiae*, which has long been or still is the dominant *Colletotrichum* species associated to OA in several olive-growing areas of the Mediterranean region, including Andalusia (Spain), Greece, southern Italy and northeastern provinces of Portugal [1,11,15]. However, in contrast *C. acutatum* and *C. nymphaeae*, in this study both *C. gloeosporioides* and *C. godetiae* were not able to infect unwounded olive drupes. This, at least in part, contradicts the results of Moral et al. [27] and Talhinhas et al. [6], who reported that wounding is not necessary to have infections by *Colletotrichum* species on detached olives. A possible explanation is that green olive drupes used in this study were less mature than those used by Moral et al. [27] and Talhinhas et al. [6]. Moreover, both Moral et al. [27]and Talhinhas et al. [6] did not use 'Coratina' in their tests on detached olive drupes. Consistently with Moral et al. [27] and Talhinhas et al. [6], results of the present study confirm that the susceptibility of detached olive drupes to artificial inoculations with *Colletotrichum* species correlated with in-field susceptibility to OA as olive cultivars used as references behaved as expected. However, consistently with Schena et al. [17], wounding was a prerequisite to have successful infections also with less virulent *Colletotrichum* species and repeatability of results. Another crucial point is the evaluation of the symptoms early after the inoculation (3, 5 and 7dpi). This could have implied an underestimation of the severity of symptoms in green olives. However, on the other hand, an early rating of symptoms on wounded ripening drupes provides a more reliable assessment of the disease severity, especially in comparative studies. The rating of the severity of symptoms

from 14 up to 35 dpi could result in an overestimation as well as a bias of interpretation of the severity of symptoms, as even less aggressive *Colletotrichum* strains could colonize senescent tissues.

In accordance with the results of Talhinhas et al. [6], who evaluated the susceptibility of eight olive cultivars commonly grown in Portugal to diverse *Colletotrichum* species, results of pathogenicity assays in this study revealed a considerable variability in both the susceptibility of olive cultivars and the virulence of diverse *Colletotrichum* species tested as well as a significant interaction between olive cultivar x *Colletotrichum* species. This last case was clearly exemplified by the cultivars Picual and Dolce Agogia, which were resistant to *C. godetiae* but susceptible to *C. acutatum*. It can be inferred that cultivars considered resistant in a geographical area where a weakly pathogenic *Colletotrichum* species prevails may behave as susceptible in areas where a more aggressive *Colletotrichum* species is dominant, provided that environmental conditions are conducive to the disease.

A better understanding of both the population structure of *Colletotrichum* in an olive growing area and the susceptibility of olive cultivars to the infections by different *Colletotrichum* species are prerequisites for developing an effective management strategy of OA. However, there is evidence that the structure of *Colletotrichum* population in a given area may change and even rapidly over time, depending on several factors, such as climate, disease management strategies, the introduction of more aggressive exotic *Colletotrichum* species from other olive growing areas, a host jump of a *Colletotrichum* species already present on other plants or the replacement of local olive cultivars with new, more susceptible cultivars. With regards to Italy, there is evidence that *C. nymphaeae*, the most common causal agent of strawberry anthracnose in this country and originally misidentified as *C. acutatum* and later as *C. simmondsii* [14,47], has only recently been reported as causal agent of OA [12]. Moreover, recent findings show an ever-mounting presence of *C. acutatum*, which is outcompeting the less aggressive *C. godetiae* and becoming the prevalent *Colletotrichum* species associated with OA in the Gioia Tauro Plane (southern Italy) [23]. An additional explanation of the displacement of *C. godetiae* by *C. acutatum* in southern Italy could also be related to the faster growth, especially at high temperatures, of *C. acutatum* with respect to *C. godetiae*. It can be envisaged that the severity of OA outbreaks in olive growing areas of Italy will increase in the coming years as a consequence of the emergence of these two aggressive *Colletotrichum* species. Perhaps it is not accidental that 'Leccino', 'Frantoio', 'Dolce Agogia' and 'S. Agostino', all relatively resistant to *C. godetiae*, are widespread in central Italy where only this *Colletotrichum* species was reported to be associated to occasional OA outbreaks [1]. Another *Colletotrichum* species in the gloeosporioides species complex, *C. theobromicola*, which was found

to be associated with anthracnose outbreaks in Australia and in artificial inoculations on detached olive drupes was demonstrated to be very aggressive (Schena et al., 2015), has been recently reported to be responsible for anthracnose outbreaks in Uruguay in association with *C. acutatum* [16]. *Colletotrichum theobromicola* has not be reported so far in olive growing regions of Northern hemisphere and its introduction into the Mediterrranean region might have phytosanitary implications.

Overall results of this study reinforced the need to identify precisely the *Colletotrichum* species in a given olive growing area and to screen the local olive cultivars for OA-susceptibility accordingly. However, the genetic resistance of cultivars might become ineffective if there is a shift in the *Colletotrichum* population, which according to the abovementioned literature is not a remote hypothesis. This implies a continuous monitoring of *Colletotrichum* populations in olive orchards and a more integrated and flexible OA management strategy.

Author Contributions: Conceptualization, M.R., E.S. and S.O.C.; methodology, A.P., S.O.C. and M.R.; software, M.R.; validation, S.O.C., S.M. and E.S..; formal analysis, M.R.; investigation, M.R., and A.P.; resources, S.O.C. and A.P.; data curation, M.R.; writing—original draft preparation, M.R. and S.O.C.; writing—review and editing, S.O.C., S.M. and E.S..; visualization, S.O.C.; supervision, S.O.C., A.P. and E.S.; project administration, S.O.C. and A.P.; funding acquisition, S.O.C. A.P. and M.S.

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3. Wilting of twigs and shoots caused by *Colletotrichum gloeosporioides* and *Colletotrichum karsti*, a new citrus disease in Italy

Mario Riolo[1,2,3], Francesco Aloi[1,4], Antonella Pane[1], Santa Olga Cacciola[1]*

[1] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[2] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[3] Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus crops (CREA- OFA), 87036 Rende, Cosenza, Italy;

[4] Department of Agricultural, Food and Forest Sciences, University of Palermo, 90128 Palermo, Italy;

*Corresponding author: Santa Olga Cacciola, E-mail olga.cacciola@unict.it

3.1. Abstract

This study was aimed at identifying the *Colletotrichum* species associated with twig and shoot dieback of citrus**,** a new syndrome occurring in the Mediterranean region and also reported as emerging in California. Overall, 89 *Colletotrichum* isolates were characterized, and they were recovered from symptomatic trees of sweet orange, mandarin and mandarin-like during a survey of citrus groves in Sicily (southern Italy). The isolates were grouped into two distinct morphotypes. The grouping of isolates was supported by phylogenetic sequence analysis of two genetic markers, the internal transcribed spacer regions of rDNA (ITS) and β-tubulin (TUB2). The groups were identified as *Colletotrichum gloeosporioides* and *C. karsti*, respectively. The former accounted for more than 91% of isolates, while the latter was retrieved only occasionally. Both species induced symptoms on artificially wound inoculated twigs. *Colletotrichum gloeosporioides* was more aggressive than of *C. karsti.* Winds and prolonged drought were the factor predisposing to Colletotrichum twig and shoot dieback. This is the first report of *C. gloeosporioides* and *C. karsti* as causal agents of twig and shoot dieback disease in Italy.

Keywords: *Colletotrichum gloeosporioides*; *Colletotrichum karstii*; ITS, TUB2, pathogenicity, citrus.

3.2. Introduction

Italy ranks second among European citrus producer countries, with Sicily and Calabria regions (southern Italy) accounting for approximately 63 and 19% of total domestic production of oranges and 53 and 20% of production of tangerines, respectively [1]. Sicily alone accounts for around 86% of total domestic production of lemons [1]. Moreover, Italy is the leading producer country of organic citrus fruit globally, with over 35,000 ha, corresponding to about one third of the total

domestic citrus growing area [2]. Over the last years in southern Italy, outbreaks of citrus twig and shoot dieback were observed to occur in major orange and mandarin growing areas following extreme weather events whose frequency in the Mediterranean region has increased due to climate change [3]. The disease, here named twig and shoot dieback of citrus or Colletotrichum twig and shoot dieback to stress its association with pathogenic *Colletotrichum* species, was recently described as an emerging new disease of citrus in Central Valley, California, distinct from the typical anthracnose of fruit, leaves and twigs [4,5]. Symptoms of dieback caused by *Colletotrichum* included chlorosis, crown thinning, necrotic blotches on leaves, blight and frequently gummosis of apical twigs, shoot and branch dieback [5]. Two *Colletotrichum* species, *C. gloeosporioides* and *C. karsti*, were associated with *Colletotrichum* dieback in California. Overall, neither species clearly prevailed over the other and, in pathogenicity tests, both species were able to infect twigs although not all field symptoms were reproduced [4]. In pathogenicity tests performed in California, *C. karsti* was more aggressive than *C. gloeosporioides* [4]. Symptoms similar to twig and shoot dieback described in California were reported from countries of the Mediterranean region, including Algeria, Marocco and Tunisia, but they were always associated with other symptoms typical of anthracnose on fruits and leaves [6–8]. In a recent survey of citrus groves in Portugal aimed at identifying the *Colletotrichum* species associated with typical anthracnose on fruits, leaves and twigs, *C. gloeosporioides*, the dominant species, was isolated preferentially from fruits and leaves while *C. karsti* was isolated preferentially from twigs and leaves, suggesting the hypothesis of differences in host-plant organ specificity between these two species [9]. Differences in host-plant organ preference between *Colletotrichum* species complex have long been known [10]. Two of the best known examples of host and organ specificity are provided by *C. abscissum* and *C. limetticola*, both in the *C. acutaum* complex, associated to post-bloom fruit drop (PFD) of citrus and Key Lime Anthracnose (KLA), respectively, occurring in the Americas [11–15]. According to the new molecular taxonomy of the genus *Colletotrichum*, based on multi-locus phylogeny and a polyphasic approach, 25 distinct *Colletotrichum* species have been so far identified to be associated to *Citrus* and allied genera worldwide. Seven of these, including *C. aenigma*, *C. ciggaro* (syn. *C. kahawae* subsp. *ciggaro*), *C. gloeosporioides sensu stricto (s.s.)* and *C. hystricis*, within the *C. gloeosporioides* species complex, *C. catinaense* and *C. karsti*, within the *C. boninense* species complex, and *C. acutatum s.s.*, within the *C. acutatum* species complex, have been reported from Italy [8,9,24–27,16–23]. The objectives of this study were to identify the *Colletotrichum* species associated with the twig and shoot dieback of citrus in Sicily and evaluate the pathogenicity of these *Colletotrichum* species on different plant organs, including fruit, leaves and shoots.

3.3. Materials and Methods

3.3.1. Sampling and isolation

During 2017 and 2018, citrus orchards were surveyed for twig and shoot dieback in six municipalities (Augusta, Lentini, Mineo, Misterbianco, Ramacca and Scordia) of the provinces of Catania and Syracuse (Sicily, southern Italy). *Colletotrichum* isolates were obtained from twigs with symptoms of blight and gumming and from leaves showing necrotic patches and mesophyll collapse. Overall, samples were collected from six citrus groves (one for each municipality) in Sicily and from diverse citrus species and cultivars, including mandarin (*C. reticulata*) 'Clementine', three cultivars of sweet orange (*Citrus ×sinensis*), 'Tarocco Lempso', 'Tarocco Scirè' and 'Lane Late', and two mandarin-like hybrids, 'Fortune' (*C. ×clementina* × 'Orlando' tangelo) and 'Mandared' (*C. ×clementina* 'Nules' × *C. ×sinensis* 'Tarocco').

To obtain fungal isolates, twig and leaf fragments (5 mm) were washed with tap water, surface sterilised with a sodium hypochlorite solution (10%) for 1 min, immersed in 70% ethanol for 30 s and rinsed in sterile distilled water (s.d.w.). After disinfection, the fragments were blotted dry on sterilised filter paper, placed in Petri dishes on potato-dextrose-agar (PDA; Oxoid Ltd., Basingstoke, UK) amended with 150 μg/mL streptomycin and incubated at 25°C until typical *Colletotrichum* colonies were observed. Sporulating conidiomata from subcultures were collected, crushed in a drop of s.d.w. and spread in Petri dishes over the surface of PDA amended with streptomycin to obtain single-conidium isolates. After 24 h incubation at 25°C, germinating conidia were individually transferred to Petri dishes onto PDA. Stock cultures of single-conidium isolates were mantained on PDA slants under mineral oil at 5-10°C in the culture collection of the Molecular Plant Pathology laboratory of the Department of Agriculture, Food and Environment of the University of Catania, Italy.

3.3.2. Fungal isolates

A total of 89 single-conidium isolates of *Colletotrichum* from citrus, representing a population of mass isolates around ten fold larger, was characterised in this study (**Table 1**). Mass isolates were obtained from symptomatic twigs and leaves and were preliminarly separated into two groups on the basis of morphotype, i.e. colony morphology, radial growth rate on PDA at 25°C and microscopical traits, such as the shape and size of conidia and the presence of setae. About 10% of mass isolates of each morphotype and from each site were selected randomly and a single-condium isolate was obtained from each selected mass isolate. A *C. acutatum s.s.* isolate (UWS 149) from olive (*Olea europaea*) sourced in Australia and a *C. gloeosporioides s.s.* isolate (C2) from lemon (*Citrus ×limon*), sourced in Calabria [28], as well as a *C. karsti* (CAM) from *Camellia* sp., sourced in Sicily [21], were used as references in growth and pathogenicity tests.

Table 1. Isolates of *Colletotrichum* sourced from different cultivars of *Citrus* species in Sicily characterized in this study.

Isolate code	Species	Host (species and cultivar)	Organ	Geographical origin	GenBank Accession	
					ITS-rDNA	ß-tubulin 2
AC1-AC34	*Colletotrichum gloeosporioides*	Sweet orange 'Tarocco Lempso'	twig	Ramacca (Ct) - Sicily	MT997785-MT997818	MW001517- MW001550
AC35-AC38	*C. gloeosporioides*	Sweet orange 'Tarocco Lempso'	leaf	Ramacca Ct) - Sicily	MT997819-MT997822	MW001551-MW001554
ALL1A-ALL1D	*C. gloeosporioides*	Sweet orange 'Lane late'	leaf	Augusta (Sr) - Sicily	MT997843-MT997846	MW001575-MW001578
ALL1E-ALL1L	*C. gloeosporioides*	Navel Lane Late	twig	Augusta (Sr) - Sicily	MT997847-MT997852	MW001579-MW001584
ALL2A	*C.karsti*	Navel Lane Late	twig	Augusta (Sr) - Sicily	MT997853	MW001545
ALL2B-ALL2H	*C. gloeosporioides*	*C. sinensis* "Lane Late"	twig	Augusta (Sr) - Sicily	MT997854-MT997860	MW001585-MW001591
ALL2I-ALL2T	*C. karsti*	*C. sinensis* "Lane Late"	twig	Augusta (Sr) - Sicily	MT997861-MT997870	MW001546-MW001555
ALL3A-ALL3C	*C. gloeosporioides*	*C. sinensis* "Lane Late"	twig	Augusta (Sr) - Sicily	MT997871-MT997873	MW001592-MW001594
ALL4A-ALL4D	*C. gloeosporioides*	*C. sinensis* "Lane Late"	twig	Lentini (Sr) - Sicily	MT997874-MT997877	MW001595-MW001598
F169	*C. gloeosporioides*	* mandarin	twig	Scordia (Ct) - Sicily	MW506960	MW513961
F172	*C. gloeosporioides*	*C. reticulata* "Fortune"	twig	Scordia (Ct) - Sicily	MW506962	MW513963
F189-F191	*C. gloeosporioides*	*C. reticulata* "Fortune"	twig	Scordia (Ct) - Sicily	MW506963-MW506965	MW513964-MW513966
F224-F227	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Misterbianco (Ct) – Sicily	MW506966-MW506969	MW513967-MW513970
F239	*C. gloeosporioides*	*C. sinensis* "Moro"	twig	Carlentini (Sr) - Sicily	MW506970	MW513971
F253-F256	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Misterbianco (Ct) – Sicily	MW506971-MW506973	MW513972-MW513972
SR5-SR6	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Mineo (Ct) - Sicily	MW506974-MW506975	MW513975-MW513976
SR8	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Mineo (Ct) - Sicily	MW506976	MW513977
SR12	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Mineo (Ct) - Sicily	MW506977	MW513978
SR15	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Mineo (Ct) - Sicily	MW506978	MW513979
SR19	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	twig	Mineo (Ct) - Sicily	MW506979	MW513980
SF2-SF3	*C. gloeosporioides*	*C. sinensis* "Tarocco Scirè"	leaf	Mineo (Ct) - Sicily	MW506980-MW506981	MW513981- MW513982
SR21	*C. gloeosporioides*	"Mandared"**	twig	Mineo (Ct) - Sicily	MW506982	MW513983
SR23	*C. gloeosporioides*	"Mandared"**	twig	Mineo (Ct) - Sicily	MW506983	MW513984
SR25	*C. gloeosporioides*	"Mandared"**	twig	Mineo (Ct) - Sicily	MW506984	MW513985
SR28	*C. gloeosporioides*	"Mandared"**	leaf	Mineo (Ct) - Sicily	MW506985	MW513986
UWS 149	*C. acutatum*	*Olea europaea*	fruit	Agonis Ridge WA Australia	JN121186	JN121273

C2	*C. gloeosporioides*	*Citrus xlimon*	fruit	Lamezia Terme (CZ) Calabria	JN121211	JN121298
CAM	*C. karsti*	*Camellia* sp.	leaf	Sicily	KC425664	KC425716

3.3.3. Morphological characteristics and optimum growth temperature of isolates

Agar plugs (5-mm-diam) were taken from the edge of actively growing cultures on PDA and transferred to the centre of 9-cm diameter Petri dishes containing PDA. Dishes were incubated at 25 °C either in the dark for 10 d to determine both the colony morphology and radial growth rate or with continuous fluorescent light to observe microscopical morphological traits. Conidial and mycelial suspensions were prepared in s.d.w. from 10-days-old cultures and examined microscopically.

Optimum temperature for radial growth was determined only for a restricted number of isolates of the two morphotypes and also for isolates of *C. acutatum* (UWS 149), *C. gloeosporioides* (C2) and *C. karsti* (CAM) used as references. Agar plugs (5-mm-diam) were taken from the edge of actively growing cultures on PDA and transferred to the centre of 9-cm diam Petri dishes containing PDA. Dishes were incubated at 20, 25 and 30 °C in the dark (three replicates per each temperature and per each isolate). Two orthogonal diameters were measured per each colony after 3 and 7 d incubation. The experiment was repeated once with similar results, so results of only one experiment are reported.

3.3.4.DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from single-conidium *Colletotrichum* isolates using the method described in Cacciola et al. [29]. The ITS1–58S–ITS2 region and the fragment of the β-tubulin 2 gene (TUB2) between exons 2 and 6 were amplified and sequenced from a complete panel of isolates as described in a previous paper [29]. Amplified products were analyzed by electrophoresis and single bands of the expected size were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with both forward and reverse primers by Macrogen Europe (Amsterdam, The Netherlands). The ChromasPro v. 1.5 software [30] was used to evaluate the reliability of sequences and to create consensus sequences. Unreliable sequences in which both forward and reverse sequences, or one or the other, were not successful or contained doubtful bases were re-sequenced. The ITS and TUB2 sequences obtained in the present study were deposited in GenBank and the relative accession numbers are reported in Table 1. Validated sequences representative of all species identified within the *C. boninense* and *C. gloeosporioides* species complexes were phylogenetically analysed to determine the relationship between different isolates and define their taxonomic status. Sequences from ex-type or authentic culture were included in the analysis as a reference (**Table 2**). Phylogenetic analysis was

conducted for the ITS and TUB2 sequences as well as for the combined data set of the two markers using maximum likelihood and Bayesian methods. TOPALi v2 [36] was used to determine the substitution model that best fitted the data. The model HKY + I + G was selected for the Bayesian and maximum likelihood phylogenetic analysis using MrBayes v. 3.1.1 and PhyML v. 2.4.5, respectively, implemented in TOPALi. Bayesian analysis was performed with four runs conducted simultaneously for 500,000 generations with 10% sampling frequency and burn-in of 30%. Maximum likelihood was performed with 100 bootstrap replicates.

Table 2. GenBank accession numbers of sequences of the isolates of worldwide origin used as references in phylogenetic analyses; *holotype; **epitype.

Species	Isolate	Clade	Origin	Host	Source	GenBank accession number	
						ITS-rDNA	ß-tubulin 2
C. acutatum	IMI 117620	acutatum	Australia	*C.papaya*	[31]	FJ788417	FJ788419
C. aenigma	ICMP 18608	gloeosporioides	USA	*Persea americana*	[18]	JX010244	JX010389
C. aeschynomenes	ICMP 17673	gloeosporioides	USA	*A. virginica*	[18]	JX010176	JX010392
C. alatae	CBS 304.67	gloeosporioides	India	*Dioscorea alata*	[18]	JX010190	JX010383
C. alienum	ICMP 12071	gloeosporioides	New Zealand	*Malus domestica*	[18]	JX010251	JX010411
C. annellatum	CBS 129826	boninense	Colombia	*Hevea brasiliensis*	[13]	JQ005222	JQ005656
C. aotearoa	ICMP 18537	gloeosporioides	New Zealand	*Coprosma sp*	[18]	JX010205	JX010420
C. beeveri	CBS 128527	boninense	New Zealand	*B. repanda*	[13]	JQ005171	JQ005605
C. boninense	CBS 123755	boninense	Japan	*C. asiaticum sinicum*	[13]	JQ005153	JQ005588
C. brasiliense	CBS 128501	boninense	Brazil	*Passiflora edulis*	[13]	JQ005235	JQ005669
C. brisbaniense	CBS 292.67	acutatum	Australia	*C. annuum*	[17]	JQ948291	JQ949942
C. carthami	SAPA100011	acutatum	Japan	*C. tinctorium*	[32]	AB696998	AB696992
C. clidemiae	ICMP 18658	gloeosporioides	USA	*Clidemia hirta*	[18]	JX010265	JX010438
C. colombiense	CBS 129818	boninense	Colombia	*Passiflora edulis*	[13]	JQ005174	JQ005608
C. fioriniae	CBS 128517	acutatum	USA	*Fiorinia externa*	[17]	JQ948292	JQ949943
C. gloeosporioides	CBS 112999	gloeosporioides	Italy	*Citrus sinensis*	[13]	JQ005152	JQ005587
C. gloeosporioides	STE-U4295	gloeosporioides	Italy	*Citrus sp.*	[33]	AY376532	AY376580
C. godetiae	CBS 133.44	gloeosporioides	Denmark	*Clarkia hybrida*	[13]	JQ948402	JQ950053
C. karsti	CBS 132134/ CORCG6	boninense	China	*Vanda sp.*	[34]	HM585409	HM585428
C. paspali	MAFF 305403	graminicola	Japan	*P. notatum fluegge*	[35]	EU554100	JX519244;
C.truncatum	CBS 151.35	truncatum	USA	*Phaseolus lunatus*	[35]	GU227862	GU228156

3.3.5.Pathogenicity test

The pathogenicity of all 89 single-conidium isolates of *Colletotrichum* from citrus and the reference isolates of *C. acutatum*, *C. gloeosporioides* and *C. karsti* was tested on mature apple fruits (*Malus domestica*) 'Fuji' and 'Cripps Pink'. The assay on apples was included in this study as it was often used as a standard method to evaluate the pathogenicity of *Colletotrichum* spp. from various host-plants [37]. Apples (three apples per fungal isolate) were wound inoculated by removing

aseptically a piece of tissue (3 mm side) using a scalpel, then inserting a mycelim plug of the same size upside down into the pulp of the fruit and putting back in place the piece of tissue. A sterile agar plug was inserted into control apples. Apples were incubated at 25 °C and the area of the external lesion was measured seven days post inoculation (d.p.i.). In a preliminary test using a set of isolates, including the reference isolates of *C. acutatum* (UW 149), *C. gloeosporioides* (C2) and *C. karsti* (CAM), no difference in susceptibility was observed between 'Fuji' and 'Cripps Pink' apples, so in subsequent tests of the 119 *Colletotrichum* isolates sourced from citrus during the survey and the reference isolates of the three *Colletotrichum* species fruits of the two apple cultivars were used indifferently depending on the availability.

The pathogenicity of a more restricted subset of isolates, including four *C. gloeosporioides* isolates from citrus (AC5, AC24 from twigs and AC38 from leaf) two *C. karsti* isolates (ALL2I and ALL2S sourced in Italy) and the reference isolates of *C. acutatum* from olive (UWS 149), *C. gloeosporioides* (C2) from lemon and *C. karsti* (CAM) from camellia, was tested on different citrus plant organs, including young twigs of sweet orange 'Tarocco Scirè', lemon (*C.* ×*limon*) 'Femminello 2Kr' and bergamot (*C.* ×*bergamia*) 'Fantastico', young and mature expanded leaves of sweet orange 'Moro' and 'Navelina', mature fruit of sweet orange 'Tarocco Meli' and lemon 'Femminello 2Kr' as well as green fruitlets of lemon 'Femminello 2Kr'.

On June 1st 2020, twigs (around 0.5 cm diameter) were wound inoculated using a scalpel to lift a strip of bark and insert under the bark, upside down and in contact with the cambium, a mycelium plug (3 mm side) taken from the edge of an actively growing culture on PDA. A plug of sterile agar was inserted under the bark of control twigs. The wounds were sealed tightly with Parafilm®. Inoculations were carried out on four-year-old trees in an experimental field in the municipality of Mineo. Six twigs on each tree were inoculated and six served as a control. The length of necrotic lesions was recorded 14 d.p.i. The experimental design was a complete randomized block with three replicates (trees) per each citrus variety and *Colletotrichum* isolate combination. The experiment was repeated on June 30th and the data of the two experiments were analyzed separately.

The same *Colletotrichum* isolates were used to inoculate fruits and leaves, to test their ability to produce symptoms of anthracnose. Ripe sweet orange and lemon fruits were surface disinfected with 70% ethanol, rinsed with s.d.w., blotted dry and inoculated by wounding and without wounding. Unwounded fruits were inoculated by putting mycelium plugs (3 mm side) directly on the peel (two plugs on the upper side of each fruit placed horizontally, four cm apart from each other). Control fruits received sterile agar plugs. The same number of fruits was inoculated using a scalpel to remove aseptically two small pieces (3 mm side) of peel, four cm apart from

each other. Mycelium plugs of the same size were inserted upside down into the albedo and the peel pieces were replaced to cover the wounds. Sterile agar plugs were inserted into the albedo of control fruits. After inoculation, fruits were placed in humid chambers (plastic boxes with air-tight lid) on plastic rings to avoid direct contact with the humid paper and incubated at 25°C under 16/8 h light/dark photoperiod and 90% RH. Symptoms were recorded three, six and 12 d.p.i. Four replicated fruits for each inoculation method (wounding and without wounding) and *Colletotrichum* isolate combination were included in each of the two separate experiments, one with mature sweet orange fruit and the other with mature lemon fruit, respectively. In a separate trial the same *Colletotrichum* isolates were inoculated on unripe fruitlets of lemon 'Femminello 2kr'. Differently from tests on mature fruit, each fruitlet was inoculated with a single plug instead of two. Symptoms were recorded three and six d.p.i.

Expanded young (from summer vegetative flushing) and mature (from spring or previous year vegetative flushing) leaves of sweet orange 'Moro' and 'Navelina' were collected on October 2nd 2020 in the same citrus orchard, surface disinfected with 70% ethanol, washed with s.d.w., blotted dry and transferred to a humid chamber (plastic boxes with air-tight lid) on blotting paper soaked with s.d.w. and covered with aluminium foil to avoid direct contact between leaves and water. Leaves were inoculated on the abaxial side by wounding and without wounding. A razor blade was used to gently scrape the surface of the leaf lamina so as to create small (2 mm side) superficial lesions (six lesions per leaf, three on each side of the midrib). A mycelium plug (3 mm side) taken from the edge of an actively growing culture on PDA was put on each lesion with the side covered by mycelium in contact with the leaf surface. Six mycelium plugs (three on each side of the midrib) were placed on the lamina of unwounded leaves. Sterile agar plugs were placed on both wounded and unwounded leaves used as controls. Leaves were incubated in humid chamber at 25°C under 16/8 h light/dark photoperiod and 90% RH and symptoms were recorded five d.p.i. The experimental design was a complete randomized block with four replicates (leaves) for each citrus variety, type of leaf (young or mature), inoculation method (wounded or unwounded) and *Colletotrichum* isolate combination.

Colletotrichum isolates used in pathogenicity tests were re-isolated from the lesions and identified based on colony morphology to fulfill Koch's postulates. The identity of the isolates obtained from artificially inoculated symptomatic twigs, fruit and leaves was also confirmed by sequencing the ITS and TUB2 regions.

3.3.6. Statistical analysis

Data from pathogenicity tests were analyzed using RStudio v.1.2.5 (R) [38]. The means of surface areas of necrotic lesions induced by different *Colletotrichum* isolates were compared and analyzed

by one-way ANalysis Of Variance (ANOVA) coupled with Tukey-Kramer Honestly Significant Difference (HSD) test. Likewise, ANOVA and Tukey-Kramer Honestly Significant Difference (HSD) test were applied for statistical analysis of the differences between mean colony diameters in radial growth tests of isolates at different temperatures. When comparing independent groups, Student's t-test was used. Levene's test was used to determine the homogeneity of variance between independent trials. No heterogeneity was detected and data from independent trials were combined.

3.4. Results

In all surveyed orchards, outbreaks of Colletotrichum twig and shoot dieback of citrus were observed from April to October on both young and mature trees and always following strong winds, occurring on trees suffering because of water stress after prolonged drought. Symptoms included blight and gumming of twigs (Figure 1 B,C), defoliation and crown thinning as well as necrotic blotches, russetting of the abaxial side and mesophyll collapse of leaves remained still attached to the twigs (Figure 1 D). In most severe cases, dieback of entire branches (Figure 1 A) and death of young (one- to three-year-old) trees were observed. The incidence of the disease in a single orchard varied greatly irrespective of the age of the trees. In most orchards, affected trees were scattered and only a few twigs or shoots were symptomatic within a tree while in a few orchards more than 80% of trees were more or less seriously affected.

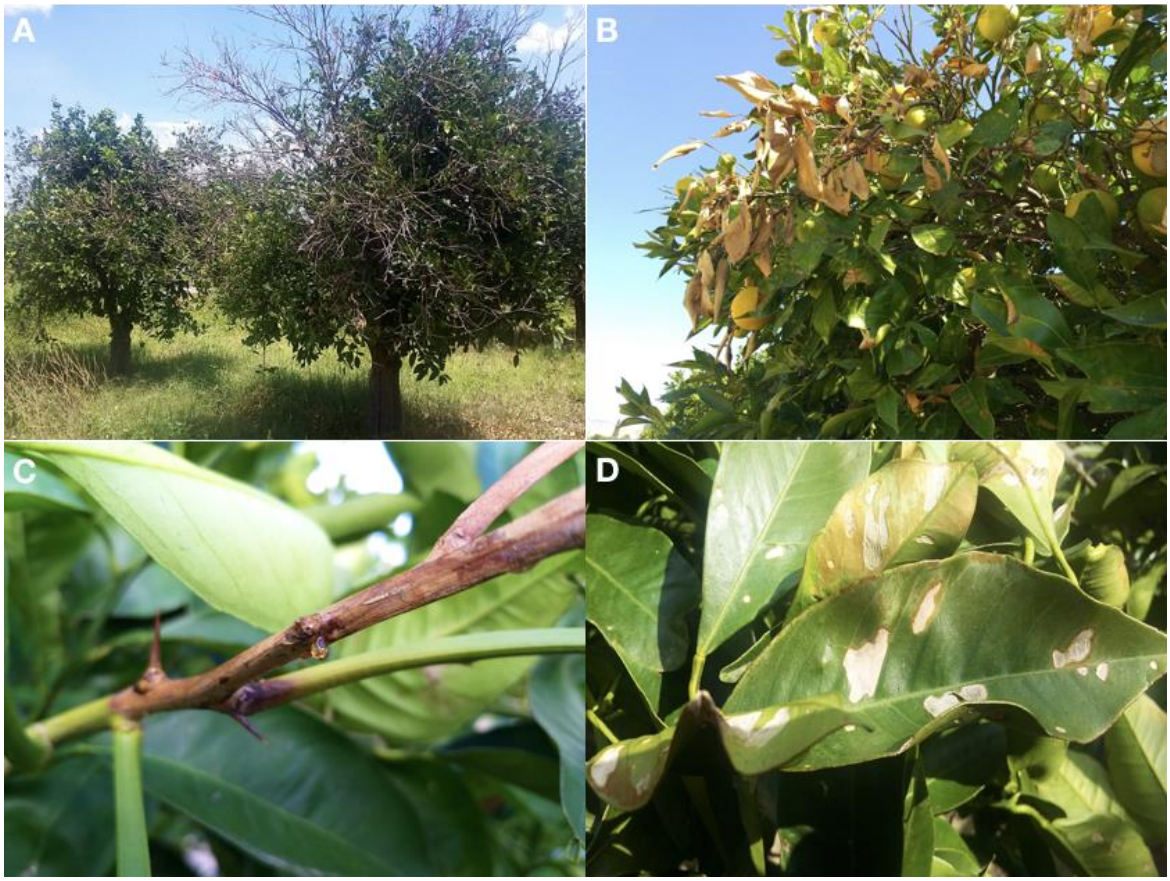

Figure 1. (**A**). Shoot blight and dieback of entire branches on Sweet orange 'Tarocco Scirè' injured by wind. (**B**) Symptoms of shoot blight incited by *Colletotrichum* infections on Sweet orange 'Tarocco Scirè' injured by wind. (**C**) Gumming associated with citrus shoot blight on Sweet orange 'Tarocco Scirè'. (**D**) Leaf mesophyll collapse and necrosis caused by wind on leaves of 'Tarocco Scirè' sweet orange. Conidiomata of *Colletotrichum* are visible on necrotic lesions as black pin point-dots.

Symptoms were often severe on the top of the canopy of mature trees and on trees exposed to wind and suffering because of drought. Overall, about 800 *Colletotrichum* isolates were sourced in Sicily, 93% from twigs and 7% from leaves.

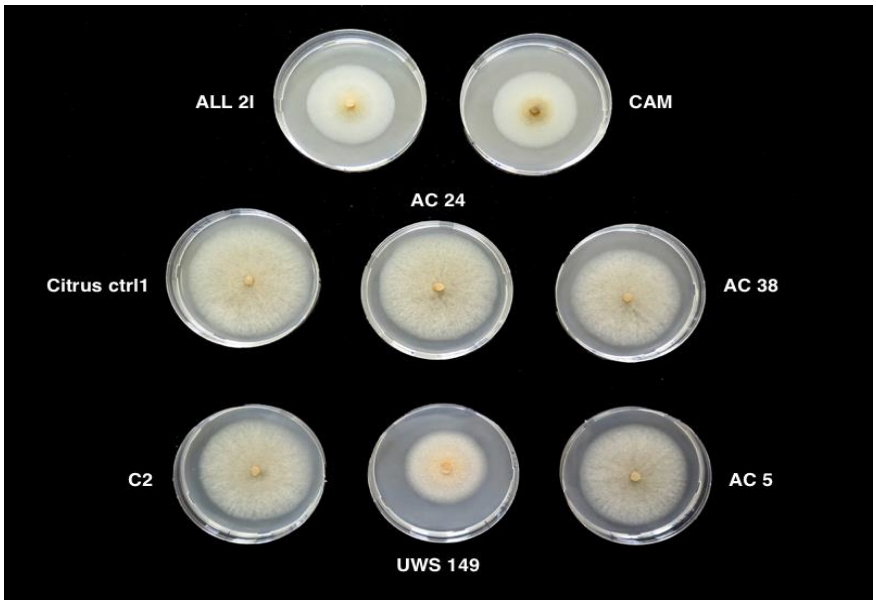

Figure 2. Morphology of 6-day-old colonies of *Colletotrichum gloeosporioides* (AC24, AC38, AC5, C2 and Citrus ctrl1), *C. acutatum* (UWS 149) and *C. karsti* (ALL 2I and CAM) grown on potato-dextrose-agar at 25 °C in the dark.

Colonies of these isolates on PDA were low-convex, fast-growing (10-11 mm average growth per day at 25°C), with entire margin and dense, cottony, aerial mycelium, initially white turning to pale grey and salmon pink conidial mucilaginous masses in the centre of the colony, dark acervuli scattered over all the surface in old colonies. Colony reverse was pale orange to uniformly grey. Single-celled conidia were, hyaline, smooth, cylindrical with both ends rounded; the range of their dimensions was 11-15 x 4-6 µm. Setae were common in most isolates. *Colletotrichum* isolates were separated into two clearly distinct groups on the basis of morphotype (Figure 2). Colonies on PDA were less fast growing (8 mm average growth per day at 25°C) and flat, with entire margin, mycelium appressed and moderately dense, white-orange to pale gray-orange, minute salmon orange conidium masses scattered over all the surface. The colony reverse was pale orange. Conidia were single-celled, hyaline, smooth, cylindrical with both ends rounded; the range of their dimensions was 10-17 x 4-6 µm. Setae were very rare or absent in most isolates.

The phylogenetic analysis of the combined data set of sequences from ITS and TUB2 regions of all single-conidium *Colletotrichum* isolates from citrus sourced in southern Italy (Table 1), along with sequences of the isolates of *C. acutatum* (UWS 149), *C. gloeosporioides* (C2) and *C. karsti* (CAM) used as references and the reference sequences of *Colletotrichum* species separated within the *C. gloeosporioides* and *C. boninense* species complexes, produced a phylogenetic tree (Figure 3) with a similar topology and high concordance with those reported by the authors who revised the systematics of these species complexes using multigene sequence analysis [13,17,18]. All the isolates with the same morphotype were identified as *C. gloeosporioides* because they clustered

(bootstrap values 100%) with the ex-type isolate of this species. Conversely, the 10 single-conidium isolates from the municipality of Augusta showing a distinct morphotype clustered (bootstrap values 100%) with ITS and TUB2 region sequences of reference isolates of *C. karsti*, including the culture from holotype isolate CBS 132134/CORCG6 and the isolate CAM from camellia sourced in Sicily. Sequences of isolates of both *C. gloeosporioides* and *C. karsti* were clearly distinct from reference sequences of *C. acutatum*. All selected isolates of both *C. gloeosporioides* and *C. karsti* as well as the reference isolate of *C. acutatum* grew faster at 25 than at 30 and 35 °C. However, at 30 and 35° C the *C. gloeosporioides* isolates were less inhibited than isolates of the other two species, indicating they were more thermofilic. In particular, the radial growth of *C. gloeosporioides* isolates at 30° C was reduced only by 7 to 11% compared to the growth at 25°C, and by 16 to 30% at 35°C. Conversely, the growth of *C. karsti* isolates was reduced by 55 to 60% at 30 °C and by 75 up to 79 % at 35 °C. The growth of the reference isolate of *C. acutatum* was dramatically reduced at 30 and 35°C, by 79 and 88% respectively (Table 3).

Table 3. Mean radial growth rates of colonies of *Colletotrichum* spp., isolates on PDA at three different temperatures, as determined after 7 d of incubation.

Colletotrichum spp.	Isolate	25°C (mm d^{-1}) mean ± S.D. [a]	30°C (mm d^{-1}) mean ± S.D. [a]	35°C (mm d^{-1}) mean ± S.D. [a]
C. acutatum	UWS 149	57 ± 0.58	12 ± 0.5	8 ± 0.29
C. gloeosporioides	AC 24	79 ± 0.5	70 ± 0.8	64 ± 1.15
C. gloeosporioides	F227	76 ± 0.58	70 ± 1.6	53 ± 17.61
C. gloeosporioides	AC 5	75 ± 0.58	69 ± 0.6	63 ± 1.76
C. gloeosporioides	C2	75 ± 0.76	68 ± 0.3	63 ± 0.29
C. gloeosporioides	AC 38	73 ± 1.04	67 ± 1.3	62 ± 0.58
C. karsti	ALL 2I	57 ± 5.35	26 ± 1.9	12 ± 0
C. karsti	CAM	56 ± 1.04	22 ± 0.8	25 ± 0.29

[a] Mean of three replicate Petri dishes.

