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Microbial community of olives and its potential for biological control of olive anthracnose

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“The little I know I owe to my ignorance”
Orville Mars

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Abstract

Olive anthracnose is an important fruit disease in olive crop worldwide and no effective method is currently available for their control. Fruit-associated microorganisms may be explored for designing new strategies for the biological control of this disease. The main aim of this work was to assess the diversity of fungal endophytes and epiphytes inhabiting olive fruits of two cultivars with different susceptibilities to olive anthracnose, and select the strains with the greatest antagonistic effect against *Colletotrichum acutatum*, the main causal agent of this disease. Culture-dependent method was used to assess fungal diversity in olives from cvs. Madural (susceptible) and Verdeal Transmontana (moderately tolerant), and the isolates obtained were identified for molecular identificantion using internal transcribed spacer (ITS1, 5.8, ITS2) region of the rDNA. The *in vitro* effect of the isolates against *C. acutatum* was analysed by the dual-culture method. Results revealed that only endophytic fungal communities composition differ significantly between cultivars. Cultivar Madural was distinguished by the higher abundance of *Gibberella* spp. whereas cv. Verdeal Transmontana was characterized by the exclusive occurrence of *Neofabraea vagabunda*. Although epiphytic community of both cultivars overlapped, several fungal genera preferred either olives from cv. Madural (e.g. *Chaetomium*) or from cv. Verdeal Transmontana (e.g. *Cytospora*, *Epicoccum* and *Quambalaria*). All the seven fungal tested were able to inhibited *C. acutatum* growth (inhibition coefficients up to 30.9), and caused morphological changes in its hyphae. Some fungi also inhibited significantly *C. acutatum* sporulation (from 46-86%) and germination (from 21-74%). Altogether, the results offer new insights into plant-microbe-microbe interactions and highlighted the potential use of these antagonistic fungi in the biocontrol of olive anthracnose.

Keywords: Olive anthracnose; endophytes; epiphytes; *Olea europaea*; biocontrol.

Resumo

A antracnose da oliveira é uma doença com grande importância a nível mundial, e não existe nenhum método eficaz para o seu controle. Os microrganismos existentes nas azeitonas podem ser explorados na tentativa de encontrar novas estratégias de luta biológica contra esta doença. Este trabalho teve como objetivo avaliar a diversidade de fungos endofíticos e epifiticos existentes nas azeitonas de duas cultivares com diferentes suscetibilidades à antracnose, e selecionar os isolados que apresentam maior atividade antagonista contra o *Colletotrichum acutatum,* principal agente causal desta doença. A diversidade de fungos nas azeitonas das cultivares Madural (suscetível) e Verdeal Transmontana (moderadamente tolerante) foi avaliada através da obtenção de isolados em meio de cultura, seguida pela sua identificação molecular através da sequenciação da região ITS (ITS1, 5.8S, ITS2) do rDNA. A atividade antagonista dos isolados contra *C. acutatum* foi avaliada usando o método de co-cultura. Os resultados obtidos evidenciaram que apenas a composição da comunidade de fungos endofíticos diferiu significativamente entre as cultivares. A cv. Madural caracterizou-se pela elevada abundância de *Gibberella* spp., enquanto a cv. Verdeal Transmontana destacou-se pela ocorrência exclusiva de *Neofabraea vagabunda.* Apesar da composição de fungos epifíticos ser similar entre as cultivares, verificou-se que alguns géneros de fungos ocorriam exclusivamente na cv. Madural (*Chaetomium*) ou na cv. Verdeal Transmontana (*Cytospora*, *Epicoccum* e *Quambalaria*). Os sete fungos testados foram capazes de inibir o crescimento de *C. acutatum* (coeficiente de inibição superiores a 30,9), e causar alterações morfológicas nas suas hifas. Alguns fungos ainda inibiram significativamente a esporulação (46-86%) e germinação (21-74%) de *C. acutatum*. Os resultados contribuíram para um melhor conhecimento das interações planta-microrganismo-microrganismo, e evidenciam o potencial uso destes fungos como antagonistas na luta biológica da antracnose da oliveira.

