ESCUELA SUPERIOR Y TÉCNICA DE INGENIERÍA AGRARIA
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Endophytic fungi of olive tree and their exploitation in the biological control of olive anthracnose

Doctoral Thesis

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Hongos endofíticos del olivo y su aprovechamiento para el control biológico de la antracnosis del olivo

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Abstract

Olive anthracnose, caused by several species of *Colletotrichum* genus, is the most economically harmful fruit disease of olive crop worldwide. Management of this disease is very difficult, being relied on the application of fungicides, which is not compatible with the Sustainable Development Goals of the 2030 Agenda. This work aim to elucidate the potential role of fungal endophytes colonizing the olive tree in protecting host plant from anthracnose, and identify candidate endophytes with biocontrol potential against *Colletotrichum* spp.. A culture-dependent approach was used to describe the fungal communities in vegetative (root, leaf, twig) and reproductive (inflorescence, fruit) organs of olive cultivars with different susceptibilities to anthracnose, over the phenological stages from floral buds to fruits. Some of the isolates obtained were screened for their *in vitro* antagonistic activity against *Colletotrichum* spp.. The biocontrol efficacy of the most promising isolate was evaluated through in fruit assays. The endophytic community of olive tree was rich and abundant, being most of the isolates from Ascomycota phylum, Sordariomycetes class and genera *Phomopsis*, *Fusarium*, *Alternaria* and *Penicillium*. Fungal assemblages in reproductive organs differed in size and composition between plant organ, phenological stage, disease incidence, and to a lesser extent host cultivar. Despite this, some fungi of reproductive organs showed specificity to a particular cultivar, suggesting that the host plant may play an important role in the recruitment of these endophytes. Similarly, a set of fungal endophytes were specific to orchards with either high or low anthracnose incidence, which suggest that they might be important in increasing (facilitation) or decreasing (antagonism) anthracnose development. The lifestyle transition in *Colletotrichum* spp. from latent to pathogen seemed also to be related to the endophytic fungal community structure of fruits. Endophytes from the most anthracnose-tolerant cultivar exhibited the greatest *in vitro* inhibitory effect on *C. acutatum* growth, sporulation and germination, when compared to the ones isolated from anthracnose-susceptible cultivar. In detached fruits, *Hypocrea lixii* reduced olive anthracnose incidence and severity (up to 75% and 30%, respectively), suggesting to be a promising biocontrol candidate. More research is still required to identify the functional role of these fungi and the mechanisms involved in conferring host plant protection to anthracnose disease in *in planta* assays.

Keywords: *Colletotrichum*, *Olea europaea* L., plant-pathogen-endophyte interactions, disease susceptibility, antagonism.

Resumen

La antracnosis es una enfermedad que aparece en todas las regiones olivicultoras, desde la cuenca mediterránea hasta América y Asia. Adquiere especial importancia en ciertos países mediterráneos, como Portugal, España e Italia (Cacciola et al., 2012). En Portugal, se considera la enfermedad más importante de los olivares, con una mayor incidencia en la franja de la costa oeste y en algunos municipios de Alentejo, siendo responsables de importantes pérdidas que se reflejan en la cantidad y calidad de la producción (Talhinhas et al., 2018). Esta enfermedad afecta todos los órganos del árbol, siendo los frutos los más afectados especialmente cuando están casi maduros (Cacciola et al., 2012). Los primeros síntomas de las aceitunas infectadas son pequeñas manchas de color marrón en el epicarpio que posteriormente se hunden. A medida que las aceitunas maduran, el centro de estas manchas hundidas se cubre con masas gelatinosas rosadas/anaranjadas de conidios que a menudo se producen en un patrón de anillo concéntrico. En las partes vegetativas, los síntomas incluyen clorosis foliar, defoliación y muerte de brotes y ramas. Las flores infectadas muestran tizón en flor, se secan y caen rápidamente (Moral et al., 2008).

Esta enfermedad puede ser devastadora, dependiendo del nivel de susceptibilidad de los cultivares, las condiciones ambientales, la presión del inóculo y la virulencia de las cepas patógenas (Talhinhas et al., 2018). En condiciones favorables, toda la producción puede ser destruida. Por ejemplo, en algunos países productores de olivos, la antracnosis del olivo fue considerado el agente causal de pérdidas de rendimiento superiores al 80% (Cacciola et al., 2012). Además, esta enfermedad puede reducir la calidad del aceite de oliva, originando en general aceites lampantes, con características sensoriales y organolépticas negativas.

Los responsables por esta enfermedad son especies de hongos pertenecientes a los complexos *Colletotrichum acutatum sensu lato* (s.l.) y *C. gloeosporioides* s.l. De estos dos complejos, *C. acutatum* s.l. es el más predominante, causando explosiones epidémicas de antracnosis en la mayoría de los países olivicultores (Talhinhas et al., 2005). La epidemiología y el ciclo de vida de la antracnosis del olivo aún no se conocen bien, especialmente en lo que respecta a la propagación y el mantenimiento del inóculo en los olivares, requiriendo más estudios. En las regiones mediterráneas, se ha observado que las infecciones comienzan durante la primavera en las flores y en los frutos jóvenes (infección primaria). El modo de supervivencia y la fuente de este inóculo primario aún

no se han determinado. Se cree que los principales reservorios de inóculo primario son las aceitunas momificadas que permanecen en el árbol o en el suelo de una estación a otra (Moral et al., 2009). También es plausible que la fuente de inóculo en la primavera pueda originarse de hongos que hibernan en material leñoso y hojas del árbol (Talhinhas et al., 2018). Después de la infección primaria, el hongo deja de crecer y permanece latente hasta que los frutos empiezan a madurar. En ese momento, con condiciones ambientales favorables, se producen masas pegajosas de esporas en acérvulo. Estas esporas se propagan posteriormente por la lluvia a nuevos frutos y otras partes de los árboles, dando lugar a infecciones secundarias (Moral et al., 2009). La antracnosis del olivo alcanza la mayor incidencia y severidad en áreas con humedad relativa elevada (más del 93%) y temperatura del aire cálida (entre 10 y 30ºC) (Cacciola et al., 2012). La ocurrencia de precipitación también es crucial para la separación de los conidios de la masa gelatinosa de los acérvulos y para su dispersión. Además, la infección de los frutos depende del grado de madurez de la cáscara. Las aceitunas en las últimas etapas de la maduración son más propensas a la infección por hongos que las verdes (Silva 2016). La gravedad de los síntomas varía ampliamente con el cultivar (i. e., su susceptibilidad a la antracnosis) y la virulencia de la cepa (Talhinhas et al., 2015).

Por lo general, la penetración y colonización de tejidos vegetales por especies de *Colletotrichum* comprende un conjunto secuencial de etapas. Comúnmente, comienza con la fijación y germinación de los conidios en la superficie del huésped, seguido por el desarrollo de un apresorio, que facilita la entrada a través de la epidermis (Wharton y Diéguez-Uribeondo 2004). También se cree que la penetración de hongos ocurre a través de estomas o lenticelas, así como heridas causadas por ataques de insectos, por ejemplo, *Bactrocera oleae* (Rossi) (Cacciola et al., 2012). Después de la penetración en los frutos, *Colletotrichum* sp. puede utilizar diferentes estrategias de infección. Estas estrategias varían desde el modo hemibiotrófico intracelular hasta el modo necrotrófico subcuticular intramural siendo el hemibiotrófico el más común (De Silva et al., 2017).

El manejo de la antracnosis del olivo es muy difícil, ya que su propagación y desarrollo depende en gran parte de las condiciones climáticas. Por lo tanto, hasta ahora no se han propuesto medidas de control efectivas para su gestión. En general, las medidas de control aplicadas se basan en un enfoque integrado que combina varios medios y herramientas, ya sea para prevenir como para proteger el cultivo del olivo contra la antracnosis (Cacciola et al., 2012). Las medidas indirectas o preventivas de la antracnosis del olivo se centran en prácticas destinadas a reducir los niveles iniciales de inóculo o

reducir la tasa de propagación del patógeno. Estas prácticas incluyen técnicas agronómicas como la poda, el drenaje y el riego, la fertilización, el uso de cultivares tolerantes/resistentes a la antracnosis o el control de insectos que potencialmente pueden propagar el patógeno, entre otros. Los métodos y herramientas para la protección del olivo incluyen el uso de fungicidas y, más recientemente, de productos naturales y agentes de control biológico. Los fungicidas recomendados para controlar la antracnosis del olivo son fungicidas protectores basados en compuestos de cobre, como el oxicloruro de cobre, el sulfato de cobre y el hidróxido de cobre (Cacciola et al., 2012). También se ha demostrado que los productos químicos más recientes, como las estrobilurinas, aumentan la efectividad de los fungicidas a base de cobre contra la antracnosis de la aceituna en los olivos cuando se usan en combinación (Moral et al., 2018). Del mismo modo, productos naturales, como productos botánicos (i. e., extractos de plantas) y productos de origen mineral (i. e., compuestos ricos en calcio) se han explorado recientemente en el control de la antracnosis del olivo (Moral et al., 2018). Se ha demostrado que los compuestos ricos en calcio inhiben la formación del apresorio de *Colletotrichum* sp. en pruebas *in vitro*, pero su aplicación en el campo no siempre fue efectiva en el control de la antracnosis (Xaviér, 2014). Asimismo, el extracto obtenido de la cáscara de granada (*Punica granatum* L.) ha mostrado ser efectivo contra *Colletotrichum* sp. en condiciones de laboratorio y para controlar la antracnosis del olivo en ensayos de campo (Pangallo et al., 2017).

El control biológico (CB) es otra alternativa para el manejo de la antracnosis del olivo, aunque este enfoque no ha sido tan efectivo como el control químico (Holt et al., 2009). Segura (2003) ilustró por primera vez las posibilidades de utilizar agentes de control biológico (ACB) para controlar el patógeno de la antracnosis del olivo. En inoculaciones artificiales de aceitunas, demostró que los microorganismos testados redujeron la gravedad de los síntomas producidos por *C. acutatum* en más del 50%. Desde entonces, se han realizado pocos estudios sobre el CB de la antracnosis del olivo y esta estrategia no se ha utilizado contra esta enfermedad en condiciones de campo. Aunque los diversos esfuerzos para comprender mejor la epidemiología y la genética de poblaciones de las diferentes especies patógenas, la antracnosis del olivo sigue siendo una "enfermedad compleja" de percibir. De hecho, no está claro cómo interactúa el patógeno con la planta huésped, cuál es la variabilidad de las especies de *Colletotrichum* en algunas regiones olivícolas, así como cuáles son las mejores estrategias de control contra esta enfermedad.

En este sentido, el uso de hongos endofíticos para controlar la antracnosis del olivo podría suponer un enfoque prometedor (Preto et al., 2017). Estos hongos poseen un papel importante en la aptitud de las plantas hospederas, protegiéndolas de microorganismos patógenos y plagas de insectos y mejorando su crecimiento (Jia et al., 2016). Pueden habitar el mismo nicho y en el mismo ambiente que el *Colletotrichum* spp., favoreciendo su potencial como agentes de CB contra la antracnosis del olivo. Por otro lado, las características benéficas de los endofíticos nativos también ofrecen ventajas dado que ya se encuentran adaptados a los cultivares/localización. Además, se trata de una alternativa que contribuye a la reducción del uso de pesticidas químicos, colaborando para la mejora de la calidad de los productos agrícolas, la reducción de la contaminación ambiental, la preservación de los recursos naturales y, por tanto, el aumento de la sostenibilidad de los agroecosistemas. Sin embargo, hasta donde sabemos, existen pocos estudios sobre el uso de hongos endofíticos como ACB contra la antracnosis del olivo.

En este contexto, el objetivo principal de este trabajo fue evaluar la comunidad de hongos endofíticos asociada a cultivares de olivo con susceptibilidad variable a *C. acutatum* y seleccionar cepas potenciales para el control de la antracnosis del olivo en el agroecosistema. En concreto, en el **Capítulo - 2** se evaluó la sucesión de la comunidad de hongos endofíticos en órganos reproductores de dos cultivares con diferentes susceptibilidades a la antracnosis (*Cobrançosa* y *Madural*). Para ello, se investigaron las diferencias en la composición de la comunidad endofítica durante el desarrollo de la inflorescencia y fruto. Específicamente, este estudio tuvo como objetivo elucidar: i) la contribución relativa del órgano vegetal (inflorescencia y fruto) y el genotipo del hospedero (cultivar) en el ensamblaje de hongos endofíticos; ii) patrones de asociación planta-endófito en diferentes estados de desarrollo de la inflorescencia y el fruto; iii) la relación entre la variabilidad en la susceptibilidad a la antracnosis de los diferentes cultivares y la comunidad endofítica presente naturalmente en la inflorescencia y el fruto.

A continuación, fue caracterizada la estructura de la comunidad endofítica de olivares con elevada y reducida incidencia de antracnosis **(Capítulo - 3)**. En este trabajo se compararon las comunidades endofíticas de botones florales, flores abiertas y frutos en olivares con elevada y reducida incidencia de antracnosis, con el fin dilucidar el papel potencial del microbiota vegetal en el proceso de patogénesis. Específicamente, el objetivo fue responder las siguientes preguntas: i) ¿Cómo varían las comunidades entre olivares con diferente incidencia de enfermedad? ii) ¿Cómo varían las comunidades en función de las fases de vida de los diferentes patógenos (i. e., desde las primeras etapas

de floración hasta el desarrollo de frutos)? iii) ¿Existe algún consorcio de hongos específicamente asociado a una incidencia de antracnosis elevada ("hongos promotores de la enfermedad") o reducida ("hongos supresores de la enfermedad"), así como con las fases vida de un patógeno específico?

Por último, se caracterizó la diversidad de hongos en órganos vegetativos y se evaluó la actividad antagónica de aislados contra *Colletotrichum* spp. **(Capítulo 4)**. El aislamiento se llevó a cabo a partir de raíces, ramas y hojas de tres cultivares con diferente susceptibilidad a la enfermedad: *Galega* (susceptible), *Picual* (tolerante) y *Cobrançosa* (moderadamente tolerante).

Capítulo 2 - Sucesión de la comunidad de hongos endofíticos en órganos reproductores de dos cultivares de olivo.

Material y métodos

El muestreo se realizó de mayo a noviembre de 2017 en un olivar localizado en Mirandela (Noreste de Portugal). Este olivar presentaba olivos de los cultivares (cvs) *Cobrançosa* y *Madural* separados 7 m entre sí y con aproximadamente 60 años de edad. Se muestrearon inflorescencias - en los estadios fenológicos D-E-F-G-H - y frutos - en los estadios fenológicos H-I-J - de siete olivos de cada cultivar seleccionados al azar. En cada momento de muestreo se recogieron 25 muestras por árbol de plantas asintomáticas. Estas muestras fueron esterilizadas, cada una se cortó en seis piezas (aprox. 2-4 mm de longitud) y se depositaron en medio de agar papa dextrosa (APD) suplementado con cloranfenicol al 0,01% (p/v). a 25 ± 2 °C en oscuridad. Los aislados obtenidos se identificaron molecularmente por la región de los ITS.

La abundancia y diversidad de endófitos se midió a través de la frecuencia de colonización (FC, %), abundancia relativa (RA, %), riqueza de especies, e índice de abundancia y diversidad. El escalamiento multidimensional no métrico (NMDS) con el índice Bray-Curtis y un análisis de similitud (ANOSIM) determinó las diferencias de la comunidad entre cvs, órganos y estadios fenológicos. Para explorar la relación entre las variables explicativas (órgano y estadio fenológico) y variables respuesta (abundancia y riqueza) se utilizaron modelos lineales generalizados mixtos (GLMMs). Se realizó un análisis de partición de la varianza (*Varpart)* para determinar los factores más importantes en la configuración de la comunidad de hongos. La relación entre el ensamblaje de hongos y la planta o estadio fenológico, en inflorescencias y frutos se estudiaron mediante un

análisis de co-inercia (CIA). Las unidades taxonómicas operativas (OTUs) indicadoras de cada cultivar y estadio se determinaron utilizando un análisis de valor indicador (*IndVal)*. El procedimiento utilizado se encuentra más detallado en el material y métodos del Capítulo 2.

Resultados

En todos los tejidos recolectados en los dos cvs se obtuvo un total de 466 aislados pertenecientes a 106 OTUs, de los cuales se identificaron 86 OTUs en inflorescencias y 42 OTUs en frutos. Las 106 OTUs fueron asignadas a 87 especies, 65 géneros y 33 familias, todas de los filos Ascomycota y Basidiomycota. Ascomycota estaba compuesta principalmente por miembros pertenecientes a las familias Xilariaceae (28%) y Pleosporaceae (15%), y los géneros *Biscogniauxia* (24%) y *Alternaria* (10%). En Basidiomycota, las familias Polyporaceae (83%) y Stereaceae (17%), y los géneros *Trametes* (75%), *Stereum* (17%) y *Coriolopsis* (8%) fueron los más abundantes.

***La diversidad y la composición de los endófitos difieren entre los cultivares*:** Los cvs diferían en el porcentaje de colonización, abundancia, riqueza y diversidad de hongos. Dentro de la comunidad que habita la inflorescencia, los resultados indicaron que el cv. *Madural* presentó una tasa de colonización significativamente mayor ($p < 0,05$) (hasta 3,2 veces), abundancia (hasta 3,1 veces), riqueza (hasta 3,0 veces) y diversidad de especies (hasta 2,3 veces) que el cv. *Cobrançosa*. El mismo patrón se observó dentro de la comunidad colonizadora de frutos. En general, los frutos del cv. *Madural* mostraron una tasa de colonización significativamente mayor ($p < 0,05$) (hasta 1,1 veces), abundancia (hasta 1,3 veces), riqueza (hasta 1,5 veces) y diversidad (hasta 1,8 veces) de hongos que el cv. *Cobrançosa.* El análisis NMDS y ANOSIM confirmaron estos resultados a través de la disimilitud entre grupos. El GLMM mostró un efecto significativo ($p < 0,01$) del cultivar sobre la abundancia y riqueza de hongos en inflorescencias y frutos. De hecho, de las 106 OTUs recuperadas, 28 fueron compartidas por ambos cvs mientras que 25 fueron exclusivas del cv. *Cobrançosa* y 53 del cv. *Madural*.

Diversidad y composición de endófitos en órganos reproductivos en diferentes estadios fenológicos: Los resultados del GLMM indicaron que el órgano afectó significativamente ($p < 0,01$) a la abundancia y riqueza total de hongos. En este análisis no se incluyó el estado fenológico (H), ya que no se obtuvo crecimiento de hongos. La abundancia y riqueza fue superior en la inflorescencia que en el fruto para los dos cvs,

especialmente en el cv. *Madural*. En el cv. *Cobrançosa,* tanto la abundancia como la riqueza de hongos aumentaron con el desarrollo de la inflorescencia, alcanzando un pico significativo ($p < 0,001$) en la floración (estadio F) (un aumento de hasta 3,2 y 2,3 veces), seguido de una diminución hasta el inicio de la madurez del fruto, alcanzando un pico significativo ($p < 0,001$) cuando más de la mitad del fruto presentaba la epidermis roja o púrpura (índice de maturación 3) (un aumento a 5,0 y 4,0 veces).

La variación de la abundancia y riqueza de hongos en los órganos reproductores del cv. *Madural* sobre los diferentes estadios fenológicos siguió una tendencia similar con excepción de los frutos maduros que presentaron mayor abundancia y riqueza de hongos en el cv. *Madural* ($p < 0,001$) (hasta 7,2 y 6,5 veces). La diferencia encontrada en la comunidad endofítica de las inflorescencias fue mayor entre el estadio fenológico G y F ($R = 0,838$ y $R = 0,931$, $p = 0,001$, para los cvs. *Madural* y *Cobrançosa*) o D, ($R = 0,862$, $p = 0,001$, para cv. *Madural*). La variación en los géneros más abundantes durante los diferentes estadios podría explicar las diferencias de composición entre los estadios G y F o D. Por ejemplo, *Biscogniauxia*, aislado preferentemente en las etapas F y D, desapareció en la etapa G, donde otros géneros como *Botryotinia* y *Cladosporium* fueron los más frecuentes.

La composición de la comunidad endofítica en los frutos fue similar entre los diferentes índices de maduración (MI). Sin embargo, se encontraron diferencias significativas entre algunos pares de MI, siendo las diferencias mayores entre los pares MI 2 - MI 4 ($R = 0,666$, $p = 0,001$, para cv. *Cobrançosa;* $R = 0,916$, $p = 0.001$, para cv. *Madural*) y MI 2 - MI 3 ($R = 0,687$, $p = 0,001$, para cv. *Cobrançosa*). De hecho, se observó que los géneros más abundantes variaban durante la maduración de los frutos. Por ejemplo, *Pyrenochaeta, Fusarium* y *Hyalodendriella* fueron más abundantes en MI 2, mientras que *Cladosporium, Alternaria y Neofabraea* fueron más ricos en MI 3 y 4. Únicamente el género *Fusarium* fue compartido entre MI 2 y MI 3 o 4, lo que también podría explicar las diferencias encontradas en las comunidades endofíticas entre MIs.

El órgano vegetal y el estadio fenológico son los factores más importantes para configurar la comunidad de hongos: El órgano ($F = 2,52$, $p = 0,005$), el estadio fenológico ($F = 1,91$, $p = 0,005$) y, en menor medida, el cultivar ($F = 1,56$, $p = 0,010$), revelaron una influencia significativa sobre la estructura de la comunidad endofítica, explicando 5,9, 5,0 y 0,9% de la variación en su composición. El análisis de partición de la varianza aplicado a cada órgano mostró que los hongos asociados a la inflorescencia fueron significativamente afectados por el estadio fenológico ($F = 1,49$, $p = 0,005$) y el

cultivar (F = 1,55, p = 0,005), explicando el 11,8% y 1,6% de la variación respectivamente. En relación a la población endofítica de frutos no se vio afectada por el cultivar (F = 1,11, p = 0,165) mientras que el estadio fenológico ejerció un efecto significativo (F = 1,42, p = 0,005), explicando el 5,3% de la variación de la composición endofítica.

Preferencia de hongos endofíticos por cultivar o estadio fenológico: En relación a las inflorescencias, los resultados obtenidos del análisis (CIA) revelaron que las especies *Diaporthe rudis, Pezizomycetes* sp., *Biscogniauxia mediterranea, Botrytis cinerea, Rosellinia limonispora, Botryotinia pelargonii, Stemphylium beticola, Chalastospora gossypii* y *Colletotrichum godetiae* se correlacionaron positivamente con el cv. *Madural* mientras que *Cladosporium cladosporioides*, *Fusarium solani*, *Fusarium oxysporum* y *Phaeosphaeria avenaria,* mostraron una correlación positiva con el cv. *Cobrançosa*. Los estadios fenológicos se diferenciaron principalmente por *Pezizomycetes* sp., *C. cladosporioides* y *Botryotinia fuckeliana,* que se relacionaron positivamente con el estadio fenológico de flores abiertas y pétalos caídos.

En los frutos, también se encontró un conjunto de OTUs positivamente asociadas al cv. *Cobrançosa* (*Neofabraea* sp. y *Ochrocladosporium*) y al cv. *Madural* (*Neofabraea vagabunda, A. alternata y Coniozyma leucospermi*). Los índices de maduración se diferenciaron principalmente por *A. alternata* y *Trametes sp.* así como *Pyrenochaeta corni y F. solani*, que se correlacionaron positivamente con el MI 4 y el MI 2. *Pseudophaeomoniella oleae* y *Aspergillus versicolor* se asociaron con el MI 3. *Pezizomycetes* sp. (*IndVal* = 0,926), *D. rudis* (*IndVal* = 0,826) y *A. alternata* (*IndVal* = 0,772) fueron las especies indicadoras que mejor caracterizaron el cv. *Madural* mientras que *F. solani* fue la única especie indicadora del cv. *Cobrançosa (IndVal* = 0,614).

Discusión

En este trabajo, se investigó la comunidad endofítica cultivable asociada a inflorescencias y frutos de dos cvs de olivo, con susceptibilidades variables a la antracnosis. A pesar de las limitaciones del enfoque cultivable relacionado con la subestimación de la composición microbiana en las muestras (Rappé y Giovannoni 2003), nuestros resultados mostraron que inflorescencias y frutos de olivo fueron colonizados principalmente por miembros de Ascomycota, siendo las clases dominantes Sordariomycetes y Dothideomycetes. Este resultado es consistente con otro estudio que

utilizó secuenciación de alto rendimiento del microbioma en los mismos órganos del olivo para la identificación (Abdelfattah et al., 2015).

Los dos cultivares estudiados presentaron comunidades diferentes en relación a la abundancia y composición de especies endofíticas, con un mayor número de aislados y especies recuperadas en el cv. *Madural* que en el cv. *Cobrançosa.* Aunque el cultivar no fue el principal factor de aglomeración de hongos en los órganos reproductivos, los resultados del análisis *Varpart* sugieren un cierto control del hospedero sobre las comunidades en las inflorescencias. La planta hospedera parece reclutar y promover selectivamente el crecimiento de ciertas especies de hongos (Bálint et al., 2013). Sin embargo, los mecanismos que conducen a las diferencias en la estructura de la comunidad endofítica entre los genotipos del hospedero, a nivel de los órganos reproductivos, no se conocen bien (Aleklett et al., 2014). Estos mecanismos pueden estar asociados a propiedades fenotípicas, como la morfología, la química, la fisiología y el sistema inmune de la planta (Lemanceau et al., 2017).

El cultivar tolerante a la antracnosis (*Cobrançosa*) parece ser capaz de prevenir la invasión y colonización de inflorescencias por endófitos cuando se compara con el cultivar susceptible (*Madural*), en el que se observa una mayor tasa de colonización por hongos. Por lo tanto, es probable que las diferencias en la susceptibilidad a la antracnosis entre los dos cvs desempeñen un papel en la estructura de las comunidades de endófitos en las inflorescencias. Además, se observaron patrones de asociación entre plantas y endófitos en diferentes estadios de desarrollo de inflorescencias y frutos. Tanto la abundancia como la riqueza de hongos fueron mayores en flores abiertas que en flores cerradas. Esto podría deberse a una mayor exposición al medio ambiente o a mayores recursos nutricionales para la colonización por hongos de flores abiertas en comparación con las cerradas. Las flores abiertas reciben más visitas de polinizadores y los granos de polen pueden transportar más microorganismos (Aleklett et al., 2014). Un grupo de OTUs se asoció positivamente a flores abiertas, siendo *Pezizomycetes* la OTU más significativa. Estas especies se encuentran en los hábitats más variados, y la colonización de flores probablemente proviene del ambiente circundante.

La abundancia y riqueza de hongos florales disminuyó a medida que cayeron los pétalos, lo que sugiere que solo algunos de los hongos que emigraron a la flor pudieron crecer y persistir con éxito. Las especies *C. cladosporioides y B. fuckeliana* estuvieron fuertemente asociadas a las flores después de la pérdida de pétalos. Las diferencias en la estructura de la comunidad endofítica también se detectaron durante la maduración de los

frutos, siendo la abundancia y riqueza de hongos mayor en las aceitunas maduras que en las verdes. Este resultado puede deberse a la disminución en el contenido de fenol durante la maduración (Gouvinhas et al., 2017), lo que provocaría que los frutos fuesen más favorables a la colonización y al crecimiento de hongos. Las OTUs más asociadas con aceitunas verdes se corresponden con hongos con función biológica desconocida (*P. corni*) u hongos fitopatógenos (*F. solani*) responsables de diversas enfermedades de los cultivos, incluyendo los olivos (Trabelsi et al., 2017). Las aceitunas maduras se asociaron positivamente con UTOs descritas como fitopatógenas (*A. alternata*) (Troncoso-Rojas and Tiznado-Hernández 2014), saprobias (*Trametes sp*.) (Zmitrovich et al., 2012) o toxigénicas *(A. versicolor*) (Engelhart et al., 2002). Recientemente se ha relacionado *P. oleae* - otro hongo asociado positivamente a frutos maduros - con riesgos de madera parda y síntomas de marchitez en los olivos (Antelmi et al., 2018). Se descubrió que conjuntos de OTUs eran específicas de cultivares particulares, con una elevada asociación, lo que sugiere que podrían ser relevantes para la salud del olivo. La mayoría de los indicadores asociados positivamente al cv. *Madural* son patógenos de plantas de olivo (i e., *C. godetiae* y *N. vagabunda*) o patógenos comunes de otras especies de plantas (i e., *D. rudis*).

En el caso del cv. *Cobrançosa* también presentaba varias OTUs asociadas positivamente. Algunas de ellas se han descrito como beneficiosas para las plantas, como *C. cladosporioides* o fitopatógenas de diversos cultivos, incluidos los olivos, como *Neofabraea* sp. (Chen et al., 2016). Finalmente, su contribución para la resistencia de los olivos contra la antracnosis requiere investigación adicional. En particular, los estudios de interacciones entre estas OTUs, el olivo y el patógeno *Colletotrichum* probablemente revelarán las funciones que estos hongos poseen en la susceptibilidad/resistencia de la planta a la enfermedad de antracnosis.

Capítulo 3 - Estructura de la comunidad de hongos endofíticos de olivares con alta y baja incidencia de antracnosis

Materiales y métodos

El material vegetal se recolectó de junio a diciembre de 2016 en dos olivares, uno históricamente con alta incidencia de antracnosis y el otro con baja incidencia de antracnosis. Las diferencias entre ambos se confirmaron mediante la estimación de la incidencia y la severidad de la enfermedad en el momento de la recolección de la muestra

(consulte la sección de incidencia y severidad de la antracnosis, Capítulo 3). Los olivares se localizan en el municipio de Mirandela, Noreste de Portugal a una distancia de ≈ 15 km. El olivar 1 se encuentra en la Aldea de Abambres, a 255 m de altitud y ≈ 2 km del río en una zona húmeda, mientras que el olivar 2 se encuentra en la Aldea de Paradela, a 450 m de altitud en una zona menos húmeda (≈ 8 km del río). Ambos olivares están compuestos por árboles del cv. *Madural* (moderadamente susceptible a la antracnosis), de edad similar (< 30 años), con un marco de plantación de 7 × 7 m. De cada olivar, se seleccionaron al azar siete árboles, y se recogieron botones florales, flores abiertas y frutos asintomáticos. En total, se recogieron 50 muestras de cada órgano por árbol y se aislaron sus hongos endofíticos. Tanto la incidencia como la severidad de antracnosis se evaluaron en los mismos árboles utilizados para aislar endófitos. Para ello se recolectaron frutos en cinco fechas diferentes, de octubre a diciembre de 2016. En cada muestreo, se recolectó aleatoriamente un total de 100 frutos por árbol.

Se determinó la incidencia y la severidad, así como el área bajo la curva de progreso de la enfermedad para la incidencia (AUDPCi) y la severidad (AUDPCs) en cada árbol y fecha de muestreo como descrito por Moral et al. (2008). Los tejidos fueron esterilizados, se cortaron en segmentos de 2-4mm y se colocaron en medio de agar papa dextrosa (APD) a 25 ± 2 °C en oscuridad. Fue procesado un total de 4200 segmentos de cada órgano. Los aislados fueron identificados molecularmente mediante la región espaciadora transcrita interna (ITS) del ADN ribosómico nuclear (ADNr).

La diversidad y composición de la comunidad asociada al olivo se comparó entre olivares (con alta o baja incidencia de antracnosis) u órganos (botones florales, flores abiertas y frutos). Se determinó la abundancia y riqueza, así como el índice de diversidad de Shannon - Wiener (H '). Se realizó un análisis de la varianza unidireccional (ANOVA) El escalamiento multidimensional no métrico (NMDS) utilizando el índice Bray-Curtis, permitió determinar la similitud de los ensamblajes endofíticos en los diferentes olivares y órganos. Las diferencias entre grupos se determinaron mediante un análisis de similitud unidireccional (ANOSIM), y la composición de la comunidad endofítica se comparó a nivel funcional.

Los factores responsables de la estructura de la comunidad se determinaron mediante un análisis de partición de variación (*Varpar*t). El análisis de bosques aleatorios (*Random Forest*) permitió identificar las OTUs más importantes en la diferenciación de los olivares u órganos. Las OTUs identificadas como altamente relevantes fueron seleccionadas y sometidas a un análisis de componentes principales (PCA). También se

realizaron correlaciones de Spearman para evaluar la correlación entre las OTUs preseleccionadas por el análisis (*Random Forest*) y la abundancia relativa de *Colletotrichum* spp. En el material y métodos del Capítulo 3 se puede encontrar una descripción más detallada del procedimiento realizado.

Resultados

En este estudio, los dos olivares evaluados mostraron una clara diferencia en la progresión de la enfermedad. De hecho, la incidencia de la enfermedad (AUDPCi) y la severidad (AUDPCs) fueron significativamente ($p < 0,001$) superiores en el olivar con antecedentes de antracnosis (olivar 1 - Abambres; AUDPCi = 8,0 y AUDPCs = 8,2) que en el olivar con antecedentes de baja incidencia de antracnosis (olivar 2 - Paradela; AUDPCi = 1,3 y AUDPCs = 1,0). Estas diferencias aumentaron con el tiempo, a medida que los frutos maduraban. El olivar con alta y baja incidencia de antracnosis serán denominados a lo largo de texto como "alta incidencia" y "baja incidencia". La abundancia de *Colletotrichum* spp. de forma endofítica en botones florales y frutos fue significativamente mayor (hasta 4,0 veces y 40,0 veces, respectivamente, $p < 0,0001$) en el olivar con alta incidencia en comparación con el olivar con baja incidencia. Los resultados también mostraron la capacidad de los aislados de *Colletotrichum* para crecer de forma asintomática en el interior del huésped de una manera endofítica y/o inactiva.

Comparación de la comunidad endofítica: Incidencia de antracnosis alta versus baja: El análisis de las comunidades en los dos olivares reveló un total de 115 OTUs, pertenecientes a dos filos, 31 familias y 61 géneros. Ascomycota fue el filo más abundante, representando el 96% del total de aislados mientras que los restantes aislados pertenecieron a Basidiomycota. *Biscogniauxia* (Xylariaceae), *Colletotrichum* (Glomerellaceae), *Alternaria* (Pleosporaceae) y *Cladosporium* (Cladosporiaceae) fueron los géneros más abundantes, representando el 44% del total de aislados. La comunidad endofítica asociada al olivo varió entre los diferentes olivares (con alta y baja incidencia de antracnosis). Hubo una abundancia significativamente superior (hasta 1,3 veces; $p = 0,001$) y riqueza (hasta 1,2 veces; $p = 0,05$) de hongos en el olivar con baja *versus* con alta incidencia de antracnosis, mientras que la diversidad de Shannon ($p = 0,05$) fue similar en ambos olivares. El aumento en la abundancia de hongos fue más evidente en las flores (aumento de 1,8 veces, $p = 0,001$), mientras que en los frutos se observó un resultado opuesto (disminución de 1,5 veces, $p = 0,031$).

Los dos olivares presentaron diferencias en la riqueza de hongos principalmente en flores abiertas y frutos, siendo hasta 1,5 veces y 1,3 veces significativamente ($p < 0,05$) superiores en el olivar con baja incidencia que en el olivar con alta incidencia de antracnosis. Se observó una disminución significativa en la abundancia y riqueza de hongos benéficos y patógenos en el olivar con alta incidencia de antracnosis en relación al olivar con baja incidencia. Esta disminución fue más notoria en los endófitos que colonizan los frutos (benéficos) y los botones florales (patógenos). Los comensales aumentaron significativamente su abundancia y riqueza en el olivar con baja incidencia en relación al olivar con alta incidencia de enfermedad, en particular en los endófitos que habitan los botones florales y las flores. La composición general de la comunidad endofítica difirió significativamente entre olivares con alta y baja incidencia de antracnosis, como indican el NMDS y el análisis de similitudes (ANOSIM; Global $R = 0,76$, $p = 0,001$) basado en el índice Bray- Curtis. Estas diferencias fueron mayores para los endófitos de los frutos ($R = 0,91$, $p = 0,001$) que de las flores abiertas ($R = 0,74$, $p = 0,001$) o los botones florales ($R = 0,55$, $p = 0,001$). También se constató que la composición endofítica de los botones florales y las flores abiertas presentó una similitud superior ($R = 0,61$ y $R = 0,60$, $p = 0,001$), seguida de la de los botones florales y frutos ($R = 0,90$ y $R = 0,92$, $p = 0,001$) y la de flores abiertas y frutos ($R = 0,91$ y $R = 0,95$, $p = 0,001$).

Los árboles del olivar con alta incidencia de antracnosis fueron colonizados principalmente por *Biscogniauxia, Cladosporium* y *Colletotrichum*, infectando más del 1,4, 1,6 y 2,6% del total de los botones de flores, flores abiertas y segmentos de frutos analizados respectivamente. En los árboles del olivar con baja incidencia de enfermedad *Biscogniauxia* fue identificado en más del 1,9% y 1,6% de los botones florales y flores analizados respectivamente, y *Alternaria* colonizó más del 1,2% de los frutos. Cada olivar tenía varias OTUs exclusivas: 37 OTUs se aislaron únicamente en el olivar con alta incidencia y 33 en el olivar con baja incidencia.

Contribución de diferentes factores a la formación de comunidades endofíticas: La composición de hongos se explica principalmente por el órgano de la planta (responsable por el 24% de la variación total, $F = 2,17$, $p = 0,005$), mientras que el tipo de olivar explica el 10% de la variación total de la comunidad ($F = 1,98$, $p = 0,005$). Las OTUs que más contribuyen a la diferencia entre olivares fueron *Epicoccum nigrum* (aislado de flores y frutos), *Biscogniauxia mediterranea* y *Diaporthe rudis* (ambos aislados de botones florales y flores abiertas). Los órganos se distinguieron

principalmente por *B. mediterranea* (en olivares de alta y baja incidencia), así como por *Neofabraea vagabunda* y *Pezizomycetes* sp. aislado en olivares con alta y baja incidencia de antracnosis.

OTUs asociadas a cada olivar y órganos vegetales: Los resultados indican que un conjunto de hongos se encuentra asociados a uno de los olivares u órganos siendo que las OTUs *Pseudophaeomoniella oleae*, *N. vagabunda*, *Neofabraea* sp. y *Parastagonospora avenae* - todas aisladas de los frutos, presentan una asociación superior al olivar de alta incidencia de antracnosis. Se observó una correlación positiva entre estas especies y la abundancia de *Colletotrichum* spp. Sin embargo, *D. rudis* (de flores), *Fusarium oxysporum* (de botones florales), *Pezizomycetes* sp., *E. nigrum*, *Mollisia minutella*, *Trametes* sp. y *Sardariomycetes* sp. (todos aislados de frutos) se encontraron altamente asociados al olivar con baja incidencia de antracnosis y correlacionados negativamente con la abundancia de *Colletotrichum* spp.

Discusión

En el presente trabajo, se investigó la diversidad y composición de hongos endofíticos de los órganos reproductores del olivo localizados en áreas de alta y baja incidencia de antracnosis. Las diferencias en la incidencia y severidad de la enfermedad, así como los niveles de inóculo de *Colletotrichum*, fueron corroborados por evaluaciones de la enfermedad en el campo, que validan su idoneidad para investigar la relevancia de estas comunidades a la presión de los patógenos. El olivar 1 presentaba una mayor proximidad al río que el olivar 2 ocasionando una influencia potencial de la humedad relativa en el desarrollo de la enfermedad. Futuros estudios deberán confirmar esta hipótesis.

En este trabajo el objetivo principal fue identificar la amplia variedad de taxones a nivel de especie y obtener aislados, para producir información que permita estudiar su papel en la patogénesis de *Colletotrichum* spp. La mayoría de las OTUs pertenecen a las clases Sordariomycetes y Dothideomycetes, tal como en los estudios realizados por Abdelfattah et al. (2015). Las comunidades endófitas de los órganos reproductivos de ambos olivares variaron en su riqueza, abundancia y composición de especies. Aunque la diferencia encontrada entre olivares no proporciona información sobre la causa y el efecto del patógeno, estos resultados sugieren múltiples interacciones entre la comunidad de patógenos y hongos en olivares con alta y baja incidencia de antracnosis. Hipotéticamente, en olivares con baja incidencia de enfermedad tanto patógenos

Colletotrichum spp. como la enfermedad no puede prosperar del mismo modo que en el olivar con alta incidencia de enfermedad debido a la presencia de una comunidad endófita más rica y abundante. La tolerancia de plantas a enfermedades se ha correlacionado en estudios previos con una mayor diversidad de hongos endofíticos (Busby et al., 2015). En el olivar con una alta incidencia de antracnosis se observó una disminución en la abundancia y riqueza tanto de endófitos benéficos (i.e., *Epicoccum nigrum* y *Chaetomium Globosum*) como de patógenos, sugiriendo que estos hongos pueden desempeñar un papel importante en el desarrollo de *Colletotrichum* spp. y de la enfermedad (Preto et al., 2017). Las especies de *Colletotrichum* responsables por la antracnosis en olivos pueden ser comensales o patógenas, dependiendo de la etapa de desarrollo del hospedero. Durante la floración, el hongo se comporta como endofítico o latente. Tras la maduración de los frutos, el hongo entra en una fase necrotrófica (Sergeeva et al., 2008).

Las diferencias en la composición de especies entre olivares se observaron de una forma más pronunciada en frutos que en flores, por tanto, estos cambios podrían afectar a las fases de vida de *Colletotrichum* spp. Estudios previos indican que la transición del hongo *Moniliophthora perniciosa* a una fase más agresiva se desencadenaba por su interacción con otros microorganismos vegetales (Bezemer et al., 2006). Nuestra hipótesis se ve reforzada por la mayor diferencia entre las comunidades de flores y frutos en el olivar con una mayor incidencia de antracnosis. Por lo tanto, parece existir una relación entre la estructura de la comunidad de hongos y la incidencia de antracnosis, esto es, cuanto mayor es la composición endofítica entre frutos y flores (donde *Colletotrichum* spp. ocurre como latente), menor es la incidencia de la enfermedad. Esta hipótesis debe confirmarse en trabajos futuros.

