Proceedings

Characterization of Olive Associated Fungi of 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 in the use of copper-based fungicides. The plant-associate fungal community has been increasingly recognized for playing an important role on plant health and fitness. Thus, in this work the olive associated fungi of cultivars with different levels of resistance to anthracnose was characterized to identify potential protecting fungi for a sustainable strategy of biocontrol. A culture-dependent approach was used to assess both epiphytic and endophytic fungal communities of leaves from the 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. Host plant (at cultivar level) had a negligeable effect on the composition of fungal communities of the leaves. Future research needs to highlight the most proeminent taxa arising from different cultivars and to decipher these complex interactions between plant-fungi and assess their role in plant health.

Keywords: *Olea europaea;* fungal diversity; endophyte; epiphyte; biocontrol; *Colletotrichum* spp

1. Introduction

Olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) cultivation is ancient, and its history merges with that of the Mediterranean basin [1]. Indeed, its origin must have taken place at Middle East, near the border between Turkey and Syria [2]. Not surpsingly, olive tree cultivation still has, currently, great economic, social and ecological importance in the Mediterranean region, where 95% of world olive groves are concentrated [3]. However, 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, caused by several species of the fungal genus *Colletotrichum*, is the most damaging disease [1,4]. The disease has been reported in all olive-growing regions worldwide, where it can have high economic impact, especially in years with environmental conditions favourable to the development of the disease (i.e., high humidity) and in groves with disease-susceptible varieties [5,6]. For instances, in Italy, Spain and Portugal, yield losses due to olive anthracnose often attain 80-100% [1,5]. Its control is very difficult and none of the available control measures are effective or safe enough [4]. For instance, fungicides residues are consistently found in oil and fruits from crops treated with copper-based fungicides [7]. To circumvent these problems, some efforts have been made to manage this disease in a more effective and environmentally friendly manner [4]. One promising approach can rely on the exploitation of endophytes that naturally colonize the internal plant tissues of olive trees. To support this approach, there are evidences showing that plants and their associated endophytes interact and modulate traits in host plants [8]. Within this context, the

manipulation of endophytes to improve positive interactions with the host plant in order to modify resistance traits could be a promising tool for the control of olive anthracnose. Indeed, several endophytic fungal species isolated from olive tree leaves [9] or olive fruits [10] showed to inhibit significantly the growth of the antrachnose causal agent *Colletotrichum acutatum*, under in vitro conditions. In this work, endophytic diversity and host plant health as well as of the influence of crop management practices on endophytic composition and the underlay effect to host plant protection against diseases. This work aims to recognize taxa commonly found on the fungal community of two important Portuguese cultivars, which are differently susceptible to olive antrachnose. The results obtained will be useful to exploit the potential in using a specific olive tree genotype (at cultivar level) to model and manage host–endophytic interactions, in order to reduce incidence/severity of anthracnose disease.

2. Experiments

2.1. Sample Collection

Leave samples were collected from an organic 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. Two varieties with different susceptibilities to antrachnose were selected, the cultivars Cobrançosa (moderately tolerant) and Madural (susceptible). For each cultivar, branches from five trees were transported to the laboratory in an icebox, and stored at 4 °C until the isolation of epi- and endophytic fungi has started.

2.2. Fungal Isolation and Enumeration

From each branch, five leaves were randomly selected and used to isolate epiphytes and endophytes. To isolate fungal epiphytes, about 1 g of leaves was added to 9 mL of sterile potassium phosphate buffer pH 7.0. This suspension was placed on a rotary shaker (200 rpm) for 60 min, at room temperature, to dislodge microorganisms from the leaves surface. Aliquots of 1 mL of the resulting microbial suspensions were separately plated in triplicate on Potato Dextrose Agar (PDA, Difco) medium. Plates were incubated at 25 ± 2 °C in the dark and were observed daily for microbial growth and colony counting. Endophytic fungi were isolated from the same leaves used to isolate epiphytes. After removing epiphytes by surface disinfection through a procedure described in Martins et al. [11] each leaf was cut into segments (ca. 5 × 5 mm), which were transferred to fresh PDA medium. To validate surface sterilization procedure, the surface of sterilized plant tissues was imprinted onto a new, fresh PDA plate. Fungal colonies were subcultured until pure epi- or endophytic cultures were obtained.

