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Characterization of leaf-associated fungi from olive cultivars with different levels of resistance to anthracnose

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Abstract

Olive anthracnose, caused by *Colletotrichum* spp., is one of the most damaging fruit diseases in olive crops worldwide. Their control is very difficult and relies mostly on the use of copper-based fungicides. The plant-associate fungal community has been increasingly recognized for playing an important role in plant health. Thus, in this work, the leaf-associated fungi of olive cultivars with different levels of resistance to anthracnose were characterized to identify potential fungi implicated in host resistance. A culture-dependent approach was used to assess both epiphytic and endophytic fungal communities of leaves of olive cultivars Madural (susceptible to anthracnose) and Cobrançosa (moderately tolerant), and the isolates obtained were identified by rRNA sequencing. Overall, *Ascomycota* phylum and *Aureobasidium* genus were the most dominant, being epiphytes significantly more diverse and abundant than endophytes. Among the genera identified in the most-resistant cultivar, *Aspergillus* and *Pseudocercospora* were the most frequently isolated within epiphytic and endophytic communities, respectively; whereas in the susceptible cultivar, *Aureobasidium* and *Didymocyrtis*, were the most frequently isolated within epiphytic and endophytic communities, respectively. The host plant (at cultivar level) had a structuring effect on the composition of fungal communities of leaves, being this effect greater on epiphytes than on endophytes. Thus, it is likely that each cultivar selects specific fungal taxa, which may lead to specific feedbacks on fitness of plant genotypes. In total, 20 fungal taxa (15 endophytes and 5 epiphytes) were responsible for more than 50% of the dissimilarity found on fungal community between cultivars. Among these, three taxa from the Phaeomoniellaceae family and one unidentified fungus, were the most discriminant. Their functional role needs to be studied in the future, because they might be important in conferring host plant resistance towards anthracnose.

Keywords: *Olea europaea*, *Colletotrichum*, Endophytes, Epiphytes, Host specificity.

Resumo

A gafa, causada por diversos fungos do género *Colletotrichum*, é das doenças que mais prejuízos causa nos olivais em todo o mundo. A sua luta é muito difícil e é feita com recurso a fungicidas à base de cobre. A comunidade fúngica associada às plantas tem sido cada vez mais reconhecida por desempenhar um papel importante na sanidade das plantas. Assim, neste trabalho, foram caracterizados fungos associados a folhas de cultivares de oliveira, com diferentes níveis de suscetibilidade à gafa, para identificar fungos potencialmente responsáveis por estas diferenças de resistência. Para tal, recorreu-se ao método dependente de cultivo para avaliar as comunidades fúngicas epífitas e endófitas em folhas das cultivares Madural (suscetível à gafa) e Cobrançosa (moderadamente tolerante), tendo os isolados obtidos sido identificados pela sequenciação da região ITS do rRNA nuclear. Globalmente, o filo *Ascomycota* e o género *Aureobasidium* foram os mais dominantes, enquanto que os epífitos revelaram ser significativamente mais diversos e abundantes do que os endófitos. Entre os géneros identificados na cultivar mais resistente, *Aspergillus* e *Pseudocercospora* foram os mais abundantes dentro das comunidades fúngicas epífitas e endofíticas, respetivamente; já na cultivar suscetível, *Aureobasidium* e *Didymocyrtis*, foram os géneros mais frequentemente isolados dentro das comunidades fúngicas epífitas e endofíticas, respetivamente. O genótipo da planta (ao nível da cultivar) teve um efeito significativo na composição da comunidade fúngica das folhas, sendo este efeito superior nos epífitos face aos endófitos. Parece, assim, que cada cultivar possui a capacidade de selecione uma espécie específica, que poderá ter repercussões positivas na planta hospedeira. No total, 20 espécies fúngicas (15 endófitos e 5 epífitos) foram responsáveis por mais de 50% da dissimilaridade encontrada na comunidade fúngica entre as cultivares. De entre estas, três espécies da família Phaeomoniellaceae e um fungo não identificado, foram os mais discriminantes. A função destas espécies na promoção de resistência na oliveira deverá ser estudada no futuro.

Palavras-chave : *Olea europaea*, *Colletotrichum*, Endófitos, Epífitos, Especificidade do hospedeiro.

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I. General introduction

1. Olive tree: Geographic distribution and economic importance

Olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) cultivation is ancient, and its history merges with that of the Mediterranean basin (Cacciola et al., 2012). Indeed, its origin must have taken place at Middle East, near the border between Turkey and Syria (Besnard et al., 2018). Starting from this region, the olive spread to other places around the Mediterranean Sea, being nowadays considered the tree par excellence of the Mediterranean region (Besnard et al., 2018). The culture of the olive tree continued its expansion outside the Mediterranean with the discovery of America, throughout Peru, Chile, Uruguay, Argentina and the coastal regions of Mexico and United States - California (IOC, 2019). Nowadays, olive growing has also been introduced in traditionally non-producing countries like South Africa, Australia, New Zealand and China (Cimato and Attilio, 2011; Su et al., 2018).

Currently, olive tree cultivation still has great economic, social, and ecological importance in the Mediterranean region, where 95% of world olive groves are concentrated, ensuring almost 95% of world production for the 2018/19 crop year (Fig. 1; IOC, 2019). About 70% of the total olive production comes from the European community (Spain, Italy, Greece and Portugal), the Near East and North Africa provides around 24%, and the remaining 6% comes from the other countries of the world, mainly from Argentina, Mexico, Peru and the United States (IOC, 2019). At the European community, Spain is the largest producer ensuring 55% of world production for the 2018/19 crop year, followed by Greece (6% of world production), Italy (5% of world production) and Portugal (3% of world production) (IOC, 2019).

The olive growing area in Portugal is of 352 000 ha, of which 23% is irrigated while the rest is rain-fed (IOC, 2019). The largest region of olive production is Alentejo, accounting for 50% of the total olive growing area, followed by Trás-os-Montes (22%), Beira Interior (18%), Ribatejo (7.7%) and Algarve (2.3%) (IOC, 2019). From those, six percent of the olive growing area is used for organic olive farming (IOC, 2019). Olive crops are economically very important in Portugal, and according to the latest market report of the International Olive Council (IOC, 2017), the olive sector represents €95.5 million and accounts for 1.36% of the value of the Portuguese agricultural production.

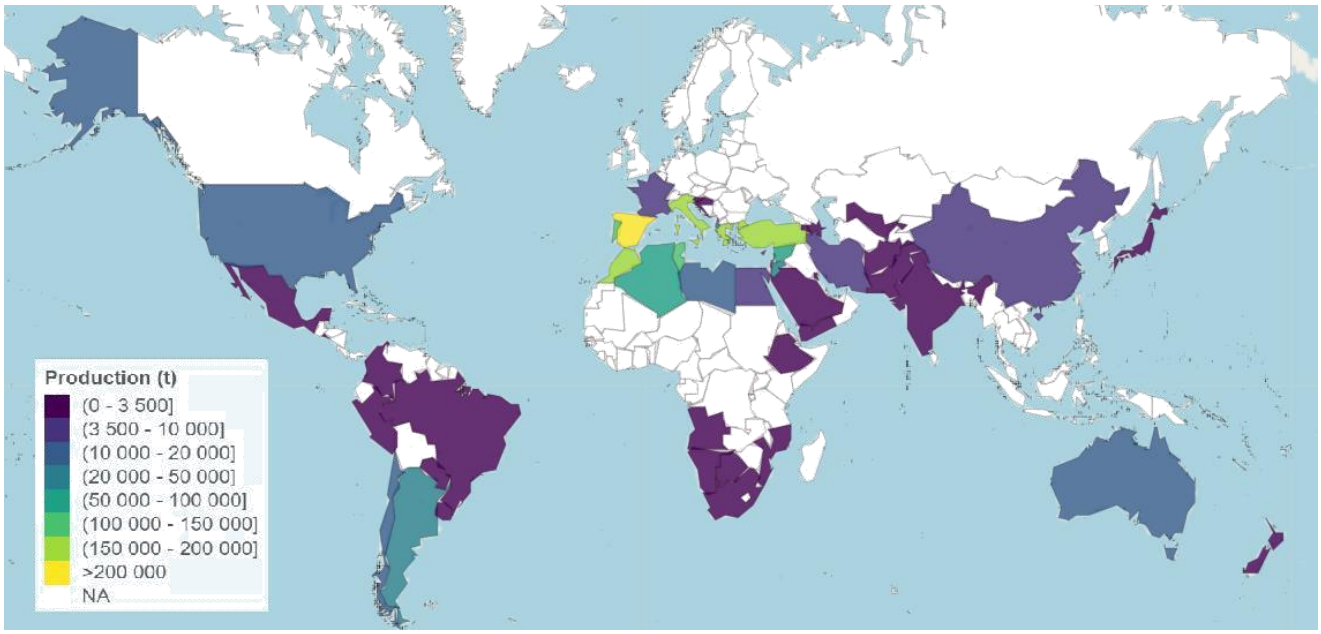

Figure 1.World olive oil production, 2018/2019 crop year. Source: IOC (2020).