Se observó que el órgano de la planta afecta significativamente a la composición de la comunidad de hongos en la endosfera de los olivos, como ha sido observado anteriormente para varias especies de plantas leñosas (Moricca et al., 2012), incluyendo el olivo (Gomes et al., 2018). La especificidad de los órganos puede estar relacionada con diferencias morfológicas y químicas entre flores y frutos, como se sugirió para los órganos vegetativos (Gomes et al., 2018). Dado que el cambio de *Colletotrichum* spp. del modo endofítico al patogénico ocurre preferiblemente en los frutos, planteamos la hipótesis de que los cambios en la composición de la comunidad endofítica entre flores y frutos pueden desempeñar un papel importante en el desarrollo de la antracnosis. Por lo tanto, la capacidad de cada órgano de la planta para configurar su comunidad endofítica parece ofrecer una defensa adicional para *Colletotrichum* spp.

Nuestros datos revelaron consorcios de OTUs específicas asociadas a cada órgano en olivares con diferentes grados de incidencia de antracnosis. Estos consorcios podrían influir en la disminución (antagonismo) o aumento (facilitación) del desarrollo de la enfermedad. Las OTUs que mejor se correlacionan con la alta incidencia de antracnosis son patógenas de olivos (*Neofabraea* sp., *N. vagabunda* y *P. oleae*) (Romero et al., 2015; Nigro y Antelmi, 2015) o de plantas de trigo (*P. avenae*) (Cunfer 2009). Estos pueden caracterizarse como "facilitadores de patógenos", ayudando al patógeno a infectar con éxito la planta o aumentar la severidad de la enfermedad. Por otro lado, *D. rudis* (de flores), *F. oxysporum* (de botones florales), *Pezizomycetes* sp., *E. nigrum, M. minutella, Trametes* sp. y *Sardariomycetes* sp. (de frutos) se encontraban altamente asociados al olivar con una baja incidencia de antracnosis, se pueden describir como protectores (Alabouvette y Olivain 2018). Por tanto, estudios futuros deberían analizar el papel de estos hongos en el desarrollo de la antracnosis del olivo

Capítulo 4 - Diversidad y actividad antagónica de hongos endofíticos asociados con cultivares de olivo

Materiales y métodos

El muestro fue realizado en tres cultivares (cvs) con diferente susceptibilidad a la antracnosis: *Galega vulgar* (susceptible), *Cobrançosa* (moderadamente susceptible) y *Picual* (resistente) procedentes de cinco olivares. En cada olivar, se seleccionaron aleatoriamente siete árboles sanos y se recolectaron cinco muestras de raíces, ramas y hojas por árbol entre enero y agosto de 2014. Las muestras se esterilizaron superficialmente de acuerdo con el procedimiento descrito por Martins et al. (2016). Las raíces y las ramas se cortaron en segmentos (4-5 mm de longitud). De las hojas se cortó un segmento de la lámina (5 × 5 mm) y uno del pecíolo (5 mm de longitud). Los segmentos se colocaron en medio de cultivo. En total, se usaron 7875 segmentos para aislar endófitos. Se extrajo el ADN de los cultivos puros y la región de los ITS fue amplificada y secuenciada. La diversidad endofítica se evaluó determinando la abundancia, riqueza y el índice recíproco de Simpson (1/D). El escalamiento multidimensional no métrico (NMDS), utilizando el índice Bray-Curtis y un análisis de similitud (ANOSIM) permitió describir las diferencias en la composición de las comunidades. A partir del análisis Co-inercia (CIA) se estimó la asociación de los hongos con un órgano/cultivar.

Ensayos antagonistas in vitro: cinco hongos del cv. *Galega* (*Trichoderma gamsii, Fusarium oxysporum, Macrophomina phaseolina, Penicillium purpurogenum y Phomopsis columnaris)* y cinco de cv. *Cobrançosa* (*Penicillium commune, Penicillium roseopurpureum, Paecilomyces lilacinus, Fusarium oxysporum y Hypocrea lixii*) se seleccionaron para detectar posibles antagonistas del patógeno *Colletotrichum acutatum*. *Penicillium roseopurpureum, M. phaseolina* y *P. columnaris* se aislaron de raíces, *P. lilacinus* y *F. oxysporum* de ramas (de cvs. *Galega* y *Cobrançosa*) y *H. lixii, P. commune, T. gamsii, P. purpurogenum y F. oxysporum* de hojas (de cvs. *Cobrançosa* y *Galega)*. La cepa *C. acutatum* (COCa2011) se obtuvo de la colección microbiana del Instituto Politécnico de Bragança (Portugal). Los patógenos se aislaron de aceitunas infectadas naturalmente del cv. *Cobrançosa* y se amplificaron y secuenciaron por la región de los ITS. La interacción de hifas entre endófitos y *C. acutatum* se estudió a través del establecimiento de dos cultivos. Se evaluó el crecimiento, la esporulación y la viabilidad de las esporas de *C. acutatum,* así como la morfología de las hifas en la zona de interacción con el endófito. La actividad inhibitoria de cada endófito se calculó de acuerdo con la ecuación de Cray et al. (2015). La esporulación y germinación de *C. acutatum* así como el registro de las características macroscópicas y microscópicas se determinaron tras la finalización de los ensayos. La relación de la inhibición *in vitro* del crecimiento, esporulación y germinación de *C. acutatum* con el origen de los aislados se determinó mediante un análisis factorial múltiple (AFM).

Ensayos de antagonistas in vivo: Los resultados *in vitro* identificaron a *H. lixii* como el hongo que presentaba más antagonismo, por lo que fue seleccionado para investigar su capacidad para suprimir el desarrollo de la antracnosis causada por tres especies del complejo *C. acutatum* (*C. acutatum, C. godetiae y C. fioriniae*). Para ello, aceitunas sin síntomas del cv. *Cobrançosa,* con tres índices de maduración (MI) 2-3-4 fueron esterilizadas superficialmente y colocadas en matraces de vidrio con papel de filtro estéril. Posteriormente, se inocularon 4 ml de suspensión de esporas endofíticas (10^6 esporas/ml), sobre las aceitunas, y después de 3 días se adicionaron 4 ml de suspensión de esporas de patógenos (10^6 esporas/ml). Los controles se realizaron inoculando aceitunas con 4 ml de solución estéril de Tween 80 al 0,02% (v/v). Tanto la incidencia como la severidad de la enfermedad se evaluó a los 7, 14 y 21 días después de la inoculación del patógeno. Finalmente, se calculó el área bajo la curva de progreso de la enfermedad para la incidencia (AUDPCi) y la severidad (AUDPC) (Moral et al. (2008).

El procedimiento utilizado se encuentra más detallado en el material y métodos del Capítulo 4.

Resultados

De todos los olivos se identificaron 1911 aislados pertenecientes a 205 OTUs, siendo mayoría del filo Ascomycota (97%), clase Sordariomycetes (59%) y familias Nectriaceae (28%), Diaporthaceae (19%) y Trichocomaceae (8%). *Fusarium, Phomopsis* y *Penicillium* fueron los géneros más abundantes, representando en conjunto un 54% del total de aislados. El número de hongos fue superior en las raíces (132 OTUs, 66 géneros, 42 familias, 13 órdenes, 4 clases y 2 filos) que en las ramas (101 OTUs, 16 géneros, 12 familias, 9 órdenes, 5 clases y 2 filos) u hojas (41 OTUs, 16 géneros, 12 familias, 9 órdenes, 5 clases y 3 filos).

Las raíces exhibieron una abundancia de hongos significativamente superior (hasta 2,4 y 5,8 veces), riqueza (hasta 1,3 y 3,6 veces) y diversidad (hasta 2,3 y 3,7 veces) en comparación con ramas y hojas. Estas diferencias fueron mayores para los endófitos que colonizan los cvs. *Picual* y *Cobrançosa* que para los que colonizan el cv. *Galega vulgar*. Los resultados fueron confirmados por el NMDS y el análisis ANOSIM con (Global R = 0,88; p = 0,001); para los cvs. *Picual* (R = 0,78, p = 0,001) y *Cobrançosa* (R = 0,73, p = 0,001) y para el cv. *Galega vulgar* (R = 0,45, p = 0,001). La composición de la hoja y la rama mostraron una mayor similitud, mientras que los órganos de la raíz y los órganos aéreos (rama y hoja) mostraron más diferencias. Del total de UTOs, un 44% fueron exclusivas de raíces, con solo un 9% compartido entre los tres órganos.

Comunidad endofítica de cultivares de olivo asociada a cada órgano vegetal: Los endofitos *Bionectria, Chalastospora, Fusarium, Macrophomina, Phomopsis, Penicillium y Trichoderma* se asociaron positivamente con la raíz. Los endofitos *Chaetomium, Epicoccum, Hypocrea y Pseudocercospora*, se asociaron positivamente con la hoja, mientras que los géneros más importantes asociados con la rama fueron *Aureobasidium, Alternaria, Biscogniauxia, Phaeosphaeria, Cladosporium, Purpureocillium y Ochrocladosporium.* Los géneros que mostraron una asociación superior con el cv. *Galega vulgar* fueron *Chromelosporium, Fusarium, Phomopsis, Penicillium y Trichoderma,* mientras que en el cv. *Cobrançosa* fueron *Chalastospora, Cladosporium, Hypocrea y Purpureocillium*, y finalmente en el cv. *Picual* fueron *Aureobasidium, Epicoccum, Crinipellis, Ilyonectria y Phaeosphaeria.*

Interacción in vitro entre hongos endófitos y C. acutatum: Los diez hongos testados inhibieron el crecimiento de *C. acutatum*, siendo *M. phaseolina, H. lixii, F. oxysporum y P. lilacinus* los que mostraron un efecto antagonista mayor (IC > 60). Estos antagonistas se aislaron principalmente del cv. *Cobrançosa* (tres de cada cuatro). La actividad antagonista de los endófitos aislados del cv. *Galega vulgar* fue menos notable. Esta inhibición fue promovida en las proximidades de las colonias de *C. acutatum.* De los cinco aislados del cv. *Galega*, solo *T. gamsii y P. purpurogenum* redujeron significativamente ($p < 0,05$) la esporulación de *C. acutatum* en 70% y 58%. Estos dos, junto con *F. oxysporum*, también mostraron capacidad para inhibir significativamente ($p < 0,05$) la germinación de *C. acutatum* (del 23 al 36%). De los cinco aislados del cv. *Cobrançosa, P. commune, P. lilacinus, F. oxysporum* y *H. lixii* redujeron significativamente ($p < 0,05$) la esporulación (hasta 76%) y la germinación, variando del 24 al 82%, en comparación con el control de *C. acutatum*.

La inhibición por contacto fue la interacción antagónica más observada seguida del crecimiento excesivo de un micelio sobre el otro (*P. columnaris; H. lixii*) y la mezcolanza de ambos micelios, conllevando la formación de una barrera pigmentada (*P. purpurogenum*). El borde de las colonias de *C. acutatum* se tornó rojo u amarillo pigmentado en la zona de interacción con *P. purpurogenum* y *P. rosesospurpurem*, y marrón oscuro en contacto con *P. lilacinus*. A diferencia de lo observado en el control, en el cultivo con *P. commune* y *F. oxysporum* en la zona de interacción las hifas de *C. acutatum* se colapsaron, hincharon y se distendieron, mientras que con *H. lixii* y *T. gamsii* se retorcieron y enrollaron. En las proximidades de *P. roseopurpureum* y *P. lilacinus* se observó necrosis y vacuolación citoplasmática. En cultivo con estos dos hongos también se formaron cristales.

El efecto antagonista producido por los hongos testados está relacionado con su origen (órgano y cultivar). De acuerdo con el análisis de AFM, tanto la hoja – en relación a los órganos (coeficiente estimado = 1,53, $p < 0,001$), como el cv. *Cobrançosa* – en relación a los cultivares (coeficiente estimado = 0,69, $p < 0,001$), mostraron una asociación superior con la inhibición de la esporulación y germinación de *C. acutatum*. La inhibición del crecimiento de *C. acutatum* reveló estar significativamente asociada con el *cv. Galega vulgar* (coeficiente estimado = 0,66, $p < 0,001$) y la rama (coeficiente estimado = 0,54, $p < 0,01$). La raíz mostró ser el órgano con una asociación negativa superior en relación a todos los parámetros evaluados (coeficiente estimado = -1,12 y -0,56, $p < 0,001$).

Bioensayos con aceituna: El hongo *H. lixii* fue identificado como el agente de control biológico de *Colletotrichum* spp. más prometedor. *H. lixii* redujo significativamente ($p < 0,05$) la incidencia y la severidad de la antracnosis del olivo en comparación con el control (en ausencia de *H. lixii*), principalmente a los 14 y/o 21 días después de la inoculación. La eficacia de *H. lixii* como agente de control biológico contra *C. acutatum* y *C. fioriniae* fue más evidente en frutos con MI = 2 y MI=4, mientras que la reducción en el desarrollo de antracnosis causada por *C. godetiae* fue evidente en frutos con los tres índices de maduración.

Discusión

En general, los endófitos que colonizaron los órganos vegetativos del olivo mostraron una elevada diversidad (un total de 205 OTUs), siendo la mayoría de los aislados pertenecientes al filo Ascomycota y la clase Sordariomycetes. Estudios previos, tanto con un enfoque culturalmente dependiente (Gomes et al., 2018) como independiente (Fernández-González et al., 2019), encontraron resultados similares. Los tres órganos albergaron diferentes comunidades, siendo la diversidad y abundancia de hongos superior en las raíces, seguida por ramas y hojas. Este resultado está de acuerdo con estudios previos que sugieren la especificidad de los órganos de los endófitos en el olivo (Gomes et al., 2018). A pesar de esta diferencia, la mayor similitud en la composición de ramas y hojas de la comunidad endofítica en comparación con las comunidades de raíces y los órganos aéreos sugiere que algunos hongos podrían crecer de ramas a hojas. En contraste, la mayoría de los hongos que colonizaron las raíces de olivo parecieron ser incapaces de moverse a los tejidos vegetales aéreos, tal como fue sugerido por Martins et al. (2016).

Se observó una asociación positiva de un conjunto de hongos con el cultivar de olivo y, por lo tanto, a su susceptibilidad subyacente a la antracnosis. Los endófitos con una asociación positiva mayor al cv. susceptible pertenecen a géneros que incluyen: (i) patógenos de plantas, como *Chromelosporium* (Mukobata y Saioto 1996), *Fusarium* (Moretti, 2009) y *Phomopsis* (Udayanga et al., 2011), (ii) hongos ubicuos presentes en diversos procesos, desde la patogenicidad necrotrófica hasta el mutualismo endofítico (*Penicillium*) (Visagie et al., 2014), y (iii) en menor medida agentes de control biológico (*Trichoderma*) (Carrero-Carrón et al., 2016). En cambio, los géneros con una asociación positiva mayor a los cvs. moderadamente susceptibles o resistentes incluyen miembros con reconocidas habilidades de control biológico contra enfermedades de plantas (*Epicoccum, Aureobasidium, Cladosporium* e *Hypocrea*) (Musetti et al., 2011; Elgorban

et al., 2014; Khan et al., 2016; Rathnayake et al., 2018) y contra plagas de insectos (*Purpureocillium*) (Medeiros et al., 2018), y en menor medida patógenos de plantas (*Crinipellis, Ilyonectria y Phaeosphaeria*) (Da Silva, 2004; Cunfer, 2009; Lombard et al., 2013). Por lo tanto, planteamos la hipótesis de que estos grupos de hongos podrían contribuir de forma determinante a la resistencia contra la antracnosis, que es una enfermedad que infecta los órganos aéreos del olivo. Sin embargo, aún se requiere trabajo adicional para confirmar dicha hipótesis.

Nuestro estudio fue el primero en mostrar que la actividad antagónica de los endófitos contra los patógenos depende de su origen (cultivar u órganos). Los endófitos del cv. *Cobrançosa* fueron más efectivos para reducir el crecimiento, la esporulación y la germinación de *C. acutatum* que los del cv. *Galega vulgar*. Entre los aislados, *H. lixii*, *F. oxysporum* y *P. lilacinus* fueron los más eficaces contra *C. acutatum*. Estos hongos son bien conocidos por su efectividad en el control biológico de fitopatógenos (Lorito et al., 2010), contra las plagas de insectos (Sharma y Marques, 2018) y en el control de nematodos parásitos de plantas (Khan et al., 2006). Además del cultivar, también se ha demostrado que el órgano de la planta del que se aisló el endófito influencia el efecto antagonista sobre el patógeno. Los endófitos foliares mostraron una mayor capacidad para inhibir la germinación y la esporulación de *C. acutatum*, mientras que los endófitos de ramas fueron los más efectivos para inhibir el crecimiento de *C. acutatum*. La antracnosis del olivo es una enfermedad transmitida por el aire; por lo tanto, suponemos que las especies que colonizan estos órganos pueden tener consecuencias importantes en el desarrollo de la enfermedad.

La supresión exhibida *in vitro* por todos los endófitos testados se promovió principalmente en las proximidades de las colonias de *C. acutatum*. Esto podría indicar la participación de metabolitos difusibles y la competencia por el espacio/nutrientes en la inhibición (Latz et al., 2018). La inhibición del crecimiento de *C. acutatum* producida por algunos endófitos también se debió al crecimiento excesivo de un micelio sobre el otro o a la mezcolanza de ambos micelios, originando la formación de una barrera. La observación de anormalidades morfológicas en las hifas de *C. acutatum* en cultivo dual con los endófitos corrobora esta hipótesis. Las alteraciones en las hifas de *C. acutatum* resultaron probablemente de la acción de enzimas líticas o toxinas, segregadas por los endófitos. Nuestros resultados indicaron *H. lixii* como un importante agente de control biológico potencial para la antracnosis del olivo. La inoculación de aceitunas con *H. lixii* se mostró eficaz en la reducción de la incidencia y la severidad de la antracnosis.

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List of Abbreviations

1/D	Simpson`s Reciprocal Index
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
Ca	*Colletotrichum acutatum*
CIA	Co-inertia analysis
cvs.	Cultivars
DNA	Deoxyribonucleic Acid
EF	Endophytic fungi
FAOTAST	Food and Agriculture Organization Corporate Statistical Database
H´	Shannon index
IndVal	The indicator value
ITS	Internal transcribed spacer
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional analysis
OA	Olive anthracnose
ºC	Celsius degrees
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rDNA	Ribossomal Deoxyribonucleic acid
SE	Standard error
sp.	Species (singular)
spp.	Species (plural)
SPSS	Statistical Package for the Social Sciences
ul	Microliter
v/v	volume/volume

Chapter 1

General Introduction

Part of this chapter is an adapted version of the published book chapter:

Martins F, Pereira JA, Baptista P (2019). Olive anthracnose and its management by fungal endophytes: An overview. *In* Varma A, Tripathi S, Prasad R (Eds.) Plant Microbe Interface. Springer, pp 253-269. doi.org/10.1007/978-3-030-19831-2.

1.1 Framework and objectives

Throughout the last two decades, an increased incidence of attacks by air- and soil-borne fungi in olive orchards has been reported. Olive anthracnose caused by species of the *Colletotrichum* complex is, among olive diseases, the most important worldwide and its spread into new areas represents a high risk to olive growing (Talhinhas et al., 2018). This disease is reported to infect almost all aerial organs of the olive tree (Cacciola et al., 2012), being however the fruits the most attacked. Once infected, the fruits show brownish-colored depressed marks with an oily appearance, becoming shrivelled, humidified and eventually may fall off from the tree. Under severe epidemics, defoliation and death of branches of the affected tree can also occur (Talhinhas et al., 2018). This can cause total or partial destruction of the fruits and reduce olive tree vigour, due to leaves drop and branches death, leading to important economic yield losses (Cacciola et al., 2012). Quality characteristics of olive oil are compromised as well. Anthracnose increases the degree of acidity and the olive oil loses much of its pleasant organoleptic characteristics (Silva, 2016).

It is believed that the fungus *Colletotrichum* spp. survives on mummified fruits that remain on the tree or in the soil, from one season to the next, restarting its activity as soon as the conditions are favourable (Talhinhas et al., 2018). The first infections start during the spring, in young flowers and fruits, where *Colletotrichum* spp. remains unnoticed in a quiescent phase; And, in the autumn, the pathogen switch to a necrotrophic lifestyle in which the disease symptoms became visible and secondary infections begin, lasting until the full maturation of the fruit (Sergeeva et al., 2008; Moral et al., 2009).

The anthracnose is difficult to control, being prevention the only reliable strategy. This is mainly based on multiple applications of fungicide (Cacciola et al., 2012), posing risks to the environment and non-target organisms, including humans. With the new trend to a more sustainable and health-oriented living, the demand for more eco-friendly control alternatives is high. A potential solution could be the use of native endophytic fungi to control olive anthracnose. These fungi live asymptomatically within plant tissues and have been increasingly recognized to provide protection against diseases (Latz et al., 2018). The exploitation of beneficial characteristics of native endophytic fungi also offers the advantage of using microorganisms already adapted to the target niche (*i.e*., pathogen) and host plant. Therefore, the main aim of this work is to study the endophytic fungal community associated to the olive tree and elucidate their influence on

the health of the tree in order to designed new strategies for the control of olive anthracnose. Specific objectives are:

1. Characterized the fungal endophytic community inhabiting roots, twigs, leaves, flowers and fruits of olive tree (*Olea europaea* L.) of four cultivars with different susceptibilities to olive anthracnose: *Galega vulgar* and *Madural* (susceptible), *Cobrançosa* (moderately susceptible) and *Picual* (resistant) (Moral et al., 2017). **Who is there?**

2. Understand the factors controlling endophytic fungal community assemblage in olive tree, such as plant organ, host genotype (at cultivar level), host phenology and anthracnose incidence. **Which factors contribute to their shaping?**

3. Disclose the role that endophytic fungal community naturally present in olive tree may have on host plant health, in particular in anthracnose disease suppression and lifestyle transition of the pathogen *Colletotrichum* spp. **What can they do?**

4. Identify and select autochthonous endophytic fungi that could be used as biocontrol agents against olive anthracnose disease. **Could they be useful?**

To achieve these objectives, this thesis is divided in five chapters. **Chapter 1** describes the objectives of this thesis and contains a general introduction to the olive anthracnose, covering the causal agents of this disease, epidemiology and life cycle. Disease control strategies were also described, with emphasis on biological control and on the exploitation of endophytic fungi in the development of new tools/approaches to manage olive anthracnose. In **chapter 2**, the endophytic fungal community in inflorescence and fruit of two olive cultivars is evaluated over different time points of reproductive organs development, and the possible drivers of fungal assemblages, in particular the host genotype (at cultivar level), plant organ and host phenology, were identified (objectives 1 and 2). In **chapter 3**, the endophytic fungal community structure in reproductive organs of olive tree from two orchards with contrasting anthracnose incidence is compared, in order to elucidate the potential role of endophytes on disease suppression and on lifestyle transition of the pathogen (objectives 2 and 3). In **chapter 4,** the endophytic fungi associated to olive tree cultivars with different susceptibilities to anthracnose were studied and the antagonistic activity of the isolates obtained against *Colletotrichum* spp. was tested using both *in vitro* duo-culture methods and bioassays with olive fruits. The relationship between olive tree cultivars (and their underlying susceptibility to anthracnose) with the level of antagonism displayed by their fungal endophytes is evaluated, in order to reveal the influence of the endophyte community on

the health of the tree. Several species were also identified as potential biocontrol agents of anthracnose (objective 4). Finally, the conclusions of the results obtained in this study and perspectives for future work are presented **in chapter 5.**

1.2 Olive Anthracnose: A general Overview

The European olive, *Olea europaea* subsp. *europaea* L., is one of the major cultivated species in countries surrounding the Mediterranean Sea. In 2016, approximately 9.2 million ha of land in this region were planted with olive trees (FAOTAST, 2018). Several insect pest and diseases attack the olive crop, reducing its yield both in terms of quantity and quality. Among diseases, anthracnose is the major causes of olive crop damage worldwide (Talhinhas et al., 2018). It was first described in Portugal in 1899 by J.V. d'Almeida (1899) and rapidly expanded to all continents (Cacciola et al., 2012) becoming a serious economic constraints to olive crop production (Mosca et al., 2014; Iliadi et al., 2018). This disease affects different organs of the olive tree, including flowers, buds, shoots, leaves, and twigs, being the fruits the most severely affected by the disease (Cacciola et al., 2012). Thus, characteristic anthracnose symptoms arise mostly on the fruits, especially when they are nearly ripened. The first symptoms of infected olives are small and brown-colored spots in the epicarp that become later sunken. As the fruits ripe, the center of these sunken spots becomes covered with pink/orange gelatinous masses of conidia that are often produced in a concentric ring pattern (Talhinhas et al., 2011). This cause mummification, rotting and premature drop of fruits, leading to significant crop losses (Fig. 1.1). The attacks can occur on any part of the fruit, but they are more frequent at the apex, because it stays wet longer time (Cacciola et al., 2012). In some cases, infected fruits may persist on the tree, becoming an inoculum reservoir of olive anthracnose (Sergeeva, 2011a). In the vegetative parts, the symptoms include leaf chlorosis, defoliation and dieback of shoot and twigs (Cacciola et al., 2012). These effects are due to production of toxins by the pathogen (Cacciola et al., 2012). The infected flowers display blossom blight, dry out and drop quickly (Moral et al., 2008; Sergeeva et al., 2008). Infections are usually most severe on the lower branches, inside the canopy on the north side, where moisture tends to remain for longer periods of time (Cantero, 1997).

The disease can be devastating, depending on the level of susceptibility of the cultivars, the environmental conditions, the inoculums pressure and the virulence of the

pathogenic strains (Talhinhas et al., 2018). Under favorable conditions, all production can be destroyed. For instances, in some olive-growing countries, the olive anthracnose was described to cause yield losses above 80% (Cacciola et al., 2012). In addition, this disease can reduce the quality of olive oil. The oils' peroxide content and acidity value from anthracnose-infected fruits sometimes can be higher than the maximum legal limit to be considered as virgin olive oil (Silva, 2016). Most of these olive oils show negative sensory and organoleptic characteristics, being classified as lampante (Silva, 2016).

Fig. 1.1 Characteristic symptoms of anthracnose on olive tree fruits of cv. *Madural* (a) production of orange/pink sticky masses of conidia in olives surface; (b) rot, mummification and dehydration of fruits; (c) symptoms appear mostly in mature fruits but, in favorable environmental conditions, green fruits may also be infected; (d) affected fruits fall prematurely to the ground (photos: Fátima Martins, IPB-ESA).

1.3 Anthracnose is caused by a complex of *Colletotrichum* species

Anthracnose in olive tree is associated with at least eight *Colletotrichum* species, belonging to two heterogeneous fungal species complexes, namely *C. acutatum* sensu lato (s.l.) and C. *gloeosporioides* s.l. (Damm et al., 2012). Of these two complexes, *C. acutatum* s.l. is the most predominant, causing epidemic explosions of anthracnose in most olive-growing countries (Talhinhas et al., 2005). Multilocus molecular phylogenetic analysis revealed that there are six species in the *C. acutatum* complex considered to be causal agents of olive anthracnose, namely *C. fioriniae*, *C. simmondsii*, *C. nymphaeae*, *C.*

acutatum sensu stricto (s.s.), *C. godetiae* (syn. *C. clavatum*) and *C. rhombiforme* (Talhinhas et al., 2018). The same study also revealed that species belonging to the '*gloeosporioides*' complex are two, *C. gloeosporioides* s.s and *C. theobromicola* (Talhinhas et al., 2018). *Colletotrichum boninenses* (syn. *C. karstii*) is a third species complex that was recently related with olive anthracnose (Schena et al., 2014). However, this complex does not appear to threaten olive production due to their weakly pathogenicity (Schena et al., 2014). Similarly, other fungal species belonging to *C. gloeosporioides* complex (*i.e*., *C. aenigma*, *C. queenslandicum*, *C. siamense* and *C. kahawae* ssp. *ciggaro*) were isolated from symptomatic fruits, but their pathogenicity in olives has not yet been confirmed (Schena et al., 2014). Among all these fungal species identified, *C. acutatum* s.s., *C. godetiae* and *C. nymphaeae*, have been recognized as major causative agents of olive anthracnose in most olive-growing countries (Mosca et al., 2014). For instances, the majority of strains examined from South Africa, Australia and Tunisia belonged to *C. acutatum* s.s. (Cacciola et al., 2012). In other studies performed in Montenegro, Greece, Italy and Spain, *C. godetiae* was identified as the most prevalent species (Moral et al., 2008, 2009, 2014). In Portugal, primarily three important species have been related to the olive anthracnose, with *C. godetiae* causing major damage in the northern region whereas *C. nymphaeae* and *C. acutatum* s.s. have been identified as the most prevalent species in the southern regions (Talhinhas et al., 2009).

Species of *Colletotrichum* have a teleomorph or sexual stage, *i.e. Glomerella* sp. (Wharton and Diéguez-Uribeondo, 2004). Nevertheless, in olive crops, the teleomorph of the pathogen, has not yet been detected in field conditions (Cacciola et al., 1996), suggesting the imperfect stage *i.e*., *Colletotrichum* sp. as the main responsible of olive anthracnose.

1.4 Epidemiology and Life cycle

Epidemiology and life cycle of olive anthracnose are still poorly understood, especially in what concerns propagation and inoculum maintenance in the olive groves, which required more studies (Moral et al., 2008; Cacciola et al., 2012). In Mediterranean regions, it has been reported that infections begin during the spring in flowers and in young fruits (primary infection; Fig. 1.2) (Moral et al., 2009). The mode of survival and the source of this primary inoculum have yet to be determined (Moral et al., 2009). It is thought that the major primary inoculum reservoirs are mummified fruits that remain on

the tree or on the ground, from one season to the next (Moral et al., 2009). It is also plausible that the source of inoculum in spring may originate from fungi that overwinter in woody material and leaves of the tree (Talhinhas et al., 2018). After primary infection, the fungus stops growing and remains dormant until fruit begins to ripen (Moral et al., 2009). At that time, with favorable environmental conditions, sticky masses of spores are produced in acervuli. These spores are then spread by rain splash to newly fruits and other tree parts, giving rise to secondary infections (Moral et al., 2009). The spread of the pathogen and infection of olive tree depend heavily on the climatic conditions (Talhinhas et al., 2015). Olive anthracnose reaches highest disease incidence and severity in areas where relative humidity is highest (over 93%) and the air temperature is warm (ranging from 10 to 30ºC) (Cacciola et al., 2012). The occurrence of precipitation is also crucial for the conidia separation from the gelatinous mass of the acervuli and for their dispersion (Cacciola et al., 2012). Also, the infection of fruits depends on the extent of peel ripeness. Olives at later stages of ripening are more prone to fungal infection than green fruits (Silva, 2016).

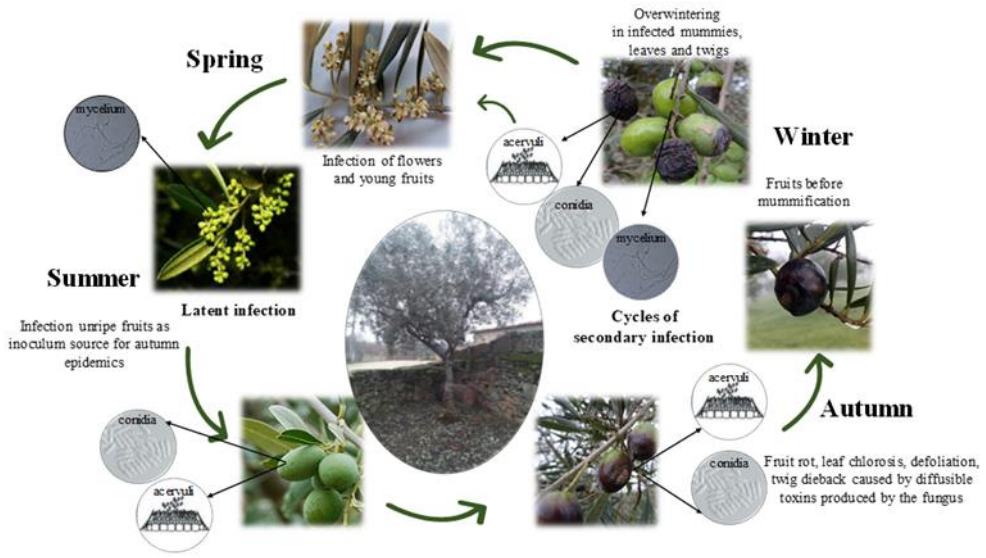

Fig. 1.2 Diagrammatic representation of disease cycle of olive anthracnose in the Mediterranean region (photos: Fátima Martins, IPB-ESA).

The severity of symptoms varies widely with the cultivar (*i.e.*, their susceptibility to anthracnose) and the virulence of the strain (Talhinhas et al., 2015). Recent studies showed that, in several olive-growing countries, the pathogen populations are particularly

adapted to both environmental conditions and the host, but severe infections occur when only virulent populations of the pathogen are present (Moral et al., 2017).

Usually, penetration and colonization of plant tissues by *Colletotrichum* species comprises a sequential set of stages. Generally, it starts with the fixation and germination of the conidia on the host surface, followed by appressorium development, which facilitates entry through the host epidermis (Wharton and Diéguez-Uribeondo, 2004). A detailed study of *C. acutatum* infection on olives showed that after spores' germination, a germ tube is produced and differentiated in an appressorium, which facilitated the penetration of the fungus into the host cells (Gomes et al., 2009). This process occurs within a few hours (48 to 72h), and consequently, the infections can occur rapidly under favorable conditions (Gomes et al., 2009). Fungal penetration is also believed to occur through stomas or lenticels as well as wounds caused by insects (*e.g. Bactrocera oleae*) attack (Cacciola et al., 2012).

After penetration on fruit, *Colletotrichum* sp. can follow different infection strategies. These strategies can be range from intracellular hemibiotrophic mode (colonizes living plant tissue and obtains nutrients from living host cells) to the subcuticular intramural necrotrophic (infects and kills host tissue and extracts nutrients from the dead host cells) mode of nutrition (Gomes et al., 2009), being hemibiotrophic the most common (De Silva et al., 2017). The infection and colonization strategy of *C. acutatum* sp. on olive fruits of both susceptible (cv. *Galega Vulgar*) and tolerant (cv. *Picual*) cultivars, was identified as intracellular hemibiotrophic, followed by a necrotrophic phase (Gomes et al., 2009).

1.5 Management strategies for olive anthracnose

Management of olive anthracnose is very difficult, because its spreading and development relies greatly on the climatic conditions. Thus, no effective control measures have been proposed so far for its management. Generally, those measures rely on an integrated approach that combines several means and tools, either to prevent (indirect method) or to protect (direct method) olive crop againts anthracnose (Cacciola et al., 2012; Moral et al., 2018). Indirect or preventive measures of olive anthracnose relies mostly on practices aiming either reduce the initial levels of inoculum or reduce the rate of spread of the established pathogen. These practices include agronomic techniques such as pruning, drainage and irrigation, fertilization, use of varieties tolerant/resistant to

anthracnose, control of insects that potentially may spread the pathogen, among others. Pruning of olive trees can be an effective way to eliminate sources of fungal inoculum, by removing diseased twigs of infected olive trees. After pruning, the plant material should be removed from the grove and destroyed. Olive pruning also promotes aeration and light penetration in the canopy, helping to reduce the severity of the disease (Sergeeva, 2011a). Irrigation management has a strong impact on the olive anthracnose disease severity and epidemic progress rates, since *Colletotrichum* sp. are greatly dependent not only on high humidity levels for all stages of their life cycle, but also on available free water for conidia dispersion, which is a process of great epidemiological consequence (Cacciola et al., 2012). Thus, over watering should be avoided in the grove where anthracnose is present in order to prevent the outbreak of the disease (Sergeeva, 2011a). Due to the dependence of *Colletotrichum* sp. to water splash for dispersion, the choice of irrigation method could be extremely important to avoid infections of epidemic-like proportions. Adequate nutrition may also have an important role in reducing the severity of olive anthracnose. Previous studies performed in strawberry showed that the source and level of nitrogen in fertilizers had a great effect on severity of anthracnose (Smith, 2009). As far as we know, no studies have been carried out to evaluate the influence of nitrogen fertilization on incidence and development of olive anthracnose. However, a balanced fertilization is frequently recommended for management of olive anthracnose (Sergeeva, 2011b). In general, a balanced fertilizer with fairly low nitrogen content will be ideal, since over-application of nitrogen fertilisers have been reported to increase the incidence of diseases on olive tree canopy (Roca et al., 2018). Use of olive cultivars resistant to the anthracnose pathogens is one of the most successful approaches to the control of this disease (Moral and Tapero, 2009). Numerous studies, carried out in several olive-growing countries, have already identified olive tree varieties with different levels of susceptibility to anthracnose, ranging from highly susceptible (*e.g*. cv. *Galega vulgar*) to highly resistant (*e.g*. cv. *Frantoio*) (*e.g.* Talhinhas et al., 2015; Moral et al., 2017). However, response to anthracnose of olive tree cultivars under field conditions has been showed to be dependent on the *Colletotrichum* species (Talhinhas et al., 2015) and on the climatic conditions, in particular of relative humidity (Moral et al., 2014; Moral et al., 2017). Thus, in certain humid olive-growing areas, anthracnose-resistant cultivars can still get infected (Moral et al., 2014; Moral et al., 2017). Control of olive fruit fly attacks which provides entry points for *Colletotrichum* sp. will limit the surface damage of the fruit and may also be useful to reduce the severity of anthracnose (Malacrinò et al., 2017).

Methods and tools for direct control of olive anthracnose include the use of fungicides and more recently of natural products and biocontrol agents. The fungicides generally recommended for controlling olive anthracnose are protective fungicides based on copper compounds, such as copper oxychloride, copper sulphate and copper hydroxide (Cacciola et al., 2012). Newer chemicals, such as strobilurins, have also been showed to increase copper-based fungicides effectiveness against olive anthracnose in orchards when used in combination (Moral et al., 2018). Similarly, natural products, like botanicals (*i.e.*, plant extracts) and products of mineral origin (*i.e.*, calcium rich compounds) have been recently explored in the control of olive anthracnose (Moral et al., 2018). Calcium rich compounds have been showed to inhibit *Colletotrichum* sp. appressorial formation under in *vitro* tests, but their field application was not always effective in the control of olive anthracnose (Xaviér, 2014). Extract obtained from the peel of pomegranate (*Punica granatum* L.) have proved to be effective against *Colletotrichum* sp. under laboratory conditions and to control olive anthracnose under in field trays (Pangallo et al., 2017). Biological control (BC) is another alternative for olive anthracnose management, although this approach has not been as effective as the chemical control (Holt et al., 2009). The possibilities of using biocontrol agents (BCAs) for controlling the pathogen of olive anthracnose were firstly illustrated by Segura (2003). In artificial inoculations of olives, the microorganisms *Aureobasidium pullulans*, *Curtobacterium flaccumfaciens* and *Paenibacillus polymyxa* were showed to decrease the severity of the symptoms produced by *C. acutatum* in 76.4%, 53.7% and 51.6%, respectively (Segura, 2003). Since then, few studies have been done on the BC of olive anthracnose and this strategy has not been used against this disease in field conditions.

Although the several efforts to better understand the epidemiology and populations genetics of the different pathogenic species, the olive anthracnose still remains a "complex disease" to decipher. Indeed, it remains unclear how the pathogen interact with the host plant, which is the variability of *Colletotrichum* species in some olive-growing regions, and which are the best control strategies against this disease. In this regard, the use of fungal endophytes to control olive anthracnose could be a promising approach (Landum et al., 2016; Preto et al., 2017). These microorganisms are able to inhabit the same niche in the same environment that of *Colletotrichum* spp., favouring them as potential biocontrol agents against olive anthracnose.

1.6 Fungal endophytes and their potential as biocontrol agents against *Colletotrichum* spp.

Fungal endophytes are microorganisms that inhabit the inner tissue of the plant, at some part or whole of its life cycle, without causing any apparent damage to the hosts (Busby et al., 2016). According to the mechanisms used to colonize the host plant, the fungal endophytes were classified as "obligate" or "facultative" (Andreote and Durrer, 2014). Obligate endophytes are transmitted to other plants by vertical colonization or by vectors and are strictly dependent on host cell metabolism for their survival and replication (Andreote and Durrer, 2014). Facultative endophytes have a free life, living outside of host plant, and during a certain stage of their life cycle they colonize the plant internally (Andreote and Durrer, 2014).

Overall, most endophytic fungi within plant tissues belong to Ascomycota and Basidiomycota phyla (Arnold and Lutzoni, 2007; Selosse et al., 2009). In particular, the composition of fungal endophytic community of olive tree have been only recently analyzed (Martins et al., 2016; Landum et al., 2016; Preto et al., 2017; Gomes et al., 2018). Overall, these studies showed that there is great diversity and abundance of fungal endophytes in several organs of olive tree, including leaves, twigs, fruits and roots. More than 65 genera from 33 families and 2 phyla of fungal species have been reported to be associated with olive tree (Fig. 1.3). Most of the fungal isolates belong to the phyla Ascomycota, accounting 93% of the total number of fungal isolates, followed by Basidiomycota (3.4% of the total fungal isolates) (Martins et al., 2016; Landum et al., 2016; Preto et al., 2017; Gomes et al., 2018). The most abundant fungal families are Pleosporaceae (17.1% of the total fungal isolates), Incertae sedis (13.7%) and Nectriaceae (8.5%). *Alternaria, Penicillium, Epicoccum* and *Phomopsis* were identified as the most abundant genera, accounting together 25.5% of total fungal isolates. The various olive tree organs surveyed displayed differences on endophytic fungal composition. Members of Pleosporaceaea and Incertae sedis were the most abundant in leaves and twigs of olive tree, accounting together 90.3% of the total isolates, whereas Trichocomaceae and Nectriaceae were the most abundant in roots and fruits, respectively (Fig. 1.3) (Martins et al., 2016; Landum et al., 2016; Preto et al., 2017; Gomes et al., 2018). Besides plant organ, host plant geographic location, host genetics (at cultivar level), season and climatic conditions, such as rainfall and temperature, were also showed to contribute to the shaping of fungal communities in olive tree (Martins et al., 2016; Preto et al., 2017; Gomes et al.,

2018). In general, the diversity of fungal endophytes in olive tree leaves and twigs is higher in spring than in autumn (Gomes et al., 2018). The same study also identified differences on fungal composition between spring and autumn. These seasonal shifts were found to be related to climatic factors, especially to rainfall and mean temperature (Gomes et al., 2018).