Palavras-Chave: Antracnose; endofíticos; epifíticos; *Olea europaea*; luta biológica.

1. Framework and objectives

1.1. Framework and objectives

Olive anthracnose, a disease of olive fruits mainly caused by diverse fungi clustering in the *Colletotrichum acutatum* species complex (Talhinhas et al., 2005, 2009), is one of the most serious constrains to the olive crop production worldwide (Cacciola et al., 2012). Their control has relied extensively on the use of chemical pesticides, with limited efficacy (Cacciola et al., 2012) and not compatible with sustainable production systems (organic and integrated production) which are the pillars of the European Model for Agriculture, according the Directive 2009/128/EC. In olive production, plant protection strategy must follow the Guidelines for integrated production of olives (Malavolta & Perdikis, 2012). Thus, a need to develop novel and environmental-friendly control strategies for management of olive anthracnose is an important topic for research.

Fruit-associated microorganisms may be explored, in an integrative perspective, for designing new strategies for the biological control of olive anthracnose.The aerial parts of the plants (phyllosphere) are colonized by a diverse microbial community, which can grow both epiphytically on the surface of plant tissues and/or endophytically within the tissues (Lindow & Brandl, 2003; Vorholt, 2012). Many of these microorganisms seem to play crucial roles in protecting plants from diseases and in promoting their growth (*e.g*. Bulgarelli et al., 2013). Such importance was specially recognized in leaf-associated bacteria (Ren et al., 2015). In opposite, other group of microorganisms as well as other plant organs, such as fruits, has been less studied. A better knowledge of both composition and function of microbiotas in and onolive fruits will certainly contribute to develop new successful and sustainable integrated crop protection against olive anthracnose. Actually, there is a growing desire to model and manage host–microbiota interactions (Kembel et al., 2014), to improve crop yields (Andreote et al., 2014). In this way, the development of so-called "microbiome-driven cropping systems" might result in the next revolution in agriculture, resulting in a more sustainable system for plant production (Andreote et al., 2014).

In this work we intend to evaluate both epi- and endophytic fungal community inhabiting olives from two cultivars with different susceptibilities to olive anthracnose, and select the strains with the greatest antagonistic effect against the causal agent of this disease, *C. acutatum*.

Specific objetives are:

1- Evaluate fungal composition of olives from two olive tree cultivars with different susceptibilities to olive anthracnose: cv. Madural (susceptible) and cv. Verdeal Transmontana (relative resistant), by using a culture-dependent approach;
2- Select the microorganisms with antagonist potential against *C. acutatum*, using *in vitro* cultures on agar plates;

The results from this study will form the basis for the identification of fungi from olives for the biological control of olive anthracnose disease. Results will also uncover previously unrecorded mechanisms of fungi-olive tree and microorganism-microorganism relationships, which may be of relevance for plant health and for designing a new strategy for the biological control of this disease.

This dissertation was organized in three chapters: the introduction (Chapter 2), the research work that was present in the format of a scientific research paper (Chapter 3), and finally the conclusions and recommendations for future work (Chapter 4). The introductory chapter was organized to offer an overview of the potential role played by phyllosphere microorganisms in host plant health, and described how they can be explored mostly in the protection of host plant to phytopathogen infection, as biological control agents or through their management in order to reduce phythopatogens. Particularly attention will be given to olive tree phyllosphere and their role in host protection against olive anthracnose.