2. Constraints to olive production: the case of anthracnose

Several pests and diseases cause significant yield and quality losses to olive table and olive oil production, the main products from this crop. Among them, olive anthracnose is the most damaging disease worldwide (Cacciola et al., 2012) and also in Portugal (Talhinhas et al., 2018). Typical anthracnose symptoms in olive fruits appear when they are nearly ripened and include dark necrotic lesions and rot with abundant orange masses of conidia that resulted in premature fruit drop or mummification (Fig. 2) (Talhinhas et al., 2011). Though it is chiefly a fruit disease, it may also affect flowers, leaves and shoots (Moral et al., 2009). Symptoms on leaves and shoots include chlorosis and necrosis of the leaves, severe defoliation, and dieback of twigs and branches (Fig. 2) (Cacciola et al., 2012). Infected flowers dry quickly, leading to its drop (Sergeeva et al., 2008).

Figure 2. Some symptoms of olive anthracnose: (a) defoliation of twigs and branches, (b) necrosis and production of orange gelatinous matrix embedding conidia in olives surface, (c) numerous infected fruits on the floor. Credits: José Alberto Pereira, ESA-IPB.

The disease has been reported from all olive-growing regions of the world, resulting in high economic impacts especially in years when environmental conditions are favorable to the disease (i.e., high humidity) and in groves with disease-susceptible varieties (Talhinhas et al., 2011). For instance, in Italy, Spain and Portugal, yield losses due to olive anthracnose often attain 80-100% (Talhinhas et al., 2011; Cacciola et al., 2012). In addition, the quality of the olive oil produced from diseased fruits can decrease significantly. These olive oils have frequently high acidity and peroxide values, which sometimes can surpass the maximum legal limit to be considered as virgin olive oil (Carvalho et al., 2008; Silva, 2016). Sensory attributes of these olive oils can also be negatively affected, showing smell and taste of grubby, rancidity, mold and awful smell and taste (Silva, 2016).

So far, olive anthracnose has been associated to 13 *Colletotrichum* species, primarily belonging to two complexes, *C. acutatum* sensu lato (s.l.) and *C. gloeosporioides* s.l., and in less extent to *C. boninense* s.l. complex (Talhinhas et al., 2018). From these, six species in the *C. acutatum* complex (*C. acutatum* sensu stricto (s.s.), *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. rhombiforme* and *C. simmondsii*) (Baroncelli et al., 2017) and two in the *C. gloeosporioides* complex (*C. gloeosporioides* s.s. and *C. theobromicola*) (Weir et al., 2012 ; Mosca et al., 2014; Schena et al., 2014) were identified to be the main causal agents of olive anthracnose. Fungi from four species in the *C. gloeosporioides* complex (*C. aenigma*, *C. kahawae* ssp. *ciggaro*, *C.*

queenslandicum and *C. siamense*) and one in the *C. boninense* complex (*C. karstii*) were isolated from anthracnose diseased olive fruits (Mosca et al., 2014; Schena et al., 2014), but their pathogenicity to olive fruit has not been confirmed. It is hypothesized that more species could be associated with olive anthracnose, either representing opportunistic or truly pathogenic interactions (Talhinhas et al., 2018). Both *C. godetiae* and *C. acutatum* s.s., followed by *C. nymphaeae*, are by far the most predominant in most olive-growing regions with a slight prevalence of the first over the other two species (Talhinhas et al., 2018). *Colletotrichum godetiae* is the prevalent species in most Mediterranean countries, while *C. acutatum* s.s. is the main causal agent in the Southern Hemisphere countries (Argentina, Chile, Perú and Austrália) but has becoming to emerge in several countries in the Mediterranean Basin (Talhinhas et al., 2018). The presence of *C. nymphaeae* have been mostly reported in southwest of the Iberian Peninsula (Talhinhas et al., 2018). In Portugal, the main causal agents of olive anthracnose are *C. nymphaeae* (80% of the isolates), *C. godetiae* (12% of the isolates) and *C. acutatum* s.s. (3−4% of the isolates), with *C. godetiae* causing major damage in the Trás-os-Montes region (Talhinhas et al. 2009).

Olive anthracnose incidence and severity vary considerably depending on climatic conditions (Talhinhas et al., 2011), the susceptibility of olive cultivars (Talhinhas et al., 2015 ; Moral et al., 2017), the fungal species and its prevalence (Talhinhas et al., 2015), and agronomical practices, such as density of olive tree plantation (Moral et al., 2012) and calcium fertilization (Xávier el al., 2014). In general, olive anthracnose reaches highest disease incidence and severity, when relative humidity is higher than 98% and the air temperature is warm, with values ranging from 10 to 30ºC (Cacciola et al., 2012). Differences in susceptibility of olive cultivars to anthracnose have been reported in many olive-growing regions (e.g., Moral and Trapero, 2009; Cacciola et al., 2012; Talhinhas et al., 2015; Moral et al., 2017). The susceptibility of cultivars assessed both in the field and in laboratory tests on detached fruits, allowed the classification of cultivars in five categories, namely highly resistant, resistant, moderately susceptible, susceptible and highly susceptible (Moral et al., 2017). In Portugal, the main olive oil cultivar, Galega vulgar, is highly susceptible to anthracnose, Cobrançosa is moderately susceptible, and Azeiteira is considered to be resistant (Talhinhas et al., 2015; Moral et al., 2017). However, this information has some limitations, since under favorable conditions the less susceptible cultivars can also be severely affect by anthracnose (Talhinhas et al., 2011). Indeed, it is commonly accepted that pathogen populations, causing epidemics in various olive- growing countries are particularly adapted to both the host and the environment conditions,

being the high variability of olive anthracnose incidence and severity mostly related to the variability of pathogen, host plant and environment at regional/local level (Cacciola et al., 2012). Canopy density also showed to play an important role in anthracnose epidemiology, being the disease severity higher in super-high-density orchards (2000 trees/ha) than in high-density orchards (200−800 trees/ha) (Moral et al., 2012). Similarly, there are some evidences suggesting that the low calcium content in the soil may contribute to increase the incidence of anthracnose (Xávier el al., 2014).

The complete disease cycle of olive anthracnose is still not fully understood (Cacciola et al., 2012). There are some studies showing that the pathogens overwinter on leaves, branches and mummified fruits remaining in trees (Talhinhas et al., 2011), and it is thought that the conidia produced by these fungi could be the major primary inoculum reservoirs of the disease in the next spring (Moral et al., 2009; Talhinhas et al., 2018). During this season, infection by *Colletotrichum* can take place in olive flowers from the early stages of flowering until fruit set, but normally remained latent until fruit begins to ripen in autumn (Moral et al., 2009). In the surface of ripe fruits, the pathogen sporulates, and the conidia release give rise to secondary infection cycles (Moral et al., 2009).

3. Anthracnose disease control

The control of olive anthracnose is very difficult because its spreading and development relies greatly on the climatic conditions. Hence, no effective control measures have been proposed so far to manage this disease. At present, management of anthracnose is based on the use of a combination of several approaches, including cultural, chemical and biological control.

Cultural practices rely mostly on the: i) planting of cultivars resistant to the anthracnose (Cacciola et al., 2012; Moral et al., 2017); ii) use of appropriate cultivation techniques, through balanced fertilization, irrigation (since the relative humidity helps successful colonization of the pathogen) and pruning, which contribute to eliminate sources of inoculum as well as to improve air movement in the canopy (Moral et al., 2014; Sergeeva, 2011); iii) anticipation of harvest time, which may help mature and overripe fruits to escape secondary infections (Cacciola et al., 2012); iv) and control of olive fruit fly attacks which generate entry points for *Colletotrichum* sp. on fruits (Malacrinò et al. 2017).

Chemical control of olive anthracnose is mostly based on the application of copper compounds (Cacciola et al., 2012). These are frequently applied at the beginning of spring and at

the first autumn rains, prior to the emergence of symptoms (Moral et al., 2014). However, such copper-based products are not effective against latent infection (Moral et al., 2009). In addition, the widespread use of these fungicides posed potential environmental and human health risks (Komárek et al. 2010). The European authorities would also like to ban, in the near future, copper fungicides in conventional and organic farming across the EU.

