Cork Oak Endophytic Fungi as Potential Biocontrol agents Against *Biscogniauxia mediterranea* and *Diplodia corticola*

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**Abstract:** An increase in cork oak diseases caused by *Biscogniauxia mediterranea* and *Diplodia corticola* has been reported in the last decade. Due to the high socio-economic and ecologic importance of this plant species in the Mediterranean Basin, the search for preventive or treatment measures to control these diseases is an urgent need. Fungal endophytes were recovered from cork oak trees with different disease severity levels, using culture-dependent methods. The results showed a higher number of potential pathogens than beneficial fungi such as cork oak endophytes, even in healthy plants. The antagonist potential of a selection of eight cork oak fungal endophytes was tested against *B. mediterranea* and *D. corticola* by dual-plate assays. The tested endophytes were more efficient in inhibiting *D. corticola* than *B. mediterranea* growth, but *Simplicillium aogashimaense*, *Fimetariella rabenhorstii*, *Chaetomium sp.* and *Alternaria alternata* revealed a high potential to inhibit the growth of both. *Simplicillium aogashimaense* caused macroscopic and microscopic mycelial/hyphal deformations and presented promising results in controlling both phytopathogens’ growth in vitro. The evaluation of the antagonistic potential of non-volatile and volatile compounds also revealed that *A. alternata* compounds could be further explored for inhibiting both pathogens. These findings provide valuable knowledge that can be further explored in in vivo assays to find a suitable biocontrol agent for these cork oak diseases.

**Keywords:** fungi; endophytes; *Biscogniauxia mediterranea*; *Diplodia corticola*; biocontrol; cork oak

1. **Introduction**

Cork oak (*Quercus suber* L.) is an evergreen tree species that covers between 1.7 and 2.7 million ha in the western Mediterranean, distributed among Portugal, Spain, France, Italy, Morocco, Tunisia and Algeria [1]. Cork oak forests display a high socio-economic and ecological importance, being mainly explored for cork production [2]. The synthesis of cork, a homogeneous tissue of phellem cells, depends on the activity of the cork cambium, which covers the trunk and branches of cork oak. Due to the interesting and unique set of physical, biological and chemical properties, such as sealing and insulating features, cork is mainly used for the production of bottle stoppers [3]. Every year, 80% of worldwide produced cork comes from the Iberian Peninsula, in which Portugal is responsible for almost half of the total global production [2,4].

In recent years, cork oak forests have been declining in the Mediterranean region, which will be further enhanced by the combined effect of global warming and drought [5]. Indeed, the climate change predictions reveal the Mediterranean region as one of the most affected regions, and Mediterranean forests...
as one of the most vulnerable ecosystems to the combined effect of temperature increase and precipitation decrease [6,7]. Not only the geographic distribution of plant pathogens is predicted to be reshuffled [8], but also the severity of plant diseases and the rate at which endophytes switch from mutualistic to pathogenic behavior are expected to increase [9,10]. Indeed, during the last decades, an increase in cork oak diseases has been reported [11], including charcoal disease (caused by *Biscogniauxia mediterranea* (De Not.) Kuntze; Xylariales) and bot canker (caused by *Diplodia corticola* A.J.L. Phillips, A. Alves and J. Luque; Botryosphaeriales). Both pathogenic fungi have an endophytic lifestyle and opportunistic behavior, which account for the increase in diseases incidence in cork oak trees under environmental stress [12]. These diseases cause high economic losses due to the development of symptoms in cork oak trunk that affect both cork production and quality, and eventually lead to tree death [13].

Few preventive measures for cork oak charcoal disease and bot canker are currently known. Good phytosanitary practices in cork oak forests are essential to prevent the spreading of diseases through spore release and colonization from tree wounds derived from pruning or cork extraction [11]. Thiophanate-methyl and carbedazim fungicides have been found effective as a preventive measure against *D. corticola* [14,15]. However, the application of fungicides has been increasingly restricted due to the potential negative impact on human health and the environment. Therefore, more environmentally friendly strategies, such as the use of biological control agents, are now being explored for controlling many forest tree diseases [16,17]. Indeed, promising results have been obtained for restricting cork oak diseases. For example, the use of *Fusarium tricinctum* resulted in a reduced mortality of *Q. cerris* and *Q. pubescens* seedlings inoculated with *D. corticola* [18]. Further, *Trichoderma* spp. presented a high in vitro antagonistic potential against *B. mediterranea* and *D. corticola* [19]. In particular, a cork oak endophytic strain of *Trichoderma citrinoviride* produces peptaibols capable of inhibiting cork oak pathogens' in vitro growth [20]. Spray suspensions of *T. atroviride* and *T. viride* combined with sanitation and scarification practices were recently suggested for decreasing charcoal disease incidence in *Q. castaneifolia* trees [21].

In the present study, we hypothesize that cork oak fungal endophytes are able to control cork oak pathogens' (*B. mediterranea* and *D. corticola*) growth and we intend to highlight the most promising cork oak endophytes to be used as a biocontrol strategy. Specifically, we aimed to identify endophytic fungal species of cork oak that can be used to control *B. mediterranea* and *D. corticola*, by studying their antifungal activities against both pathogens and by characterizing the interactions between endophytes and pathogens.