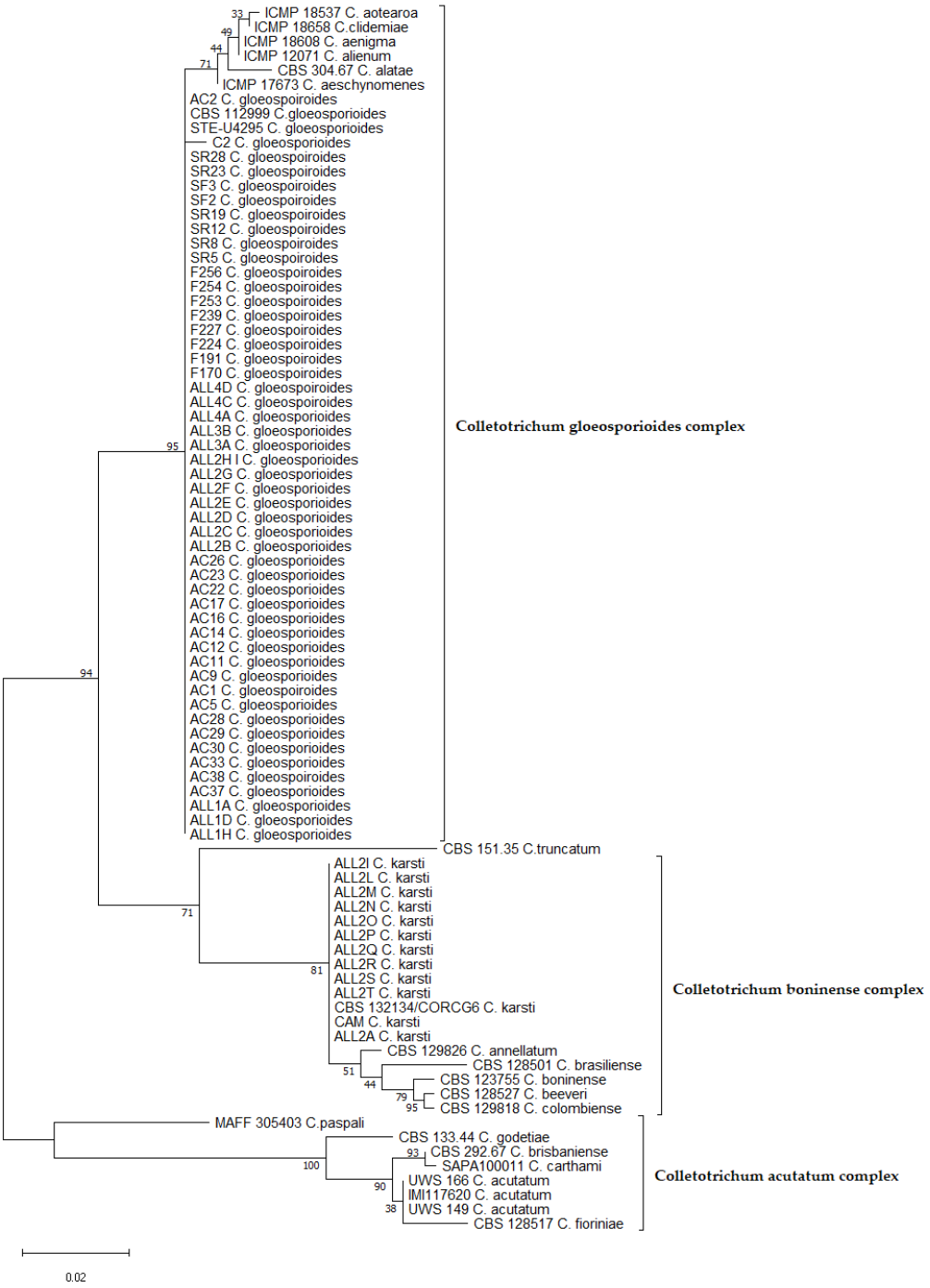

Figure 3. Phylogenetic tree obtained using combined internal transcribed spacers (ITS) and β-tubulin (TUB2) sequences of isolates of *Colletotrichum* spp. collected in the present study along with reference isolates of *C. karsti*, *C. gloeospoiroides*, and other representative species of the other *Colletotrichum boninense, C. gloeosporioides* and *C. acutatum* species complex. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model and the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

In pathogenicity tests on apples, all isolates were pathogenic. However, symptoms induced on apples by isolates of the three tested *Colletotrichum* species were different. Necrotic lesions induced by the *C. gloeosporioides* isolates were dark brown, with a definite margin and black conidiomata emerging from the surface in concentrinc rings (Figure 4 A).

Figure 4. (A) Necrotic lesion induced by a *Colletotrichum gloeosporioides* isolate on a wound inoculated apple seven d.p.i. (B) Necrotic lesion induced by the *Colletotrichum acutatum* reference isolate on a wound inoculated apple seven d.p.i. (C) Necrotic lesion induced by a *Colletotrichum karsti* isolate on a wound inoculated apple seven d.p.i.

Lesions induced by the C. *acutatum* isolate were pale brown with an irregular margin, a faint white aerial mycelium around the inoculation wound and point-like orange masses of conidia scattered over the surface of the lesion (Figure 4 B). Lesions on apples inoculated with *C. karsti* isolates were restricted, dark brown with a faint white mycelim emerging from the wound and without sign of sporulation (Figure 4 C). No significant difference in virulence was found between isolates of the same species irrespective of their origin, so data of all isolates belonging to the same species were pooled together. *C. gloeosporioides* isolates and the reference isolate of *C. acutatum* were significantly more virulent than isolates of *C. karsti* (Figure 5).

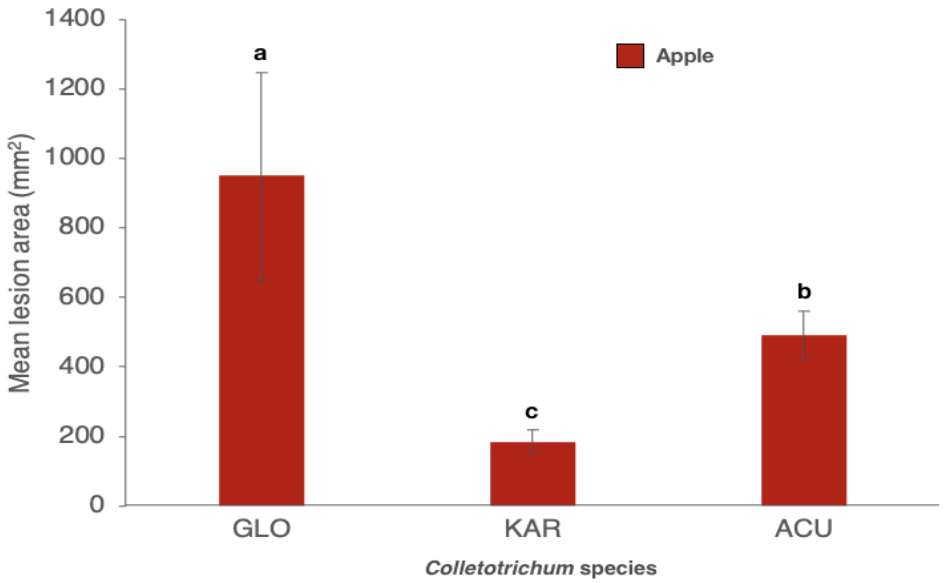

Figure 5. Mean area (± SD) of necrotic lesions (mm²) induced by *Colletotrichum gloeosporioides*[a] (GLO), *C. karsti* (KAR) and *C. acutatum* (ACU) isolates on wound inoculated apples, seven d.p.i. Values sharing same letters are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$)

In in-field tests on twigs of sweet orange, lemon and bergamot the isolates of *C. gloeosporioides* and *C. acutatum* were more aggressive than isolates of *C. karsti*. Isolates of *C. acutatum* and *C.*

gloeosporioides induced gumming in twigs of all three citrus species while isolates of *C. karsti* induced gumming only in bergamot twigs (Figure 6 A, B, C).

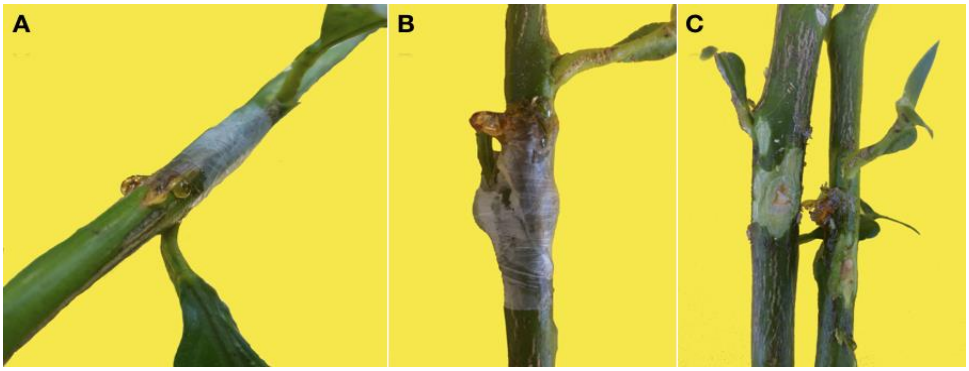

Figure 6. **(A)** Gumming on a twig of lemon 'Femminello 2Kr' wound inoculated with a *Colletotrichum gloeosporioides* isolate 14 d.p.i. **(B)** Gumming on a twig of bergamot 'Fantastico' wound inoculated with a *Colletotrichum gloeosporioides* isolate 14 d.p.i. **(C)** Twigs of bergamot 'Fantastico' wound inoculated with *Colletotrichum karsti* (left) and *C. gloeosprioides* (right) 14 d.p.i. The bark was removed to show the internal symptoms: the twig on the left shows a gum impregnation of the young xylem and a cicatrizial tissue around the inoculation point while the twig on the right shows a profuse gumming extending beyond the inoculation point.

Mean length of necrotic lesion of the bark induced by the *C. gloeosporioides* and *C. acutatum* isolates was significantly higher than the length of lesions induced by the *C. karsti* isolates **(Figure 7)**. In twigs inoculated with *C. karsti* isolates, the necrotic lesion was localized around the inoculation point and the wound healed rapidly. No significant difference in lesion size was observed between sweet orange, lemon and bergamot twigs. Likewise, no significant difference in virulence, as determined on the basis of the size of the necrotic lesion, was observed between isolates of the same *Colletotrichum* species so data of all isolates belonging to the same species were pooled together. On control twigs, inoculation wounds healed rapidly without any symptoms of necrosis or gummosis.

In tests on green and mature fruit as well as on tender and mature leaves *Colletotrichum* isolates were able to induce lesions only after wounding. In pathogenicity tests on mature fruit of sweet orange and lemon, *C. gloeosporioides* isolates were more aggressive than isolates of *C. karsti* even on fruits. Isolates of both species induced a brown necrotic halo around the inoculation wound and the necrosis extended deep into the albedo (Figure 8 A, B), but the mean size of lesions induced by the *C. gloeosporioides* isolates was greater than the mean size of lesions induced by the *C. karsti* isolates on both sweet orange and lemon (Figure 9). No significant difference in virulence, as determined based on the average size of the necrotic lesion, was observed between isolates of the same *Colletotrichum* species so data of isolates belonging to the same species were pooled together (Figure 9).

The reference isolate of *C. acutatum* induced quite peculiar symptoms on both sweet orange and lemon fruit as the inoculation wounds were covered by a white, cottony aerial mycelium, which masked the lesion. The necrosis extended into the albedo and reached its maximum extent 6 d.p.i., but did not expand further (Figure 8 C, D).

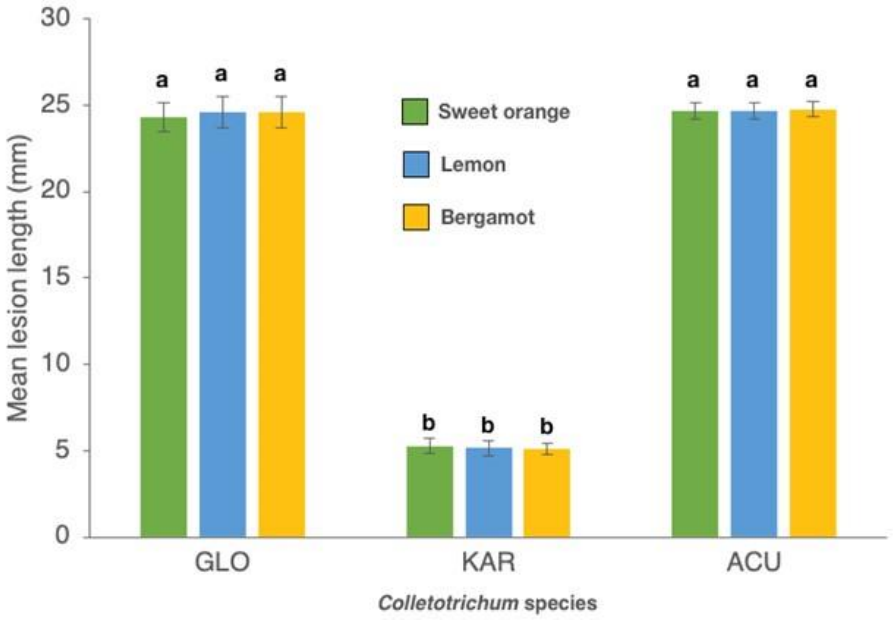

Figure 7. Mean length (mm) of necrotic lesions (± SD) [a] induced by isolates of *Colletotrichum gloeosporioides* [b] (GLO) (five isolates), *C. karsti* [c] (KAR) (three isolates) and *C. acutatum* [d] (ACU) (one isolate) on in-field artificially inoculated twigs of sweet orange 'Scirè VCR', lemon 'Femminello 2kr' and bergamot 'Fantastico', 14 d.p.i. Values sharing same letters are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$). [a] Six twigs per each of three replicated trees. [b] Means of 90 replicates. [c] Means of 54 replicates. [d] Means of 18 replicates

Figure 8. (A) Necrotic lesions around the inoculation point in a fruit of sweet orange 'Tarocco Meli' wound inoculated with an isolate of *Colletotrichum gloeosporioides* 12 d.p.i.; the peel has been removed to show that the necrosis extends into the albedo. **(B)** Necrotic lesions around the inoculation point in a fruit of lemon 'Femminello 2Kr' wound inoculated with an isolate of *Colletotrichum gloeosporioides* 12 d.p.i.; the peel has been removed to show that the necrosis extends into the albedo. **(C)** Aerial mycelium developed on the two inoculation points in a fruit of sweet orange 'Tarocco Meli' wound inoculated with the reference isolate of *Colletotrichum acutatum* 12 d.p.i.; the peel has been removed to show the necrotic lesion extending into the albedo lined by an orange coloured cicatricial tissue. **(D)**. Aerial mycelium growing on the two inoculation points in a fruit of lemon 'Femminello 2Kr' wound inoculated with the reference isolate of *Colletotrichum acutatum* 12 d.p.i.; the peel has been removed to show the necrotic lesion extending into the albedo.

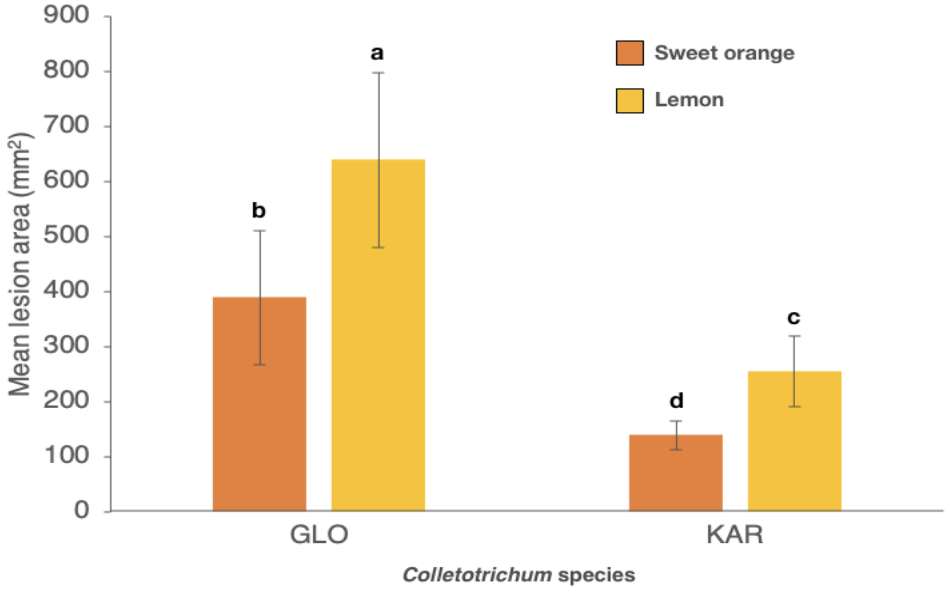

Figure 9. Mean area (mm²) of necrotic lesions (± SD) induced by isolates of *Colletotrichum gloeosporioides*[b] (GLO) (five isolates) and *C. karsti* [c] (KAR) (three isolates) [a] on wound inoculated fruits of sweet orange 'Tarocco Meli' and lemon 'Femminello 2kr', 12 d.p.i. Values sharing same letters are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$). [a] Four fruits for each citrus species and two inoculation wounds per fruit. [b] Means of 40 replicates. [c] Means of 24 replicates.

As a consequence, the area of the external necrotic lesion cannot be measured and direct comparison with isolates of the other two *Colletotrichum* species was not possible in terms of virulence. No symptoms were observed on control fruits.

On green fruitlets of lemon differences of symptoms induced by isolates of the three *Colletotrichum* species were almost exclusively qualitative. All *C. gloeosporioides* and *C. karsti* isolates induced gumming and a very restricted necrotic lesion around the inoculation point (Figure 10 A, C) while fruitlets inoculated with the *C. acutatum* isolate showed gumming and an abundant white aerial mycelium covering the lesion (Figure 10 B). Symtom appeared three d.p.i. and did not evolve further over the next three days. The only symptom on control fruitlets was the necrosis of tissue plug removed temporarily to inoculate the fruitlets and replaced to plug the inoculation wound.

Figure 10. (**A**) Aerial gray mycelium and gum exudate on green fruitlets of lemon 'Femminello 2Kr' wound inoculated with an isolate of *Colletotrichum gloeosporioides* 6 d.p.i. (**B**) Aerial white mycelium and gum exudate on green fruitlets of lemon 'Femminello 2Kr' wound inoculated with the reference isolate of *Colletotrichum acutatum* 6 d.p.i. (**C**) Aerial gray mycelium and gum exudate on green fruitlets of lemon 'Femminello 2Kr' wound inoculated with an isolate of *Colletotrichum karsti* 6 d.p.i.

All *Colletotrichum* isolates induced circular necrotic lesions on young leaves of both 'Navelina' and 'Moro' (Figure 11 A, B, C, D) while the only symptom induced on mature leaves of these two sweet orange cultivars was a translucid, very restricted dark brown halo around the inoculation point. No symtoms were observed on both young and mature control leaves.

Figure 11. (**A**) Necrotic lesions induced by the heterologous *Colletotrichum karsti* isolate from camellia on wound inoculated young leaves of sweet orange 'Navelina' 5 d.p.i. (**B**) Necrotic lesions induced by the heterologous reference isolate of *Colletotrichum acutatum* from olive on wound inoculated young leaves of sweet orange 'Navelina' 5 d.p.i. (**C**) Necrotic lesions induced by an isolate of *Colletotrichum karsti* from citrus on wound inoculated young leaves of sweet orange 'Moro' 5 d.p.i. (**D**) Necrotic lesions induced by an isolate of *Colletotrichum gloeosporioides* from citrus on wound inoculated young leaves of sweet orange 'Moro' 5 d.p.i.

Slight, albeit significant, differences in susceptibility were observed between 'Moro' and 'Navelina', the latter being more susceptible to the infection of aggressive isolates. There were, in fact, significant differences in virulence among the *Colletotrichum* isolates tested. Unespectedly, the two heterologous isolates, i.e. the *C. acutatum* reference isolate from olive and the *C. karsti* reference isolate from camellia, were the most aggressive. Both the *C. karsti* isolates recovered from 'Lane Late' showed an intermediate virulence while the *C. gloeosporioides* isolates were slightly less virulent and did not differ significantly between each other (Figure 12).

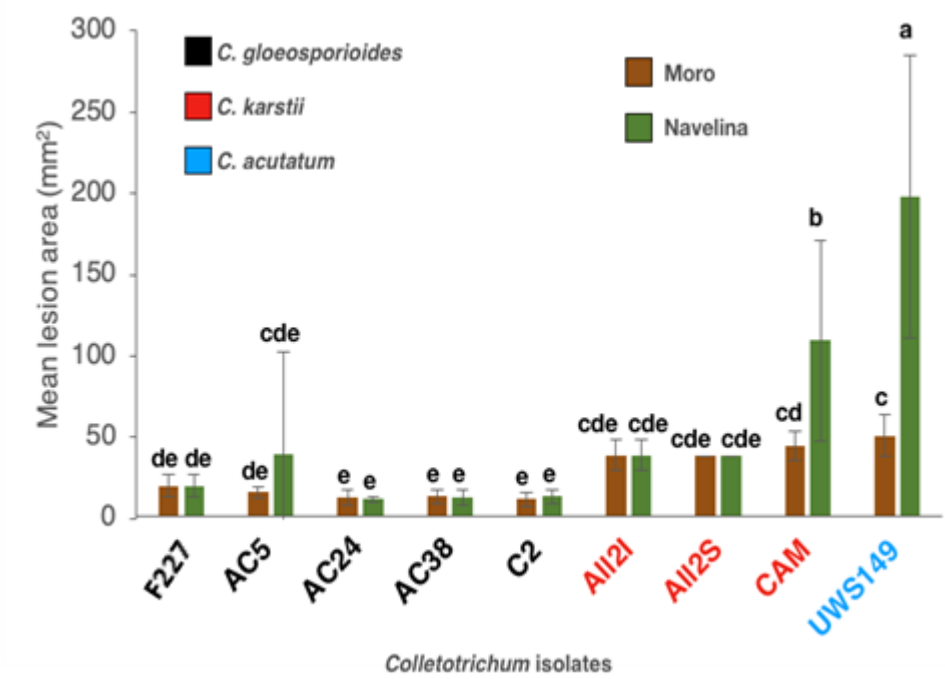

Figure 12. Mean area [a] of necrotic lesions (mm²) (±SD) incited by isolates of *C. gloeosporioides* (Citrus ctr1, AC5, AC24, AC38 and C2), *C. karsti* (All2I, All2S and CAM) and *C. acutatum* (UWS149) on wound inoculated young leaves of sweet orange 'Moro' and 'Navelina', five d.p.i. Values sharing same letters are not statistically different according to Tukey's honestly significant difference (HSD) test ($P \leq 0.05$). [a] Means of 24 replicates (four leaves with six lesions each).

3.5. Discussion

A new disease of citrus, named twig and shoot dieback or Colletotrichum twig and shoot dieback to stress its association with pathogenic *Colletotrichum* species, and recently observed in California, is reported for the first time from Italy, where it was found to be quite common and widespread. According to the modern taxonomy of *Colletotrichum* based prevalently on multilocus sequence phylogeny and consistently with the results obtained in California, the *Colletotrichum* species associated with twig and shoot dieback of citrus were identified as *C. gloeosporioides s.s.*, in the *C. gloeosporioides* species complex, and *C. karsti*, in the *C. boninense* species complex. The results of in-field tests provided evidence that both *Colletotrichum* species were pathogens on twigs of different citrus species, but *C. gloeosporioides* was more aggressive than *C. karsti*, indicating the former species was the main causative agent of twig and shoot dieback in surveyed areas. By contrast, in California *C. karsti* was proved to be more virulent than *C. gloeosporioides* [4]. To explain this discrepancy one can only speculate that populations of *C. gloeosporioides* and *C. karsti* associated to citrus in California are different from populations of these two species from the Mediterranean region. However, no general conclusion can be drawn as in pathogenicity tests performed in California only a single isolate of *C. gloeosporioides* and a single isolate of *C. karsti* were compared [4]. In agreement with our results, previous studies aimed at identifying the *Colletotrichum* species associated to citrus anthracnose in China and

Europe, showed that among the *Colletotrichum* species recovered from citrus groves *C. gloeosporioides* was the most common and the most virulent on detached citrus fruits [20,22,24]. Marked differences in pathogenicity were reported among isolates of *C. gloeosporioides* from citrus sourced in Tunisia as well as among *C. gloeosporioides* isolates from olive sourced in Italy [21,26]. In Portugal, an isolate of *C. karsti* from sweet orange was as virulent as an isolate of *C. gloeosporioides* from lemon when inoculated on sweet orange fruits and significantly less virulent when inoculated on lemon and mandarin fruits [9]. In this study, all tested isolates of *C. gloeosporioides* were more aggressive on twigs than isolates of *C. karsti* irrespective of their origin. *Colletotrichum gloeosporioides*, both in a broad (*C. gloeosporioides* species complex) and in a strict sense (*C. gloeosporioides s.s.*), has a wide host range and is the most common *Colletotrichum* species associated to symptoms of citrus anthracnose globally [7,18–20,24,39–41]. In this study, all isolates of *C. gloeosporioides* recovered from trees with symptom of twig and shoot dieback in Sicily were very aggressive and were able of inducing anthracnose symptoms on fruits and leaves as well as necrosis and gumming on twigs of different citrus species.

Colletotrichum karsti is also a polyphagous species and among the species in the *C. boninese* complex it is the most common and the one reported from a greater number of countries and different geographical areas [13]. In Italy, it was recovered from several host-plants including olive (*Olea europaea*) and there is evidence of it as a citrus inhabitant in southern Italy since the 1990s [21,42]. In this study, isolates of *C. karsti* retrieved from citrus were less virulent than isolates of *C. gloeosporioides* on artificially inoculated fruits and twigs of both sweet orange and lemon. But, isolates of *C. karsti* from citrus demonstrated to be more virulent than *C. gloeosporioides* isolates on young leaves of sweet orange. This is in agreement with previous reports indicating this *Colletotrichum* species is a common foliar pathogen of citrus [9,18–20]. In a previous study aimed at characterizing the *Colletotrichum* species in the *C. gloeosporioides* and *C. boninense* complexes associated with olive anthracnose, *C. karsti* isolates from olive showed a low level of virulence on olive drupes, suggesting this species was an occasional pathogen on olive [21].

Overall the results of this survey do not support the hypothesis of organ specificity as a factor determining the prevalence of a *Colletotrichum* species over another in citrus trees affected by twig and shoot dieback. Probably, the proportion of *C. gloeosporioides* and *C. karsti* isolates recovered in this study and the distribution of these two *Colletotrichum* species in surveyed areas depend on other factors conditioning their fitness and adaptative capacity. In the years following the revision of the systematics of *C. gloeosporioides* and *C. boninense* species complexes that led to the segregation of *C. karsti* as a distinct species within the *C. boninense* complex, *C. karsti* has been increasingly reported from several citrus growing countries, including China, Iran, Italy, New

Zealand, Portugal, South Africa, Tunisia, Turkey and USA, always in association with other *Colletotrichum* species, mainly *C. gloeosporioides* [4,9,18,19,22,23,26,27]. This does not imply necessarily that *C. karsti* is an emerging pathogen *sensu* [3]. It seems more likely that the proliferation of reports of this *Colletotrichum* species on citrus from diverse and distant geographical areas is a consequence of the taxonomic revision of the *C. gloeosporioides* and *C. boninense* species complexes based on multilocus phylogenetic analysis, which provided a framework for a correct identification of already present cryptic species.

In the present study, a third *Colletotrichum* species included as a reference, *C. acutatum s.s.*, was shown to be as virulent as *C. gloeosporioides* on artificially inoculated twigs of different citrus species while on fruits of sweet orange and lemon induced symptoms different from the typical anthracnose. Interestingly, shoot blight is one of the symptoms of KLA, a disease caused by *C. limetticola*, also a species in the *C. acutatum* complex and affecting exclusively Key lime [13,43]. The only report of *C. acutatum s.s* on citrus in Europe is from a small island of the Aeolian archipelago, north of Sicily, where it was recovered from leaves of lemon and sweet orange [24]. Yet this polyphagous *Colletotrichum* species, probably originating from the southern hemisphere, is already established in southern Italy on different host-plants and is replacing *C. godetiae* (syn. *C. clavatum*) as the main causal agent of olive anthracnose in Calabria [44–46]. In addition, there is evidence of its presence on oleander in Sicily since the 2001 [28]. The threat posed by this exotic *Colletotrichum* species as a potential citrus pathogen in the Mediterranean region would deserve particular attention.

The sudden outbreak of twig and shoot dieback in vast areas following winds and prolonged drought presupposes an inoculum already present and widespread throughout the citrus orchards. As a matter of fact, *Colletotrichum gloeosporioides* and *C. karsti*, like many other *Colletotrichum* species, may have different lifestyles. They may be latent pathogens, endophytes, epiphytes or saprobes and switch to a pathogenic lifestyle when host plants are under stress [47]. As stress factors have a fundamental role in triggering the infection by *Colletotrichum* species, twig and shoot dieback may be regarded as a complex disease, a definition also encompassing other emerging citrus diseases, such as dry root rot incited by species of *Fusarium s.l.* [48–50]. A common feature of this type of diseases is the difficulty in reproducing the syndrome in experimental conditions as even wounding may only partially substitute environmental stresses and cannot reproduce alone the effects of different stressors acting simultaneously on host-plant. This may explain the failure in reproducing all field symptoms of Colletotrichum twig and shoot dieback by artificial inoculations [4].

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4. Secondary metabolites produced by *Colletotrichum* spp. on different olive cultivars

Mario Riolo[1,2], Carlos Luz[3], Elena Santilli[4], Giuseppe Meca[3], Santa Olga Cacciola[1]*

[1.] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[2.] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[3.] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Burjassot, Spain

[4.] Council for Agricultural Research and Agricultural Economy Analysis, Research Centre for Olive, Citrus and Tree Fruit-Rende CS (CREA- OFA), 87036 Rende, Italy;

[3.] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

* Correspondence: olga.cacciola@unict.it

4.1. Abstract

This study was aimed to characterize the secondary metabolites produced by four *Colletotrichum* species, *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. karsti*, both *in vitro*, on potato desxtrose agar (PDA) and oatmeal agar (OA), and during the infection process of fruits of four olive cultivars differing in susceptibility to anthracnose, 'Coratina' and 'Ottobratica', both susceptible, 'Frantoio' and 'Leccino', both resistant. The metabolites were extracted from axenic cultures after seven days incubation and from olives at three different times, 1, 3 and 7 days post inoculation (dpi). They were identified using the HPLC-QTOF analysis method. In total, as many as 45 diverse metabolites were identified; of these 29 were detected on infected olives and 26 in axenic cultures on agar media (OA and PDA). Only 10 metabolites were present in both fruits and axenic cultures while 19 were found exclusively on olives and 16 exclusively in axenic cultures. The 45 identified metabolites comprised fatty acid, phenolics, pyrones, sterols, terpenes and miscellaneous compounds. Each *Colletotrichum* species produced a different spectrum of metabolites depending of the type of matrices. On artificially inoculated olives the severity of symptoms, the amount of fungal secondary metabolites and their number peaked 7 dpi irrespective of the cultivar susceptibility and the virulence of the *Colletotrichum* species. The metabolite profiles as represented by heat maps were the result of the interaction olive cultivar x *Colletotrichum* species.

Keywords: *Colletotrichum* spp.; olive cultivars; secondary metabolites; pathogenicity; PCA, heatmap

4.2. Introduction

Anthracnose is a major disease of olive (*Olea europaea*) in many olive growing countries worldwide [1–4]. Several species of *Colletotrichum* are responsible for olive anthracnose (OA). Overall, 18 *Colletotrichum* species have been so far reported to be associated with this disease

worldwide [2]; they vary in virulence and geographical distribution [2,4–7]. Most of these species were segregated from the *Colletotrichum acutatum* (*C. acutatum s.l.*), *C. boninense* (*C. boninense s.l.*) and *C. gloeosporioides* (*C. gloeosporioides s.l.*) species complexes and formally described as separate taxa after the acceptance of multilocus phylogenetic analysis as a taxonomic criterion. *Colletotrichum acutatum* is the most virulent species; as a pathogen responsible for OA it is widespread in the southern hemisphere and is emerging in several olive growing areas of the Mediterranean macroregion, including Albania, Greece, Italy, Portugal, Spain and Tunisia [7,8]. *Colletotrichum nymphaeae* is the dominant *Colletotrichum* species associated with OA in south-western Portugal and was reported as the second most common species associated with OA, after *C. acutatum*, in Uruguay [6,7,9]. It has been found also in other olive producing areas of the Mediterranean macroregion but has not yet been reported from olive producing areas of the southern hemisphere, except for Brazil [4]. *Colletotrichum godetiae*, along with *C. acutatum* is the most common species associated with OA in Greece, Southern Italy and Tunisia. It has been recorded also in Montenegro with the former name of *C. clavatum* and is by far the dominant species associated with OA in Andalusia, the leading olive producing region of Spain [4,7,10]. Other *Colletotrichum* species are weakly pathogenic, occur sporadically or have a more restricted geographical distribution [4,5,7,11]. *Colletotrichum fioriniae* was first reported from some regions of Portugal among the *Colletotrichum* species associated with OA [1] and subsequently as the species responsible for OA outbreaks in Uruguay and California (USA) [4,6]. In the latter country it is also common as causal agent of anthracnose of pistachio (*Pistacia vera*) [4]. Very recently this *Colletotrichu*m species has been reported from southern Italy as causal agent of olive anthracnose in association with *C. acutatum* and *C. godetiae* (Riolo and Cacciola, *Journal of Plant Pathology*, accepted). *Colletotrichum karsti*, like other *Colletotrichum* species associated with OA, is polyphagous [12] but it was proved to be a weak pathogen on olive [5,7]. *Colletotrichum siamense* was found to be associated with OA in Australia and, differently from *C. karsti*, in artificial inoculations on detached olives proved to be aggressive [4,5]. *Colletotrichum theobromicola*, alone or in association with other *Colletotrichum* species, was reported as causal agent of anthracnose in Australia and South America [5,6,13], and in artificial inoculations on detached olives proved to be very aggressive [5]. It has not been reported so far as a pathogen of olive in the northern hemisphere but has been intercepted in Israel as causal agent of fruit anthracnose on avocado (*Persea americana*) and leaf spot on cyclamen (*Cyclamen persicum*) [14,15]. Recently, *C. theobromicola*, along with *C. aenigma*, *C. alienum*, *C. perseae* and *C. siamense*, all already known as pathogens of olive [4,5], have been recognized as noxious organisms of quarantine concern for the European Union [16]. The most typical and economic relevant symptom of OA is fruit rot,

which results in yield losses due to premature fruit drop and in detrimental effects on physicochemical and organoleptic characteristics of the oil produced with infected fruits [1–4,17–23]. The severity of damage caused by OA depends on several factors, including environmental conditions, the virulence of the *Colletotrichum* species involved and the susceptibility of olive cultivar. A great variability in susceptibility to OA among olive cultivars of various geographic origin has been observed [1,2,9,11,24–26]. Among the Italian cultivars 'Frantoio' and 'Leccino' were proved to be relatively resistant while 'Carolea', 'Coratina' and 'Ottobratica' were proved to be very susceptible [7]. Moreover, a significant interaction between olive cultivar susceptibility and *Colletotrichum* species virulence was highlighted [7,9]. For example, 'Picual', a Spanish olive cultivar which is regarded as relatively resistant in the geographical area of origin where *C. godetiae* has been largely prevailing over other *Colletotrichum* species for many years [1,4], was confirmed to be resistant to *C. godetiae* but proved to be very susceptible to *C. acutatum* in laboratory tests on detached fruits [7].

In addition to fruit rot, symptoms of OA include blossom blight, leaf yellowing, wilting defoliation and dieback of twigs and branches [1,6,25]. The very low frequency of pathogen isolation from leaves and branches of adult trees with natural infections of OA, suggested the hypothesis that a single or multiple phytotoxins produced by *Colletotrichum*, could account for the induction of these symptoms on leaves and twigs (as reviewed by Cacciola et al. [1]). This hypothesis was corroborated by Moral et al. [25]. These Authors inoculated young olive plants and noticed that wilting and dieback symptoms developed only on twigs bearing rotten fruits. Twigs without fruits were also infected, but did not show symptoms, suggesting that a diffusible metabolite produced by the fungus in infected fruits is responsible for the symptoms on other organs of the plant [4]. Indeed, Ballio et al. [27] had demonstrated that the causal agent of OA produced *in vitro* aspergillomarasmin B, a phytotoxic derivative of lycomarasmin. Numerous other bioactive secondary metabolites produced *in vitro* by *Colletotrichum* species have been since isolated and identified from culture fluids, they mostly included polyketides, terpenoids, peptides and aminoacid derivatives, alkaloids, derivatives of shikimic acid and metabolites of mixed biosynthetic origin [28–38]. The interest of above mentioned studies has focused mainly on the potential applications of these metabolites either in pharmacology as drugs or in agriculture as natural substances with antifungal and herbicidal activity [32,39]. Several secondary metabolites isolated from culture fluids of *Colletotrichum* species, such as Colletotrichin E, Colletofragarone A1, Colletochlorin A and Aspergillomarasmin showed phytotoxic activity [29,39]. However, after the pioneering study of Ballio et al. [27] no other research has dealt with the characterization of secondary metabolites produced by *Colletotrichum* species infecting olive

fruits, their detrimental effects on the oil quality and their role as pathogenicity or virulence factors in the pathogenesis of OA. This study, aimed at identifying the secondary metabolites produced by diverse *Colletotrichum* species during the infection process of olive fruits, was intended as a contribute to fill this gap. In particular, its aim was to identify the secondary metabolites produced by diverse *Colletotrichum* species in vitro and during the infection process of olive fruits.

4.3. Materials and Methods

4.3.1. Fungal isolates, culture media and production of inoculum

Isolates of four different *Colletotrichum* species, *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. karsti*, were included in this study (Table 1). The isolates were Identified in previous studies based on both morphological characteristics and phylogenetic analysis of the internal transcribed spacer (ITS) ITS1–5.8S–ITS2 region of the ribosomal DNA (rDNA), and part of the β-tubulin gene (TUB2) sequences [7,40].

Table 1. Isolates of *Colletotrichum* used in this study.

Isolate code	Species	Host (species and cultivar)	Organ	Geographical origin	GenBank Accession	
					ITS-rDNA	ß-tubulin 2
UWS149	*C. acutatum*	*Olea europaea*	Fruit	Australia	MT997785	MW001517
C9D2C	*C. acutatum*	*O. europaea*	Fruit	Calabria (IT)	MZ502315	MZ508448
C2	*C. gloeosporioides*	*Citrus* x*limon*	Fruit	Calabria (IT)	JN121211	JN121298
RD9B	*C. gloeosporioides*	*O. europaea*	Fruit	Calabria (IT)	MZ502314	MZ508447
OLP 12	*C. godetiae*	*O. europaea*	Fruit	Calabria (IT)	JN121131	JN121218
OLP 16	*C. godetiae*	*O. europaea*	Fruit	Calabria (IT)	JN121137	JN121224
C12D1A	*C. karsti*	*O. europaea*	Fruit	Sicily (IT)	ON231821	ON246203
CAM	*C. karsti*	*Camellia* sp.	Leaf	Sicily (IT)	KC425664	KC425716

All isolates included in this study were sourced from the culture collection of the Molecular Plant Pathology Laboratory of the Department of Agriculture, Food and Environment of the University of Catania, Italy. Potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, UK) and oatmeal agar (OA; Sigma-Aldrich, St. Louis, Mo, USA), were used as culture media throughout this study. .

A conidial suspension of each isolate (10^6 conidia ml^{-1}) in sterile distilled water (SDW) was prepared from 10-day old cultures grown on PDA at 25 °C and used as inoculum. The concentration of conidia in the suspension was adjusted with a hemocytometer.

4.3.2. Inoculations of olives

Fruits of four olive (*Olea europaea* L.) cultivars ('Coratina', 'Frantoio', 'Leccino' and Ottobratica') differing in their susceptibility to OA were used in this study; they were collected from 15-year-old olive trees in the experimental orchard of CREA OFA (Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus Crops) in the municipality of Rende, province of Cosenza, Calabria region, Italy (DATUM WGS 84, 39°21'59.4" N 16°13'44.4" E). The ripening stage of fruits was evaluated according to the maturity index (MI) of Guzmán et al. [41], with value 0 corresponding to >50% bright green and value 4 to 100% blackish-purple or black. Fruits of the same cultivar were collected from three distinct trees at a medium ripening stage (MI 3-4) and pooled together. They were stored for 3 h inside a refrigerated bag, before being transported to the laboratory and were inoculated the day after collection. Before the inoculation, fruits were surface disinfected by immersion in a 0.5% NaOCl solution for 30 s, rinsed in SDW, blotted dry and placed in incubation trays. Olives were punctured with a sterile needle in an equatorial position and a 20 μl droplet of the conidial suspension (10^6 conidia/ml), prepared as described above, was pipetted onto the surface of the wound. Controls received a 20 μl droplet of SDW. Forty fruits were inoculated per each isolate × cultivar combination, i.e. 200 fruits per cultivar, including the controls. After inoculation, fruits were incubated for 7 days in humid chamber at 23±1 °C, with a photoperiod of 16 h of light and 8 h of dark and 80% relative humidity. Symptom severity on inoculated fruits was rated daily using an empirical scale from 0 to 6 [9] and the relative area under disease progress curve (rAUDPC) was calculated according to Riolo et al. [7]. The experiment was repeated three times. Results of the three experiments were pooled together as they did not differ significantly from each other.