Regarding the **biological control** of olive anthracnose disease, few data are available in the literature, particularly in what concern to its use in field conditions. The inoculation of detached olive fruits with *Aureobasidium pullulans*, *Curtobacterium flaccumfaciens* and *Paenibacillus polymyxa* were shown to reduce the severity of the symptoms produced by *C. acutatum* in 76.4%, 53.7% and 51.6%, respectively (Segura, 2003). In field trays was observed that the application of *A. pullulans* to olive trees significantly reduced the anthracnose severity by 40% and latent infection by 14% (Nigro et al., 2018). One of the promising approaches in the biological control of anthracnose is the use of endophytes (Landum et al., 2016; Preto et al. 2017). These microorganisms are characterized to colonize the internal tissues of plants and recognized to confer host plant protection against abiotic and biotic stresses, including plant pathogens (Bacon and White, 2015). Indeed, several endophytic fungal species isolated from olive tree leaves (Landum et al., 2016) or olive fruits (Preto et al., 2017) showed to inhibit significantly the growth of *C. acutatum* under *in vitro* conditions. Some of these fungal species also showed the capacity to reduce the sporulation of *C. acutatum* by 46–86% and the germination by 21-74%, and to induce morphological alterations on pathogen hyphae (Preto et al., 2017). A similar protective effect was demonstrated in detached olive fruits inoculated with the fungal endophyte *Trichoderma koningii* obtained from olive tree leaves, that showed the ability to reduce significantly both olive anthracnose disease incidence and severity, when compared to control (non-inoculated with *T. koningii*) (Martins et al., 2019). The degree to which fungal endophyte regulates *C. acutatum* infection, was observed to be depend on host plant (Martins et al., 2013), emphasizing the importance of the interaction between endophyte–host plant for the biocontrol of the pathogen. Indeed, under *in vitro* confrontation assays between the endophyte *Penicillium commune* and *C. acutatum*, it was observed that the presence of olive leaf (+leaf) between these two fungi increases the inhibitory effect of the endophyte over the pathogen when compared to –leaf treatment (Martins et al., 2013). Altogether, the results obtained up to date regarding the exploitation of endophytes in the biocontrol of olive anthracnose appear to be very promis

4. Olive tree and its microbiome

Plant microbial inhabitants (those near or on plant tissues) of the rhizosphere (roots) and phyllosphere (above-ground parts of plants) are considered epiphytes, whereas microorganisms in the endosphere (i.e. residing within plant tissues) are considered endophytes (Turner et al., 2013). Interactions between the host plant and its microbiome as well as between members of the microbiota are highly complex and dynamic, with great impact for plant growth and productivity (Turner et al., 2013; Trivedi et al., 2019). There are some studies indicating that within plant microbiome, pathogens can establish multiple interactions, either positive or negative, with other microorganisms that may trigger or influence the disease process (Jakuschkin et al., 2016), something that is also true for olive trees (Fausto et al., 2018). For studying plant microbiomes, the use of high-throughput sequencing (HTS) methodologies is currently widely adopted as they allow identification of thousands to millions of sequences in a single sample (Turner et al., 2013; Berg, 2020). Yet, the cultivation and isolation of plant-associated microorganisms still is a requirement to better understanding plant-microbiota interactions and their functions in such intricate systems (Sarhan et al., 2019), and likewise to exploit and develop microbial-based products for use in agricultural biotechnology (Berg et al., 2020). Moreover, Anguita-Maeso et al. (2020) have recently noticed that almost 60% of bacterial genera recovered through cultivation methods could not be captured by a HTS approach when studying olive tree endophytic communities, highlighting the importance of continuing to adopt culture-dependent approaches for the study of microbial diversity associated with this important plant crop.

After reviewing plant-microbiome interactions, Trivedi et al. (2020) recognized that, for the same plant host, microbial communities (bacteria and fungi) associated with roots differ substantially from the aboveground communities. Even though soil may act as a common reservoir for both belowground and aboveground plant microbiota, other sources may play as inoculant reservoirs for the phyllosphere community. This is especially true for the fungal community, in which it was observed a smaller overlap between the community of aerial plant tissues and that of the soil than it was for the case of bacteria (Trivedi et al., 2020). By studying the olive tree phyllosphere, Gomes et al. (2018) observed that leaves harbored a fungal endophytic community more similar to the corresponding epiphytic community than it was observed in twigs. A possible explanation why epiphytes are more prone to colonize inner tissues of leaves than those of twigs is because of the presence of stomata, which represent natural open entrances at the

leaf surface (Gomes et al., 2018). Indeed, this transmission route was already confirmed to occur with bacterial endophytes (Frank et al., 2017). It is thus important to deepen the knowledge on how related the epiphytic and endophytic communities of olive tree leaves are (e.g., to recognize common, ecologically exchangeable taxa, including pathogens), namely in different cultivars and/or agronomic management systems (Fausto et al., 2018).

5. Fungal endophytes of olive tree and their manipulation for anthracnose control

The composition of endophytic fungal community associated with leaves, twigs, fruits and roots of the olive tree was recently reviewed by Martins et al. (2019) and Nicoletti et al. (2020). According to these authors, the overall endophytic fungal community of olive tree is mostly composed by members of phylum Ascomycota, representing more than 93% of the total number of endophytic isolates (Fig. 3). Fungal members of Pleosporaceaea and *incertae sedis* taxa were the most abundant in both leaves and twigs, accounting together to 90.3% of the total isolates, whereas Trichocomaceae and Nectriaceae were the most abundant in roots and fruits, respectively (Martins et al., 2019). Members belonging to Basidiomycota accounted for only 3.4% of the total isolates associated with the olive trees (Martins et al., 2019). Overall, the fungal endophytes in olive tree seem to be extremely diverse and therefore they are likely to play a multiplicity of ecological functions as well.

Although the abovementioned studies provided important insights about the endophytic fungal composition of olive tree, there are still important knowledge gaps concerning the importance of endophytic diversity to plant health, markedly on what concerns protection to pathogens. Similarly, almost nothing is known about the impact of crop management practices on the endophytic diversity, and of their effect on plant pathogen/diseases. Such knowledge will be of utmost importance for the development of novel measures to control diseases, through the manipulation of the endophytic microbial community (termed Bioengineering). In this more sustainable approach to suppress diseases, the endophytic community can be intentionally manipulated in order to maximize the benefits of the microbial social network for the host plant (Orozco-Mosqueda et al., 2018). To the best of our knowledge, such approach has not yet been explored in woody crop trees. Manipulation of microbiota is applied most extensively in humans for the treatment of diseases (Larsen and Claassen, 2018). Recent studies regarding olive endophytic microbial communities, showed that different abiotic (e.g., climate-related, host plant location, season) and biotic (e.g., plant organ, host genotype) factors play a pivotal role in structuring these communities (Martins et al., 2016; Preto et al., 2017; Gomes et al.,

2018; Mina et al., 2020). These results emphasize the potential importance of olive orchard management practices on the manipulation of endophytic microbial communities in order to improve positive interactions with the host plant. This approach will require a thorough knowledge of which factors are shaping olive tree-endophytic assemblages, and which are them role/function in plant health.