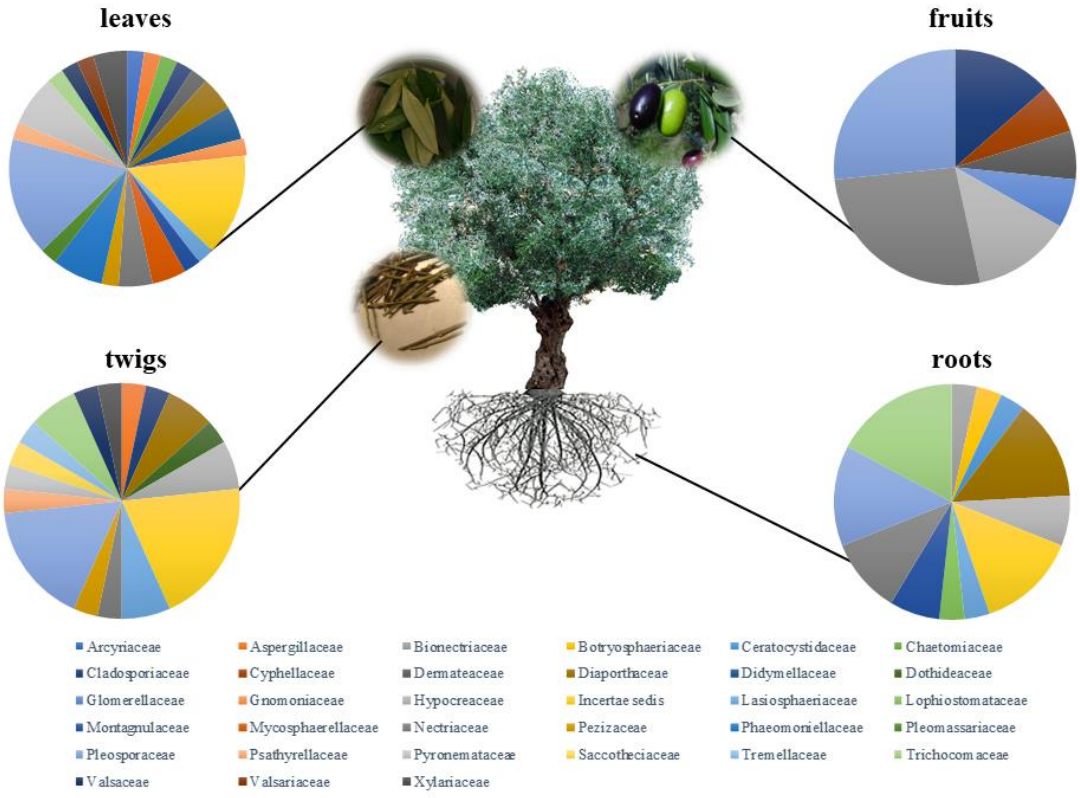

Fig. 1.3 Abundance (number of isolates) of fungal endophytes, at family level, present in leaves, twigs, fruits and roots of olive tree (*Olea europaea* L.).

Geographic distance was also found to affect the structure of fungal endophytic communities especially of roots, but also of leaves and twigs (Martins et al., 2016). An inverse relationship was noticed between the similarity of endophytic assemblages and their geographyc distance (Martins et al., 2016).

There is growing evidence that these endophytic fungi fulfill important functions for plant health and productivity (Khare et al., 2018). Endophytes can, for instances, promote plant nutrition and protection against abiotic (*e.g.* drought and extreme temperatures) and biotic stresses, such as plant pathogens (Bacon and White Jr., 2015).

In particular, the mechanisms used by endophytic fungi to protect host plant against pathogens are mostly rely on the production of secondary metabolites, such as alkaloids, peptides, steroids, terpenoids, phenols, quinines, flavonoids, siderophores and volatile organic compounds (Gao et al., 2010; Ownley et al., 2010; Speckbacher and Zeilinger, 2018). Most of these classes of compounds comprise phytohormones, mycotoxins, antimicrobial molecules as well as antibiotics that may reduce pathogen infection directly, through antibiosis, mycoparasitism and competition, and indirectly by induction of plant resistance response (Lacava and Azevedo, 2014). Endophytic fungi are also known to produce cell wall degrading enzymes (*e.g*., chitinases, proteases and glucanases) with ability to destroy pathogens' cell wall (Lorito et al., 2010; Katoch et al., 2014). The above mechanisms regularly operated simultaneously.

Till date, few studies were conducted to explore the biocontrol activities of endophytes against anthracnose disease caused by *Colletotrichum* species under *in vivo* conditions (*i.e*. in detached fruits, field and/or greenhouse) (Table 1.1). The results obtained up to date appear to be very promising being the level of disease suppression achieved by application of fungal endophytes ranging from 2.5% to 83%, depending on the fungal species (Table 1.1). According to the results showed in Table 1.1, the most promising fungal endophytes to control anthracnose diseases are *Trichoderma* spp., *Nodulisporium* sp. and *Cordana* sp., and also some yeasts belonging to the genera *Debaryomyces* and *Cryptococcus*. These strains showed to be effective in reduced *Colletotrichum* growth and disease severity in several hosts like papaya (Valenzuela et al., 2015; Hernandez-Montiel et al., 2018), mango (Bautista-Rosales et al., 2014) and wild banana (Nuangmek et al., 2008). Competition for nutrients and space, antibiosis, mycoparasitism, and production of cell wall degrading enzymes, antibiotics, and volatile organic compounds were the most important modes of action of fungal endophytes for anthracnose disease control (Table 1.1).

Table 1.1 Fungal endophytes that have been tested under *in vivo* to control anthracnose disease caused by *Colletotrichum* spp., their possible mechanisms of action and their efficacy.

Antagonistic fungal isolates	Host plant	Assays	Disease agent	Mechanism of action	Efficacy	Reference
Aureobasidium pullulans	Olive (*Olea europaea* L.)	Field assay	*Colletotrichum spp*	NA	Reduced both latent infection (14%) and disease severity (40%)	Nigro et al. 2018
Pichia kudriavzevii *Wickerhamomyces anomalus*	Olive (*Olea europaea* L.)	*In vivo* (ripe olive fruit)	*C. gloeosporioides*	Competition Antibiotic production Invasive growth	Reduced disease severity (6.99-22.05%)	Pesce et al. 2018
Debaryomyces hansenii	Papaya (*Carica papaya* L.) var. Maradol	*In vivo* (fruit)	*C. gloeosporioides*	Volatile organic compounds	Reduced pathogen growth (36%) and disease severity (83%)	Hernandez-Montiel et al. 2018
Trichoderma spp.	Papaya (*Carica papaya* L.) var. Maradol	*In vivo* (fruit)	*C. gloeosporioides*	Invasive growth Mycoparasitism	Reduced pathogen growth (50-60%), and disease severity (77.40%)	Valenzuela et al. 2015
Cryptococcus laurentii	Mango *(Mangifera indica* L.)	*In vivo* (Mango fruit)	*C. gloeosporioides*	Antibiosis Nutrient competition Hydrolytic enzymes	Reduced disease severity (75.88%)	Bautista-Rosales et al. 2014
Trichoderma viride	Bean (*Phaseolus vulgaris* L.)	*In vivo* (seeds)	*C. lindemuthianum*	Mycoparasitism Antibiosis	Reduced the growth (59.48%) and the germination (73.60%) of the pathogen, as well as disease severity (32.02%)	Padder and Sharma 2011
Cordana abramovii. *Nodulisporium* sp.	Wild Banana (*Musa acuminata* Colla)	*In vivo* (detached banana)	*C. musae*	Competition Antibiotic production	Reduced the growth (90%) and the germination (91%) of the pathogen, as well as disease severity (53%)	Nuangmek et al. 2008
Trichoderma viride	Cowpea (*Vigna unguiculata* L.)	*In vivo* (seedling)	*C. lindemuthium*	Mycoparasitism Antibiosis	Reduced disease severity (2.5%)	Adebanjo and Bankole 2004

NA: not applicable.

1.7 Fungal endophytes on the control of olive anthracnose

Despite the ability of fungal endophytes to control anthracnose disease, there are only limited studies on the use of these fungi against olive anthracnose. In addition, most of these studies were performed under controlled conditions, by using *in vitro* experiments, being field assays much more limited. Among the various endophytic fungal species tested in *in vitro* laboratory assays, *Alternaria* sp., *Diaporthe* sp. and *Nigrospora oryzae* isolated from olive tree leaves, have been showed to inhibited up to 26.8% the growth of *C. acutatum* (Landum et al., 2016). This inhibitory effect was ascribed to the production of volatile compounds by the endophyte, in particular of phenylethyl alcohol, 4-methylquinazoline, benzothiazole, benzyl alcohol, lilial and galaxolide (Landum et al., 2016). Similarly, the endophytic fungal species *Chondrostereum purpureum*, *Chaetomium globosum*, *Aspergillus westerdijkiae*, *Aspergillus* sp. 1, *Quambalaria cyanescens*, *Epicoccum nigrum* and *Aspergillus brasiliensis*, isolated from olive fruits, have been showed to inhibited *C. acutatum* growth under *in vitro* conditions, reaching inhibition values of 30.9 to 71.3% (Preto et al., 2017). Some of these endophytic fungal strains also showed to induce morphological alterations on pathogen hyphae and to reduce both the production (up to 46%) and germination (up to 21%) of *C. acutatum* spores (Preto et al., 2017). Although the exact mechanism of antagonism displayed by these fungi is not clear, it is hypothesized the involvement of antimicrobial compounds and lytic enzymes, secreted by endophytic isolates, which may act synergistically against the fungal pathogen (Preto et al., 2017). The degree to which fungal endophyte regulates *C. acutatum* infection, is dependent on both host plant and the order of arrival of the pathogen and endophyte (Martins et al., 2013). *In vitro* confrontation assays between the endophyte *Penicillium commune* and *C. acutatum* in the presence of olive leaf (+leaf) revealed a greater inhibitory effect of the endophyte over the pathogen when compared to –leaf treatment (Martins et al., 2013). This result suggests that the plant-endophyte interaction is critical for the biocontrol of the pathogen. The observed inhibitory effect on *C. acutatum* sporulation and germination was strong (around 50 and 60%, respectively) when the endophyte colonized the leaf before the pathogen (Martins et al., 2013).

In olive fruit inoculation assays, the endophytic fungi *Trichoderma koningii* have been showed to reduced significantly ($p<0.05$) both incidence (AUDPCi) and severity (AUDPCs) of olive anthracnose when compared to control (*i.e.*, in the absence of *T. koningii*), either at 14 and/or 21 days post inoculation (Fig. 1.4). The effectiveness

of this endophyte as biological control agent against olive anthracnose was most notorious on fruits that start to change skin color (maturation index 2) than on purple or black olives (maturation index). The endophyte *T. koningii* also showed the capacity to inhibited significantly the production and germination of spores produced by the pathogen *C. godetiae* in olives, either at maturation index 2 (up to 1.6- and 6.1-fold, respectively) or 3 (up to 2.1- and 5.7-fold, respectively) when compared to control (Martins et al., 2017).

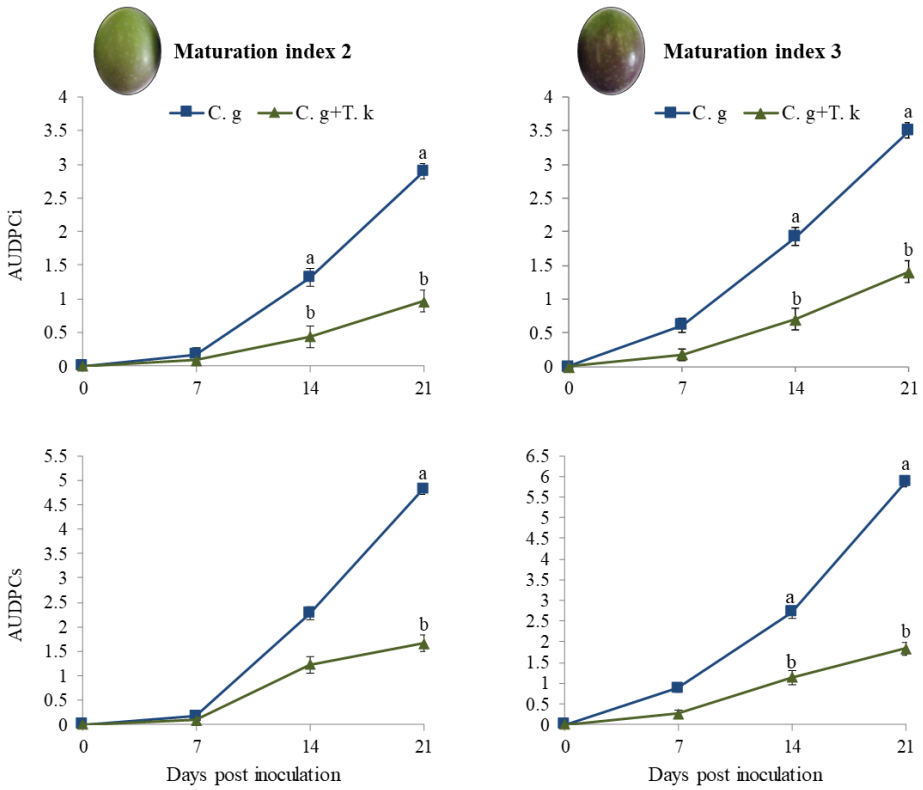

Fig. 1.4 Area under the disease progress curve of incidence (AUDPCi) and severity (AUDPCs) in olive fruits from cv. *Madural*, at maturation index 2 and 3, after 7, 14 and 21 days of inoculation only with *C. godetiae* (C.g) or in combination with the endophyte *T. koningii* (C.g + T.k). In each day, mean values followed by different letters are significantly different ($p < 0.05$).

Few studies have determined the efficacy of fungal endophytes against olive anthracnose under field conditions. Only recently, was reported that the treatment of olive tree with the endophyte *Aureobasidium pullulans* in field trays significantly reduced anthracnose severity by 40% and latent infection by 14% (Nigro et al., 2018).

1.8 Conclusions

The use of endophytic fungi for the biological control of olive anthracnose could be a sustainable alternative to olive crop production (Lugtenberg et al., 2016). Despite no effective biocontrol agents are still available against olive anthracnose, some authors have already described promising results in this area. However, most of these studies have detected the biocontrol activity of the fungal endophyte by using *in vitro* and *in vivo* tests on detached fruits, under controlled conditions. They therefore do not replicate the environment in which the biocontrol agent must function. More studies aiming the selection of fungal endophytes as biological control agents against olive anthracnose by using *in planta* assays, either in the field or greenhouses conditions, are required. Similarly, we still have incomplete knowledge on the various relationships that fungal endophytes can establish with their host and with other members of plant-associated microbial community, under natural conditions. Such studies will certainly contribute to enhance the chances to obtain competent endophytic biocontrol agent, and therefore develop new successful and sustainable integrated crop protection against olive anthracnose.

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Chapter 2

Endophytic fungal community succession in reproductive organs of two olive tree cultivars

This chapter was submitted as an original article to *Fungal Ecology*: Fátima Martins, Diogo Mina, José Alberto Pereira, Paula Baptista. Endophytic fungal community succession in reproductive organs of two olive tree cultivars.

2.1 Abstract

Studies on endophytic fungi were undertaken mostly on vegetative organs of plants. In contrast, endophytes of reproductive organs and the factors shaping their assemblages have been less evaluated. Here, the diversity and dynamic of fungal endophytes in inflorescences and fruits of two olive tree cultivars with contrasting susceptibilities to olive anthracnose, were assessed over different phenological stages, starting before flowers open and ending at fruit ripening. Assessment of fungal diversity by rDNA sequencing of cultivable isolates revealed that inflorescences harbored a higher richness and abundance of endophytes than fruits. Endophytes from Sordariomycetes were the most dominant in inflorescences while in fruits were from Dothideomycetes. Both plant organ and phenological stage, were key determinant of community assemblage of endophytes in reproductive organs. Plant cultivar only affects endophytic assemblages on inflorescences. Overall, both fungal abundance and richness increase over inflorescence development reaching a peak on the flowering, and afterwards dropped until fruit starts the maturity process and then increase reaching a peak when the fruit becomes ripe. This temporal pattern was attributed to a set of fungal taxa that were found to be positively associated to a specific phenological stage, over the inflorescence development and fruit maturation, for each cultivar. These changes in fungal community structure in olive inflorescences and fruits could provide a basis for anthracnose disease management, a topic that requires further research.

2.2 Introduction

Plants are associated to a great diversity of fungi that live within their tissues as endophytes (Sridhar, 2019). These fungi play an important role on host plant fitness, by protecting them from pathogenic microorganisms and insect pests and by improving plant growth (Jia et al., 2016). Because of their recognized importance, studies on fungal endophytes and of their interaction with the host plant have recently produced a growing body of literature (Khare et al., 2018). However, we are yet to know what forces determine specific fungal community assemblages (Compant et al., 2016), in particular in woody perennials. Endophytic fungal surveys conducted in woody perennials over the last years showed that plant organ is a significant factor in shaping endophytic fungal assemblage (Moricca et al., 2012; Abdelfattah et al., 2015; Martins et al., 2016; Singh et

al., 2017; Gomes et al., 2018; Singh et al., 2018; Ren et al., 2019). In general, the main plant tissues that have been analysed in these studies were vegetative organs, such as leaves, twigs, barks, stems and roots (Moricca et al., 2012; Abdelfattah et al., 2015; Martins et al., 2016; Singh et al., 2017; Gomes et al., 2018; Singh et al., 2018; Ren et al., 2019), and only a few studies have looked for fungal endophytes in reproductive organs (*e.g.*, flowers or fruits) (Abdelfattah et al., 2015; Singh et al., 2018; Ren et al., 2019). Furthermore, little research has been performed on the monitoring of endophytic fungal community of reproductive organs over their development, ranging from flowering to fruit ripening (Abdelfattah et al., 2015).

Plant genetics (at species or genotype level) also control endophytic fungal communities' composition of woody perennials (Moricca et al., 2012; Lamit et al., 2014; Bálint et al., 2015; Yao et al., 2019; Küngas et al., 2019). As far as we known, host plant effect on fungal assemblage have been recognized only on endophytes colonizing leaves (Moricca et al., 2012; Yao et al., 2019), roots (Andrade-Linares and Franken, 2013) and twigs (Moricca et al., 2012). By contrast, the role of host plant in shaping endophyte fungal communities in reproductive organs haven´t been revealed yet in detail. A better understanding of the role played by the host in controlling endophytic fungal communities associated with its reproductive organs, could help to identify whether the differences on host susceptibility to fruit diseases is somehow linked with the host plant capacity to recruit specific endophytic fungal species. Indeed, previous studies have shown the ability of fungal endophytes isolated from flowers (Katoch and Pull, 2017) and fruits (Mejía et al., 2008; Bautista-Rosales et al., 2014; Preto et al., 2017) to inhibit the growth of several plant pathogens, emphasizing the importance of endophytes as biocontrol agents against fruit diseases. The ability of plants to recruit protective microorganism to suppress pathogens was already reported in the rhizosphere (Berendsen et al., 2012). To the best of our knowledge, such plant effect on endophytic fungal community inhabiting the plant above ground parts is largely unknown.

Olive tree (*Olea europaea* L.) is one of the most economically important oil-producing crops in many countries of the world, particularly in the Mediterranean basin countries (Uylaşer and Yildiz, 2014). In most of these countries, the anthracnose (a disease caused by *Colletotrichum* spp.) is a key constraint to olive production through its effects on olives (Cacciola et al., 2012; Mosca et al., 2014). The infection process is believed to start in flowers, remaining quiescent until the ripening of the fruits (Sergeeva et al., 2008), where symptoms have been most noted (Cacciola et al., 2012). So far, no

anthracnose-resistant olive tree genotypes have been found, but there are cultivars that are more prone to infection than others (Moral et al., 2014; Moral et al., 2017). For example, among the most important Portuguese commercial olive cultivars, *Cobrançosa* is less susceptible to anthracnose than the cv. *Madural* (Torres, 2007; Gomes et al., 2009). Here, the compositional differences of the endophytic fungal community of different plant reproductive organs (inflorescence and fruit) of two olive cultivars with different susceptibilities to olive anthracnose (*Cobrançosa* and *Madural*) were investigated, over inflorescence and fruit development. Specifically, this study aims to decipher: i) the relative contribution of plant organ (inflorescence and fruit) and host genotype (at cultivar level) on endophytic fungal assembly; ii) patterns of plant-endophyte association over different time points of inflorescence and fruit development; iii) if the differences in susceptibility of the cultivars to anthracnose may be related to the endophytic community naturally present in inflorescence and fruit. In this study was used a culture dependent PCR-based identification approach to characterize the endophytic fungal communities, with the aim to further exploit and used these strains to improve resistance/tolerance of olive tree to anthracnose disease.

2.3 Materials and methods

2.3.1 Samples collection

The plant sampling was conduct from May to November 2017, in one olive grove located in Mirandela (northeast of Portugal) at coordinates 41° 33 08″N, 7° 07′ 24″W. This grove is mainly composed of olive trees of cultivars *Cobrançosa* (moderately susceptible to anthracnose) and *Madural* (susceptible to anthracnose), spaced 7 m from each other, and with approximately 60 years old. This grove has been managed through integrated production guidelines (Malavolta and Perdikis, 2018). From each cultivar, seven olive trees were randomly selected and used to collected inflorescence and fruit samples over different phenological stages (Sanz-Cortés et al., 2002). The collection of inflorescences was performed during inflorescence development (*i.e.*, on flower cluster development - D, and on flower cluster totally expanded - E) and flowering (*i.e.*, flowers open - F, and petals fallen or faded - G), whereas fruit collection was conducted on fruit development (*i.e.*, stone hardening - H) and ripening (*i.e.*, beginning of maturation - I, and maturation of fruit-J) (Fig. 2.1). The maturity stage of the collected fruits was estimated by a maturity index (MI) described by Hermoso et al. (2001), which were

ranged from 2 to 4. At each time point of sampling, were collected 25 asymptomatic plant samples *per* tree, around the perimeter of the tree at the operator height and placed directly into sterile bags. The samples collected were transported to the laboratory and stored at 4 °C until isolation of endophytic fungi, which was carried out within one week.

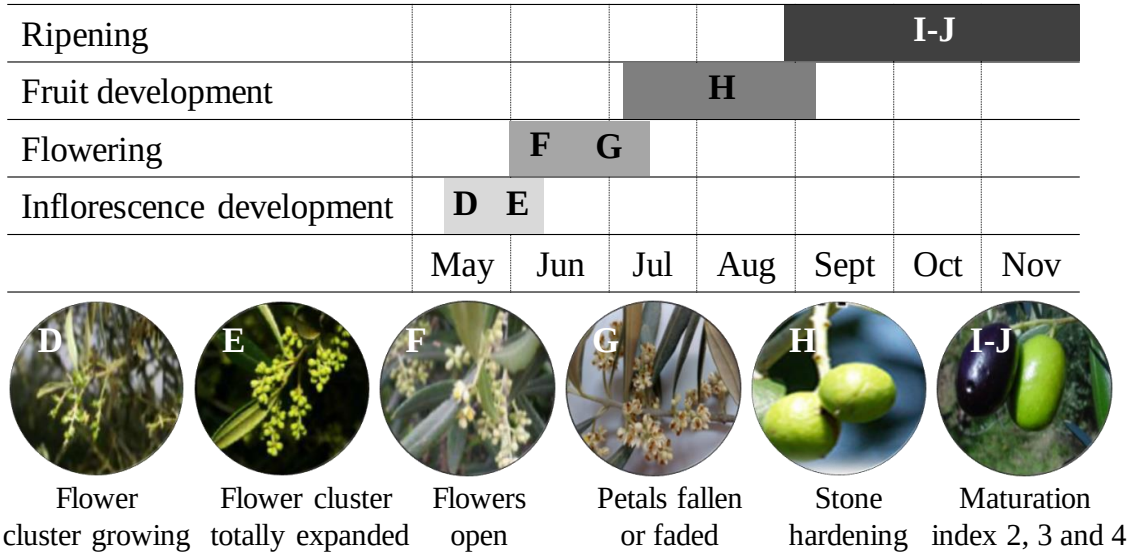

Fig. 2.1 Phenological growth stages of the olive tree cvs. *Cobrançosa* and *Madural* in which was performed the sampling of flowers and fruits, from May to November 2017. Phenological stages (from D to J) were defined by Sanz-Cortés et al. (2002) while maturation index of fruits was defined by Hermoso et al. (2001) as MI 2 (epidermis shows red spots in less than half fruit), 3 (epidermis is red or purple in more than half fruit) and 4 (black epidermis and white pulp).

2.3.2 Isolation of endophytic fungi

The plant tissues collected were rinsed with distilled water, and then surface sterilized through sequential immersion in 3% (v/v) sodium hypochlorite for 1 min (for inflorescences) or 2 min (for fruits), 70% (v/v) ethanol for 1 min and rinsed three times with sterile distilled water (1 min each). The efficiency of the surface sterilization had previously been optimized for the different olive plant tissues. The water from the final wash was collected and plated onto potato dextrose agar (Difco, PDA) as a positive control to confirm that the surface sterilization had been successful. After surface sterilization, each sample was cut into six pieces (*ca*. 2-4 mm in length) and plated onto PDA medium supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). A total of 8400 segments of inflorescences (7 olive trees × 2 cultivars

x 25 plant tissues × 4 phenological stages x 6 tissue segments) were inoculated. For fruits, a total of 6300 segments were assayed (7 olive trees × 2 cultivars x 25 fruit tissues × 3 MI × 6 tissue segments). All plates were incubated at 25 ± 2 °C in the dark and were daily observed. The fungi growing out of the tissue segments were recorded as endophytes and were sub-cultured on individual PDA plates to obtain pure isolates for subsequent identification. Pure cultures of each isolate were deposited in the culture collection of the Mountain Research Centre (CIMO), School of Agriculture - Polytechnic Institute of Bragança.

2.3.3 Identification of fungal isolates

The isolates obtained were grouped according to their morphological characteristics (colony morphology, hyphae, spores and reproductive structures). For each morphotype was selected two representative isolates for molecular identification, by using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Fungal DNA was extracted using REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) following manufacturer's instructions. The PCR reactions, with the pair of primers *ITS1* and *ITS4* (White et al., 1990), were performed using the same DNA extraction kit following the manufacturer's instructions, in the MyCycler thermal cycler (BioRad). The temperature cycle used in the amplification was 94° C for 3 min (1 cycle); 94° C for 30 sec, 53° C for 50 sec, 72° C for 2 min (35 cycles); and 72° C for 10 min (1 cycle). The amplified products (~650 bp) were purified and sequenced using Macrogen Inc. services (Madrid, Spain). The DNA sequences were analyzed with DNASTAR v.2.58 software, and fungal identification was performed using both NCBI (http://www.ncbi.nlm.nih.gov) and UNITE (https:// unite.ut.ee/) databases and BLAST algorithm. The results were sorted according to the higher identity score and the lowest *E-value*. For sequence identities > 98%, the genus and species were accepted; for sequence identities between 95% and 97%, only the genus was accepted; and for sequence identities < 95%, isolates were labeled as 'unknown' fungi. The operational taxonomic units (OTUs) identified were classified according to the Index Fungorum Database (www.indexfungorum.org).

2.3.4 Abundance and diversity of fungal endophytes

Abundance and diversity of fungal endophytes detected on inflorescence and fruit over different phenological stages were measured through frequency of colonization (FC, %), relative abundance (RA, %), species richness, abundance and diversity index. FC (%) was estimated as the total number of plant tissue segments colonized by each endophytic OTU divided by the total number of plant segments surveyed. RA (%) of a fungal OTU was determined by the total number of isolates of an OTU divided by the total number of isolates of all OTUs. Species richness (average number of OTUs *per* tree) and abundance (average number of isolates *per* tree) were estimated considering each individual tree as a sample unit, for statistical analysis purposes. The diversity was estimated by computing Shannon–Wiener (H) index in *Species Diversity and Richness* v. 4.0 software (Seaby and Henderson, 2007), and the results were presented as the mean of individual host tree or as the total number (the values of all samples lumped together). Differences among the means were determined by an analysis of variance (ANOVA) with SPSS v.18 software, and the averages were compared using Tukey's test ($p<0.05$).

2.3.5 Data analysis

Generalized linear mixed models (GLMMs) were used to test effects of host cultivar (*Cobrançosa* and *Madural*), plant organ (inflorescence and fruit), phenological stage, and their interaction on fungal abundance and richness. These models were developed in R (R Core Team, 2018) following Zuur et al. (2009). Since the number of levels of samples collected was different for inflorescences and fruits, its effect was modelled independently of the organ. Both the abundance and richness of fungi were counts; thus the Poisson distribution was used for each model as:

$$\text{Abundance}_{is} \sim \text{Poisson}(\mu_{is}) \Rightarrow \text{E}(\text{Abundance}_{is}) \sim \mu_{is}$$

$$\eta_{is} = \alpha + \beta_1 \times \text{Cultivar}_{is} + \beta_2 \times \text{Organ}_{is} + \beta_3 \times \text{Cultivar}_{is} : \text{Organ}_{is} + a_i \quad \text{[Eq.1]}$$

$$\eta_{is} = \alpha + \beta_1 \times \text{Cultivar}_{is} + \beta_2 \times \text{Index}_{is} + \beta_3 \times \text{Cultivar}_{is} : \text{Index}_{is} + a_i \quad \text{[Eq.2]}$$

$a_i \sim N(0, \sigma^2_a)$; $\log(\mu_{is}) = \eta_{is}$ and

$$\text{Richness}_{is} \sim \text{Poisson}(\mu_{is}) \Rightarrow \text{E}(\text{Richness}_{is}) \sim \mu_{is}$$

$$\eta_{is} = \alpha + \beta_1 \times \text{Cultivar}_{is} + \beta_2 \times \text{Organ}_{is} + \beta_3 \times \text{Cultivar}_{is} : \text{Organ}_{is} + a_i \quad \text{[Eq.3]}$$

$$\eta_{is} = \alpha + \beta_1 \times \text{Cultivar}_{is} + \beta_2 \times \text{Index}_{is} + \beta_3 \times \text{Cultivar}_{is} : \text{Index}_{is} + a_i \quad \text{[Eq.4]}$$

$a_i \sim N(0, \sigma^2_a)$; $\log(\mu_{is}) = \eta_{is}$

where $Cultivar_{is}$ represents the effect of the olive tree cultivar, $Organ_{is}$ represents the effect of the two olive organs considered, $Cultivar_{is}$: $Organ_{is}$ and $Cultivar_{is}$: $Index_{is}$ represent the interaction term between the two corresponding drivers, respectively, and a_i represents the random effect term for the olive tree.

Non-metric multidimensional scaling (NMDS) was carried out to explore the similarity of fungal community composition among olive tree cultivars (*Cobrançosa* and *Madural*), plant organ (inflorescence and fruit) and phenological stages. NMDS was performed by using Bray-Curtis dissimilarity matrix (Bray and Curtis, 1957), generated with OTU abundance data with the values square root transformed to limit the influence of abundant fungal OTUs. One-way analysis of similarity (ANOSIM) was then done to test for significant differences in dissimilarities of the fungal community groupings obtained in NMDS ordination, using the same Bray–Curtis distance matrices. These analyses were performed using the *Community Analysis Package* v. 5.0 (Seaby and Henderson, 2014).

To estimate the percentage of variation of endophytic fungal community explained by host cultivar, pant organ and phenological stage, was performed a variation partitioning analysis with the function *varpart* (based on redundancy analysis, RDA) in the *vegan* R package (Oksanen et al., 2018).

Co-inertia analysis (CIA) was used to explore the relationships between host cultivar or phenological stage variables and endophytic fungal assemblage in inflorescences and fruits of olive tree. This analysis also helped to identify fungal OTUs that correlate with particular host cultivar or phenological stage. The CIA was performed in the R statistical environment (R Core Team 2018), by using *coinertia* function (to do the analysis) and *table.value* function (to visualize the results in a factorial map), both available in the *ade4* package (Dray and Dufour, 2007). The statistical significance of co-inertia was evaluated with a Monte Carlo permutation test (10,000 simulations), by using *randtest.coinertia* function available in the same R package.

To identify fungal OTUs that are characteristic of each host cultivar and phenological stage, was performed an indicator value (*IndVal*) analysis (Dufrêne and Legendre, 1997), using the function *multipatt* from *indicspecies* R package (Cáceres, 2012). This method identifies indicator species based on their specificity (*i.e.*, uniqueness) to a particular habitat (A) and their frequency in that habitat (B). The *IndVal* ranges from 0 to 1 with higher values representing higher indicative power, and

significant ($p<0.05$) values greater than 0.5 indicate species that are deemed characteristic of a group (Cáceres et al., 2012).

2.4 Results

Across all the plant tissues collected in both cultivars (*Cobrançosa* and *Madural*) was obtained a total of 466 endophytic isolates belonging to 106 fungal OTUs, of which 86 OTUs were identified at the inflorescence level and 42 OTUs at the fruit´s level (Table S2.1). The 106 OUTs were assigned to 87 species, 65 genera and 33 families, all from the Ascomycota and Basidiomycota phyla (Fig. S2.1). Ascomycota was mostly composed by members belonging to families Xilariaceae (28%) and Pleosporaceae (15%), and genera *Biscogniauxia* (24%) and *Alternaria* (10%). Among Basidiomycota, both Polyporaceae (83%) and Stereaceae (17%) families, and the genera *Trametes* (75%), *Stereum* (17%) and *Coriolopsis* (8%) were the most abundant. Overall, the colonization rate of endophytic fungi was higher on inflorescence (4.2%) than on fruits (1.8%) (Table S2.1).

2.4.1 Diversity and composition of endophytes differ between cultivars

The olive cultivars studied markedly differed in their percentage of fungal colonization, abundance (average number of isolates *per* tree), richness (average number of taxa *per* tree) and diversity (H) (Table S2.1). Within endophytic communities inhabiting inflorescence, altogether the results indicated that cv. *Madural* presented significantly higher ($p<0.05$) fungal colonization rate (up to 3.2-fold), abundance (up to 3.1-fold), richness (up to 3.0-fold) and species diversity (up to 2.3-fold) than cv. *Cobrançosa*. The same pattern was observed within the endophytic community colonizing fruits. Overall, fruits of cv. *Madural* showed significantly ($p<0.05$) higher colonization rate (up to 1.1-fold), abundance (up to 1.3-fold), richness (up to 1.5-fold) and diversity (up to 1.8-fold) of fungal endophytes than cv. *Cobrançosa*. These results were corroborated by generalized linear mixed model (GLMM), which showed a significant ($p<0.01$) effect of host cultivar on the abundance and richness of fungal endophytes in inflorescence and fruit (Table S2.2).

NMDS plots, based on Bray-Curtis index, and ANOSIM analysis showed that endophytic community composition differ among olive tree cultivars (global $R=0.59$, $p=0.001$), being this dissimilarity greater for inflorescence ($R=0.41$, $p=0.001$) than for

fruit (R=0.18, p=0.001) (Fig. 2.2). In fact, only 28 fungal OTUs out of the 106 recovered in this study were isolated in both cultivars; 25 fungal OTUs were isolated only in cv. *Cobrançosa* and 53 were recovered only in cv. *Madural* (Fig. S2.2). A higher percentage of OTUs specific to one cultivar was observed in inflorescence (around 84%) when compared to fruits (around 71%).

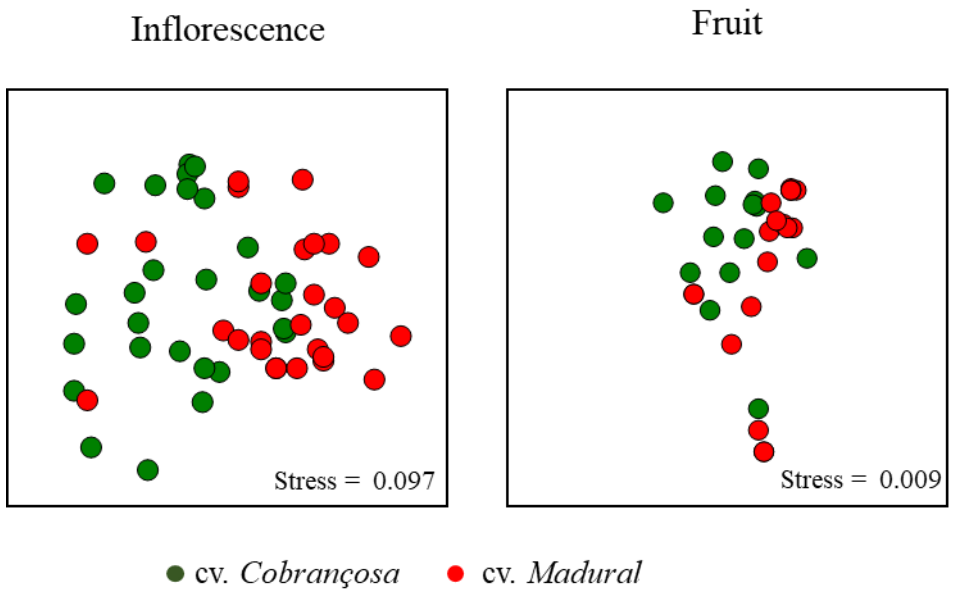

Fig. 2.2 Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of endophytic fungal communities grouped by olive tree cultivars (*Cobrançosa* and *Madural*) in each plant organ (inflorescence and fruit). Endophytes from inflorescences were recovered over different host phenological stages (D, E, F and G), while endophytes from fruits were recovered over fruit ripening (MI 2,3 and 4). For more details see Fig. 2.1. Cluster analysis was performed with Bray-Curtis coefficient. Kruskal's stress values less than 0.2 represent good ordination plots.

2.4.2 Diversity and composition of endophytes in reproductive organs over different phenological stages

A GLMM was used to explore the relationship between the explanatory (organ and phenological stage) and response (fungal abundance and richness) variables (Fig. 2.3; Table S2.2). In this analysis was not included the phenological stage correspondent to the fruit development (H), since no fungal growth was obtained in the assayed fruit explants. The reason of fungal fail to grow could be attributed to the high concentration of antimicrobial compounds, such as phenolic compounds and other secondary metabolites, in fruits at this stage (Pereira et al., 2006; Machado et al., 2015). GLMM results indicated

that the plant organ (inflorescence and fruit) affected significantly ($p<0.01$) the total endophytic fungal abundance and richness (Table S2.2). Overall, the abundance and richness of fungal endophytes were greater on inflorescence than on fruit, for both olive tree cultivars, and in particular for cv. *Madural* (Fig. 2.3). Host phenology has similarly affected significantly ($p<0.01$) both fungal abundance and richness in inflorescence and fruit. In cv. *Cobrançosa*, both fungal abundance and richness increase over inflorescence development reaching a significant ($p<0.001$) peak on the flowering, specifically in the stage when flowers are open - F (an increase up to 3.2- and 2.3-fold, respectively). Afterwards, the fungal abundance and richness dropped until fruit starts the maturity process, and then increase over the fruit maturation reaching a significant ($p<0.001$) peak when fruit epidermis is red or purple in more than half fruit - MI 3 (an increase up to 5.0 and 4.0-fold, respectively). The variation of both fungal abundance and richness on reproductive organs of cv. *Madural* over the different phenological stages followed a similar trend. The only difference found between these two cultivars was at the level of the fruits. In contrast to cv. *Cobrançosa*, both fungal abundance and richness on fruits of cv. *Madural* increase significantly ($p<0.001$) over fruit maturation (up to 7.2 and 6.5-fold, respectively).

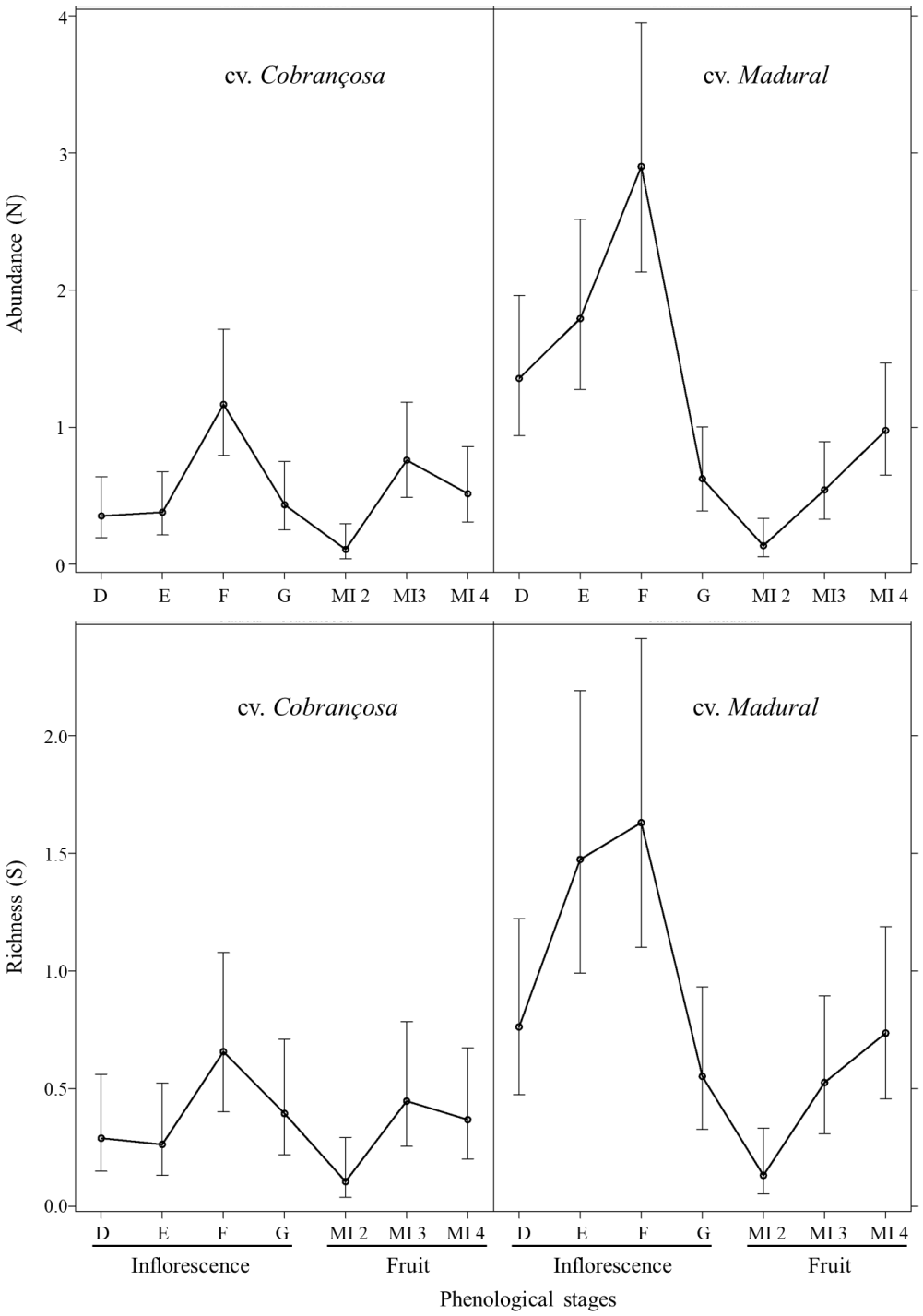

Fig. 2.3 Generalized linear mixed models in order to evaluate the effect of the plant organ (inflorescence and fruit) and phenological stage on both the abundance and species richness of the endophytic fungal community of olive tree cultivar *Cobrançosa* and *Madural*. Host phenological stage includes inflorescence development (D, E), flowering (F, G) and fruit ripening (MI 2, 3, 4). For more details see Fig. 2.1.