2. Materials and Methods

2.1. Cork Oak Forests Sampling and Endophyte Recovery

Cork oak samples were collected from eight different forests in Portugal (Table S1). Two forests were sampled from the National Park of Peneda-Gerês (PG-ER and PG-RC) and Herdade da Contenda (HC-CT and HC-MA). A single forest was sampled from Limãos (LI), Alcobaça (AL), Gavião (GV) and Grândola (GR). Between April and October of 2017, five to six trees were sampled from each cork oak forest, considering trees at different disease severity levels (Table 1; Figure 1). Disease severity levels were grouped into three categories and determined by considering defoliation (5 levels: 0–10%—no damage; 11–25%—light damage; 26–50%—moderate damage; 51–90%—severe damage; > 91%—extreme damage), as well as canopy and trunk damages (3 levels: 0—no damage; 1—moderate damage; 2—severe damage), for different factors (dried, wilting and decolorated leaves, presence of cankers, decolorated trunk, presence of exudates and visible sporulation). Trees were considered as healthy, presenting mild symptoms or declining. Healthy trees presented no or light defoliation, displaying low canopy and trunk damage (maximum of two factors with 1 damage level, but not affecting overall vigor of the tree). When tree vigor was moderately affected by a combination of factors (moderate defoliation and/or some canopy and trunk damage), trees were considered as displaying mild symptoms. Declining trees presented a clear decline in their vigor (accentuated or very accentuated defoliation that could be coupled with more than three factors classified with 1 and/or 2 damage levels).
Table 1. Collection of cork oak branches in sampled forests. The number of trees sampled from each disease severity level is presented. For more details of sampled forests, see Table S1.

| Location       | Cork Oak Stand | Collection Date | Disease Severity Level |
|----------------|----------------|-----------------|------------------------|
| Peneda-Gerês   | PG-ER          | May, 2017       | 2                      |
|                | PG-RC          | July, 2017      | 4                      |
| Limãos         | LJ             | April, 2017     | 0                      |
| Gavião         | GV             | July, 2017      | 0                      |
| Alcobaça       | AL             | May, 2017       | 2                      |
| Grândola       | GR             | May, 2017       | 0                      |
| Herdade        | HC-CT          | October, 2017   | 2                      |
| Contenda       | HC-MA          | October, 2017   | 2                      |

Five to seven branches were collected from each cork oak tree. Twigs were collected from each branch and thoroughly washed in tap water. To obtain only endophytes, a surface sterilization was performed based on the method described by Martins et al. [22]. Twigs were sequentially immersed in ethanol 70% (v/v) for 2 min, bleach (3–5% chlorine) for 6 min and ethanol 70% (v/v) for 1 min, followed by three washes in sterile deionized water (1 min each) and drying. Sterile twigs were cut into segments (4–5 cm) and transferred to Potato Dextrose Agar (PDA) medium (5 segments/plate). Three replicates were used for each branch. Sterilization controls were performed by spreading the last washing water (10 μL) onto PDA medium. Incubation was performed in the dark, at room temperature (21–23 °C). The outgrowing fungi were recognized as endophytic fungi and were successively subcultured in fresh PDA medium until pure cultures were obtained.

2.2. Endophytic Fungi Identification and Selection of Potential Antagonistic Fungi

Endophytic fungi were grouped into morphotypes, according to their cultural features (color, shape, elevation and margins) [23]. From each morphotype, at least three isolates were used for DNA extraction, using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA). The fungal rDNA-ITS region was amplified using universal primer pairs ITS1F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’), or ITS1F and ITS4 (5’-TCCTCCGCTATTGATATGC-3’) [24]. PCR mixtures (25 μL) contained 1× Complete NH₄ Reaction Buffer (BIORON GmbH, Germany), 200 μM of each dNTP (NZYTech, Portugal), 1 μM of each primer, 1 μL of DNA template and 1.25 U of DFS-Taq DNA Polymerase (BIORON GmbH, Germany). Amplifications were performed using the following protocol: initial denaturation 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 52 °C (or 54 °C with ITS1F-ITS4) and 60 s
were selected to be tested against the pathogens in antagonistic assays due to their cultural readiness, were added to both groups. For proceeding to the antagonism assays, endophytes were selected based on their potential beneficial role and availability. All groups were considered with the exception of those fungi exclusively described as pathogenic and all cork oak pathogens. Eight fungal isolates were selected to be tested against the pathogens in antagonistic assays due to their cultural readiness, namely *Simplicillium aogashimaense* isolate Gr67, *Coniothyrium carteri* isolate Gv5, *Diaportha passiflorae* isolate Erm6, *Fimetariella rabenhorstii* isolate Br33, *Fusarium oxysporum* isolate Cab77, *Chaetomium* sp. isolate Erm52, *Alternaria alternata* isolate Cab37 and *Penicillium olsonii* isolate Gv63. *B. mediterranea* and *D. corticola* isolates were inoculated on cork oak stems to confirm symptoms development, completing Koch’s postulates.