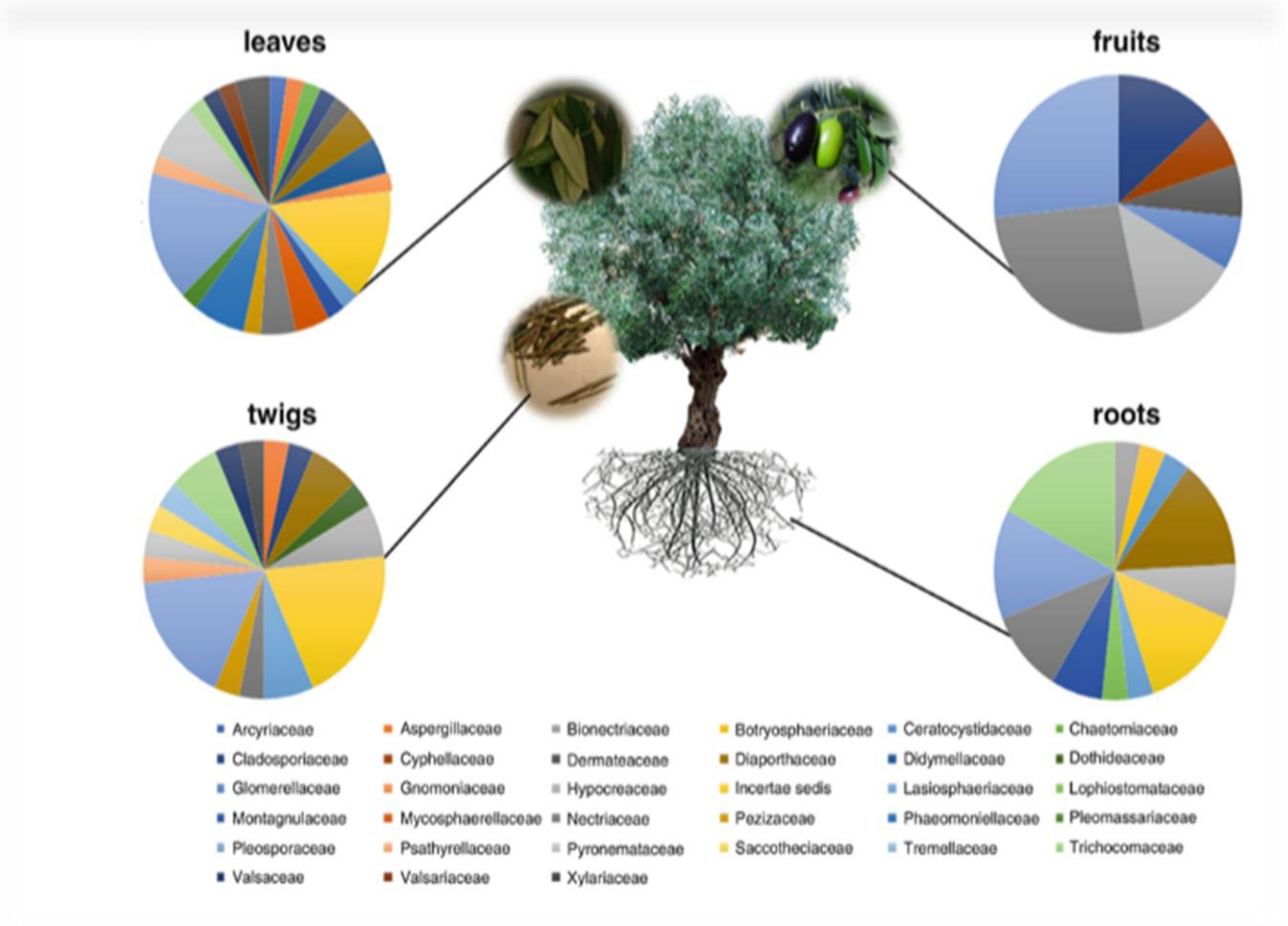

Figure 3. Abundance of fungal endophytes (number of isolates), at family level, colonizing leaves, twigs, fruits and roots of olive tree (Olea europaea L.). Source: Martins et al. (2019).

II. Objectives

The state-of-the-art literature shows a major gap in knowledge of the relationship between plant health, the associated microbial communities and the environment. This work aims to evaluate the importance of olive tree genotype (at cultivar level) in shaping the leaf-fungal composition, and their functional significance in conferring different host susceptibilities to anthracnose disease. Accordingly, both epiphytic and endophytic fungal communities of leaves collected in the exterior and interior of olive tree canopy from two cultivars with different susceptibilities to anthracnose (Madural and Cobrançosa, which are highly and less susceptible to anthracnose, respectively), were studied using culturing methods followed by sequencing of the PCR amplicons of fungal isolates. With this work we want to answer the following questions:

i) How do leaf-associated fungal communities differ in diversity and composition between the two host cultivars?

ii) May leaf location within tree canopy influence the associated fungal communities.

iii) May host cultivar shape the associated fungal community. If so, is this host effect similar for endophytes and epiphytes?

iv) Is there any fungal consortium specifically associated to each cultivar?

Such approach would allow to capture the complex plant-microbe-enviroment interactions and predict links between the cultured fungal community and host plant susceptibility to anthracnose. In particular, it is expected to elucidate wether each cultivar has a genetic system to host and nurture a specific fungal community. The results obtained will be useful to exploit the potential to use a specific olive tree genotype (at cultivar level) to model and manage host–fungal interactions, in order to reduce incidence/severity of anthracnose disease. Similarly, it is expected to identify a set of promising fungal isolates for olive crop protection against anthracnose. This knowledge will offer opportunities for controlling the olive anthracnose disease by using innovative and sustainable approaches based either on the manipulation of olive tree fungal communities, in order to improve positive interactions with the host plant, or by inoculation of olive tree with biocontrol agents.

III. Material and methods

1. Sample collection

Leaf samples were collected from one olive orchard located in Vale de Telhas, Mirandela (Northeast of Portugal, 41°36'29.0"N 7°13'27.6"W). This is a mountainous region, with altitudes ranging between 300 and 500 m, displaying a Mediterranean climate, with cold and rainy winters and long, hot, and dry summers. The average annual rainfall in Mirandela ranged from 500 to 700 mm, mainly occurring between October and February, and monthly average temperatures ranging from 3 to 26 °C. The olive orchard surveyed is organically managed, not irrigated, ploughed only to a shallow depth (10 cm) two times per year, and only treatments with copper-based products were performed once per year for fungal diseases control and no other pesticides were applied during our study. In this orchards, two common cultivars displaying different susceptibilities to anthracnose, were selected - the cultivars Cobrançosa (moderately susceptible) and Madural (susceptible). Five trees of each cultivar were used to collected asymptomatic leaves, from May to June 2019. In each tree was collected two branches with leaves, one in the exterior and the other in the interior of the canopy. The branches were transported to the laboratory and processed as expediently as possible, in order to isolate fungi from the epi- and endophytic communities.

2. Fungal isolation and enumeration

From each branch, five asymptomatic leaves were randomly selected and used to isolate epiphytes and endophytes. For isolating fungal epiphytes, around 1 g of leaves was added to 9 mL of sterile potassium phosphate buffer pH 7.0 (8 g/L NaCl; 0.20 g/L KCl; 1.4 g/L Na_2HPO_4; 0.24 g/L KH_2PO_4). This suspension was placed on a rotary shaker (200 rpm) for 15 min, at room temperature, to dislodge microorganisms from the plant surface. Aliquots of 1 mL of the resulting microbial suspensions were separately plated in triplicate on Potato Dextrose Agar (PDA; Difco, United States) medium. Endophytic fungi were isolated from the same leaves used to isolate epiphytes. For that, leaves were previously surface sterilized through a procedure described by Martins et al. (2016). It consisted in a successive immersion in 70% (v/v) ethanol for 1 min, 3-5% (v/v) sodium hypochlorite for 1 min and rinsed three times (1 min each) with autoclave-sterilized distillate water. Each leaf was then aseptically cut into five segments (c.a. 5 × 5 mm) which were transferred to PDA, the same culture medium used to isolate epiphytes. To validate surface sterilization procedure, the surface of sterilized plant tissues was imprinted

onto fresh PDA medium plates. Plates of both epi- and endophytic fungi were incubated at room temperature (22 ± 3°C) in the dark and were observed daily for microbial growth and colony counting. The enumeration of colonies was performed after acknowledging all the different fungal colony morphotypes present in plates. Fungal colonies from the different morphotypes were then subcultured on fresh medium until pure epi- and endophytic cultures were obtained. Again, isolates were first grouped according to their morphological similarity (colony morphology, hyphae, spores, and reproductive structures). One representative isolate of each fungal morphotype was then selected for further molecular identification.

3. Molecular characterization of the isolates and identification

The selected molecular marker wasp the interna transcribe Spacer (ITS) region of the nucleal ribosomal DNA (Rda), winch Is the Accepted DNA bar code for fungi (Scotch et al., 2012). Total nonunique DNA wasp extractive from Harvest éd mycelia/spores (5-10 days of culture growth) using the REDExtract-N-Amp™Plant PCR kit (Sigma, Poole, UK). The ITS region was amplified by polymerase chain reaction (PCR) using the primer set ITS1/ITS4 or, if unsuccessful, any of the other combination presented in Table 1.

Table 1. Conserved oligonucleotides used to amplify the ITS region from the fungal isolates obtained in this study.

Primer	Sequence (5’ $\rightarrow$ 3’)	Sense/anti-sense strand	Reference
ITS1*	5'TCCGTAGGTGAACCTGCGG3'	Forward	White et al., 1990
ITS2	5'GCTGCGTTCTTCATCGATGC 3'	Reverse	White et al., 1990
ITS4*	5'TCCTCCGCTTATTGATATGC 3'	Reverse	White et al., 1990
ITS5	5'GGAAGTAAAAGTCGTAACAAGG'	Forward	White et al., 1990
SR6R	5' AAGWAAAAGTCGTAACAAGG 3'	Forward	Vigalys and Hester.,1990

* By default, this was the first primer set tested (PCR product expected size: 600-700 bp). If amplification was unsuccessful, other combinations tested in further PCRs were ITS5/ITS4, SR6R/ITS4, ITS1/ITS2 or ITS5/ITS2 (whenever using the ITS2 reverse primer, the PCR product expected size was 200-300 bp).

All PCR reactions were performed using a MyCycler (BioRad, Hercules, United States) thermal cycler. PCR programs were set for an initial denaturation step at 95ºC for 5min, followed by 30 cycles of denaturation at 94ºC for 30s, primer annealing at 48-56ºC (52ºC has default temperature) for 40s and extension at 72ºC for 40s, followed by a final extension step at 72ºC for 7 min. The PCR components and respective amounts and concentrations are described in Table 2.

Table 2.PCR reagents used for 20 µL reactions.