2. Introduction

2.1. Olive anthracnose

Olive anthracnose is considered the most important fruit disease in olive crop worldwide (Cacciola et al., 2012). Firstly reported in Portugal (Almeida, 1899), this disease rapidly spread to all olive producing areas in the Mediterranean region, Asia, North and South America, South Africa and Australia (Cacciola et al., 2012). This disease affects mostly the fruits, and in less extended the flowers, leaves and shoots (Moral et al., 2009; Sergeeva, 2011). Most common disease symptoms in fruits include soft circular rotted spots with abundant pink/orange masses of spores that appeared on the surface of ripe and overripe olives (Talhinhas et al., 2011). This causes premature fruit drop and mummified fruits (Fig. 2.1). The last one may persist on the tree, providing inoculum for new infections. In severe cases the vegetative parts are also affected causing defoliation, shoot death and loss of tree vigour (Cacciola et al., 2012).

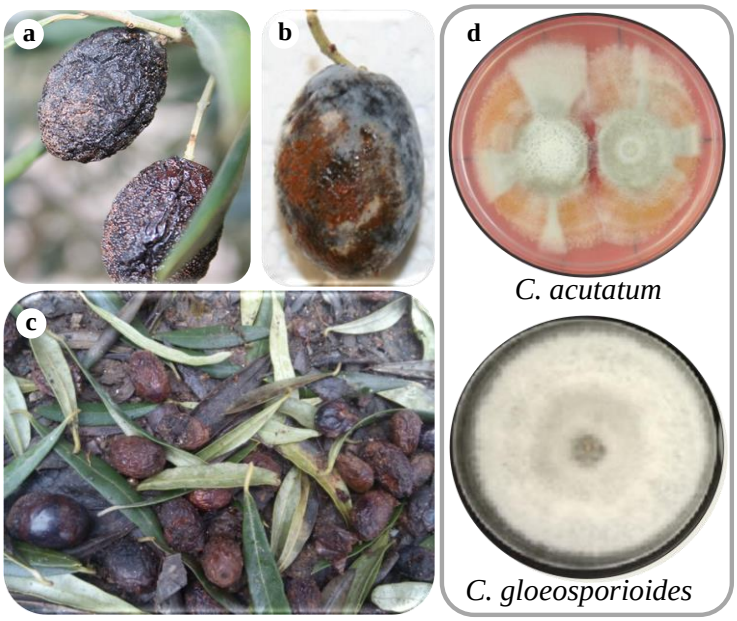


Figure 2.1. Symptoms of olive anthracnose and colonies of the causal agents. (a) mummified and dehydrate olives, (b) necrosis and production of pink/orange spores on the surface of the rotting olives, (c) numerous infected fruits on the floor, (d) and colonies (upper surface) of *Colletotrichum acutatum* and *C. gloeosporioides* growing in Potato Dextrose Agar medium (photos: Paula Baptista).

Olive anthracnose is caused by two complexes of species showing high phenotypic and genotypic diversity, *Colletotrichum gloeosporioides* sensu lato (s.l.) and *C. acutatum* s.l. (Fig. 2.1) (Sreenivasaprasad & Talhinhas, 2005; Damm et al.,

2012; Weir et al., 2012), being the latter more prevalent and aggressive than the first one (Talhinhas et al., 2005, 2011). A third complex, *C. boninense*, was recently associated with olive anthracnose, but it does not appear to represent a serious threat for olive production (Schena et al., 2014). According to Talhinhas et al. (2009), *C. acutatum* s.l. is the most prevalent in Portugal.

2.1.1. Olives production losses caused by anthracnose

Olive anthracnose is reported to cause significant yield losses, poor fruit and oil quality, in most olive-growing countries. For instances, in several Mediterranean countries, such as Italy and Spain, olive anthracnose was reported to cause 80-100% yield loss (review by Cacciola et al., 2012). According to Moral et al. (2009), only in Spain, about $93.4 million are lost each year due to anthracnose prevalence. In Portugal, severe epidemics of olive anthracnose occurs in wet regions, leading to considerable yield losses up to 100% for the susceptible olive cv. Galega Vulgar (Talhinhas et al., 2011). In Australia, under favourable environmental conditions, the disease was reported to affect up to 80% of olives in susceptible cultivars such as 'Barnea' and 'Manzanillo' (Sergeeva, 2011). Yield losses of up to 70% have also been registered in olive cv. Arauco in Argentina (review by Cacciola et al., 2012).