2.3. Antagonistic Assay In Vitro by Dual-Plate and Categorization of Fungal Interactions

The eight selected endophytic fungi were tested in vitro against phytopathogens *Biscogniauxia mediterranea* isolate Gr13 and *Diplodia corticola* isolate Gr23, both isolated from cork oak trees showing mild symptoms. Antagonistic assays were performed by the dual-culture method in PDA medium. Fungal plugs (5 mm) of an actively growing endophyte and pathogen were placed 3 cm apart from each other (using 9 cm diameter Petri dishes) and incubated in the dark, at 28 °C (±2 °C). Those endophytes displaying a slow growth rate (<0.1 cm²/h; Table S2) were inoculated 72 h before the pathogen. In parallel, control plates were similarly prepared but using a single pathogen (or endophyte) plug. All plates were photographed 72 h after being inoculated with the endophyte and/or pathogen, and growth area was measured using *ImageJ 1.50i* software [28]. Growth areas were used to determine the percentage of mycelial growth inhibition, according to the formula: \( (Ac−Ai)/Ac \times 100 \), where \( Ac \) is the area of fungal growth in the control plate and \( Ai \) is the area of fungal growth with the interacting fungus. Three independent experiments were performed (with at least 3 replicates each) for all endophyte/pathogen combinations. Statistical analysis was performed using ANOVA in GraphPad Prism 7.00 software (La Jolla, CA, USA) to determine the impact of endophytes on pathogen growth.

Fungal interactions between endophytes and pathogens were categorized based on Tuininga [29]. Considering that (-) corresponds to mycelial growth decrease, (+) to growth increase and (0) to similar mycelial growth, interactions among endophyte/pathogen could be defined as co-antagonism (-/-), antagonism (-/0), agonism (+/+), co-habitation (0/0), commensalism (0/+), and mutualism (+/+). For describing the fungal interactions, dual-culture plates were observed daily for 15 days. Mycelial interactions were also recorded based on Badalyan et al. [30], who defined the following classes: A—deadlock with mycelial contact; B—deadlock at distance; C—replacement, overgrowth without deadlock; CA1 and CA2—partial and complete replacement after initial deadlock with mycelial contact;
and CB1 and CB2—partial and complete replacement after initial deadlock at a distance. Finally, hyphal interactions were observed using mycelia from the interacting region of dual-culture plates, after 15 days of interaction, and compared with mycelia taken from controls. Photographs were taken using a Leica MC170 HD digital camera attached to a Leica S9 D stereomicroscope (Leica Microsystems, Germany) or using an automated Leica DM5000B microscope (Leica Microsystems, Germany).

2.4. Antifungal Non-Volatile Compounds Assay

The antagonistic effect of the metabolites produced by the eight selected fungal endophytes was tested using a method adapted from Campanile et al. [18]. Three mycelial fungal plugs (5 mm) of actively growing endophytes were inoculated in Erlenmeyer flasks (250 mL) containing 50 mL of sterile Potato Dextrose Broth (BD Difco™, Switzerland). Culture flasks were placed in an orbital incubator at 28 °C (±2 °C), with 150 rpm agitation, for 12 days. Liquid cultures were transferred to falcon tubes and centrifuged for 15 min at 12,000 rpm. The supernatant was collected and filtered through a 0.22 µM membrane filter to remove hyphal residues and conidia. Fungal filtrates were added to sterile warm PDA medium [20% (v/v)] and poured into 9 cm Petri dishes. A mycelial plug of an actively growing pathogen (B. mediterranea or D. corticola) was placed in the center of the plate. Control plates were similarly prepared but containing PDA without a fungal filtrate. Mycelial growth areas were determined as previously referred and mycelial growth inhibition was determined according to the formula: \( \frac{Ac-Af}{Ac} \times 100 \), where Ac is the area of fungal growth in the control plate and Af is the area of fungal growth in the presence of the filtrate. Three independent experiments were performed (with at least 3 replicates each) for all endophyte/pathogen combinations. Statistical analysis was performed using ANOVA in GraphPad Prism 7.00 software (La Jolla, CA, USA) to understand the impact of endophytic compounds on pathogen growth.

2.5. Antifungal Volatile Compounds Assay

The effect of volatile compounds produced by the eight selected fungal endophytes in inhibiting pathogen growth was evaluated by an inverted plate method [31]. For each endophyte/pathogen combination, two PDA plates were inoculated with a single mycelial plug (5 mm) of an actively growing endophyte (or pathogen). Then, the PDA plate with the pathogen was inverted on the top of the endophyte plate and both were sealed with parafilm. Incubation occurred at 28 °C (±2 °C), for 72 h. Control plates were performed without inoculation of the endophyte in the bottom plate. As previously described, those endophytes displaying a slow growth rate (<0.1 cm²/h; Table S2) were inoculated 72 h before the pathogen. The percentage of inhibition was calculated as mentioned before. Three independent experiments were performed (with at least 3 replicates each) for all endophyte/pathogen combinations. Statistical analysis was performed using ANOVA in GraphPad Prism 7.00 software (La Jolla, CA, USA) to understand the impact of endophytic volatiles on pathogen growth.

3. Results and Discussion

3.1. Endophytic Fungal Community of Cork Oak

Endophytic fungi of cork oak twigs were recovered from eight different forests in Portugal. From a total of 1117 fungal isolates, 440 were molecularly identified and grouped into 128 OTUs comprising 18 orders, 38 families, 45 genera and 39 species (Table 2). Only OTUs classified up to genus and species were considered in this work (70 OTUs). From these, and to the best of our knowledge, 54 fungal OTUs have never been reported as cork oak endophytes before (e.g., Fimetariella rabenhorstii, Discosia sp.), including 18 that have never been described as plant endophytes (e.g., Caliciopsis beckhausii, Diaporthe passiflorae, Proliferodiscus sp.) (Table 2). Among previously undescribed cork oak endophytes, 28 were only recovered from a single sampled cork oak forest, but others (26) were recovered from different locations, which strengthens their role as cork oak endophytes. For example, Discosia sp., Cryphonectria naterciae and Neocucurbitaria sp. were recovered from different Portuguese forests,
or from forests displaying the highest (e.g., *F. rabenhorstii*) or the lowest (e.g., *Plectania rhytidia*) precipitation levels.