Component	Volume (µL)	Final concentration
PCR reaction buffer (10X)	2	1X
Primer forward (10 µM)	0.4	0.2 µM
Primer reverse (10 µM)	0.4	0.2 µM
dNTPs (10 mM)[1]	0.4	0.2 mM
BSA (30 mg/mL)[2]	1	1,5 mg/mL
DNA polymerase (5 U/µl) [3]	0.1	0.025 U/µl
ddH_2O[4]	14.1	-
DNA molde (5-50 ng/µL)	1.6	0.4-4 ng/µL

[1]Deoxyribonucleotide triphosphate solution mix; equimolar solution of dATP, dCTP, dGTP and dTTP, at 2.5 mM each
[2]Bovine serum albumin
[3] DFS-*Taq* DNA polymerase (Bioron, Germany)
[4] sterile ultrapure water

All PCR products were analyzed by agarose gel electrophoresis (1.3%) in TBE buffer 1X (89 mM Tris base, 89 mM boric acid and 2 Mm EDTA, pH 7.6). PCR products (10 µL) were loaded onto the gel together with 2 µL of blue loading buffer (Bioron, Germany). The DNA Ladder 1Kb (Bioron, Germany) was used as molecular marker for DNA during gel electrophoresis, in the quantity recommended by the supplier. Electrophoretic separation was carried out at a potential difference of 100 V for 45 minutes. After the running, to stain the nucleic acid bands, a post-electrophoresis gel staining was performed by immersing it in an aqueous solution of GelRed at 3X (Biotium, United States), for 30 to 45 minutes. Gels were visualized by using the BioRad ChemiDoc^{TM} XRS system coupled with Image Lab^{TM} Software (Biorad, Hercules, United States). Valid amplified products were sent for sequencing to Macrogen Europe B.V. (Amsterdam, The Netherlands) services. Raw DNA sequences were then inspected and edited with Geneious 8.1.8 (Biomatters, New Zealand) and/or MEGA X (Kumar et al., 2018) software's. Then, the curated nucleotide sequences were used as queries in Basic Local Alignment Search Tool (BLAST) searches in GenBank, the NCBI's public nucleotide database (https://blast.ncbi.nlm.nih.gov/). To identify the fungal isolates, both the default (nr/nt) and the curated Fungi RefSeq ITS NCBI (Federhen, 2015) databases were used for nucleotide BLAST searches. In accordance to Vu et al. (2019), for sequence identities >99.6% species names were accepted; for sequence identities between 94.3% and 99.5% only the genus was accepted; and for sequence identities ranging between 88.5-94.2% and 81.2-

88.4% the family and order names were retained, respectively. If no identification could be attained at the genus level, isolates were identified as “unassigned” adding the corresponding lowest possible taxonomic rank classification. Fungal morphotypes for which it was not possible to obtain a molecular-based classification were distinguished but generically identified as “unknown fungi”. Pure cultures of each identified isolate were deposited in the CIMO culture collection at the Polytechnic Institute of Bragança, being preserved in 30% glycerol (v/v) at -80º C.

4. Diversity and composition of fungal communities

The diversity of fungal community was evaluated at the level of their richness (number of different taxa), evenness, and also Shannon–Wiener (H) index species diversity, using PAST (Paleontological Statistics) software v4.03 (Hammer et al., 2001). The results are presented as the mean of replicates (considering each individual tree as a sample unit), displaying respective SD values. To determine differences among the means, a one-way analysis of variance (ANOVA) with PAST v4.03 software was carried out, and the averages were compared using Tukey’s test ($p<0.05$). Non-metric multidimensional scaling (NMDS) was carried out to explore the similarity of fungi’s community composition among the surface and inner leaf tissues (epiphytes and endophytes, respectively), interior and exterior of tree canopy, and olive tree cultivars (Madural and Cobrançosa). This analysis ranks fungal communities of each sample (represented by squares) in ordination space in a way that the distance between the two squares is inversely proportional to their similarity. NMDS was performed by using Bray-Curtis similarities matrices (Bray and Curtis, 1957). NMDS calculates a stress value (Kruskal's stress), which assesses how well the derived ordination fits the given dissimilarities. According to Clarke (1993), Kruskal's stress values less than 0.2 represent plots with good ordination. Analysis of similarity (ANOSIM) was used to test for significant differences ($p<0.05$) between fungal community groupings obtained in NMDS ordination, using the Bray–Curtis distance matrices. This analysis compares the species composition between-groups (fungal community, canopy location and olive tree cultivars) and generates an R-value that gives the degree of discrimination between groups, associated to a p-value (significant when lower than 0.05). R-values range from 0 (completely similar) to 1 (completely dissimilar) (Clarke and Gorley, 2001). When a significant difference was observed, similarity percentage analyses (SIMPER) were performed to reveal which fungal taxa contributed to the dissimilarity between olive tree cultivars. All multivariate analyses were done using the *Community Analysis Package* v. 3.0 (Henderson and Seaby, 2007).

IV. Results and discussion

1. General description of the fungal community

The isolation and characterization of fungi from the olive leaves allowed to distinguish 153 taxa, mostly belonging to the Ascomycota (relative abundance of 89%) phylum (Figure 4), which is consistent with early studies conducted on olive leaves and twigs using both culture- (Martins et al., 2016; Gomes et al., 2018) and uncultured-dependent methods (Abdelfattah et al., 2015; Giampetruzzi et al., 2020; Vergine et al., 2020). The Basidiomycota represented 3% of the entire community, while an unassigned Mucoromycota species (0.4%) was found to be present in both endophytic and epiphytic communities. Twenty-six of the isolates have been identified to the species level, while the remaining could not be assigned at this taxonomic rank, nor even at the genus level (additional 63 isolates). The high number of unclassified and unidentified isolates agrees with the evidence that there is still novel olive tree-associated fungal diversity to be uncovered, as previously suggested for the endophytic fungal communities of leaves and twigs of some olive cultivars (Giampetruzzi et al., 2020; Vergine et al., 2020). The identified species belonged to 26 genera and 20 families, all from the Ascomycota (20 genera) and the Basidiomycota (6 genera) phyla. The more abundant class was Dothideomycetes (41.2%), followed by Eurotiomycetes (34.8%) (Figure 4), as previously reported in leaves of other olive cultivars, using metabarcoding approaches (Abdelfattah et al., 2015). At the order level, Phaeomoniellales (28.1%), Pleosporales (22.4%), and Dothideales (11.9%) were the most abundant taxa.

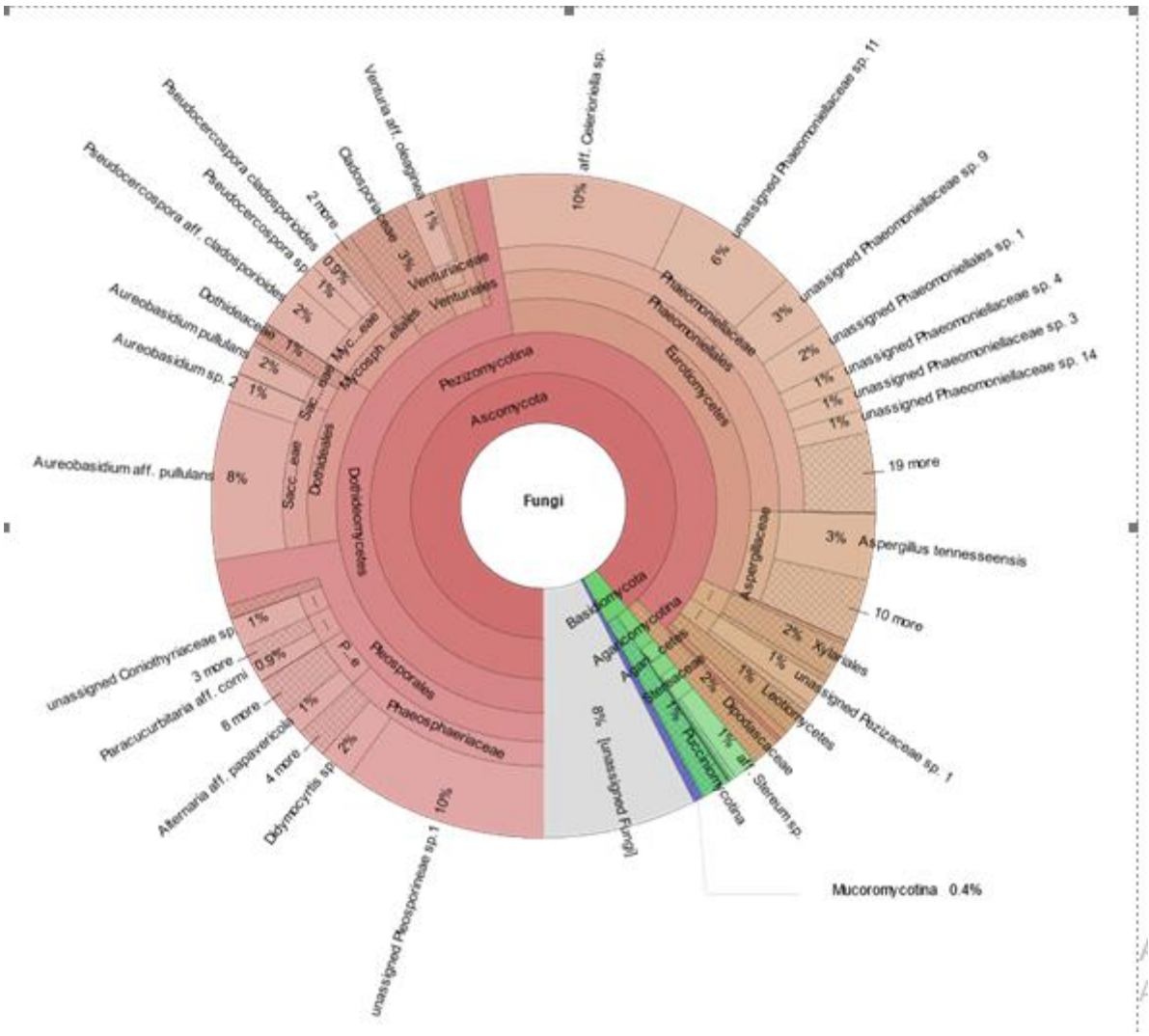

Figure 4.Krona chart showing the relative abundance of total fungi (endophytic and epiphytic), at the species level, detected on leaves of cvs. Cobrançosa and Madural olive trees. Figure was constructed using Krona interactive tool (Ondov et al., 2011).