The NMDS ordination, based on Bray-Curtis index, revealed that each organ and phenological stage formed individual cluster (Fig. 2.4).

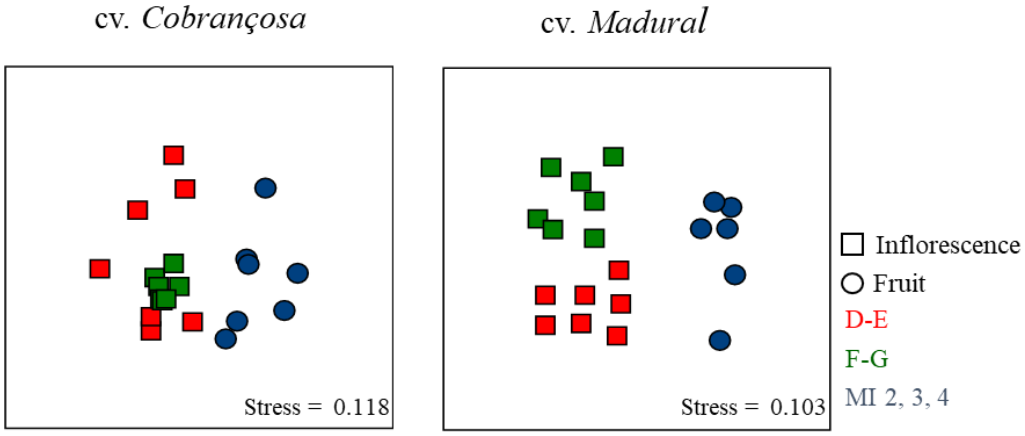

Fig. 2.4 Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of endophytic fungal communities grouped by plant organ (show by different symbols) and phenological stage (show by different colors) in olive tree cultivars *Cobrançosa* and *Madural*. Host phenological stage includes inflorescence development (D, E), flowering (F, G), and fruit ripening (MI 2, MI 3, MI 4). For more details see Fig. 2.1. Kruskal's stress values less than 0.2 represent good ordination plots.

The ANOSIM test confirmed the significant differences in the whole fungal community structures among plant organs (global R = 0.850, p=0.001) and phenological stages (global R = 0.500, p= 0.001). These differences were greater in cv. *Madural* (R = 0.860 and R = 0.690, p = 0.001, for plant organ and phenological stage, respectively) than in cv. *Cobrançosa* (R = 0.520 and R = 0.670, p = 0.001, respectively). In particular, inflorescences had different fungal communities across the phenological stages (Global R = 0.412, p=0.001). The endophytic community colonizing this organ displayed the greatest differentiation among phenological stage G (petals fallen or faded) and F (flowers open) (R=0.838 and R=0.931, p=0.001, for cvs. *Madural* and *Cobrançosa*, respectively) or D (flower cluster growing) (R=0.862, p=0.001, for cv. *Madural*) (Table S2.3). There was some variation in the most abundant genera during the different phenological stages, which could account for the differences found on fungal composition among stages G and F or D (Fig. 2.5).

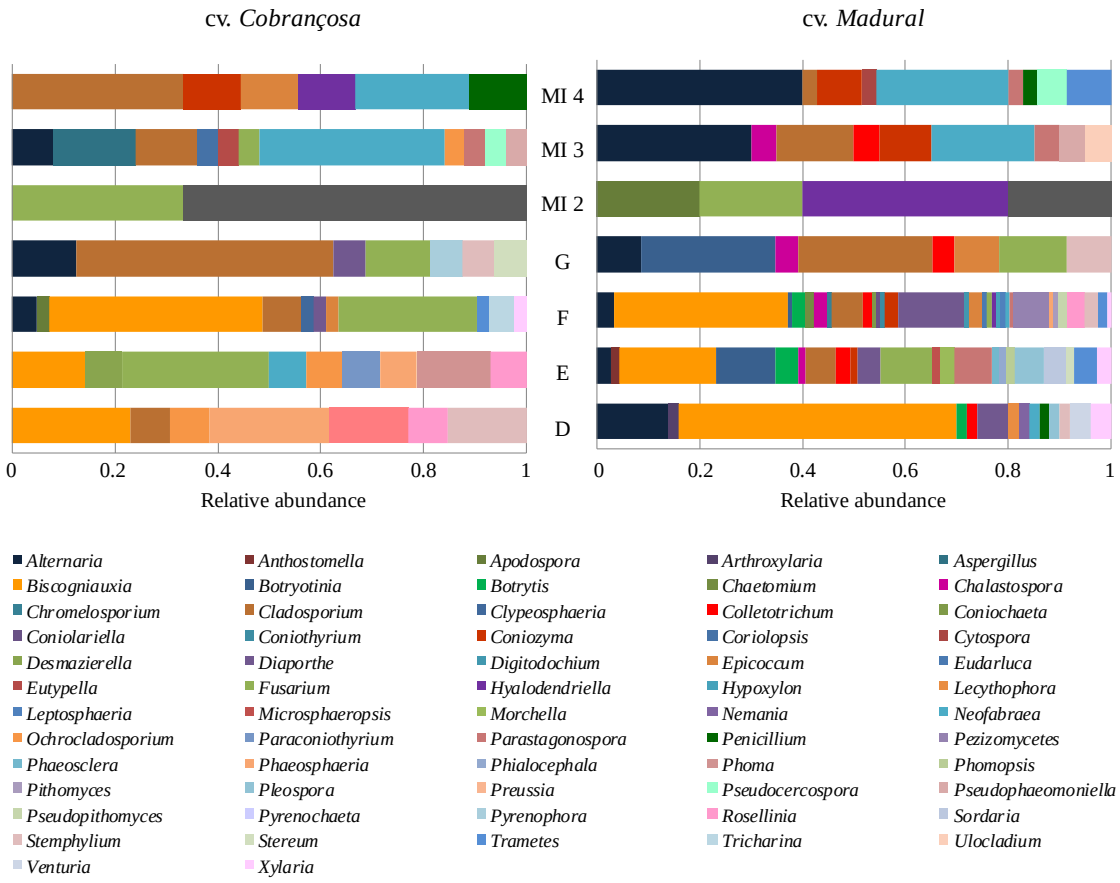

Fig. 2.5 Relative abundance of endophytic fungal genera associated with inflorescence and fruit of cultivars *Cobrançosa* and *Madural* throughout the different phenological stages. Host phenological stage includes inflorescence development (D, E), flowering (F, G), and fruit ripening (MI 2, MI 3, MI 4). For more details see Fig. 2.1.

For instances, *Biscogniauxia* that was isolated preferentially in F and D stages, disappeared in stage G where other genera, such as *Botryotinia* and *Cladosporium*, were the most frequent. In addition, endophytic fungi of twenty-four genera, which appeared in stage F, disappeared in stage G, contrasting with specific genera only found in stage G (*Pyrenophora* and *Stereum*). In contrast to inflorescences, fungal communities' composition of fruits was similar among the different maturation indexes (Global R = 0.209, p=0.001). Despite this, there were significant differences between some pairs of MI, with the strongest separation occurring between the MI 2 and MI 4 (R=0.666, p=0.001, for cv. *Cobrançosa*; R=0.916, p=0.001, for cv. *Madural*) or MI 3 (R=0.687, p=0.001, for cv. *Cobrançosa*) (Table S2.3). Indeed, was noticed that the most abundant genera varied over fruit ripening (Fig. 2.5). For instances, *Pyrenochaeta*, *Fusarium* and *Hyalodendriella* were most abundant in MI 2, while *Cladosporium*, *Alternaria* and

Neofabraea were richest in MI 3 and 4. There is only one genus (*Fusarium*) shared between MI 2 and MI 3 or 4, which could also account for the differences found in the fungal communities among MI.

2.4.3 Plant organ and phenological stage are the most important factors for shaping the fungal community

Overall, plant organ (F=2.52, p=0.005), phenological stage (F=1.91, p=0.005) and in less extent the cultivar (F=1.56, p=0.010), revealed to influence significantly the structure of the entire fungal community, explaining 5.9, 5.0 and 0.9% of the variation in their composition, respectively. Variance partitioning analysis applied to each organ showed that endophytes-associated to inflorescence were significantly affected by both phenological stage (F=1.49, p=0.005) and cultivar (F=1.55, p=0.005), explaining 11.8% and 1.6% of composition variation, respectively. In contrast, the endophytic population of fruits was not affected by the cultivar (F=1.11, p=0.165), but the phenological stage exerted a significant effect (F=1.42, p=0.005), explaining 5.3% of endophytic composition variation.

2.4.4 Cultivar- and phenological stage preference of endophytic fungi

Co-inertia analysis was used to find relationships between endophytic fungi assemblage and their plant host or phenological stage, in inflorescence and fruit (Fig. 2.6 and Fig. 2.7). This analysis intends to identify specific fungal OTUs associated to each host cultivar and phenological stage, with an attempted to elucidate their potential role in cultivar differences in anthracnose disease susceptibility. At the level of inflorescence, the results indicated that *Diaporthe rudis, Pezizomycetes sp., Biscogniauxia mediterranea, Botrytis cinerea, Rosellinia limonispora, Botryotinia pelargonii, Stemphylium beticola, Chalastospora gossypii* and *Colletotrichum godetiae*, were positively associated to cv. *Madural*, while *Cladosporium cladosporioides*, *Fusarium solani, Fusarium oxysporum* and *Phaeosphaeria avenaria,* were positively correlated with cv. *Cobrançosa* (Fig. 2.6). The phenological stages were mostly differentiated by *Pezizomycetes sp.*, *Cladosporium cladosporioides* and *Botryotinia fuckeliana*, which were positively related with the stage of flowers open (the first one) and petals fallen (the last two ones).

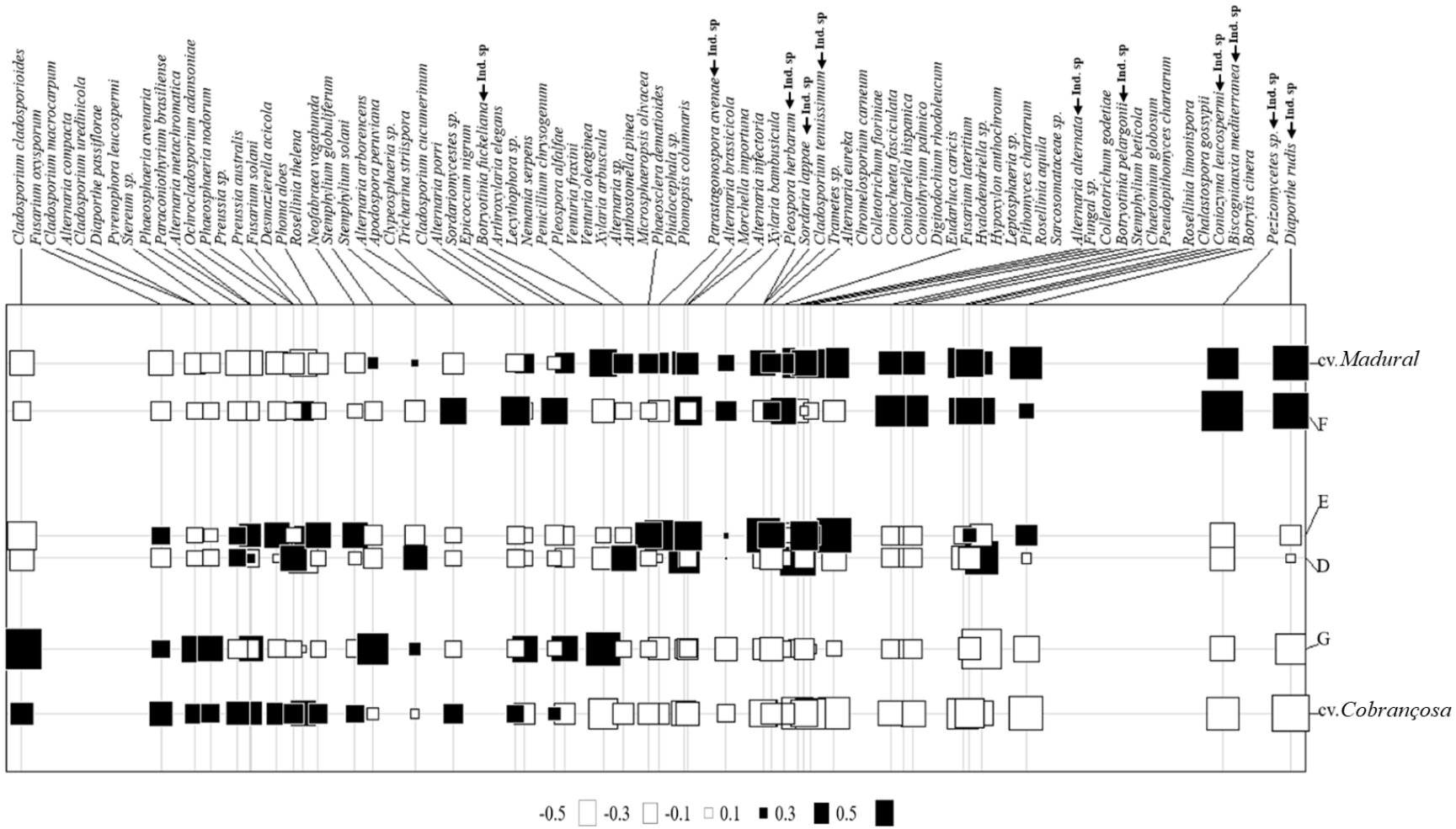

Fig. 2.6 Co-inertia factorial map showing positive (■) and negative (□) relationships between fungal communities inhabiting inflorescence and olive tree cultivar (*Cobrançosa* and *Madural*) and phenological stage. The square size indicates the degree of relatedness between variables (host cultivar or phenological stage) and fungal community. Arrows showed the indicator species associated with each cultivar or phenological stage. Host phenological stage includes inflorescence development (D, E) and flowering (F, G). For more details see Fig. 2.1.

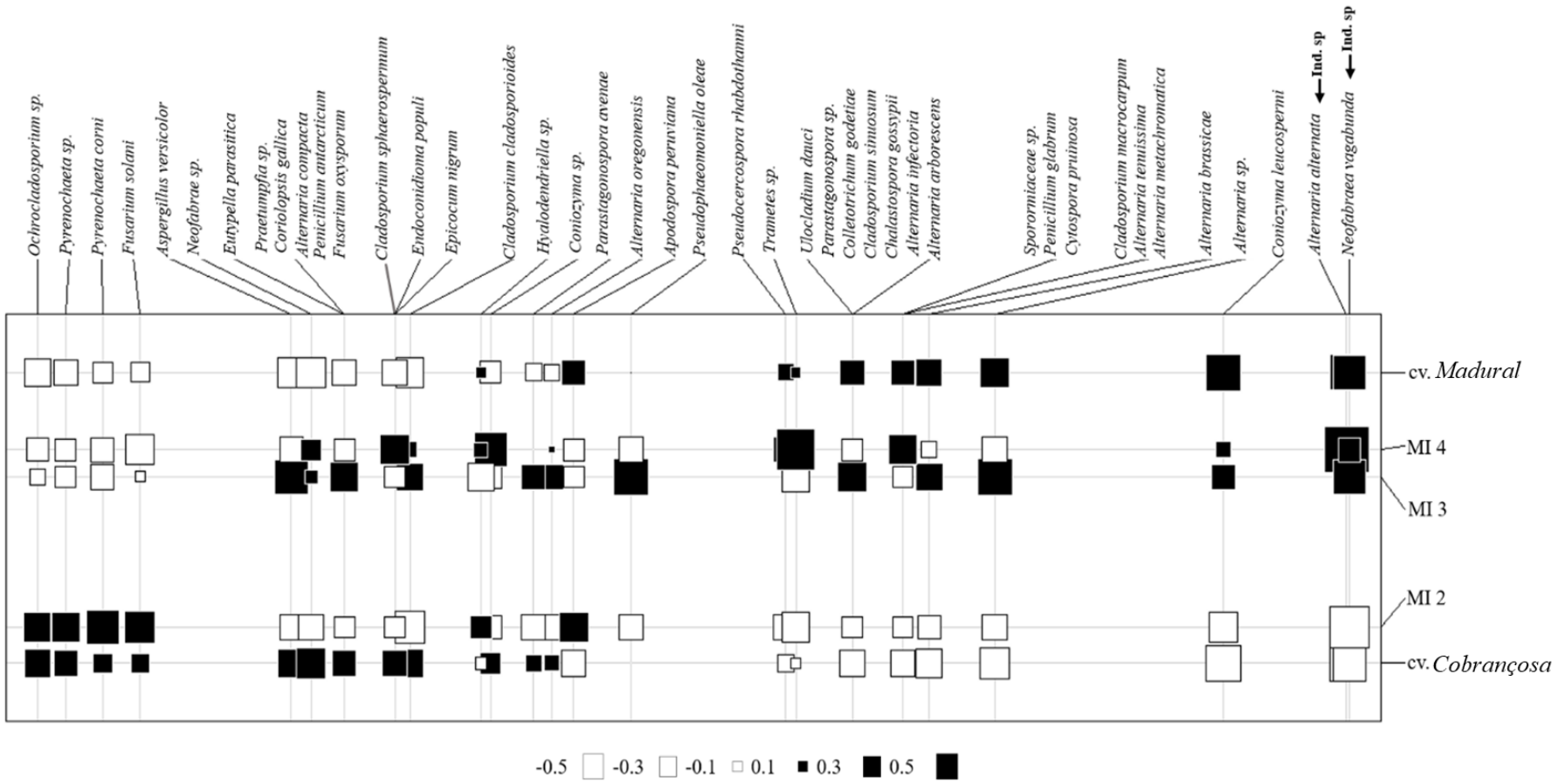

Fig. 2.7 Co-inertia factorial map showing positive (■) and negative (□) relationships between fungal communities inhabiting fruit and olive tree cultivar (*Cobrançosa* and *Madural*). The square size indicates the degree of relatedness between variables (host cultivar or maturation index) and fungal community. Arrows showed the indicator species associated with each cultivar or maturation index. Host maturation indices includes, fruit ripening (MI 2, MI 3, MI 4). For more details see Fig. 2.1.

Within the endophytic community of fruits, it was also found a set of fungal OTUs highly positively associated either to cv. *Cobrançosa* (*Neofabraea* sp. and *Ochrocladosporium*) or to cv. *Madural* (*Neofabraea vagabunda*, *Alternaria alternata* and *Coniozyma leucospermi*) (Fig. 2.7). The maturation indexes were mostly differentiated by *Alternaria alternata* and *Trametes* sp. as well as *Pyrenochaeta corni* and *Fusarium solani*, which were positively correlated with the MI 4 and MI 2, respectively. The fungal OUTs *Pseudophaeomoniella oleae* and *Aspergillus versicolor*, were linked with MI 3.

Species indicator analyses were further used to identify which fungal OTUs best characterized host cultivar (and their underlying susceptibility to anthracnose disease). Results revealed thirteen indicator OTUs of cv. *Madural* (eleven at the inflorescence level and two at the fruits level), and one indicator OTUs of cv. *Cobrançosa* (at fruit level) (*IndVal* > 0.56, $p<0.05$; Table S2.4). From these, the best indicator fungal OTUs that characterized cv. *Madural* were *Pezizomycetes sp.* (*IndVal* = 0.926), *Diaporthe rudis* (*IndVal* = 0.826) and *Alternaria alternata* (*IndVal* = 0.772). In cv. *Cobrançosa*, the only species indicator was *Fusarium solani* (*IndVal* = 0.614).

2.5 Discussion

The endophytic fungal community associated to reproductive organs has been rarely studied, in particular in woody plant species. In this work, was investigated the cultivable endophytic fungal community associated to inflorescence and fruits of two olive tree cultivars, with contrasting susceptibilities to olive anthracnose. Special focus was given to the cultivable endophytes, so that we could test further their ability in conferring host plant protection against anthracnose. It is well-known that cultivable approach has some limitations mainly related with the underestimation of the microbial composition in samples (Rappé and Giovannoni, 2003). However, it has been demonstrated that all frequently endophytic fungal genera detected from the culture-dependent approach were also detected in culture-independent (*e.g.*, next generation sequencing) method (Dissanayake et al., 2018). Indeed, here was found that inflorescences and fruits of olive tree were colonized by fungal members mostly from Ascomycota with dominant classes being Sordariomycetes and Dothideomycetes (Fig. S2.1). This result was consistent with other study using high-throughput sequencing of fungal microbiome in the same olive tree plant organs (Abdelfattah et al., 2015). Hence, it is likely that these particular fungi were dominant in olive tree reproductive organs and

can be considered as the key classes (Sordariomycetes for flowers and Dothideomycetes for fruits). Curiously, the present study revealed that members of Xilariaceae were the most abundant in reproductive organs, while previous studies focused on endophytes isolated from vegetative organs of olive tree reported Leptosphaeriaceae, Pleosporaceae, Pyronemataceae, Trichocomaceae, and Diaporthaceae as the most abundant (Martins et al., 2016; Gomes et al., 2018). Thus, it is likely that endophytic community composition of reproductive organs could be different from the one of vegetative organs of olive tree, as previously suggested for other plant species (Compant et al., 2011).

2.5.1 Contribution of plant organ and host genotype on endophytic fungal assembly

The two olive tree cultivars surveyed have different fungal endophyte communities, in terms of both abundance and species composition, with greater number of isolates and species recovered from cv. *Madural* than from cv. *Cobrançosa*. The effect of the host genotype in shaping the composition of fungal endophytic communities has been reported mostly in vegetative organs (*e.g.*, Cordier et al., 2012; Bálint et al., 2013; Qian et al., 2018). Here, although the plant cultivar is not identified as the main driver of fungal assemblage in reproductive organs, the results from the variation partitioning analysis suggests a degree of host control over fungal communities in inflorescences. Thus, as previously reported for vegetative organs (Bálint et al., 2013; Qian et al., 2018; Gomes et al., 2019), also in inflorescences the host plant seems to selectively recruited and promoted the growth of certain fungal species. A similar host-cultivar effect on endophytic fungal structure was reported on flowers of Pinggu peach trees (Ren et al., 2019). It is still not clear the mechanisms leading to differences in the endophytic fungal community structure among host genotypes (Bálint et al., 2013), in particular at the level of reproductive organs (Aleklett et al., 2014). These mechanisms might be associated with phenotypic properties, such as morphology, chemistry and physiology (Lindow and Brandl, 2003; Hoffman and Arnold, 2008; Lemanceau et al., 2017), and immune system (Saunders and Kohn, 2009) of the host plant. The ability of microorganisms to invade and colonize host plants depends on their evasion of host defense (Jones and Dangl, 2006). Here, the anthracnose-moderately susceptible cultivar (i.e., *Cobrançosa*) seems to be able to prevent the invasion and colonization of inflorescences by fungal endophytes when compared to the anthracnose-susceptible cultivar (i.e., *Madural*), because of the higher

fungal colonization rate observed in the latter cultivar. Thus, differences in anthracnose-susceptibility among the two surveyed olive cultivars are likely play a role in the structure of fungal endophytic communities of inflorescences. However, the contribution of host cultivar on fungal assemblage was found to differ among reproductive organs. In contrast to inflorescence, the fruit-associated fungal community assemblage was not influenced by the host cultivar. Thus, it is possible that host plant effect may vary due to other factors. Previous studies have already showed that patterns of phenotypic response within a given plant genotype may vary due to the surrounding environment, that can amplify, overwhelm or mask the effects of host genes, influencing fungal communities (Wagner et al., 2016). The collection of inflorescences and fruits for the isolation of endophytes was performed in different weather conditions (at spring and autumn, respectively). Thus, it is likely that variation on host plant effect on fungal assemblage among these two reproductive organs may be dependent on specific environmental variables. However, a more systemic investigation would be required to confirm if this hypothesis is true.

A higher endophytic fungal abundance and diversity was observed in inflorescences than in fruits. There are few studies that directly compare the endophytic microbial communities of flowers to those of other parts of the same plant. The few examples investigating the endophytic community in grapevine (Compant et al., 2011) and in olive tree (Abdelfattah et al., 2015) have found a higher bacterial and fungal population in flowers than in fruits, respectively, as observed in our study. This result may be related to differences between these two organs on their chemical composition. Olive fruits are rich on phenol compounds (Pereira et al., 2006; Machado et al., 2015), being most of them with antimicrobial activity (Marković et al., 2019), which could prevent the colonization of fruits by fungi. In contrast, flowers are report to be a nutrient-rich environment to the microorganisms (Ferrante et al., 2012). In particular, flower stigmas exude sugars and amino acids creating a wet and sticky environment (Ngugi and Scherm, 2006), and thus it is likely that support a relatively large microbial load compared to that of other plant organs. In fact, the variation partitioning analysis showed that the composition of fungal community of reproductive organs was primarily impacted by plant organ. In agreement with our data, both flowers and fruit of olive tree have showed to host different endophytic fungal communities (Abdelfattah et al., 2015).

2.5.2 Patterns of plant-endophyte association over different time points of inflorescence and fruit development

Analysis of temporal patterns showed that endophytic fungal community of both inflorescence and fruit exhibited temporal patterns. Both abundance and richness of fungal endophytes were higher on open flowers than on closed ones, as previously showed in the apple tree flowers (Shade et al., 2013). This is probably due to the higher exposition to the environment or to the greater nutritional resources for fungal colonization of open flowers when compared to closed ones. Moreover, in comparison to closed flowers, the open ones receive more pollinator visits and pollen grains that can carry microorganisms to the flower as discussed in Aleklett et al. (2014). There was a group of fungal OTUs positively associated to flowers when opened, being *Pezizomycetes* sp. the most significant. Species of Pezizomycetes class occur in a broad range of habitats, including soil, wood decay and plants, either as saprobic, mycorrhizal or endophyte (Hansen et al., 2013). According to Rodriguez et al. (2009) the Pezizomycetes belongs to class 3 endophytes, which are characterized to be horizontally transmitted by spores and hyphal fragments from plant to plant, by biotic or abiotic dispersion agents. Thus, we hypothesized that *Pezizomycetes* sp. colonizing open olive tree flowers probably came from surrounding environment. After opening, the fungal abundance and richness of flowers decreased to petal fall, suggesting that only some of the fungi that immigrate to the flower were able to successfully growth and persist in it. It is possible that some of the fungi that arrive on open flowers are opportunistic or transient endophytes, remaining only a small fraction in the flower tissues (Moricca and Ragazzi, 2008). Both *Cladosporium cladosporioides* and *Botryotinia fuckeliana* were strongly positively associated to flowers after the loss of petals. The first fungal OUT has been described as antagonists toward plant pathogens (Köhl et al., 2015) or to have insecticidal proprieties against insect pests (Shaker et al., 2019), while *B. fuckeliana* (sexual stage of *Botrytis cinerea*) is a well know plant pathogen (Boddy, 2016).

There were also detected differences in fungal community structure across fruit ripening, being fungal abundance and richness found to be higher in ripe olives than in unripe ones. This result may be due to the decreased on phenols content along ripening (Gouvinhas et al., 2017), making the fruit more favourable to fungal colonization and growth. A set of fungal OTUs were found to be positively associated to either unripe (MI 2) or ripe fruits (MI 3 and 4). The OTUs most associated to unripe fruits are fungi with

unknown biological function (*Pyrenochaeta corni*) or phytopathogenic fungus (*Fusarium solani*) responsible to cause several crop diseases (Coleman, 2016), including on olive tree (Chliyeh et al., 2017; Trabelsi et al., 2017). Ripe fruits were positively associated to fungal OTUs described to be phytopathogens (*Alternaria alternata*) (Troncoso-Rojas and Tiznado-Hernández, 2014), saprobic (*Trametes* sp.) (Zmitrovich et al., 2012) or toxigenic (*Aspergillus versicolor*) (Engelhart et al., 2002). *Pseudophaeomoniella oleae*, another fungus positively associated to ripe fruits, was recently reported to be associated with brown wood streaking and wilting symptoms on olive tree (Antelmi et al., 2018).

2.5.3 Can differences in susceptibility of the cultivars to anthracnose be related to the endophytic community naturally present in inflorescence and fruit?

A set of fungal OTUs was found to be specific to a particular cultivar, suggesting that they might be relevant to olive tree health. Otherwise, these fungi would not be maintained highly associated to these different olive cultivars. Most of the indicator/positively associated fungal OTUs to anthracnose-susceptible cv. *Madural* are plant pathogens of olive tree (*Colletotrichum godetiae* and *Neofabraea vagabunda*) causing fruit rot diseases (Cacciola et al., 2012; Romero et al., 2016), or are common pathogens of other plant species, such as *Diaporthe rudis* (Guarnaccia et al., 2018), *Alternaria alternata* (Troncoso-Rojas and Tiznado-Hernández, 2014), *Biscogniauxia mediterranea* (Linaldeddu1 et al., 2010), *Botrytis cinerea* (Williamson et al., 2007) and *Stemphylium beticola* (Hanse et al., 2015), being only one fungal OTU (*Pezizomycetes* sp.) been reported to offer benefits to their host plants (Hansen et al., 2013). However, there were also found a set of fungal OTUs positively associated to cv. *Madural*, which biological function is unknown (*e.g.*, *Rosellinia limonispora*, *Chalastospora gossypii*, *Coniozyma leucospermi*). Their role in conferring olive tree resistance against anthracnose disease remains a topic for further research.

The most anthracnose-resistant cv. *Cobrançosa* had also several indicator/positively associated fungal OTUs, being some of them described as plant beneficial fungus, such as *C. cladosporioides* (Köhl et al., 2015; Shaker et al., 2019) or phytopathogens of several crops, including of olive tree, such as *Neofabraea* sp. (Chen et al., 2016), *Phaeosphaeria avenaria* (Bathgate and Loughman, 2001) and *Fusarium solani* (Chliyeh et al., 2017; Trabelsi et al., 2017). Other isolates associated to *Cobrançosa*

cultivar were *Fusarium oxysporum*, which include both pathogenic and nonpathogenic strains (Bao et al., 2014), and *Ochrocladosporium* with unknown biological function.

In conclusion, the endophytic fungal communities of olive tree were primarily impacted by plant organ and phenological stage, and to a lesser extent by host cultivar. Indeed, host cultivar effect on fungal community composition was observed only on inflorescences. This organ was also showed to harbour a greater diversity and abundance of fungal endophytes than fruits. Diverse and specific fungal OTUs were found to be associated to each olive cultivar/plant organ/phenological stage, being most of them pathogens of other plant species or fungi with unknown biological function. If such fungi contribute to olive tree resistance to anthracnose disease is a topic that requires further research. In particular, studies of interactions between these fungal OTUs and the olive tree and the pathogen *Colletotrichum* will likely reveal function roles of these fungal endophytes on host susceptibility/resistance to anthracnose disease.

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2.7 Supporting Information

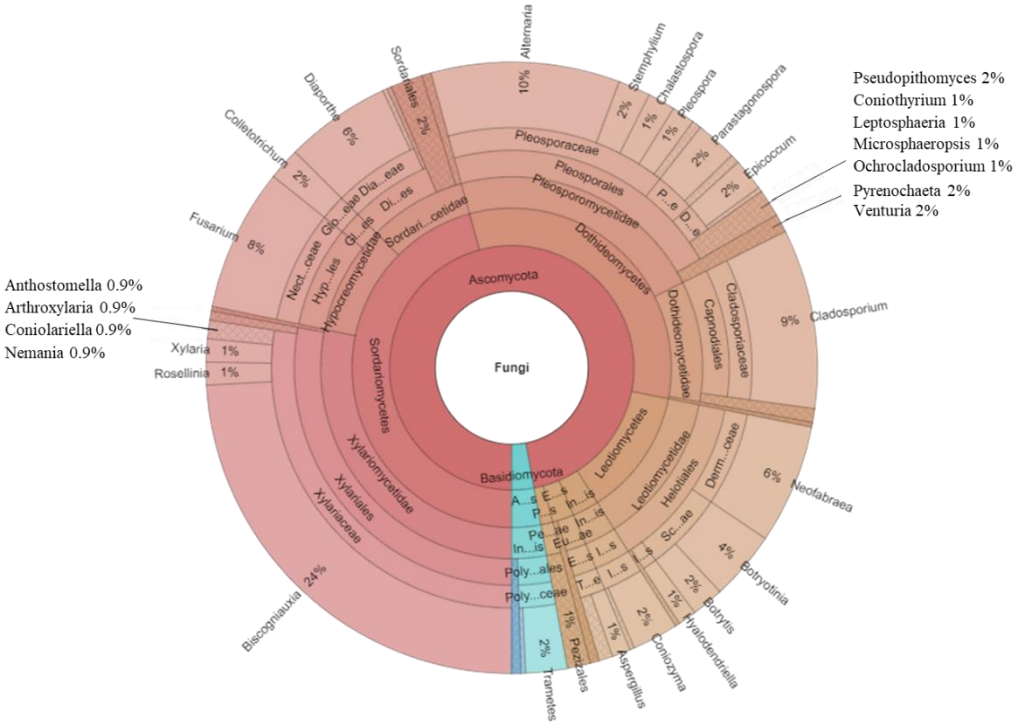

Fig. S2.1 Krona chart showing the relative abundance of endophytic fungi, at the genus level, detected on inflorescence and fruit of olive tree of cvs. *Cobrançosa* and *Madural*.

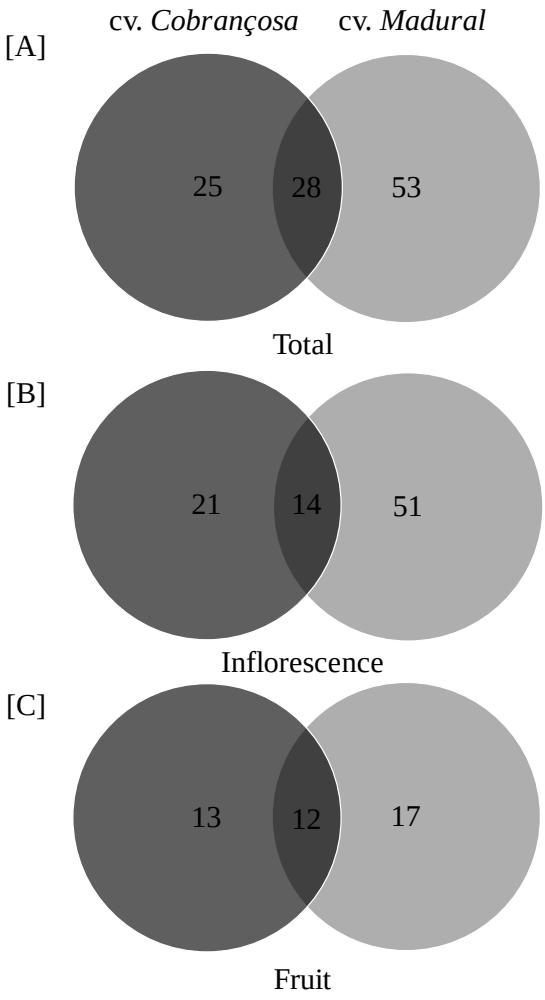

Fig. S2.2 Venn diagrams representing the total number of fungal OTUs shared between *Cobrançosa* and *Madural* olive tree cultivars when considering the [A] total, [B] inflorescence and [C] fruit samples.

Table S2.1 Abundance and diversity of fungal endophytes detected on inflorescence and fruit over different phenological stages of cultivars *Cobrançosa* and *Madural*. Species diversity index (H) is presented as mean values ± SE (n=7) and as total number (in brackets). Different superscript lowercase letters in each row denote a statistically significant difference ($p<0.05$) within inflorescence or fruit. Host phenological stage includes inflorescence development (D, E), flowering (F, G), and fruit ripening (MI 2-4). For more details see Fig. 2.1.

		Inflorescence					**Fruits**			
	Parameters	**D**	**E**	**F**	**G**	**Total**	**MI 2**	**MI 3**	**MI 4**	**Total**
cv. *Cobrançosa*	Total nº of isolates	13	14	43	16	86	4	29	19	52
	Average nº of isolates *per* tree	1.9±0.3^{a}	2.0±0.4^{a}	6.1±0.6^{b}	2.3± 0.3^{a}	3.1±0.4^{c}	0.6±0.2^{a}	4.1±0.8^{b}	2.7±0.6^{c}	2.5±0.5^{c}
	Total no. of OTUs	10	10	14	11	35	4	14	12	25
	Average no. of OTUs *per* tree	1.6±0.3^{a}	1.4±0.2^{a}	3.6±0.4^{b}	2.1± 0.3^{c}	2.2±0.2^{c}	0.6±0.2^{a}	3.0±0.3^{b}	2.0±0.3^{c}	1.9±0.3^{c}
	Total frequency of colonization (%)	1.2	1.3	4.1	1.5	2.0	0.4	2.8	1.8	1.7
	Shannon-Wiener index (H)	0.5±0.2^{a,c} (2.2)	0.3±0.1^{a} (2.2)	1.1± 0.1^{b} (1.9)	0.7±0.1^{c} (2.1)	0.6±0.1^{c} (2.8)	n.d.	0.8±0.2^{a} (2.4)	0.6±0.2^{a,b} (2.3)	0.4±0.1^{b} (2.8)
cv. *Madural*	Total nº of isolates	51	71	121	24	267	5	20	36	61
	Average nº of isolates *per* tree	7.3±1.8^{a}	10.1±1.7^{a,b}	17.3±1.8^{c}	3.4±0.4^{d}	9.6±1.2^{b}	0.7±0.4^{a}	2.9±0.7^{b}	5.1±1.1^{c}	3.2±0.6^{b}
	Total no. of OTUs	18	26	41	12	65	4	14	15	29
	Average no. of OTUs *per* tree	4.3±1.1^{a}	8.7±2.4^{b}	10.6±2.8^{b}	3.0±1.3^{a}	6.6±1.1^{c}	0.7 ±0.4^{a}	3.4±0.7^{b}	4.0±0.9^{b}	2.8± 0.5^{c}
	Total frequency of colonization (%)	4.9	6.8	11.5	2.3	6.4	0.5	1.9	3.4	1.9
	Shannon-Wiener index (H)	1.1±0.3^{a} (1.9)	1.8±0.3^{b} (2.8)	1.9±0.2^{b} (2.9)	0.8±0.3^{a} (2.2)	1.4±0.2^{c} (3.2)	n.d.	0.9±0.3^{a} (2.5)	1.3±0.3^{a} (2.2)	0.7±0.2^{b} (2.9)
Total	Total nº of isolates	64	85	164	40	353	9	49	55	113
	Average nº of isolates *per* tree	4.6±1.2^{a}	6.1±1.4^{a}	11.7±1.8^{b}	2.9±0.3^{c}	6.3±0.8^{a}	0.6±0.2^{a}	3.5±0.5^{b,c}	3.9±0.7^{c}	2.7±0.4^{b}
	Total no. of OTUs	27	34	46	20	86	7	25	23	42
	Average no. of OTUs *per* tree	3.0±0.7^{a,b}	5.1±1.5^{a,c}	7.1±1.7^{c}	2.6±0.6^{b}	4.4±0.6^{a}	0.6±0.2^{a}	3.7±0.4^{b}	3.0±0.6^{b,c}	2.5±0.3^{c}
	Total frequency of colonization (%)	3.0	4.0	7.8	1.9	4.2	0.4	2.3	2.6	1.8
	Shannon-Wiener index (H)	0.7±0.2^{a} (2.3)	1.1±0.3^{a} (2.8)	1.5±0.2^{b} (3.1)	0.7±0.2^{a} (2.5)	1.0±0.1^{a} (3.3)	n.d.	0.8±0.2^{a} (2.9)	0.9±0.2^{a} (2.6)	0.6±0.1^{b} (3.3)

n.d. - Not determined

Table S2.2 Summary outputs of the developed generalized linear mixed models for the effect of the olive tree cultivar (*Cobrançosa* and *Madural*), organ (inflorescence and fruit), and host phenological stage on the abundance and species richness of the fungal endophytic community. Host phenological stage includes inflorescence development, flowering, and fruit ripening. For more details see Fig. 2.1.