Some OTUs were more widespread throughout Portuguese cork oak forests than others. *B. mediterranea* was the only fungus identified in all cork oak stands and *Fusarium* sp. was present in all forests, with the exception of LI and GR. Further, *Penicillium* sp., *Sarocladium kiliense* and *Neocucurbitaria* sp. were recovered from almost all cork oak stands. In contrast, there were fungi (such as *Diplodia corticola*, *Nonappendiculata quercina*, *Ciboria* sp. and *Pezicula cinnamomea*) that were only recovered from one sampling location. Since many fungi are difficult to be cultured, culture-dependent methods are known to underestimate fungal communities [32]. The used approach only targeted those endophyte fungi that can be easily cultured on artificial media and have a rapid growth rate. Furthermore, as many endophytes do not sporulate in culture, their morphotype discrimination through cultural features is challenging and might have been underestimated. For these reasons, we are aware that the performed endophyte survey through cultural methods (and based on morphotypes discrimination) misrepresented the endophyte diversity in cork oak forests, as already reported elsewhere for other plant hosts [32]. Accordingly, our study reported a strong dominance of fungi belonging to Ascomycota (only one OTU belonged to the Basidiomycota phylum), like previously reported in other studies using culture-dependent methods, e.g., [33,34]. Culture-independent methods could have provided a different picture of fungal communities. For example, in grapevine, besides Ascomycota (described using cultural methods), Basidiomycota and Zygomycota fungi were additionally detected recurring to a metabarcoding approach [33]. Despite the recognized limitations, the used culture-dependent approach provided the availability of endophyte isolates to proceed in searching for biocontrol strategies.

The ecological role of each identified fungal species was determined based on the literature. As the functional role of certain endophytes could change according to their plant host genotype, during different stages of the plant life cycle or in extreme conditions [26], many endophytes were included in mixed groups (such as the pathogenic–beneficial group). Furthermore, within certain genera, there are species known to be pathogenic and others beneficial, being impossible to consider a single functional role. Despite these constrains, the number of identified OTUs displaying a phytopathogenic role was higher, when compared to other functional groups (Table 2). A total of 39 OTUs were considered as displaying a phytopathogenic role (including exclusive phytopathogenic [26], phytopathogenic–beneficial [10] and phytopathogenic–other [3] functional groups), while only 21 displayed a beneficial role (including beneficial [10], phytopathogenic–beneficial [10] and beneficial–other [1] functional groups). The richness of fungi displaying a potential phytopathogenic role was also higher than any other functional group in all cork oak stands and whatever the cork oak tree disease severity level (Figure 2). Among them, few cork oak pathogens were found: *B. mediterranea* [12], *Coryneum* sp. [35], *Cryptonectria naterciae* [36], *D. corticola* [37], *D. quercivora* [38], *Discula quercina* [39] and *Neofusicoccum parvum* [35]. Interestingly, *B. mediterranea*, *Coryneum* sp. and *C. naterciae* were isolated from trees in all disease severity levels, while *D. corticola*, *D. quercivora* and *Discula quercina* were only isolated from trees with declining symptoms (Figure 3). In agreement, cork oak pathogens (*B. mediterranea* and *Coryneum* sp.) were isolated from healthy cork oak trees in Italy [12,40]. The presence of *D. corticola*, *D. quercivora* and *Discula quercina* in declining cork oak trees agrees with their role as emerging pathogens to *Quercus* spp. in different regions of the world [11,41], which is emphasized by their risk to cork oak health as previously reported [39,42]. Concerning the fungi displaying a beneficial role, AL forest presented the highest number of OTUs with a potential beneficial role (all described as pathogenic–beneficial), but GV forest was the richest with exclusive beneficial fungi (Figure 2A). GV forest corresponded to one of the forests displaying the highest number of declining trees (results not presented), which agrees with the finding that trees with declining symptoms displayed a higher richness of exclusive beneficial fungi (a non-significant 1.5-fold increase in relation to healthy trees; Figure 2B). The role of stress-affected plants in recruiting beneficial microorganisms is still under debate and a “cry-for-help” hypothesis was recently proposed, in which plants are able to recruit plant-protective microbes when they are under attack by pathogens [43,44]. For example, tomato plants...
under stress produce root exudates to signal the beneficial *Trichoderma harzianum* T22 strain to direct growth toward the plant host [45]. However, further studies on endophyte distribution among healthy and diseased cork oak trees are still needed for providing clear evidence that support a “cry-for-help” strategy for the sustainability of threatened cork oak forests.

**Figure 2.** Cork oak endophytic fungal functional groups presented in cork oak forests (A) and disease severity level (B). Operational taxonomic units (OTUs) belonging to more than one functional group were added to both, except for the pathogenic–beneficial group that is represented as such.