4.3.3. Secondary metabolites analysis by HPLC-QTOF

To extract the secondary metabolites produced by *Colletotrichum* species on olives during the infection process, flesh fragments (5 mm diameter) were sampled from inoculated olives at three different time intervals, i.e. 1, 3 and 7 days post inoculation (dpi). Fragments were excised from around the inoculation site and transferred into 15 ml Eppendorf tubes (three fragments from three distinct olives of the same batch per tube). For each batch, three replicates (tubes) were processed at each sampling time. Pulp fragments were weighted and a volume of methanol (MeOH) was added in a 1:5 (w/v) ratio. The tubes were incubated at room temperature and continuous stirring (150 rpm) for 24 hours. After incubation, the extract was centrifugated at 4,000 rpm for 15 min at 4°C, the supernatant was filtered through a 0.22 μm filter and injected for HPLC–QTOF analysis. All the analyses were performed in triplicate. The extraction of metabolites produced by *Colletotrichum* species on agar media (PDA and OA) was performed with the same method with a few modifications. Fungal isolates were grown in Petri dishes at 23

±1 °C for 7 days. Then dishes were transferred into sterile urine beakers and methanol was added in a 1:2 (w/v) ratio. Then the samples were incubated at room temperature and stirring (150 rpm) for 24 hours. After incubation, the extract was centrifugated at 4000 rpm for 15 min at 4°C, the supernatant was filtered through a 0.22 μm filter and injected for HPLC–QTOF analysis. All the analyses were performed in triplicate. An HPLC Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) was used for the chromatographic analysis, it consisted of an automatic sampler, a binary pump and a vacuum degasser. Injection volume was 5 μL and the analysis was performed in 25 minutes. The separation of the analytes was performed using a Gemini C18 column (50 mm × 2 mm, 110 Å and particle size 3 μm) (Phenomenex, Palo Alto, CA, USA). Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), both with 0.1% formic acid. The elution flow rate was 0.3 mL/min and the elution gradient was as follows: 0 min, 5% B; 30 min, 95% B and 35 min, 5% B. Mass spectrometry analyses were performed using a QTOF (6540 Agilent Ultra High-Definition Accurate Mass, Agilent Technologies, Santa Clara, CA, USA), coupled to an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface operating in positive ion mode. Optimized mass spectrometry parameters included: capillary voltage 3.5 kV; fragment voltage 175 V; drying gas flow (N2) 8 L/min, temperature 350 °C; collision energy 10, 20 and 40 eV, nebulizer pressure 30 psi. Data analysis was performed by MassHunter Qualitative Analysis Software B.08.00 (Agilent Technologies, Santa Clara, CA, USA).

4.3.4. Statistical analysis

To compare the severity of symptoms on olives of different cultivars all the data were normalized by square root transformation and then subjected to analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test as a post hoc test (R software). The analytical data obtained by HPLC-QTOF were log10 transformed before statistical analysis. The secondary metabolites obtained from olive cultivar x *Colletotrichum* species combinations at each sampling time (1, 3 and 7 dpi) were compared using the LSD test at $P \leq 0.05$, after analysis of variance (one-way ANOVA). Relationships among olive cultivar x *Colletotrichum* species combinations were analyzed using Pearson's correlation coefficient analysis. All the above statistical analyses were performed using RStudio v.1.2.5 (R). MetaboAnalyst 5.0 software [42] was used for principal component analysis (PCA) using log10 transformed data. The features included were log transformed and mean centred.

4.4. Results

In artificial inoculation assays *Colletotrichum acutatum* was the most virulent and *C. karsti* the least virulent of the four *Colletotrichum* species tested, as expected (Figure 1).

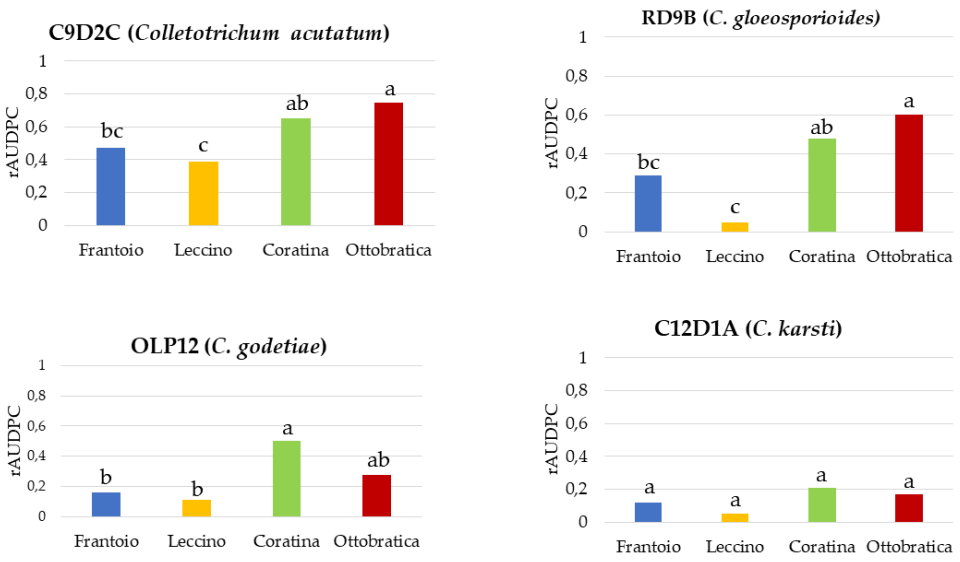

Figure 1. Mean olive anthracnose intensity over time (relative area under the disease progress curve [rAUDPC]) on drupes of nine olive cultivars inoculated with isolates of *Colletotrichum acutatum*, *C. gloeosporioides*, *C. godetiae* or *C. karsti* (one isolate per species). Severity of symptoms on inoculated olive drupes was assessed daily for 7 days and rated on a 0 to 6 scale according to the size of necrotic lesion and the abundance of fungal sporulation. Values sharing a common letter are not statistically different according to Tukey's honestly significant difference (HSD) test ($p \leq 0.05$).

Moreover 'Ottobratica' and 'Coratina' were confirmed to be very susceptible to the infection of *Colletotrichum* species, while both 'Leccino' and 'Frantoio' were once more confirmed to be relatively resistant also to the most aggressive *Colletotrichum* species. No significant difference in susceptibility was noticed among the four olive cultivars inoculated with *C. karsti* as this *Colletotrichum* species was poorly aggressive on all tested cultivars.

Overall, the analysis by HPLC-QTOF revealed 45 secondary metabolites on both fruits of different olive cultivars inoculated singularly with four *Colletotrichum* species and axenic cultures of *Colletotrichum* isolates on agar media (Table 2). Not all secondary metabolites produced on olives were detected in vitro and viceversa. Overall 29 metabolites were detected in extracts from infected olives, 26 in extracts from pure cultures of *Colletotrichum* species on agar media and of these only 10 were found in extracts from both inoculated olives and pure cultures while 19 were detected exclusively on olives. Only 16 metabolites were found exclusively in pure cultures on artificial media; of these four were detected in both OA and PDA extracts, 10 exclusively in PDA extracts and two exclusively in OA extracts. The identification of the metabolites was supported by a purpose-built database, based on the secondary metabolites produced by *Colletotrichum* species reported in the literature (Table 2). The absence of these metabolites in extracts from

control non-inoculated olive fruits confirmed the 45 identified metabolites were of fungal origin (Figure 2 and Figure 3).

Table 2. Secondary metabolites of *Colletotrichum* identified in this study.

Secondary metabolites	Indentificative code	Source[a]	References[b]
(+) - (3R,4S)-cis-4-hydroxy-6-deoxyscytalone	M1	COR, FR, OTT, PDA	[43]
(22E,24R) - 19(10→ 6) - abeoergosta-5,7,9,22-tetraen-3β-ol	M2	COR, FR, LEC, OTT, OA, PDA	[44]
4-Hydroxy-benzamide	M3	PDA	[29]
3b-Hydroxy-ergosta-5-ene	M4	PDA	[44]
Akodionine	M5	PDA	[45]
Aspergillomarasmin B	M6	PDA, OA	[27]
Brevianamide F	M7	PDA	[45]
Collectotrichin C	M8	PDA, OA	[46]
Colletodiol	M9	PDA	[47]
Colletofragarone A1	M10	COR, FR, LEC, OTT, OA, PDA	[48]
Colletoic acid	M11	FR, LEC, OTT	[49]
Colletol	M12	PDA	[35]
Colletotricone B	M13	PDA, OA	[44]
Colletotrilactam A	M14	PDA, OA	[50]
Colletotrilactam C	M15	COR, OTT, PDA	[50]
Diketopiperazines brevianamide F	M16	PDA	[45]
Gloeosporone	M17	PDA	[51]
Hydroheptelidic acid	M18	COR, OTT, PDA	[52]
Colletochlorin E	M19	PDA	[39]
Novae-zelandin A	M20	COR, FR, LEC, OTT, OA, PDA	[53]
Phenethyl 2-hydroxypropanoate	M21	COR, FR, LEC, OTT, PDA	[54]
Pyrenocine B	M22	COR, FR, LEC, OTT, PDA	[55]
Colletotryptin A	M23	PDA	[56]
Colletomellein B	M24	OA	[45]
Monocerin	M25	COR, FR, LEC, OTT, OA	[57]
Fusarentin 6,7-dimethyl ether	M26	OA	[57]
(4R)-4,8-dihydroxy-α-tetralone	M27	COR, FR, LEC, OTT, OA	[58]
3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene	M28	FR, LEC, OTT	[44]
4-Hydroxybenzoic acid	M29	COR, FR, LEC, OTT	[45]
2- (4-Hydroxyphenyl) acetic acid	M30	COR, LEC, OTT	[29]
Altenuene	M31	COR, LEC	[59]
Chermesinone B	M32	COR, FR, LEC, OTT	[60]
Collectotrichin A	M33	COR, FR, LEC, OTT	[46]
Colletoketol	M34	COR, LEC	[35]
Colletonoic acid	M35	COR, LEC	[61]
Colletotrialide	M36	COR, LEC, OTT	[57]
Colletotrichine A	M37	FR	[62]
Hederagonic acid	M38	COR, FR, LEC, OTT	[45]
Monopalmitolein	M39	COR, FR, LEC, OTT	[45]
Pyrenocine N	M40	LEC	[53]
Lumichrome	M41	COR, LEC, OTT	[63]
Monocillin I	M42	OTT	[64]
Colletotrichine B	M43	FR, LEC, OTT	[31]
Pyrenocine O	M44	FR, LEC, OTT	[53]
Piperin	M45	LEC	[65]

[a] Matrices on which the metabolites were identified in this study. COR= Coratina; FR= Frantoio; LEC= Leccino; OTT= Ottobratica; OA= Oatmeal Agar; PDA= Potato Dextrose Agar

[b] Original reports of the metabolites.

The semi-quantitative heat map method made it possible to compare the spectra of secondary metabolites produced by the *Colletotrichum* species on each olive cultivar at three different time intervals after inoculation. In general, the production of secondary metabolites in all olive cultivars inoculated singularly with each of the four *Colletotrichum* species peaked at 7 dpi while the metabolite profiles of non-inoculated controls, i.e. olives treated only with water, at three time intervals after wounding, clustered together, as no detectable amounts of fungal metabolites were recorded on these olives (Figure 2 and Figure 3). At T1 (1 dpi), the metabolite profiles of 'Ottobratica', 'Coratina' and 'Frantoio' olives inoculated with *C. acutatum*, *C. gloeosporioides*, *C. godetiae* or *C. karsti*, showed traces of one or few of the fungal metabolites found in the respective metabolite profiles at T2 (3 dpi) and T3 (7dpi). Conversely, the metabolite profiles of 'Leccino' olives inoculated with *C. karsti*, at both T1 and T2 did not differ from the non-inoculated controls as no amount of fungal metabolites was detected, while the profile of olives of this olive cultivar inoculated with *C. acutatum*, *C. gloeosporioides* or *C. godetiae*, at T1 showed only traces of one (*C. gloeosporioides* and *C. godetiae*) up to a maximum of three (*C. acutatum*) of the fungal metabolites found in the respective metabolite profiles at T2 and T3. In detail, at T3 (7 dpi) in olives of 'Ottobratica' inoculated with *C. acutatum* and *C. godetiae* the most abundant secondary metabolites were M32, M38, M33, M36 and M10. (Figure 2). Monocerin (M25), together with Lumichrome (M41) were recorded on drupes inoculated with *C. acutatum* or *C. karsti*. High amounts of simple phenolic compounds, such as 4-hydroxybenzoic acid (M29) and 2-(4-Hydroxyphenyl) acetic acid (M30), were detected on olives of this cultivar inoculated with *C. acutatum* or *C. godetiae.* At T3, particularly high amounts of M28, M10, M15, M22, M1 and M13 metabolites were detected in olives inoculated with *C. godetiae*, while M27, M39 and M44 were the prevalent fungal metabolites in olives inoculated with *C. gloeosporioides*. At T3 the metabolite M10 (Colletofragarone A1) was detected on all olives inoculated with one of the four *Colletotrichum* species. On olives inoculated with *C. godetiae* this last metabolite was already detected at T1 (1 dpi). As for 'Coratina' (Figure 2), the metabolites M2, M10, M20, M27, M31, M35 and M38 were detected in all the metabolite profiles of olives inoculated with *C. acutatum*, *C. gloeosporioides*, *C. godetiae* or *C. karsti* at T3 (7 dpi) as well as in the metabolite profile of olives inoculated with *C. godetiae*, at T2 (3dpi). All these metabolites, with the only exception of M27, were also detected at T2 in the profile of olives inoculated with *G. gloeosporioides* and *C. karsti*, and all, except M27 and 35, were present in the profile of olives inoculated with *C. acutatum*, at T2. In

the metabolite profile of olives inoculated with *C. godetiae* at T3, two fungal metabolites, M1 [(+) - (3R,4S)-cis-4-hydroxy-6-deoxyscytalone)] and M21 (Phenethyl 2-hydroxypropanoate), stood out for their high amount. In the profile of these olives also the metabolites M22 (Pyrenocine B), M33 (Collectotrichin A) and M43 (Colletotrichine B) were highly represented. Collectotrichin A was extracted in a relatively high amount from olives inoculated with *C. godetiae* even at T2 (3 dpi). The metabolites M22 and M43 were also detected in a relative high amount in the profile of olives inoculated with *C. gloeosporioides*, at T3. The metabolites M30 and M36 were the most abundant in the metabolite profile of olives inoculated with *C. karsti*. In the profile of these olives also the metabolites M15, M29, M34 and M39 were detected in a relative high amount. At T3, the last four metabolites and the metabolite M25 were also abundant in the profile of olives inoculated with *C. gloeosporioides*. The most abundant fungal metabolite in the profile of olives inoculated with *C. acutatum* at T3 was M25 (Monocerin), which at T3 was also present in a relatively high amount in the metabolite profile of olives inoculated with *C. gloeosporioides*. Overall, the highest number of fungal metabolites was detected in the profile of olives inoculated with *Colletotrichum gloeosporioides* and *C. godetiae*.

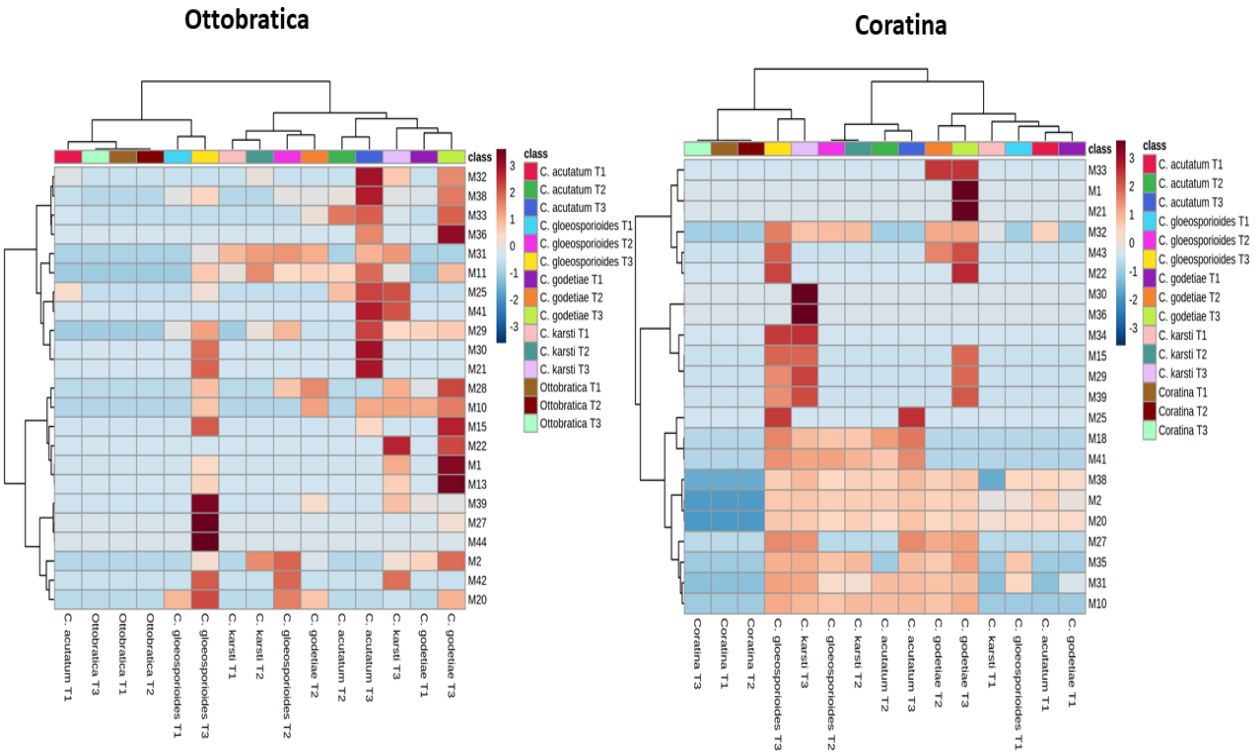

Figure 2. Heat map of metabolites produced by *Colletotrichum* species on olives of 'Ottobratica' and 'Coratina'. Colors indicate the relative abundance (logarithmic scale) of metabolites produced in each olive cultivar x *Colletotrichu*m species combination during the infection process, at different time intervals after inoculation (T1, T2 and T3, corresponding to one, three and seven days post inoculation, respectively).

Brown and blue represent high and low abundance, respectively. Non inoculated olives of each cultivar at T1, T2 and T3 were included as controls.

As for 'Leccino', the most resistant cultivar tested, at T3 (7 dpi) the metabolite profiles of olive batches inoculated with the four *Colletotrichum* species were substantially different as regards the relative abundance of each fungal metabolite (Figure 3). The highest number of metabolites (in total 18 per profile) was found in the profile of olives inoculated with *C. acutatum*, *C. gloeosporioides* and *C. godetiae*, while the profile of olives inoculated with *C. karsti* consisted of only 15 metabolites. The metabolite profile of olives inoculated with *C. acutatum* was characterized by a high amount of M36 (Colletotrialide) and M41 (Lumichrome). Also high amounts of metabolites 29, 35, 43 and 45 were detected. The metabolite profile of olives inoculated with *C. gloeosporioides* was characterized by a high amount of M40 (Pyrenocine N). Other metabolites of this profile present in a relatively high amount included M21, M22, M32, M33 and M44. The metabolite profile of olives inoculated with *C. godetiae* was characterized by a high amount of M30 [2-(4-Hydroxyphenyl) acetic acid]. Other metabolites of this profile present in a relatively high amount included M10, M11 and M35. Finally, the most abundant fungal metabolites in the profile of extracts from olives inoculated with *C. karsti* were M25, M27, M34 and M45.

Also for 'Frantoio', a resistant cultivar like 'Leccino', the metabolite profiles of olives inoculated with the four *Colletotrichum* species at three diverse time intervals after inoculation (T1, T2 and T3, corresponding to 1, 3 and 7 dpi) can be separated into three distinct groups (Figure 3). The first group comprised the profiles of non-inoculated controls at T1, T2 and T3, which did not show the presence of any fungal secondary metabolite. The second group comprised the profile of olive batches inoculated with *C. acutatum*, *C. gloeosporioides*, *C. godetiae* or *C. karsti* at T1, which showed traces of only one or a maximum of four fungal secondary metabolites. The third group comprised the profiles of all other olive batches. Also for this olive cultivar the highest amount of fungal metabolites was detected in olives inoculated with the four *Colletotrichum* species processed 7 dpi (T3). The richest in fungal metabolites (17 in total) was the profile obtained from olives inoculated with *C. acutatum*, at T3. This profile was characterized mostly by high levels of M13 (Colletotrichone B), M21 (Phenethyl 2-hydroxypropanoate), M33 (Colletotrichin A) and M37 (Colletotrichine A). Relatively high amounts of M22, M29, M30, M32 and M40 were also present in this profile. The profile obtained from olives inoculated with *C. godetiae* comprised 15 diverse fungal metabolites and was characterized by high levels of M1 [(+) - (3R,4S)-cis-4-hydroxy-6-deoxyscytalone] and M43 (Colletotrichine B). Relatively high amounts of M11, M27 and M39 were also present in this profile. Similar to the profile associated to the infection by *C. godetiae*,

the metabolite profile from olives inoculated with *C. gloeosporioides* at T3 comprised in total 15 metabolites and only traces of a sixteenth metabolite (M43). The most abundant metabolite of this profile was M22 (Pyrenocyne B). Other metabolites detected in a relatively high amount included M27, M28, M31 and M32. The most abundant fungal metabolite in the profile of olives 'Frantoio' inoculated with *C. karsti*, at T3 was M28 (3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene). The metabolite profile of olives inoculated with *C. karsti* comprised in total 14 diverse metabolites and shared with the profile from olives inoculated with *C. gloeosporioides* 13 metabolites, including M2, M10, M11, M20, M22, M27, M28, M29, M31, M32, M38, M39 and M44.

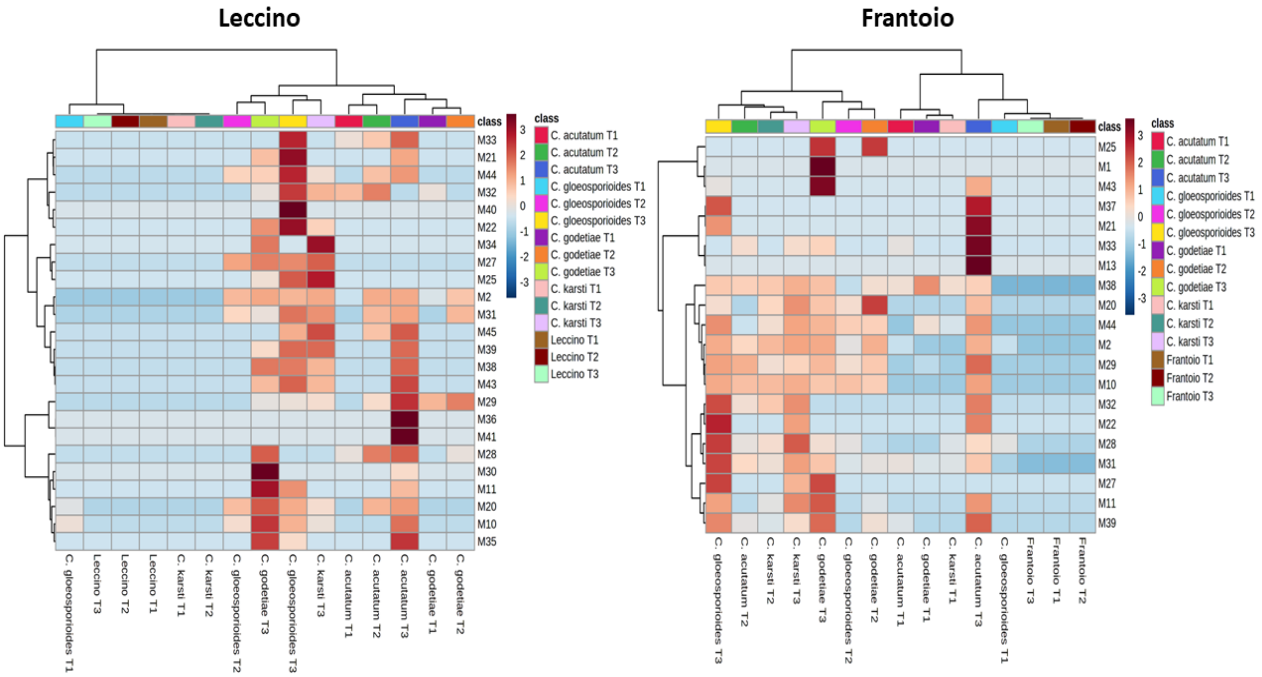

Figure 3. Heat map of metabolites produced by *Colletotrichum* species on olives of 'Leccino' and 'Frantoio'. Colors indicate the relative abundance (logarithmic scale) of metabolites produced in each olive cultivar x *Colletotrichu*m species combination during the infection process, at different time intervals after inoculation (T1, T2 and T3, corresponding to one, three and seven days post inoculation, respectively). Brown and blue represent high and low abundance, respectively. Non inoculated olives of each cultivar at T1, T2 and T3 were included as controls.

In order to evaluate the production of secondary metabolites by various *Colletotrichum* species on different olive cultivars, a PCA analysis of data was performed, based on the secondary metabolites identified (Table 2) on olive cultivars inoculated with the four diverse *Colletotrichum* species, at three distinct time intervals after inoculation (T1, T2 and T3).

Figure 4 shows the clustering of the inoculated olives at different time intervals (score plot) and the metabolite trends (loading plot) in different quadrants (I, II, III, IV); the sum of the principal

component values reached 61.8% of the total variance. PC1 represented 44.5% and PC 2 17.3% of the total variance.

In the score plot of the cultivar Ottobratica (Figure 4), the value 0 was recorded for all samples at both T1 (1 dpi) and T2 (3 dpi), while each group was split at T3 (7 dpi), based on the secondary metabolites produced. Samples infected by *Colletotrichum acutatum* (Ca_1,2,3_T3), *C. karsti* (Ck_1,2,3_T3) and *C. godetiae* (Cgo_1,2,3_T3) clustered in the quadrant II. Samples infected by *C. gloeosporioides* (Cg_1,2,3_T3), with the only exception of replicate 1, tend towards quadrant IV. In the loading plot, metabolites M44, M39, M20 and M42 tend towards quadrant IV, clustering the *C. gloeosporioides* samples, at T3 (7 dpi). By contrast, metabolites M32, M33, M41, M25, M36, M38, M22, M11, M19, M1and M29 cluster the samples infected by *C. acutatum*, *C. godetiae* and *C. karsti* at T3 (Figure 4). As for the cultivar Coratina, the sum of the principal component values reached 67% of the total variance. PC1 represented 48.4% and PC 2 18.6% of the total variance. On the score plot, drupes inoculated with *C. godetiae* at T2 and T3 were grouped in the II quadrant. By contrast, olives inoculated with *C. karsti*, *C. gloeosporioides* and *C. acutatum* at T3. clustered in the IV quadrant. The rest of the samples tended towards the centre. The loading plot indicates that the secondary metabolites M21, M35, M10, M38, M34, M30, M18 and M41 clustered the samples at infected by *C. karsti*, *C. acutatum* and *C. gloeosporioides*, at T3. The rest of the samples cluster around the value 0 (Figure 5).

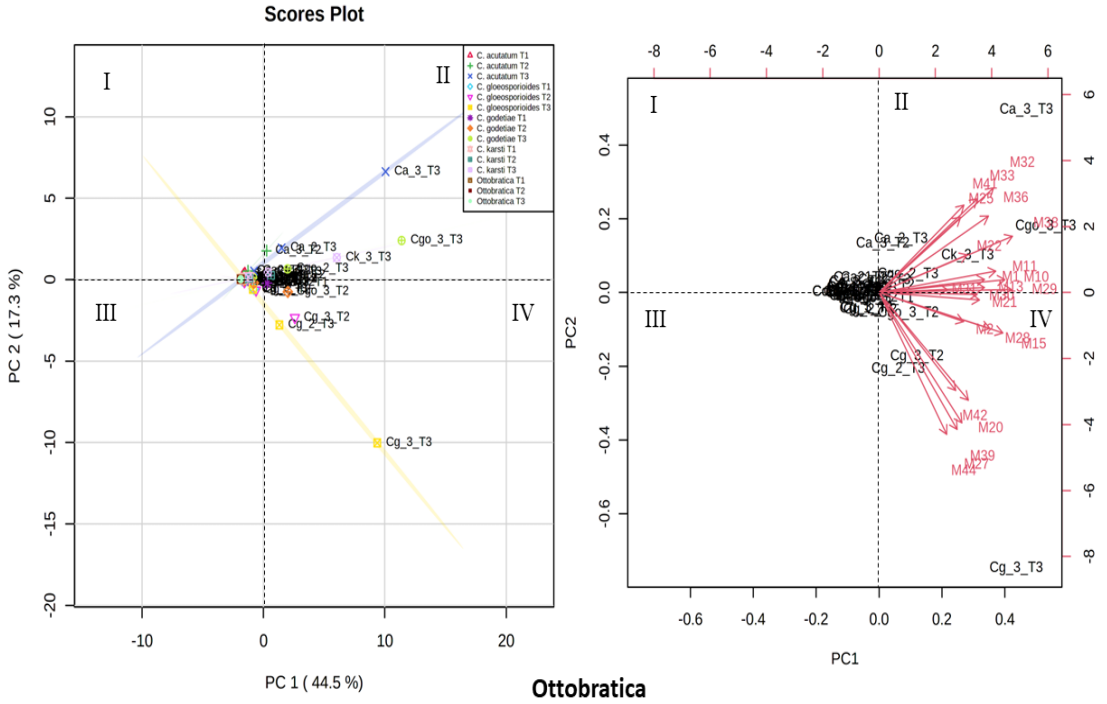

Figure 4. Principal Component Analysis (PCA), scores plot (a) and loading plot (b), based on secondary metabolites produced by *C. acutatum* (Ca), *C. gloeosporioides* (Cg), *C. godetiae* (Cg) and *C. karsti* (Ck) on the cultivar Ottobratica at different time intervals after inoculation (T1, T2 and T3).

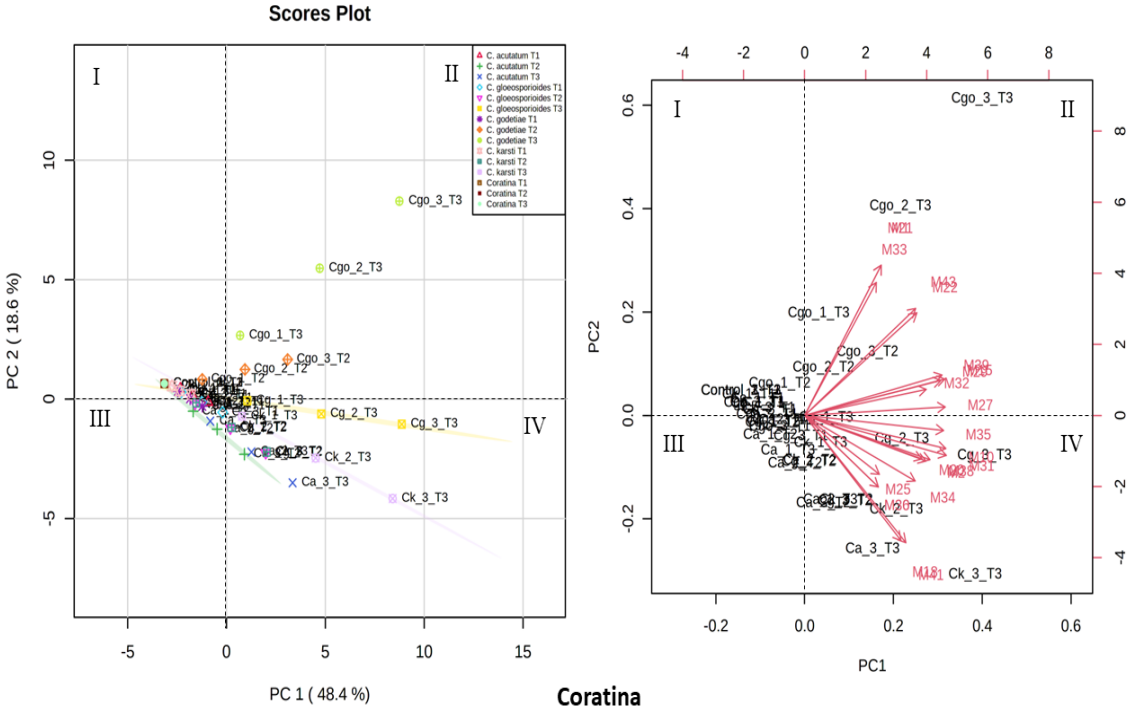

Figure 5. Principal Component Analysis (PCA), scores plot (a) and loading plot (b), based on secondary metabolites produced by *C. acutatum* (Ca), *C. gloeosporioides* (Cg), *C. godetiae* (Cg) and *C. karsti* (Ck) on the cultivar Coratina at different time interval after inoculation (T1, T2 and T3).

The PCA of the Frantoio cultivar recorded a sum of components equal to 71.4% of the total variance, of which PC1 represented 54.2% and PC2 17.2%. Analysing the score plot in Figure 6, samples inoculated with *C. godetiae* at T2 and T3 cluster within quadrant II, while olives inoculated with *C. gloeosporioides* and those inoculated with *C. acutatum*, at T3 cluster in quadrant IV. The rest of the samples, at T1 and T2, tend to cluster around the 0 point. As regards the direction of the secondary metabolites in the loading plot, M11 and M25 tend to cluster the samples infected by *C. godetiae* (C.go_1,2,3_T2,T3) at T2 and T3 towards quadrant II. By contrast, secondary metabolites M38, M2, M11, M44, M39, M10, M31 and M29 cluster samples infected by *C. karsti* at T3 in quadrant II. Morover, secondary metabolites M28, M33, M22, M32, M13, M21 and M37 tend towards quadrant IV, clustering the samples inoculated with *C. acutatum* (Ca_1,2,3_T3) and *C. gloeosporioides* (Cg_1,2,3_T3) at T3 (Figure 6).

Finally, as regards the cultivar Leccino, it presented a sum of components equal to 69.1% of the total variance, with PC1 and PC2 percentage values of 52.2 and 16.9, respectively (Figure 7). Analysing the quadrants of the score plot, samples inoculated with *C. acutatum* and *C. godetiae* at T3 clustered in quadrant II, differently from olives inoculated with *C. karsti* and *C. godetiae* at T3, which grouped in quadrant IV. Conversely, olives inoculated with *C. godetiae* at T2 clustered in

quadrant I. Olives inoculated with *C. gloeosporioides* at T2 and *C. acutatum* at T1 grouped in quadrant III. Loading plot analysis revealed that the secondary metabolites M36, M41, M28, M29, M35, M30, M10, M11, M20, M43 and M38 tended towards quadrant II, grouping the drupes inoculated with *C. acutatum* and *C. godetiae* at T3, whereas olives inoculated with *C. karsti* and *C. gloeosporioides* at T3 grouped on the basis of secondary metabolites M38, M45, M39, M31, M44, M21, M34, M27, M22, M32, M40 and M25.

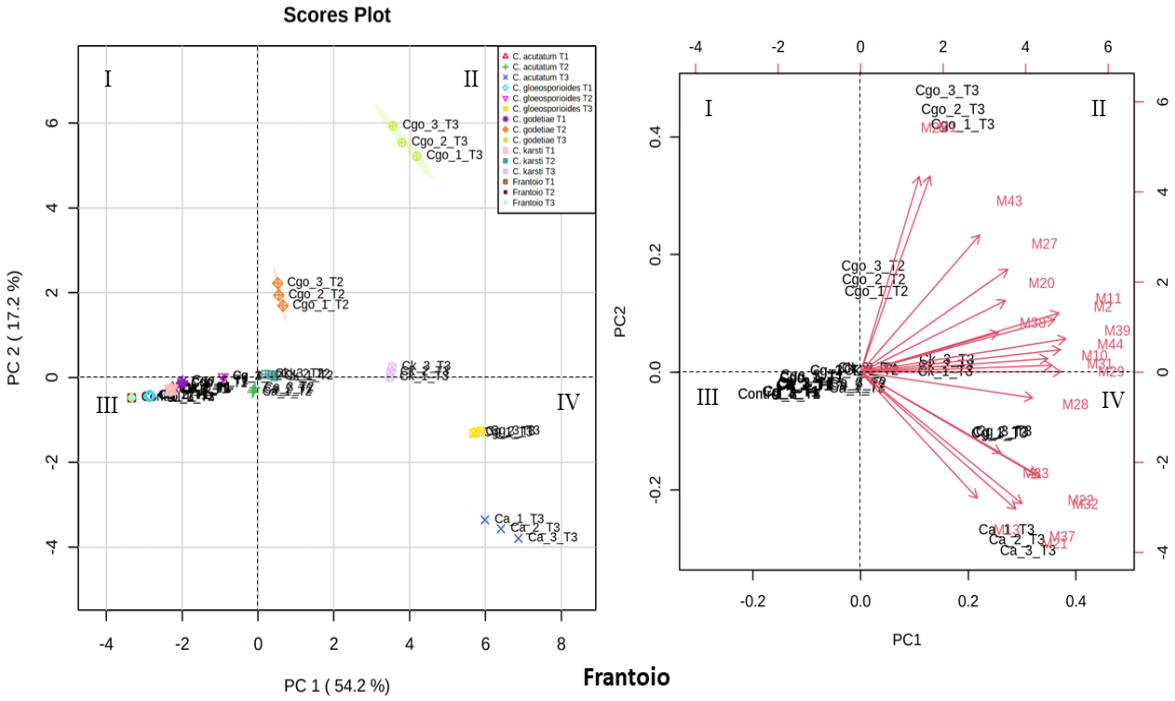

Figure 6. Principal Component Analysis (PCA), scores plot (a) and loading plot (b), based on secondary metabolites produced by *C. acutatum* (Ca), *C. gloeosporioides* (Cg), *C. godetiae* (Cg) and *C. karsti* (Ck) on olives of the cultivar Frantoio at different time intervals after inoculation (T1, T2 and T3).

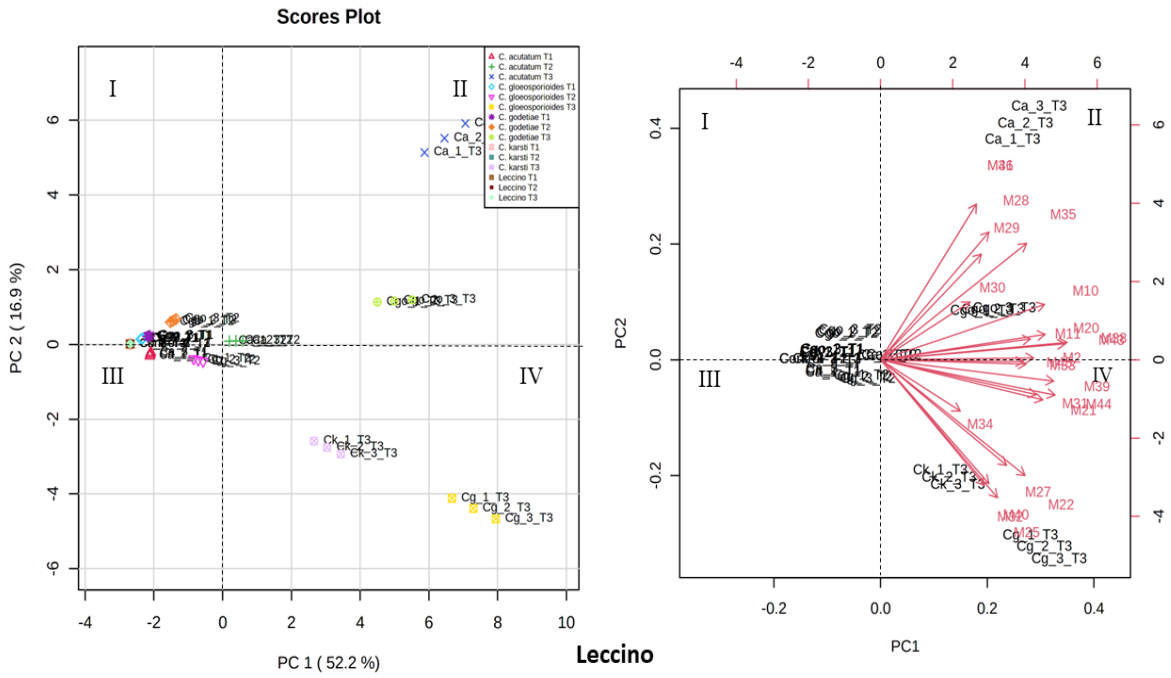

Figure 7. Principal Component Analysis (PCA), scores plot (a) and loading plot (b), based on secondary metabolites produced by *C. acutatum* (Ca), *C. gloeosporioides* (Cg), *C. godetiae* (Cg) and *C. karsti* (Ck) on olives of the cultivar Leccino at different time intervals after inoculation (T1, T2 and T3).

The metabolites produced by *C. acutatum*, *C. gloeosporioides*, *C. godetiae* and *C. karsti* on olives of different cultivars were compared with those produced by these four species of *Colletorichum* on agar media (PDA and OA). On PDA, heat map analysis revealed abundant production of the metabolites M4 and M8 by *C. karsti* isolates (CAM and C12D1A, respectively). In contrast, the secondary metabolites M3 and M12 were only produced by *C. karsti* isolate C12D1A sourced from olive drupes. Medium to high values of the secondary metabolites M19, M5, M13 and M14 were recorded from *C. godetiae* isolates OLP10 and OLP12. M14 was also detected in medium to high quantities in the extracts of the *C. karsti* isolate C12D1A. The metabolites M5, M13, M17 and especially M1 were produced from the *C. acutatum* isolate UWS149. Both *C. acutatum* isolates produced in abundance the metabolites M23, M18, M9, M15 and M20. In contrast, the *C. gloeosporioides* isolates were characterized by the production of M2, M6, M21, M22, M17, M20 and M19. On Oatmeal agar (OA), a lower number of metabolites were produced than on PDA and some of these metabolites were different from those on PDA. *Colletotrichum karsti*, for example, produced M13 on OA but not on PDA. Conversely, both isolates of *C. gloeosporioides* produced M20 on PDA but not on OA. This secondary metabolite was also produced by isolates of *C. karsti* on OA (Figure 8).