Regarding isolates identified at least at the genera level (expressed as total number of isolates), the most abundant identified taxa in the endophytic community (Figure 5a) were *Pseudocercospora* (9.6%), *Cladosporium* (4.8%) and *Didymocyrtis* (3.3%). However, there were three abundant taxa that could not be assigned at the genus level (which are included in the label "Others", in Figure 5), two unassigned Phaeomoniellaceae species (11.5% and 5.6%) and one unidentified fungus (8.1%). Considering different cultivars, for cv. Cobrançosa, *Pseudocercospora* (with 13%), *Cladosporium* (8%) and *Alternaria* (7%) were the most abundant identified genera. For cv. Madural, the identified genera *Didymocyrtis*

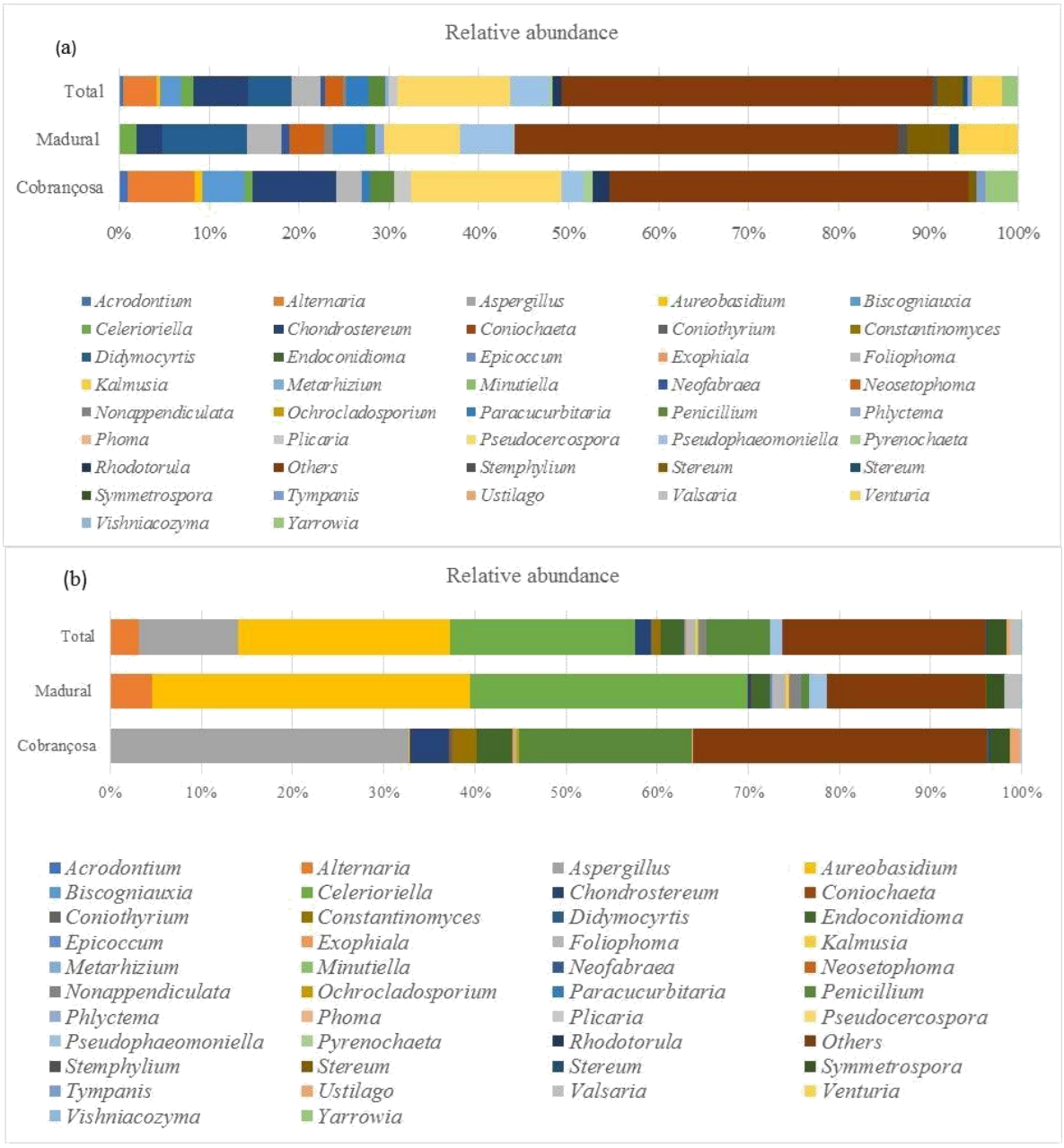

Figure 5.Relative abundances of genera from the (a) endophytic and (b) epiphytic communities associated with leave tissues of Olea europaea L. (cvs. Madural and Cobrançosa).

In relation to the epiphytic community (Figure 5b), *Aureobasidium* (21%) was the most abundant taxon, followed by an unassigned Phaeosphaeriaceae species (19.4%), *Celeriorella* (18.3%), *Aspergillus* (6.5%), and *Penicillium* (4.3%). Taking into account the epiphytic community in each cultivar, the most abundant genera for cv. Cobrançosa were *Aspergillus* (16%) and *Penicillium* (10%), while for cv. Madural were *Aureobasidium* (35%) and *Celerioriella* (31%). The genera *Aureobasidium*, *Pseudocercospora*, *Cladosporium* and *Alternaria* seem to be ubiquitous in olive tree leaves, as they were identified as the most

abundant in several other studies, using both culture-dependent (Martins et al., 2016; Gomes et al., 2018) and culture-independent (Abdelfattah et al., 2015) methods.

2. Comparison of fungal diversity

The fungal diversity, determined by the species richness (number taxa), evenness (homogeneity or even of a community in terms of the abundance of its species) and Shannon-Wiener diversity index, was compared between the two fungal communities (Epiphytic vs. Endophytic), the position of leaves within tree canopy (Inside vs. Outside) and the cultivars (Cobrançosa vs. Madural) (Fig. 6). Although the similar Shannon-Winer index between endophytic and epiphytic communities, the average number of epiphytic species per tree was significantly higher (up to 1.5-fold, $p < 0.001$) than endophytic. Moreover, endophytic community exhibited significantly more evenness (up to 1.4-fold; $p < 0.001$) than the epiphytic community. In previous studies, the same result was observed either within fungal (Gomes et al., 2018) or bacterial (Mina et al., 2020) communities colonizing olive tree leaves. The above-ground plant surface, or phyllosphere, is considered to be a harsh environment for microorganisms, since they are exposed to adverse environmental conditions, such as shifts in temperature, limited moisture, and solar radiation (Whipps et al., 2008). Nevertheless, epiphytic microorganisms can thrive on plant leaf surfaces by producing several biostructures or compounds. For instance, they can form aggregate structures to maintain a hydrated surface (Whipps et al., 2008) or produce surfactants to increase the diffusion and solubilization of substrates from the plant (Burch et al., 2012). On the other hand, endophytes have a more sheltered habitat, but they may have the competence to penetrate and colonize the internal tissues of plants and overcome the defense reactions of host plant (Rastogi et al., 2013). Many fungi land on leaves surface, but only a few enter the tissues via natural openings such as stomata and hydathodes (Volholt, 2012). These dissimilar stress factors faced by epiphytes and endophytes may explain why the fungal diversity on the surface of leaves was much higher than those colonizing the endosphere. In contrast to previous studies (Gomes et al., 2018), no differences were observed on the whole fungal diversity among cultivars or position of leaves within the tree canopy (Fig. 6). The contrasting results in our study and that of Gomes et al. (2018) can be due to differences in sampling sites and data collection, as these factors can affect fungal endophytic (Martins et al., 2016) and epiphytic (Gomes et al., 2018) communities.