Anthacnose is also responsible for quality degradation of olive oils (Moral et al., 2014), which was reported to be directly proportional to the infection levels (Iannotta et al., 1999; Carvalho et al., 2008). Olive oils extracted from infested olives showed higher peroxide value and free acidity, and lower oxidative stability and phenolic compounds (Iannotta et al., 1999; Carvalho et al., 2008). At infection rates up to 30%, olive oil quality is severely affected and fresh olive oils cannot be classified as extra-virgin olive oils, being considered in the majority of the cases as lampante due to both quality and sensorial defects (Iannotta et al., 1999).

2.1.2. Disease management

Olive anthracnose is difficult to control after the symptoms appear, particularly when environmental conditions are favourable for infection (Sergeeva, 2011). Thus, control is essentially based on preventive methods, such as the use of resistant cultivars, spray of trees with copper compounds (*e.g.* Bordeaux mixture, copper

oxychloride), and cultural control (*e.g.* pruning the trees, early fruit harvesting) (Cacciola et al., 2012). All these methods, with exception to the first one, are not effective in suppressing olive anthracnose (Moral et al., 2014). There are also environmental and toxicological concerns about the use of copper compounds, because of possible copper residues in olive fruits and consequently in oil (Soares et al., 2006). Therefore, there is a need to develop novel and environmental-friendly control strategies for management of olive anthracnose. One of those strategies can be the use of antagonist-mediated biological control which, to the best of our knowledge, was never explored in olive anthracnose. Only recently, some fungi of *Olea europaea* L. showed the hability to limit the growth of *C. acutatum* under *in vitro* conditions (Landum et al., 2016). In this study were isolate epiphytic and endophytic fungi, from olive leaves of cv. Galega Vulgar, and the fungi that inhibited mycelia growth of *C.acutatum* were *A. niger, N. oryzae* and one unclassified endophytic fungus.

Although the use of less susceptible or resistant cultivars is an effective way to control this disease, it has not been conveniently explored so far (Cacciola et al., 2012). In addition costs associated with replacing an established crop with these cultivars are very high (Landum et al., 2016). In what concerns Portuguese cultivars, some are traditionally considered susceptible to anthracnose (*e.g.* Galega Vulgar) whereas others are considered as resistant (*e.g.* Negrinha de Freixo) (Talhinhas et al., 2015). In Trás-os-Montes region, severe anthracnose infections were observed in cv. Madural, and in opposite cv. Verdeal Transmontana was less affected (Pereira JA, personal communication).

2.2. The Phyllosphere

The phyllosphere, defined as the above-ground plant parts, represents a habitat for diverse organisms (Newton et al., 2010), including bacteria, yeast, filamentous fungi, oomycetes, archaea and algae (Lindow & Brandl, 2003) and it represent the largest biological habitat on Earth (Delmotte et al., 2009). Bacteria are, by far, the most common (Lindow & Brandl, 2003) and therefore most studies focus on these. In the aerial parts of the plants, these microorganisms can live on the surface of plant tissues (epiphytes) and/or within living plant tissues (endophytes) (Newton et al., 2010). Many of these microorganisms interact with their plant host intimately

improving plant growth and promoting protection against abiotic and biotic stresses, such as pathogen infection (Fig. 2.2) (Lindow & Brandl, 2003; Muller & Ruppel, 2014). Most of the research on this topic has focused on the composition and functional roles of bacteria inhabiting leaves (Whipps et al., 2008). In opposite, other aerial plant parts (*e.g.* fruits) as well as other group of microorganisms (*e.g.* filamentous fungi and yeasts) were less studied.