DV	M	IV	Type II Wald chisquare	df	p
Abundance (N)	Organ	Organ	47.425	1	<0.01
		Cultivar	57.224	1	<0.01
		Organ:Cultivar	14.847	1	<0.01
	Phenological stage	Cultivar	52.41	1	<0.01
		Phenological stage	136.434	6	<0.01
		Cultivar: Phenological stage	28.052	6	<0.01
Richness (S)	Organ	Organ	27.2714	1	<0.01
		Cultivar	45.3546	1	<0.01
		Organ:Cultivar	5.2573	1	0.022
	Phenological stage	Cultivar	39.565	1	<0.01
		Phenological stage	67.589	6	<0.01
		Cultivar: Phenological stage	14.303	6	0.026

DV: Dependent variable; M: Model; IV: Independent variable, df: Degrees of freedom; p - Probability.

Table S2.3 Results of ANOSIM analysis for the fungal endophytic assemblages in inflorescence and fruit of olive trees from cvs. *Cobrançosa* (dark gray color) and *Madural* (light gray color), considering the different phenological stages. ANOSIM test showed the R-statistics (R) and the statistical significance, which is denoted by asterisks (* p<0.05; ** p<0.01; *** p<0.001). Host phenological stage includes inflorescence development (D, E), flowering (F, G), and fruit ripening (MI 2-4). For more details see Fig. 2.1.

	D	**E**	**F**	**G**	**MI 2**	**MI 3**	**MI 4**
D	-------	0.575**	0.396**	0.862***	0.916**	0.609***	0.908***
E	0.5*	-------	0.761***	0.728***	0.904***	0.634***	0.979***
F	0.527 **	0.523**	-------	0.838***	0.914***	0.596***	0.998***
G	0.531*	0.658**	0.931***	-------	0.631***	0.449**	0.878***
MI 2	0.5 n.s	0.125 n.s	0.619 n.s	0.811*	-------	0.375***	0.916***
MI 3	0.738**	0.626**	0.851***	0.785**	0.687*	-------	0.193*
MI 4	0.555**	0.555*	0.995***	0.932**	0.666**	0.242 n.s	-------

n.s. - not significant.

Table S2.4 Endophytic fungal indicator OTUs detected on inflorescence and fruit over different phenological stages of cultivars *Cobrançosa* and *Madural*. A - Specificity (*i.e.* uniqueness to a particular habitat), B - Sensitivity (i.e. frequency within that particular habitat). Host phenological stage includes inflorescence devlopment (D, E), flowering (F, G), and fruit ripening (MI 2-4). For more details see Fig. 2.1.

	Indicator fungal OTUs	Phenological stage/MI	A	B	Frequency of occurrence (%)	*IndVal*	p-value
cv. *Cobrançosa*	*Fusarium solani*	F	0.440	0.857	85.7	0.614	0.001
cv. *Madural*	*Alternaria alternata*	D	0.714	0.571	57.1	0.639	0.005
	Pleospora herbarum	E	1.000	0.571	57.1	0.756	0.002
	Botryotinia pelargonii	E	0.714	0.714	71.4	0.714	0.001
	Cladosporium tenuissimum	E	1.000	0.428	43.0	0.655	0.009
	Sordaria lappae	E	1.000	0.428	43.0	0.655	0.011
	Parastagonospora avenae	E	0.714	0.571	57.1	0.639	0.008
	Pezizomycetes sp.	F	1.000	0.857	85.7	0.926	0.001
	Diaporthe rudis	F	0.681	1.000	100	0.826	0.001
	Biscogniauxia mediterranea	F	0.386	1.000	100	0.621	0.001
	Coniozyma leucospermi	F	0.750	0.428	43.0	0.567	0.043
	Botryotinia fuckeliana	G	0.625	0.7143	71.4	0.668	0.003
	Alternaria alternata	MI 4	0.833	0.7143	71.4	0.772	0.001
	Neofabraea vagabunda	MI 4	0.391	1.000	100	0.626	0.009

Chapter 3

Endophytic fungal community structure in olive orchards with high and low incidence of olive anthracnose

This chapter was submitted as an original article to *Frontiers in Plant Science*: Fátima Martins, Diogo Mina, José Alberto Pereira, Paula Baptista. Endophytic fungal community structure in olive orchards with high and low incidence of olive anthracnose.

3.1 Abstract

Fungal endophytes have been increasingly recognized to promote host plant protection to pathogens, but knowledge of the multiple effects that they could have in crop diseases is still scarce. This work attempts to understand the role of fungal endophytes in crop diseases, specifically in reducing disease development and interfering on lifestyle transition of the pathogen. To accomplish this, the endophytic fungal community of reproductive organs of olive tree from two orchards showing different levels of anthracnose incidence, a major disease of olive fruits, was characterized and compared. The surveyed of endophytes was started before flowers open (latent pathogen) and ended at fruit ripening (necrotrophic pathogen). The two orchards showed distinct endophytic communities, differing in species richness, abundance and composition, with highest isolation rates and richness of endophytes in the orchard with low anthracnose incidence. These differences among orchards were greater on fruits than on flowers, suggesting that these changes in endophytic fungal composition may influence the lifestyle shifts in pathogen (from latent to pathogen). A number of fungal taxa were found to be positively associated to one of the two orchards. The fungal endophytes best correlated with high incidence of anthracnose are pathogens, while endophytes-associated to low anthracnose incidence are described to protect plants. Altogether, the results suggest varying pathogen-endophyte interactions among the two orchards. Probably, in the orchard with low disease incidence both the pathogen and the disease cannot thrive so well as in the orchard with high disease incidence due to the richer and more abundant endophyte community with protective effect.

3.2 Introduction

In nature, every plant species have been found to cohabit with a great diversity of endophytic microorganisms, including fungi (Hardoim et al., 2015). The members of these complex fungal communities continually interact among each other and with their hosts, sometimes conferring benefits (mutualistic) and at other times causing harm (pathogenic) by contributing to disease in plants (Compant et al., 2019). However, the importance of multispecies interaction on plant diseases has been mostly recognized from studies involving pathogen-pathogen interaction (Lamichhane and Venturi, 2015; Abdullah et al., 2017). In contrast, the relevance for plant health of the interaction

between the pathogen and other microbial groups associated to plants is still scarce (Lamichhane and Venturi, 2015). Mounting evidence shows that specific microbial taxa that co-occur with the pathogen may impact the pathogenic process (Kemen, 2014; Vayssier-Taussat et al., 2014). For example, some non-pathogenic bacteria that co-occur with the pathogen *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) have been showed to increase the severity of olive knot disease (Hosni et al., 2011; Passos da Silva et al., 2014). Besides bacteria, also fungi have been recently suggested to interact with *Psv* in olive tree twigs with important implications for the development of the olive knot disease (Gomes et al., 2019). Despite these evidences, we are only just beginning to understand the importance of pathogen-microbial interaction for plant health (Kemen, 2014).

Other challenging question that may have significant implications on pathogenesis is related to the pathogen-microbial interaction importance in the change of the pathogen's lifestyle. Most phytopathogenic fungi start their lifecycle with a quiescent phase, remaining unnoticed, before switching to an active necrotrophic lifestyle in which the disease symptoms became visible (Alkan and Fortes, 2015). Studies addressing the factors underlying this switch of lifestyle have been only focused on host plant (*e.g.*, physiological, chemical and defense response), pathogen (*e.g.*, virulence genes, effectors) or environmental aspects (Reviewed by Prusky et al., 2013), completely disregarding the plant microbiota. The potential importance that this microbial community might have in maintaining or facilitating the pathogen transition from quiescence to necrotrophic colonization is still unknown.

The anthracnose, caused by several species of the *Colletotrichum* genus, is considered to be one of the most globally-distributed and economically-important disease, responsible for significant losses in many crops (De Silva et al., 2017), including olive (Cacciola et al., 2012). In the olive tree, this disease affects mostly the fruits, especially when they are nearly ripened (Cacciola et al., 2012). Several species of *Colletotrichum* spp. responsible to cause olive anthracnose have been reported to exhibited different lifestyles, ranging from latent infections, at the flowering stage, to a necrotrophic phase upon fruit ripening (Sergeeva et al., 2008). Before this devastating stage these fungi can also adopt endophytic- or hemibiotrophic-like lifestyle before fruit ripening (De Silva et al., 2017). These characteristics make the olive tree and the anthracnose disease a good model system for studying the influence of the plant microbiota on the outcome of plant–pathogen interactions.

In light of the above, this work compared the endophytic fungal communities of flower buds, flowers and fruits of olive tree between orchards with high and low anthracnose incidence, in order to elucidate the potential role of plant microbiota on the pathogenesis process. Specifically, we want to answer the following questions: i) In what way do the endophytic fungal communities differ between orchards with different disease incidence? ii) In what way do the endophytic fungal communities differ among the different pathogen's lifestyle (*i.e.*, from early flowering stages until fruit set)? iii) Is there any fungal consortium specifically associated with high ("disease-promoting fungi") or low ("disease-suppression fungi") anthracnose incidence as well as with a specific pathogen's lifestyle? A better knowledge of the importance of plant fungal endophytes on the anthracnose incidence may help to develop holistic management strategies against this disease. In this work, was used a culturable approach to evaluate the endophytic fungal community, which allows the further study of the functions and application of the isolates identify to limit or prevent anthracnose disease.

3.3 Materials and methods

3.3.1 Study area and plant material collection

The plant material was collected from June to December 2016 in two olive orchards, one historically with high anthracnose incidence (orchard 1 - 41° 33 45"N, 7° 10' 58"W) and the other with low incidence of anthracnose (orchard 2 - 41° 33 08″N, 7° 07′ 24″W) (Fig. 3.1a). These differences between the two orchards were confirmed by estimating the disease incidence and severity simultaneously to sample collection (please see anthracnose incidence and severity section). Both orchards are located in the same region (*i.e.*, Mirandela, Northeast of Portugal) at ≈15 km apart, but in areas with different altitudes and probably of humidities (Fig. 3.1a). The first orchard is located in Abambres at 255 m altitude and at ≈2 Km from the river, while the second orchard is located in Paradela at 450 m altitude (≈8 Km from the river). Both orchards are composed of olive trees from cv. *Madural* (which is moderately susceptible to anthracnose), with a similar age (< 30 years old), planted at 7 × 7 m spacing and managed through integrated production guidelines (Malavolta and Perdikis, 2018). From each orchard, seven olive trees were randomly selected, and asymptomatically flower buds, flowers and fruits were collected around the perimeter of the tree at the operator height and used for isolation of fungal endophytes. The sampling was conduct in several dates spanning the entire period

from flower bud until fruit set. The plant material collected were divided into three groups: 1) flower buds, which comprised different development stages (*i.e.*, petals just visible, green petals longer than sepals and petals whitening); 2) flowers having different stages of development (*i.e.*, on flower cluster development, and on flower cluster totally expanded, flowers open and petals fallen or faded); 3) fruits, which were picked at veraison (fruit matures from yellow-green) and mature (fruit skin turns from purple to black and the flesh darkens). In total, 50 samples of each flower buds, flowers and fruits, were collected *per* tree, being each tree corresponding to a biological replicate. The samples collected were placed directly into sterile bags, transported to the laboratory and stored at 4 °C until isolation of endophytic fungi, which was carried out within one week.

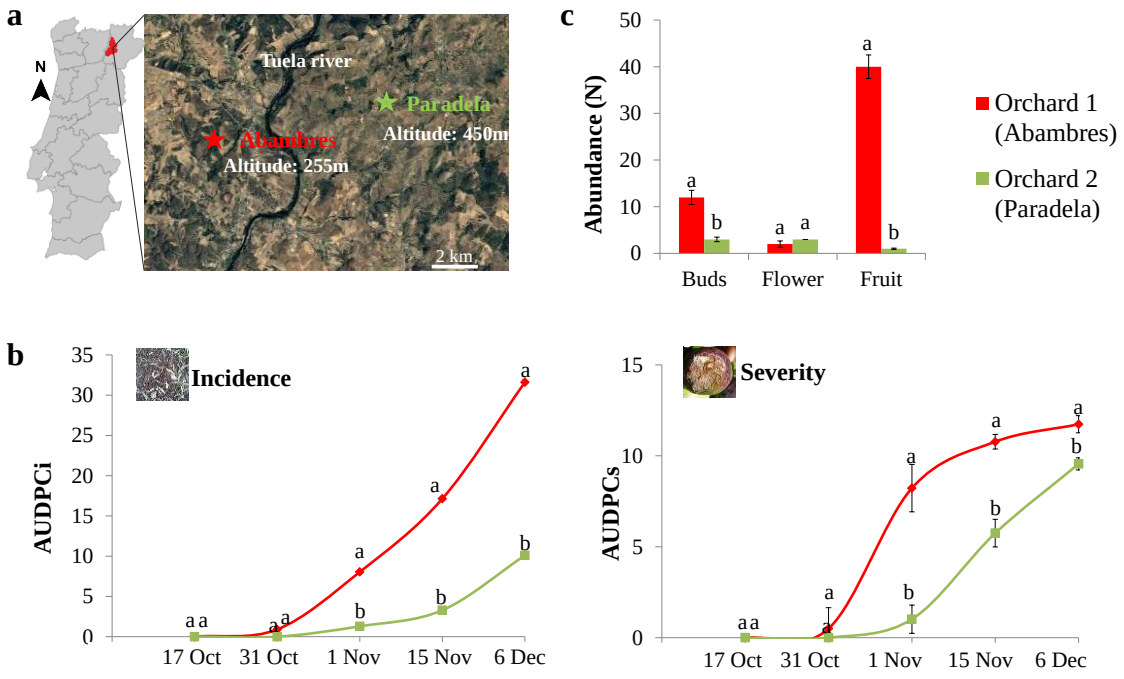

Fig. 3.1 Olive orchards used for the isolation of endophytic fungi from flower buds, flowers and fruits of olive tree. (**a**) Localization of the two olive orchards – orchard 1 (Abambres) and 2 (Paradela) in the Northeast of Portugal, (**b**) Area under the disease progress curve of incidence (AUDPCi) and severity (AUDPCs) of olive anthracnose evaluated in both orchards during the autumn-winter period of 2016, (**c**) Abundance of *Colletotrichum* spp. in flower buds, flowers and fruits of olive trees from the two orchards. Data points with different letters indicate statistically significant differences (at least $p < 0.05$) between the two olive orchards.

3.3.2 Anthracnose incidence and severity

Both incidence (*i.e.* percentage of infected fruits) and severity (*i.e.* proportion of fruit area that is affected) of anthracnose were assessed in the same trees used to isolated fungal endophytes. This was done simultaneously to olive fruits collection, at five different dates, from October-December 2016. At each time, was randomly collected a total of 100 fruits *per* tree around the canopy at 1.5–2 m above ground height and placed directly into sterile bags. The fruits were transported to the laboratory in an icebox, and after one week of incubation in a wet chamber (80% relative humidity, 22°C), the fruits were examined for the appearance of symptoms. The disease incidence was determined by the percentage of infected fruits, and severity was determined by using a 0 to 5 rating scale, where 0 = no visible symptoms, 1 = visible symptoms affecting <25% of the fruit surface, 2 = 25–49%, 3 = 50–74%, 4 = 75–100%, and 5 = soapy fruit (Moral et al., 2008). The area under disease progress curve for disease incidence (AUDPCi) and severity (AUDPCs) was further calculated for each tree and sampling date, following the procedure described by Moral et al. (2008).

3.3.3 Endophytic fungi isolation

The collected plant tissues were briefly washed under distilled water and further subjected to surface sterilization through sequential immersion in a series of solutions as follows: sodium hypochlorite 3% (v/v) for 1 min (for flower buds and flowers) or 2 min (for fruits), 70% (v/v) ethanol for 1 min, and sterile distilled water (three times, 1 min each). The sterilized flower buds, flowers and fruits were cut by surgical blade into 2-4 mm segments and plated onto Difco potato dextrose agar (PDA) medium supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). The success of surface sterilization method was confirmed by imprinting the surface of the plant segments onto PDA medium. In total 4200 segments of each flower buds, flowers and fruits were inoculated (2 orchards x 7 olive trees x 50 plant tissues x 6 tissues segments). The plates were incubated at 25 ± 2°C in the dark and were daily observed to check the growth of endophytic fungi from the plant segments. All endophytic fungal isolates obtained were purified through single spore isolation technique in fresh PDA medium for later identification.

3.3.4 Identification of endophytic fungi

Endophytic fungi were first grouped into morphotypes according to their morphological features (*i.e*., colony appearances, characteristics of the hyphae, spores and reproductive structures). After that, two representative isolates of each morphotype were selected for molecular identification, using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). The genomic DNA was extracted from fungal spores/mycelium, using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) following the manufacturer's instructions. The PCR reactions, with the pair of primers ITS1 and ITS4 (White et al., 1990) were performed using the same DNA extraction kit following the manufacturer's instructions, in the MyCycler thermal cycler (BioRad). The temperature cycle used in the amplification was 94° C for 3 min (1 cycle); 94° C for 30 sec, 53° C for 50 sec, 72° C for 2 min (35 cycles); and 72° C for 10 min (1 cycle). The amplified products (~650 bp) were purified and sequenced using Macrogen Inc. services (Madrid, Spain). The obtained DNA sequences were analyzed with DNASTAR v.2.58 (Lasergene) software, and fungal identification was performed using the NCBI (http://www.ncbi.nlm.nih.gov) and UNITE (https://unite.ut.ee/) databases and BLAST algorithm. The blast results were analyzed according to the parameters described by Raja et al. (2017). All operational taxonomic unit (OTU) was taxonomically classified at species or genus level according to the Index Fungorum Database (www.indexfungorum.org). For preservation purpose, all isolates identified were deposited in the culture collection of the Mountain Research Centre (CIMO), School of Agriculture - Polytechnic Institute of Bragança.

3.3.5 Diversity and structure of fungal community

The diversity and composition of endophytic fungal community associated to olive tree was compared between orchards (with high and low incidence of olive anthracnose) or plant organs (flower buds, flowers, and fruits). In this analysis, the *Colletrotrichum* was excluded from the database in order to reflect the true fungal community changes (*i.e.*, that is not due to an overabundance of the pathogen in the orchard with high anthracnose incidence). Fungal diversity was assessed by evaluating the abundance (average number of isolates *per* tree) and richness (average number of OTUs *per* tree), and by computing Shannon–Wiener (H') diversity index with *Species Diversity and Richness* v. 4.0 (Henderson and Seaby, 2007). The results are presented as the mean of replicates (N=7), displaying respective SE values. To determine differences

among the means, a one-way analysis of variance (ANOVA) with SPSS v.18 software was done, and the averages were compared using Tukey's test ($p<0.05$).

Non-metric multidimensional scaling (NMDS) was carried out with Bray-Curtis index, in order to assess the similarity of endophytic assemblages with respect to orchards (with high and low incidence of olive anthracnose) and plant organ (flower buds, flowers, and fruits). Significant differences between fungal community groupings obtained in NMDS ordination was assessed by a one-way analysis of similarity (ANOSIM), using Bray–Curtis distance matrices (obtained from raw abundance data). ANOSIM generates an R-value ranging from 0 (completely similar) to 1 (completely dissimilar) and a p-value (significant level below 0.05) (Clarke and Gorley, 2015). Both NMDS and ANOSIM were performed using *Community Analysis Package* v.4.0 (Henderson and Seaby, 2007).

Composition of fungal community was also compared at functional level. For this, the identified fungal OTUs were firstly classified into functional categories according to Hardoim et al. (2015) and based on literature (Table S3.1). This classification included commensal (fungi with not apparent effect on host plants), beneficial (fungi that confers host plant protection and promotes plant growth) and pathogenic (that included latent pathogens) fungal groups. Fungal OTUs belonging to other groups or not able to be identified into a functional group were categorized as "other" and "unknown fungi", respectively. After grouping, the changes on fungal abundance and richness of each functional group across plant organs were determined among orchards (*i.e.*, with high and low anthracnose incidence). A one-way analysis of variance (ANOVA) was performed as previously described, to determine differences on fungal functional groups among orchards.

3.3.6 Factors driving fungal community structure

To estimate the relative contribution of the type of olive orchard (*i.e.*, high and low anthracnose incidence) and plant organ (flower buds, flowers and fruits) in shaping the endophytic community composition a variation partitioning (*Varpart*) analysis was performed with the vegan package of *R* software using *varpart* function (R Core Team, 2018). The significance of each fraction was tested using the *anova.cca* function.

3.3.7 Identification of fungal OTUs associated with each orchard or plant organ

Random forest analysis was performed to identify which fungal OTUs were the most important in differentiating orchards (high *vs*. low incidence of anthracnose) or olive tree organs (flower buds, flowers, and fruits). The importance of OTUs to distinguishing fungal community was measure by considering the decrease in mean Gini: a higher decrease will imply a higher importance (Breiman, 2001). This analysis was computed with the *RandomForest* package (Cutler et al., 2007) from R (R Core Team, 2018). The fungal OTUs that were identified to be highly relevant to differentiated orchards or plant organs were selected and subject to principal component analysis (PCA). The PCA aimed to identify the fungal OTUs consortium associated to each orchard and plant organ. This analysis was performed by using the *psych* package (Revelle, 2017) from R (R Core Team, 2018). *Spearman* correlations were also performed in the R *corrplot* (Wei and Simko, 2017) package to assess the correlation between the fungal OTUs preselected by the random forest with the relative abundance of *Colletotrichum* spp.

3.4 Results

3.4.1 Anthracnose incidence and *Colletotrichum* abundance

In this study was evaluated the endophytic fungal community associated to olive trees from two orchards with a different historical record of anthracnose incidence. The results from disease progression curves also clearly differentiated the two orchards regarding the levels of anthracnose (Fig. 3.1b). Indeed, both disease incidence (AUDPCi) and severity (AUDPCs) were significantly ($p<0.001$) higher in the orchard with a history of anthracnose (orchard 1 - Abambres; AUDPCi=8.0 and AUDPCs =8.2) than in the orchard with a historically low incidence of anthracnose (orchard 2 – Paradela; AUDPCi=1.3 and AUDPCs =1.0). These differences were noticed after the 1st November and becoming higher for incidence over time as fruit matured. For simplification, the orchard with high and low incidence of anthracnose will be termed from now as “high incidence” and “low incidence”, respectively.

The isolation of *Colletotrichum* spp. from the interior of asymptomatic reproductive organs of trees located in both orchards, also confirms the high prevalence of the pathogen in the orchard with high incidence (Fig. 3.1c). Indeed, the abundance of *Colletotrichum* spp. in flower buds and fruits was significantly higher (up to 4.0-fold and

40.0-fold, respectively, $p<0.001$) in the orchard with a high incidence when compared to the orchard with low incidence. More importantly, the results also showed the ability of these *Colletotrichum* isolates to grow asymptomatically inside the host in an endophytic and/or quiescent manner. The identified endophytic *Colletotrichum* strains belonging to *C. acutatum* complex - *C. godetiae*, which was found in all organs surveyed, and *C. fioriniae*, that was detected in both flowers and fruits (data not showed).

3.4.2 Fungal community comparison: High *versus* low anthracnose incidence

Analysis of fungal communities in olive trees from the two orchards revealed a total of 115 OTUs, belonging to two phyla, 31 families and 61 genera. Ascomycota was the most abundant phylum, accounting for 96% of the total isolates and the remaining isolates belonged to Basidiomycota (Fig. S3.1). *Biscogniauxia* (Xylariaceae), *Colletotrichum* (Glomerellaceae), *Alternaria* (Pleosporaceae) and *Cladosporium* (Cladosporiaceae) were the most abundant genera, accounting together with 44% of total isolates.

The endophytic fungal community associated with olive tree varied between the two different orchards (*i.e.*, with high and low anthracnose incidence). Overall, there was a significantly greater abundance (up to 1.3-fold; $p=0.001$) and richness (up to 1.2–fold; $p=0.05$) of fungal endophytes in the orchard with low *versus* high anthracnose incidence, while Shannon diversity ($p=0.05$) were similar among orchards (Fig. 3.2a). The increase in fungal abundance was more evident within endophytes of flowers (1.8-fold increase, $p=0.001$); whereas an opposite result was observed in fruits (1.5-fold decrease, $p=0.031$). Similarly, fungal richness in flowers and fruits was up to 1.5-fold and 1.3-fold significantly ($p<0.05$) higher in the orchard with low anthracnose incidence than in the ones with high incidence, respectively.

The fungal functional categories that changed more between the two orchards were beneficial and pathogenic fungi, and in less extent commensals (Fig. 3.2b). A significant decline in abundance and richness of beneficial and pathogenic fungi was observed in the orchard with high anthracnose incidence in relation to the orchard with low incidence of anthracnose. This decreased was most notorious within the endophytes colonizing the fruits (for beneficials) and the flower buds (for pathogens). Commensals increased significantly their abundance and richness in the orchard with low incidence in

relation to the orchard with high disease incidence, in particular within endophytes inhabiting flower buds and flowers.

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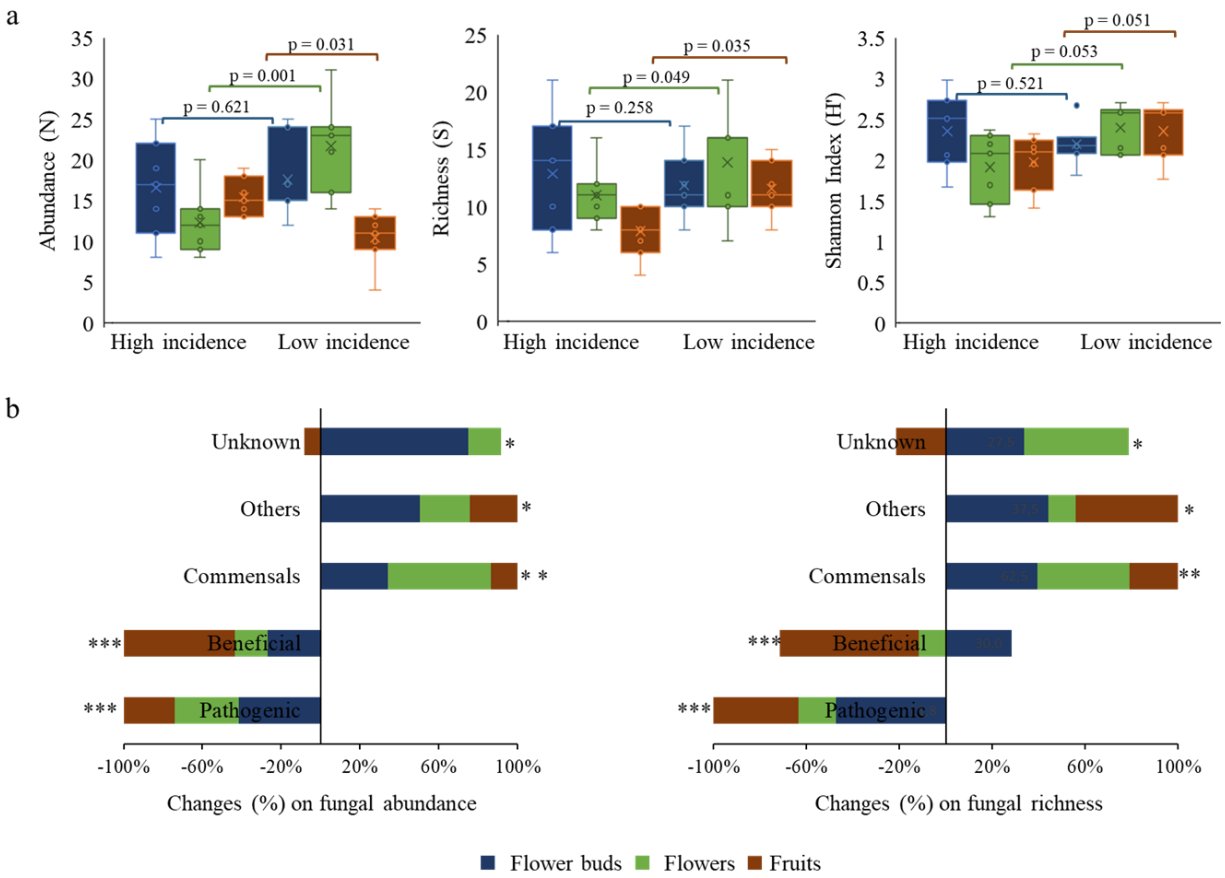

Fig. 3.2 Comparison of endophytic fungal diversity in reproductive organs of olive tree between orchards with high (High incidence) and low (Low incidence) incidence of olive anthracnose. (**a**) Diversity at community level by determining abundance, richness and Shannon–Wiener index. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (dots). Statistically differences between pairs of values are showed over horizontal lines. (**b**) Changes (%) on endophytic fungal abundance and richness between orchards with high and low incidence of anthracnose for each functional group. Asterisks indicate statistically significant differences between the two orchards (*p < 0.05, **p< 0.01, ***p < 0.001).

The whole fungal community composition significantly differs between orchards with high and low incidence of anthracnose, as revealed by the non-metric multidimensional scaling (NMDS) plots and analysis of similarities (ANOSIM; Global R=0.76, p=0.001) based on Bray-Curtis index (Fig. 3.3). These differences were higher within endophytes of fruits (R=0.91, p=0.001) than of flowers (R=0.74, p=0.001) or flower buds (R=0.55, p=0.001).

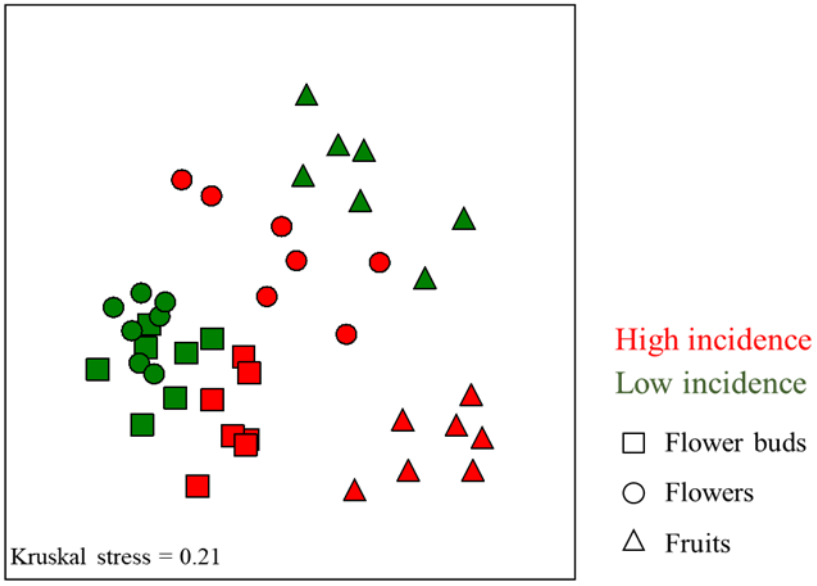

Fig. 3.3 Non-metric multidimensional scale (NMDS) plots for the endophytic fungal assemblages in olive tree due to different olive orchards (with high or low incidence of olive anthracnose) and organ (flower buds, flowers and fruits). Cluster analysis was performed with Bray-Curtis coefficient (raw abundance data). Kruskal's stress values are presented (values less than 0.2 represent good ordination plots).

For each orchard was similarly found differences on fungal endophytic composition between the three olive tree organs (Fig. 3.3), being these differences slightly greater in the orchard with high anthracnose incidence (Global R=0.77, p=0.001) than in the orchard with low incidence (Global R=0.70, p=0.001). Pairwise comparisons performed in orchards with high and low anthracnose incidence indicated that the fungal composition in the flower buds and flowers were the most similar (R=0.61 and R=0.60, p=0.001, respectively), followed by the flower buds and fruits (R=0.90 and R=0.92, p=0.001, respectively) and flowers and fruits (R=0.91 and R=0.95, p=0.001, respectively).

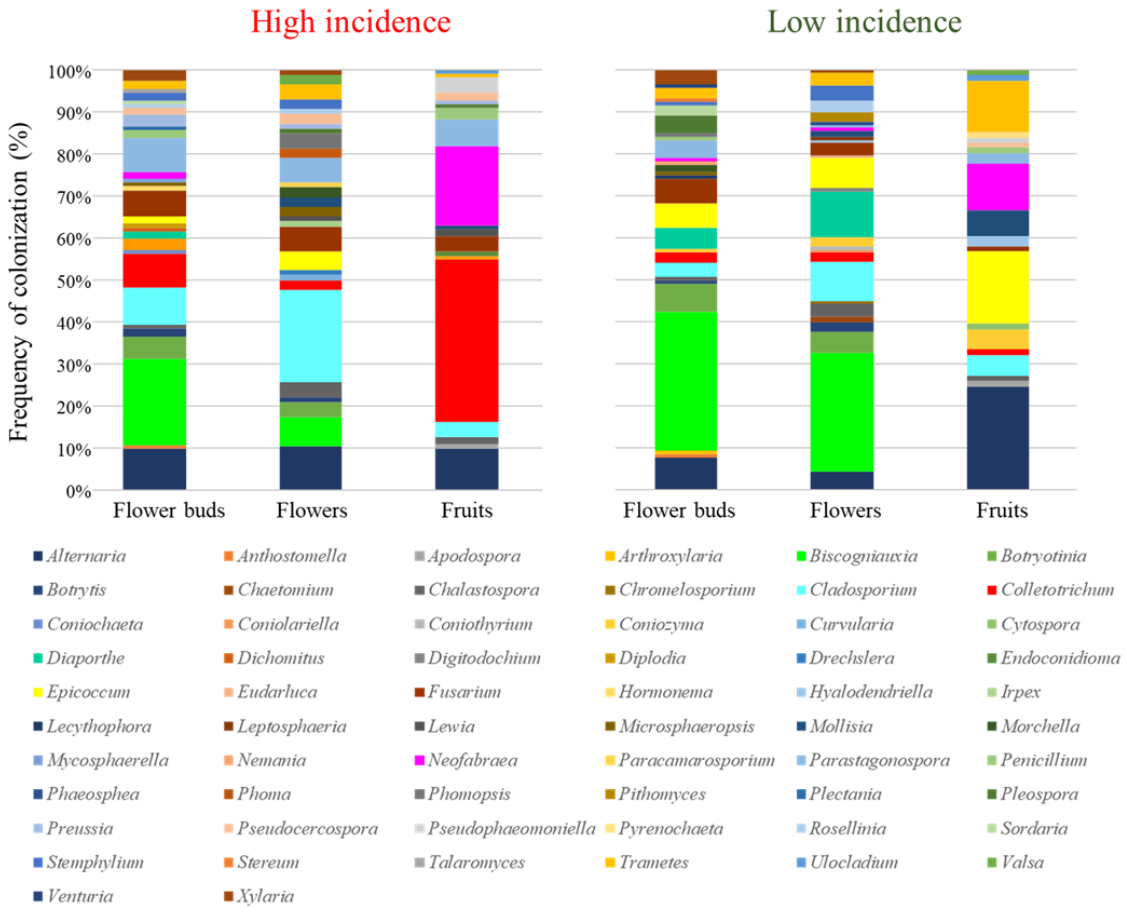

Fig. 3.4 Frequency of colonization (%) of fungal endophytes in flower buds, flowers and fruits of olive tree growing in orchards with high (High incidence) and low (Low incidence) incidence of olive anthracnose.

The trees growing in the orchard with high anthracnose incidence were colonized mostly by *Biscogniauxia*, *Cladosporium* and *Colletotrichum*, infecting more than 1.4, 1.6 and 2.6% of the total flower buds, flowers and fruits segments surveyed, respectively (Fig. 3.4). The most dominant fungi in trees from orchard with low disease incidence were *Biscogniauxia*, which occurred on more than 1.9% and 1.6% of the flower buds and flowers analysed, respectively, and *Alternaria* that colonized more than 1.2% of the fruits. Likewise, each orchard had several OTUs that were unique: 37 fungal OTUs were isolated only in the orchard with high incidence and 33 were recovered only in the orchard with low incidence (Fig. S3.2).

3.4.3 Contribution of different drivers for fungal community shaping

The relative contribution of the type of olive orchard (*i.e.*, high and low anthracnose incidence) and plant organ (flower buds, flowers and fruits) to the assembly of endophytic fungal community was assessed by using a variation partitioning (*Varpart*) analysis. Results revealed that fungal composition was mainly explained by the plant organ (responsible for 24% of the total variation, $F = 2.17$, $p = 0.005$), while the type of orchard explained 10% of the total community variation ($F = 1.98$, $p = 0.005$). A random forest analysis was then performed to identify which fungal OTUs were most important in differentiating either orchards (*i.e.*, with high and low anthracnose incidence; Fig. S3.3) or olive tree organs (*i.e.*, flower buds, flowers and fruits; Fig. S3.4). This analysis identified a total of ten and seventeen different fungal OTUs as being important in distinguishing the two olive orchards (Fig. S3.3) and plant organs (Fig. S3.4), respectively. The fungal OTUs that most differentiate orchards were *Epicoccum nigrum* (isolated from flowers and fruits), *Biscogniauxia mediterranea* and *Diaporthe rudis* (both isolated from flower buds and flowers) (Fig. S3.3). Plant organs were distinguished mostly by the fungal OTUs *B. mediterranea* (in both high and low incidence orchards) as well as *Neofabraea vagabunda* and *Pezizomycetes* sp. isolated in olive orchards with high and low anthracnose incidence, respectively (Fig. S3.4)

3.3.4 OTUs associated with each olive orchard and plant organ

In our study, the results show that both plant organ and type of olive orchard play an important role in fungal community assembly, suggesting the existence of an endophytic consortium associated with each organ or olive orchards. With an attempt to identify the composition of such consortium, a PCA was performed by using the fungal OTUs previously selected by the random forest analysis (Figs. S3.3 and S3.4).

Results revealed that the fungal OTUs *Pseudophaeomoniella oleae*, *Neofabraea vagabunda*, *Neofabraea* sp. and *Parastagonospora avenae* were the most associated with high anthracnose incidence, being all of them isolated from fruits (Fig. 3.5). This result is corroborated by the significantly positive correlation found between these fungal OTUs with *Colletotrichum* spp. abundance (Table S3.2). In contrast, *Diaporthe rudis* (from flowers), *Fusarium oxysporum* (from flower buds), *Pezizomycetes* sp., *Epicoccum nigrum*, *Mollisia minutella*, *Trametes* sp. and *Sardariomycetes* sp. (all isolated from fruits) were found to be highly associated to orchard with low anthracnose incidence (Fig.

3.5). Some of these fungal OTUs were also found to be significantly negatively correlated with *Colletotrichum* spp. abundance, as well as many other fungal OTUs (Table S3.2).

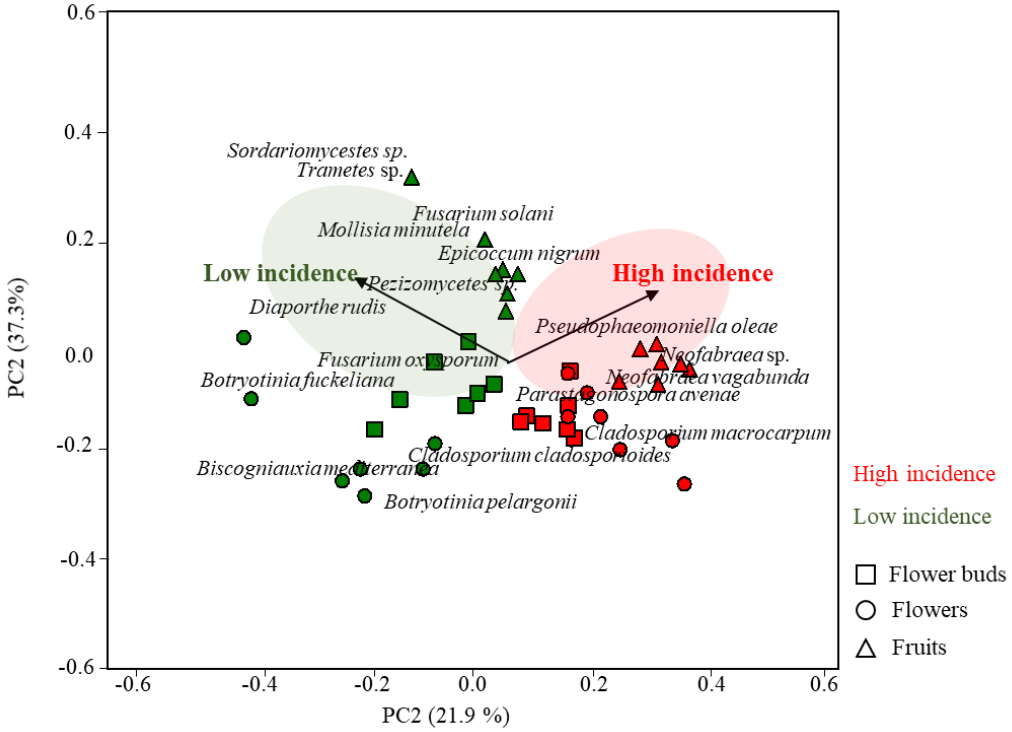

Fig. 3.5 Principal component analysis (PCA) of endophytic fungal community inhabiting flower buds, flowers and fruits of olive tree growing in orchards with high incidence (High incidence) and low (Low incidence) incidence of olive anthracnose. This analysis was performed with preselected fungal OTUs by the random forest analysis.

3.5 Discussion

In the present work, the diversity and composition of endophytic fungi of reproductive organs of olive tree from olive orchards with high and low incidence of olive anthracnose was investigated. Differences between these two orchards on disease incidence and severity as well as on *Colletotrichum* inoculum levels were corroborate by field anthracnose disease assessments, which validate their suitability to investigate the relevance of the endophytic fungal communities of olive tree on pathogen pressure. The olive cultivar surveyed was the same in both orchards, but probably there could be some differences in the environmental conditions due to the higher proximity of the orchard 1 (Abambres) to the river when compared to orchard 2 (Paradela). Thus, it is likely that the

relative humidity, which is a very important factor for anthracnose development (Talhinhas et al., 2018), can be slightly higher on orchard 1 than on orchard 2. This hypothesis needs to be confirmed. Apart from differences on *Colletotrichum* abundance and probably on relative humidity values, both orchards are within the same edapho-climatic zone and are managed in the same way.