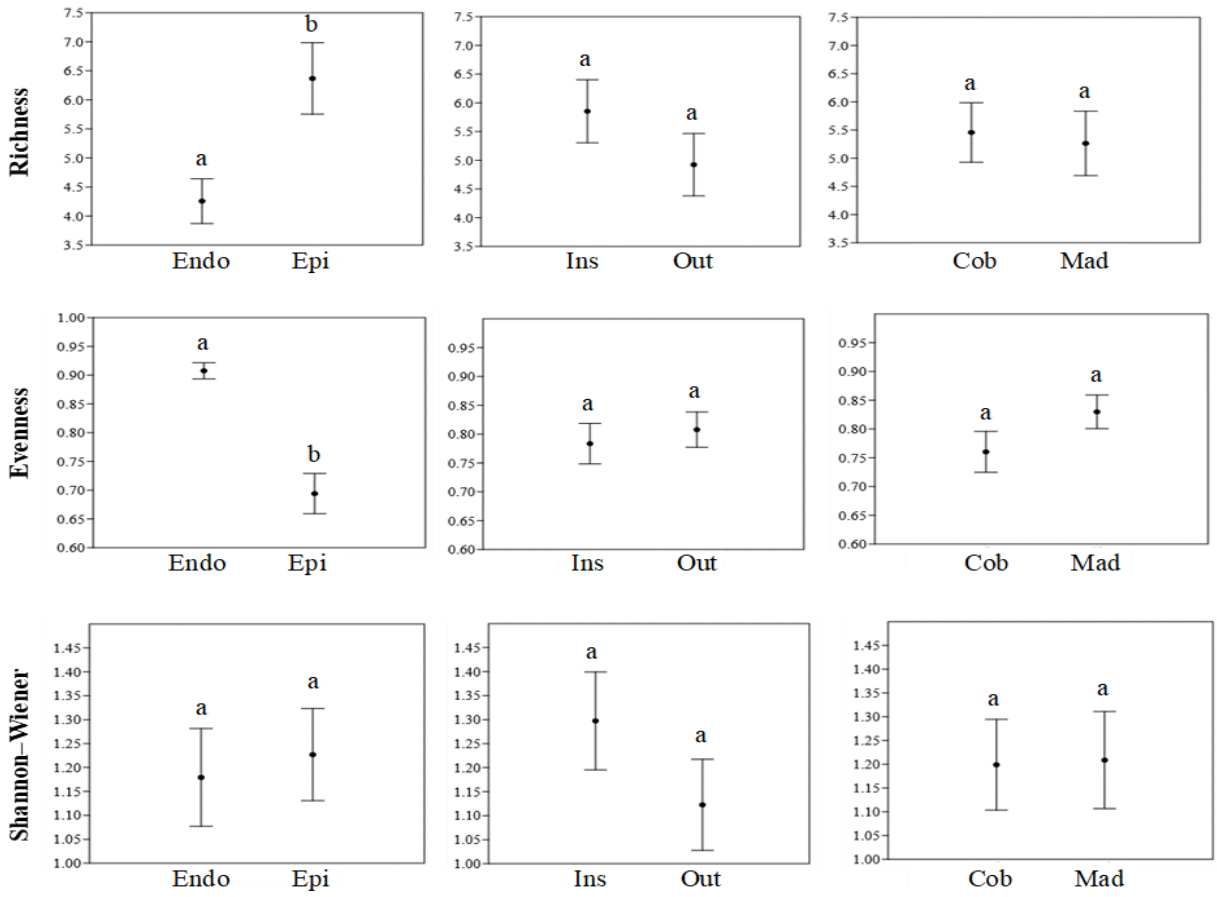

Figure 6. Comparison of fungal diversity of olive tree leaves between plant habitat (Endophytes - Endo vs. Epiphytes - Epi), position within tree canopy (Inside – Ins vs. Outside – Out) and cultivars (Cobrançosa – Cob vs. Madural – Mad). Diversity at community level was evaluated by determining fungal richness, evenness and Shannon–Wiener index. Graphics depict the mean (dot) and the standard deviation (vertical lines), being olive tree used as a replicate (N=5). Different letters among each variable denote statistically significant differences (at $p<0.05$; one-way ANOVA, Tukey test).

3. Comparison of fungal composition

The composition of the entire endophytic and epiphytic fungal communities present on olive tree leaves were similar, as indicated by the non-metric multidimensional scaling (NMDS) plot (Fig. 7a), based on Bray-Curtis index. Although the overlapping of these two fungal communities, the ANOSIM test revealed that their composition is significantly different ($R = 0.242$, $p=0.001$; Table 3). The importance of the plant habitat (internal *vs*. external plant tissues) for the fungal community structure of leaves have been previously described in several plant species, including olive tree (Gomes et al., 2018). Differences on nutrients and/or

environmental conditions between internal and external olive tree leaves could have influenced the selection of specific fungal taxa, giving rise to different fungal communities within epiphytes and endophytes. According to SIMPER analysis, 8 fungal taxa explained up to 50% of the dissimilarity found between endophytic and epiphytic fungal community's composition (Fig. 7b). These differences were due to the higher occurrence of Pleosporineae sp. 1 and/or the exclusive occurrence of *Aureobasidium* sp. 2, *Aspergillus tennesseensis*, *Aureobasidium* aff. *pullulans*, Phaeomoniellales sp. 1 and *Endoconidioma* aff. *populi* in epiphytic fungal community.

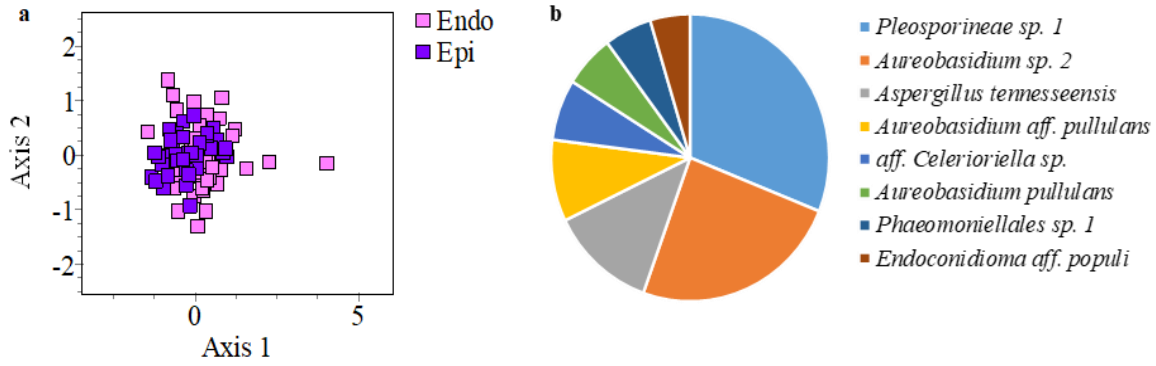

Figure 7. Structure of the fungal community associated to olive tree leaves. (a) Non-metric multidimensional scale (NMDS) plot corresponding to the clustering of fungal communities isolated from the surface (Epiphytes – Epi) and endosphere (Endophyte – Endo) of olive tree leaves**. (b)** List of the fungal taxa that explained up to 50% the dissimilarity found between endophytic and epiphytic fungal communities, obtained by the SIMPER analysis. Cluster analysis was performed with Bray-Curtis coefficient, being the Kruskal's stress value 0.098 (≤ 0.2, which represent good ordination plot).

The composition of fungal communities on leaves collected in the interior and exterior of olive tree canopy was very similar, as revealed by the overlapping of the samples in the NMDS plots (Fig. 8) and results of ANOSIM analysis (Table 3). Within epiphytic community, this phenomenon could be explained by the relatively similar environment conditions between the exterior and interior of the olive tree canopy. Accordingly, the observed results for the endophytic assemblage are probably due to the similarities of endospheric environment among leaves from the exterior and interior of canopy.