**Figure 3.** Endophytic fungal OTUs recovered from cork oak trees at different disease severity levels. Black/white color indicates fungal isolation/no isolation from trees with different disease severity levels. Isolates used in this work are depicted in bold.
Table 2. Endophytic fungal OTUs recovered from twigs of cork oak trees located in different forests. Taxonomic classification (with closest match identity in brackets) and their respective functional group are revealed. Reports about their endophytic behavior are referred to. When existing, references related to cork oak are presented (bold). Isolates used in this work are depicted in red. Functional groups are represented as: P—pathogenic; B—beneficial; O—other; U—unknown.

| Taxonomic Classification | Closest Match GenBank | Cork Oak Forests | Functional Group | Identified Endophyte |
|--------------------------|-----------------------|------------------|------------------|---------------------|
| Ascomycota               |                       |                  |                  |                     |
| Amphiphaeriales          |                       |                  |                  |                     |
| Discosia sp.             | KU325138.1 (100%)     | x                | P [46]           |                     |
| Nonappendiculata quercina| MH554025.1 (98.78%)   | x                | P [46]           |                     |
| B. Botryosphaeria        |                       |                  |                  |                     |
| Diplodia corticola       | MT015621.1 (100%)     | x                | P [37]           |                     |
| Diplodia quercivora      | JX894205.1 (97.72%)   | x                | P [38]           |                     |
| Dothiorella iberica      | MT261024.1 (100%)     | x                | P [39]           |                     |
| Neofusicoceum parsum     | MT645697.1 (99.3%)    |                  | P [40]           |                     |
| C. Capnodiales           |                       |                  |                  |                     |
| Cladosporium berbericum  | LT854669.1 (99.22%)   | x                | P [51]           |                     |
| Cladosporium perangustum | MK111614.1 (99.10%)   | x                | P [52]           |                     |
| Cladosporium sp.         | MN879328.1 (100%)     | x                | P [53]           |                     |
| Cladosporium sphaeroergum| MT645920.1 (99.51%)   |                  | B [54]           |                     |
| C. Coryneliales          |                       |                  |                  |                     |
| Calicopsis beckhausenii  | NR_132090.1 (99.57%)  | x                | U [55]           |                     |
| Calicopsis sp.           | NR_132090.1 (91.91%)  | x                | U [56]           |                     |
| C. Diaporthales          |                       |                  |                  |                     |
| Coryneum sp.             | MH647330.1 (95.54%)   | x                | x                | x                 |
| Cryphoeum natercine      | MT645942.1 (100%)     | x                | x                | x                 |
| Cytospora cedri          | MN871816.1 (100%)     | x                | P [57]           |                     |
| Cytospora cinnereomorpha | KY051946.1 (100%)     | x                | P [58]           |                     |
| Diaporthes sp.           |                       |                  |                  |                     |
| Dothideales              |                       |                  |                  |                     |
| Aureobasidium pullulans  | MT645930.1 (99.57%)   | x                | O [60]           |                     |
Table 2. Cont.

| Taxonomic Classification | Closest Match GenBank | PG-BR | PG-RC | Li | Al | GV | GR | HC-CT | HC-MA | Functional Group | Identified Endophyte |
|--------------------------|-----------------------|-------|-------|---|----|----|----|-------|-------|------------------|----------------------|
| Cork Oak Forests         |                       |       |       |   |    |    |    |       |       |                  |                      |
| Eurotiales               |                       |       |       |   |    |    |    |       |       |                  |                      |
| Penicillium galatum      | MT582777.1 (100%)     | x     | x     | x |    |    |    |       |       |                  | P [70]               |
| Penicillium olsonii      | MT582783.1 (100%)     | x     |       |   |    |    |    |       |       |                  | B [72]               |
| Penicillium sp.          | LN901128.1 (99.54%)   |       |       |   |    |    |    | x     |       |                  | O [74] P [76] B [72] |
| Helotiales               |                       |       |       |   |    |    |    |       |       |                  |                      |
| Ciboria sp.              | KF45322.1 (94.59%)    | x     |       |   |    |    |    |       |       |                  | P [75]               |
| Pezicula cinnamomea      | MK097714.1 (100%)     | x     |       |   |    |    |    |       |       | Pezicula sp.     | P [76] B [72]        |
| Pezicula aerosporulosa   | KR857921.1 (100%)     | x     |       |   |    |    |    |       |       | Pezicula sp.     | P [76] B [72]        |
| Pezicula aerosporulosa   | MH862573.1 (98.66%)   | x     |       |   |    |    |    |       |       | Pezicula sp.     | O [80]              |
| Helotiales               |                       |       |       |   |    |    |    |       |       |                  |                      |
| Hypocreales              |                       |       |       |   |    |    |    |       |       |                  |                      |
| Clonostachyus sp.        | MK789204.1 (91.84%)   | x     |       |   |    |    |    |       |       |                  | B [83]               |
| Fusarium compactum       | KJ626364.1 (98.53%)   | x     |       |   |    |    |    |       |       |                  | P [85]               |
| Fusarium oxysporum       | MT530243.1 (100%)     | x     |       |   |    |    |    |       |       |                  | P [87] B [88]        |
| Fusarium sp.             | MT645120.1 (100%)     | x     |       |   |    |    |    |       |       |                  | P [87] B [88]        |
| Sarcocladium kiliense    | MK789203.1 (100%)     | x     |       |   |    |    |    |       |       |                  | P [90] B [91]        |
| Sarcocladium sp.         | MT645143.1 (99.36%)   | x     |       |   |    |    |    |       |       |                  | P [90] B [91]        |
| Simplicitium aogashimaense| MK685280.1 (99.82%)   | x     |       |   |    |    |    |       |       |                  | U                    |
| Simplicitium sp.         | MH859771.1 (99.12%)   | x     |       |   |    |    |    |       |       |                  | U                    |
| Tolypocladium sp.        | KX003436.1 (100%)     | x     |       |   |    |    |    |       |       |                  | O [96]               |
| Pezizales                |                       |       |       |   |    |    |    |       |       |                  |                      |
| Plectania rhizidula      | MH003435.1 (98.99%)   | x     | x     |   |    |    |    |       |       |                  | U [27]               |
| Pseudoplectania ericae   | MT498082.1 (99.65%)   | x     |       |   |    |    |    |       |       |                  | U                    |
| Pyrenula sp.             | MT556695.1 (100%)     | x     | x     |   |    |    |    |       |       |                  | O [98]               |
| Phaeomoniellales         | Neophaeomoniella sp.  |       |       |   |    |    |    |       |       |                  |                      |
| Alternaria alternata     | MT635274.1 (100%)     | x     | x     |   |    |    |    |       |       |                  | P [102] B [103]      |
| Alternaria sp.           | MT557456.1 (100%)     | x     | x     |   |    |    |    |       |       |                  | P [104] B [103]      |
| Angustimassarina sp.     | MN693699.1 (100%)     | x     | x     |   |    |    |    |       |       |                  | U                    |
| Coniothyrium ferrisianum | MH586054.1 (100%)     | x     |       |   |    |    |    |       |       |                  | U [107]              |
| Coniothyrium palmicola   | JX681086.1 (99.53%)   | x     |       |   |    |    |    |       |       |                  | U                    |
| Epicoccum nigrum         | MT548679.1 (100%)     | x     |       |   |    |    |    |       |       |                  | B [27]               |
| Kalmusia sp.             | MK796143.1 (100%)     | x     |       |   |    |    |    |       |       |                  | P [108]              |
| Neocucurbitaria cast      | MK796144.1 (100%)     | x     |       |   |    |    |    |       |       |                  | U [110]              |
### Table 2. Cont.