In this study the endophytic fungal community associated to reproductive organs of olive tree growing in the two orchards was evaluated by using a culture-dependent method. Such approach may introduce biasness in estimating fungal diversity, being recently deduced a ratio of 1:8.8 of the numbers of cultured fungi and the OTUs detected by different culture-independent methods (Wu et al., 2019). However, culture-independent techniques may also have disadvantages. For example, most of the fungal OTUs identified by this technique were only assignable to order, family or genus level (Dissanayake et al., 2018; Jayawardena et al., 2018). In the culture-dependent method, most of the fungal taxa can be identify to species level (Jayawardena et al., 2018). Our priority in this work was to identify the wide variety of fungal taxa at the species level and get the cultures of specimens, in order to study in future works their role in the pathogenesis of the *Colletotrichum* spp., the causative agent of olive anthracnose. Although there are several growth media for cultivating fungal endophytes (Murphy et al., 2015), we chose to use basic nutrient-rich media (PDA), so that the beneficial endophytes could be easily mass-produced for agriculture use. Although the limitation of culture-dependent methods in recovering a small portion of the diversity (Wu et al., 2019), most of the fungal OTUs detected in the present work belong to Sordariomycetes and Dothideomycetes classes, as previously reported by Abdelfattah et al. (2015) who studied the fungal community associated to flowers and fruits of olive tree by 454-pyrosequencing.

3.5.1 In what way do the endophytic fungal communities differ between orchards with different disease incidence?

The endophyte communities of reproductive organs of olive tree at the two orchards were distinct, differing in species richness, abundance and composition. Although this variation between orchards did not provide information regarding the pathogen cause and effect, these results suggest varying pathogen-fungal community interactions in the orchard with high and low disease incidence. Perhaps, in the olive

orchard with low disease incidence both the pathogens *Colletotrichum* spp., and the disease cannot thrive so well as in the orchard with high disease incidence due to the richer and more abundant endophyte community. Indeed, in many cases the tolerance of host plants to diseases was correlated with increased endophytic fungal diversity (Busby et al., 2015). Additionally, in the orchard with high disease incidence was observed a decline in abundance and richness of beneficial endophytic fungi, which may probably contain antagonistic fungi that limit the development of *Colletotrichum* spp.. and of disease. Some of the declined fungal OTUs we identified have previously been reported to inhibit *Colletotrichum* spp. in detached olive fruits, such as *Epicoccum nigrum* and *Chaetomium globosum* (Preto et al., 2017). Similarly, many other studies have already showed the capacity of fungal endophytes to interact directly with the pathogen in a way that can affect the progress of the disease in several pathosystems (for review, see Busby et al., 2015). Besides beneficial fungi, the abundance and richness of pathogenic fungi of other plant species was also observed to decline in the orchard with high disease incidence. These results suggest that this group of fungi might also have an important role in the development of olive anthracnose. Indeed, the interspecific competition between pathogenic fungi colonizing various plant parts have been already showed to play an important role in pathogenesis (for review, see Abdullah et al., 2017). Besides *Colletotrichum*-endophytic interaction, the differences on relative humidity values that probably occur among olive orchards might not be disregard as a valid explanation for the differences in endophyte communities. There has been little work studying the context-dependency of endophyte-mediated disease outcomes in field/realistic conditions (Whitaker and Bakker, 2019). The few studies available in controlled conditions showed that plant-pathogen-endophyte interaction outcomes are dependent on the environmental conditions (Whitaker and Bakker, 2019). Therefore, in our work the environment cannot be excluded as an important factor in determining changes in endophytic fungal community.

3.5.2 In what way do the endophytic fungal communities differ among the different pathogen's lifestyle (*i.e.*, from early flowering stages until fruit set)?

Colletotrichum species responsible to cause olive anthracnose may be either commensal or pathogenic depending on the developmental stage of the host (Sergeeva et al., 2008). During the flowering, the fungus adopts an endophytic- or latent-lifestyle, and

upon fruit ripening the fungus shift to a necrotrophic phase (De Silva et al., 2017; Sergeeva et al., 2008). Since differences on endophytic species composition among orchards with high and low anthracnose incidence were mostly noticed within fruits than in flowers, we hypothesized that these changes in endophytic composition may influence the lifestyle shifts in *Colletotrichum* spp.. The transition of the fungus *Moniliophthora perniciosa* to a more aggressive lifestyle was previously suggested to be triggered by their interaction with other plant microbes (Bezemer et al., 2006). Our hypothesis is reinforced by the fact that a higher difference on fungal endophytic composition among flowers and fruits is observed at the orchard with the higher anthracnose incidence. Thus, it seems that there is a relationship between the fungal community structure and anthracnose disease incidence: the greater similarity on fungal composition between fruits and flowers (where *Colletotrichum* spp. occurs as latent), the lesser is disease incidence. Such hypothesis should be confirmed in future work.

Plant organ was also found to significantly affect the composition of endophytic fungal community in the olive tree endosphere as reported previously for several woody plant species (Moricca et al., 2012), including olive tree (Martins et al., 2016; Gomes et al., 2018). However, most of these studies have been focused on vegetative organs, being less studied the microbiota that inhabits the reproductive organs of woody species. As far as we known, only Abdelfattah et al. (2015) have similarly found differences on fungal composition (both epiphytes and endophyte) between fruits and flowers of olive tree. The organ-specificity of fungal endophytes observed in our work could be related to morphological and chemical differences between flowers and fruits, as previously suggested for vegetative organs (Gomes et al., 2018). Flowers and fruits seem to produce distinct microhabitats, potentially selecting for specific microbial colonizers. Since the switching of *Colletotrichum* spp. from endophytic to pathogenic mode occur preferably on fruits, we hypothesized that the changes in endophytic community composition between flowers and fruits may play an important role in the anthracnose development. Thus, the ability of each plant organ to shape its endophytic community seems to serve as an additional layer of defence to *Colletotrichum* spp.. Indeed, flower microbiota has been increasingly recognized as important agents of disease control (Aleklett et al., 2013). Such hypothesis needs however to be confirmed.

3.5.3 Is there any fungal consortium specifically associated with high ("disease-promoting fungi") or low ("disease-suppression fungi") anthracnose incidence as well as with a specific pathogen's lifestyle?

Our data revealed that a consortium of fungal OTUs is associated with each organ in orchards with different anthracnose incidence. It is assumed that these consortia probably have relevance to the pathogenesis of *Colletotrichum* spp. and consequently to disease incidence. The fungal OTUs best correlated with high incidence of anthracnose are pathogens of olive tree (*Neofabraea* sp., *Neofabraae vagabunda* and *Pseudophaeomoniella oleae*) (Romero et al., 2015; Nigro and Antelmi, 2015) or of wheat plants (*Parastagonospora avenae*) (Cunfer, 2009). These fungi are positively correlated with *Colletotrichum*, and can be characterized as "pathogen facilitators", helping the pathogen to successfully infect the plant or increase the severity of the disease. In contrast, *Diaporthe rudis* (from flowers), *Fusarium oxysporum* (from flower buds), *Pezizomycetes* sp., *Epicoccum nigrum*, *Mollisia minutella*, *Trametes* sp. and *Sardariomycetes* sp. (all isolated from fruits) were found to be highly associated to orchard with low anthracnose incidence. Most of these fungal OTUs have been reported as protective. For example, *E. nigrum* isolated from rye and wheat grains demonstrated to be effective against *Fusarium* spp., limiting its growth under *in vitro* conditions (Ogórek and Plaskowska, 2011). The capacity of non-pathogenic strains of *F. oxysporum* to control *Fusarium* diseases has been also demonstrated (Alabouvette and Olivain, 2018). Species of the genus *Trametes* are well known for their ability to produce enzymes, which could assist in the defence of the plant against pathogens (Fonseca et al., 2016). Similarly, species from Pezizomycetes have been reported to offer benefits to their host plants (Hansen et al., 2013).

Overall, the abundance, richness and composition of fungal endophytes inhabiting the reproductive organs of olive tree differ between orchards with variable anthracnose incidence, suggesting varying *Colletotrichum*-endophytic interactions in the orchard with high and low disease incidence. These differences were greater on fruits than on flowers, which suggested that the lifestyle transition in *Colletotrichum* spp. from latent (during flower stage) to pathogen (during fruit stage) might be related to the endophytic fungal community structure. A set of fungal OTUs were found to be associated to orchards with either high or low anthracnose incidence, which their role to decrease (antagonism) or increase (facilitation) olive anthracnose development should be study in a near future.

Acknowledgments

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3.7 Support information

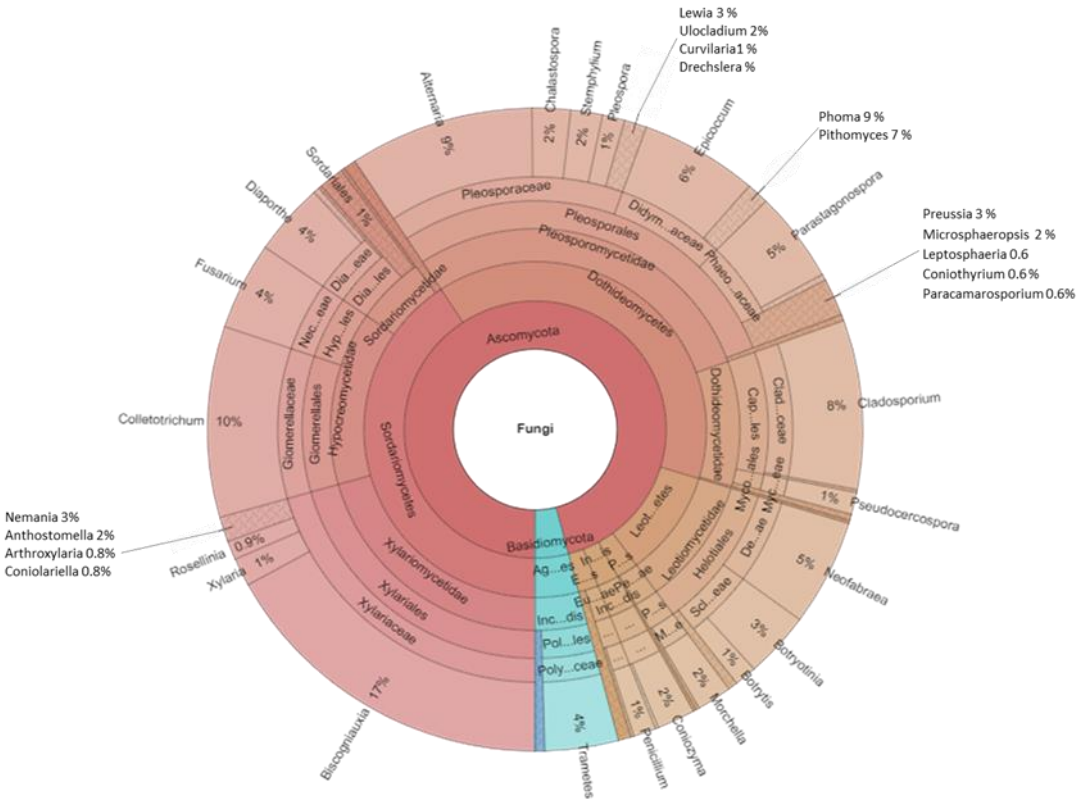


Fig. S3.1 Krona chart of the taxonomic affiliation down to the genus level of the endophytic fungal community associated to olive tree from the two orchards surveyed.

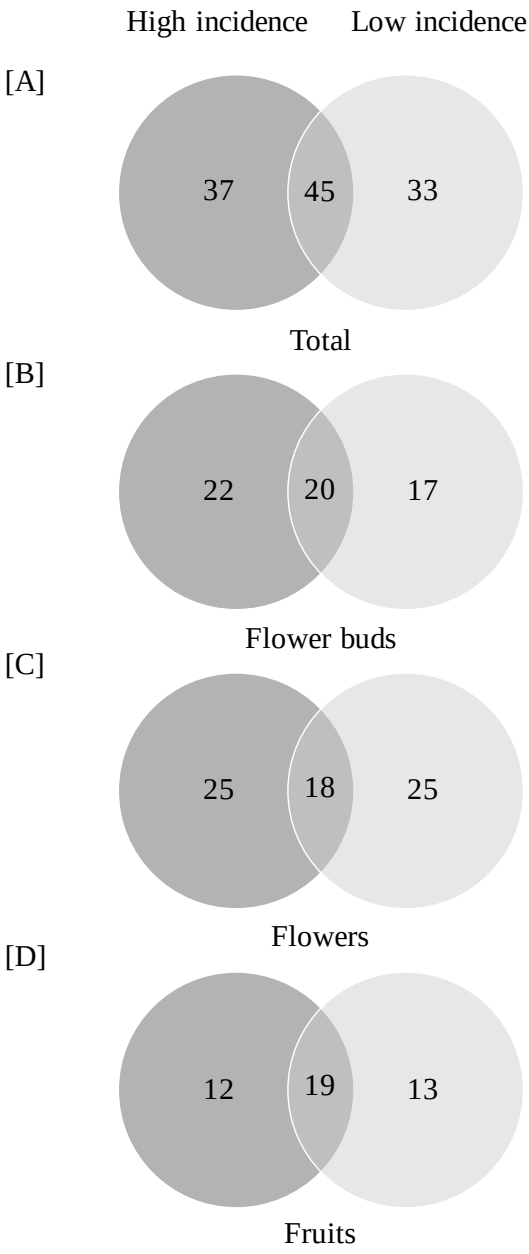


Fig. S3.2 Venn diagrams representing the total number of fungal OTUs shared between high (High incidence) and low (Low incidence) incidence of olive anthracnose when considering the [A] total, [B] flower buds, [C] flowers and [D] fruits samples.

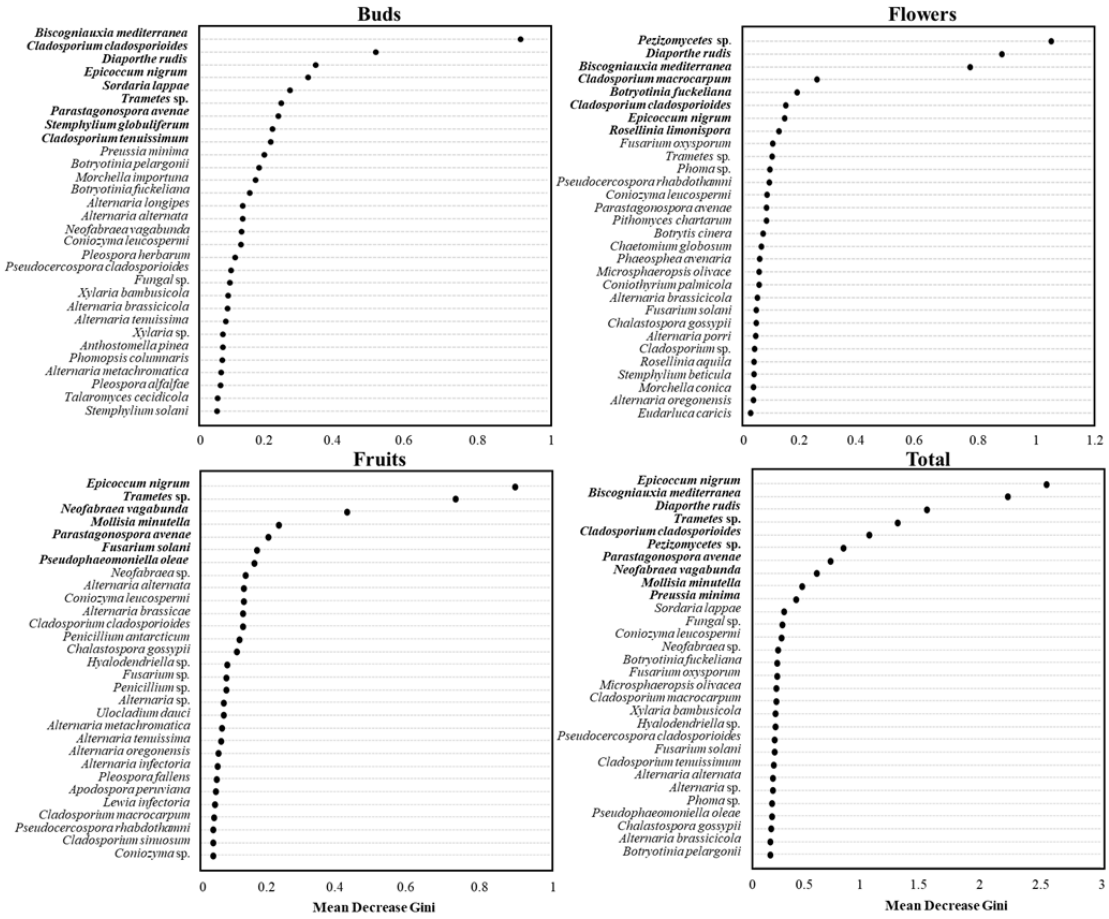


Fig. S3.3 Ranking of the relative importance of each fungal OTUs to distinguish between the high (High incidence) and low (Low incidence) incidence of olive anthracnose in each olive tree organ (flower buds, flowers and fruits) or in total organs. Mean Decrease Gini value measure the importance of OTUs, with highest values representing the best predictors. The OTUs in bold were considered as the main relevant to distinguish olive orchards with high and low incidence of olive anthracnose.

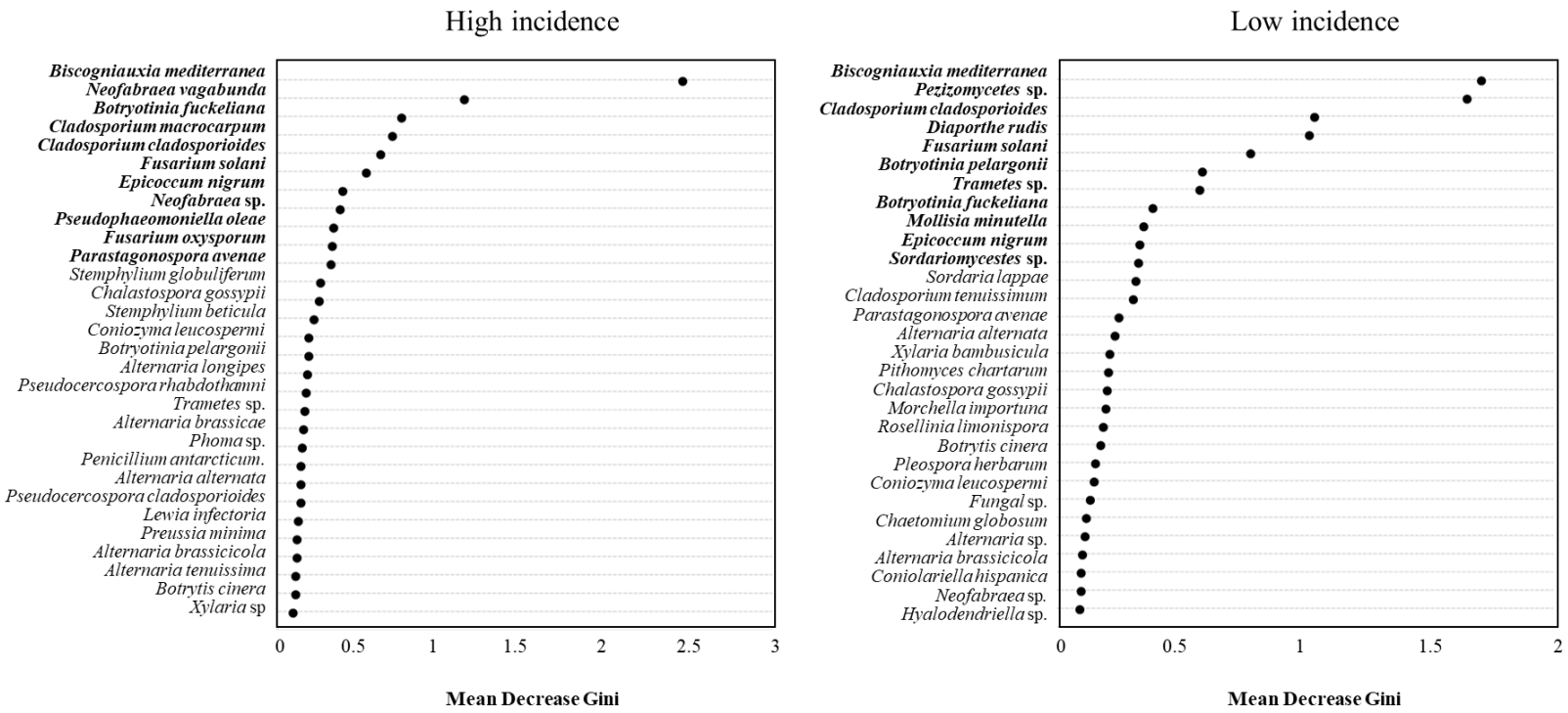


Fig. S3.4 Ranking of the relative importance of each fungal OTUs to distinguish between the olive organs (flower buds, flowers, and fruits) within each olive orchard, high (High incidence) and low (Low incidence) incidence of olive anthracnose. Mean Decrease Gini value measure the importance of OTUs, with the highest values representing the best predictors. The OTUs in bold were considered as the main relevant to distinguish the olive tree organs in each orchard (high and low incidence of olive anthracnose).

Table S3.1 Functional categories of the several fungal operational taxonomic unit (OTU) identified in flower buds, flowers and fruits from *cv. Madural*.

Operational taxonomic unit (OTU)	**Functional Categories**
Alternaria alternata	Plant pathogen/Commensalistic
Alternaria arborescens	Plant pathogen
Alternaria brassicae	Plant pathogen/Commensalistic
Alternaria brassicicola	Plant pathogen
Alternaria eureka	Plant pathogen
Alternaria infectoria	Plant pathogen
Alternaria longipes	Plant pathogen
Alternaria metachromatica	Plant pathogen
Alternaria oregonensis	Plant pathogen/Others
Alternaria porri	Plant pathogen
Alternaria sp.	Plant pathogen/Others
Alternaria tenuissima	Plant pathogen/Others
Alternaria triticina	Plant pathogen
Alternaria consortialis	Plant pathogen
Anthostomella pinea	Others
Apodospora peruviana	Unknown
Apodospora sp.	Unknown
Arthroxylaria elegans	Commensalistic
Biscogniauxia mediterranea	Plant pathogen
Botryotinia fuckeliana	Plant pathogen
Botryotinia pelargonii	Plant pathogen
Botrytis cinera	Plant pathogen
Chaetomium globosum	Beneficial
Chalastospora gossypii	Beneficial
Chromelosporium carneum	Unknown
Cladosporium cladosporioides	Plant pathogen/Beneficial
Cladosporium cucumerinum	Plant pathogen
Cladosporium herbarum	Plant pathogen/Commensalistic
Cladosporium iridis	Plant pathogen
Cladosporium macrocarpum	Others
Cladosporium sinuosum	Plant pathogen
Cladosporium sp.	Plant pathogen/Commensalistic
Cladosporium tenellum	Unknown
Cladosporium tenuissimum	Plant pathogen
Colletotrichum clavatum	Plant pathogen
Colletotrichum fioriniae	Plant pathogen
Colletotrichum godetiae	Plant pathogen
Coniochaeta nepalica	Others
Coniolariella hispanica	Unknown

Coniothyrium palmicola	Unknown
Coniozyma leucospermi	Unknown
Coniozyma sp.	Unknown
Curvularia trifolii	Plant pathogen
Cytospora pruinosa	Plant pathogen
Diaporthe rudis	Plant pathogen
Dichomitus squalens	Plant pathogen
Digitodochium rhodoleucum	Unknown
Diplodia mutila	Plant pathogen
Drechslera dematioidea	Plant pathogen
Endoconidioma populi	Unknown
Epicoccum nigrum	Plant pathogen/Beneficial
Eudarluca caricis	Commensalistic
Fungal sp.	Unknown
Fusarium lateritium	Plant pathogen
Fusarium oxysporum	Plant pathogen
Fusarium solani	Plant pathogen
Fusarium sp.	Plant pathogen/Commensalistic
Hormonema carpetanum	Unknown
Hyalodendriella sp.	Unknown
Irpex lacteus	Plant pathogen
Lecythophora sp.	Plant pathogen
Leptosphaeria sp.	Plant pathogen
Lewia infectoria	Plant pathogen
Microsphaeropsis olivace	Plant pathogen/Beneficial/Others
Mollisia minutella	Unknown
Morchella conica	Beneficial
Morchella importuna	Beneficial
Mycosphaerella aurantia	Plant pathogen
Nemania serpens	Plant pathogen
Neofabraea vagabunda	Plant pathogen
Neofabraea sp.	Plant pathogen
Paracamarosporium leucadendri	Unknown
Parastagonospora avenae	Plant pathogen
Parastagonospora sp.	Plant pathogen
Penicillium antarcticum	Commensalistic
Penicillium chrysogenum	Commensalistic
Penicillium glabrum	Plant pathogen
Penicillium sp.	Plant pathogen/Beneficial
Pezizomycetes sp.	Commensalistic
Phaeosphaeria avenaria	Plant pathogen
Phoma herbarum	Plant pathogen
Phoma macrostoma	Plant pathogen
Phoma sp.	Plant pathogen
Phomopsis columnaris	Plant pathogen/Others

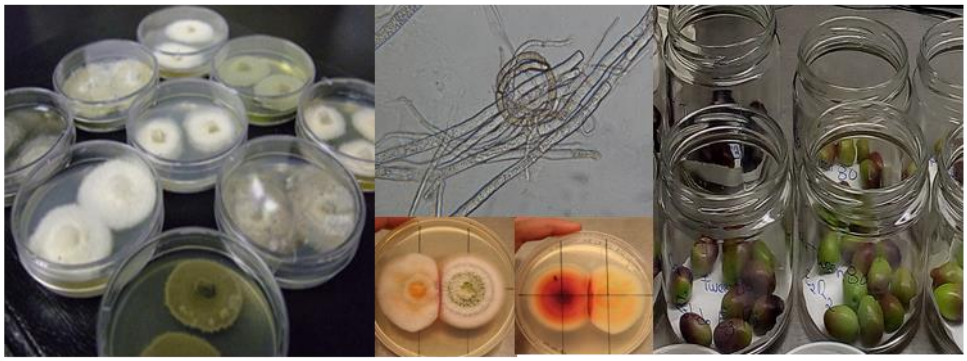
Pithomyces chartarum	Plant pathogen
Plectania sp.	Unknown
Pleospora alfalfae	Plant pathogen
Pleospora fallens	Plant pathogen
Pleospora herbarum	Plant pathogen
Preussia minima	Plant pathogen
Pseudocercospora cladosporioides	Plant pathogen
Pseudocercospora rhabdothamni	Plant pathogen
Pseudophaeomoniella oleicola	Plant pathogen
Pyrenochaeta corni	Unknown
Rosellinia aquila	Unknown
Rosellinia limonispora	Unknown
Rosellinia sp.	Unknown
Rosellinia thelena	Unknown
Sordaria lappae	Other
Sordaria macrospora	Plant Pathogen
Stemphylium beticola	Plant Pathogen
Stemphylium globuliferum	Plant Pathogen
Stemphylium solani	Plant Pathogen
Stereum sp.	Commensalistic
Talaromyces cecidicola	Unknown
Trametes sp.	Unknown
Ulocladium dauci	Plant pathogen
Valsa cypri	Plant pathogen
Venturia fraxini	Plant pathogen
Xylaria arbuscula	Others
Xylaria bambusicola	Others
Xylaria sp.	Others

Table S3.2 Correlation coefficient (Cr) between the abundance of endophytic fungal OTUs preselected by the random forest analysis with the abundance of the pathogen *Colletotrichum godetiae* or *Colletotrichum fioriniae*. All Cr values are significant at ($p<0.001$). A correlation of -1.0 shows a perfect negative correlation, while a correlation of 1.0 shows a perfect positive correlation. A correlation of 0.0 shows no relationship between the two variables. Positive correlations are indicated in bold.

Endophytic fungi	**Pathogenic fungi**	**Correlation coefficient**
Biscogniauxia mediterranea	*Colletotrichum fioriniae*	-0.206
Botryotinia fuckeliana	*Colletotrichum fioriniae*	-0.141
Cladosporium cladosporioides	*Colletotrichum fioriniae*	-0.013
Cladosporium macrocarpum	*Colletotrichum fioriniae*	-0.108
Biscogniauxia mediterranea	*Colletotrichum godetiae*	-0.409
Botryotinia fuckeliana	*Colletotrichum godetiae*	-0.153
Cladosporium cladosporioides	*Colletotrichum godetiae*	-0.466
Cladosporium macrocarpum	*Colletotrichum godetiae*	-0.458
Epicoccum nigrum	*Colletotrichum fioriniae*	-0.141
Epicoccum nigrum	*Colletotrichum godetiae*	-0.480
Fusarium oxysporum	*Colletotrichum fioriniae*	-0.091
Fusarium oxysporum	*Colletotrichum godetiae*	-0.370
Fusarium solani	*Colletotrichum fioriniae*	0.193
Fusarium solani	*Colletotrichum godetiae*	-0.109
***Neofabraea* sp.**	***Colletotrichum fioriniae***	**0.501**
***Neofabraea* sp.**	***Colletotrichum godetiae***	**0.488**
Neofabraea vagabunda	*Colletotrichum fioriniae*	-0.135
Neofabraea vagabunda	***Colletotrichum godetiae***	**0.657**
Parastagonospora avenae	***Colletotrichum fioriniae***	**0.010**
Parastagonospora avenae	*Colletotrichum godetiae*	-0.097
Pseudophaeomoniella oleae	***Colletotrichum fioriniae***	**0.810**
Pseudophaeomoniella oleae	***Colletotrichum godetiae***	**0.569**

Chapter 4

Diversity and antagonistic activity of endophytic fungi associated with olive tree cultivars



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4.1 Abstract

Olive anthracnose is a devastating fungal disease caused by species of the genus *Colletotrichum*, which currently has no effective control strategies. The use of naturally fungal endophytes as biological control agents could be an environmental-friendly control strategy for integrated management of this disease. This study aims to disclose the potential role of fungal endophytes on olive tree resistance to anthracnose and select the strains with the greatest ability to antagonized *Colletotrichum* sp. To this end, the endophytic fungal communities from roots, twigs, and leaves of three olive cultivars with variable susceptibility to anthracnose (ranging from susceptible to resistant), were characterized by rDNA sequencing of the cultivable isolates, and the antagonistic effect of some of these isolates against *Colletotrichum* sp. was assessed by dual culture and fruit inoculations assays. Overall, roots had a higher richness and abundance of fungal endophytes than twigs and leaves, being this difference greatest in the most resistant cultivars. Co-inertia analysis showed that the endophytes most associated to susceptible cultivar comprise plant pathogens, while the ones most associated to resistant cultivars are recognized biocontrol fungi. All the endophytes tested in dual culture assays were able to inhibited *C. acutatum* growth, sporulation and germination, and to cause abnormalities in pathogenic hyphae. Following multivariate analyses, this level of inhibition was dependent on endophytic origin. Endophytes from twigs of susceptible cultivar had the highest capacity to inhibit the pathogen growth, while inhibition of germination and sporulation of *C. acutatum* were associated to endophytes from leaves of moderately susceptible cultivar. *Hypocrea lixii* was the most effective antagonistic strain, showing capacity to reduce anthracnose incidence (up to 2.6-fold) and severity (up to 1.9-fold), but with variable efficiency according to the pathogen species and fruit maturation index. Altogether, results suggest that the fungal communities inhabiting each of the cultivars might be an important determinant of host plant resistance to olive anthracnose.

4.2 Introduction

Plants are colonized by complex communities of fungal endophytes that inhabit the interior of both below- and above-ground tissues (Martins et al., 2016). Studies carried out in the last decade have demonstrated that these microorganisms may benefit the host plant by enhancing its growth, yield and resistance to pests and diseases (*e.g.*, Lata et al., 2018; Khare et al., 2018). Plant protection against diseases is mainly attributed to endophytic production of secondary metabolites in colonized plants (Lacava and Azevedo, 2014). These metabolites may directly suppressed pathogens or may indirectly enhance host plant resistance/defense (Eyles et al., 2010; Gao et al., 2010). Because of the beneficial impact of endophytes on host plant, the bioprospecting of fungal endophytes for application in agriculture have been increasing (Lugtenberg et al., 2016)

The olive anthracnose, a disease caused by several species of the genus *Colletotrichum*, is one of the most serious constrains to the olive production worldwide (Cacciola et al., 2012). This disease affects aboveground olive tree organs, but mostly the fruits, by causing rotting, mummification and premature fall of fruits (Cacciola et al., 2012; Mosca et al., 2014). In all of the countries where it occurs severe damage has been registered on both yield and oil quality (Cacciola et al., 2012). Control measures of the disease relied extensively on the application of fungicides, with narrow efficacy (Cacciola et al., 2012) and not compatible with sustainable production systems, which is one of the main Sustainable Development Goal of the 2030 Agenda. Therefore, the use of fungal endophytes in the biological control of olive anthracnose could be a very promising approach (Landum et al., 2016; Preto et al., 2017). There are extremely few studies to date on this topic, being only few fungal endophytes demonstrated the capacity to reduce the mycelial growth, sporulation and viability of *Colletotrichum acutatum*, under *in vitro* conditions (Ladum et al., 2016; Martins et al., 2017; Preto et al., 2017). In field situation, the application of fungal endophytes showed to be less effective in reducing olive anthracnose disease (Nigro et al., 2018). The bioprospecting of naturally occurring endophytic fungi in olive tree might increase the biocontrol efficacy. Indeed, these fungi are more adapted to the host plant, the resident microbiota and the environment, which may be important to prevent failures (Köhl et al., 2019). In this respect, the bioprospecting of fungal endophytes in olive tree cultivars resistant or less susceptible to olive anthracnose seems to be a promising approach. These plants may host a microbial community that might be potentially important in conferring host resistance to disease.

This suggestion is supported by studies that have shown a link between endophytic communities and the susceptibility of host plant to diseases (Podolich et al., 2015; Gomes et al., 2019). For example, the endophytic fungal community of disease-resistant olive cultivar *Cobrançosa* was observed to be higher in abundance and richness, in particular of "protective" endophytes, and to be differently affected by pathogen invasion as compared to endophytic fungal communities of susceptible cultivars (Gomes et al., 2019). Besides host plant genotype, the biocontrol provided by the fungal endophytes might also depend on the plant organ from which they are isolated. Indeed, it was demonstrated previously that organs of a single plant may differ in both fungal endophytic fungal community composition (Martins et al., 2016; Gomes et al., 2018) and bioactive secondary metabolites (Porras-Alfaro and Bayman, 2011). Therefore, the rational selection of host plant and organ type for endophyte isolation might be an important step to maximize the possibility for discovering endophytes with biocontrol abilities. As far as we known, this plant and organ selection strategy has never been exploited.

In this work, the endophytic fungal community associated to roots, twigs and leaves of three olive cultivars of varying susceptibilities to anthracnose was characterized by DNA sequencing of the internal transcribed spacer region of the isolates. The ability to antagonize the pathogen *C. acutatum* of several fungi isolated from the three organs of both anthracnose-susceptible and -moderately susceptible cultivars was then evaluated through *in vitro* assays. The ability of the most antagonistic isolate to reduced anthracnose disease development was then evaluated in olive fruit inoculation assays. With this work we intend to answer the following questions: i) Is the difference in the susceptibility of cultivars to anthracnose associated with a particular endophytic fungal consortia naturally present in roots, twigs and leaves?; ii) Is the antagonistic effect of native endophytes against *C. acutatum* linked to their origin in terms of the plant host (*i.e.*, susceptibility to anthracnose) and plant organ (*i.e.*, root, twig and leaf)?; iii) Which mechanisms are involved in the antagonistic effect displayed by native endophytes against *C. acutatum*?

4.3 Materials and methods

4.3.1 Plant sampling

The olive trees surveyed were from three different cultivars (cvs.) with variable susceptibility to olive anthracnose: *Galega vulgar* (susceptible), *Cobrançosa* (moderately susceptible) and *Picual* (resistant) (Moral et al., 2017). Five orchards, which geographic location is indicated in Table 4.1, were selected for each cultivar to collect the plant material for endophytic isolation. The selected orchards encompasses olive trees with ages ranging from 30 to 40 years, planted at a density of 7x7 m, and have been managed by following the integrated production guidelines (Malavolta and Perdikis, 2018). In each orchard, seven healthy trees were randomly selected, and five samples of roots, twigs and leaves were collected *per* tree between January and August of 2014. Roots were collected from the upper part of the A horizon, 5 cm below the soil surface. All samples were placed in individual plastic bags, labelled, and transported to the laboratory in an icebox. The plant material was stored at 4ºC for fungal isolation.

Table 4.1 Location of the olive orchards where the plant organs of the olive tree cultivars *Galega vulgar*, *Cobrançosa* and *Picual* were collected during the year of 2014.

Olive cultivar	Olive orchard	Location	GPS Coordinates
Galega vulgar	1	Castelo Branco, Portugal	39º 49' 16.475"N, 7º 27' 34.672"W
	2	Castelo Branco, Portugal	39º 49' 14.801"N, 7º 27' 37.292"W
	3	Castelo Branco, Portugal	40º 12' 1.555"N, 7º 33' 36.713"W
	4	Castelo Branco, Portugal	40º 11' 48.271"N, 7º 33' 43.607"W
	5	Castelo Branco, Portugal	40º 10' 52.558"N, 7º 33' 8.507"W
Cobrançosa	1	Bragança, Portugal	41º 19' 50.880"N, 7º 4' 20.640"W
	2	Bragança, Portugal	41º 19' 24.960"N, 7º 4' 21.720"W
	3	Bragança, Portugal	41º 29' 30.84"N, 7º 15' 18.84"W
	4	Bragança, Portugal	41º 29’ 490"N, 7º 15' 413"W
	5	Bragança, Portugal	41º 26' 38"N, 7º 13' 16"W
Picual	1	Deifontes, Spain	37º 19' 52.5"N, 3º 33' 43.32"W
	2	Deifontes, Spain	37º 19' 12.78"N, 3º 34' 14.82"W
	3	Generalife, Spain	37º 10' 13.74"N, 3º 34' 24.96"W
	4	Jaén Spain	37º 56' 53.94"N, 3º 14' 7.92"W
	5	Jaén Spain	37º 56' 53.22"N, 3º 14' 1.14"W

4.3.2 Plant material sterilization and fungal isolation

The collected roots, leaves and twigs were washed with abundant distillate water, and the roots and twigs were cut into 4 cm long segments. Samples were surface sterilized according to a procedure previously optimized for the different olive plant tissues and

described by Martins et al. (2016). The success of surface sterilization method was confirmed for each set of five plant tissues segments by plating the last washing water of the sterilization procedure onto DifcoTM potato-dextrose agar (PDA) medium. Each sterilized root and twig was cut into five segments (4-5 mm in length), and for each leaf, four segments (5 × 5 mm) from the lamina and one from the petiole (5 mm in length) were excised. The segments were immediately transferred to 9 cm diameter Petri plates, containing 10 ml of PDA medium supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). Each plate contained five tissue segments of the total 25 fragments assayed *per* plant organ and tree. In total, 7875 segments were used in this study to isolate endophytes (5 orchards x 3 cultivars x 7 trees x 3 plant organs x 25 tissue segments). Petri plates were sealed with parafilm and incubated in the dark at 25 ± 2ºC. Fungi growing out of the segments were recorded as endophytic fungi and were sub-cultured on individual PDA plates for subsequent identification. Pure cultures of each isolate were deposited in the culture collection of the Mountain Research Centre (CIMO), School of Agriculture - Polytechnic Institute of Bragança.

4.3.3 Identification of isolates

Fungal isolates growing in PDA plates for two weeks were grouped according to their macro and micromorphological characteristics. Two isolates representative of each morphotype were selected for further molecular identification using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). DNA was extracted from fungal spores/mycelium, using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK), in accordance to manufacturer's instructions. The PCR was performed with the primers *ITS1* and *ITS4* (White et al., 1990), by using the same DNA extraction kit following the manufacturer's instructions, in the MyCycler thermal cycler (BioRad). The temperature cycle used in the amplification was 94°C for 3 min (1 cycle); 94°C for 30 sec, 53°C for 50 sec, 72°C for 2 min (35 cycles); and 72°C for 10 min (1 cycle). PCR products were purified and sequenced by Macrogen (Madrid, Spain). Sequence analysis of the ITS sequences was carried out using DNASTAR v.2.58 (Lasergene) software. The search for homologous sequences was done using Basic Local Alignment Search Tools at the NCBI (http://www.ncbi.nlm.nih.gov) and UNITE (https://unite.ut.ee/) databases. The blast results were analysed according to the parameters defined by Raja et al. (2017). All operational taxonomic unit (OTU) was

taxonomically classified at species or genus level according to the Index Fungorum Database.

4.3.4 Diversity and composition of fungal community

The diversity of fungal endophytes within the plant organs (root, twig, leaf and total) of each olive tree cultivar were evaluated by determining the abundance (average number of isolates *per* tree), richness (average number of OTUs *per* tree) and Simpson's Reciprocal Index (1/D) index, which was estimated in *Species Diversity and Richness v. 4.0* software (Seaby and Henderson, 2006). The results are presented as the mean of seven independent experiments (= tree) displaying the respective SE values. Differences among the means were determined by an analysis of variance (ANOVA) with SPSS v.18 software, and the averages were compared using Tukey's test ($p<0.05$). A non-metric multidimensional scaling (NMDS) was performed using Bray-Curtis index obtained from a normalized abundance OTU matrix, in order to describe differences on the fungal endophyte community composition between plant organs (root, twig, and leaf) for each olive tree cultivar (*Galega vulgar*, *Cobrançosa* and *Picual*). To test significant differences between the fungal community groups observed in NMDS ordinationan, an analysis of similarity (ANOSIM) was performed using Bray–Curtis distance matrices, with a significance level of 0.05. This analysis originates an R-value ranging from 0 (completely similar) to 1 (completely dissimilar) (Clarke and Gorley, 2015). Both NMDS and ANOSIM analyses were conducted on the *Community Analysis Package v. 5.0* (Henderson and Seaby, 2014).

A co-inertia analysis (CIA) was performed in order to identify the fungal endophytes (at genus level) that are more associated to a specific plant organ and cultivar. This analysis was performed in R software (R Core Team, 2018), using the *coinertia* function in the "*ade4*" package and the *table.value* function to visualize the results.