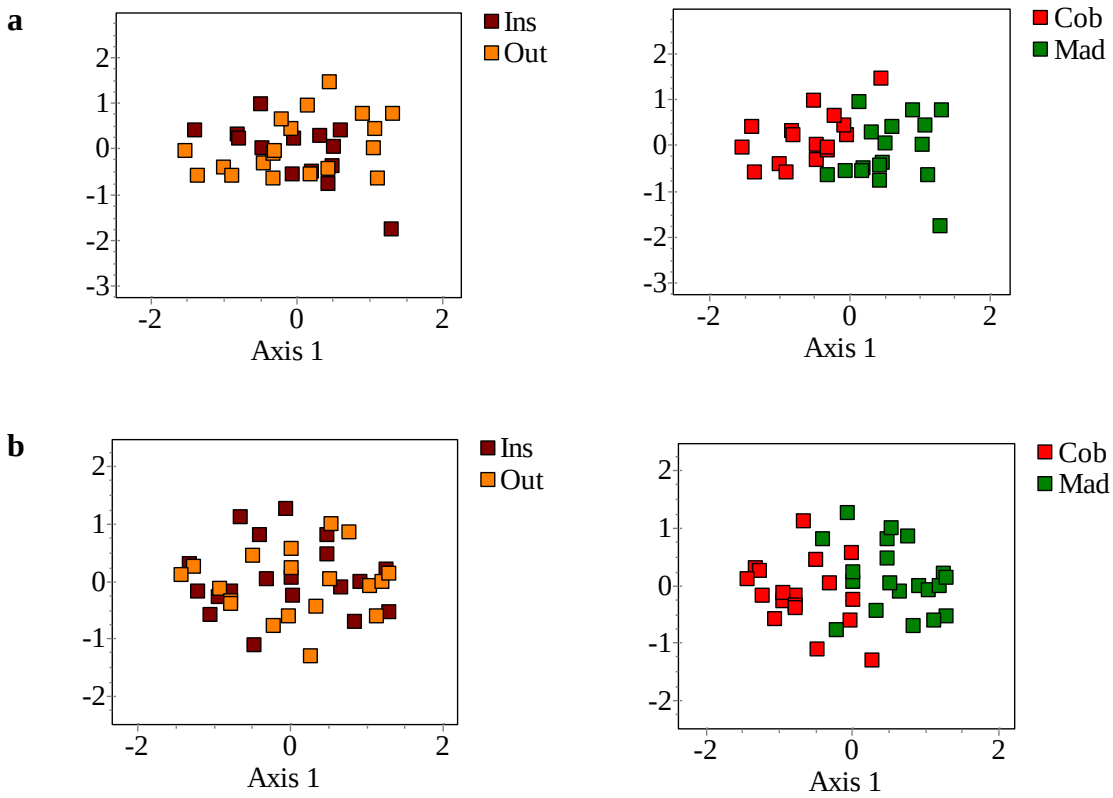

Figure 8. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of endophytic **(a)** or epiphytic **(b)** fungal communities grouped by position within tree canopy (Inside – Ins vs. Outside – Out) and cultivars (Cobrançosa – Cob vs. Madural – Mad). Cluster analysis was performed with Bray-Curtis coefficient, being Kruskal's stress values 0.086 and 0.113 for endophytic and epiphytic communities, respectively (≤0.2, which represent good ordination plots).

The ANOSIM analysis revealed that host cultivar significantly affected the composition of total fungal communities of leaves (R=0.247, p=0.001; Table 3). This host effect was greater within the epiphytic (R=0.450, p=0.001) than the endophytic (R=0.260, p=0.001) community. The NMDS plots corroborate these results by showing two clusters of fungal communities associated with different olive tree cultivars (Cobrançosa and Madural), being their separation slightly greater at the level of epiphytes than of endophytes (Fig. 8). This implied that endophytic and, in particular, epiphytic community structure of olive tree leaves can be influenced by host cultivar, which is consistent with previous studies focusing on fungal(Gomes et al., 2019) or bacterial (Mina et al, 2020) communities of olive tree twigs and leaves. Altogether, it is likely that each olive cultivar can apparently select specific foliar fungal strains from the environment, as previously suggest for other plant species (Bálint et al., 2013). From our findings, the fungal recruitment occurring in the leaves is for both epiphytes and

endophytes; and we hypothesized that this phenomenon may lead to specific feedbacks on fitness of plant genotypes as suggested previously by Mina et al. (2020). This assumption still needs to be confirmed with further work. Still, the SIMPER analysis revealed that up to 50% of the dissimilarity found on fungal community among cultivars is due to 15 endophytes and 5 epiphytes taxa (Fig. 9). Within endophytes, two taxa from the Phaeomoniellaceae family and one unidentified fungus, accounted for 19% of the divergence found among cultivars (Fig. 9a). One of these taxa (Phaeomoniellaceae sp.11) occurred in higher abundance in cv. Madural, while the remaining two taxa were exclusively found in cv. Cobrançosa. At the level of the epiphytic fungal community, a single taxon (Pleosporineae sp. 1) was responsible for more than 20% of the dissimilarity found on fungal community among cultivars, due to its highest occurrence in the cv. Cobrançosa (Fig. 9b). We hypothesized that this cultivar-specific fungal community might be determinant for host resistance/susceptibility to anthracnose. The functional role of these fungi, and in particular for the most 5 discriminant taxa here identified, needs to be studied in the future, because they might be important in conferring host plant resistance towards anthracnose.

Table 3. Analysis of similarity (ANOSIM), based on Bray-Curtis distance, comparing the composition on fungal communities between the endophytic (End) and epiphytic (Epi) communities, position within tree canopy (Inside – Ins vs. Outside – Out) and cultivars (Cobbrançosa – Cob vs. Madural – Mad). Included are the results for the whole fungal community (total) as well as for the endophytic or epiphytic communities.

Fungal community	Comparison	R-statistics	P-value
Total (Endo + Epi)	Endo vs. Epi	0.242	0.001
	Ins vs. Out	-0.017	0.329
	Cob vs. Mad	0.247	0.001
Endophytic	Ins vs. Out	0.028	0.192
	Cob vs. Mad	0.260	0.001
Epiphytic	Ins vs. Out	-0.036	0.442
	Cob vs. Mad	0.450	0.001

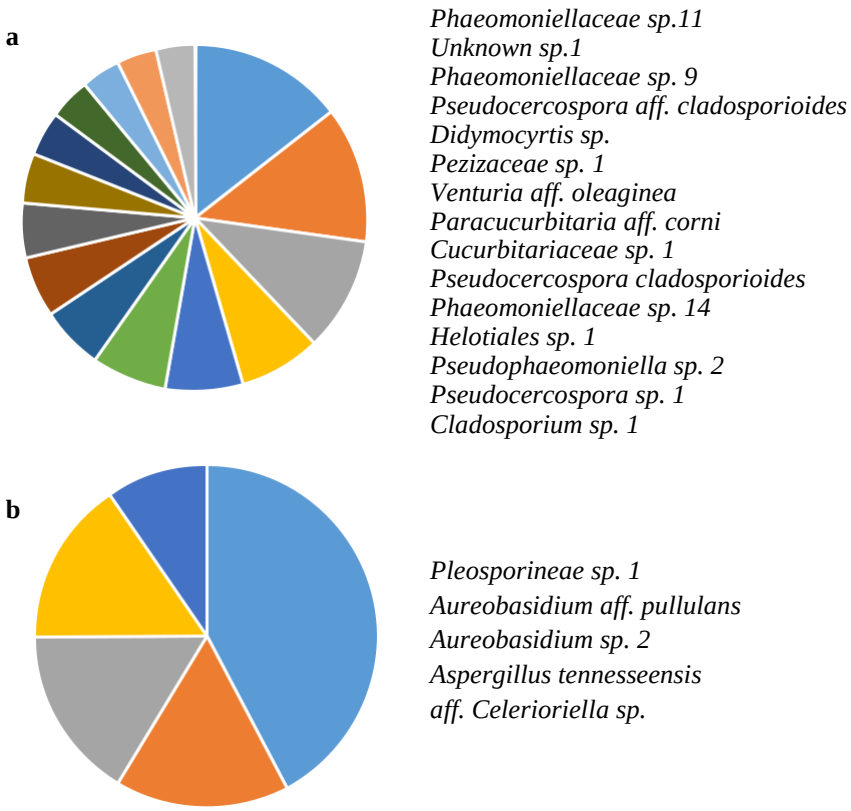

Figure 9.Fungal taxa at the level of the endophytic (a) and epiphytic (b) communities that explained up to 50% the dissimilarity found between cultivars (Cobrançosa and Madural), obtained by the SIMPER analysis.

V. Conclusion and future perspectives

This study provided some new insights on the fungal diversity composition of endophytic and epiphytic communities associated with leaves from olive trees. It was shown that the epiphytic community of the studied organic olive orchard is largely dominated by three abundant taxa (*Aureobasidium*, a *Phaeosphaeriaceae* unassigned species and *Celeriorella*; representing almost 60% of the total epiphytic isolates). The endophytic community shown to be less diverse but more even than the epiphytic community, a pattern previously shown for fungi associated to olive tree leaves and to other plant organs (Martins et al., 2016; Preto et al., 2017). Some relevant, but unassigned taxa (i.e., not identified at least at the genus level), present in both endophytic and epiphytic olive tree leaves communities are likely to represent novel taxa, something that deserve further investigation. This is the case of two *Pleosporineae* and *Phaeomoniellales* species, that contributed to explain the dissimilarity between the observed endophytic and epiphytic fungal communities; and several endophytic *Phaeomoniellaceae* and other unassigned species and an epiphytic *Pleosporineae* species, that contributed to explain the dissimilarity between cultivars. The host specificity demonstrate in this study was greater on epiphytes than on endophytes, and independent on the position of the leaf within tree canopy. Thus, the taxonomic revision and characterization of the function role of these taxa, some of them likely to represent cultivar-specific fungal components, are of high interest in the field of olive crop protection. For instance, it will be important to explore the potential of these fungi to act as biocontrol agents able to confer host plant resistance towards anthracnose. Future research needs to decipher these complex interactions between plant-fungi-causal agent of anthracnose and assess their role in plant health.

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