In detail the only metabolites produced by *C. gloeosporioides* on OA were M6 and M25. By contrast, both species of *C. karsti* on OA produced abundantly the metabolites M26, M8, M2 and M24, *Colletotrichum acutatum* producesd more metabolites on PDA, while *C. karsti* produced more metabolites on OA (Figure 8).

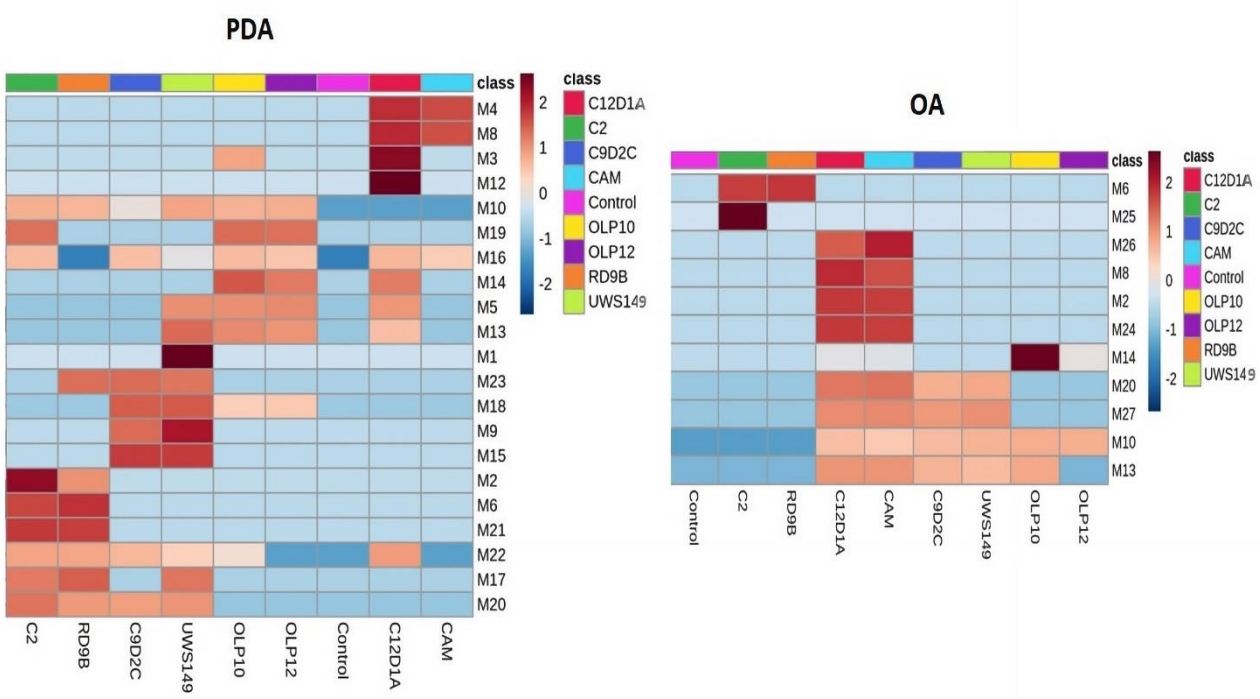

Figure 8. Heat map of metabolites produced by *Colletotrichum* species in axenic cultures on two diverse agar media (PDA and OA). Colors indicate the relative abundance (logarithmic scale) of metabolites produced in each medium. Brown and blue represent high and low abundance, respectively.

4.5. Discussion

This study is the first to characterize the secondary metabolites released by diverse *Colletotrichum* species associated with OA both *in vitro* and during the infection process of olive fruits. It showed that these fungal pathogens produce a wide array of secondary metabolites both *in vitro* and *in planta*. In total, 45 diverse metabolites were identified; of these 29 were detected on infected fruits, 26 in axenic cultures and 10 on both fruits and axenic cultures. Overall, 10 out of 26 secondary metabolites produced *in vitro* were found exclusively in axenic cultures grown on PDA, while two were found exclusively in axenic cultures on OA and four on both culture media, indicating the metabolite profile of *Colletortrichum* species was strongly influenced by the matrice. Each *Colletotrichum* species produced a different spectrum of metabolites. Moreover the metabolite profile of each species varied both qualitatively and quantitatively depending of the type of matrices. Substantial differences in the metabolic profile of the same *Colletotrichum* species were observed in extracts from both different artificial culture media and olive cultivars. The

secondary metabolites identified on olive fruits inoculated with *Colletotrichum* included compounds with phytotoxic and/or cytotoxic activity such as Chermesinone B and Colletonoic acid, produced in a relatively high amounts in olives infected by *C. acutatum* and *C. godetiae* and possessing antibacterial, antifungal and antialgal properties [60,61]. Other secondary metabolites with phytotoxic and/or cytotoxic activity identified in this study, included Colletofragarone A1 and Pyrenocine B, also among the secondary metabolites produced by *C. godetiae* on infected olives, which according to the literature are phytotoxic [31,53], Pyrenocine O, produced by *C. gloeosporioides* on olives of 'Ottobratica' and belonging to a class of compounds with several biological properties, including phytotoxicity, cytotoxicity and antifungal efficacy [53], Pyrenocine B and Colletotrichine B, produced in abundance by both *C. gloeosporioides* and *C. godetiae* on olives of 'Coratina' and known for their phytotoxicity [31,53], the polyketide metabolite Monocerin produced by *C. godetiae* on olives of 'Leccino' and reported originally as a phytotoxic metabolite produced by the fungus *Exserohilum turcicum* (formerly known as *Helminthosporium turcicum*), the causal agent of northern corn leaf blight disease in maize [66]. This corroborates the hypothesis of an involvement of secondary metabolites of *Colletotrichum* species in the pathogenesis of OA. Furthermore, if it were demonstrated that biologically active fungal secondary metabolites produced on infected olives would contaminate the oil during the extraction process this might have toxicological implications. Examining the variability of secondary metabolite profiles resulting from the interaction *Colletotrichum* species x olive cultivar and comparing the differences between susceptible and resistant cultivars or between aggressive and less virulent *Colletorichum* species, no obvious difference in the ability of producing specific metabolites can be correlated with the symptom severity. By contrast, substantial differences were observed in the dynamics of metabolite profiles in resistant ('Leccino' and 'Frantoio') and susceptible ('Ottobratica' and 'Coratina') olive cultivars, irrespective of the *Colletotrichum* species involved. Not surprisingly, the amount and the number of metabolites produced in infected olives increased dramatically over the course of infection but this process has been considerably slowed down in resistant olive cultivars. These trends were confirmed my multicomponent analysis. In the light of the results of this study showing that several different bioactive secondary metabolites are produced on olives infected by *Colletotrichum* species the assumption that Aspergillomarasmin produced in rotten fruits is responsible for the symptoms on twigs and leaves [4] appears as an oversimplification. As a matter of fact, Aspergillomarasmin, first identified by Ballio et al. [27] in culture fluids of *C. gloeosporioides s.l.*, in this study was extracted (in the form of Aspergillomarasmin B) in high quantity from axenic cultures of *C. gloeosporioides* on both PDA and OA but it was never found, even in trace amounts, in the olives infected by

Colletotrichum species irrespective of the cultivar and the *Colletotrichum* species tested, suggesting this toxin is not a key factor in the pathogenesis of OA. Overall, results of this study highlight the diversity and complexity of biochemical interactions between *Colletotrichum* species and olive and stress the importance of *in planta* studies to get a better insight into the pathogenesis mechanisms of OA.

Author Contributions: conceptualization, S.O.C., G.M, C.L. and M.R.; methodology, C.L., G.M., and M.R.; analysis of results C.L., and M.R.; resources S.O.C., E.S. and G.M.; original draft preparation, M.R. and S.O.C.; editing., M.R. and S.O.C.; supervision, S.O.C., G.M. and E.S. All authors have read and agreed to publish this version of the manuscript.

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5. First report of root rot caused by *Phytophthora bilorbang* on *Olea europaea* in Italy

Elena Santilli[1,†], Mario Riolo[1,2,3,†], Federico La Spada[2], Antonella Pane[2*], Santa Olga Cacciola[2]

[1] Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus crops (CREA- OFA), 87036 Rende, Cosenza, Italy;

[2] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[3] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[*] Correspondence: olga.cacciola@unict.it (S.O.C.); apane@unict.it (A.P.).

[†] These authors are equally contributed.

5.1 Abstract

Leaf chlorosis, severe defoliation and wilt associated with root rot were observed on mature olive trees cv. Nera di Gonnos in an experimental orchard at Mirto Crosia (Calabria, southern Italy). An oomycete was consistently isolated from rotten roots of symptomatic olive trees. It was identified as *Phytophthora bilorbang* by morphological characters and sequencing of Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA). Pathogenicity was verified by inoculating potted two-month-old rooted cuttings of *Olea europaea* var. Nera di Gonnos in a soil infestation trial. *P. bilorbang* was re-isolated from roots of symptomatic, artificially inoculated olive cuttings to fulfill Koch's postulates. This is the first report of *P. bilorbang* on *O. europaea* L. and on a species of the Oleaceae family worldwide.

Keywords: Oomycete; pathogenicity, phylogenetic analyses; olive; first report

5.2 Introduction

Olive (*Olea europaea L.*, family Oleaceae) originated in the Near East and spread westward in the Mediterranean basin where it is widely cultivated as a fruit tree[1,2]. Based on data from the International Olive Oil Council (IOOC), more than 10 million hectares are cultivated with olive globally and 95% of them are in the Mediterranean basin [3]. With ca. 1.1 million ha of olive groves, Italy is the second largest olive growing country in the world, with Apulia, Calabria, and Sicily regions of southern Italy accounting for about 70% of the production [4]. As a typical Mediterranean plant, olive has been traditionally cultivated in arid lands. However, during the last decades in many olive growing countries, including Italy, the olive cultivation has been extended to different types of soil and irrigation has become a common practice in olive orchards.

An emerging phytopathological problem of olive trees growing in wet or waterlogged soils is root rot caused by *Phytophthora* spp. [5].

The genus *Phytophthora* (*Pythiaceae*, Peronosporales, Oomycota, and Chromista) comprises more than 180 described taxa[6]. With the advent of DNA sequencing the systematics of the genus evolved from morphological criteria to molecular phylogeny. Therefore, the species of *Phytophthora* were identified using molecular markers and grouped into 12 phylogenetic clades some of which have subclades[7–10]. Several *Phytophthora* species of different phylogenetic clades have been reported as causative agents of leaf chlorosis, wilting, defoliation, and twig dieback, as a consequence of root rot and basal stem cankers on olive worldwide, including *Phytophthora acerina, Phytophthora cactorum,*

Phytophthora cinnamomi, Phytophthora citricola sensu lato, Phytophthora cryptogea, Phytophthora drechsleri, Phytophthora inundata, Phytophthora megasperma, *Phytophthora nicotianae, Phytophthora oleae, Phytophthora palmivora, Phytophthora pini,* and *Phytophthora plurivora* [5,11,20,21,12–19]. These species differ in aggressiveness, temperature requirements for growth, geographical distribution, and ecology. *Phytophthora palmivora*, alone or in association with *Verticillium dahliae*, was reported as causal agent of rot of fine roots and wilt of young olive trees in nurseries and new plantings in Italy [12,22]. *Phytophthora oleae* was recovered from soil and roots of wild olive trees in protected natural areas in Spain and Sicily (southern Italy) and is widespread in soil of commercial olive orchards in Calabria (southern Italy) [20,23]. *Phytophthora inundata* is responsible for root rot and wilt of olive trees in clay soils after flooding, acting as an opportunistic albeit aggressive root pathogen [15,18]. In moist environments, some soil-inhabiting species, like *P. nicotianae* and *P. oleae* have occasionally adapted to an aerial lifestyle and may infect aboveground parts of olive trees such as drupes, leaves, and twigs causing fruit rot, leaf drying, filloptosis, and twig dieback [24,25]. The main aim of the present study was to identify and characterize the causative agent of root and crown rot in olive tree of the cv. Nera di Gonnos in Calabria.

5.3. Materials and Methods

5.3.1. Isolation and morphological identification of isolates

In autumn 2019, symptoms of defoliation, wilt, and root rot were observed on 15-year-old olive trees in an experimental orchard at Mirto Crosia, in Calabria (Figure 1a). Trees were watered in summer using a drip irrigation system. About 40% of the trees of the cv. Nera di Gonnos, originating from Sardinia (southern Italy), were affected. Symptoms were indicative of *Phytophthora* root rot (PRR). Necrotic fine roots were sampled from three distinct symptomatic olive trees cv. Nera di Gonnos in the experimental orchard at Mirto Crosia characterized by a soil

with silty loam texture (Geographic Coordinates (DATUM WGS 84) 39°61′59.0″ N, 16°76′11.4″ E, Cosenza, Calabria, southern Italy). Roots were thoroughly washed in tap water, superficially disinfected in 1% NaClO for 2 min, then immersed in 70% EtOH for 30 s, rinsed in sterile distilled water, dipped dry, and plated on selective PARPNH V8-agar [26]. After an incubation period of 24–48 h in the dark at 25° C, pure cultures were obtained by transferring outgrowing single hyphae onto V8-juice agar (V8A) [11].

Purified cultures were finally obtained by single hyphal culture on V8-agar. Colony morphology and morphological features of isolates, including the morphology and dimensions of reproductive structures, were determined on colonies grown on V8A at 20–22 °C in the dark according with standard procedures [11]. Sporangia production was stimulated following the method described by Jung et al. [27]. Small fragments (size 2 mm) were cut from the growing edge of 5 to 7-d-old cultures grown in Petri dishes (15 mm diam.) on V8A at 20 °C in the dark, they were placed in a 5 cm diameter Petri dish and flooded with non-sterile soil extract water (200 g soil suspended in 1 L of de-ionized water for 24 h at room temperature and then filtered). After incubation at 20 °C in the dark for 24–72 h, dimensions, and morphological features of 50 mature sporangia of each isolate were determined at x400 magnification

5.3.2. Molecular identification of isolates

Species were molecularly identified by the amplification and analysis of Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA). To this aim, total DNA was extracted from 7-d-old cultures grown on V8-agar at 20 °C by using the PowerPlant® Pro DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. The PCR amplification was performed by using the primer pairs ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) [28] and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [29] in a 25 µl reaction mix containing PCR Buffer (1X), dNTP mix (0.2 mM), MgCl2 (1.5 mM), forward and reverse primers (0.5 µM each), Taq DNA Polymerase (1 U),

and 100 ng of DNA. The thermocycler conditions were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min. Amplicons were detected in 1% agarose gel and sequenced in both directions by an external service (Macrogen, Amsterdam, The Netherlands). All the sequences were analyzed by using FinchTV v.1.4.0. Species identification was performed by blast searches in GenBank and in a local database containing sequences of ex-type or key isolates from published studies. Isolates were assigned to a species when the respective consensus sequence was between 99 and 100% identical to that of a reference isolate. Validated sequences representative of *Phytophthora* species identified within the ITS clade 6 were phylogenetically analyzed. Sequences from ex-type or authentic culture were

included in the analysis as a reference [30]. Phylogenetic analysis was conducted for the ITS sequences by the maximum likelihood method, based on the Tamura–Nei model (the software MEGA-X was used).

5.3.3. Pathogenicity test

Pathogenicity of *Phytophthora* specie identified, isolated from roots of *O. europaea*, was tested using a soil infestation method according to Jung et al. (2017) [7]. The isolate CBS 146531 (GenBank accession number MT103546), sourced from symptomatic olive trees cv. Nera di Gonnos in an experimental orchard at Mirto Crosia, was used in pathogenicity tests. Ten potted 2-month-old rooted cuttings of olive cv. Nera di Gonnos were transplanted into free-draining pots containing a mixture of autoclaved universal potting soil (©Cifo Srl, Giorgio di Piano, Bologna, Italy) and inoculum (20 cm^3 of inoculum per 1000 cm3 of potting mixture). Inoculum consisted of a 21-day-old culture of the isolate CBS 146531 grown in the dark at 25 °C in a 750 mL jar containing a sterilized medium made of 50 mL of wheat seeds and 50 mL V8-juice broth. Ten control plants were transplanted in free-draining pots containing non-infested potting mixture. After transplanting, all plants were maintained in saturated soil for 48 h and then transferred to a growth chamber at 23 °C, 80% relative humidity, and a photoperiod of 16 h of light and 8 h of dark. The trial was considered concluded when inoculated plants showed severe symptoms of decay (6 weeks post inoculation). At the end of the test, the isolate was re-isolated from necrotic roots using the selective PARPNH V8-agar and sequenced.

Symptoms were assessed visually in accordance with Engelbrecht et al. [31]. The wilting categories reported were 1) normal (no signs of wilting or drought stress), 2) slightly wilted (slight leaf angle changes but no folding, rolling, or changes in leaf surface structure), 3) wilted (strong leaf angle change but no cell death), 4) severely wilted (very strong change of leaf angle or protrusion of veins on the leaf surface with beginning necrosis), 5) nearly dead (most leaves necrotic, some young leaves still green near the midrib, leaf angles mostly near 0°), and 6) dead (all above-ground parts dead). Data from pathogenicity test were analyzed by a two sample t-test performed by using the software RStudio for $P = 0.01$.

5.4. Results

Isolations from rotten roots of symptomatic olive trees sampled in the experimental orchard of Mirto Crosia found consistently a homothallic *Phytophthora* taxon with a notable colony morphology (Figure 1c, d). Eighteen single-hypha isolates of this *Phytophthora* taxon, obtained from three independent trees (six from each tree), were characterized. They formed stellate to petaloid colonies on V8 juice-agar (V8A) and dense-felty, chrysanthemum-like and dome shaped

in the center colonies on potato-dextrose-agar (PDA). Extreme temperatures for growth were 4 (minimum) and 32 °C (maximum), with an optimum at 25 °C. Sporangia formed on V8A were persistent, non-papillate, limoniform to ellipsoid and internally proliferating. Their dimensions were 45.0 to 55.0 × 25.2 to 30.2 μm, with a mean length to breadth ratio of 1.8. Globose oogonia (diameter ranging from 27.3 to 32.0 μm), paragynous antheridia and plerotic oospores (diameter ranging from 28.2 to 35.3 μm) with a thick wall (2.5 to 3.0 μm) were also observed in single cultures on V8A.

Amplification and sequencing of Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) of all 18 isolates revealed 100% identity with the sequence of *Phytophthora bilorbang* ex-type CBS 161653 (GenBank Accession Number JQ256377 [30]). The phylogenetic analysis of the combined data set of sequences from ITS region of 10 out of 18 isolates recovered from olive at Mirto Crosia along with reference sequences of *Phytophthora* species within the ITS clade 6 produced a phylogenetic tree with a similar topology and high concordance with the one reported in the original description of *P. bilorbang* [30]. All isolates from olive clustered (bootstrap values of 1000 replicate) with the ex-type isolate of this species (Figure 2). The ITS sequences of all isolates from olive were deposited in GenBank (the respective GenBank accession numbers are given in Figure 2).

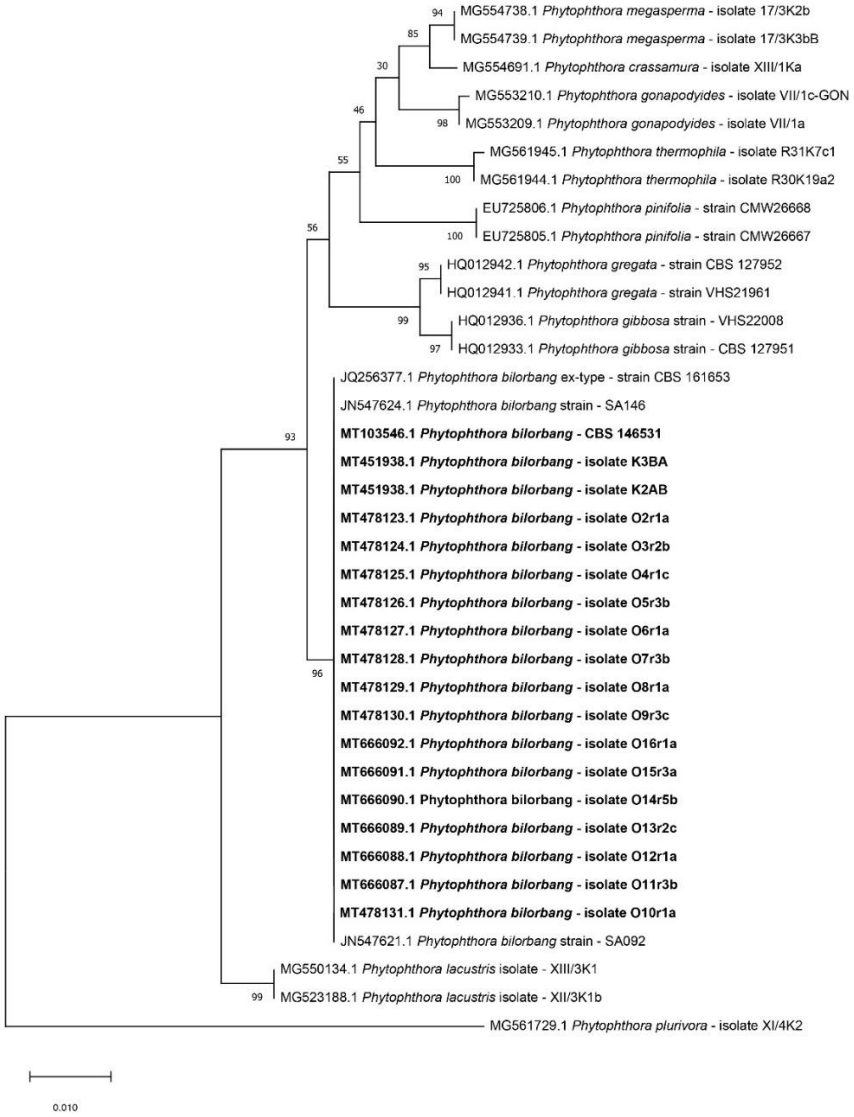

Figure 2. Phylogenetic tree for the ITS loci obtained by the maximum likelihood method, based on the Tamura–Nei model. Relationships between the 18 *Phytophthora bilorbang* isolates from olive (in bold), the ex-type isolate of *P. bilorbang* from European raspberry and other *Phytophthora* species within the ITS Clade 6. *P. plurivora* was used as outgroup taxon. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches).

The *P. bilorbang* isolate CBS146531 from olive proved to be pathogenic on olive cuttings cv. Nera di Gonnos. All 10 rooted cuttings transplanted into pots filled with infested soil developed severe symptoms of root rot, leaf chlorosis, defoliation, wilt, and final death of the whole cutting within six weeks after the transplant (Figure 1b). Mean severity of symptoms (± S.D.) in inoculated cuttings as evaluated according to Engelbrecht et al. [31] was 4.3 ± 1.16. Conversely, control cuttings grown in non-infested potting mixture showed no aerial symptoms and no necrotic fine roots. Difference between mean severity of symptoms of inoculated and control cuttings was significant for P=0.01. *Phytophthora bilorbang* was re-isolated only from roots of symptomatic

cuttings; thus, fulfilling Koch's postulates. The identity of isolates obtained from necrotic roots of symptomatic, artificially inoculated cuttings, was determined by the colony morphology, microscopy observations, and ITS rDNA sequencing.

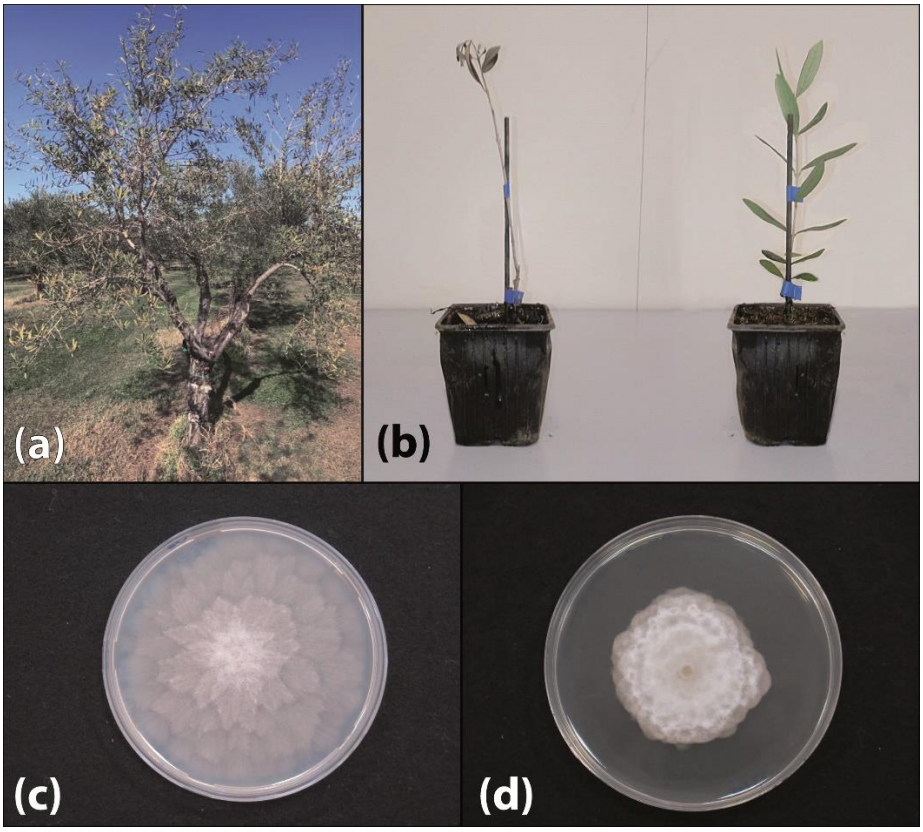

Figure 1. (a) Decline symptoms on a tree of olive (*Olea europaea*) cv. Nera di Gonnos incited by *Phytophthora bilorbang* in Calabria. (b) Wilt of a potted rooted cutting of olive cv. Nera di Gonnos (on the left) artificially inoculated with *P. bilorbang* through the soil, 6 weeks after transplanting into infested soil, and control non-inoculated cutting (on the right). (c) Morphology of 6-day-old colonies of *P. bilorbang* grown on V8 juice-agar and (d) on potato-dextrose-agar at 25 °C in the dark.

5.5. Discussion

Phytophthora bilorbang was described in 2012 in Western Australia as a new species in ITS clade 6 sub-clade II, and as a pathogen of European raspberry (*Rubus anglocandicans*) [30]. Its role as the main causative agent of "raspberry decline" syndrome in Australia was further confirmed in a later study [32]. The ITS sequences of *P. bilorbang* are identical to the corresponding sequences of several isolates deposited as *Phytophthora* taxon oaksoil and an isolate deposited as *Phytophthora* taxon riversoil, whose provisional names refer to their origin, i.e., soil of oak forests and riparian ecosystems, respectively [15,30,33]. The relationship between *P. bilorbang*, *Phytophthora* taxon oaksoil, and *Phytophthora* taxon riversoil is still debated [34]. However, as the most distinctive

character separating *P. bilorbang* from the other two taxa is homothallism [34], the isolates obtained from olive in Calabria were confidently referred to this species. In general, *Phytophthora* species of clade 6 have been found in forests and riparian ecosystems and, with some exceptions such as *Phytophthora asparagi*, *Phytophthora crassamura*, *P. megasperma* and, *Phytophthora rosacearum*, showing only a limited association with agriculture [34–41]. Additionally, *Phytophthora* species of clade 6 are predominantly sterile or homothallic in culture and appear functionally adapted to survive and thrive in aquatic environments and inundated soils [34,42]. The function of most of these species within the ecosystems is not yet fully understood. It has been hypothesized they have a prevalently saprotrophic lifestyle and their common presence and even dominance in environmental water surveys have been assumed as evidence for this hypothesis [36]. However, some members of ITS Clade 6, such as *Phytophthora pinifolia*, *P. inundata*, *Phytophthora* taxon Pgchlamydo, and *Phytophthora gonapodyides*, can be opportunistic and sometimes aggressive tree pathogens [36–39,42]. Results of pathogenicity tests on olive cuttings confirm that *P. bilorbang*, which has a prevalently aquatic lifestyle and is frequently recovered from streams and irrigation reservoirs, can be included in this group of opportunistic aggressive pathogens. It is hypothesized that in the experimental plot of Mirto Crosia soil waterlogging and asphyxiation as a consequence of flooding events or excessive irrigation predisposed the olive trees to *P. bilorbang* infections. In an extensive survey of European nurseries, *P. bilorbang* was found in rhizosphere soil of potted plants suggesting this species, like other soilinhabitant *Phytophthora* species, can be spread worldwide through the trade of nursery plants [43]. *Phytophthora bilorbang*, like most other soil-borne *Phytophthora* species, is a polyphagous pathogen whose host-range comprises, besides *Rubus anglocandicans* (Rosaceae), plants of different families including *Alnus glutinosa* (Betulaceae), *Juniperus phoenicea* (Cupressaceae), and *Pistacia lentiscus* (Anacardiaceae) [30,39,44]. In Italy, it was recovered from rhizosphere soil and plants of the Mediterranean maquis in Sardinia (southern Italy) [39,44]. However, to the best of our knowledge, this is the first report of *P. bilorbang* as a pathogen of olive and other plants in the Oleaceae family worldwide.

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6. *Phytophthora heterospora* sp. nov., a new conidia-producing sister species of *P. palmivora*

Bruno Scanu[1,*], Thomas Jung[2,3], Hossein Masigol[4], Benedetto Teodoro Linaldeddu[5], Marília Horta Jung[2,3], A. Brandano[1], Reza Mostowfizadeh-Ghalamfarsa[4], Josef Janoušek[2], Mario Riolo[6], Santa Olga Cacciola[7]

[1] Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy; abrandano@uniss.it (A.B.)
[2] *Phytophthora* Research Centre, Faculty of Forestry and Wood Technology, Mendel University in Brno, 613 00 Brno, Czech Republic;
[3] *Phytophthora* Research and Consultancy, 83131 Nußdorf, Germany;
[4] Department of Plant Protection, School of Agriculture, Shiraz University, 7144165186 Shiraz, Iran;
[5] Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova, 35020 Legnaro, Italy;
[6] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;
[7] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;
* Correspondence: bscanu@uniss.it

6.1. Abstract

Since 1999, an unusual *Phytophthora* species has repeatedly been found associated with stem lesions, root and collar rot on young olive trees in Southern Italy. In all cases, this species was obtained from recently established commercial plantations or from nursery plants. Morphologically the *Phytophthora* isolates were characterized by the abundant production of caducous, non-papillate conidia-like sporangia and caducous, papillate sporangia, resembling *P. palmivora* var. *heterocystica*. Additional isolates with similar features were obtained from nursery plants of *Ziziphus spina-christi* in Iran, *Juniperus oxycedrus* and *Capparis spinosa* in Italy, and mature trees in commercial farms of *Durio zibethinus* in Vietnam. In this study, morphology, breeding system and growth characteristics of these unusual *Phytophthora* isolates were examined and combined mitochondrial and nuclear multigene phylogenetic analyses were performed. The proportion between conidia-like and sporangia varied amongst isolates and depending on the availability of free water. Oogonia with amphigynous antheridia and aplerotic oospores were produced in dual cultures with an A2 mating type strain of *P. palmivora*, indicating all isolates were A1 mating type. Phylogenetically, these isolates grouped in a distinct well-supported clade sister to *P. palmivora*, thus they constitute a separate species. The new species, described here as *Phytophthora heterospora* sp. nov., proved to be highly pathogenic to both olive and durian plants in artificial stem inoculations.

Keywords: Oomycete; Peronosporaceae, Clade 4; taxonomy; multigene phylogenetic analyses; olive; durian; conidia

6.2. Introduction

The genus *Phytophthora* de Bary (Peronosporaceae, Peronosporales, kingdom Stramenipila) is one of the most important groups of plant pathogens causing a range of diseases in agricultural, horticultural, forest and natural ecosystems worldwide [1–3]. Initially, the taxonomic description of *Phytophthora* species was exclusively based on morphological analyses of reproductive structures, such as sporangia, chlamydospores, hyphal swellings, oogonia, oospores, antheridia, as well as colony morphology and growth rate at different temperatures [1]. With the advent of molecular DNA techniques and the development of phylogenetic inference, our understanding of the systematic and diversity of *Phytophthora* species has changed considerably over time [3–5]. Currently almost 200 species are officially described, grouped into 12 distinct and well supported phylogenetic clades and numerous sub-clades [4,6].

The majority of the described *Phytophthora* species are soilborne and waterborne, primarily responsible for root and collar rots, and occasionally bleeding stem cankers on several plant hosts [1,3,5,7]. These species are characterized by the production of persistent sporangia and their infection occurs through the release of biflagellate zoospores into soil or surface water, which are then attracted by chemical or electrical signals generated by the plant host [8]. Other *Phytophthora* species, namely airborne, produce almost exclusively caducous sporangia and primarily infect aerial parts of plants, causing leaf necroses, shoot blights, fruit rots and bleeding bark cankers on stem and branches [1,3,5]. In this case infection occur through detached sporangia spread by wind and rain splash that can either germinate directly to produce mycelia (which can in turn produce further sporangia) or indirectly by releasing zoospores [3,7,9]. Finally, there are some species with a mixed epidemiological strategy, having both caducous and persistent sporangia, thereby behaving as soil- and airborne pathogens [3,5,7,10].

In 2010, a new disease caused by *Phytophthora* was reported on 3-4 years old olive (*Olea europaea* L. cv. Bosana) trees in recently established plantations and commercial nurseries in Sardinia, Italy [11]. Specific symptoms consisted of leaf chlorosis, wilting, defoliation and dieback, eventually followed by plant death (Figure 1A–B). This syndrome was associated with orange-brown and flame shaped necrosis in the inner bark originating from root or collar infections and developing up to 50 cm in the stem (Figure 1C–F). Single necrotic spots, unconnected to collar lesions, often occurred along stems (Figure 1G), indicating that bark necrosis might also originated from aerial infections. The disease resulted in severe dieback and mortality of olive trees, with an incidence ranging from 20 to 60 %. Isolations from infected bark tissues consistently yielded a *Phytophthora* species which, based on morphological characters and 99.62 % similarity of the internal transcribed spacer (ITS) sequences, was initially identified as *Phytophthora palmivora* (E.J. Butler)

E.J. Butler [11]. Most of the isolates, however, behaved unusually by producing, in addition to the typical papillate sporangia of *P. palmivora*, also conidia-like sporangia that exclusively germinated directly via germ tubes. This unique feature was already previously reported from a few isolates obtained from root rot of young olive trees in Calabria, Italy, also identified as *P. palmivora*, although no DNA sequences could be generated at that time [12,13]. Due to their resemblance to the conidia of downy mildews (DM) the conidia-like sporangia of this unusual *Phytophthora* species hereafter referred to as conidia.

Similar symptoms to those described on olive in Italy were detected in 2013 in the Mekong River delta (Vietnam) on mature, fruit-bearing trees of durian (*Durio zibethinus* L.), one of the most appreciated and profitable fruit crops in this country. Durian trees showed aerial stem cankers with gum exudates and longitudinal cracking of the bark (Figure 1H), from which a *Phytophthora* species was consistently isolated and morphologically identified as *P. palmivora*. Additional isolates, with identical ITS sequences to those of the isolates recovered from olive trees, were detected from crown and root rot of Christ's thorn jujube (*Ziziphus spina-christi* (L.) Desf.) nursery seedlings in Kazerun County (Fars Province, Iran) in 2011 and from juniper (*Juniperus oxycedrus* L.) and capper (*Capparis spinosa* L.) nursery plants in Italy in 2013 and 2014, respectively.

In all cases, the isolates were characterized by the production of conidia that resembled the sporocysts described for *P. palmivora* var. *heterocystica* Babacauh from *Theobroma cacao* L. in Ivory Coast in 1983 [1,14], for which, unfortunately, no specimens and DNA sequence data are available. Therefore, in this study extensive morphological, physiological and phylogenetic analyses of the unusual *Phytophthora* isolates from Italy, Iran and Vietnam were performed and comparisons made to *P. palmivora*, resulting in the description of *Phytophthora heterospora* sp. nov.

Figure 1. Disease symptoms caused by *Phytophthora heterospora* on *Olea europaea* in Italy (**A–G**): (**A**) 3-4 years old olive trees showing chlorosis and increased transparency of the crown; (**B**) A five years-old plantation with dying and dead trees; (**C**) necrotic outer bark lesion on the lower part of the stem (arrow); (**E**) Orange-brown and flame shaped lesions on the stem; (**E,F**) Necrotic bark lesions girdling the collar; (**G**) Single-spot bark lesions on the main stem (arrow); (**H**) Old stem canker and scaffold branches on a mature tree of *Durio zibethinus* in Vietnam. (**I**) A dead 1-year-old seedling of *Ziziphus spina-christi* in a nursery in Iran. (**J**) Two years old *Juniperus oxycedrus* seedlings with dieback and mortality in a nursery in Italy.

6.3. Materials and Methods

6.3.1. Phytophthora *isolation and culture maintenance*

From olive trees *Phytophthora* isolations were mostly made from necrotic bark lesions on stems, collars and roots. Small pieces from the margins of fresh lesions were cut aseptically and plated onto synthetic mucor agar (SMA) [15]. Soil and fine roots of infected trees were also collected and baited with *Rhododendron* sp. and citrus leaves using the method originally described by Jung et al. [16]. From *C. spinosa*, *D. zibethinus*, *J. oxycedrus* and *Z. spina-christi* seedlings *Phytophthora* isolates were obtained by both direct isolation from stem lesions, infected roots and collar tissues and baiting of the rhizosphere soil using fresh cork oak and citrus leaves as baits and selective SMA and CMA-PARP agar [17]. Any colonies developing from infected tissues and leaf baits on selective media were subcultured onto carrot agar (CA; Oxoid® n°3 agar 16 g L^{-1}, $CaCO_3$ 3 g L^{-1}, blended carrots 200 g L^{-1}) and cornmeal agar (CMA; Oxoid® ground corn extract 40 g L^{-1}, agar 15 g L^{-1}), incubated at 20 °C and examined within 4 days using morphological characters for identification [15].

Six isolates of *P. palmivora*, including the representative specimen type 'S' (CBS 179.26) proposed by Brasier and Griffin [18], were included for interspecific comparisons (Table 1, Table S1). Stock cultures were maintained on CA slopes at 15 °C in the dark and preserved in the culture collections of the University of Sassari, Italy, and the University of Catania, Italy. Dried culture holotypes were lodged with the CBS Herbarium, and ex-type and paratype cultures were deposited at the Westerdijk Fungal Biodiversity Institute (CBS; Utrecht, The Netherlands) and the novel taxonomic description and nomenclature submitted to MycoBank.

Table 1. Details of *Phytophthora* isolates used in the morphological, growth-temperature and pathogenicity studies.

***Phytophthora* taxa**	**Isolate codes** [1]	**Country, region**	**Year**	**Host**
P. heterospora	PH047	Italy, Sardinia	2010	*Olea europaea*
P. heterospora	PH051 (CBS 148035)	Italy, Sardinia	2010	*Olea europaea*
P. heterospora	PH052	Italy, Sardinia	2010	*Olea europaea*
P. heterospora	PH054 (CBS 148034) (T)	Italy, Sardinia	2010	*Olea europaea*
P. heterospora	PH057	Italy, Sardinia	2010	*Olea europaea*
P. heterospora	PH211	Italy, Sardinia	2013	*Juniperus oxycedrus*
P. heterospora	317-A12 (CBS 148036)	Italy, Sicily	2014	*Capparis spinosa*
P. heterospora	Palm2 [2]	Italy, Calabria	1999	*Olea europaea*
P. heterospora	Campobello 2b	Italy, Sicilia	2005	*Olea europaea*
P. heterospora	DB2	Vietnam, Mekong Delta	2013	*Durio zibethinus*
P. heterospora	A1A	Vietnam, Mekong Delta	2013	*Durio zibethinus*
P. heterospora	A1B1	Vietnam, Mekong Delta	2013	*Durio zibethinus*
P. heterospora	C2B1	Vietnam, Mekong Delta	2013	*Durio zibethinus*
P. palmivora	CBS 179.26 [3]	Sri Lanka, n.a.	1979	*Theobroma cacao*

P. palmivora	Phoenix4 [4]	Italy, Sicily	2005	*Phoenix canariensis*
P. palmivora	PhoenixF [4]	Italy, Sicily	2005	*Phoenix canariensis*
P. palmivora	GRE1 (IMI 390579) [5]	Italy, Sicily	2002	*Grevillea rosmarinifolia*
P. palmivora	MD5 (IMI 503890) [6]	Vietnam, Mekong Delta	2013	*Artocarpus heterophyllus*
P. palmivora	MD6 (IMI 503891) [6]	Vietnam, Mekong Delta	2013	*Artocarpus heterophyllus*
P. taxon *palmivora*-like	PH083	Italy, Sardinia	2011	*Arbutus unedo*
P. taxon *palmivora*-like	PH090	Italy, Sardinia	2011	*Arbutus unedo*

[1] Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; IMI = CABI Bioscience, United Kingdom; other isolate names and numbers are as given by the collectors; T = ex-type culture. [2] Isolate from Cacciola et al. [12]. [3] Isolate from Brasier and Griffin [18]. [4] Isolates from Pane et al. [19]. [5] Isolate from Cacciola et al. [20]. [6] Isolates from Van Tri et al. [21].