2.3. Fungal Identification

After morphological characterization, isolates were grouped by fungal colony types as described in Gomes et al. [12]. A representative isolate of each morphotypewas then selected for a molecular-based identification using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (ITS1, 5.8S, ITS2). Mycelia and/or spores from each isolate were harvested, and the total genomic DNA extracted using the REDExtract-N-Amp™Plant PCR kit (Sigma, Poole, UK). The ITS region was PCR amplified using the primer sets ITS1/ITS4, ITS5/ITS4 or ITS2/ITS4 [13]. The amplified products were sequenced by Macrogen Europe B.V. (Amsterdam, The Netherlands). The obtained raw DNA sequences were analyzed and edited with the software Geneious 8.1.8. (Auckland, New Zealand). In order to identify the fungal isolates, the default (*nr*/nt) and the curated Fungi RefSeq ITS NCBI [14] databases were used for nucleotide BLAST searches. In accordance to Vu et al. [15], 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. Pure cultures of each identified isolate were preserved and deposited in the CIMO culture collection, at the Polytechnic Institute of Bragança.

3. Results

3.1. 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 1). 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 (63 isolates). The identified species belonged to 26 genera and 20 families, all from the Ascomycota (20 genera) and the Basidiomycota (6) phyla. The more abundant class was Dothideomycetes (41.2%), followed by Eurotiomycetes (34.8%) (Figure 1), while at the order level, Phaeomoniellales (28.1%), Pleosporales (22.4%), and Dothideales (11.9%) were most abundant taxa.
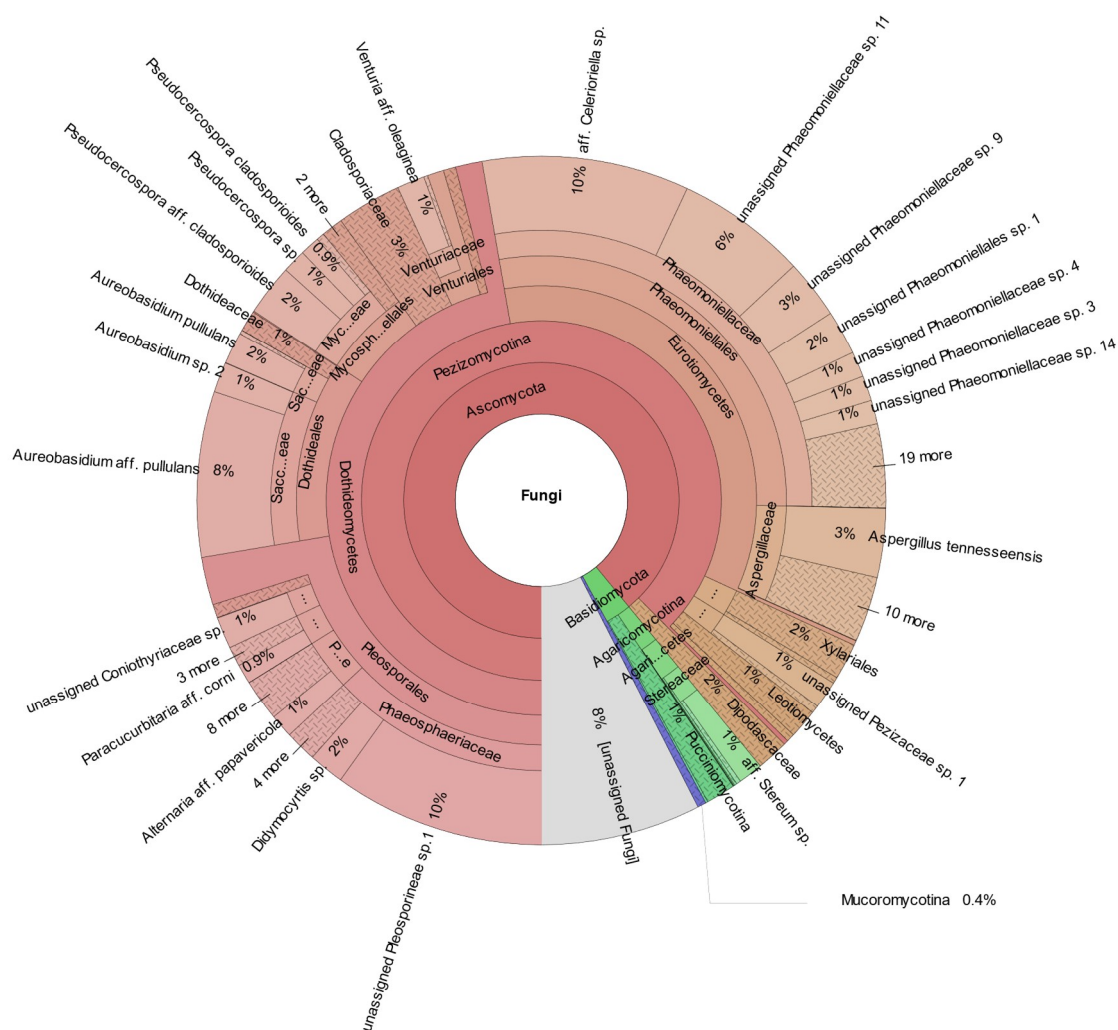