| Taxonomic Classification | Closest Match GenBank | Cork Oak Forests | Functional Group | Identified Endophyte |
|--------------------------|-----------------------|------------------|------------------|---------------------|
|                          |                       | PG-ER | PG-RC | Li  | AL  | GV  | GR  | HC-CT | HC-MA |                     |
| Neocucurbitaria sp.      | MH858303.1 (93.78%)   | x     | x     | x   | x   | x   | U   |        |       | [110]                |
| Phoma sp.                | KX815489.1 (100%)     | x     | x     | x   |     |     | P   | [111]  | [40]               |
| Preussia sp.             | MN696547.1 (100%)     | x     | x     | x   |     |     | B   | [112]  |       |
| Pyrenophora bisporalata  | MH864748.3 (100%)     | x     | x     |     |     |     | P   | [114]  | -                 |
| Sordariales              |                       |       |       |     |     |     |     |        |       |
| Chaetomium globosum      | MT588864.1 (100%)     | x     | x     |     |     |     | B   | [115]  | [116]             |
| Chaetomium sp.           | MN139302.1 (100%)     | x     | x     | x   |     |     | B   | [117]  | [118]             |
| Copromyces sp.           |                       |       |       |     |     |     | U   | -      |       |
| Fimetariella ravenhorstii| MN555335.1 (100%)     | x     | x     | x   |     |     | O   | [119]  | [120]             |
| Xylariales               |                       |       |       |     |     |     |     |        |       |
| Biscogniauxia mediterranea| MT86230.1 (100%)     | x     | x     | x   | x   | x   | x   | P     | [121]             |
| Daldinia sp.             | MN341734.1 (97.83%)   | x     | x     |     |     |     |     | B     | [122]             |
| Xylaria sp.              | JQ661730.1 (99.78%)   | x     | x     |     |     |     | P   | [124]  | [125]             |
| Basidiomycota            |                       |       |       |     |     |     |     |        |       |
| Atheliales               | Athelia arachnoida    | x     | x     |     |     |     | P   | [127]  | -                 |
3.2. Interactions of Endophytes against B. mediterranea and D. corticola

Eight endophyte isolates were selected for testing their ability to inhibit B. mediterranea and D. corticola growth, based on their potential beneficial ecological role, availability and culture readiness. D. corticola mycelial growth was persistently inhibited by the presence of these cork oak endophytes, while B. mediterranea was differentially inhibited by endophytes (Figure 4). Dual-plate experiments revealed that B. mediterranea growth was inhibited by F. rabenhorstii (33.4%, \( p \leq 0.001 \)), A. alternata (19.3%), Chaetomium sp. (13.6%) and S. aogashimaense (12.6%), whereas the other fungal endophytes promoted or did not affect pathogen growth (Figure 4). Regarding endophytes inhibiting B. mediterranea and considering the effect of pathogens on endophyte growth (Figure S1), S. aogashimaense was the only endophyte not inhibited by this pathogen, displaying a typical antagonist interaction (0/0) (Table 3). Although F. rabenhorstii strongly inhibited B. mediterranea, there was a negative impact of the pathogen on this endophyte growth (co-antagonism; \(-/\)). Both endophytes displayed a similar interaction with the D. corticola pathogen, resulting in antagonism/co-antagonism with pathogen inhibition (40.8%, \( p \leq 0.001 \)) by S. aogashimaense; 42.2%, \( p \leq 0.001 \) by F. rabenhorstii). In contrast, A. alternata and Chaetomium sp. increased their growth while inhibiting B. mediterranea, thus displaying agonist interactions (+/+) with this pathogen. Both endophytes displayed a distinct interaction with D. corticola. A. alternata was not affected (antagonism; 0/0) and Chaetomium sp. was inhibited by this pathogen (co-antagonism; \(-/\)). Except for F. oxysporum (agonism; 0/0), all the other tested endophytes also revealed a co-antagonism interaction with D. corticola.