6.3.2. DNA extraction, amplification and sequencing

Extraction of mycelial DNA of the *P. heterospora* and *P. palmivora* isolates used on the morphological studies was performed using the InstaGene Matrix (BioRad Laboratories, Hercules, CA) and the DNGTM-PLUS (Cinnagen, Tehran, Iran) kits following the manufacturer's instructions. The resulting DNA was stored at -20 °C, and its quantity and quality were determined using a MD-100 Nanodrop machine (NanoDrop Technologies, Wilmington, DE, USA). The Internal Transcribed Spacers of the ribosomal RNA (ITS) was amplified and sequenced using primers ITS-6 [22] and ITS-4 [23]. Additional gene regions were amplified and sequenced; (1) β-tubulin (*Btub*) was amplified using primers Btub F1 and Btub R1 [24], (2) the mitochondrial genes cytochrome c oxidase subunit 1 (*cox*1) was amplified with primers FM83 and FM84 [25], and (3) NADH dehydrogenase subunit 1 (*nadh*1) was amplified with NADHF1 and NADHR1 primer [26]. PCR conditions and reaction mixture were as described previously [24,26], except for the amplification conditions for the *cox*1 that consisted of 1 cycle of 95 °C for 2 min followed by 35 cycles of 94 °C for 40 s, 55 °C for 50 s, 72 °C for 1 min and a final extension step of 7 min at 72 °C. The PCR products were purified using the EUROGOLD gel extraction kit (EuroClone S.p.A., Italy) following manufacturer's instructions. All gene regions were sequenced in both directions with primers used in amplification by the BMR Genomics DNA sequencing service (www. bmrgenomics.it). DNA sequence chromatograms were viewed and edited using BioEdit v. 5.0.6 software [27]. DNA isolation, amplification and sequencing of additional loci of oomycete isolates needed for phylogenetic analysis was done as described previously [28]. Heterozygous sites observed were labelled according to the IUPAC coding system. All sequences derived in this study were submitted to GenBank.

6.3.3. Phylogenetic analyses

For phylogenetic analyses, the sequences obtained in this study were complemented with publicly available sequences of *Phytophthora* isolates representative of all main phylogenetic clades. Furthermore, loci of representative DM, including grass-infecting graminicolous downy

mildews (GDM), downy mildews with pyriform haustoria (DMPH), downy mildews with coloured conidia (DMCC) and brassicolous downy mildews (BDM) were added to the sequence's dataset. The source databases were the GenBank Nucleotide Collection and GenBank Whole-Genome Shotgun contigs. In some cases, sequences from two isolates from the same species were combined into a single sample because neither of them had all desired loci available in GenBank; this was only performed if the two isolates shared at least one identical sequence. Sequences of *NothoPhytophthora valdiviana* were included as outgroup taxon.

The sequences of the loci used in the analyses were aligned using the online version of MAFFT v. 7 [29] by the E-INS-I strategy (ITS) or the G-INS-I strategy (all other loci). Many of the DM species are known to have extremely long ITS sequences of up to more than 3,000 bp, caused by long repetitive insertions, which can affect both ITS1 and ITS2 [30]. The ITS alignment in this study was manually edited and adjusted and all insertions longer than 50 bp present exclusively in DM isolates were removed (in total 5,729 characters). To sort out the phylogenetic position of *P. heterospora* within phylogenetic Clade 4, a concatenated 4-partition dataset of the nuclear (ITS, *Btub*) and mitochondrial loci (*cox*1, *nadh*1), consisting of 4,161 characters, was analysed.

Bayesian Inference (BI) analysis was performed using MrBayes version 3.2.7 [31,32] into partitions with the GTR Gamma + I nucleotide substitution model. Four Markov chains were run for 20 M generations, sampling every 1,000 steps, and with a burn in at 9,000 trees. Maximum-Likelihood (ML) analysis was carried out using the raxmlGUI v. 2.0 [33] implementation of RAxML [34] with a GTR Gamma + I nucleotide substitution model. There were 10 runs of the ML and bootstrap (thorough bootstrap) analyses with 1,000 replicates used to test the support of the branches. Phylogenetic trees were visualized in Mesquite version 3.61 [35] and/or MEGA X version 10.2.6 [36] and edited in figure editor programs. Datasets presented and original trees deriving from BI and ML analyses are available from DRYAD.

6.3.4. Morphological characterisation and cardinal temperature for growth

Ten isolates of *P. heterospora* and four isolates of *P. palmivora* were included in the morphological studies. Measurements and photos of morphological structures were made at 400x magnification and recorded using a digital camera Leica DFC495 connected to a Leitz Diaplan compound microscope (Leitz, Germany) and Leica Application Suite imaging software v.4.5.0 (Leica Microsystems, Switzerland). Conidia and sporangia were examined both on solid CA after 7 days at 20 °C and from CA plugs (10 mm diameter) placed in 60 mm Petri dishes flooded with nonsterile soil extract water. Chlamydospores and hyphal swellings were examined directly on CA plates, if present. Length (l), breadth (b), l/b ratio, pedicel length and characteristic features of conidia and sporangia, as well as shape and diameters of chlamydospores and hyphal

swellings of 50 mature structures randomly selected were recorded for each isolate [37]. In addition, the proportion between conidia and sporangia was assessed across the isolates, both in water and on solid CA. Specifically, four 1 mm^2 discs were taken from each *P. heterospora* isolates listed in table 1 and mounted on glass slides with sterile water. The number of sporangia contained in each 1 mm^2 was counted using the Leitz Diaplan compound microscope.

Sexual compatibility type of all *P. heterospora* isolates was determined in dual culture with known A1 and A2 tester strains of *P. cinnamomi* (P904, P1889) on CA [15]. Selfed gametangia of the six representative isolates were induced on 35-mm plates containing 10 ml CA in polycarbonate membrane tests (Whatman Nuclepore™ Track-Etched Membranes, Sigma-Aldrich, St. Louis, Missouri, USA) with opposite mating type tester strains of *P. palmivora* (CBS 179.26). Paired cultures were incubated at 20 °C in darkness and scored for gametangial formation after 15–20 days. Fifty gametangia were chosen *at random*, and dimensions and characteristic features of antheridia, oogonia and oospores were measured and recorded at 400x magnification. The oospore aplerotic index and wall index were calculated according to Dick [38].

Colony morphologies were characterized from 4-day-old cultures incubated at 20 °C in the dark on CA, V8-juice agar (V8A; filtered V8 juice 100 mL L^{-1}, $CaCO_3$ 0.1 g L^{-1}) [15], potato dextrose agar (PDA; 39 g L^{-1}) and malt extract agar (MEA; 16 g L^{-1}) (all agar media were sourced from Oxoid®, Basingstoke, UK). Temperature-growth rate studies were performed on CA according to Scanu et al. [15]. Each isolate was incubated with three replicates at 5, 10, 15, 20, 25, 27.5, 30, 32.5, 35 and 40 °C (all ± 0.5°C).

6.3.5. Pathogenicity test

In order to fulfil Koch's postulates, pathogenicity of three representative isolates of *P. heterospora* was tested against 2 years-old olive (*Olea europaea* cv. Bosana) saplings. One isolate of *P. palmivora* and one isolate of *P.* taxon *palmivora*-like were included in the trial. The experimental design consisted in five replicates (saplings) per isolate, and five saplings, inoculated with a CA plug, used as negative controls. A 5 mm diameter hole through the bark to the sapwood surface was made using a cork borer. Then a 5 mm agar plug taken from the margin of 4-days-old culture grown on CA was inserted into the wound. Soon after inoculation, wounds were wrapped with sterile damp cotton wool and covered with aluminum foil and Parafilm. Saplings were maintained in the laboratory for 2 months in daylight and at a temperature ranging between 25 and 30 °C. At the end of the experiment, the outer bark was stripped and the necrotic lesion area surrounding each inoculation point was traced and then measured using APS Assess 2.0 software (APS Press) as described by Scanu and Webber [10]. Re-isolation was attempted by transferring

ten pieces taken from the margin of each lesion to SMA and incubating the plates at 20 °C in the dark.

The pathogenicity of the *P. heterospora* isolate DB2 was tested on 12-year-old durian trees in a commercial farm in the Dong Nai province (southern Vietnam). Inoculation was performed in December 2013 by underbark-inoculation. A colony plug (5 mm diam) was inserted under the bark of branches of the trees (two trees and three branches for each tree). The bark disk was put back in place to plug the wound which was covered with a sterile moist cotton plug and sealed with adhesive tape. Branches inoculated with sterile agar served as a control. During the experiment temperature ranged between 25 °C (night) and 32.5 °C (day). Natural light with around 12 h of light. Symptoms were first recorded 15 days after inoculation. For measuring the size of the necrotic lesions, the bark was removed 30 days after inoculation.

6.4. Results

The aligned datasets for the nuclear (ITS, *Btub*) and mitochondrial (*cox*1, *nadh*1) genes consisted of 1,101, 918, 1346, and 796 characters, respectively. Across the concatenated 4,141 characters alignment of all four gene regions, *P. heterospora* showed 21–28 unique polymorphisms. All isolates of *P. heterospora* were identical across all four loci apart from positions 307 and 438 in *Btub* which were heterozygous in several isolates and position 660 in *Btub* where isolate PH211 had an A instead of a G. The mitochondrial sequences of the two isolates PH083 and PH090 from *A. unedo*, informally designated here as *P.* taxon *palmivora*-like, were identical to those of *P. heterospora*.

To resolve the phylogenetic positions of *P. heterospora* within the genus *Phytophthora* and its separation from *P. palmivora*, a concatenated nuclear and mitochondrial 4-partitions dataset (4,141 characters) was analysed. Support for terminal clades and their clustering was similar in both BI and ML analyses and the ML analysis is presented here with both ML bootstrap and BI Posterior Probability values included (Figure 2, Dryad Dataset, https://doi.org/10.5061/dryad.1jwstqjvx). The isolates of *P. heterospora* formed a fully supported monophyletic cluster (BI posterior probability = 1.00, ML bootstrap = 99 %), which resided in Clade 4 in a close position to *P. palmivora*. Interestingly, isolate PH090 clustered in a strongly supported basal position to *P. heterospora*, intermediate between the latter and *P. palmivora* and, hence, is designated here as *P.* taxon *palmivora*-like (Figure 2). Amongst the isolates of *P. palmivora*, there was considerably higher intraspecific variability than in *P. heterospora*, with the two isolates from *D. zibethinus* (MD5 and MD6) forming a distinct lineage (Figure 2). The phylogenetic analyses of the 65-taxon dataset, which included *Phytophthora*, *NothoPhytophthora* and representatives of all DM groups, placed the

latter into two fully supported monophyletic clades nested within the genus *Phytophthora*. The DMCC, GDM and BDM clade resided in sister position to a large cluster comprising *Phytophthora* Clades 1-5, 12 and 14 and the DMPH whereas the latter clustered together with the obligate biotrophic *Phytophthora cyperi* from Clade 14 in a sister position to Clade 1 (Figure 2).

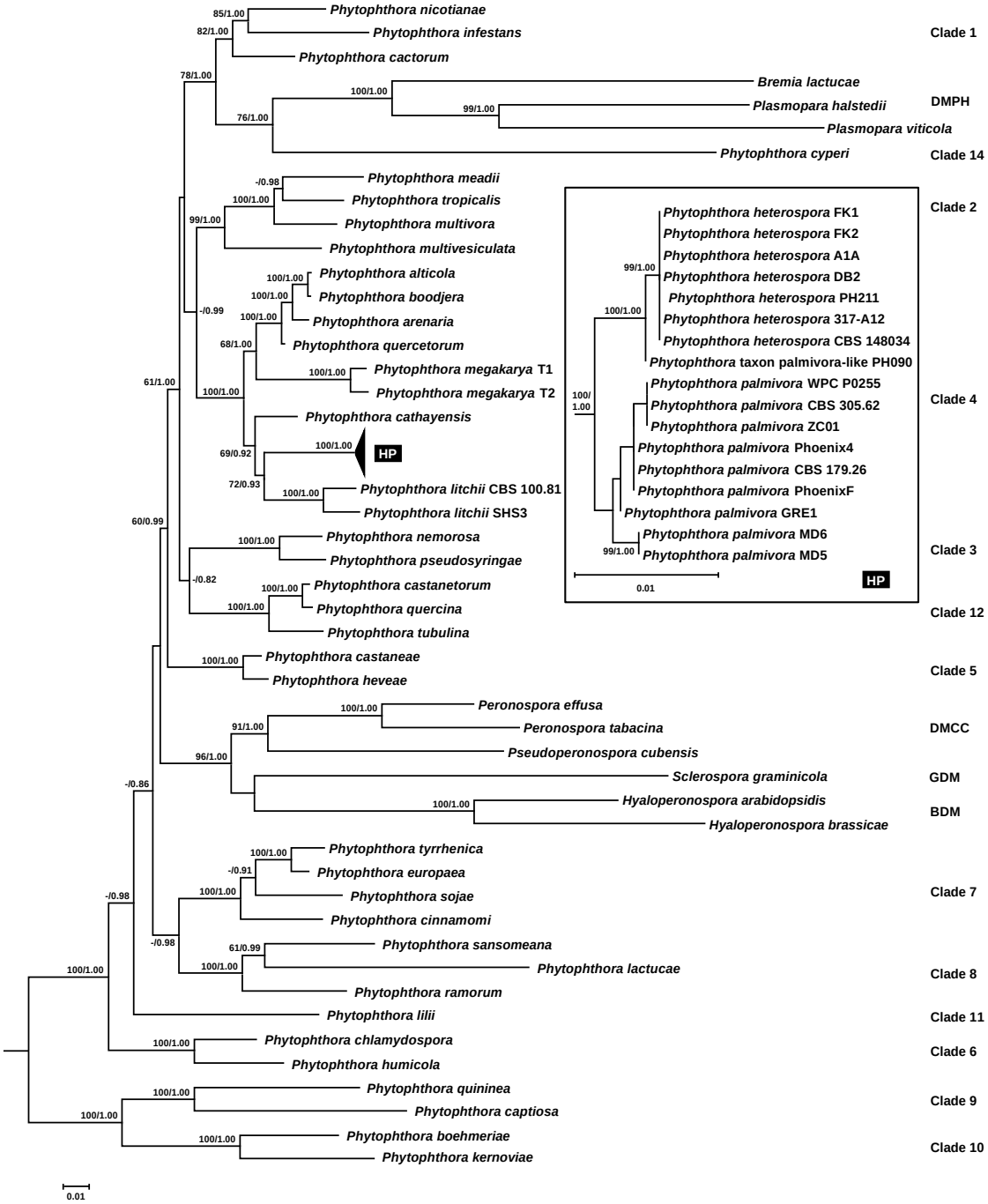


Figure 2. Fifty percent majority rule consensus phylogram derived from maximum likelihood analysis of a concatenated four-locus (ITS, *Btub*, *cox*1, *nadh*1) dataset of representative species from phylogenetic clades 1–12 and 14 of *Phytophthora* and the four downy mildew groups DMPH, DMCC, GDM, and BDM. Maximum likelihood bootstrap values and Bayesian posterior probabilities are indicated but not shown below 60% and 0.80, respectively. *NothoPhytophthora valdiviana* was used as outgroup taxon (not shown). Scale bar = 0.01 expected changes per site per branch.

Phytophthora heterospora Scanu, Cacciola, Linaldeddu & Jung sp. nov. (Figures 3–5)

MycoBank MB 841284.

Etymology: name refers to the production of a variety of different spores including conidia, zoospore-releasing sporangia, chlamydospores and oospores.

Holotype: Italy, Sardinia, Sorso; isolated from a bark lesion on a young olive tree. Collected: B. Scanu, 2010; CBS H-24777 (holotype, dried culture on CA, Herbarium CBS-KNAW Fungal Biodiversity Centre), CBS 148034 = PH054 (ex-holotype culture). ITS, *Btub*, *cox*1 and *nadh*1 sequences GenBank MT232393, MZ782807, MZ782828, MZ782849, respectively.

Description: conidia were abundantly produced in solid media (CA and V8A), but less frequently formed in non-sterile soil extract water (Figure 3A–G). Shapes of conidia showed a wide variation ranging from ellipsoid (over all isolates 76.4 %; Figure 3A–B, E–G) or subglobose to globose (14.8 %; Figure 3 C–D), or ovoid (6.8 %; Figure 3C–D) and limoniform (2.0 %), sometimes containing a large vacuole at maturity (Figure 3F–G). conidia had mostly round or less frequently tapered bases, proliferating externally with hyphae arising close to the base and forming mostly monochasial helicoid sympodia (Figure 3A–C). Empty conidia were characterized by a thick wall. All conidia were caducous, showing a short pre-formed pedicel < 5 μm (av. 3.9 ± 0.4 μm), which breaks off from the bearing conidiophore (Figure 4B). Nodose swellings were common at the insertion points of the conidia to the conidiophore (Figure 3A–D). The conidia dimensions of the 10 isolates of *P. heterospora* averaged 37.2 ± 1.8 μm in length (l) and 24.0 ± 1.1 μm in breath (b) (overall range 34.7–39.3 × 22.7–25.8 μm) with a l/b ratio of 1.6 ± 0.1 (range of isolate means 1.4–1.7). Conidia did not release zoospores but germinated directly with one or more germ tubes which usually emerged through the conidial wall and originate new conidia or papillate microsporangia (Figure 3L–N).

Sporangia of *P. heterospora* were readily produced in non-sterile soil extract water, but more rarely observed in solid agar (CA and V8A) (Figure 3H–J, L–N), typically borne terminally on unbranched sporangiophores or in irregular lax or regular dense sympodia. Sporangia often originated from a conidium (Figure 3L–N) and proliferated externally (Figure 3M). Sporangial shapes were diverse ranging from ovoid (over all isolates 68.4 %; Figure 3H, N) or elongate-ovoid to elongate-obpyriform (14.8 %; Figure 3J), or limoniform (6.8 %; Figure 3I, L–N), mouse to distorted shapes (6.3 %) and subglobose (3.7 %), often with laterally attached sporangiophore (48.6 %), occasionally with markedly curved apex (8.6 %) and presence of a vacuole (8.0 %; Figure 3I, N) and rarely with a hyphal appendix. Sporangia were exclusively caducous, with a short pedicel < 5 μm (av. 3.6 ± 0.5 μm), papillate or very rarely bipapillate (over all isolates <1 %). The sporangial dimensions averaged 41.0 ± 4.2 μm in length (l) and 23.1 ± 2.3 μm in breath (b) (overall

range 34.6–45.2 × 18.8–24.6 μm) with a l/b ratio of 1.8 ± 0.1 (range of isolate means 1.6–1.9). Sporangia germinated directly by forming a germ tube (Figure 3J) that originated a new sporangium or indirectly by releasing zoospores (Figure 3K). Zoospores limoniform to reniform whilst motile, becoming spherical (av. diam = 8.8 ± 1.2 μm) on encystment. The proportion of conidia vs sporangia varied based on the isolates and cultural conditions, with two major groups behaving differently (Table 2).

Table 2. Proportion (%) of conidia, sporangia and chlamydospores produced by *Phytophthora heterospora*, *P.* taxon *palmivora*-like and *P. palmivora* at 20 °C on solid carrot agar (CA) and on CA submerged in nonsterile soil extract water.

***Phytophthora* spp.**	**Conidia/Sporangia/Chlamydospores**	
	CA	**Water**
P. heterospora [1]	86/8/6	74/24/2
P. heterospora [2]	34/56/10	26/74/8
P. taxon palmivora-like	-/82/18	-/85/15
P. palmivora	-/78/22	-/80/20

[1] Group of isolates from *Capparis spinosa*, *Juniperus oxycedrus* and *Olea europaea* in Italy. [2] Group of isolates from *Durio zibethinus* in Vietnam.

Figure 3. Conidia and sporangia of *Phytophthora heterospora*. (**A–G**) Conidia formed on CA; (**A,B**) Ovoid conidia forming mostly monochasial helicoid sympodia, still attached to the conidiophores or recently detached with nodose swellings (arrows) at the insertion points of the conidia to the conidiophores; (**C**) Spherical to ovoid conidia with short pre-formed pedicels (arrows); (**D,E**) Detached conidia with short pedicels (arrows); (**F**) Ellipsoid conidium, with large lipid globule, tapering base and external proliferation; (**G**) Catenulate ellipsoid conidia with large lipid globules, forming a monochasial sympodium; (**H**) Ovoid, laterally attached papillate sporangium; (**I**) Limoniform papillate sporangium with short pedicel; (**J**) Empty sporangium with direct germination through the apex; (**K**) Sporangium releasing zoospores; (**L–M**) Conidia germating by forming papillate microsporangia (L-N) and a new conidium (arrow). Scale bar: 25 μm.

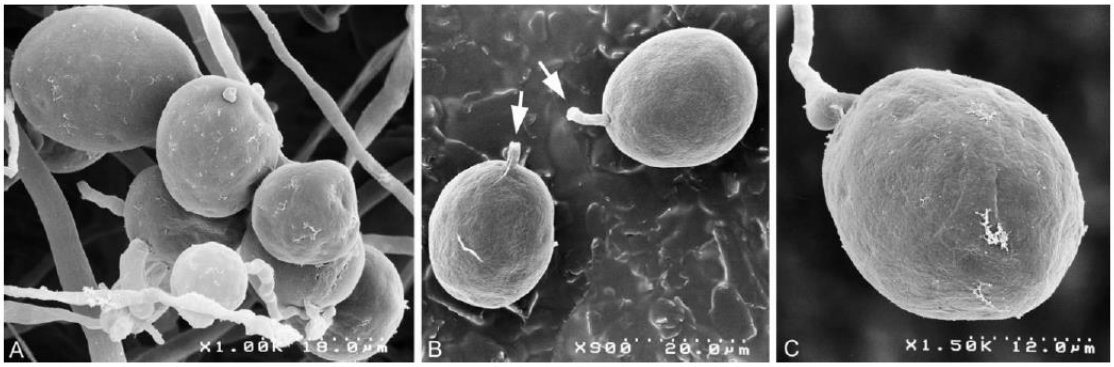


Figure 4. Scanning electron microscopy (SEM) images of pseudoconidia formed by *Phytophthora heterospora* on carrot agar: (A) cluster of pseudoconidia; (B) detached globose pseudoconidia with short pedicels (arrows); (C) ovoid pseudoconidium still attached to the conidiophore. Magnifications and scale bar dimensions are given in the right-bottom corners.

Hyphal swellings frequently occurring along the sporangiophores and conidiophores, subglobose to mostly globose, intercalary, catenulate averaging 10.4 ± 3.6 μm. Diameters of primary hyphae ranged from 3.2 to 6.8 μm. Chlamydospores were abundantly produced on CA and V8A (Figure 5A–F); they were thick walled, globose, and terminally (Figure 5A, D), intercalary (Figure 5B) or laterally (Figure 5C) inserted, with 28.4 ± 4.4 μm diameter (overall range 19.3–43.0 μm). Chlamydospores often germinated with one or more germ tubes originating new smaller chlamydospores (Figure 5E, F).

All *P. heterospora* isolates were self-sterile and produced gametangia readily when paired with A2 isolates of *P. palmivora* using the polycarbonate membrane test on CA (Figure 5K–N). No gametangia were produced when isolates of *P. heterospora* were paired with each other or with A1 mating-type tester strains of *P. cinnamomi*. Oogonia were globose, sometimes slightly comma-shaped (Figure 5M), often with short tapering bases (av. 74.6 %; Figure 5G–K), or elongated with long, and often distorted bases (av. 25.4 %; Figure 4L–M), produced in pairs or clumps (Figure 5G–I) or single and borne laterally (Figure 4J–M). Oogonial walls were always smooth and occasionally turned golden-brown within 4 weeks (Figure 5G, H). Oogonial diameters averaged 30.5 ± 2.2 μm with a total range of 24.3–37.4 μm. Oospores were aplerotic (av. 94.7 %; Figure 5G, L–M) or infrequently plerotic (av. 5.3 %; Figure 5H–K), contained large ooplasts, occasionally excentric, with thin walls (on av. 1.6 ± 0.3 μm) and a mean oospore wall index of 0.33 ± 0.06.

Oospore abortion rates averaged 60 % (Figure 5G, I). Antheridia were exclusively amphigynous, 51.4 % unicellular (Figure 5G–K) and 48.6 % bicellular (Figure 5L–M), averaging 15.3 ± 2.8 μm in length and 13.8 ± 1.5 μm in breath, with a l/b ratio of 1.1 ± 0.1, often with one or more finger-like projections.

Colony morphology and growth rates: *P. heterospora* colonies were radiate with limited aerial mycelium on CA and V8A, uniform and slightly woolly on PDA and poorly developed, dense-felty with irregular margin on MEA (Figure 6). Temperature-growth relations are shown in Figure 7A. All eight isolates included in the growth test had similar growth rates. The maximum growth temperature was around 32.5 °C, while the minimum temperature was above 10 °C. The average radial growth rate at the optimum temperature of 27.5 °C was 12.8 mm/d (Figure 7A).

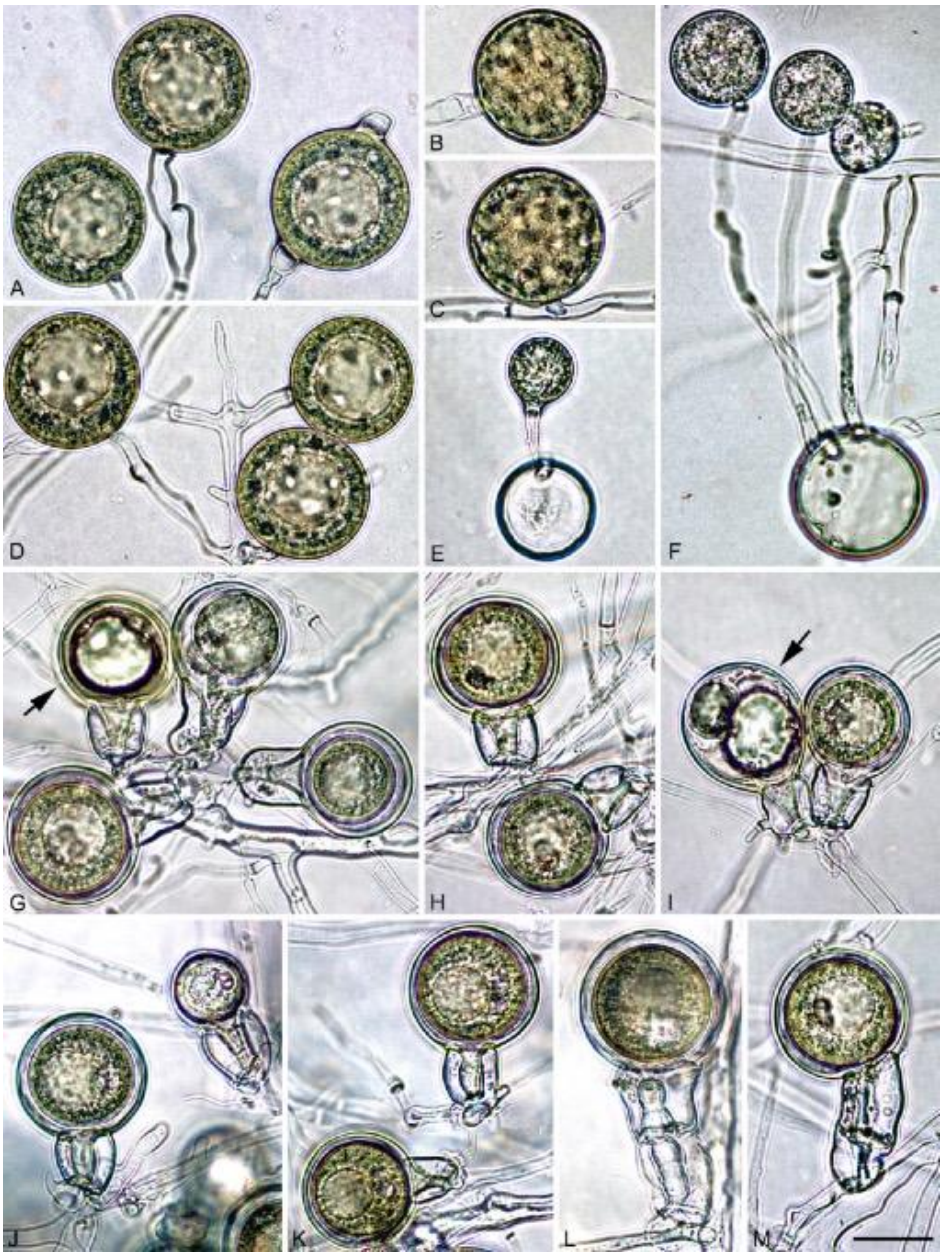


Figure 5. Chlamydospores and gametangia of *Phytophthora heterospora* formed on CA. (**A–F**) Thick walled, globose chlamydospores; (**A,D**) Terminal with lipid globules; (**B**) Intercalary; (**C**) Laterally attached chlamydospores; (**E,F**) Germinating chlamydospores with one or more germ tubes forming new chlamydospores; (**G–M**) Globose oogonia with aplerotic oospores containing large lipid globules, and amphigynous antheridia; (**G**) Oogonia with short tapering bases formed in a clump, one with aborted oospores (arrow); (**I**) Oogonia with short tapering bases, one with aborted oospore and finger-like projection on the antheridium (arrow); (**J,K**) Oogonia with short unicellular antheridia; (**L**) Oogonia with very long bicellular antheridium; (**M**) Comma-shaped oogonium with long bicellular antheridium. Scale bar: 25 μm.

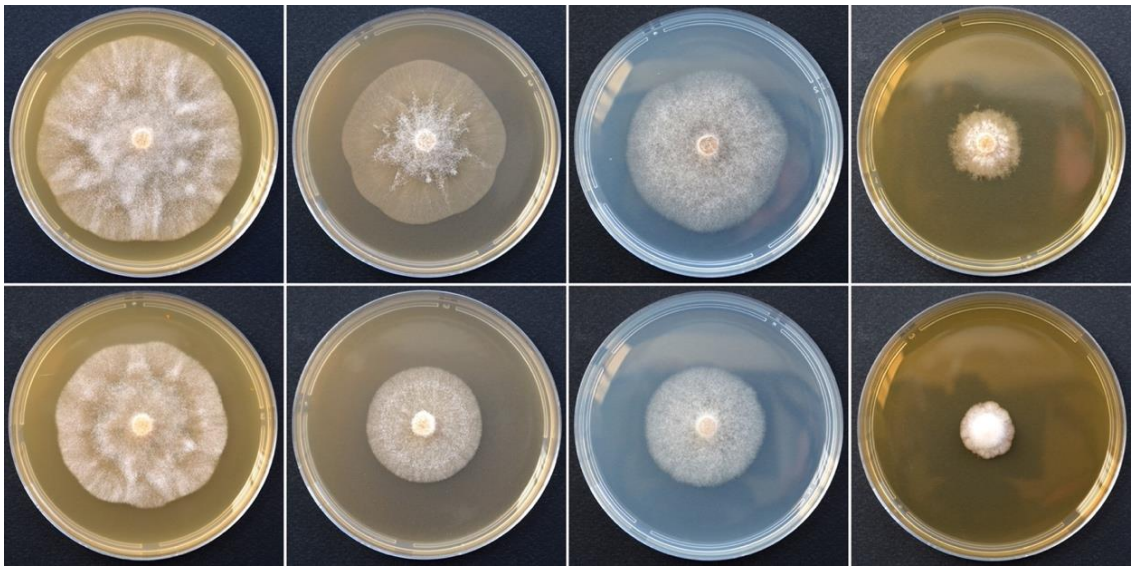
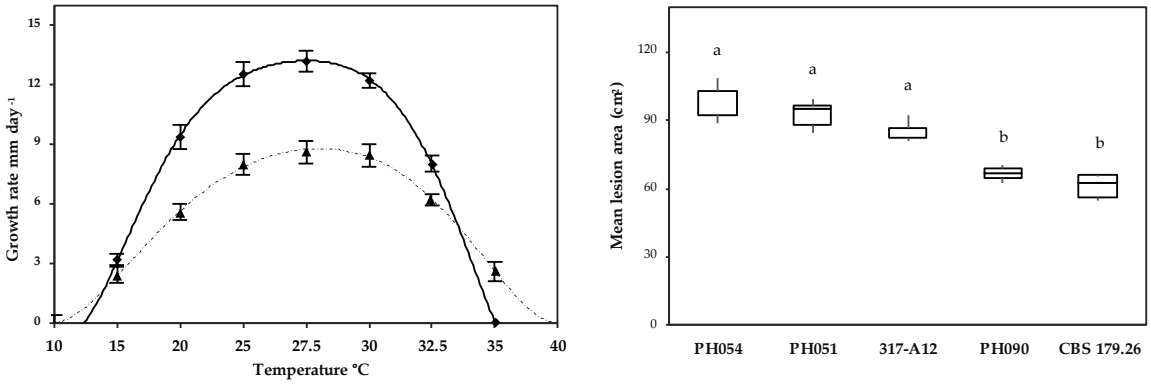


Figure 6. Colony morphologies of *Phytophthora heterospora* (upper row; isolate PH054) and *P. palmivora* (bottom row; isolate CBS 179.26) after 4 days growth at 20 °C in the dark on CA, V8A, PDA and MEA (from left to right).

Other specimens examined: Italy, Sardinia, Villamar. Isolated from a collar lesion of a young *Olea europaea* tree. Collected by: B. Scanu, 2010; CBS H-24778, CBS 148035 = PH051. Sardinia, Villamar. Isolated from rhizosphere soil of *Olea europaea* saplings. Collected by: B. Scanu, 2010; PH052; PH057. Sardinia, Sorso. Isolated from rhizosphere soil of a young *Olea europaea* trees. Collected by: B. Scanu, 2010; PH047. Italy, Sicily, Catania. Isolated from *Capparis spinosa*. Collected by: S.O. Cacciola, 2014; CBS H-24779, CBS 148036 = 317-A12. Italy, Calabria, Lamezia Terme. Isolated from *Olea europaea*. Collected by: S.O. Cacciola, 1999; Palm2. Italy, Sicily, Campobello di Mazara. Isolated from *Olea europaea*. Collected by: S.O. Cacciola, 2005; Campobello 2b. Vietnam, Mekong Delta, Vinh Long. Isolated from necrotic bark lesions on *Durio zibethinus* trees. Collected by: S.O. Cacciola, 2013; DB2; A1A; A1B1; C2B1.



(a) (b)

Figure 7. Temperature-growth relations and pathogenicity of *Phytophthora* taxa: (a) Radial growth rates of *P. heterospora* (continuous line; 10 isolates) and *P. palmivora* (dashed line; 4 isolates) on CA at different temperatures. The data are plotted as average ± SD; (b) Box and whiskers diagram showing mean lesion areas produced by three isolates of *P. heterospora* (PH054, PH051 and 317-A12), *P. taxon palmivora*-like (PH090) and *P. palmivora* (CBS 179.26) on 2 years-old olive saplings after 2 months. Control saplings did not show any necrotic lesions around the inoculation points and were not included. Different letters above bars indicate significant differences according to Fisher's LSD test (P = 0.05).

Host/distribution: *Olea europaea*, *Juniperus oxycedrus* and *Capparis spinosa* (Italy), *Ziziphus spina-christi* (Iran) and *Durio zibethinus* (Vietnam).

Notes: *Phytophthora heterospora* and *P. palmivora* are very similar in terms of colony morphology and sporangia, chlamydospores and gametangia characteristics. However, *P. heterospora* can be easily distinguished by the production of conidia on solid agar media. Other key differences between *P. heterospora* and *P. palmivora* are: (i) the lower maximum temperature for growth and faster growth rates at most temperatures of *P. heterospora*; (ii) the higher oospore abortion rate of *P. heterospora* (60 % vs 48 %). Phylogenetically, *P. heterospora* differs from *P. palmivora* in ITS, *Btub*, *cox*1 and *nadh*1 by 3-4, 11-16, 1-2 and 6 fixed polymorphisms, respectively. Although *P. heterospora* is present in four continents, its geographic distribution, presently seems to be restricted to a few countries and mainly to nursery plants and new plantations, contrasting the global distribution of *P. palmivora* in a wide range of natural, horticultural and ornamental ecosystems and nurseries [1,18,39].

In the inoculation tests, both *P. heterospora* and *P. palmivora* were shown to be pathogenic to *O. europea* (Figure 7b). All isolates used in the experiments were able to cause necrotic lesions significantly larger ($P < 0.0001$) than those of the negative controls. Overall, with mean lesion areas of 92.4 ± 8.2, *P. heterospora* was significantly ($P < 0.05$) more aggressive than *P. palmivora* with 64.1 ± 5.2 cm^2, respectively. The isolate PH054 was the most aggressive among the *P. heterospora* isolates, causing a mean lesion area of 98.2 ± 8.2, but intraspecific differences were statistically not significant ($P < 0.05$). Saplings inoculated with *Phytophthora* species developed the typical symptoms observed in the field, such as leaf chlorosis, wilting and defoliation. *Phytophthora* taxon *palmivora*-like (PH090) were also pathogenic with necrotic lesions comparable to *P. palmivora*. All *Phytophthora* taxa were re-isolated from necrotic stem lesions.

On durian branches wound-inoculated with the Vietnamese *P. heterospora* isolate DB2 from durian, the first symptoms, consisting in gum exudation and bark necrosis, appeared 15 days after inoculation. After 30 days the mean area of necrotic lesions in inoculated branches was 96 ± 10 mm^2. No symptoms were observed on the control branches.

6.5. Discussion

Species in the genus *Phytophthora* are characterized by the production of asexual sporangia releasing biflagellate zoospores into an evanescent vesicle that soon breaks allowing zoospores to swim into the water [1]. The occurrence of an additional asexual dissemination structure in *P. heterospora*, named conidia as in DM and fungi, which germinate directly instead of releasing zoospores, represents a unique feature for the genus *Phytophthora.* This trait was previously reported by Babacauh in 1983 on isolates obtained from *T. cacao* in Ivory Coast, designated as *P. palmivora* var. *heterocystica* because of the differentiation of unusual conidia-like sporangia [1,14]. Since no specimens linked to the original description are available and no further reports of this variety have been published, it remains unclear whether *P. heterospora* and *P. palmivora* var. *heterocystica* belong to the same taxon, although it cannot rule out.

As stated by Babacauh [1,14], the occurrence of conidia may be an evolutionary trend towards less dependence on free water, as shown for various DM groups. Similar transitional phenotypic and ecological characters between the hemibiotrophic or necrotrophic *Phytophthora*s and the obligate biotrophic DM have been reported in other species and genera [1,40,41]. This is the case with *Phytophthora litchii*, the taxonomic position of which has long been controversial due to the differentiation of *Peronospora*-like sporangiophores [42,43], and all the unculturable obligate biotrophic *Phytophthora* species, including *P. cyperi, P. leersiae*, *P. lepironiae* and *P. polygoni* [4,44,45]. Other examples include the genera *Viennotia*, *Poakatesthia* and *Sclerophthora*, which possess similar features as *Phytophthora* species, although they are placed among the DM [46–48]. Already in 1952, Gäumann [49] proposed that *Phytophthora* and DM were likely to be related, but their taxonomic relationship has long been a matter of debate [50,51]. The first phylogenetic studies using nuclear ITS rDNA suggested that the two taxonomic groups potentially form a monophyletic clade [22,52]. This hypothesis was corroborated by subsequent multigene phylogenetic and phylogenomic studies, with all DM unambiguously residing within the genus *Phytophthora* [4,40,41,53–55]. Similar results were obtained in the phylogenetic analyses of the present study using a concatenated nuclear and mitochondrial 4-locus dataset. DM were nested within the *Phytophthora* clades. As shown before by Bourret et al. [4], in addition to *Phytophthora* Clades 1-12 and 14, two further clades were identified, one accommodating the DMCC, GDM and BDM and another one the DMPH. Apparently, in the genus *Phytophthora* an obligate biotrophic lifestyle evolved several times independently since the DMCC, GDM and BDM share a common ancestor with *Phytophthora* Clades 1-5, 12 and 14 whereas the DMPH together with the obligate biotrophic *P. cyperi* from Clade 14 share a common ancestor with *Phytophthora* Clade 1. No close phylogenetic relationship was found between *P. heterospora* and any of the DM included

in the analyses, although in previous phylogenetic studies, using only single genes, Clade 4 species were several times shown to be the nearest relatives to the DM [4,22,43]. However, as reported for the downy mildew-like species *P. litchii* (also Clade 4), *P. heterospora* may have some genomic characteristics with DM forced by environmental and host adaptation during its evolution [43].

Phylogenetically, *P. heterospora* is closely related to *P. palmivora* from which it can be distinguished by 3-4 fixed polymorphisms in ITS sequences and a total of 18-25 bp differences across the *Btub*, *cox*1 and *nadh*1 gene regions. Nonetheless, due to the high intraspecific variability [18, this study] and the lack of any type material or key specimens for *P. palmivora* [56,57] that could serve as DNA sources for robust molecular identification and phylogenetic studies, isolates belonging to *P. heterospora* have often been misidentified in the past. A search of ITS sequences in the National Center for Biotechnology Information (NCBI) database and previous publications revealed that isolates with sequences identical to *P. heterospora* have been recorded from almost all continents and different hosts. The crown and root rot of pomegranate reported in young plantations in Italy and Turkey appear to be most likely caused by *P. heterospora* [58,59]. The lower maximum temperature (<35 °C) for mycelium growth of Turkish isolates and their phylogenetic position indicate they did not belong to *P. palmivora* [59]. Similarly, the dieback and mortality of young olive trees in a nursery and in plantations in Souk El Arbaa (Morocco) attributed to *P. palmivora* [60] were presumably also caused by *P. heterospora*, as demonstrated by the occurrence of subglobose and non-papillate sporangia, which resemble conidia. Unfortunately, the identification of the isolates was based exclusively on morphological characters and no DNA sequences are available [60]. All these records, together with the finding of *P. heterospora* from olive plantations in this study, suggest that the pathogen is well established in agricultural tree crops in areas with Mediterranean and semi-arid climatic conditions, most likely favored by the intensive cultivation systems [61,62]. The clonal nature and high virulence of *P. heterospora*, as well as its restricted distribution to nurseries and new plantings, indicate it is an emerging and potentially invasive pathogen recently introduced via infected plants [63,64]. This hypothesis is supported by the abundant detection of *P. heterospora* in asymptomatic potted plants of ten different hosts in two large European retail nurseries using a qPCR technique [65].