Figure 1. Krona chart showing the relative abundance of total fungi (endophytic and epiphytic), at the genus level, detected on leaves of olive tree of cvs. Cobrançosa and Madural.

Regarding isolates identified at least at the genera level (expressed as total number of isolates and average number of isolates per tree), the most abundant identified taxa in the endophytic community (Figure 2a) 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 2), 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* (8%), *Pseudocercospora* (7%) and *Venturia* (5%) were the most abundant. In relation to the epiphytic community (Figure 2, b), *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%).
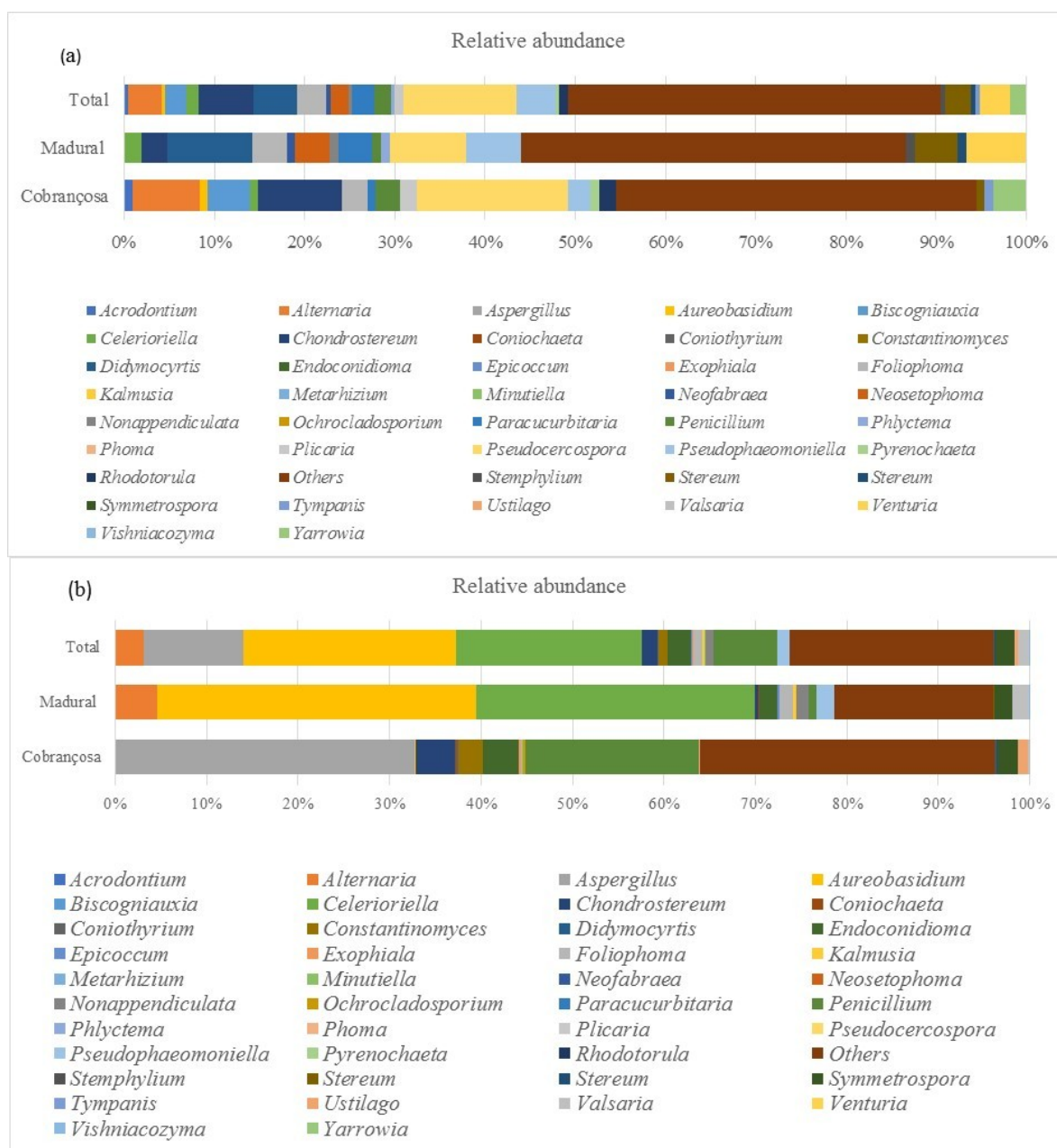