![Figure 4](image)

**Figure 4.** Inhibition of B. mediterranea and D. corticola growth caused by the tested endophytic fungi in dual-culture assay. Negative values indicate that the area of the plate covered by the interacting pathogen was higher than that of the control. Different letters represent statistical significance \((p \leq 0.005)\) between endophytes in each antagonistic assay and *** \((p \leq 0.001)\) represents statistical significance between the control and co-culture of a pathogen.

Fungal interactions were further evaluated by following the macro- and microscopic modifications of mycelia in the interaction region. When interacting with both pathogens, S. aogashimaense revealed a deadlock at distance (B) interaction type (Figure S2A,B) and caused visible modifications on hyphae of both pathogens (Figure 5A–D). In the presence of this endophyte, B. mediterranea showed typical hyphal deformations caused by the interacting partner, such as the presence of coiled hyphae and production of vesicle-like structures (Figure 5A,B). Further, D. corticola suffered mycelial modifications caused by interaction with S. aogashimaense, which included hyphal coiling and vacuolization, as well as the production of vesicle-like structures (Figure 5C,D). Such alterations have been frequently reported in different incompatibility systems [128,129] and have been related to programmed cell death (PCD) events occurring during interaction [130]. The A. alternata–B. mediterranea interaction also revealed hyphal deformations, such as hyphal vacuolization, production of vesicle-like structures and
hyphal penetration that resemble a mycoparasitism interaction (Figure 5E–G). Accordingly, instead of a deadlock at distance interaction type, in this interaction, there was a partial replacement of mycelia after an initial deadlock with mycelial contact (CA1; Table 3 and Figure S2C). Interestingly, although the inhibitory activity of an *A. alternata* isolate from *Q. cerris* against *D. corticola* has been reported, both in dual-culture and in planta [18], we have not detected hyphal distortions in the *A. alternata–D. corticola* interaction. All the other studied interactions did not reveal hyphal distortions, even though similar interaction types have been detected after 15 days of interaction (partial replacement after initial deadlock with mycelial contact; CA1), as well as a deadlock with mycelial contact (A) (Table 3).

**Table 3.** Classification of fungal interactions occurring between tested endophytes and pathogens on dual-culture assay. The interaction type was classified based on endophyte/pathogen growth by “+” for higher growth, “−” for less growth and “0” for equal growth in relation to control. Underlined mycelial interactions denote endophyte replacement by the pathogen.

| Endophyte               | *B. Mediterranea* | *D. Corticola* |
|-------------------------|-------------------|----------------|
|                         | Type of Interaction | Mycelial Interaction | Type of Interaction | Mycelial Interaction |
| *S. aogashimaense*      | antagonism (0/-)   | B               | antagonism (0/-)   | B                 |
| *C. carteri*            | agonism (+/+)     | CA2             | co-antagonism (+/-)| CA2               |
| *D. passiflorae*        | agonism (+/+)     | CB1             | co-antagonism (+/-)| CB1               |
| *F. rabenhorstii*       | co-antagonism (+/-)| A              | co-antagonism (+/-)| CA1               |
| *F. oxysporum*          | commensalism (+/0)| CA1             | antagonism (0/-)   | CA1               |
| *Chaetomium* sp.        | agonism (+/-)     | CA1             | co-antagonism (+/-)| CA1               |
| *A. alternata*          | agonism (+/-)     | CA1             | antagonism (0/-)   | CA1               |
| *P. olsonii*            | agonism (+/-)     | CB1             | co-antagonism (+/-)| CA1               |

**Figure 5.** Hyphal modifications produced in the interaction between *S. aogashimaense* and *B. Mediterranea* (A,B), *S. aogashimaense* and *D. corticola* (C,D) and *A. alternata* and *B. Mediterranea* (E–G). Single arrows designate coiled hyphae, double arrows vesicle-like structures and triple arrows hyphal vacuolization. Black line represents 50 µM scale.
3.3. Fungal Inhibitors Production by Cork Oak Endophytes

For understanding the production of inhibitors by the tested endophytes, their non-volatile and soluble compounds (produced in liquid culture) and volatile emissions were tested against *B. mediterranea* and *D. corticola* pathogens. Endophytic soluble compounds seem to have more impact on inhibiting mycelial growth than volatile compounds, mainly against *B. mediterranea* (Figure 6). However, this primary conclusion should be taken with some precaution as these inhibitory compounds were tested by using different strategies for their collection and assay. The endophytes that revealed the greatest inhibitory activity against *B. mediterranea* in the dual-culture method (*F. rabenhorstii, A. alternata, Chaetomium sp.* and *S. aogashimaense*) produced a soluble extract with high anti-fungal activity against both pathogens. These endophytes inhibited from 28% to 51% of *B. mediterranea* growth (*F. rabenhorstii p ≤ 0.05* and *A. alternata p ≤ 0.01*) and from 26% to 72% of *D. corticola* growth (*S. aogashimaense* and *A. alternata p ≤ 0.001; *F. rabenhorstii p ≤ 0.01*). The volatile emissions from the same endophytes also revealed inhibitory activities against *B. mediterranea* and *D. corticola* (ranging from 1% to 8% for *B. mediterranea*, and 18% to 39% for *D. corticola*). Multiple studies have already revealed the antifungal activity of genera from tested endophytes, in particular of *Chaetomium* spp., e.g., [131], *Coniothyrium* spp., e.g., [132], *Diaporthe* spp., e.g., [133], *Penicillium* spp., e.g., [134], and *Simplicillium* spp., e.g., [135], as well as their ability to produce a battery of antifungal compounds. However, less information is available on the inhibitory activity of *Fimetariella* spp. or the antifungal activities of *A. alternaria* and *F. oxysporum* species, which have been mainly recognized as important plant pathogens. This work provides new information related to the production of antimicrobial compounds, mainly from *F. rabenhorstii* and *A. alternata*, against cork oak pathogens.