The current distribution of *P. heterospora* in the field, however, may be limited by hot temperatures and drought in summer which are not favorable to requirements of the pathogen for growth and sporulation. Similar to its closest relative *P. palmivora*, also *P. heterospora* might be more adapted to an aerial lifestyle requiring high humidity [39,66], as revealed by the findings from durian trees in tropical Southeast Asia, including Indonesia, Malaysia, Thailand, and

Vietnam (this study). The morphological and physiological attributes of *P. heterospora* support its ability to behave either as an aerial or soilborne pathogen. The production of both caducous conidia and sporangia provide *P. heterospora* with an ecological advantage in durian farms in southern Vietnam as during monsoon season conidia are transported aerially by rain and wind and may infect stems and branches. During the dry season water splashes caused by sprinkler irrigation systems may also favor aerial infections [39,66]. The ability of causing cankers on fruit-bearing trees of durian suggests *P. heterospora* may be an aggressive pathogen provided that environmental conditions are conducive, i.e., in tropical and subtropical regions. In addition, some key morphological properties such as a heterothallic breeding system, an oospore wall index of only 0.33, caducity of conidia and sporangia and thick-walled chlamydospores, indicate a potential center of origin of *P. heterospora* in tropical regions, most likely Southeast Asia, Central America or West Africa, as suggested for most Clade 4 species [67,68]. The finding of an isolate with identical ITS sequence to *P. heterospora* (GenBank accession: KY475630) from *T. cacao* in Ghana, a neighbor country to Ivory Coast where *P. palmivora* var. *heterocystica* was originally described, supports the hypothesis of a West African origin [1,14]. However, to ascertain the evolutionary and geographic origin of *P. heterospora* further studies are needed, including surveys in yet non-surveyed natural environments to detect the pathogen on co-evolved hosts without causing diseases [69,70] followed by phenotyping and genotyping studies using a global population [71–73].

The differences in the proportion of conidia and sporangia production recorded (1) for the same *P. heterospora* isolates between solid dry agar and water culture, and (2) between *P. heterospora* isolates from Mediterranean regions in Italy and humid tropical Vietnam are of high interest. The ability of *P. heterospora* to partially switch between asexual structures depending on the substrate of growth, i.e. increase conidia production on solid agar and sporangia production in water, respectively, indicates that this trait evolved as an adaptation to specific environmental conditions [18]. Therefore, isolates of *P. heterospora* from Mediterranean and semi-arid regions may be on the evolutionary path towards a lifestyle as conidial plant pathogen with reduced dependence on free water, while isolates from Vietnam apparently prefer sporangia due to the conducive conditions in tropical humid environments [39]. Spore formation and germination involve the staged expression of a large subset of the transcriptome, commensurate with the importance of spores in the life cycle. Therefore, further studies, using new technologies such as RNA-seq may be effective in understanding spore biology and the pathogenic mechanisms of *P. heterospora* [74].

Remarkably, isolates PH083 and PH090 from *A. unedo* in Italy belonged to a discrete lineage in an intermediate evolutionary position between *P. heterospora* and *P. palmivora* and, consequently, were designated as *P.* taxon *palmivora*-like. Both nuclear gene sequences and the absence of conidia characterize these isolates as *P. palmivora*. However, their *cox*1 and *nadh*1 sequences are identical to *P. heterospora*, i.e. they share the same maternal line. This feature was previously reported for the three hybrid species in Clade 7a, namely *P.* ×*cambivora*, *P.* ×*heterohybrida* and *P.* ×*incrassata*, which share the same *nadh*1 genotype and, hence, most likely the same maternal parent. Interestingly, the occurrence of multiple heterozygous sites in the *Btub* sequences of both *P.* taxon *palmivora*-like (7) and *P. palmivora* (10) suggests they may originate from sexual hybridisation rather than somatic fusion, a feature common to all known *Phytophthora* hybrids [75,76]. However, further molecular analyses, such as cloning, sequencing of other nuclear and mitochondrial genes, and estimation of nuclear DNA content and ploidy level by flow cytometry are required to confirm this hypothesis [77].

Our finding of high phenotypic and genetic variability amongst *P. palmivora* isolates is in accordance with previous studies [18,67,78]. The two isolates from jackfruit in Vietnam [21] were genetically different from all the other *P. palmivora* isolates. Interestingly, they originate from the same area of the Mekong River Delta as the *P. heterospora* isolates from durian trees [21]. Further investigations are urgently needed to characterize the global population structure of *P. palmivora* considering its panglobal distribution and high impact on a wide range of horticultural and ornamental plant species [39].

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7. Diversity of *Phytophthora* communities across different types of Mediterranean vegetation in a nature reserve area

Mario Riolo[1,2,3,†], Francesco Aloi[1,4,†], Federico La Spada[1], Saverio Sciandrello[5], Salvatore Moricca[6], Elena Santilli[2], Antonella Pane[1*], Santa Olga Cacciola [1*]

[1.] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[2.] Council for Agricultural Research and Agricultural Economy Analysis, Research Centre for Olive, Citrus and Tree Fruit-Rende CS (CREA- OFA), 87036 Rende, Italy;

[3.] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[4.] Department of Agricultural, Food and Forest Sciences, University of Palermo, 90128 Palermo, Italy;

[5.] Department of Biological, Geological and Environmental Sciences, University of Catania, Italy;

[6.] Department of Agricultural, Food, Environmental and Forestry Science and Technology, University of Florence, Italy;

[*] Correspondence: olga.cacciola@unict.it (S.O.C.); apane@unict.it (A.P.).

[†] These authors are equally contributed.

7.1. Abstract

Protected natural areas are a reservoir of *Phytophthora* species and represent the most suitable sites to study their ecology, being less disturbed by human activities than other environments. The specific objective of this study was to correlate the diversity and distribution of *Phytophthora* species with the vegetation in aquatic, riparian and terrestrial habitats within a protected area in eastern Sicily, southern Italy. Environmental samples (water and soil) were sourced from two streams running through the reserve and six different types of vegetation, including *Platano-Salicetum pedicellatae*, *Sarcopoterium spinosum* community, *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis, Oleo-Quercetum virgilianae* and gallery forest dominated by *Nerium oleander* (Natura 2000 classification of habitats). *Phytophthora* species were recovered from samples using leaf baiting and were classified on the basis of morphological characteristics and sequencing of Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA). Overall, 11 *Phytophthora* species, within 5 different ITS Clades, were identified, including *P. asparagi*, *P. bilorbang*, *P. cryptogea*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. nicotianae*, *P. oleae*, *P. parvispora*, *P. plurivora*, and *P. syringae*. No *Phytophthora* species were found in the *Sarcopoterium spinosum* comm. *Phytophthora asparagi*, *P. lacustris* and *P. plurivora* were the prevalent species in the other five plant communities, but only *P. plurivora* was present in all of them. Overall aquatic species from Clade 6 (100 out of 228 isolates) were the most common; they were recovered from all five types of vegetation, streams and riparian habitats. *Phytophthora* populations found in the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* show the highest diversity while no

correlation was found with the physicochemical characteristics of the soil. The vegetation type and the aquatic or terrestrial habitat were identified as major environmental factors influencing the diversity of *Phytophthora* communities in this reserve.

Keywords: leaf baiting; rDNA ITS regions; soil; water; ITS clades; Mediterranean vegetation; ecology; soil inhabitants; aquatic species

7.2. Introduction

The genus *Phytophthora* (Pythiaceae, Peronosporales, Oomycota, Chromista) comprises to date over 180 described species while according with a conservative estimate the actual number of species in this genus is at least double if not triple [1,2]. Many *Phytophthora* species, such as *Phytophthora infestans*, *P. cinnamomi* and *P. ramorum*, are destructive plant pathogens causing severe crop losses and tree decline worldwide [3–10]. Most plant pathogenic *Phytophthora* species are polyphagous, with a host range encompassing plants of different families [11–15], and are typically soil-inhabitants, although a number of them that produce deciduous sporangia have partially or temporarily adapted to an aerial lifestyle [16]. A more restricted number of species in the phylogenetic ITS clade 6 recovered from water courses, lakes and irrigation basins are functionally more adapted to aquatic habitats [17–21]. In general, these aquatic *Phytophthora* species are weakly aggressive as plant pathogens and, consequently, it has been assumed they behave as saprotroph in plant debris in water and as opportunistic pathogens in riparian habitats. However, their ecological role in ecosystems is not fully understood.

Human-mediated transport, mainly the trade of nursery plants, has been identified as a major pathway for the introduction of non-native *Phytophthora* species into new areas [22–26]. It was demonstrated, e.g., that there is a causal link between the ornamental plant industry and the introduction of the destructive oak pathogen *P. ramorum* in the wildland in North America [27]. The use of nursery plants for forest restoration and afforestation is a way to introduce and spread exotic *Phytophthora* species in natural habitats and forests [28–30]. In a survey of protected natural areas in Sicily 13 out of 20 *Phytophthora* recovered species were putatively exotic and only seven could be considered endemic to Europe [31]. In many cases, *Phytophthora* species found in forests and natural or naturalized ecosystems included aggressive plant pathogens of cultivated plants suggesting that these ecosystems may act, in turn, as a potential sources and reservoirs of *Phytophthora* inoculum for agricultural crops [32]. As a consequence, monitoring of forest and natural ecosystems should be included in *Phytophthora* species surveillance and biosecurity schemes. The establishment of alien invasive *Phytophthora* species in natural ecosystems has destabilizing effects as it affects the ecosystem homeostasis and resilience. The invasion of natural and semi-natural ecosystems by these pathogens may endanger native and rare plant species and

are a threat to the diversity of plant communities [27,32–34]. As a consequence, the knowledge of resident *Phytophthora* population should be a prerequisite for a rational management strategy of protected natural areas (PNAs).

The refinement of baiting and sampling methods together with rapid advances in molecular diagnostics and DNA-sequencing technology [35–54] facilitated the detection of *Phytophthora* in environmental samples and stimulated the study of *Phytophthora* communities in forest and natural ecosystems all over the world, including watercourses and still unexplored areas [55–67]. These surveys revealed the richness of *Phytophthora* diversity in these ecosystems and led to the discovery of an impressive number of cryptic new species in this genus. They contributed to a better understanding of the global diversity of *Phytophthora,* the geographic radiation pathways of single *Phytophthora* species and clades from their centers of origin, their lifestyle and, in particular, their reproductive behavior and adaptation to different environments [2]. Despite numerous surveys, the environmental factors shaping the *Phytophthora* populations and driving their compositional changes in the wild have been poorly investigated. Yet PNAs, being less disturbed by human activities, are the most suitable context for studying the ecology of *Phytophthora* species. The main objective of this study was to investigate if the diversity of *Phytophthora* species and their frequency and spatial distribution across a small nature reserve in Sicily (southern Italy) are correlated with the type of vegetation and other environmental factors.

7.3. Materials and methods

7.3.1. Sampling area

Sampling activities were carried out in the "Complesso Speleologico Villasmundo- S. Alfio" Regional Nature Reserve (Strict Nature Reserve), part of the Special Areas of Conservation (SAC) (ITA090024) "Cozzo Ogliastri" in the municipality of Melilli (Sicily, Italy) (Figure 1). The reserve is managed by the CUTGANA (University of Catania) and was established in 1998 in order to protect one of the most important karst systems of the hyblean area. The reserve covers a surface of 72 hectares, in the north-eastern sector of Climiti Mountains, between the "Belluzza" and "Cugno di Rio" streams. Along the river "Cugno di Rio", there are caves entrances (A zone, the core zone of the Reserve). The buffer zone of the Reserve (B zone) hosts several natural ecosystems of great importance.

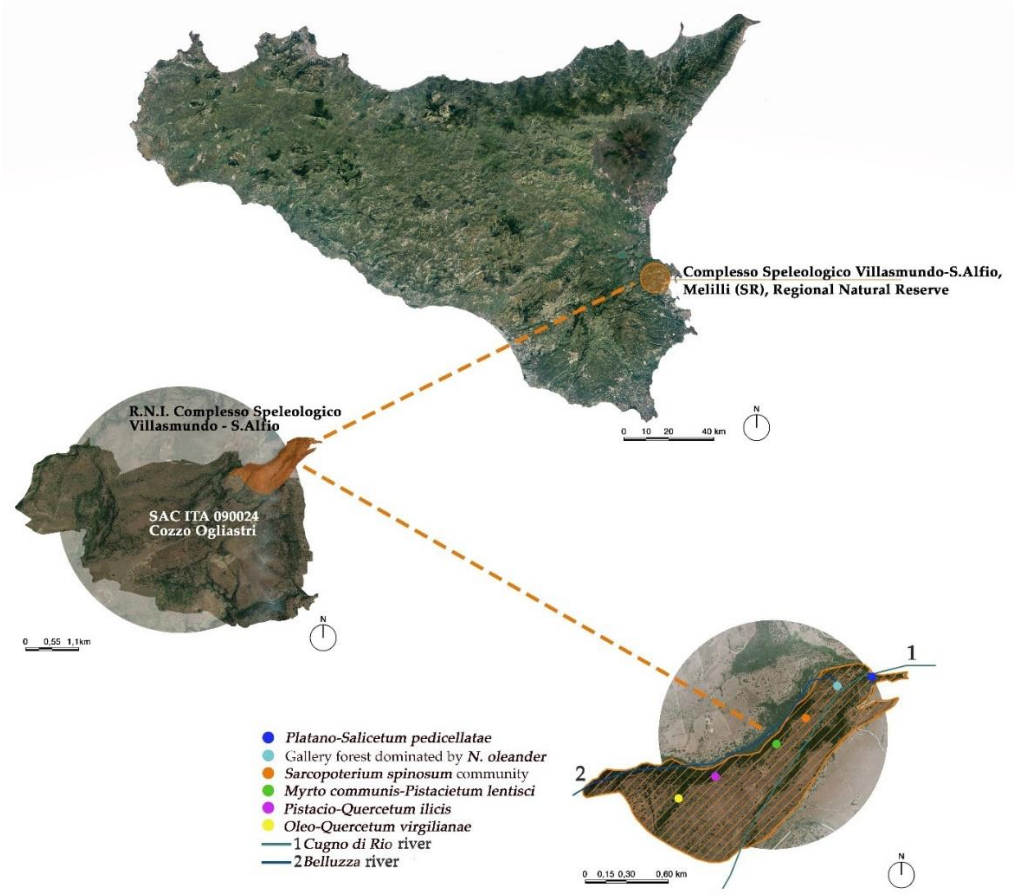


Figure 1. Geographical location of "Complesso Speleologico Villasmundo- S. Alfio" Regional Nature Reserve (RNR) and plant communities within the reserve.

The plant communities and their respective habitats (according to the Habitats of Directive 92/43/EEC) where samples were collected are located in the B zone of the Reserve and are described herewith. *i) Platano-Salicetum pedicellatae* Barbagallo, Brullo & Fagotto (Nature CODE 92C0 I): forests and woods, prevalently riparian, dominated by oriental plane (*Platanus orientalis* L.) and willow (*Salix pedicellata* Desf.), probably a relic of a more extended plane tree wood. *ii) Sarcopoterium spinosum* community (Nature CODE 5420): low, thorny formations of hemispherical shrubs of the coastal thermo-Mediterranean zone of Aegean islands, of mainland Greece and the Ionian islands, of coastal Anatolia, much more widespread and diverse than the western Mediterranean formations. *iii) Myrto communis-Pistacietum lentisci* (Molinier) Rivas-Martínez (Nature CODE 9320): thermo-Mediterranean woodland dominated by arborescent mastic (*Pistacia lentiscus* L.) and myrtle (*Myrtus communis* L.). *iv) Pistacio-Quercetum ilicis* Brullo & Marcenò (Nature CODE 9340): Mediterranean oak stand characterized by holm oak (*Quercus ilex* L.). *v) Oleo-Quercetum virgilianae* Brullo (Nature CODE:91AA): mature climax community typified by southern live oak *(Quercus virgiliana* (Ten.) Ten.) in association with cork oak (*Q. suber* L.), holm oak (*Q. ilex* L.), carob (*Ceratonia siliqua* L.), wild olive (*Olea europaea* L. subsp. *sylvestris* (Mill.)

Hegi) and mastic (*P. lentiscus* L.). *vi)* Gallery forest dominated by oleander (*Nerium oleander* L.) (Nature CODE 92CO I): thermo-Mediterranean community dominated by oleander (*N. oleande*r L.) in association with willows (*Salix* spp.) and poplars (*Populus* spp.). Sampling activities were carried out during the autumn of 2015/2016 and 2017/2018. Plant nomenclature follows Pignatti [68], while the syntaxa classification follows Biondi *et al*. [69]. For the correlation between plant communities and habitat types, we referred to the Italian Interpretation Manual for the Habitats of Directive 92/43/EEC [70].

7.3.2. Sampling and Phytophthora *isolation*

Twenty rhizosphere soil samples, including fine roots, were collected from 20 mature trees and shrubs growing in all six plant communities.

Soil sampling and isolation were performed in accordance with Jung et al. [31]: four soil cores were collected under each tree or shrub, 50–150 cm away from the stem base, and rhizosphere soil from all four cores were bulked together (about 1L).

For each sample, subsamples of 400 mL were used for baiting tests that were performed in a walk-in growth chamber with 12 h natural daylight at 20 °C. Young leaves of *C. siliqua* and *Quercus* spp. floated over flooded soil were used as baits. After 24-48 h incubation, necrotic segments (2×2 mm) from symptomatic leaves were plated in Petri dishes onto selective PARPNH-agar medium [71]. Petri dishes were incubated at 20 °C in the dark. Outgrowing *Phytophthora* hyphae were transferred onto V8-juice agar (V8A) under the stereomicroscope. All the *Phytophthora* isolates were maintained on V8-agar in the dark at the temperature of 6 °C.

Additional isolations were performed directly from river water by using an *in situ* bating technique. To this aim, 10 non- wounded young leaves of *C. siliqua* and *Quercus* spp. were arranged in a mesh-bag styrofoam raft (25 x 30 cm) fixed to float on the water surface (Figure 2). In total, five mesh-bag-styrofoam rafts were placed, two on the surface of the Belluzza stream and three on the Cugno di Rio river, the two water courses crossing the reserve. The rafts were collected after 3 days. All obtained isolates were maintained on V8A and stored at 6°C in the dark.

Figure 2. Mesh-bag-styrofoam raft: **A** raft placed in Cugno di Rio river; **B** Raft floating on the water surface of the Belluzza river.

7.3.3. Morphological characterization of isolates

Cultures of seven days, grown on V8A at 20°C in the dark, were used to group all isolates into morphotypes on the basis of their colony growth patterns. For each host-plant and plant community, the different morphological types have been labelled with progressive numbering (Roman numbering); then, isolates belonging to the same sampling hosts have been tagged with the relative type-number.

Moreover, morphological features of chlamydospores, sporangia, oogonia, antheridia, hyphal swellings, were carefully analyzed and compared with species descriptions in the literature [72].

7.3.4. Molecular identification of isolates

The DNA of the pure cultures of isolates obtained from soil and rafts was extracted by using PowerPlant® Pro DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. The DNA was preserved at -20°C. The identification of *Phytophthora* species was performed by the analysis of Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA). DNA was amplified using forward primers ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') [73] and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [74]. The PCR amplification mix and thermocycler conditions were in accordance with Cooke et al. [73]. All PCRs were carried out in a 25 µl reaction mix containing PCR Buffer (1X), dNTP mix (0.2 mM), MgCl2 (1.5 mM), forward and reverse primers (0.5 mM each), Taq DNA Polymerase (1 U) and 100 ng of DNA. The thermocycler conditions were as follows: 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and then 72°C for 10 min.

Amplicons were detected in 1% agarose gel and sequenced in both directions by an external service (Amsterdam, The Netherlands). Derived sequences were analyzed using FinchTV v.1.4.0

[75]. For species identification, blast searches in GenBank[76], in a local database containing sequences of ex-type or key isolates from published studies and in *Phytophthora* Database [77] were performed. Isolates were assigned to a species when their sequences were at least 99-100% identical to a reference isolate.

7.3.5. Analysis of Phytophthora *diversity*

The *Phytophthora* diversity from soil samples sourced from the six plant communities was assessed by using the Shannon Diversity (H = −Σpiln(pi)), the Pielou evenness (J = H/lnS) and the Simpson dominance ($\lambda = 1/\Sigma pi2$) indices, where pi represents the frequency of each species and S the number of different species per plant communities. Since the assumption of normal distribution was violated (the Shapiro−Wilk test was applied), the statistical differences in the diversity among sampling areas were assessed by the Chi-square non parametric test of Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test (the R software [79] was used).

7.3.6. Soil analysis and USDA classification

Additional twenty rhizosphere soil samples were collected from plants within the selected sampling areas. The soil analysis was performed by a private laboratory (Progetto Ambiente & C. s.a.s., Catania, Italy) following the "Official method of soil chemical analysis" in accordance with standard protocols defined by D.M. 13/09/1999, G.U. n°248, 21/10/99 and D.M. 25/03/2002, G.U. n°84, 10/04/2002. The following characteristics of the soil were determined: pH-H_2O, electrical conductivity at 25°C, active limestone, organic matter content, nitrates and soil texture. To define the soil texture of each sample, USDA classification method [80] was used. The percentage of each soil component (sand, clay and silt) has been used in order to assign each sample to a textural class.

7.4. Results

Molecular analyses were performed on 228 isolates, of which 45 from rivers and 183 from soil of the reserve. These isolates represented all morphotypes recovered in soil samples and baiting rafts. Morphological and ITS sequence analyses revealed the occurrence of multiple *Phytophthora* species in each type of plant community with the only exception of the *Sarcopoterium spinosum* vegetation, where no *Phytophthora* species was detected. ITS sequence analyses showed that all 228 isolates (65.9%) matched with 99–100% identity reference sequences of 11 known *Phytophthora* species belonging to five different ITS Clades. ITS sequences of isolates of this study were deposited at GenBank, and, since isolates of the same species were all the same, only some sequences were deposited in GenBank. Among the isolates, 100 belonged to species in ITS Clade 6 (i.e.: *P. bilorbang, P. asparagi, P. lacustris* and *P. gonapodydes*), 72 to species in ITS Clade 2 (i.e.: *P.*

multivora, *P. oleae* and *P. plurivora*), 20 to species in ITS Clade 8 (i.e.: *P. cryptogea* and *P. syringae*) and 36 to *P. parvispora* and *P. nicotianae,* in ITS Clades 7 and 1, respectively. The distribution of each *Phytophthora* species in each plant community type is shown in Table 1.

Table 1. Diagrammatic representation of the diversity and distribution of *Phytophthora* species recovered from six plant communities in the ''Complesso Speleologico Villasmundo -S. Alfio" RNR. The proportion of isolates of each *Phytophthora* species recovered from each plant community is reported on the same lane. The color intensity indicates the frequency of each species in each plant community.

Plant community	***Phytophthora* species recovered in the reserve**										
	Clade 1	**Clade 2**				**Clade 6**			**Clade 7**	**Clade 8**	
	NIC	MUL	OLE	PLU	ASP	LAC	GON	BIL	PAR	CRY	SYR
Platano - Salicetum pedicellatae	16.7%	6.7%		3.3%		36.7%	3.3%	3.3%	6.7%	23.3%	
Sarcopoterium spinosum comm.											
Myrto communis - Pistacietum lentisci				34.4%	59.4%						6.25%
Pistacio - Quercetum ilicis				81.8%	4.5%	13.6%					
Oleo - Quercetum virgilianae	48%		8.7%	4.3%	35%					4.3%	
Gallery forest dominated by *N. oleander*				14.3%		71.4%		14.3%			

ASP= *P. asparagi*; BIL= *P. bilorbang*; CRY= *P. cryptogea*; GON= *P. gonapodyides*; LAC= *P. lacustris*; MUL= *P. multivora*; NIC= *P. nicotianae*; OLE= *P. oleae*; PAR= *P. parvispora*; PLU= *P. plurivora*; SYR= *P. syringae*. % isolated species in each plant community

With the only exception of the *Sarcopoterium spinosum* community, *Phytophthora* species from the ITS Clade 6 were isolated from all the plant communities (Figure 3). The most common *Phytophthora* species were aquatic species from the ITS Clade 6, such as *P. lacustris*, which were recovered from all river and riparian systems. *P. gonapodyides* and *P. bilorbang* were isolated from rhizosphere soil of willow while *P. lacustris* was recovered from river water and rhizosphere soil of mastic. With regard to species from Clade 2, *P. oleae*, *P. multivora* and *P. plurivora*, they were detected in five out of six surveyed plant communities. In particular, *P. oleae* was recovered from rhizosphere soil of southern live oak; *P. multivora* from river water and *P. plurivora* from river water and rhizosphere soil of mastic, cork oak, southern live oak and holm oak. The Clade 8 species, *P. cryptogea* and *P. syringae,* were found in three out of six plant communities. *P. cryptogea* was recovered from rhizosphere soil of willow, plane tree and southern live oak while *P. syringae* was recovered only from rhizosphere soil of mastic. *Phytophtora parvispora* (ITS Clade 7) was isolated exclusively from rhizosphere soil of mature willow trees in the *Platano-Salicetum pedicellatae* plant community. Finally, the ITS Clade 1 species *P. nicotianae* was found in the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virigilianae* plant communities and was isolated from rhizosphere soil of plane, willow, southern live oak and cork oak (Table 2). *Phytophthora plurivora*, *P. asparagi*, *P. lacustris and P. nicotianae* were the prevalent species accounting for 28%, 25%, 17% and 14% of all the isolates, respectively. Conversely, *P. syringae*, *P. gonapodyides*, *P. bilorbang*, *P. multivora*, *P. oleae* and *P.parvispora* were represented by less than 3% of isolates.

The structure of the community *of Phytophthora* species in soil samples differed significantly between the vegetation types. Among 11 *Phytophthora* species detected, only *P. plurivora* was found in all five plant communities. By contrast, *P. oleae* was detected exclusively in the *Oleo-Quercetum virgilianae, P. syringae* in the *Myrto Communis- Pistacietum lentisci*; *P. gonapodyides*, *P. bilorbang* and *P. parvispora* in the *Platanum-Salicetum pedicellatae.*

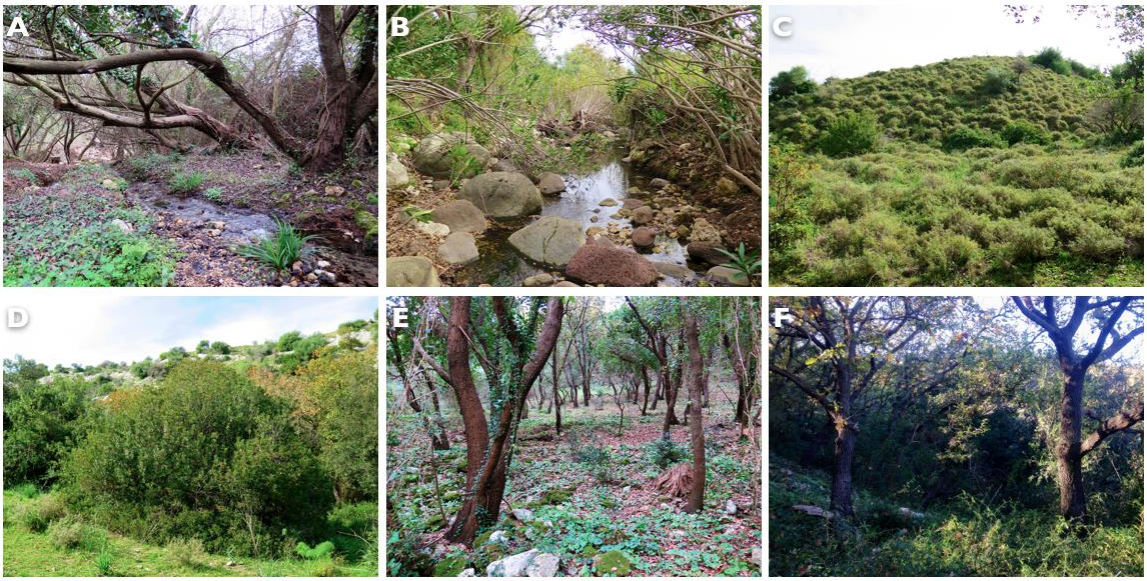
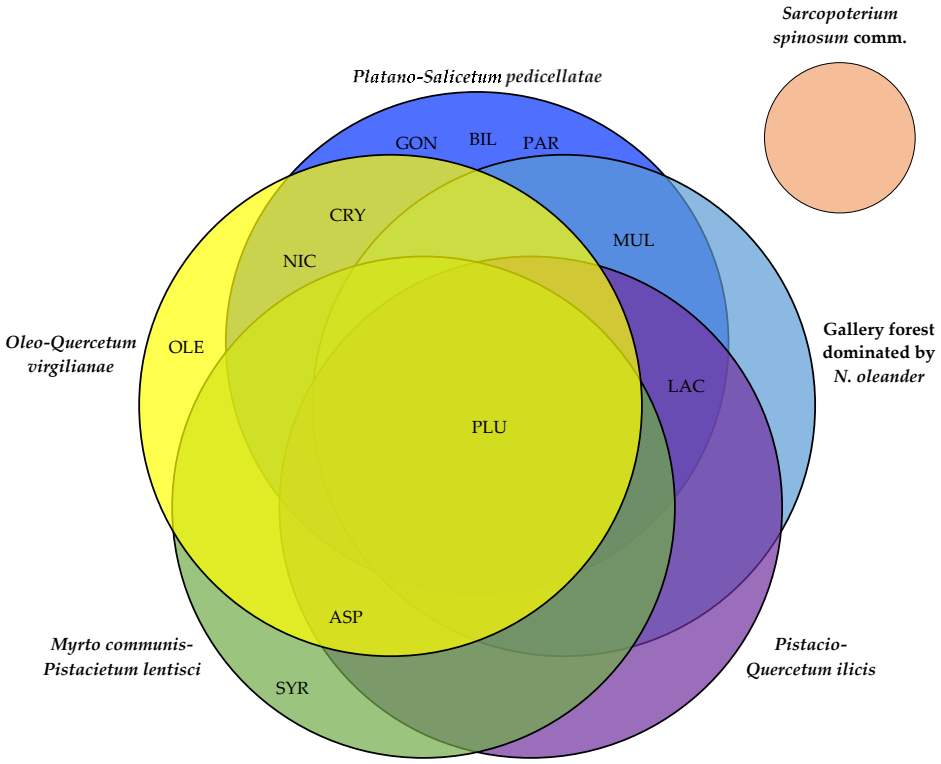


Figure 3. Plant communities: **(A)** *Platano-Salicetum pedicellatae*; (**B)** Gallery forest dominated by *Nerium oleander*; **(C)** *Sarcopoterium spinosum* community; **(D)** *Myrto communis-Pistacietum lentisci*; **(E)** *Pistacio-Quercetum ilicis*; **(F)** *Oleo-Quercetum virgilianae*.

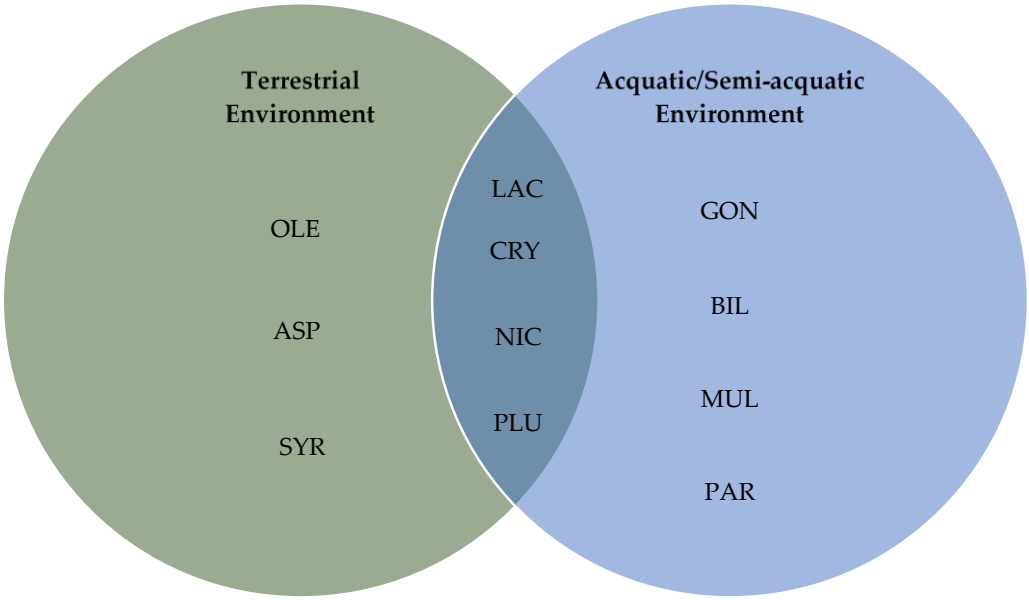


ASP= *P. asparagi*; BIL= *P. bilorbang*; CRY= *P. cryptogea*; GON= *P. gonapodyides*; LAC= *P. lacustris*; MUL= *P. multivora*; NIC= *P. nicotianae*; OLE= *P. oleae*; PAR= P. *parvispora*; PLU= *P. plurivora*; SYR= *P. syringae*

Figure 4. Venn diagram showing the distribution of *Phytophthora* species in six different plant communities in the "Complesso Speleologico Villasmundo- S. Alfio" RNR.

Overall, the sets of *Phytophthora* species from different plant communities strongly overlapped (Figure 4).

Comparing the different environments from which the species have been isolated, it can be observed that *P. cryptogea, P. lacustris*, *P. nicotianae*, and *P. plurivora* were recovered from both terrestrial and aquatic or semi-aquatic environments, while *P. asparagi*, *P. oleae* and *P. syringae* were found exclusively in terrestrial environments, *P. bilorbang*, *P. gonapodyides*, *P. multivora* and *P. parvispora* only in aquatic or semi-aquatic environments. Overall, the sets of species from terrestrial habitats and from aquatic or semi-aquatic (riparian) habitats were distinct (Figure 5).



ASP= *P. asparagi*; BIL= *P. bilorbang*; CRY= *P. cryptogea*; GON= *P. gonapodyides*; LAC= *P. lacustris*; MUL= *P. multivora*; NIC= *P. nicotianae*; OLE= *P. oleae*; PAR= P. *parvispora*; PLU= *P. plurivora*; SYR= *P. syringae*

Figure 5. Venn diagram showing the distribution of *Phytophthora* species in terrestrial and aquatic or semi-aquatic environments in the ''Complesso Speleologico Villasmundo-S. Alfio" RNR.

Results of soil analyses are schematically summarized in Table 3. Values of soil pH from all plant communities were above 7.5 and not significantly different from each other. Concerning the electrical conductivity at 25°C, moderately high values were found in soil samples from the *Platano-Salicetum pedicellatae* (1,100 ± 48 μS/cm), *Sarcopoterium spinosum* comm. (973 ± 45 μS/cm) and *Myrto communis-Pistacietum lentisci* (894 ± 43 μS/cm) plant communities, high values in soil of the *Pistacio-Quercetum ilicis* (1,414 ± 63 μS/cm) and significantly lower values in soil from the *Oleo-Quercetum virgilianae* (439 ± 36 μS/cm) plant community. As far as the active limestone is concerned, a high value was found in soil of the *Oleo-Quercetum virgilianae* (127 ± 5 g/Kg), while a relatively low value was recorded in soil from *Pistacio-Quercetum ilicis* (31 ± 2 g/Kg). The amount of nitrates was relatively high in soil from *Pistacio-Quercetum ilicis* and *Oleo-Quercetum virgilianae* plant communities (12.8 ± 1 and 11.59 ± 1 mg/Kg, respectively) and significantly lower in soil taken from *Platano-Salicetum pedicellatae*, *Sarcopoterium spinosum* comm. and *Myrto communis-Pistacietum lentisci*. Soils from all plant communities were rich in organic matter. The highest content of organic matter was found in soil from *Pistacio-Quercetum ilicis* (15%).

According to the USDA soil textural classification, soils from *Platano-Salicetum pedicellatae*, *Sarcopoterium spinosum* comm. and *Oleo-Quercetum virgilianae* were sandy clay loam; soil from *Pistacio-Quercetum ilicis* was clay loam and soil from the *Myrto communis-Pistacietum lentisci* was

sandy clay. No obvious correlation was found between soil characteristics and *Phytophthora* species diversity.

Table 2. Soil characteristics in each plant community type of the ''Complesso Speleologico Villasmundo-S. Alfio" RNR.

Soil properties	**Plant community[a]**				
	Platano-Salicetum pedicellatae	*Sarcopoterium spinosum* comm.	*Myrto communis-Pistacietum lentisci*	*Pistacio-Quercetum ilicis*	*Oleo-Quercetum virgilianae*
pH	7.5 ± 0.1	7.4 ± 0.1	7.3± 0.1	7.4± 0.1	7.6± 0.1
Electrical conductivity at 25° C (μS/cm)	1.100 ± 48	973 ± 45	894 ± 43	1.414 ± 63	439 ± 36
Active limestone (g/Kg)	69 ± 3	86 ± 4	81 ± 4	31 ± 2	127 ± 5
Soil texture	Sandy clay loam	Sandy clay loam	Sandy clay	Clay loam	Sandy Clay loam
Nitrates (mg/Kg)	6.7 ± 0.7	6.8 ± 0.6	4 ± 0.5	12.8 ± 1	11.59 ± 1
Organic matter (%)	5 ± 0.3	6 ± 0.5	8.2 ± 0.7	15 ± 2	6.1 ± 0.5

[a] Only rafts were placed in the Gallery forest dominated by Nerium oleander and no soil sample was collected from this vegetation community.

The analysis of diversity of *Phytophthora* populations from different plant communities showed a high variability of evenness (Table 4). Significantly higher values of Shannon and Pielou eveness diversity indexes were observed in the *Phytophthora* populations from the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* plant communities, while the evenness was moderate to low in *Phytophthora* populations from *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilici* and Gallery forest dominated by *N. oleander*. By contrast, values of Simpson dominance index were significantly higher in *Phytophthora* populations from Gallery forest dominated by *N. oleander* and *Pistacio-Quercetum ilicis*, intermediate in the *Phytophthora* population from *Myrto communis- Pistacietum lentisci* and significantly lower in the *Phytophthora* populations from *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* plant communities.

Table 3. Values of the diversity indices, Shannon diversity, Evenness and Simpson dominance of *Phytophthora* populations from six different plant communities in the "Complesso Speleologico Villasmundo-S. Alfio" RNR. Data were analyzed with the Kruskal–Wallis test. Different letters indicate significant differences according to Dunn's multiple comparison tests (P≤0,01).

Plant communities	**Diversity indexes**					
	Shannon index		**Pielou evenness**		**Simpson dominance**	
Platano-Salicetum pedicellatae	1.707	a	0.821	a	0.229	c
Sarcopoterium spinosum comm.	-		-		-	
Myrto communis- Pistacietum lentisci	0.760	b	0.692	b	0.563	b
Pistacio-Quercetum ilicis	0.652	bc	0.593	c	0.649	a
Oleo-Quercetum virgilianae	1.205	a	0.749	ab	0.361	c
Gallery forest dominated by *N. oleander*	0.451	c	0.650	b	0.722	a

7.5. Discussion

As many as 11 *Phytophthora* species, including putatively endemic and exotic species as well as pathogens associated prevalently to agriculture, such as *P. nicotianae* [14,81], were found in the ''Complesso Speleologico Villasmundo-S. Alfio" RNR, a relatively high number compared to the limited extension of the reserve (0.72 km^2). This can be explained with the quite recent establishment of the reserve (D. ARTA n° 616 04/11/1998), which up to 22 years ago also comprised tree crops whose relics still survive, and the high variability of plant communities included within the boundaries of the reserve, each occupying a different ecological niche and constituting a distinct ecosystem. The variability of ecosystems within the reserve is further increased by the presence of two streams, which favor the settlement of species with aquatic or semi-aquatic habitats along the banks and in the rhizosphere soil of the riparian vegetation.

Phytophthora plurivora, *P. asparagi* and *P. lacustris* were the most widespread species in the "Complesso Speleologico Villasmundo—S. Alfio" RNR, whereas the other species had a scattered distribution. Among the species recovered from "Complesso Speleologico Villasmundo-S. Alfio" RNR, some, such as *P. cryptogea*, *P. gonapodyides, P. plurivora* and *P. syringae*, are common in natural and forest ecosystems throughout Europe [35,60,61,82], while others, like *P. bilorbang P. oleae* and *P. parvispora,* occur more sporadically. Both *P. plurivora* and *P. gonapodyides* were reported in a previous survey of protected natural areas in Sicily [31]. In this study, *P. oleae* was isolated only from rhizosphere soil of southern live oak *(Q. virgilianae* (Ten.) Ten.) in the *Oleo-Quecetum virgilianae* plant community. It is the first time this recently described species is reported from a host plant other than olive (*O. europaea* L.) [83,84]. *Phytophthora bilorbang* is a prevalently aquatic species, but it has been occasionally reported as an opportunistic, aggressive plant pathogen [85]. *Phytophthora parvispora* (formerly, *P. cinnamomi* var. *parvispora*), a species in clade 7a, is an aggressive plant pathogen with a prevalently terrestrial habitat. In this survey, it was recovered from the rhizosphere of willow (*S. pedicellata* Desf.) in a riparian semi-aquatic environment. In a previous study, it had been reported as a pathogen of strawberry tree (*Arbutus unedo*) in Sardinia (central Italy) and described as a new species distinct from *P. cinnamomi* [86]. The first record of this species in Italy dates back to 2010 and was from ornamental plants in nursery [87].