Figure 2. Relative abundances of genera from the (**a**) endophytic and (**b**) epiphytic communities associated with leave tissues of *Olea europaea* L. (cvs. Madural and Cobrançosa).

4. Discussion and Conclusions

This study provided some new insights on the fungal diversity composition of endophytic and epiphytic communities associated with leaves from olive trees cultivated in an organic orchard. It was shown that the epyphitic community is largely dominated by 2–3 abundant taxa (*Aureobasidium*, a Phaeosphaeriaceae unassigned species and *Celeriorella*), and it seems that the composition may differ between cultivar, at least regarding the more abundant taxa. On the other hand, for the endophytic community, the most abundant taxa were not so dominant and there is not an obvious discrepancy between the taxa composition in cvs. Cobrançosa and Madural. These patterns were already shown in previous studies on olive trees, in leaves and in other plant organs [8,9]. Also, some abundant, but unassigned species that are present in both endophytic and epiphytic olive tree leaves communities are likely to represent novel taxa, something that deserve further investigation. We prospect that the determination of indicator value indices should help to expose the preference/specificity of a set of fungal species with the most-resistant cultivar. Preliminary data from ecological diversity and multivariate statistical analyses (data not shown), however, indicate that the host plant (at cultivar level) had a negligeable effect on the composition of fungal communities of the leaves. On the other hand, they also highlight that *Phaeomoniella*, *Pseudocercospora*, *Paracucurbitaria*, and two unidentified species are the five taxa that contributed the most for the differences between the endophytic and epiphytic composition. Future research needs to decipher these complex interactions between plant-fungi-pathogen and assess their role in plant health.

Author Contributions: J.A.P. and P.B. conceived and designed the experiments; H.B, V.R. and D.M. performed the experiments; H.B., V.R. and P.B. analyzed the data; J.A.P. and P.B. contributed with reagents, materials and analysis tools; V.R. and P.B. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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