4.3.5 *In vitro* antagonistic assays

From the identified endophytic fungi, five OTUs from cv. *Galega vulgar* (namely *Trichoderma gamsii*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Penicillium purpurogenum* and *Phomopsis columnaris*) and five OTUs from cv. *Cobrançosa* (namely *Penicillium commune*, *Penicillium roseopurpureum*, *Paecilomyces*

lilacinus, *Fusarium oxysporum* and *Hypocrea lixii*) were selected for the screening of potential antagonists of the pathogen *Colletotrichum acutatum*. These endophytes were selected based on their high abundance, exclusively occurrence in a specific organ, and capacity to growth on artificial media. *P. roseopurpureum*, *M. phaseolina* and *P. columnaris* were isolated from the roots, while *P. lilacinus* and *F. oxysporum* from the twigs, and *H. lixii, P. commune, T. gamsii, P. purpurogenum* and *F. oxysporum* from the leaves. *Colletotrichum acutatum* strain (COCa2011) was obtained from the microbial collection of the Polytechnic Institute of Bragança (Portugal). This fungus, which is one of the main causal agents of olive anthracnose, was isolated from the inner tissues of naturally infected olives of cv. *Cobrançosa* (Mirandela, Portugal) and previously molecularly identified by sequencing the ITS region of rDNA using both the universal *ITS1* and *ITS4* primers (White et al., 1990), and degenerate primers *Coll1F* and *Coll3Rb* (Mosca et al., 2014).

The hyphal interaction between endophytes and *C. acutatum* was studied by the establishment of dual cultures in Petri dishes (9 cm diameter) containing 10 ml of PDA. Petri dishes were inoculated with 10 µl of spore suspension (10^6 spores/ml) of each fungus, on opposite sides of the plate (about 3 cm apart). The spore suspension was obtained by transferring mycelial plugs removed from two-week-old pure cultures, into a tube with 1 ml of sterile 0.025% (v/v) Tween 80. For the fungi that did not sporulate (*i.e.*, *P. columnaris*), the inoculation of Petri dishes was performed by placing mycelial plugs (5 mm diameter) removed from two-week old pure cultures. As controls, fungal pairings of the same isolate were prepared. Five replicates of each combination were performed, and the plates were sealed with parafilm and incubated in the dark at 25 ± 2ºC. Several parameters were evaluated in the antagonistic assays, namely the growth, sporulation and spore viability of *C. acutatum* as well as hyphae morphology of the pathogen at the interaction zone with the endophyte.

Pathogen growth. During interaction, the internal radial growth of the pathogen towards the interacting endophyte and the distance between colonies of each other, were measured daily by using a graduated ruler, until the pathogen/endophyte had reached the edge of the plate. With the data obtained was calculated the inhibitory activity of each tested endophyte according to the equation devised by Cray et al. (2015). This equation originates an inhibition coefficient, which was estimated based on the growth inhibition of the pathogen at distance (prior to contact with the endophyte), in the vicinity of the

endophyte and in a zone-of-mixed culture (after contact with the endophyte). Significant differences on the inhibition coefficient between the tested endophytes were determined by one-way ANOVA with a Tukey test at p-value<0.05, conducted with SPSS v.21 software.

Pathogen sporulation and germination. The number of spores produced by *C. acutatum* as well as their viability was determined at the end of the dual culture assays. For this, a spore suspension was obtained by aseptically removing three mycelial plugs from the interaction zone, that were transfer into a tube with 1 ml of sterile 0.025% (v/v) Tween 80. After vortexing for 1-2 min, the concentration of spores on the suspension was estimated in a Neubauer counting chamber and results expressed in spores/mL. Spores' viability was assessed by calculating the percentage germination of spores. For this, 9 cm Petri dishes containing water agar (15 g/L agar-agar) were inoculated with the same spore suspension used to quantify sporulation. After incubation, at 25±2°C in the dark for 16h, the percentage of germination was evaluated by counting the number of germinated and non-germinated spores, from a total of 300 spores *per* Petri dish. The ability of the tested endophytes to reduced *C. acutatum* sporulation and viability was assessed by calculating the percentage of inhibition in relation to control. This was estimated by dividing the difference between control and treatment, by the control value and, multiplied by 100. Significant differences on the inhibition of sporulation and spore germination between the tested endophytes were determined by one-way ANOVA with a Tukey test at p-value<0.05, conducted with SPSS v.21 software.

Macro and microscopic characterization of dual cultures. Macroscopic characteristics of the colonies were also registered and include color and border appearance of the colony, aerial growth, medium coloration and exudates production. The outcome of interspecific interactions was additionally assessed based on the terminology proposed by Tuininga (2005): i) contact inhibition, when growth of both species stops at the line of contact, and no clear zone is formed between them; ii) inhibition at distance, when neither species can enter the area inhabited by the other, and a clear zone is formed; iii) overgrowth of a mycelium over the other; and iv) intermingling of both mycelia, with the formation of a barrage in the contact zone between species. Hyphae morphology in the interaction zone was evaluated under light microscopy at the end of the assay, in order to provide a complete description of the mechanisms that lead to a concrete outcome. For this, portions of mycelium from the interaction zone were mounted on a glass slide in

distilled water and examined in a LeitzLaborlux 12 microscope. Pictures were captured with a Wild Leitz MPS46/52 Photoautomat camera.

4.3.6 Relationship between antagonistic activity and origin of the endophyte

To determine whether the *in vitro* inhibition of growth, sporulation and germination of *C. acutatum* are related with the origin of the endophytic isolates (*i.e.*, plant organ or cultivar), a Multiple Factor Analysis (MFA) was performed in R software (R Core Team, 2018) using *FactoMineR* for the analysis and data visualization (Le et al., 2008) and *factoextra*. This analysis reveals the most important variables (plant organ or cultivar) that contribute the most in explaining the variations in the inhibition of growth, sporulation and germination of *C. acutatum.*

4.3.7 *In vivo* antagonistic assays

Results from the *in vitro* antagonistic assays identified *H. lixii* as the most antagonistic fungal isolate (see results and discussion section). This isolate was selected, and its ability in suppressing the development of olive anthracnose was investigated by using an olive fruit bioassay. The biocontrol ability of this endophyte was tested against three species in the *C. acutatum* complex, namely *C. acutatum*, *C. godetiae* and *C. fioriniae*, which are considered to be the main causal agents of olive anthracnose (Talhinhas et al., 2018). These pathogens, obtained from the microbial collection of the Polytechnic Institute of Bragança (Portugal), were isolated from naturally infected olives of cv. *Cobrançosa* and cv. *Madural* (Mirandela, Portugal) and were molecularly identified following the same procedure previously described for *C. acutatum*. For the establishment of the assays, was used symptomless olive fruits from cv. *Cobrançosa*, with three different maturation stages/index (MI): 2 (epidermis shows red spots in less than half fruit), 3 (epidermis is red or purple in more than half fruit) and 4 (black epidermis and white pulp) (Hermoso et al., 2001). After washing in running water, the olives were surface sterilized through sequential immersion in 70% (v/v) ethanol for 1 min, 3-5% (v/v) sodium hypochlorite for 2 min, and then rinsed three times (1 min each) with sterile distilled water. Then, ten disinfested fruits were placed on round glass flasks (7 cm of diameter and 8 cm of height) containing sterilized filter paper (Whatman nº 4). Fruit inoculations were performed by adding to each flask 4 ml of endophytic spore suspension

(10^6 spores/ml), that was evenly distributed over the olives, and after 3 days were additionally inoculated with 4 ml of pathogen spore suspension (10^6 spores/ml). Spore suspensions were obtained following the same procedure previously described in *in vitro* antagonistic assays. Controls were performed by inoculating olives with 4 mL of sterile aqueous solution of 0.02% (v/v) Tween 80 or pathogen spore suspension (10^6 spores/ml). For each maturation index were performed five replications each containing ten fruits. Flasks were incubated at room temperature (20 ± 4°C), under daylight regime, and the filter paper was kept wet during the experiment to maintain high humidity conditions necessary for infection. Both disease incidence (*i.e.*, the percentage of infected fruits) and disease severity (*i.e.*, the proportion of fruit area that is affected) was assessed at 7, 14 and 21 days after pathogen inoculation. The disease incidence was determined by the percentage of infected fruits, and severity was determined by using a 0 to 5 rating scale, where 0 = no visible symptoms, 1 = visible symptoms affecting <25% of the fruit surface, 2 = 25–49%, 3 = 50–74%, 4 = 75–100%, and 5 = soapy fruit (Moral et al., 2008). The area under the disease progress curve for the disease incidence (AUDPCi) and severity (AUDPCs) was further calculated for each assay following the procedure described by Moral et al. (2008). Significant differences on the AUDPCi and AUDPCs between endophytic inoculated fruits and control were determined by one-way ANOVA with a Tukey test at p-value<0.05, conducted with SPSS v.21 software.

4.4 Results

4.4.1 Fungal community associated to each plant organ across the olive tree cultivars

The isolation of endophytic fungi from all olive trees allowed the identification of 1911 isolates belonging to 205 OTUs. Most of these isolates belong to the phylum Ascomycota (97% of the total number of fungal isolates), class Sordariomycetes (59%) and families *Nectriaceae* (28%), *Diaporthaceae* (19%) and T*richocomaceae* (8%) (Fig. S4.1). *Fusarium, Phomopsis* and *Penicillium,* were identified as the most abundant genera, accounting together 54% of total fungal isolates. Overall, was found a larger consortium of fungal endophytes in roots (132 OTUs, 66 genera, 42 families, 13 orders, 4 classes, and 2 phyla) when compared to twigs (101 OTUs, 16 genera, 12 families, 9

orders, 5 classes, and 2 phyla) and leaves (41 OTUs, 16 genera, 12 families, 9 orders, 5 classes, and 3 phyla) regardless of cultivar (Fig. S4.1).

In total, roots exhibited significantly ($p<0.001$) higher fungal abundance (up to 2.4- and 5.8-fold), richness (up to 1.3- and 3.6-fold) and diversity (up to 2.3- and 3.7-fold) when compared to twigs and leaves, respectively (Fig. 4.1). These differences among plant organs were higher for endophytes colonizing cvs. *Picual* and *Cobrançosa* than cv. *Galega vulgar* (Fig. 4.1). The NMDS plots (Fig. 4.2) and ANOSIM analysis (Table S4.1) also indicated differences on fungal community composition among the three plant organs surveyed (Global $R = 0.88$; $p=0.001$), being higher in both cvs. *Picual* ($R=0.78$, $p=0.001$) and *Cobrançosa* ($R=0.73$, $p=0.001$) than in cv. *Galega vulgar* ($R=0.45$, $p=0.001$). In general, the composition in the leaf and twig was the most similar, while both root and aerial organs (twig and leaf) displayed the most dissimilar ones (Table S4.1). Indeed, out of total fungal OTUs (205), around 44% was unique to the roots, being only 9% shared between the three organs surveyed (Fig. S4.2).

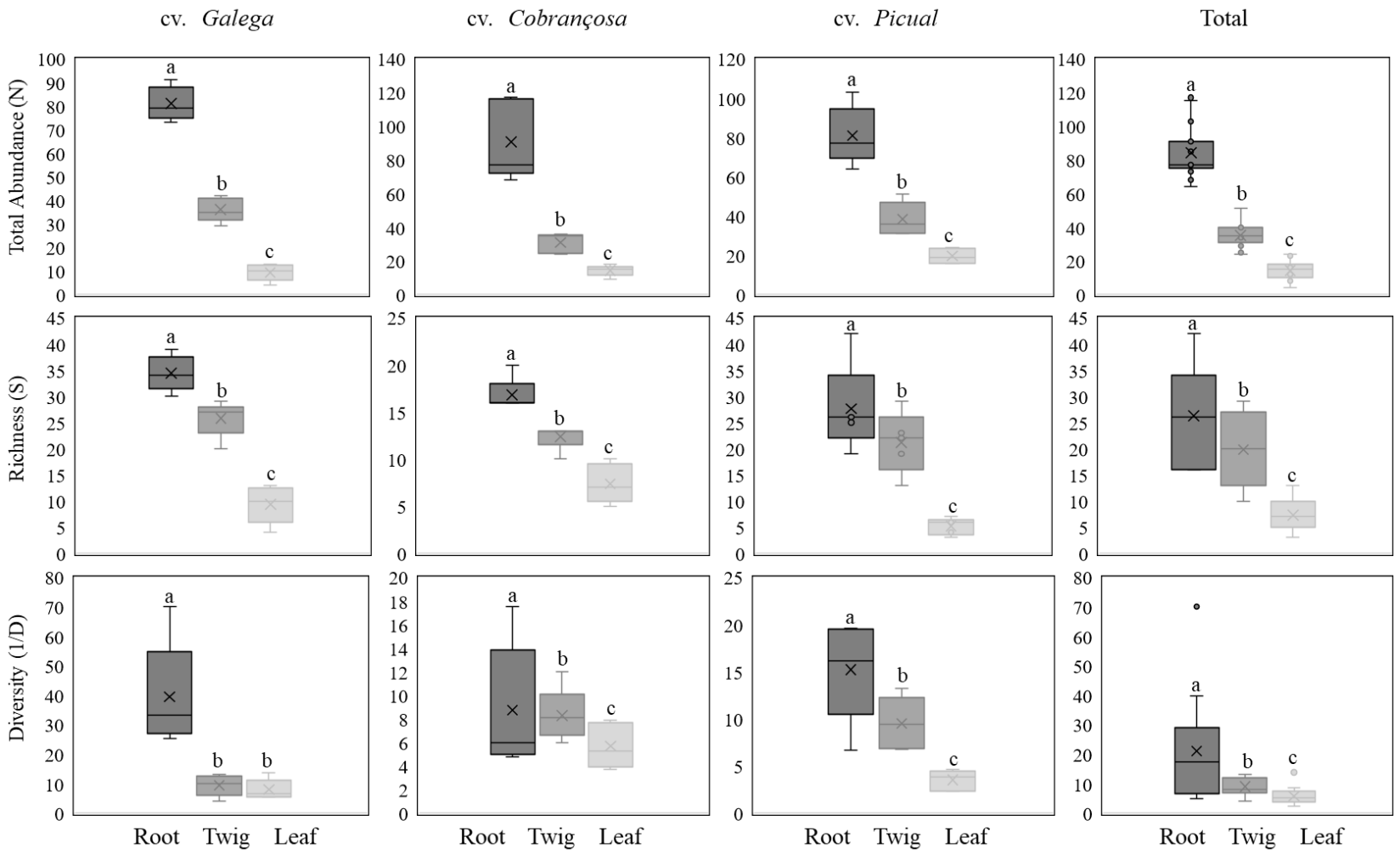

Fig. 4.1 Abundance (N), richness (S) and diversity (1/D) of fungal endophytes detected on root, twig, and leaf of olive tree cultivars *Galega vulgar*, *Cobrançosa* and *Picual* and, in all cultivars (Total). Different superscript lowercase letters denote a statistically significant difference ($p<0.05$) among plant organs. Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers), and outliers (dots).

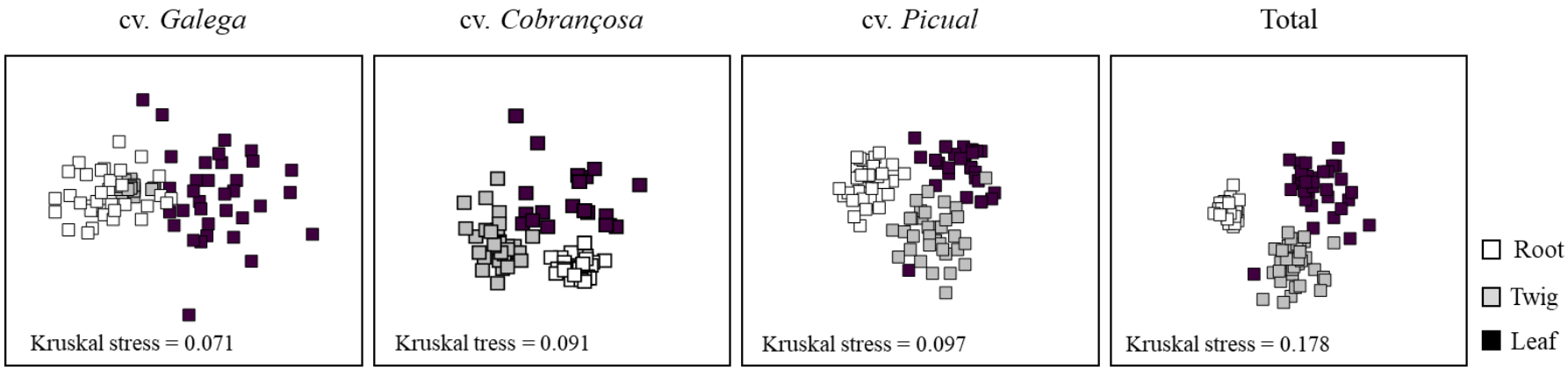

Fig. 4.2 Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of fungal endophyte communities grouped by plant organ (root, twig and leaf) for each olive tree cultivar and, in all olive tree cultivars (Total). Cluster analysis was performed with Bray-Curtis coefficient. Kruskal's stress values less than 0.2 represent good ordination plots.

Co-inertia results revealed that specific fungal genera were positively associated with a particular plant organ or cultivar (Fig. 4.3). The endophytes *Bionectria, Chalastospora, Fusarium, Macrophomina, Phomopsis, Penicillium* and *Trichoderma* were positively associated with the root. The endophytes *Chaetomium, Epicoccum, Hypocrea* and *Pseudocercospora,* were positively associated with leaf, while the most important genera associated to twig were *Aureobasidium, Alternaria, Biscogniauxia, Phaeosphaeria, Cladosporium, Purpureocillium* and *Ochrocladosporium.* In terms of cultivars, the genera most associated with cv. *Galega vulgar* were *Chromelosporium, Fusarium, Phomopsis, Penicillium* and *Trichoderma,* whereas in cv. *Cobranços*a were *Chalastospora, Cladosporium, Hypocrea* and *Purpureocillium*, and finally in cv. *Picual* were *Aureobasidium, Epicoccum, Crinipellis, Ilyonectria* and *Phaeosphaeria*.

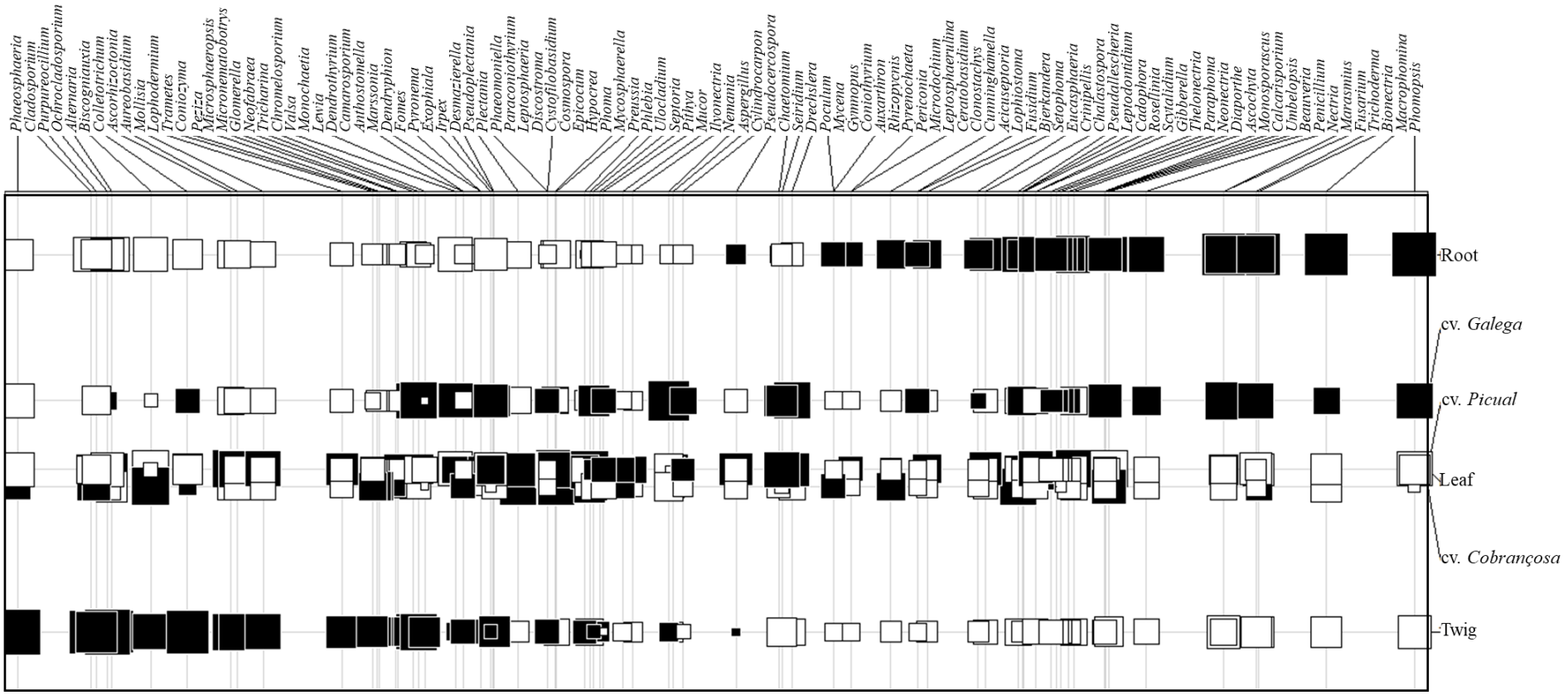

Fig. 4.3 Co-inertia factorial map showing positive (■) and negative (□) relationships between fungal communities inhabiting organ (root, twig and leaf) and olive tree cultivar (*Galega vulgar, Cobrançosa* and *Picual*). The square size indicates the degree of relatedness between variables (organ and cultivar) and fungal community.

4.4.2 *In vitro* interaction between fungal endophytes and *C. acutatum*

All the ten fungal endophytes tested showed capacity to inhibit the growth of *C. acutatum*, being *M. phaseolina*, *H. lixii*, *F. oxysporum* and *P. lilacinus*, the isolates with the greatest antagonistic effect (IC>60; Fig. 4.4). These antagonists were mostly isolated from the moderately susceptible cv. *Cobrançosa* (three out of four). In contrast, the antagonist activity displayed by most of the endophytes isolated from the susceptible cv. *Galega vulgar* was less noticeable. The inhibition exhibited by all the tested fungi was mainly promoted in the vicinity of *C. acutatum* colonies, having the inhibition at distance and after contact between colonies a lower contribution.

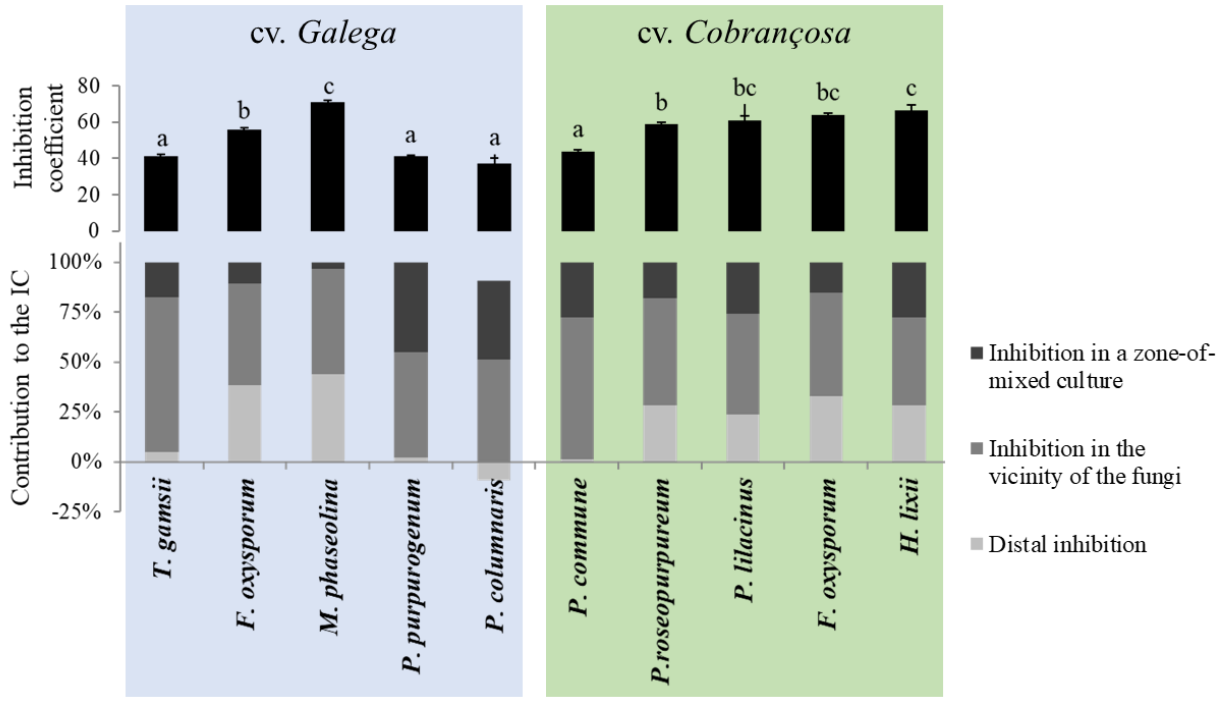

Fig. 4.4 Inhibition coefficients (IC) of fungal isolates obtained from cvs. *Galega vulgar* and *Cobrançosa* against *Colletotrichum acutatum* in dual culture assay and percentage contribution of each parameter to the isolate's inhibitory efficacy. IC values are expressed as mean ± SE (n = 5). Negative values indicate that colony development of the pathogen was promoted. Bars with different lowercase letters indicate significant differences ($p < 0.05$).

Marked differences were also observed among the endophytic fungal isolates tested on their capacity to inhibit both sporulation and germination of *C. acutatum* (Fig 4.5). Out of the five isolates from the susceptible cv. *Galega vulgar,* only two (*i.e.*, *T. gamsii* and *P. purpurogenum*) significantly ($p<0.05$) reduced the sporulation of *C. acutatum* by 70% and 58%, respectively, in relation to the control. These two isolates, together with *F. oxysporum*, also showed the capacity to inhibit significantly ($p<0.05$) the germination of *C. acutatum,* from 23 to 36%. On the other hand, four isolates of the moderately susceptible cv. *Cobrançosa* (*i.e.*, *P. commune, P. lilacinus, F. oxysporum* and *H. lixii*) significantly reduce ($p<0.05$) the sporulation of *C. acutatum* (up to 76% when compared to the control); And all of the five isolates tested from this cultivar inhibit significantly ($p <0.05$) the germination of *C. acutatum*, ranging from 24 to 82%.

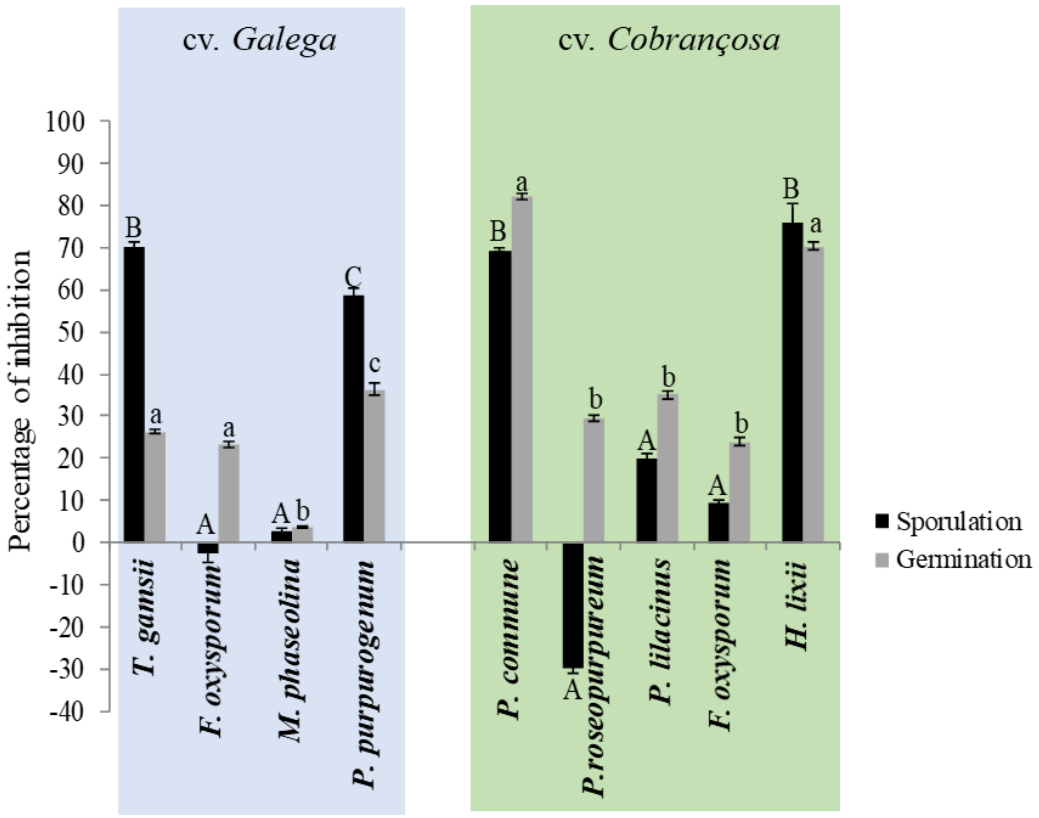

Fig. 4.5 Percentage inhibition of sporulation and conidial germination of *Colletotrichum acutatum* in dual culture with endophytic fungi isolated from cvs. *Galega vulgar* and *Cobrançosa.* Negative values indicate that sporulation of the pathogen was promoted. Each value is expressed as mean ± SE (n = 5). Bars with different uppercase (sporulation) and lowercase (germination) letters indicate significant differences ($p<0.05$).

Macroscopic observation of colonies during interaction was made in order to evaluate the outcome of the interaction as well as the occurrence of morphologic alterations (Fig. 4.6). Different types of antagonistic interaction were observed depending on the species paired. Contact inhibition was the most observed in dual cultures with endophytic species from cv. *Galega vulgar*, followed by overgrowth of a mycelium over the other (*P. columnaris*) and intermingling of both mycelia, with the formation of a pigmented barrage (*P. purpurogenum*). Contact inhibition and overgrowth of a mycelium over the other (*H. lixii*) were the most observed in endophytic species from cv. *Cobrançosa.* In the interaction zone, some endophytic fungi produce compounds that cause color changes on mycelium pigmentation of *C. acutatum*, an effect that was not observed in the control. Border of *C. acutatum* colonies become red or yellow pigmented in the interaction zone with *P. purpurogenum* and *P. rosesospurpurem*, respectively, and dark brown in contact with *P. lilacinus* (Fig. 4.6).

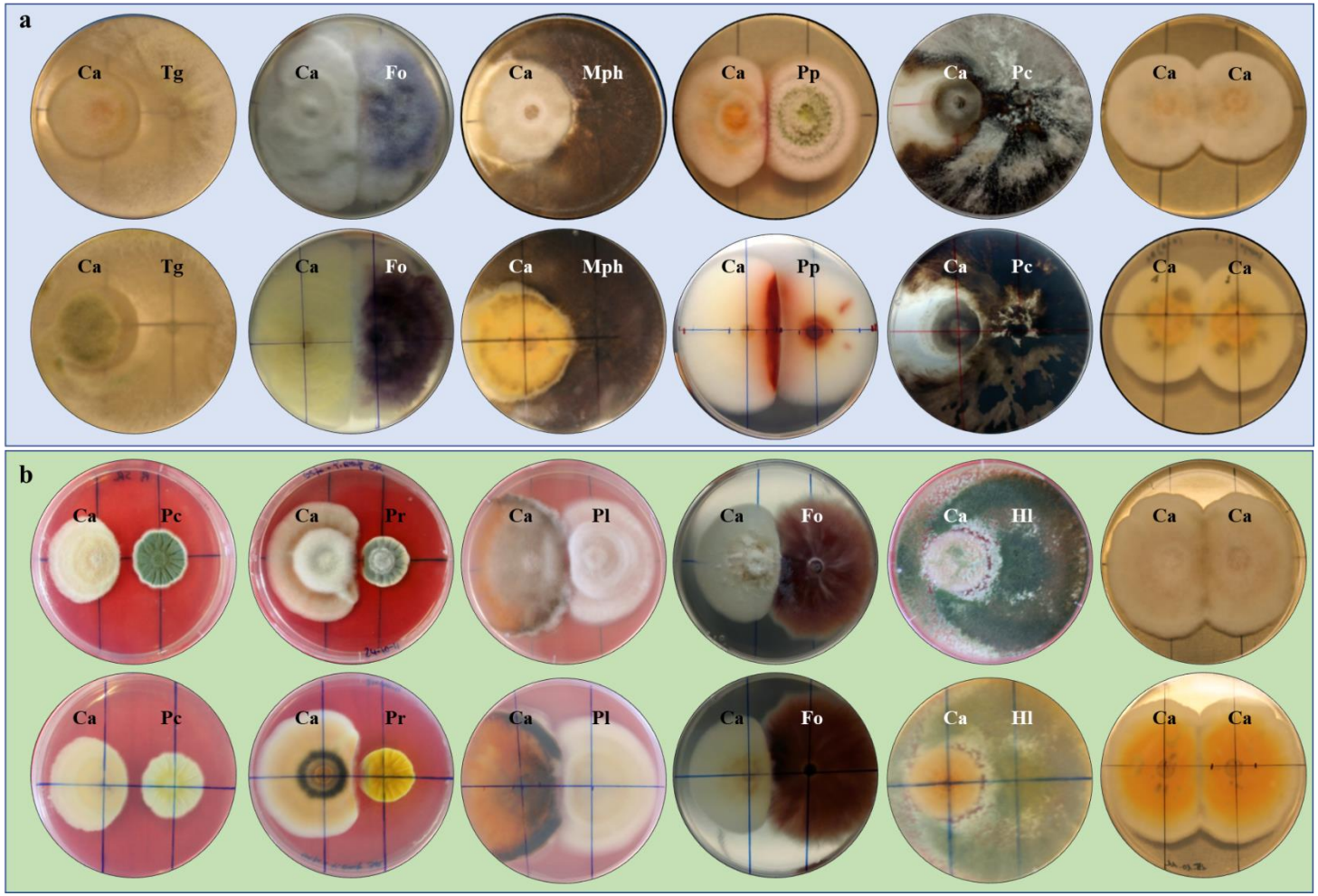

Fig. 4.6 Macroscopic mycelial interaction on potato dextrose agar between *Colletotrichum acutatum* (ca) and endophytic fungi obtained from cvs. *Galega vulgar* (A) and *Cobrançosa* (B), on the 15th day of dual culture, in the upper sides and on the reverse sides. Fungal isolates from cv. *Galega vulgar*: *Trichoderma gamsii* (Tg), *Fusarium oxysporum* (Fo), *Paecilomyces lilacinus* (Pl), *Macrophomina phaseolina* (Mph), *Penicillium purpurogenum* (Pp) *Phomopsis columnaris* (Pc); and from cv. *Cobrançosa: Penicillium. Commune*, (Pc) *Penicillium roseopurpureum* (Pr), *Paecilomyces lilacinus*, (Pl), *Fusarium oxysporum* (Fo) and *Hypocrea lixii* (Hl). .

All dual cultures were observed microscopically in the interaction zone, being possible to detect several changes on *C. acutatum* hyphae morphology (Fig. 4.7). In contrast with control, hyphae of *C. acutatum* become collapsed, swollen and distended in the dual culture with *P. commune* (a) and *F. oxysporum* (b), or twisted and coiled in the dual culture with *H. lixii* (c) and *T. gamsii* (d). Necrosis and cytoplasmic vacuolation were also common alterations observed in *C. acutatum* hyphae in the vicinity of *P. roseopurpureum* (e) and *P. lilacinus* (f). There were also observed the formation of crystals in dual culture with *P. purpurogenum* (g) and *P. lilacinus* (h).

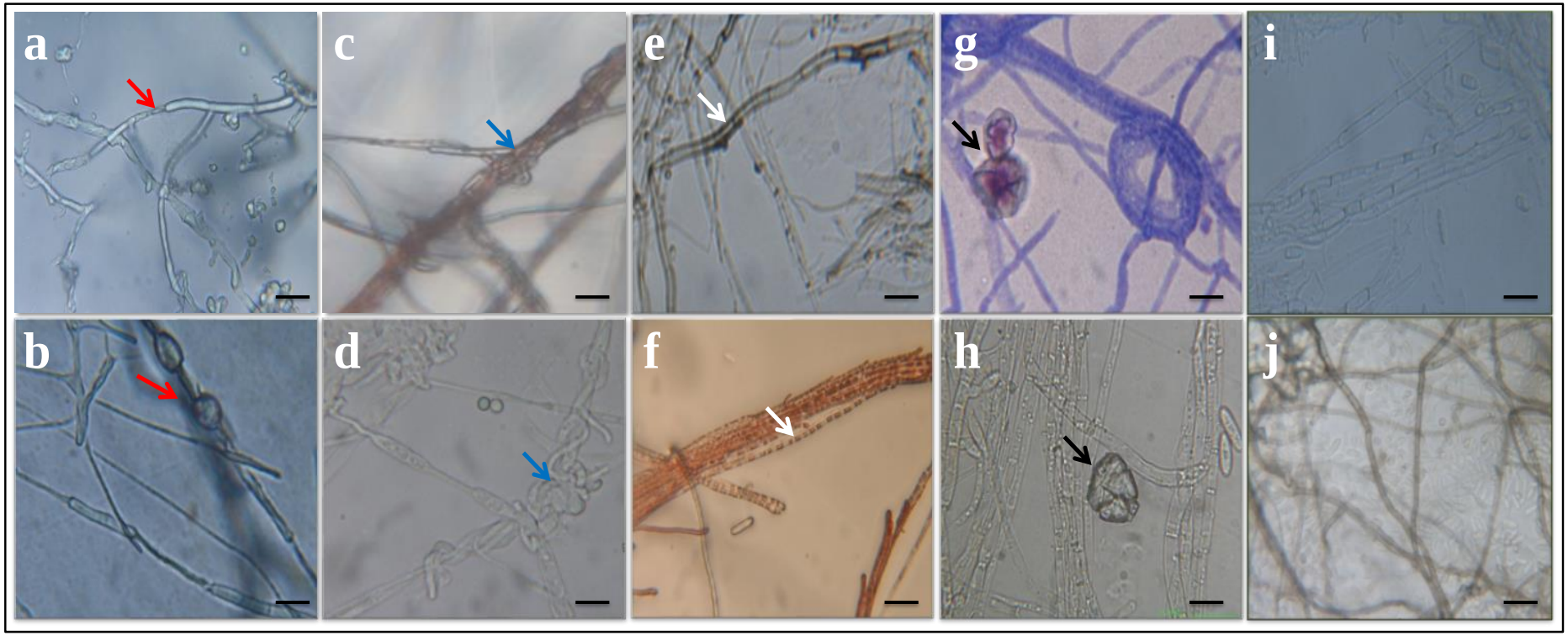

Fig. 4.7 Hyphal morphology of *Colletotrichum acutatum* in dual culture with fungal isolates obtained from cvs. *Galega vulgar* and *Cobrançosa*. Collapse, swollen and distended (red arrow) of hyphae in the dual culture with *Penicillium commune* (a) and *Fusarium oxysporum* (b); twisting coiled (blue arrow) of hyphae in the dual culture with *Hypocrea lixii* (c) and *Trichoderma gamsii* (d); necrosis and vacuolization (white arrow) of hyphae in the dual culture with *Paecilomyces lilacinus* (e) and *Penicillium purpurogenum* (f); formation of crystals (black arrow) in the dual culture with *Penicillium roseopurpureum* (g) and *Phomopsis columnaris* (h); Normal *C. acutatum* hyphae (i and j) in control plates. Bar = 15 µm.

4.4.3 Association between endophytic origin and the inhibition of *C. acutatum*

According to the MFA analysis, the antagonistic effect displayed by the endophytic fungi tested is linked to their origin in terms of plant organ and cultivar (Fig 4.8). Based on this analysis, the variables that are most correlated to the Dimension 1 are the inhibition of sporulation and spore germination of *C. acutatum* by the fungal endophytes (R^2=0.78 and 0.58, respectively; $p<0.001$), the plant organ (R^2=0.75; $p<0.001$) and cultivar (R^2=0.25; $p<0.001$). Among plant organs and cultivars, both leaf (estimated coefficient=1.53; $p<0.001$) and cv. *Cobrançosa* (estimated coefficient =0.69; $p<0.001$) revealed to be significantly associated to Dimension 1. The inhibition of *C. acutatum* growth was more explained by Dimension 2 (R^2=0.61; $p<0.001$), which revealed to be significantly associated with cv. *Galega vulgar* (estimated coefficient=0.66, $p<0.001$) and twig (estimated coefficient=0.54, $p<0.01$). Root showed to be the most negatively associated organ with dimension 1 and 2 (estimate coefficient=-1.12 and -0.56, respectively; $p<0.001$). Altogether, the results indicate that the high values of inhibition of germination and sporulation of *C. acutatum* are more associated with endophytes isolated from leaves of cv. *Cobrançosa,* while the highest capacity to inhibit the growth of *C. acutatum* are more associated to endophytes obtained from twigs of cv. *Galega vulgar*. The roots do not appear to be an important source of antagonistic fungal endophytes of *C. acutatum*.