Phytophthora gonapodyides is a species with a prevalently aquatic lifestyle, but may be an aggressive opportunistic plant pathogen [88]. It inhibits seed germination and causes root rot and stem lesions in *Quercus robur* L. and *Q. ilex* L.; also in association with other species of *Phytophthora* it was recovered from declining oak stands even in xerophytic environments [71,89,90]. According with Erwin and Ribeiro [90] the damage caused by this species is

underestimated and is overshadowed by that of other *Phytophthora* species, since it is traditionally regarded a minor pathogen. Presumably, the role of this and other *Phytophthora* species with a prevalently aquatic lifestyle in natural ecosystems is more complex than that of mere plant pathogens and deserves to be further investigated.

Phytophthora plurivora is a very polyphagous pathogen whose host range encompasses more than 80 woody host species including oaks (*Quercus* spp.), willows (*Salix* spp.), oleander (*N. oleander*) and oriental plane (*P. orientalis* L.) [31,92]. In the 'Complesso Speleologico Villasmundo-S. Alfio" RNR, it was the species with the widest distribution and was isolated from rhizosphere soil of mastic (*P. lentiscus* L.), southern live oak (*Q. virgilianae* (Ten.) Ten.), holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.) as well as from river water of *Platano-Salicetum pedicellatae*. Its polyphagia and the ability to produce resting spore, such as thick-walled oospores, might explain the cosmopolitan attitude of this species and its widespread occurrence in Mediterranean natural ecosystems. Interestingly, both *P. plurivora* and the other three species found exclusively in terrestrial habitats, i.e. *P. asaparagi*, *P. oleae* and *P. syringae*, are homothallic and produce oospores with thick walls. This could be a common adaptive strategy of these species to cope with adverse environmental conditions typical of many ecosystems in Mediterranean climate, such as long periods of drought alternating with intense rainfall, mild wet winters, high temperatures in summer, wide excursion of daily temperature and rapid fluctuation of air and soil humidity. In general, the ability to produce resting structures increases the competitiveness of *Phytophthora* spp. in terrestrial environments. All the species isolated from rhizosphere soil samples collected from diverse plant communities and streams in the ''Complesso Speleologico Villasmundo-S. Alfio" RNR, with the only exception of *P. lacustris*, produce at least one kind of resting structures. However, only *P. asparagi*, *P. bilorbang*, *P. multivora*, *P. plurivora* and *P. syringae* are homothallic. In addition, *P. nicotianae, P. oleae, P. parvispora* and possibly *P. syringae* are able to produce chlamydospores.

Phytophthora lacustris is an ubiquitous species in riparian ecosystems, such as reed belts and riparian alder stands, throughout Europe and North America [20,93]. Like other species in clade 6, this species tolerates high temperatures, is sexually sterile and produces a great amount of zoospores [20]. For the clade as a whole, this combination of characters has been interpreted as an adaptation to riparian environments [17,94]. However, although *P. lacustris* is significantly less aggressive than other *Phytophthora* spp. with a terrestrial habitat, it can seriously infect fine roots of trees stressed by episodes of flooding or drought [20]. Accordingly, in the present study, *P. lacustris* was isolated prevalently from the stream crossing the Gallery forest plant community which is dominated by oleander (*N. oleander*), and from the rhizosphere soil of willow (*S.*

pedicellata) trees in the *Platano-Salicetum pedicellate* riparian plant community, but *P. lacustris* was recovered even from the rhizosphere soil of southern live oak (*Q. virgilianae*) in the xerophytic *Pistacio-Quercetum ilicis* plant community.

Phytophthora asparagi, another species included in clade 6 but in a separate subclade [95], was recovered exclusively from terrestrial environments and was common in different plant communities, including *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis* and *Oleo-Quercetum virgilianae*. In a previous survey of the National Park of La Maddalena archipelago (Sardinia)., this species was isolated frequently from the rhizosphere soil of typical plants of the Mediterranean maquis vegetation showing symptoms of decline, including white asparagus (*Asparagus albus L.), Phoenician juniper (Juniperus turbinata Guss.) and mastic (P. lentiscus L.) [65]*. In the ''Complesso Speleologico Villasmundo-S. Alfio" RNR, *P. asparagi* was recovered from the rhizosphere soil of mastic as well as from the rhizosphere soil of evergreen oaks, including southern live oak (*Q. virginiana* (Ten.) Ten.), holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.), as a result the list of known host-plants to which this species is associated has expanded.

In the present survey, two other species from clade 6, *P. gonapodiydes* and *P. bilorbang*, were detected. They were found exclusively in riparian ecosystem and rivers, confirming a prevalently aquatic lifestyle [20,65,94,96]. Very probably, the high number of isolates of clade 6 species obtained in this study and their widespread occurrence is related to the high adaptability of these species to different environmental conditions and their ability to produce numerous zoospores, which in natural ecosystems are easily transported and spread by water courses.

The diversity and species richness of *Phytophthora* populations associated to different plant communities, as measured using three distinct indexes, Shannon diversity, Pielou eveness and Simpson dominance, were significantly higher in *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae*, very probably reflecting the richness of host-plant species and the complexity of these termo-mesophilous communities. Moreover, the higher richness and diversity of species in the *Platano-Salicetum pedicellatae* might be explained by the presence of both aquatic and terrestrial environments within this plant community. The value of Pielou evenness index also suggests that *Phytophthora* community in the *Platano-Salicetum pedicellatae* was more balanced than in the *Oleo-Quercetum virgilianae*, due to the dominance of *P. nicotianae*, an aggressive and polyphagous plant pathogen, in the latter community. Considering the proportions of isolates recovered from each of the abovementioned plant communities regarded together as an ecological succession, it can be supposed that the progressive decrease of complexity causes both the reduction in the diversity of *Phytophthora* communities and the progressive unbalance in their composition, resulting in the dominance of the most aggressive

Phytophthora species. In accordance with this hypothesis, the very low complexity of the garrigue of *Sarcopoterium spinosum* (L.) Spach is the extreme ecological limit for the establishment of *Phytophthora* communities.

Nevertheless, the sets of *Phytophthora* species in each plant community, as represented by the Venn diagram, showed the tendency to cluster together, probably due to the dominant presence of two invasive species, such as *P. plurivora* and *P. asparagi*, and the proximity in the space of different ecosystems. The strong overlapping of *Phytophthora* species sets also indicates that environmental conditions are conducive to these oomycetes in all types of plant communities examined in this study. In particular, no obvious correlation was observed between the diversity of distribution of *Phytophthora* populations and major soil properties that may influence the ecology of *Phytophthora* species, their aggressiveness and ability to survive, such as pH, salinity, textural class, active limestone, nitrates and organic matter content [97–103]. In all plant community types, the content of organic matter was relatively high, but particularly in the *Pistacio-Quercetum ilicis*. This might explain the occurrence of *P. lacustris* in rhizosphere soil of trees in this vegetation community. *Phytophthora* species, in fact, including several aggressive plant pathogens, have the ability to either survive or complete their lifecycle as saprobes, despite their poor ability to compete with other saprophytic organisms. This saprophytic attitude is more pronounced in clade 6 species, like *P. lacustris* [20]. Therefore, higher levels of organic matter favor the establishment and survival of *Phytophthora* spp., in general, and of clade 6 species, in particular.

Also other soil parameters, with the only exception of the high level of active limestone in the *Oleo-Quercetum virgilianae*, which could be a limiting factor, can be considered within the optimum range in all plant community types, including the *Sarcopoterium spinosum* comm., where no *Phytophthora* species was found. This indicates that soil properties were not a limiting factor for the settlement and survival of *Phytophthora* spp. in this xerophytic plant community type. Conversely, this confirms a prevalent direct or indirect major role of the vegetation type as driving factor shaping the *Phytophthora* communities in natural ecosystems. Overall, comparing *Myrto communis- Pistacietum lentisci, Pistacio-Quercetum ilicis* and *Sarcopoterium spinosum* comm., with *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae*, the *Phytophthora* diversity shows a trend that could be related with the degree of maturity of plant communities. Results of this study are in agreement with those of a similar study carried out in forests of South Africa [104]. Conversely the geographic distribution of *P. cinnamomi* in the Iberian Peninsula turned out to be influenced primarily by abiotic factors, soil texture and climate, followed by land use and lastly by the presence of main host-plant species [105]. However, comparisons with the results of

this last study are impaired by the scale of the survey, the heterogeneity of environments investigated and the fact that only one *Phytophthora* species was involved.

When the *Phytophthora* species found in the "Complesso Speleologico Villasmundo-S. Alfio" RNR were grouped on the basis of the type of environment from which they were recovered, Venn diagram clearly separated them into two distinct sets, including species with a prevalent or exclusive terrestrial habitat, such as *P. asparagi*, *P. oleae* and *P. syringae,* and species with a prevalent or exclusive aquatic or semi-aquatic habitat, such as *P. bilorbang* and *P. gonapodyides*, respectively. The first group was characterized by homothallic species, able to produce thick-walled oospores allowing them to survive adverse soil conditions; the second one comprised species that have adapted to thrive in aquatic and semi-aquatic environments. The presence of prevalently aquatic species in the first set and vice versa, the presence of typically terrestrial species in the second set, may be explained with the proximity of vegetation to water streams, flooding events and runoff of rain water. Consistently with our results, in a very recent study a correlation was found between the *Phytophthora* community and both the type of vegetation and environment in the French Guiana rainforest, which like other neotropical forests is considered a major plant diversity hotspot [105]. However, unexpectedly the *Phytophthora* community in this forest showed a low diversity compared to the richness of species recovered from the "Complesso Speleologico Villasmundo-S. Alfio" RNR.

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8. Antifungal activity of selected lactic acid bacteria from olive drupes

Mario Riolo[1,2], Carlos Luz[3], Elena Santilli[4], Giuseppe Meca[3], Santa Olga Cacciola[1]*

[1.] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

[2.] Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[3.] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Burjassot, Spain

[4.] Council for Agricultural Research and Agricultural Economy Analysis, Research Centre for Olive, Citrus and Tree Fruit-Rende CS (CREA- OFA), 87036 Rende, Italy;

[3.] Department of Agricultural Science, Mediterranean University of Reggio Calabria, 89122 Reggio Calabria, Italy;

* Correspondence: olga.cacciola@unict.it

8.1. Abstract

In this study, 16 Lactobacilli (LABs) isolated from the drupes of olive (*Olea europaea*) oil varieties were identified as *Lactiplantibacillus plantarum* (seven isolates), *Pediococcus pentosaceus* (six isolates), *Enterococcus faecium* (two isolates) and *Streptococcus salivarius* (a single isolate) by peptide mass fingerprinting and sequencing of the 16S rRNA. Antifungal activity of LABs and their cell-free fermentates (CFSs) against several plant pathogenic fungi and oomycetes (fungi sensu lato), including *Alternaria*, *Aspergillus*, *Colletotrichum*, *Penicillium*, *Plenodomus* and *Phytophthora*, was evaluated in vitro using the culture overlaying and the agar diffusion tests. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined. LABs showed antifungal activity against the fungi sensu lato tested. The most noticeable inhibitory activity was shown by isolates of *L. plantarum* and *P. pentosaceus* against *Fusarium oxysporum*, *Colletotrichum* species and *Penicillium nordicum*. Chemical analysis revealed CFSs contained acid lactic and variable quantities of 14 diverse phenolic acids and 26 volatile organic compounds (VOCs). No obvious correlation was found between the metabolic profile of LABs and their antifungal efficacy. However, it is the first time that the potential of fermentates of LABs, recovered from drupes of olive oil varieties, as natural fungicides, was demonstrated.

Key words: LABs, VOCs, Organic Compounds; Phenolic Acids; olive diseases

8.2. Introduction

Several airborne and soilborne fungi and oomycetes cause diseases on Olive (*Olea europaea* L., family Oleaceae) [1–4]. Some of these pathogens, such as species of *Alternaria*, *Aspergillus*, *Colletotrichum*, *Fusarium* and *Penicillium*, are responsible for decay of olive drupes and deterioration of oil quality [5–10]. The damage caused by these fungi has particular economic relevance in countries where high quality standards of olive oil are pursued, such as Spain and

Italy, that with Greece are the three major olive producer countries worldwide [11]. Presently, the management of olive diseases caused by fungi and oomycetes, including fruit rots, largely relies on pre-harvest treatments with copper and synthetic fungicides. However European Directive 2009/128/EC, aiming at reducing substantially the use of pesticides in agriculture, has fostered the search of alternative, environmentally friendly and toxicologically more safe strategies, including the use of generally recognized as safe (GRAS) natural substances and microrganisms or their metabolites inciting natural plant defence mechanisms [12–20]. Lactobacilli (LABs) is the term commonly adopted to refer to Gram-positive bacteria formerly grouped in the genus Lactobacillus (family, *Lactobacillaceae*), the largest genus of lactic acid bacteria that recently was split into several genera, according to new taxonomic criteria [21]. LABs are found in a wide variety of environments, including soil (most commonly associated with the rhizosphere), plants (particularly decaying plant material), animals and humans (especially the oral cavity, intestinal tract and vagina). LABs are widely applied in food processing as starter cultures of fermentations and additives. In fruit and vegetable supply chains LABs have been exploited as food preservative for their ability to prevent the microbe dependent food spoilage [22]. The efficacy of LABs in prolonging the fruit shelf-life depends on their ability to produce antibacterial, antioxidant and antifungal secondary metabolites. LABs are also used to improve the flavour and taste and as probiotics to enhance the nutritional value of foods [23–25]. Previous studies highlighted the diversity of populations of LABs in the microbioma of olive fruit and showed it was dependent on the olive cultivar and the geographical origin [26–28], however predominant species of LABs in most olive fermentations are *Lactiplantibacillus plantarum* and *Pediococcus pentasaceus* [26,29–36]. LABs comprise numerous GRAS species including both *L. plantarum* and *P. pentosaceus* [37,38].

The objective of this study was to characterize various strains of lactic acid bacteria (LABs) isolated from different olive varieties and test their antifungal activity against the main olive pathogens. Moreover, the variability between the isolated and tested LABs strains was investigated with multicomponent analysis (PCA) based on phenolic, organic acids and volatile organic compounds (VOC) of each LABs.

8.3. Materials and Methods

8.3.1. Bacterial and fungal strains

LABs characterised in this study were obtained from different olive varieties (var) or cultivars (cv) sourced in Italy and Spain. The isolation was performed using sterile urine baker containing olives and Man Rogosa and Sharpe broth (MRS-B) (Oxoid, Dublin, Ireland) (ratio 1:2). Three replicates were performed for each olive sample. They were incubated at 37°C for 72 h in an

anaerobic atmosphere, using an Anaerocult® system (Millipore, Milan, Italy). Serial dilutions (10^{-4}, 10^{-5}, 10^{-6}) of the fermented media were sprayed on Man Rogosa and Sharpe agar (MRS-A) in Petri dishes and kept as before for 48 h. For each plate, five colonies were isolated and grown individually on MRS-A (Oxoid, Dublin, Ireland) in the same incubation conditions. To obtain pure cultures, different colony forming units were sub-cultured on MRS-A. Gram stain was performed on isolated cultures to select only Gram-positive bacteria [39]. Gram-positive, catalase-negative bacilli were identified as putative LABs. Selected cultures were preserved in cryotubes on MRS-B submerged with 30% glycerol at −20°C.

To restore the cultures, frozen bacteria were added to MRS-B and incubated for 24 h at 37°C, then 1 mL aliquots were transferred to MRS-B and incubated as above.

To evaluate the antifungal activity of isolates, LABs were tested *in vitro* against pathogenic fungi *s.l.*, i.e. *Alternaria alternata* (isolates CECT 646 and ITEM 8121), *Aspergillus flavus* (ITEM 8111), *Colletotrichum acutatum* (UWS149 and C9D2C), *C. fiorinae* (ER2147 and C15D6A), *C. gloeosporioides* (C2 and RD9B), *C. nymphaeae* (RB 012 and RB 428), *Fusarium oxysporum* (COAL 68), *Penicillium digitatum* (P1PP0 and N2F1), *P. expansum* (COAL 95), *P. nordicum* (CECT 2320), *Plenodomus tracheiphilus* (Pt2), *Phytophthora nicotianae* (P4K3F8 and T-2K1A) and *Ph. oleae* (V-2K10A). Those isolates were sourced from the collection of the Molecular Plant Pathology laboratory of the Department of Agriculture, Food and Environment, Catania University, Italy, the Colección Española de Cultivos Tipo (CECT), Valencia University, Spain, and ITEM Collection from Istituto di Scienze delle Produzioni Alimentari (ISPA), National Research Council, Bari, Italy. Most of them were characterized in previous studies [16,40–44].

8.3.2. Bacterial identification

LAB strains characterization was performed by extraction of bacterial cultures, as reported by [45]. The method was performed with MALDI-TOF MS using a Microflex L20 mass spectrophotometer (Bruker Daltonics, Billerica, MA, USA) equipped with an N2 laser. Spectra were acquired in positive linear ion mode. Voltage acceleration was 20 kV and the mass range for the analysis was delimited from 2000 to 20000 Da. For each sample, three spectra were obtained following the method MALDI Biotyper Realtime Classification (RTC). Identification corresponded to the largest log score. Results were compared with the database MBT 7854 y MBT 7311_RUO (Bruker Daltonics).

8.3.3. Fungal inoculum

Ascomycota isolates were grown on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, UK) at 25°C in the dark until the mycelium covered the Petri dish. A conidial suspension (104 ml^{-1}) of each isolate in sterile distilled water (SDW), used as inoculum, was determined with a Neubauer

chamber. *Phytophthora nicotianae* and *Ph. oleae* were grown on Oat Meal Agar (OMA, Thermo Fisher, Waltham, Massachusetts, USA) at 22°C in the dark for 7 days. Zoospore production was performed according to Aloi et al. [46]. Zoospore concentration was determined with a Neubauer chamber and adjusted to 10^4 zoospores/mL.

8.3.4. Preparation of CFS (culture-free supernatants)

The preparation of culture-free supernatants (CFSs) was performed according Dopazo et al. [24]. Then, the medium was centrifuged at 4°C and 4000 rpm for 15 min, in an Eppendorf 5810 R centrifuge (Eppendorf, Hamburg, Germany). CFS was recovered, frozen at −80°C, lyophilized in a FreeZone 2.5 L Benchtop Freeze Dry System, (Labconco, Kansas City, MO, USA) and preserved at −20 °C for further analysis. To be tested lyophilized CFSs were resuspended in SDW.

8.3.5. Qualitative assay of antifungal activity in solid medium

The effect of LABs and their CFSs on the growth of tested fungi *s.l.* was preliminarily evaluated using two distinct qualitative methos, the culture overlay, and the diffusion agar tests, respectively. In the first test, LAB cultures, grown for 24 h at 37ºC, were inoculated in the centre of MRS-A dishes containing 15 ml of medium and incubated at 37ºC for 72 h. Conidia or zoospores were then suspended in sterile 0.1% Tween–water and their concentration was adjusted using a Neubauer chamber. After 24 h incubation, dishes inoculated with the LAB suspension were covered with 15 mL of PDA (45ºC) containing 104 spores/mL and incubated at 25ºC for another 72 h. After incubation the fungal growth inhibition was determined according to Guimarães et al. [47]. The diffusion agar test was used to assay the inhibitory effect of bacterial CFSs on fungi s.l. tested according to Dopazo et al. [24]. Halos larger than 8 mm were considered positive for antifungal activity [48]. The results of the test, performed in triplicate, were scored based on an empirical scale where: (+) mean diameter of inhibition halo <8 mm; (++) mean diameter of inhibition halo 8-10 mm; (+++) mean diameter of inhibition halo >10 mm.

8.3.6. Quantitative assay of antifungal activity

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the CFS were determined in sterile 96-well microplates using the method described by Luz et al [49]. For each well, 100 μL of CSF were dispensed at doses between 0.1 and 100 g/L. Then, 100 μL of conidium/zoospore sospensions (5×10^4 spores/mL) in PDB were added to each well. A negative control was obtained by adding 200 μL PDB to a well and a positive one by adding 100 μL PDB and 100 μL of the spore suspension. This method was applied for each bacterial strain tested in this study. Then, microplates were incubated at 25 °C for 72 h. For each pathogen four replicas were performed. The MIC was considered the lowest concentration of CFS at which fungi did not grow. To determine MFC, 10 μL of the concentration corresponding to the MIC and higher

concentrations were sub-cultured on PDA dishes and incubated at 25° C for 72h. Accordingly, the MFC was defined as the lowest extract concentration which prevented any mycelium growth.

8.3.7. Identification of organic and phenolic acids in CFS

To identify organic acids produced by LABs selected on the basis of their antifungal activity, CFS were diluted in water (ratio 1/20) and injected into the high-performance liquid chromatography (HPLC) system (Agilent 1100 Series 203 HPLC System, Agilent Technologies, Palo Alto, CA, USA), equipped with a diode array and quaternary pump, using a 20 μL sample injection loop [50]. The analytical separation was achieved with a Rezex ROA-Organic Acid H+ (8%) (150 x 7.8 mm) ion exclusion column (Phenomenex, Torrance, CA, USA) using an isocratic mobile phase of acidified water (pH 2.1) at a flow rate of 0.6 mL/min for 25 min. The chromatogram was monitored at 210 nm and results were expressed as g/L. Data were acquired by the HP209 CORE ChemStation system (Agilent Technologies, Santa Clara, CA, USA).

The QuEChERS method supported the recognition of phenolic acids of CFS isolates eliminating possible interferents before the chromatographic analysis [51]. Extract was prepared for each fermented CFS, bringing ten millilitres with 10 mL ethyl acetate, 4 g MgSO4, 1% formic acid, 1 g NaCl and then vortexed for 1 min, and then it was centrifugated at 4000 rpm for 15 minutes. Next step was the integration of 150 mg C18 and 900 mg MgSO4 with the supernatant and then vortex for 1 min. The extract was centrifuged again, and the supernatant was evaporated into Turbo vapor a nitrogen flow. Before chromatographic analysis, the purified extract was resuspended in 1 mL of H2O:ACN (90:10 v/v).

For the determination, HPLC system Agilent 1200 (Agilent Technologies, Santa Clara) equipped with a vacuum degasser, autosampler and binary pump was used. The column was a Gemini C18 (50 mm × 2 mm, 100 Å, particle size of 3-μm; Phenomenex). The mobile phases consisted of water (A) as solvent, ACN as solvent B, both acidified (0.1% formic acid), with gradient elution (0 min, 5% B; 30 min 95% B; 35 min, 5% B). Before every analysis, the column was equilibrated for 3 min. 20 μL of sample was injected and the flow rate was 0.3 mL/min. Q-TOF-MS (6540 Agilent Ultra High Definition Accurate Mass) was used to conduct Mass spectrometry (MS) analysis equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) interface in negative ionisation mode following conditions: drying gas flow (N2), 8.0 L/min; nebuliser pressure, 30 psig; gas drying temperature, 350°C; capillary voltage, 3.5 kV; fragmentor voltage, 175 V; scan range, m/z 20–380. Targeted MS/MS experiments were carried out using collision energy values of 10, 20 and 40 eV. Integration and data elaboration were managed using MassHunter Qualitative Analysis software B.08.00 [52]. Results were expressed with a relative abundance (Log scale 2 to -2).

8.3.8. Analysis of the main volatile organic compounds (VOCs) from CFS

The determination of volatile organic compounds (VOCs) was performed by HS-SPME (headspace solid-phase microextraction) and subsequent analysis by gas chromatography coupled to mass spectrometry (GC-MS), following the methodology described by Luz et al. [24] with slightly modifications.

Samples were prepared for analysis by adding 10 mL of fermented CFS in 20 mL glass vial; they were then transferred to a 55 °C bath for 45 min with constant stirring with a crystal rod throughout the whole incubation period. The SPME holder (Supelco, Bellafonte, PA, USA) contained a fused-silica fibre of 80μm x 10 mm coated with a layer of divinylbenzene–carboxen–polydimethylsiloxane (DVB/CWR/PDMS) (Agilent Technologies, Santa Clara, California, US). The fibre was introduced into the Agilent 7890A GC system coupled to an Agilent 7000A triple quadrupole mass spectrometer with an electronic impact (EI) sensor. Thermal desorption of the analytes was performed at 250 °C for 10 min. Injection was performed in splitless mode. The capillary column (J&W Scientific, Folsom, CA, USA), with an HP-5MS (30 m × 0.25 mm, 0.25 μm 5% diphenyl–95% dimethylpolysiloxane) (Agilent Technologies, Santa Clara, California, US) was used for the analysis. The carrier gas was helium (99.99%), flowing at a rate of 1 mL min^{-1}. The program started at 40 °C for 2 min, then increased to 160 °C at 6 min; finally, the temperature was raised to 260 °C at 10 °C min^{-1} and remained constant for 40 min. Flow in the column was transferred to an Agilent 5973 MS detector. The ion source temperature was set at 230 °C, the ionizing electron energy was 70 eV and the mass range was 40–450 Da in full-scan acquisition mode. Compound identification was performed comparing their mass spectra with those recorded in an NIST Atomic Spectra Database version 1.6 (Gaithersburg, MD, USA), using 95% spectral similarity. Three replicas of each analysis were carried out. In addition, linear retention indices (LRI) were calculated based on the retention time of a solution of alkanes (C8-C20) tested under the same conditions as the samples and compared with the existing literature. Results were given as a percentage of each VOC in the CFS by dividing each analyte area by the total area.

8.3.9. Statistical analysis

Analysis was performed using RStudio v.1.2.5 (R). The assays were performed in triplicates, and the differences between control and treated groups were analyzed by Student's t-test, while the differences between the groups were analyzed by one-way ANOVA test. The significance levels were set HSD post hoc test at $p < 0.05$. Volatile compounds were analyzed by ANOVA test and the significance levels were set at $p \leq 0.01$. Moreover, to correlate the metabolites produced by each CFSs with the species of LAB identified, a Principal component analysis (PCA) was realised

using MetaboAnalyst 5.0 software [45]. The features included were log transformed and mean centred.

8.4. Results

Bacterial isolates (Table 1) were identified by peptide mass fingerprinting (Microflex L20) MALDI TOF MS. Identifications with a Log (score) higher than 2 were considered at species level. The isolates were identified as *Lactiplantibacillus plantarum* OV 20246 (OV8, OV9, OV16, OV17, OV20), *L. plantarum* OV 1055 (OV11), *Pediococcus pentosaceus* OV 20280 (LEC3, COR6, OT3 and OT5), *Streptococcus salivarius* OV 140417 (FR2), *Enterococcus faecium* (FR6 and COR6). Moreover, the identity of the strains was confirmed by the full sequence of the 16S rRNA obtained and compared to other verified isolates deposited in NBCI using BlastN tool.

Table 1: Isolates of Lactic Acid Bacteria (LAB) sourced from oleaster (*Olea europaea oleaster*) and different cultivars of olive (*O. europaea*) in Valencia (Spain) and Calabria (Italy), respectively, and characterized in this study.

9Isolate code	Species	Host (Species and Cultivar)	Geographical Origin
OV7	*Lactiplantibacillus plantarum*	*Olea europaea oleaster*	Villar del Arobispo, Valencia, Spain
OV8	*L. plantarum*	*O. europaea oleaster*	Villar del Arobispo, Valencia, Spain
OV9	*L. plantarum*	*O. europaea oleaster*	Villar del Arobispo, Valencia, Spain
OV11	*L. plantarum*	*O. europaea oleaster*	Higueruelas, Valencia, Spain
OV16	*L.plantarum*	*O. europaea oleaster*	Higueruelas, Valencia, Spain
OV17	*L. plantarum*	*O. europaea oleaster*	Higueruelas, Valencia, Spain
OV20	*L. plantarum*	*O. europaea oleaster*	Higueruelas, Valencia, Spain
FR2	*Streptococcus salivarius*	*O. europaea* 'Frantoio'	Mirto-Crosia, Cosenza, Italy
FR6	*Enterococcus faecium*	*O. europaea* 'Frantoio'	Mirto-Crosia, Cosenza, Italy
LEC3	*Pediococcus pentosaceus*	*O. europaea* 'Leccino'	Mirto-Crosia, Cosenza, Italy
OT3	*P. pentosaceus*	*O. europaea* 'Ottobratica'	Mirto-Crosia, Cosenza, Italy
OT5	*P. pentosaceus*	*O. europaea* 'Ottobratica'	Mirto-Crosia, Cosenza, Italy
COR2	*E. faecium*	*O. europaea* 'Coratina'	Mirto-Crosia, Cosenza, Italy
COR3	*P. pentosaceus*	*O. europaea* 'Coratina'	Mirto-Crosia, Cosenza, Italy
COR5	*P. pentosaceus*	*O. europaea* 'Coratina'	Mirto-Crosia, Cosenza, Italy
COR6	*P. pentosaceus*	*O. europaea* 'Coratina'	Mirto-Crosia, Cosenza, Italy

In *in vitro* assays, all 16 LABs showed antifungal activity. However, there was a consistent variability in the inhibitory efficacy among bacterial isolates against the diverse fungi *s.l.* tested. In the overlay test (Table 2), both isolates of *E. faecium* (FR6 and COR2) showed no antifungal activity and none of the bacterial isolates inhibited the mycelium growth of *A. flavus* and *Plenodomus tracheiphilus*. The most noticeable inhibitory activity was shown by isolates of *L. plantarum* and *P. pentosaceus*. Isolates of these two bacterial species exerted a relevant inhibitory activity versus *Fusarium oxysporum* and species of *Colletotrichum* with inhibition halos in most cases larger than 10 mm. Large (diameter > 10 mm) and medium (diameter 8÷10 mm) inhibition

halos were also observed in tests against *P. nordicum*. The inhibitory activity versus other *Penicillium* species was less consistent. All *L. plantarum*, *P. pentosaceus* and *S. salivarius* isolates showed inhibitory activity against *A. alternata*. However, the most effective were isolates OV8, OV17, COR3 and LEC3, whose inhibition halos were medium (diameter 8-10 mm) to large (diameter > 10 mm) in size. In tests against *Phytophthora* isolates the maximum inhibitory activity (mean diameter of inhibition halo > 10 mm) was shown by the bacterial isolate COR6 (*P. pentosaceus*). The inhibition halos induced by the other *L. plantarum*, *P. pentosaceus* and *S. salivarius* isolates were medium (diameter 8-10 mm) to low (diameter < 8mm) in size.

In the agar diffusion test (Table 3) all LABs, including the isolates of *E. faecium* (FR6 and COR2), showed antifungal activity. With few exceptions, results of this test agreed with those of the culture overlay test. None of the CFSs, in fact, inhibited the mycelial growth of *P. tracheiphilus* and CFSs of most bacterial isolates showed no antifungal activity against *A. flavus*. Only CFSs of bacterial isolates OV8, OV20 and FR6 inhibited the growth of this fungus and in any case the mean diameter of inhibition halos was < 8 mm. It was confirmed a noticeable inhibitory activity of *L. plantarum* and *P. pentosaceus* on *Colletotrichum* species and *F. oxysporum*. Also, the fermentate of FR6 isolate (*E. faecium*) showed a high inhibitory activity against *Colletotrichum* species and *F. oxysporum*. Although this LAB did not show any antifungal activity in the culture overlay test, in the agar diffusion tests its fermentate exerted a notable inhibition versus *Colletotrichum* species and *F. oxysporum* (diameter of inhibition halos >10 mm). Consistently with the results of the culture overlay test, *P. nordicum* was remarkably inhibited by LAB fermentates. Lab isolates with the highest inhibitory effect (diameter of inhibition halos >10 mm) were OV7, OV8 and OV11, all identified as *L. plantarum*. Inhibition halos of all other *Penicillium* species never exceeded 10 mm in diameter, in most cases they were <8 mm in diameter or no inhibitory effect was detected. CFSs of all bacterial species showed an inhibitory effect on one or both isolates of *A. alternata*, with the only exception of the CFS of COR 6 *P. pentosaceus* isolate. However, the diameter of inhibition halos was always <10 mm. CFSs of three *L. plantarum* isolates (OV8, OV11 and OV20) showed a relevant inhibitory activity against *P. nicotianae* and *P. olea*e (diameter of inhibition halos 8-10 mm), while CFSs of other bacterial isolates exerted only a low inhibitory activity (diameter of inhibition halos <8mm) or did not exert any inhibitory activity on these oomycetes.

The results of assays, aimed at determining the values of MIC and MFC and thus quantifying the antifungal activity of CFSs of LABs, are summarized in Table 4. In general, the values of MIC and MFC were in the same order of magnitude and in most cases overlapped. Consistently with the results of both the culture overlay and the diffusion tests, the CFSs that showed high antifungal activity, i.e. low values of MIC and MFC, included the CFSs of five LAB isolates, OV7, OV8, OV9,

OV11 and OV20, identified as *L. plantarum*), LEC3 (*P. pentosaceus*) and FR6 (*E. faecium*). Particularly, the MIC and MFC values of OV7 bacterial isolate were in the range of 12.5 to 100 g/L. The highest MIC and MFC values (100 g/L) for CFS of this *L. plantarum* isolate were recorded against *Ph. nicotianae*. Conversely, the lowest value (12.5 g/L) was recorded in assays versus isolates of *Colletotrichum*. However, differently from the other isolates of *Colletotrichum*, both MIC and MFC values against C2 and RD9B isolates of *C. gloeosporioides,* recovered from distinct host plants, were relatively high, i.e., 100 g/L (for both MIC and MFC against C2 isolate) and 50 g/L (for both MIC and MFC against isolate RD9B). Isolate OV8 of *L. plantarum*, which in general showed a comparable antifungal activity to that of isolate OV7, was more effective in inhibiting the mycelium growth of both C2 and RD9B isolates of *C. gloeosporioides*. MIC and MFC values against these two fungus isolates were 25 versus 12.5 g/L and 50 versus 50, respectively. Moreover, while MIC and MFC values against *Phytophthora* isolates ranged between 25 and 50 g/L for the LAB isolate OV8, they ranged between 10 and 100 g/L for isolate OV7. The CFS of *E. faecium* FR6 isolate, which showed no antifungal activity in the overlay test, but showed a consistent inhibitory activity in the agar diffusion test, inhibited all tested pathogens at concentrations ranging from 12.5 to 50 g/L for MIC and 25 to 50 g/L for MFC. MIC and MFC values for the CFS of this LAB isolate against isolates of *Phytophthora* were relatively high (100 g/L). Finally, FR6 isolate showed a noticeable antifungal activity against *P. nordicum* (MIC and MFC 12.5 and 25 g/L, respectively) and a relatively low antifungal activity against *P. expansum* and *P. digitatum* (MIC and MFC values between 50 and 100 g/L). The CFS of *P. pentosaceous* isolate LEC 3 showed a consistent inhibitory activity against all fungi and oomycetes tested, with MIC and MFC values ranging from 25 and 50 g/L.

General trends, confirming in part the results of culture overlay and agar diffusion tests, were observed. The antifungal activity against *A. flavus* of CFSs of all LABs was very low (values of both MIC and MFC ≥ 100 g/L) or in many cases out of the detectable range. CFSs of all LABs showed a clear antifungal activity against *A. alternata* and *F. oxysporum*, with MIC values ranging between 25 and 50 g/L. The antifungal activity of CFSs against *Colletotrichum* species was even more pronounced. With few exceptions, including the two above mentioned isolates of *C. gloeosporioides* (C2 and RD9B) and isolates of *C. godetiae*, MIC values against isolates of this fungal genus ranged between 12.5 and 50 g/L. MIC values of CFSs against *P. nordicum* were in the same range, confirming the high sensitivity of this isolate to the inhibitory effects of LABs. However, the antifungal activity of CFSs against the other two *Penicillium* species, *P. digitatum* and *P. expansum*, was less remarkable and lower compared to species of *Colletotrichum*. MIC values for the last two species of *Penicillium* ranged between 25 and 50 g/L. In general, isolates of

Phytophthora showed a lower sensitivity to all tested LAB fermentates compared to species of *Colletotrichum* and *Penicillium*. MIC and MFC values for these oomycetes were in the range between 25 and 100 g/L and in a few cases exceeded 100 g/L.

Table 2. Antifungal activity of lactic acid bacteria (LABs) against *Colletotrichum*, *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, *Plenodomus* and *Phytophthora* species in the culture overlay test. The antifungal activity was represented as follows: (+) means of inhibition zone between the well and fungal growth 8 mm, (++) means of inhibition zone between the well and fungal growth 8–10 mm, (+++) means of inhibition zone between the well and fungal growth > 10 mm. The well radius was 5 mm.

Pathogen	Isolate code	Lactic Acid Bacteria (LABs)															
		OV7	OV8	OV9	OV11	OV16	OV17	OV20	COR2	COR3	COR5	COR6	OT3	OT5	LEC3	FR2	FR6
Alternaria alternata	ITEM 8121	+	+++	+	+	+	++	+	-	++	+	+	+	+	++	+	-
A. alternata	CECT 646	+	+++	+	+	+	+++	+	-	++	+	+	+	+	+++	+	-
Aspergillus flavus	ISPA 8111	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum acutatum	C9D2C	+++	+++	+++	+++	+++	++	+++	-	++	+	+++	++	+++	+++	+	-
C. acutatum	UWS 149	+++	+++	+++	+++	+++	+	+++	-	++	+	+++	++	+++	+++	+	-
C. fiorinae	C15D6A	++	+++	+++	++	+++	+	+	-	+	++	+++	++	+++	+++	++	-
C. fiorinae	ER2147	++	++	+++	++	+++	+	+	-	+	++	++	++	+++	++	++	-
C. gloeosporioides	RD9B	++	+++	+	+	+++	+++	+	-	+	-	++	+	++	++	++	-
C. gloeosporioides	C2	++	++	+	+	+++	++	+	-	++	-	++	+	++	++	++	-
C. nymphaeae	RB012	++	+++	++	++	++	+++	-	-	+	+++	+++	++	+++	++	++	-
C. nymphaeae	RB428	++	+++	++	++	++	+++	-	-	+	+++	+++	++	+++	++	++	-
Fusarium oxysporum	COAL 68	+++	+++	++	+++	+++	+++	++	-	+	+	++	+	++	+++	++	-
Penicillium digitatum	P1PP0	+	+	++	-	+	+	+	-	+	++	-	+	+	-	+	-
P. digitatum	N2F1	+	+	+	-	+	+	+	-	+	++	-	+	+	-	+	-
P. expansum	COAL 95	+	+	+	+	+	++	++	-	-	-	-	-	+	-	-	+
P. nordicum	CECT 2320	++	++	+++	+	+++	-	+++	-	+++	+	+++	+	+	+	+	-
Phytophthora nicotianae	P4K3F8	++	++	++	++	+	+	++	-	+	++	++	+	+	++	+	-
Ph. nicotianae	T-2K1A	++	++	++	++	+	+	++	-	+	++	+++	+	+	++	++	-
Ph. oleae	V-2K10A	++	++	++	+	+	+	++	-	+	+	++	+	+	++	+	-
Plenodomus tracheiphilus	Pt.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Antifungal activity of sixteen cell-free supernatants (CFS) of lactic acid bacteria (LABs) against *Colletotrichum*, *Penicillium*, *Aspergillus*, *Fusarium*, *Alternaria*, *Plenodomus* and *Phytophthora* species in the diffusion agar test. The antifungal activity was represented as follows: (+) means 8 mm of inhibition zone between the well and fungal growth, (++) means 8–10 mm of inhibition zone between the well and fungal

growth, (+++) means > 10 mm of inhibition zone between the well and fungal growth. The well radius was 5 mm. A CFS concentration of 400 mg/m was used. MRS medium was used as control.