![Figure 6](image-url)

**Figure 6.** Inhibition of *B. mediterranea* and *D. corticola* growth caused by non-volatile (A) and volatile compounds (B) produced by the tested endophytic fungi. Negative values indicate that the area of the plate covered by the interacting pathogen was higher than that of the control. Different letters represent statistical significance (*p < 0.005*) between endophytes in each antagonistic assay and * (p ≤ 0.05), ** (p ≤ 0.01) and *** (p ≤ 0.001) represent statistical significance between the control and co-culture of a pathogen.

Interestingly, endophytes with no inhibitory activity against *B. mediterranea* in dual-plate methods also revealed the production of inhibitors for this phytopathogen. For example, the soluble compounds of *P. olsonii* revealed the highest inhibition (65%, p ≤ 0.001) of all against *B. mediterranea*, and volatiles from *C. carteri* and *F. oxysporum* also exhibited high inhibitory activity against the same pathogen (8% and 12%, respectively). These results suggest that the inhibitory effect of a specific fungal isolate against a phytopathogen may be due to the production of multiple compounds that could act in a synergistic or antagonistic way [136]. Accordingly, the production of inhibitory compounds such as volatiles, antibiotics and other secondary metabolites is gaining biotechnological interest for the control of phytopathogens. For example, the potential of *S. coffeanum* volatile compounds against *Aspergillus* species was reported by Gomes et al. [95] and *S. lamellicola* was used to produce...
a fungicide against Botrytis cinerea [137]. The production of inhibitory compounds is gaining interest when produced by potential pathogens. For example, the potential of Alternaria sp. was already reported for the control of fungal and bacterial growth [103], but some species are widely known as phytopathogens [104], representing a disadvantage for field application. The same is described for the Fusarium oxysporum phytopathogen [88,138]. Therefore, the recognition of non-volatile or volatile inhibitory compounds could represent a biotechnological advantage for using those isolates as biocontrol agents.

4. Conclusions

The use of naturally adapted endophytes (for a specific plant host/environment) in a biocontrol strategy has gained increasing interest for restricting plant diseases. In this cork oak endophyte survey, we detected a high number of fungal OTUs from trees displaying different disease severity levels. The number of OTUs belonging to the pathogenic functional group (including described pathogens to other plant species) was high, even when considering healthy trees. Isolates of potential pathogens (namely, Alternaria alternata and Fusarium oxysporum) have revealed a strong in vitro inhibitory effect against cork oak pathogens (B. mediterranea and D. corticola). In particular, an A. alternata isolate revealed a high inhibitory activity against both pathogens, promoting hyphal deformations on B. mediterranea. Although this was not the case when interacting with D. corticola, this pathogen was similarly inhibited by A. alternata and F. oxysporum, displaying a mycelial interaction type based on the partial replacement of mycelia after an initial deadlock with mycelial contact. Non-volatiles and volatiles obtained from these isolates (particularly from A. alternata) revealed inhibitory activity and their potential to be used in a biocontrol strategy for restraining cork oak diseases should be further explored.

The colonization of plants by beneficial endophytes has been a useful biocontrol strategy. This work suggests Simplicillium aogashimaense as an antagonistic fungus towards B. mediterranea and D. corticola with potential to be used as a biocontrol agent against cork oak diseases. Indeed, S. aogashimaense presented promising results inhibiting both pathogens’ growth, which was reinforced by the promotion of pathogens’ hyphae deformations during interaction (deadlock at distance). A high ecological and economical value has been given to Simplicillium species due to their biocontrol role and production of bioactive compounds. For example, soybean plants when inoculated with S. lanosoniveum before infection with soybean rust pathogen (Phakopsora pachyrhizi) revealed reduced disease severity [139]. Although we believe S. aogashimaense could similarly be explored to control cork oak diseases, better understanding of its potential role as a biocontrol agent is still required. We thus conclude that cork oak endophytes could be further explored as biocontrol agents against cork oak diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2309-608X/6/4/287/s1, Table S1: Characterization of sampled cork oak stands; Table S2: Growth rates of cork oak endophytic fungi used for the antagonistic assays; Figure S1: Inhibition of fungal endophytes growth caused by the B. mediterranea and D. corticola in dual culture assay; Figure S2: Interaction in dual culture of S. aogashimaense-B. mediterranea (A), S. aogashimaense-D. corticola (B) and A. alternata- B. mediterranea (C) 72 h and 15 days after inoculation.

Author Contributions: Conceived and designed the experiments: T.L.-N., P.B., R.M.T. and D.C. Sampling: T.L.-N. and D.C. Conducted the main experiments: D.C. Analyzed the data: T.L.-N. and D.C. Wrote the original draft: T.L.-N. and D.C. Reviewed, edited and prepared the MS for submission: T.L.-N., P.B. and R.M.T. All authors have read and agreed to the published version of the manuscript.

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