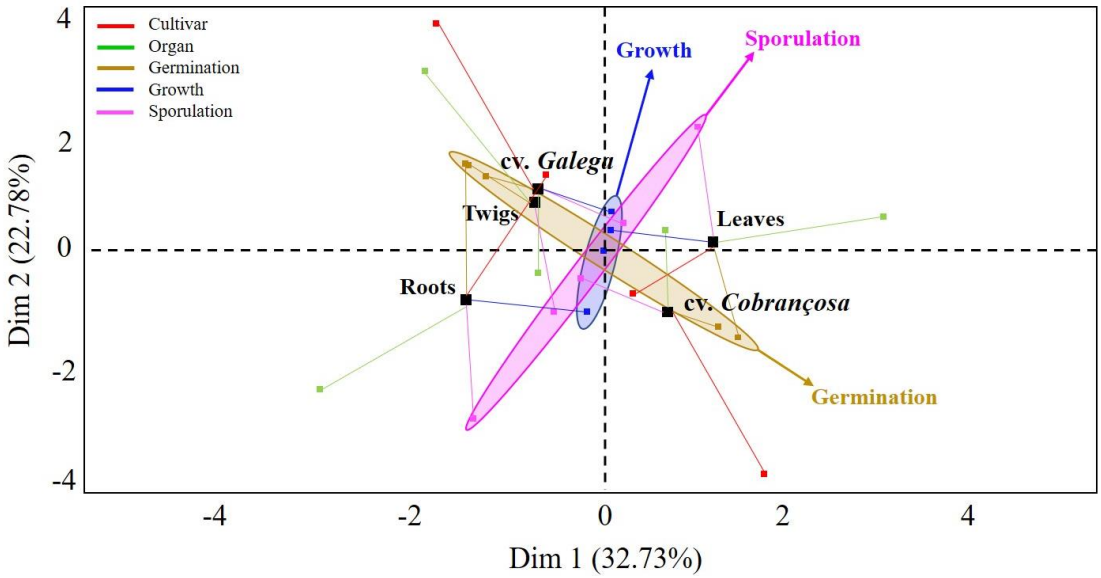

Fig. 4.8 Individual factor map obtained with the Multiple Factor Analysis (MFA) representing the association of the inhibition of growth, sporulation and spore germination of *Coletotrichum acutatum* by different fungal endophytes obtained from different plant organs (root, twig and leaf) of two olive cultivars (cv. *Galega vulgar* and cv. *Cobrançosa*). Dimension 1 and 2 represent 55.51% of the total variance. Inhibition of germination (contribution of 77.9%, $p<0.001$) and sporulation (contribution of 57.6%, $p<0.001$) are more associated to Dimension 1, while growth inhibition is associated to Dimension 2 (contribution of 61.0%, $p<0.001$).

4.4.4 Olive fruit bioassays

Based on the results from *in vitro* assays, the fungus *H. lixii* was identified as the most promising biocontrol agent of *Colletotrichum* spp.. Therefore, its efficacy in reducing incidence (AUDPCi) and severity (AUDPCs) of anthracnose, caused by the pathogens *C. acutatum*, *C. godetiae* and *C. fioriniae*, was evaluated by artificial inoculation of detached fruits. This study was conduct with olives with three different maturation indexes (2, 3 and 4), in order to assess the effect of fruit ripening on the interaction pathogen-endophyte. Results showed that *H. lixii* reduced significantly ($p<0.05$) both incidence and severity of olive anthracnose when compared to control (*i.e.*, in the absence of *H. lixii*), mostly at either 14 and/or 21 days post-inoculation (Fig. 4.9). Overall, the antagonistic effect of *H. lixii* was significantly ($p<0.001$) higher against the pathogen *C. acutatum* followed by *C. godetiae* and *C. fioriniae*, being this effect variable according to the maturation index of fruits. For instances, the effectiveness of *H. lixii* as biological control agent against *C. acutatum* and *C. fioriniae* was most notorious on fruits at maturation index 2 and at maturation index 4, respectively, while the reduction of anthracnose development caused by *C. godetiae* was effective in the fruits at the three maturation indexes. Disease incidence and severity was also found to increased significantly over fruit ripening, being purple or black (MI 3 and 4, respectively) olives developed more significantly ($p<0.05$) anthracnose symptoms than green/yellowish green olives.

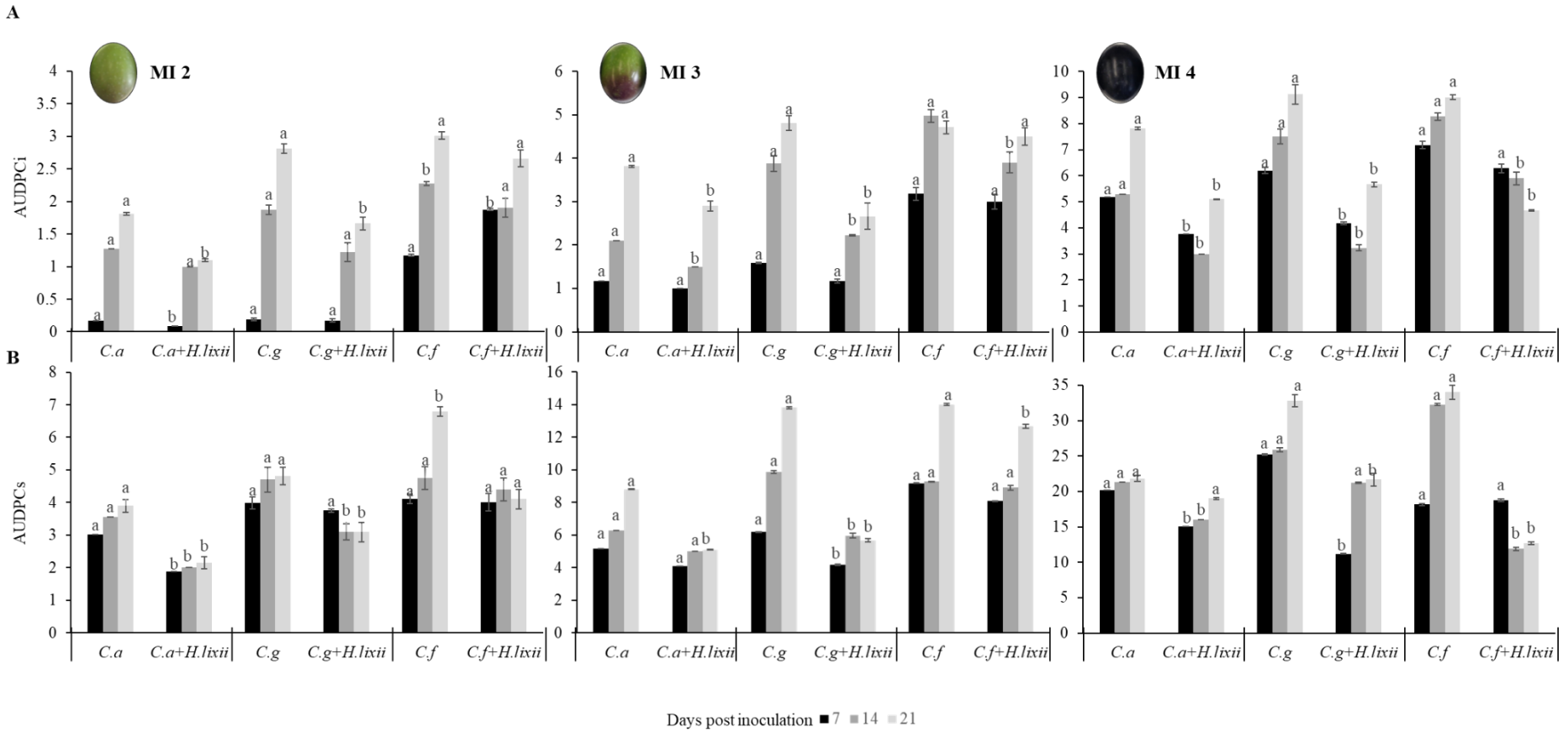

Fig. 4.9 Area under the disease progress curve of incidence (AUDPCi) and severity (AUDPCs) in olive fruit with three different maturation indexes (MI 2, MI 3 and MI 4, respectively) inoculated with the endophyte *Hypocrea lixii* and one of the pathogens *Colletotrichum acutatum* (C.a), *Colletotrichum godetiae* (C.g) and *Colletotrichum fioriniae* (C.f), after 7, 14 and 21 days. In each day, mean values of the control (pathogen) and of the treatment (pathogen+endophyte) followed by different letters are significantly different ($p<0.05$). Pictures depict an example of olives at the different maturation stage.

4.5 Discussion

Overall, the fungal endophytes colonizing the vegetative organs of olive tree were highly diverse (total of 205 OTUs), being most of the isolates belonging to phylum Ascomycota and class Sordariomycetes. Similarly, previous studies using both cultural-dependent (Martins et al., 2016; Gomes et al., 2018) and –independent approach (Abdelfattah et al., 2015; Fernández-González et al., 2019) have identified this fungal class as the most abundant in olive roots, leaves and twigs. Therefore, isolates from Sordariomycetes seems to be a general characteristic of the endophytic community of olive tree tissues. At the genus level, *Fusarium* and *Phomopsis*, were found as the most abundant in the root and aboveground organs (twig and leaf) of olive, respectively, as previously reported by Martins et al. (2016) who used a similar approach to the present study to characterize the endophytic fungal community of olive tree. However, using a culture-independent approach, Fernández-González et al. (2019) did not found *Fusarium* as the most representative of olive roots of several cultivars present at the World Olive Germplasm Collection (in Spain). Without excluding the potential biases related to culture-dependent method (Wu et al., 2019), this variation on the most abundant genera in roots could be related with differences on sampling sites and sampling time, which were recognized to influence endophytic fungal assemblage in olive tree (Martins et al., 2016; Gomes et al., 2018).

The three different organs surveyed showed to harbour different endophytic fungal communities with higher fungal diversity and abundance observed in roots, followed by twigs and leaves. This result is in agreement with previous studies suggesting organ-specificity of fungal endophytes in olive tree (Martins et al., 2016; Gomes et al., 2018). As previously suggested for other woody species (Küngas et al., 2020), it is likely that the growth of endophytes in olive is restricted by the features of a particular organ. Despite this, the greater similarity in the fungal community composition of twigs and leaves, when compared to roots and aboveground organs, suggests that some fungi are still able to grow from twigs to leaves. In contrast, most of the fungi colonizing the olive roots seem to be unable to move to aboveground plant tissues, as previously suggested by Martins et al. (2016).

4.5.1 Is the difference in the susceptibility of cultivars to anthracnose associated with a particular endophytic fungal consortium naturally present in roots, twigs and leaves?

A set of fungal genera were identified to be positively associated to a specific olive tree cultivar and thus to their underlying susceptibility to anthracnose. It is thus tempting to speculate that the fungal communities inhabiting each of the cultivars might be an important determinant of host plant resistance to olive anthracnose. Indeed, the endophytes most positively associated to the anthracnose-susceptible cultivar are from genera that include plant pathogens, such as *Chromelosporium* (Mukobata and Saioto 1996), *Fusarium* (Moretti, 2009) and *Phomopsis* (Udayanga et al., 2011), as well as ubiquitous fungi widely present in various processes from necrotrophic pathogenicity to endophytic mutualism (*Penicillium*) (Visagie et al., 2014), and in less extent biocontrol agents (*Trichoderma*) (Carrero-Carrón et al., 2016). In contrast, the fungal genera most positively associated to anthracnose-moderately susceptible or –resistant cultivars, include members with recognized biocontrol abilities against plant diseases (*Epicoccum*, *Aureobasidium, Cladosporium* and *Hypocrea*) (Musetti et al., 2011; Elgorban et al., 2014; Khan et al., 2016; Rathnayake et al., 2018) and insect pests (*Purpureocillium*) (Medeiros et al., 2018), and in less extent plant pathogens (*Crinipellis*, *Ilyonectria* and *Phaeosphaeria*) (Da Silva, 2003; Cunfer, 2009; Lombard et al., 2013). Curiously, these genera used as biocontrol agents were also found to be positively associated with aboveground olive organs. Thus, we hypothesize that these fungal groups could be an important determinant of anthracnose resistance, which is a disease that infects aboveground olive organs. However, further work is still required to confirm this.

4.5.2 Is the antagonistic effect of native endophytes against *C. acutatum* linked to their origin in terms of the plant host (*i.e.*, susceptibility to anthracnose) and plant organ (*i.e.*, root, twig and leaf)?

Our study was the first showing that antagonistic activity of fungal endophytes against pathogens, depend on their origin in terms of host cultivar and plant organ. Overall, the endophytes from the anthracnose-moderately susceptible cultivar were more effective in reducing the growth, sporulation and germination of *C. acutatum* than the ones from the anthracnose-susceptible cultivar. Among the screened isolates, *H. lixii*, *F. oxysporum* and *P. lilacinus*, were the most effective against *C. acutatum*. *Hypocrea lixii*, the teleomorph of *Trichoderma harzianum* (Chaverri and Samuels, 2002), is known for

its effectiveness in biocontrol of numerous phytopathogenic fungi (Lorito et al., 2010), such as *Fusarium graminearum* (Saravanakumar et al., 2017), *Bipolaris oryzae* (Abdel-Fattah et al., 2007), *Sclerotinia sclerotiorum* (Zhanga et al., 2016), including several species of *Colletotrichum* (Freeman et al., 2004; Alvindia, 2018), and to induce systemic resistance against pathogen (Saravanakumar et al., 2016). Fungi of the *Fusarium oxysporum* species complex are well-known for inducing wilt or root rots on several plants (Abdel-Azeem et al., 2019), but others are considered as nonpathogenic and used as biocontrol agents against insect pests (Sharma and Marques, 2018) and phytopathogens (Kaur et al., 2010), including *Colletotrichum* (Bonatelli et al., 2016). *Paecilomyces lilacinus*, is a nematophagous fungus, which has been used for the biological control of a wide spectrum of plant-parasitic nematodes (Khan et al., 2006; Anastasiadis et al., 2008). In addition, its ability to inhibit the growth of plant pathogens, such as *Mucor piriformis*, *Trichothecium roseum*, *Rhizoctonia solani* and *Verticillium dahliae*, was verified under *in vitro* conditions (Lan et al., 2017).

Besides host cultivar, also the plant organ from which the endophyte was isolated showed to influence the antagonistic effect over the pathogen. Endophytes from leaves exhibited the highest capacity to inhibit both germination and sporulation of *C. acutatum*, whereas endophytes from twigs were the most effective in inhibit the growth of *C. acutatum*. In contrast, endophytic fungal strains from olive roots were less effective against *C. acutatum*. Olive anthracnose is an airborne disease; therefore we hypothesize that these endophytic species colonizing the aboveground olive organs might have important consequences for disease development. However, further studies are still needed to confirm the role of these fungi in the susceptibility of olive tree cultivars to anthracnose disease.

Our results showed that *H. lixii* holds great promise as biocontrol agent of olive anthracnose. The inoculation of olives with *H. lixii* was effective in reducing the incidence and severity of anthracnose. Previous studies have also pointed out the biocontrol ability of this species against anthracnose caused by *Colletotrichum truncatum* on soybean (Lalhruaitluangi and Sinha, 2019), *Colletotrichum acutatum* on strawberry (Freeman et al., 2001) and *Colletotrichum gloeosporioides* in pepper and cherry tomato (Kim et al., 2014), and in papaya fruit (De la Cruz-Quiroz et al., 2018). The biocontrol efficiency of *H. lixii* was observed to be dependent on the pathogen species they are confronted with as well as on the fruit maturation index. It is especially effective against *C. acutatum* and

C. fioriniae if applied in fruits at MI 2 and 4, respectively, and against *C. godetiae* in any of the MI studied.

4.5.3 Which mechanisms are involved in the antagonistic effect displayed by native endophytes against *C. acutatum*?

The suppression exhibited *in vitro* by all the tested fungal endophytes was mainly promoted in the vicinity of *C. acutatum* colonies. This could indicate the involvement of diffusible metabolites and competition for space/nutrients in the inhibition, which are well-known antagonistic mechanisms (Heydari and Pessarakli et al., 2010; Latz et al., 2018). The most inhibitory endophytes *H. lixii*, *F. oxysporum* and *P. lilacinus*, have been already reported to produce antimicrobial compounds (*e.g.*, Liu et al., 2012; Bun, 2013).

Inhibition of *C. acutatum* growth by some endophytes was also due to overgrowing of a mycelium over the other or intermingling of both mycelia, with the formation of a barrage. These effects, which rely on physical contact between fungi, are frequently associated to the occurrence of mycoparasitism (Pal and Gardener, 2006; Latz et al., 2018). The observation of morphological abnormalities in *C. acutatum* hyphae when in co-culture with the endophytes, corroborates this hypothesis. Alterations in *C. acutatum* hyphae were presumably the result of lytic enzymes or toxins, secreted by the endophytes, that may act on the pathogen, as previously reported for other antagonists (Li et al., 2004). These compounds may disrupt the host wall and the internal organization of the hyphae causing coagulation, cytoplasmic vacuolation, among other abnormalities (Viterbo et al., 2002). *H. lixii, F. oxysporum* and *P. lilacinus* species are known to produce lytic enzymes, such as chitinases, proteases and β-glucanases (López-Mondéjar et al., 2011).

In conclusion, the internal tissues of olive tree harbor a number of endophytic fungal strains which is likely to play an important role in conferring host plant resistance to anthracnose. The strongest antagonistic potential against *C. acutatum* was ascribed to fungi inhabiting the aboveground organs, in particular of anthracnose-moderately susceptible cultivars. In particular, *H. lixii*, showed great potential in the management of olive anthracnose disease. Although promising results were obtained from using this strain, further experiments are needed to determine its effectiveness under field conditions, and with different cultivars. The biocontrol mechanisms displayed by this strain also need to be deeply investigated.

Acknowledgments

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4.7 Support information

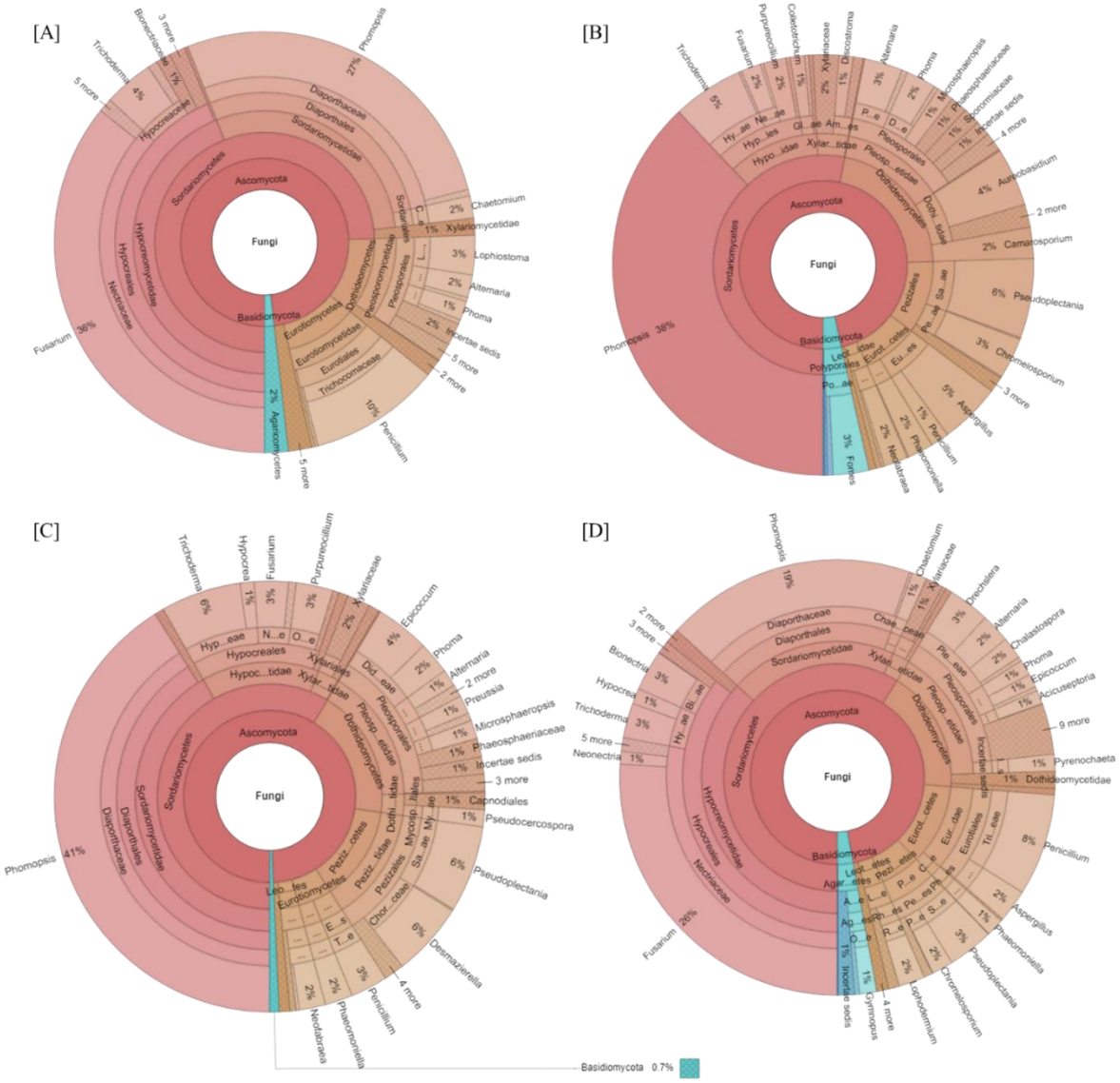

Fig. S4.1 Krona charts showing the relative abundance of endophytic fungi, at the genus level, detected on [A] root, [B] twig, [C] leaf and [D] total organs of all olive tree cultivars surveyed (*Galega vulgar*, *Cobrançosa* and *Picual*).

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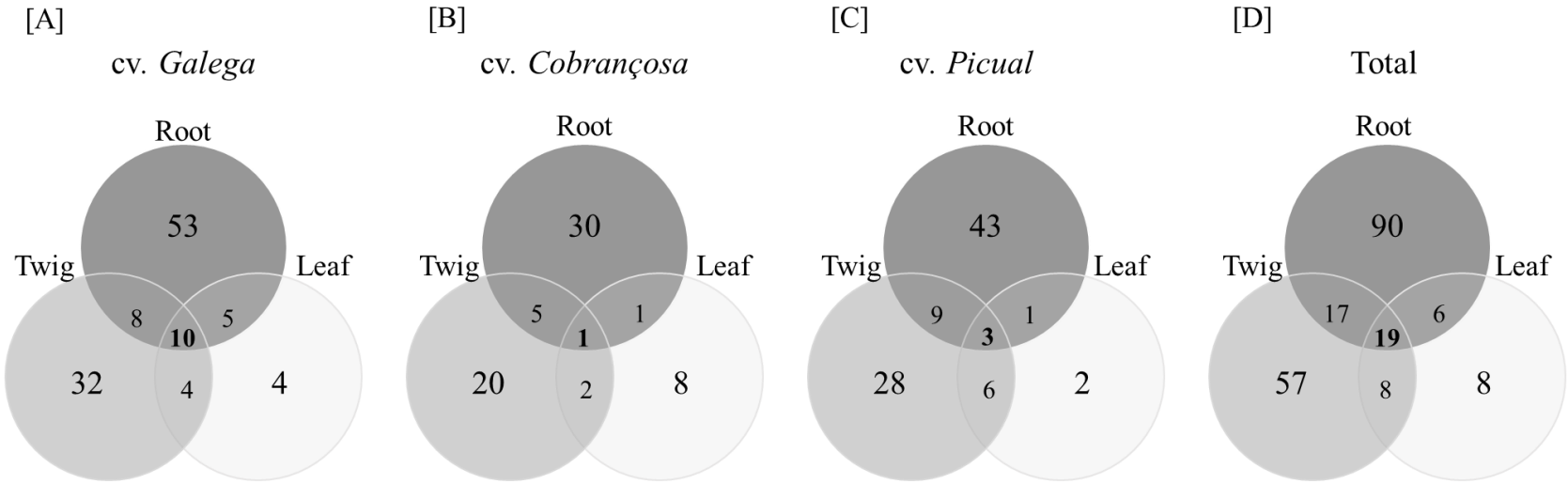

Fig. S4. 2 Venn diagrams representing the total number of fungal OTUs shared between root, twig and leave of olive tree cultivars [A] *Galega vulgar*, [B] *Cobrançosa*, [C] *Picual* and [D] total of cultivars.

Table S4.1 Similarity analysis (ANOSIM) based on the Bray-Curtis distance between fungal communities inhabiting root, twig and leaf of cvs. *Galega vulgar*, *Cobrançosa* and *Picual*. *R*-statistics values are displayed (all are significant at p=0.001).

Pairwise comparison	*Galega vulgar*	*Cobrançosa*	*Picual*
Root *vs*. Twig	0.560	0.924	0.868
Root *vs*. Leaf	0.417	0.871	0.834
Twig *vs*. Leaf	0.172	0.644	0.663

Chapter 5

General Conclusions

5.1 General Conclusions

Olive anthracnose, caused by different species of the genus *Colletotrichum*, is the most important fruit disease of olive fruit worldwide (Cacciola et al., 2012). This disease is difficult to control, being based on the use of chemical fungicides, with limited efficacy (Cacciola et al., 2012) and not compatible with sustainable production systems (organic and integrated production) which are Sustainable Development Goals (SDGs) of the 2030 Agenda agreed at the United Nations. Thus, there is a need to develop novel and environmental-friendly control strategies for management of olive anthracnose. In recent years, there has been an increasing number of studies recognizing the tremendous potential of plant-associated endophytes to improve plant resilience and yields in several farming systems (Latz et al., 2018). These microorganisms, living in the internal tissues of the host plants, can improve plant growth and health, by protecting plants from the deleterious effects of biotic and abiotic stresses (Latz et al., 2018). Therefore, the main aim of this PhD thesis was to evaluate the endophytic fungal community associated to the olive tree and elucidate their possible role on plant defense against olive anthracnose. Therefore, at first, studies were conducted to characterize the fungal endophytes inhabiting several plant organs of olive tree cultivars with contrasting susceptibilities to anthracnose ("Who is there?") and evaluate whether factors such as plant organ, host genotype, host phenology and diseases incidence affect endophytic fungal assemblages ("Which factors contribute to their shaping?"). In subsequent studies, endophytic fungal communities were related with plant susceptibility to anthracnose disease ("What can they do?"), and their potential used as biocontrol agents against olive anthracnose was evaluated in *in vitro* and in fruits bioassays ("Could they be useful?").

Who is there?

The endophytic fungal community associated with roots, twigs, leaves, inflorescences and fruits of olive tree, was rich and abundant, comprising species belonging mainly to Ascomycota phylum and Sordariomycetes class, being Basidiomycota the less represented fungal phylum in all samples. Endophytic communities differed according to the olive tree organ, being members of the genera *Phomopsis* (Diaporthaceae) the most dominant endophytes in leaves and twigs, whereas *Fusarium* (Nectriaceae), Biscogniauxia (Xylariaceae) and Alternaria (Pleosporaceae) were the most abundant in roots, inflorescences and fruits, respectively.

Which factors contribute to their shaping?

The significance of different biotic factors, such as plant organ, host genotype (at cultivar level), host phenology and anthracnose disease incidence, on endophytic fungal assemblages in reproductive organs of olive tree was investigated. Results from **chapter 2** revealed that plant organ, phenological stage and in less extent the cultivar, influence significantly the structure of the entire fungal community inhabiting reproductive organs of olive tree, in particular the inflorescences. The endophytic fungal population of fruits were only affected by the phenological stage. Overall, both fungal abundance and richness increase over inflorescence development reaching a peak on the flowering, and afterwards dropped until fruit starts the maturity process and then increase reaching a peak when fruit epidermis is red or purple. In general, the inflorescences harboured a greater diversity and abundance of fungal endophytes than fruits. Results from **chapter 3** indicated that the level of anthracnose incidence also contribute to the shaping of fungal communities in olive tree reproductive organs, in particular of fruits.

What can they do?

The endophytic fungal community associated to reproductive organs of olive tree was compared between orchards with contrasting anthracnose incidence over different phenological stages, in order to elucidate the potential role of endophytes on disease suppression and on lifestyle transition of the pathogen *Colletotrichum* spp. (**chapter 3**). The endophyte communities of reproductive organs at the two orchards were distinct, differing in species richness, abundance and composition. Overall, a greater abundance and richness of fungal endophytes was observed in the orchard with low anthracnose incidence when compared to the ones with high incidence. In particular, a reduction on abundance and richness of beneficial (in the fruits) and pathogenic (in the flower buds) fungi was observed in the orchard with high anthracnose incidence in relation to the orchard with low incidence of anthracnose. A number of fungal OTUs were found to be positively associated to one of the two orchards. While *Pseudophaeomoniella oleae*, *Neofabraea vagabunda*, *Neofabraea* sp. and *Parastagonospora avenae* were the most associated with high anthracnose incidence, *Diaporthe rudis*, *Fusarium oxysporum*, *Pezizomycetes* sp., *Epicoccum nigrum*, *Mollisia minutella*, *Trametes* sp. and *Sardariomycetes* sp. were highly associated to orchard with low anthracnose incidence. Altogether, these results suggest varying *Colletotrichum* spp.-fungal community interactions in the orchard with high and low disease incidence, which outcome seems to

be critical for the establishment of fungal communities (and the underlying pathogen) in olive tree. Probably, in the olive orchard with low disease incidence both the pathogens *Colletotrichum* spp., and the disease cannot thrive so well as in the orchard with high disease incidence due to the richer and more abundant endophyte community, in particular of antagonistic fungi. Differences on fungal community composition between the two orchards were greater on fruits than on flowers, suggesting that the lifestyle transition in *Colletotrichum* spp. from latent (during flower stage) to pathogen (during fruit stage) might be related to the endophytic fungal community structure.

Could they be useful?

The endophytic fungal community associated to olive tree cultivars of varying susceptibilities to olive anthracnose (*Galega vulgar:* susceptible, *Cobrançosa*: moderately susceptible, and *Picual*: resistant) were studied and the antagonistic activity of the fungal isolates from *Galega vulgar* and *Cobrançosa* were evaluated against *Colletotrichum* spp. (**chapter 4**). Overall, was found a higher richness and abundance of fungal endophytes in roots when compared to twigs and leaves. These differences among organs were higher in the most resistant cultivars (*Picual* and *Cobrançosa*) than in the most susceptible cultivar *Galega vulgar*. Dual-culture experiments showed that endophytes from the anthracnose- moderately susceptible cultivar displayed the greatest antagonistic effect against *C. acutatum,* by reducing its sporulation, germination and growth, than the ones from the susceptible cultivar. The most potent antagonists were *Penicillium commune, Paecilomyces lilacinus, Fusarium oxysporum* and *Hypocrea lixii*, which were also showed to cause morphological changes in *C. acutatum* hyphae. Multivariate analysis of the data obtained from the dual-culture experiments showed that the highest capacity to inhibit the growth of *C. acutatum* is associated to endophytes isolated from twigs of the most susceptible cultivar, while inhibition of germination and sporulation of *C. acutatum* are more associated with endophytes isolated from leaves of the moderately susceptible cultivar. In contrast, roots of both cultivars do not appear to be an important source of antagonistic endophytes of *C. acutatum*. In artificial inoculation of detached fruits, the endophyte *H. lixii* showed to reduced significantly the incidence and severity of olive anthracnose caused by the pathogens *C. acutatum, C. godetiae* and *C. fioriniae*. The effectiveness of this endophyte as biological control agent varied according to the fruit maturity, being most notorious on fruits that start to change skin color (maturation index 2-3) than on purple or black olives (maturation index 4).

Altogether, the results suggest that differences on endophyte community composition between olive cultivars are somehow correlated with plant susceptibility to anthracnose disease.

Overall, the community of fungal endophytes inhabiting the olive tree was shown to result from a complex interaction established between plant-*Colletotrichum*-resident endophytic microbiota. This study also brings into focus the importance of these interactions to olive tree health and, in particular, to the development of olive anthracnose disease. Such knowledge can be used to model the desired activities of beneficial endophytes in order to improve crop protection. Based on our results, we believe that olive tree microbiota can be optimized for anthracnose disease resistance by the application of fungal endophytes. These studies indicate that *H. lixii*, *F. oxysporum* and *E. nigrum*, holds great promise for this purpose. The time of their application could be also an important factor to generate and maintain beneficial microbes at the right time, either at the inflorescences or on the fruit. It is expected that endophytic application during flowering will reduced *Colletotrichum* spp. pressure while during the fruit maturation, the lifestyle transition of *Colletotrichum* spp. from latent to pathogen could be compromised. However, it is still required further studies to unravel the intricacies of communication between all members of this multipartite interaction, that lead to assembly the pathobiome in olive fruits. In particular, the exact mechanism by which *H. lixii*, *F. oxysporum* and *E. nigrum*, interfered with *Colletotrichum* spp. should be studied and carefully examined by using metatranscriptome and metaproteome analysis.

5.2 Conclusiones Generales

La antracnosis del olivo, causada por diferentes especies del género *Colletotrichum*, es la enfermedad de este cultivo más importante en todo el mundo (Cacciola et al., 2012). El control de esta enfermedad se basa principalmente en el uso de productos químicos que presentan una efectividad limitada (Cacciola et al., 2012) y no es compatible con sistemas de producción sostenibles (producción orgánica e integrada), que se incluyen en los Objetivos de Desarrollo Sostenible (ODS) de la Agenda 2030 acordada por las Naciones Unidas.

Por lo tanto, es necesario desarrollar nuevas estrategias de control sostenibles para el manejo de la antracnosis del olivo. En los últimos años, un número creciente de estudios han reconocido el enorme potencial de los endófitos asociados a las plantas para mejorar su resistencia y rendimiento en varios sistemas agrícolas (Latz et al., 2018). Estos microorganismos, que viven en los tejidos internos de las plantas hospederas, pueden mejorar su crecimiento y salud protegiéndolas de los efectos nocivos del estrés biótico y abiótico (Latz et al., 2018). Por lo tanto, el objetivo principal de esta tesis doctoral fue evaluar la comunidad endofítica asociada al olivo y dilucidar su posible papel en la defensa de las plantas contra la antracnosis del olivo.

En primer lugar, se llevaron a cabo estudios para caracterizar los endófitos que habitan varios órganos de cultivares de olivo con diferente susceptibilidad a la antracnosis ("¿Quién está allí?") y para evaluar el efecto sobre los ensamblajes de hongos endófitos de factores como el órgano de la planta, el genotipo del huésped, la fenología del huésped y la incidencia de enfermedad ("¿Qué factores contribuyen a su conformación?"). A continuación, se relacionaron las comunidades endofíticas con la susceptibilidad de las plantas a la enfermedad ("¿Qué pueden hacer?"), y se evaluó su potencial como agentes de control biológico contra la antracnosis del olivo tanto in *vitro* como en bioensayos de frutos ("¿Podrían ser útiles?").

¿Quién está ahí?

La comunidad endofítica asociada a raíces, ramas, hojas, inflorescencias y frutos de olivo se mostró rica y abundante, comprendiendo especies pertenecientes principalmente a el filo Ascomycota y a la clase Sordariomycetes, siendo Basidiomycota el filo menos representado en todas las muestras. Las comunidades endófitas diferían según el órgano del olivo, siendo los miembros de los géneros *Phomopsis* (Diaporthaceae) los endófitos predominantes en hojas y ramas, mientras que *Fusarium*

(Nectriaceae), *Biscogniauxia* (Xylariaceae) y *Alternaria* (Pleosporaceae) resultaron más abundantes en las raíces, inflorescencias y frutos.

¿Qué factores contribuyen a su conformación?

Se investigó la importancia de diferentes factores bióticos, como el órgano de la planta, el genotipo del huésped (a nivel del cultivar), la fenología del huésped y la incidencia de la enfermedad de antracnosis, sobre los ensamblajes de hongos endofíticos en los órganos reproductivos del olivo. Los resultados del **capítulo 2** revelaron que el órgano de la planta, la etapa fenológica y, en menor medida, el cultivar, influyen significativamente en la estructura de toda la comunidad que habita los órganos reproductores del olivo, en particular las inflorescencias. La población de hongos endofíticos en frutos solo se vio afectada por la etapa fenológica. En general, tanto la abundancia como la riqueza de hongos aumentaron cuando el desarrollo de la inflorescencia alcanzó un pico en la floración, y disminuyendo posteriormente hasta que el fruto comienzó el proceso de madurez. A continuación, aumentó hasta alcanzar un pico cuando la epidermis del fruto alcanzó el color rojo o púrpura. En general, las inflorescencias albergaban una mayor diversidad y abundancia de endófitos que los frutos. Los resultados del **capítulo 3** indicaron que el nivel de incidencia de antracnosis también contribuye a la conformación de las comunidades de hongos en los órganos reproductores del olivo, en particular de los frutos.

¿Qué pueden hacer?

Con el objetivo de dilucidar el papel potencial de los endófitos en la supresión de la enfermedad y en la transición de fases de vida del patógeno *Colletotrichum* spp. se comparó la comunidad endofítica asociada a los órganos reproductivos del olivo entre olivares con diferente incidencia de antracnosis en diferentes etapas fenológicas. (**Capítulo 3**). Las comunidades endófitas de los órganos reproductivos en los dos olivares se mostraron distintas, difiriendo en la riqueza, abundancia y composición de las especies. En general, se observó una mayor abundancia y riqueza de hongos endófitos en el olivar con baja incidencia de antracnosis en comparación con los de alta incidencia. En particular, se observó la abundancia y riqueza de hongos benéficos (en los frutos) y patógenos (en los botones florales) menor en el olivar con alta incidencia de antracnosis que en el olivar con baja incidencia de antracnosis. Se observó que una relación positiva entre varias OTUs y uno de los dos olivares. Mientras que *Pseudophaeomoniella oleae*, *Neofabraea vagabunda*, *Neofabraea* sp. y *Parastagonospora avenae* fueron los más asociados con el olivar de alta incidencia de antracnosis, *Diaporthe rudis*, *Fusarium*

oxysporum, Pezizomycetes sp., *Epicoccum nigrum, Mollisia minutella, Trametes* sp. y *Sardariomycetes* sp. mostraron una elevada asociación al olivar con baja incidencia de antracnosis. Estos resultados sugieren múltiples interacciones de la comunidad de hongos y *Colletotrichum* spp. en el olivar con alta y baja incidencia de enfermedad, cuyo efecto parece ser crítico para el establecimiento de comunidades de hongos (y el patógeno subyacente) en el olivo. Probablemente, en el olivar con baja incidencia de enfermedad tanto los patógenos *Colletotrichum* spp., como la enfermedad no pueden prosperar tan satisfactoriamente como en el olivar con alta incidencia de enfermedad debido a la presencia de una comunidad endófita más rica y abundante, principalmente de hongos antagonistas. Las diferencias en la composición de la comunidad endofítica entre los dos olivares fueron mayores en los frutos que en las flores, lo que sugiere que la transición de fases de vida en *Colletotrichum* spp. desde latente (durante la etapa de la flor) hasta patogénica (durante la etapa del fruto) podría estar relacionada con la estructura de la comunidad endofítica.

¿Podrían ser útiles?

Se estudió la comunidad endofítica asociada a cultivares de olivo de diferente susceptibilidad a la antracnosis (*Galega vulgar*: susceptible, *Cobrançosa*: moderadamente susceptible y *Picual*: resistente) y se evaluó la actividad antagonista de los aislados endofíticos de *Galega vulgar* y *Cobrançosa* contra *Colletotrichum* spp. **(Capítulo 4**). En general, se encontró una mayor riqueza y abundancia de hongos endófitos en las raíces en comparación con las ramas y las hojas. Estas diferencias entre los órganos fueron mayores en los cultivares más resistentes (*Picual* y *Cobrançosa*) que en el cultivar *Galega vulgar*, más susceptible. Los experimentos de cultivo dual realizados con endófitos aislados del cultivar moderadamente susceptible a antracnosis mostraron el mayor efecto antagonista contra *C. acutatum*, al reducir su esporulación, germinación y crecimiento. Los antagonistas más potentes fueron *Penicillium commune, Paecilomyces lilacinus*, *Fusarium oxysporum* e *Hypocrea lixii*, que también mostraron cambios morfológicos en las hifas de *C. acutatum*. El análisis multivariado de los datos obtenidos de los experimentos de cultivo dual mostró que la mayor capacidad para inhibir el crecimiento de *C. acutatum* está asociada a endófitos aislados de ramas del cultivar más susceptible, mientras que la inhibición de la germinación y la esporulación de *C. acutatum* manifestó una asociación superior a endófitos aislados de las hojas del cultivar moderadamente susceptible.

Sin embargo, las raíces de ambos cultivares no parecen ser una fuente importante de endófitos antagonistas de *C. acutatum*. En la inoculación artificial de frutos separados, el endófito *H. lixii* mostró una reducción significativa de la incidencia y severidad de la antracnosis del olivo causada por los patógenos *C. acutatum*, *C. godetiae* y *C. fioriniae*. La efectividad de este endófito como agente de control biológico varió según la madurez del fruto, siendo más notoria en frutos que comienzan a cambiar el color de la piel (índice de maduración 2-3) que en las aceitunas moradas o negras (índice de maduración 4). En conjunto, los resultados sugieren que las diferencias en la composición de la comunidad endófita entre los cultivares de olivos están de alguna manera correlacionadas con la susceptibilidad de las plantas a la enfermedad de antracnosis.

En general, se demostró que la comunidad de endófitos que habitan en el olivo es el resultado de una interacción compleja establecida entre la microbiota endofítica residente en las plantas y *Colletotrichum.* Este estudio también destaca la importancia de estas interacciones para la salud de los olivos y, en particular, para el desarrollo de la enfermedad de la antracnosis del olivo. Este conocimiento se puede utilizar para direccionar las actividades deseadas de los endófitos benéficos y mejorar la protección de los cultivos. En base a nuestros resultados, creemos que la microbiota del olivo puede ser optimizada para la resistencia a la antracnosis mediante la aplicación de hongos endófitos. Estos estudios indican que *H. lixii*, *F. oxysporum* y *E. nigrum* son muy promisorios para este propósito. El momento de su aplicación también puede ser un factor importante para generar y mantener microbios benéficos en el momento adecuado, en las inflorescencias o en los frutos. Se espera que la aplicación endofítica durante la floración reduzca la presión de *Colletotrichum* spp. durante la maduración de los frutos, la transición del modo de vida de *Colletotrichum* spp. de latente a patógeno podría verse comprometida. Sin embargo, aún se necesitan más estudios para esclarecer las complejidades de la comunicación entre todos los miembros de esta interacción múltiple, que condicionan el ensamblaje del patobioma en el olivo. En particular, el mecanismo exacto por el cual *H. lixii*, *F. oxysporum* y *E. nigrum* interfieren con *Colletotrichum* spp. debe estudiarse y examinarse cuidadosamente mediante el análisis de metatranscriptomas y metaproteínas.