Pathogen	Isolate code	CFS of Lactic Acid Bacteria (LABs)															
		OV7	OV8	OV9	OV11	OV16	OV17	OV20	COR2	COR3	COR5	COR6	OT3	OT5	LEC3	FR2	FR6
Alternaria alternata	CECT 646	++	++	+	+	-	+	++	++	-	+	-	+	-	+	++	+
A. alternata	ITEM 8121	++	++	+	++	+	+	++	++	+	+	-	-	+	+	+	+
Aspergillus flavus	ITEM 8111	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Colletotrichum acutatum	UWS149	+++	+++	+++	++	++	+++	+++	++	+++	++	++	++	++	++	+	++
C. acutatum	C9D2C	+++	+++	+++	++	++	+++	+++	++	+++	++	++	++	++	++	+	++
C. fiorinae	ER2147	++	+++	+++	++	++	+++	+++	++	+	++	+	++	+	-	++	+++
C. fiorinae	C15D6A	+++	+++	+++	+++	++	+++	+++	++	+	++	+	++	+	+	++	+++
C. gloeosporioides	C2	+	++	++	+++	++	+	+++	++	+	+	+	+	++	+	+	++
C. gloeosporioides	RD9B	++	+++	++	+++	++	+	+++	++	+	++	+	++	++	+	+	++
C. godetiae	OLP10	++	++	+	+++	++	+	+++	++	+	+	+	++	+++	+	++	++
C. godetiae	OLP12	++	++	+	+++	++	+	+++	++	+	+	+	++	+++	+	++	++
C. nymphaeae	RB428	++	+++	++	++	++	++	+++	++	+	++	+	++	++	++	++	+++
C. nymphaeae	RB012	++	+++	++	++	++	++	+++	++	+	++	+	++	++	++	++	+++
Fusarium oxysporum	COAL 68	+++	+++	++	++	++	++	+++	+	++	+	-	++	++	+	++	+++
Penicillium digitatum	N2F1	+	+	+	+	+	+	+	++	-	+	+	-	-	-	-	-
P. digitatum	P1PP0	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-
P. expansum	COAL 95	+	+	+	+	+	++	++	-	-	-	-	-	+	-	-	+
P. nordicum	CECT 2320	+++	+++	++	+++	++	++	++	++	++	+	+	+	++	+	++	++
Phytophthora nicotianae	T-2K1A	+	++	+	++	+	-	++	+	-	-	-	+	-	-	-	+
Ph. nicotianae	P4K3F8	+	++	+	++	+	-	++	+	-	-	-	-	-	-	-	-
Ph. oleae	V-2K10A	+	++	+	++	+	-	++	-	-	-	-	-	-	-	-	-
Plenodomus tracheiphilus	Pt 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of lyophilised CFSs of lactic acid bacteria expressed in g/L. nd = not detected.

Pathogens and isolate code	**CFS of Lactic Acid Bacteria (LAB)**													
	OV7		**OV8**		**OV9**		**OV11**		**OV16**		**OV17**		**OV20**	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus flavus ITEM 8111	Nd	Nd	100	200	200	200	100	100	100	200	Nd	Nd	100	100
Alternaria alternata CECT 646	25	25	25	25	50	50	25	25	50	100	50	50	25	50
A. alternata ITEM 8121	25	25	25	50	25	50	25	25	50	50	50	50	25	25
Colletotrichum acutatum UWS149	12.5	12.5	12.5	12.5	25	25	25	50	50	100	12.5	12.5	25	25
C. acutatum C9D2C	12.5	12.5	12.5	12.5	12.5	25	50	50	25	25	12.5	25	12.5	25
C. fiorinae ER2147	25	25	25	25	25	50	25	25	25	25	25	25	25	25
C. fiorinae C15D6A	12.5	12.5	12.5	12.5	25	25	12.5	12.5	25	25	25	25	25	25

C. gloeosporioides C2	100	100	25	25	25	25	25	25	50	50	50	50	25	50
C. gloeosporioides RD9B	50	50	12.5	25	25	25	25	25	50	25	50	50	12.5	12.5
C. godetiae OLP10	25	25	25	25	50	50	12.5	12.5	25	25	50	50	25	25
C. godetiae OLP12	25	25	25	25	50	50	25	25	25	25	50	100	25	25
C. nymphaeae RB428	25	25	25	25	25	25	25	25	25	25	25	25	25	25
C. nymphaeae RB012	25	25	25	25	25	25	25	25	25	25	25	25	25	25
Fusarium oxysporum COAL 68	50	50	50	50	50	50	25	25	25	25	50	50	25	50
Penicillium digitatum N2F1	25	50	50	50	50	100	50	50	50	100	50	50	50	50
P. digitatum P1PP0	25	50	50	50	50	100	50	50	50	100	50	50	50	50
P. expansum COAL 95	25	25	50	50	50	50	50	50	50	100	25	25	50	50
P. nordicum CECT 2320	12.5	12.5	12.5	12.5	25	25	25	25	12.5	25	25	25	12.5	25
Phytophthora nicotianae T-2K1A	100	100	25	50	50	50	50	100	100	100	Nd	Nd	25	50
Ph. nicotianae P4K3F8	50	100	25	50	50	50	50	50	100	100	Nd	Nd	25	25
Ph. oleae V-2K10A	50	50	25	25	50	50	50	50	50	50	Nd	Nd	25	50

Table 4. (Continue)

Pathogens and isolate code	**Lactic Acid Bacteria (LABs)**																	
	COR2		**COR3**		**COR5**		**COR6**		**OT3**		**OT5**		**LEC3**		**FR2**		**FR6**	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus flavus ITEM 8111	Nd	Nd	100	100	100	100	Nd	Nd	Nd	Nd	Nd	Nd	100	100	Nd	Nd	Nd	Nd
Alternaria alternata CECT 646	25	25	50	50	50	100	50	50	50	100	50	50	50	50	50	50	50	50
A. alternata ITEM 8121	50	50	50	50	50	50	25	25	50	50	50	50	50	50	50	50	50	50
Colletotrichum acutatum UWS149	50	50	50	50	50	50	50	50	25	50	25	25	50	50	50	50	50	50
C. acutatum C9D2C	50	50	25	50	50	50	50	50	50	100	25	50	50	50	50	50	50	50
C. fiorinae ER2147	50	50	50	50	50	50	50	50	50	50	50	100	50	50	50	50	12.5	25
C. fiorinae C15D6A	50	50	50	50	50	50	50	50	25	25	100	100	50	50	50	50	25	25
C. gloeosporioides C2	50	50	100	100	100	100	100	100	100	100	50	50	25	25	50	50	25	25
C. gloeosporioides RD9B	25	25	50	50	50	50	50	50	50	100	50	50	25	50	50	50	25	25
C. godetiae OLP10	50	50	25	25	50	100	50	100	25	50	25	50	50	50	50	50	50	50
C. godetiae OLP12	50	50	25	50	50	100	50	100	50	100	25	25	50	50	50	50	50	50
C. nymphaeae RB428	25	25	25	50	25	50	50	50	25	50	25	50	50	50	50	50	25	50
C. nymphaeae RB012	25	25	25	50	25	50	50	50	25	25	25	50	50	50	50	50	25	25
Fusarium oxysporum COAL 68	25	25	50	50	50	50	50	100	25	25	25	50	50	50	50	50	50	50
Penicillium digitatum N2F1	50	100	50	100	50	100	50	100	50	50	50	100	50	50	50	50	50	100
P. digitatum P1PP0	50	100	50	100	50	100	50	100	50	50	50	100	50	50	50	50	50	100
P. expansum COAL 95	50	50	50	50	50	50	50	100	50	50	50	50	50	50	50	50	100	100
P. nordicum CECT 2320	12.5	12.5	25	50	25	25	25	25	12.5	12.5	25	25	25	50	50	50	12.5	25
Phytophthora nicotianae T-2K1A	100	100	100	100	50	50	50	50	100	100	25	50	50	50	50	50	100	100
Ph. nicotianae P4K3F8	100	100	100	100	50	100	50	50	100	100	50	50	50	50	50	50	100	100
Ph. oleae V-2K10A	100	100	100	100	50	50	50	50	100	100	50	50	50	50	50	50	100	100

Overall, an organic acid (lactic acid) and 14 phenolic acids were characterized and quantified in CFSs of LABs isolates. Comparing the data by ANOVA there were significant differences in

metabolite profile among the 16 bacterial isolates. All isolates produced lactic acid, as expected, with values ranging from 18.4 to 32.8 g/L (Table 5). The highest concentrations of lactic acid were recorded in CFSs of OV8, OV7, OV9, OV11, OV17 and OV20 *L. plantarum* isolates. Conversely, the lowest concentration of lactic acid (18.4 ± 1.6 g/L) was found in CFS of FR2 isolate (*S. salivarius*) (Table 5). The phenolic acids detected and quantified in the CFSs were 1-2-dihydroxybenzene, 3-(4-hydroxy-3-methoxyphenyl) propionic, 3-4-dihydroxyhydrocinnamic, benzoic acid, caffeic acid, DL-3-phenyllactic acid (PLA), ferulic acid, hydroxicinnamic acid, P- coumaric acid, salicilic acid, sinapic acid, syringic acid, vanillic acid and vanillin. Only *L. plantarum* OV8 strain produced high values of all acids (Figure 1). PLA was recorded with a high relative abundance (positive log scale) in CFS s of OV8, OV9, OV11, OV16, OV20 (*L. plantarum*) and FR6 (*E. faecium*). Moreover, high relative abundance of benzoic and 3-4-dydroxyhydrocinnamic acids were found in CFSs of *L. plantarum* OV11, OV8 and OV9 isolates. Benzoic acid was not recorded in CFSs of OV7, OV17, COR3 and LEC3 *L. plantarum* and *P. pentosaceus* isolates. Caffeic acid, vanillic acid and vanillin were produced in abundance by all LABS, except for OV 16 and FR 6 for vanillin, COR 3 for vanillic acid and OV17 for caffeic acid. The volatile metabolites extracted from CFSs were characterized using the HS-SPME/GC-MS technique. A total of 26 VOCs from CFSs and the control (MRS-B) were detected and quantified. The VOCs were divided into seven classes based on their chemical properties: acids (0% - 15.7%), alcohols (0% - 13.6%), aldehydes (0% - 3.2 %), alkanes (4.1% - 15.2%), alkenes (24.7% - 40.3%), ketones (6.1%- 42.4%) and esters (0 – 5.4%) (Table 6). Compared to the other samples, the control showed a different spectrum of compounds with the prevalence of an alkene (2,4 dimethyl-1-heptene and 1-undecanol) and a ketone (acetophenone). Fermentation of the MRS-B by the bacterial isolates determined an increase of the repertoire of compounds, including alcohols, aldehydes, esters and acids. In particular, the concentrations of acids and alcohols increased significantly, especially in CFSs of isolates identified as *L. plantarum* and *P. pentosaceus*. Interestingly, all CFSs produced decanal, a compound of the aldehyde class. It was detected in high proportion in CFSs of isolates FR6 and COR2 of *E. faecium* as well as in isolates OV16 and OV17 of *L. plantarum*. Differently from all the other classes of compounds, after the fermentation in all CFSs the percentage of ketones increased. The 4-nonanone was the ketone identified in greatest quantity. Control samples showed lower value of ketones, except for the acetophenone.

Table 5. Quantification of lactic acid (g/L) produced in CFSs by bacterial strains isolated from olives. Data are means ± standard deviation. Letters indicate statistically significant differences using one-way ANOVA Tukey HSD post hoc test ($p < 0.05$).

Isolate code	Strain	Lactic acid (g/L)
OV7	*Lactiplantibacillus plantarum*	31.4 ± 1.0 ^{ef}
OV8	*L. plantarum*	32.8 ± 0.8 ^{f}
OV9	*L. plantarum*	32.6 ± 1.3 ^{f}
OV11	*L. plantarum*	31.6 ± 0.3 ^{ef}
OV16	*L. plantarum*	29.7 ± 1.1 ^{def}
OV17	*L. plantarum*	31.6 ± 0.7 ^{ef}
OV20	*L. plantarum*	30.6 ± 0.4 ^{ef}
LEC3	*Pediococcus pentosaceus*	25.9 ± 0.3 ^{bcd}
OT3	*P. pentosaceus*	27.6 ± 1.3 ^{bcde}
OT5	*P. pentosaceus*	23.7 ± 1.4 ^{b}
COR3	*P. pentosaceus*	28 ± 2.4 ^{cde}
COR5	*P. pentasaceus*	26 ± 2.7 ^{bcd}
COR6	*P. pentasaceus*	24 ± 0.6 ^{bc}
FR2	*Streptococcus salivarius*	18.4 ± 1.6 ^{a}
FR6	*Enterococcus faecium*	23.4 ± 1.6 ^{b}
COR2	*E. faecium*	26 ± 2.4 ^{cde}

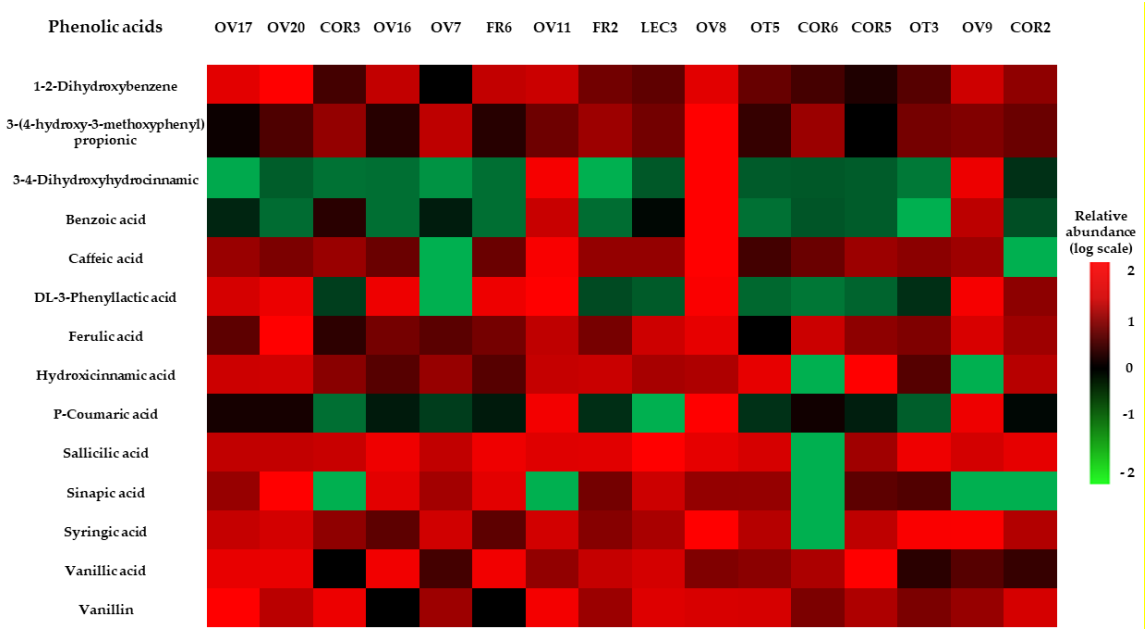

Figure 1. Heat map of phenolic acid produced by tested bacterial strains. Colors are based on relative abundance (logarithmic scale) of produced metabolites, independently for each bacterium: red represents high abundance and green low abundance.

To further characterize the isolates recovered from drupes, a PCA analysis based on VOC, organic and phenolic data of all CFSs was performed. The sum of all these components (PC) accounted for 45.8% of the total variance, while PC1 and PC2 represented 28.2% and 17.6% of the total variances, respectively. Fig. 2 shows that the samples grouped according to their metabolic profile rather than the species as identified by peptide mass fingerprinting and sequencing of the 16S

rRNA. The CFSs analysed were split into four different clusters, identified in relation to the relative abundance of a metabolite. The PC1 distributed the OV8, OV11 and COR 3 isolates on positive axis. These latter isolates on the loading plot (Fig. 2B) showed a cluster in relation to the production of benzonic acid (V 30), octane (V 20), 2-dodecanone (V 24), decane (V 13), undecanoic acid (V 3). Conversely, isolates of *L. plantarum* (OV 20, OV16, OV9) showed an aggregation type for the production. of 1-decene, 9-methyl (V 17), DL-3 phenyllatic acid (V 27) and 1,2-dihydroxybenzene (V 28).

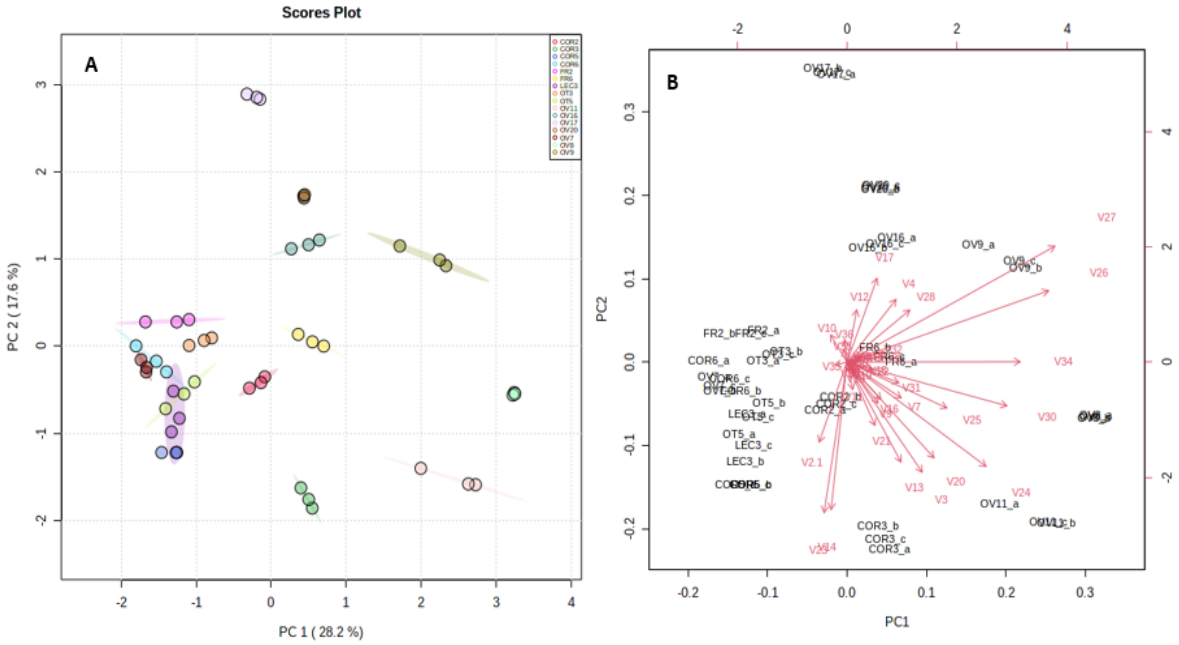

Figure 2. Principal Component Analysis (PCA), scores plot (a) and loading plot (b), based on volatile compounds, organic and phenolic acids of CFSs.

Table 6 Identification and quantification as % area of VOCs from each CFS sample analysed. Results are expressed as mean ± standard deviation. Different letters indicate significant differences among treatments ($P \leq 0.01$). The experiment was performed in triplicate (n=3).

Nº	Rt	Compound	Control	COR2	COR3	COR5	COR6	LEC3	FR2	FR6	OT3
Acid			0.0^{e}	10.3^{abcd}	8.9^{cd}	14.6^{ab}	9.0^{cd}	6.4^{d}	9.4^{bcd}	9.2^{bcd}	10.6^{abcd}
1	3,87	Acetic acid	n.d	8.07 ± 1.35	6.80 ± 3.84	12.84 ± 2.8	9.01 ± 0.68	4.85 ± 4.13	9.13 ± 0.36	7.03 ± 2.13	10.04 ± 1.8
2	7,93	Hexanoic acid	n.d	0.96 ± 0.20	0.89 ± 0.08	1.30 ± 0.02	n.d	n.d	n.d	0.92 ± 0.37	n.d
3	19,66	Undecanoic acid	n.d	1.28 ± 0.03	1.22 ± 0.67	0.49 ± 0.12	n.d	1.55 ± 1.09	0.24 ± 0.01	1.21 ± 0.26	0.53 ± 0.02
Alcohol			0.0^{f}	7.1^{e}	7.7^{de}	9.2^{de}	8.0^{de}	13.6^{a}	10.4^{bcd}	8.2^{de}	12.6^{abc}
4	13,44	1-Nonanol	n.d	n.d	1.22 ± 0.11	1.85 ± 0.16	1.44 ± 0.43	n.d	n.d	1.31 ± 0.01	n.d
5	15,34	1-Decanol	n.d	4.30 ± 0.66	3.01 ± 0.33	4.56 ± 0.11	3.88 ± 0.29	7.54 ± 0.55	6.67 ± 0.19	3.78 ± 0.31	6.88 ± 0.45
6	16,19	3-Undecanol	n.d	1.84 ± 0.27	1.64 ± 0.24	1.85 ± 0.11	1.75 ± 0.23	3.60 ± 0.56	2.87 ± 0.12	2.11 ± 0.16	3.62 ± 0.83
7	18,02	1-Undecanol	n.d	0.93 ± 0.53	1.84 ± 0.05	0.90 ± 0.07	0.94 ± 0.08	2.44 ± 0.50	0.84 ± 0.17	0.99 ± 0.13	2.11 ± 1.03
Aldehyde			0.0^{g}	2.8^{abcd}	2.6^{abcd}	1.5^{ef}	1.3^{f}	2.5^{abcd}	1.3^{f}	3.0^{abc}	2.6^{abcd}
8	14,01	Decanal	n.d	2.83 ± 0.94	2.55 ± 0.05	1.45 ± 0.04	1.25 ± 0.43	2.48 ± 0.19	1.29 ± 0.12	3.04 ± 0.04	2.58 ± 0.66
Alkane			7.7^{def}	15.2^{a}	10.3^{bcd}	10.2^{bcd}	13.4^{abc}	6.1^{def}	5.1^{ef}	9.3^{bcde}	10.4^{bcd}
9	3,54	Heptane, 4-methyl	n.d	7.34 ± 3.51	3.92 ± 0.54	2.86 ± 0.16	3.36 ± 1.51	1.20 ± 0.83	n.d	4.24 ± 0.94	4.86 ± 3.31

10	3,68	Pentane, 2,3-dimethyl	n.d	n.d	0.83 ± 0.08	n.d	4.09 ± 3.34	n.d	n.d	n.d	n.d
11	4,42	Heptane, 2,4-dimethyl	n.d	n.d	0.46 ± 0.27	n.d	n.d	n.d	n.d	n.d	n.d
12	7,57	Nonane, 4-methyl	0.60 ± 0.06	2.44 ± 0.55	1.70 ± 0.25	1.68 ± 0.23	1.38 ± 0.38	1.54 ± 0.38	2.20 ± 0.45	1.63 ± 0.31	1.61 ± 0.88
13	8,57	Decane	4.78 ± 0.22	1.98 ± 0.47	1.63 ± 0.01	2.41 ± 0.27	1.64 ± 0.63	n.d	n.d	1.71 ± 0.30	n.d
14	10,19	Decane, 2-methyl	2.34 ± 0.09	1.66 ± 0.14	0.66 ± 0.12	1.70 ± 0.02	1.62 ± 0.42	1.16 ± 0.69	1.23 ± 0.13	0.56 ± 0.04	2.23 ± 0.04
15	13,75	Dodecane	n.d	1.81 ± 0.49	1.05 ± 0.16	1.50 ± 0.12	1.30 ± 0.15	2.16 ± 0.13	1.66 ± 0.01	1.14 ± 0.20	1.65 ± 0.16
Alkene			28.2^{efg}	33.7^{abcdef}	34.3^{abcde}	33.4^{abcdef}	37.2^{abc}	34.8^{abcde}	40.3^{a}	37.3^{ab}	26.6^{fg}
16	4,99	2,4-Dimethyl-1-heptene	10.57 ± 1.3	16.49 ± 0.4	23.21 ± 0.9	20.55 ± 0.1	27.33 ± 6.5	16.35 ± 3.4	22.33 ± 0.3	23.81 ± 3.3	9.13 ± 0.6
17	10	1-Decene, 9-methyl	6.71 ± 0.84	0.70 ± 0.16	0.76 ± 0.01	0.50 ± 0.06	n.d	0.90 ± 0.21	1.40 ± 0.51	0.95 ± 0.17	0.81 ± 0.45
18	10,87	1-Undecene	10.93 ± 1.2	15.41 ± 0.7	9.15 ± 0.62	11.14 ± 0.8	8.84 ± 4.0	14.17 ± 1.7	15.31 ± 1.6	11.32 ± 1.5	14.18 ± 2.8
19	15,66	1-Tridecene	n.d	1.09 ± 0.71	1.15 ± 0.07	1.24 ± 0.41	1.02 ± 0.22	3.41 ± 0.13	1.26 ± 0.26	1.16 ± 0.13	2.48 ± 1.01
Ketone			6.1^{a}	28.0^{c}	32.4^{c}	31.2^{c}	31.2^{c}	36.6^{bc}	32.1^{c}	29.3^{c}	36.0^{bc}
20	8,06	2-Octanone	1.30 ± 0.05	0.49 ± 0.19	2.37 ± 0.07	0.85 ± 0.28	0.96 ± 0.05	1.00 ± 0.47	0.65 ± 0.13	0.58 ± 0.21	0.62 ± 0.07
21	9,22	4-Nonanone	5.98 ± 2.63	20.16 ± 2.1	15.32 ± 0.5	21.46 ± 0.6	21.83 ± 1.9	20.19 ± 1.4	17.41 ± 0.7	18.17 ± 4.1	23.22 ± 0.8
22	10,63	Acetophenone	55.46 ± 1.2	5.83 ± 0.72	5.47 ± 2.16	8.03 ± 1.10	7.99 ± 0.70	13.49 ± 0.50	13.76 ± 0.67	9.88 ± 0.90	11.41 ± 1.82
23	13,37	2-Decanone	1.33 ± 0.32	1.52 ± 0.45	5.46 ± 0.68	0.60 ± 0.03	n.d	1.33 ± 0.04	n.d	0.33 ± 0.00	n.d
24	18,28	2-Dodecanone	n.d	n.d	2.81 ± 0.23	0.24 ± 0.02	0.38 ± 0.21	0.61 ± 0.12	0.29 ± 0.04	0.33 ± 0.05	0.77 ± 0.30
25	22,67	2-Tetradecanone	n.d	n.d	0.95 ± 0.07	n.d	n.d	n.d	n.d	n.d	n.d
Ester			0.0^{e}	2.9^{cd}	3.9^{abc}	0.0^{e}	0.0^{e}	0.0^{e}	1.5^{de}	3.8^{abc}	1.3^{de}
26	17,86	Neryl acetate	n.d	2.90 ± 2.12	3.93 ± 0.12	n.d	n.d	n.d	1.48 ± 0.32	3.79 ± 0.40	1.26 ± 0.74

Table 6 (Continue)

Nº	Rt	Compound	OT5	OV7	OV8	OV9	OV11	OV16	OV17	OV20
Acid			8.1^{cd}	5.9^{d}	6.4^{d}	8.8^{cd}	10.1^{bcd}	12.4^{abc}	7.7^{cd}	15.7^{a}
1	3,87	Acetic acid	7.60 ± 0.59	5.53 ± 0.30	3.59 ± 2.74	8.80 ± 0.06	9.48 ± 0.67	11.75 ± 1.5	7.69 ± 3.12	15.10 ± 2.7
2	7,93	Hexanoic acid	n.d	0.42 ± 0.16	n.d	n.d	n.d	n.d	n.d	n.d
3	19,66	Undecanoic acid	0.51 ± 0.19	n.d	2.76 ± 0.14	n.d	0.59 ± 0.09	0.64 ± 0.44	n.d	0.63 ± 0.08
Alcohol			13.2^{ab}	10.1^{cd}	10.3^{bcd}	9.9^{cde}	8.9^{de}	8.4^{de}	8.7^{de}	13.1^{ab}
4	13,44	1-Nonanol	n.d	0.64 ± 0.01	1.06 ± 0.31	1.47 ± 0.17	n.d	1.84 ± 0.28	1.75 ± 0.25	1.94 ± 0.06
5	15,34	1-Decanol	7.11 ± 0.49	5.69 ± 0.92	4.26 ± 0.28	4.27 ± 0.17	4.28 ± 0.18	2.83 ± 1.15	4.09 ± 0.70	6.59 ± 0.34
6	16,19	3-Undecanol	3.70 ± 0.59	2.81 ± 0.50	1.97 ± 0.22	2.23 ± 0.19	1.96 ± 0.46	2.66 ± 0.30	1.99 ± 0.54	3.23 ± 0.01
7	18,02	1-Undecanol	2.39 ± 0.13	0.99 ± 0.32	3.05 ± 1.06	1.92 ± 0.15	2.65 ± 0.13	1.04 ± 0.38	0.82 ± 0.31	1.35 ± 0.19
Aldehyde			2.2^{bcde}	2.1^{def}	2.2^{cdef}	2.5^{abcd}	2.2^{bcde}	3.2^{a}	3.1^{ab}	2.7^{abcd}
8	14,01	Decanal	2.24 ± 0.06	2.09 ± 0.22	2.16 ± 0.32	2.50 ± 0.26	2.24 ± 0.07	3.23 ± 0.57	3.15 ± 0.45	2.69 ± 0.42
Alkane			9.2^{cde}	8.3^{def}	8.9^{cde}	8.5^{def}	13.8^{ab}	9.6^{bcde}	8.4^{def}	4.1^{f}
9	3,54	Heptane, 4-methyl	3.23 ± 0.38	2.95 ± 0.92	2.50 ± 1.30	3.80 ± 0.08	1.39 ± 0.08	2.91 ± 1.38	4.45 ± 2.14	n.d
10	3,68	Pentane, 2,3-dimethyl	n.d	n.d	n.d	n.d	n.d	1.49 ± 1.25	0.77 ± 0.06	n.d
11	4,42	Heptane, 2,4-dimethyl	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
12	7,57	Nonane, 4-methyl	1.89 ± 0.55	1.50 ± 0.28	1.90 ± 0.47	2.78 ± 0.37	1.58 ± 0.08	1.14 ± 0.82	1.79 ± 0.58	1.92 ± 0.56
13	8,57	Decane	n.d	1.95 ± 0.22	2.26 ± 0.36	n.d	2.17 ± 0.08	2.43 ± 0.68	n.d	n.d
14	10,19	Decane, 2-methyl	1.60 ± 0.90	0.65 ± 0.08	0.60 ± 0.04	n.d	6.94 ± 0.30	n.d	n.d	n.d
15	13,75	Dodecane	2.45 ± 0.13	1.24 ± 0.22	1.68 ± 0.12	1.87 ± 0.08	1.70 ± 0.09	1.60 ± 1.16	1.37 ± 0.47	2.14 ± 0.09
Alkene			36.3^{abcd}	31.1^{bcdef}	33.7^{abcdef}	38.9^{a}	24.7^{g}	28.9^{defg}	37.9^{ab}	29.8^{cdefg}
16	4,99	2,4-Dimethyl-1-heptene	15.86 ± 0.3	15.98 ± 3.01	17.66 ± 0.4	19.90 ± 1.7	14.38 ± 1.3	20.63 ± 2.7	22.37 ± 4	10.02 ± 3.1
17	10	1-Decene, 9-methyl	1.11 ± 0.49	0.93 ± 0.02	1.12 ± 0.14	1.33 ± 0.31	0.82 ± 0.03	1.04 ± 0.30	1.00 ± 0.08	1.19 ± 0.25
18	10,87	1-Undecene	16.80 ± 2.15	13.23 ± 1.7	13.77 ± 1.0	16.40 ± 1.5	8.36 ± 0.54	5.80 ± 1.41	13.6 ± 3.43	16.4 ± 1.33

19	15,66	1-Tridecene	2.51 ± 0.10	0.97 ± 0.24	1.19 ± 0.03	1.31 ± 0.02	1.11 ± 0.09	1.41 ± 0.43	0.99 ± 0.23	2.16 ± 0.28
Ketone			30.7^{c}	42.4^{b}	33.3^{c}	27.8^{c}	36.0^{bc}	33.1^{c}	31.6^{c}	28.9^{c}
20	8,06	2-Octanone	0.53 ± 0.01	0.61 ± 0.26	4.19 ± 0.22	2.90 ± 0.34	3.42 ± 0.12	0.61 ± 0.19	n.d	0.44 ± 0.01
21	9,22	4-Nonanone	18.99 ± 1.30	23.74 ± 4.3	13.76 ± 0.9	13.99 ± 1.9	11.71 ± 0.1	20.44 ± 5.9	21.91 ± 0.4	17.08 ± 2.3
22	10,63	Acetophenone	9.66 ± 0.90	17.70 ± 3.3	7.75 ± 0.7	6.40 ± 0.5	12.03 ± 0.1	11.64 ± 2.3	9.65 ± 1.2	11.01 ± 3.3
23	13,37	2-Decanone	0.84 ± 0.17	n.d	n.d	n.d	2.40 ± 0.27	n.d	n.d	n.d
24	18,28	2-Dodecanone	0.72 ± 0.14	0.38 ± 0.04	5.77 ± 1.22	3.64 ± 0.17	4.91 ± 0.20	0.38 ± 0.09	n.d	0.34 ± 0.06
25	22,67	2-Tetradecanone	n.d	n.d	1.84 ± 0.71	0.91 ± 0.01	1.48 ± 0.19	n.d	n.d	n.d
Ester			0.3^{e}	0.0^{e}	5.1^{ab}	3.6^{bc}	4.4^{abc}	4.5^{abc}	2.6^{cd}	5.8^{a}
26	17,86	Neryl acetate	0.28 ± 0.03	n.d	5.09 ± 2.06	3.58 ± 0.37	4.39 ± 0.28	4.51 ± 0.85	2.61 ± 0.62	5.75 ± 1.17

8.5. Discussion

In this study, it was demonstrated that fermentates of LABs isolated from olives exert a noticeable antifungal activity against a wide range of fungi *s.l.* pathogenic to olive or producing mycotoxins that may contaminate food products of olive industry, such as olive oil. They were effective against *A. alternata, A. flavus, C. acutatum*, *C. fiorinae*, *C. gloeosporioides*, *C. nymphaeae*, *F. oxysporum*, *P. digitatum*, *P. expansum*, *P. nordicum*, *Ph. nicotianae* and *Ph. oleae*. LABs isolated from olives in this study were identified as *L. plantarum*, *P. pentosaceus*, *E. faecium* and *S. salivarius*. There are several reports of the presence of *L. plantarum*, *P. pentosaceus* and *Enterococcus* sp. in different matrixes including table olive fruits [26,36,54]. In particular, previously articles highlighted *L. plantarum* and *P. pentosaceus* showed antimicrobial activity [24,25,55]. However, all previous studies focused on LABs isolated from fruits or brine of olive table cultivars and their biotechnological applications in the food industry. By contrast, LABs characterised in this study were from olive oil varieties. Moreover, to the best of our knowledge, this is the first report of antifungal activity of *S. salivarum* and *E. faecium* and the first time that it is envisaged a potential application of LABs isolated from olive fruits and their fermentates in agriculture as potential BCAs or natural fungicides, respectively. As many of the fungi *s.l.* inhibited by LABs isolated from olive drupes, such as *A. alternata*, *C. acutatum, C. gloeosporioides*, *F. oxysporum* and *Ph. nicotianae*, are very polyphagous pathogens [42,46,56–59] it can be hypothesized that their antifungal potential could be exploited to manage diseases of several other crop plants, besides olive. Chemical analyses of CFSs from liquid cultures of all LABs revealed they produce a large repertoire of active metabolites, including lactic acid, numerous phenolic acids and VOCs, that might be valued also in food industry with the same purposes for which the use of metabolites of other LABs is a consolidated technique. The antifungal activity of LABs recovered from olives varied considerably depending on the bacterial isolate and the fungi tested. For instance, no or a scarce antifungal activity was shown by all LABs tested against *Pl. tracheiphilus* and *A. flavus*. Most LAB isolates tested in this study inhibited significantly the *in vitro* growth of *Colletotrichum*

species, but there were exceptions, such as those of C2 and RD9B isolates of *C. gloeosporioides* and *C. godetiae*. Similarly, *Penicillium* species showed a lower sensitivity than *Colletotrichum* species, but *P. nordicum* was an exception as its sensitivity to CFSs of most bacterial isolates was even lower than the sensitivity showed by *Colletotrichum* species. This sensitivity of *P. nordicum* to fermentates of LABs is consistent with the results of Guimarães et al. [47]. In this study, three distinct methods were used to assay the antifungal activity of LAB fermentates, the culture overlay, the agar diffusion and the serial dilution tests, the last one to determine both MIC and MFC values of CFSs. In general, results of the three tests showed a similar trend. However, also in this case there were exceptions. The most relevant one was the *E. faecium* strain FR6. In the culture overlay test this isolate showed no antifungal activity against *C. fioriniae, C. nympheae* and *F. oxysporum,* but exerted a high inhibitory activity against these fungi in the agar diffusion and the serial dilution tests, suggesting the inhibitory activity was due to diffusible metabolites released by the bacterium in the culture medium. Some of the metabolites recovered from the culture liquids in this study have been previously reported as antifungal agents produced by LABs [61–63]. For instance, benzoic acid, cinnamic acid and its derivatives were reported in the literature to exert antifungal activity [64,65]. Also caffeic acid, vanillic acid and vanillin were mentioned as phenolic acids with antifungal activity [66–68]. The presence of hydroxycinnamic acids, p-coumaric, caffeic acid and ferulic acid is correlated to the capacity of *L. plantarum* to metabolize these phenolic compounds [69] while the lower level of acetophenone in CFSs, compared to the control samples, was probably correlated to the reduced potential of LABs to produce chiral alcohols [70]. Interestingly, all CFSs produced decanal, a compound of the aldehyde class. Decanal was reported to possess antifungal activity against *P. digitatum, P. italicum* and fungi of other genera such as *Aspergillus* and *Geotrichum* [67,71–73]. Isolates of the genus *Lactiplantibacillus* characterised in this study produced a greater amount of lactic acid than LABs of other genera. Moreover, the chemometric analysis separated he CFSs of the LABs isolated from olives into four distinct clusters based on the relative abundance of specific metabolites. For instance, isolates OV8, OV11 (*L. plantarum)* and COR 3 *(P. pentosaceus),* formed a cluster in relation to the production of benzonic acid, octane, 2-dodecanone, decane and undecanoic acid. Conversely, the isolates OV 20, OV16 and OV9 (as *L. plantarum)* grouped because of their ability to produce 1-decene, 9-methyl, DL-3 phenyllatic acid and 1,2-dihydroxybenzene. However, no obvious correlation was found between both the level and spectrum of antifungal activity showed by LAB isolates characterised in this study and their metabolic profiles.

The discovery that LABs isolated from drupes of olive oil varieties exert an effective inhibitory activity on a wide range of fungi *s.l.* is not only a novelty but open the way to potential biotechnological applications. Their characterization and the identification of active metabolites present in their fermentates are a preliminary step toward this objective.

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9. General considerations

This PhD thesis, which has focused on emerging and re-emerging diseases of olive caused by *Colletotrichum* and *Phytophthora* species in southern Italy, has dealt with diverse case studies and many fundamental aspects of Plant Pathology, including disease etiology, conventional and molecular diagnosis, taxonomy of bacteria and fungi based on both conventional morphological characters and multiloci phylogenetic analysis, ecology, epidemiology, biochemical pathogen/plant interactions and disease management strategies. First of all it has to be emphasized that such an articulated study with so many objectives was possible thanks to the financial and scientific support, laboratory facilities and expertise provided by four scientific institutions and their commitment in the tutorial role, the Department of Agriculture of the University Mediterranea of Reggio Calabria (RC, Italy), responsible for coordinating the PhD course, the Molecular Plant Pathology Laboratory of the Department of Agriculture, Food and Environment of the University of Catania (CT, Italy), where most of the laboratory activity was carried out, the Council for Agricultural Research and Agricultural Economy Analysis, Research Centre for Olive, Citrus and Tree Fruits of Rende (CS, Italy), whose facilities and olive cultivar collections were invaluable tools for field research and experimental activity, and the Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia (Burjassot, Spain) where most of microbiological test and biochemical analyses were performed.

As for diseases incited by soilborne *Phytophthora* species major contributions have concerned: i. The characterization and formal description of a new species, *P. heterospora*, which has peculiar morphological and ecological features; *P. heterospora* causes root and basal stem rot of young olive trees and is closely related to the well known and extremely polyphagous *P. palmivora*. ii. The recovery of *P. oleae*, a recently described species already reported as a causal agent of rot of olive fruits in Italy and root rot of wild olives in Spain, in a nature reserve in Sicily; *P. oleae*, along with other soilborne *Phytophtora* species, was associated to the rhizosphere of *Oleo-Quercetum virgilianae*, a Mediterranean type of vegetation characterized by the presence of olive; iii. The first report of olive as a new host of *P. bilorbang* worldwide.

As for olive fruit anthracnose (OA) incited by diverse *Colletotrichum* species major contributions have concerned: i. The evaluation of the susceptibility of a few of the most popular Italian olive cultivars to infections by diverse *Colletotrichum* species; a great variability in virulence among the *Colletotrichum* species tested and a significant interaction *Colletotrichum* species x olive cultivar were observed, which has epidemiological implications; the olive cultivars tested were separated into very susceptible, moderately susceptible and resistant based on the pathogenicity assays. ii.

The identification of *Colletotrichum* species responsible for a new syndrome of citrus called twig and shoot dieback; the causal agents of the disease were identified as *C. gloeosporioides* and *C. karsti*, two species associated occasionally with OA that are ubiquitous in citrus orchards of the Mediterranean region. iii. The selection of lactobacilli isolated from olive fruits based on their antifungal activity in view of the application as candidate BCAs against plant diseases, and evaluation of the antifungal activity of metabolites extracted from fermentates of selected lactobacilli, in view of their exploitation in agriculture as natural fungicides. iv. The identification of the numerous secondary metabolites produced by *Colletotrichum* species associated with OA and the comparison of the metabolite profiles showed by diverse *Colletotrichum* species *in vitro* on two different culture media and on infected olives of four cultivars differing in susceptibility to OA during the infection process; interestingly the dynamics of metabolite profiles was correlated with the susceptibility of cultivars to OA. Should new financial resources become available and if I was offered the opportunity to continue the research in this field, the following research lines would deserve to be developed: i. Application of metabarcoding approaches to study the diversity of *Phytophthora* communities associated with the rhizosphere of olive. ii. Further selection of lactobacilli isolated from olives and characterization of biologically active metabolites extracted from their fermentates. iii. Genomics and metabolomics of the interaction *Colletotrichum* species/olive fruits. iv. Toxicological implications of secondary metabolites released by *Colletotrichum* species infecting olives and role of these fungal metabolites as virulence factors in the pathogenesis of OA.