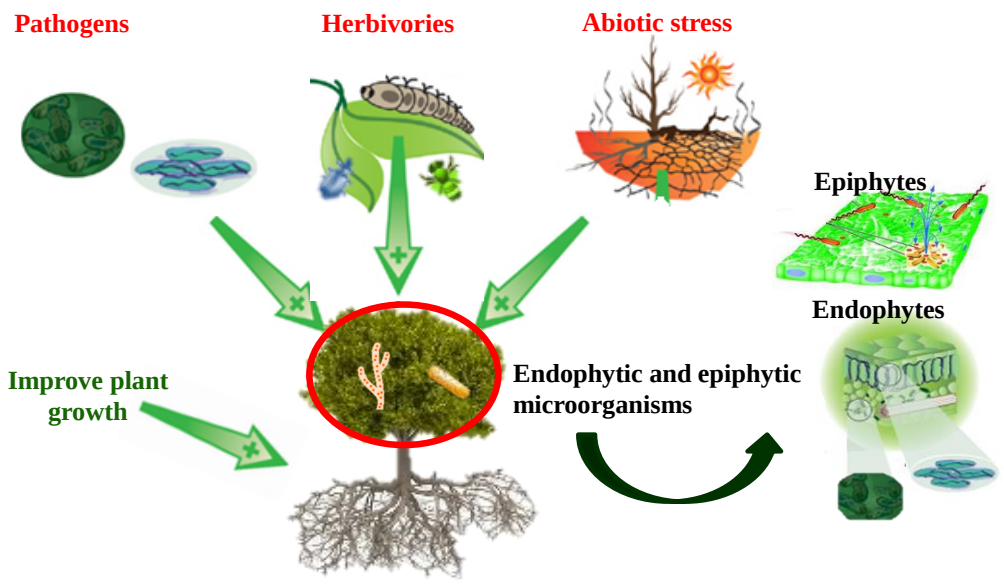


Figure 2.2. .Effect of endophytic and epiphytic microorganisms inhabiting phyllosphere on plant host growth and protection against biotic and abiotic stress (Adapted from Partida-Martínez & Martin, 2011).

Phyllosphere associated-microorganisms promote plant growth from multiple mechanisms such as i) the direct acquisition of mineral nutrients; ii) the production of plant hormones like cytokinins (Ivanova et al., 2000) and auxins (Omer et al., 2004); iii) and indirectly by protecting plants against plant pathogens (Wang et al., 2009), through the production of antimicrobial compounds (antibiosis), and competition for nutrients and space (Berg, 2009).

Plant defense via endophytes are more commonly described than via epiphytes. Endophytes can reduce the infection of plant tissues by pathogens either directly, through the production of antagonistic molecules (antibiosis), mycoparasitism and competition for resources (for niche, infection site or nutrients), or indirectly by induction of plant resistance response (Fig. 2.3) (Lacava et al., 2014). These

mechanisms frequently operated simultaneously. The mechanism of antibiosis includes production of antibiotics (*e.g.* terpenoids, alkaloids and polypeptides), bioactive volatile organic compounds (*e.g.* acids, alcohols, alkyl pyrones, ammonia, esters, hydrogen cyanide, ketones, and lipids), and enzymes (reviewed by Gao et al., 2010 and Ownley et al., 2010). These enzymes are distinctly different from those involved in mycoparasitism of phytopathogens, since they are mostly involved in the suppression of pathogen activity and/or in the pathogen´skill (Ownley et al., 2010). Mycoparasitism is a process by which endophyte act as mycoparasites and directly parasitize phythopathogen. This process occurs in four steps: 1) endophyte growth toward the pathogen that produces chemical stimuli (*e.g.* volatile chemoattractant); 2) recognition between endophyte and pathogen, which was tought to be mediated by lectins of pathogens and carbohydrate receptors on the surface of the endophyte; 3) attachment of endophyte to pathogen either by coil around host hyphae or grow alongside it, and cell wall degradation of pathogen through the production of lytic enzymes by the endophyte like chitinases, ß-1,3 gluconases, proteases and lipases; 4) and finally, penetration of endophyte into the pathogen (Lo, 1998; Gao et al., 2010; Ownley et al., 2010).

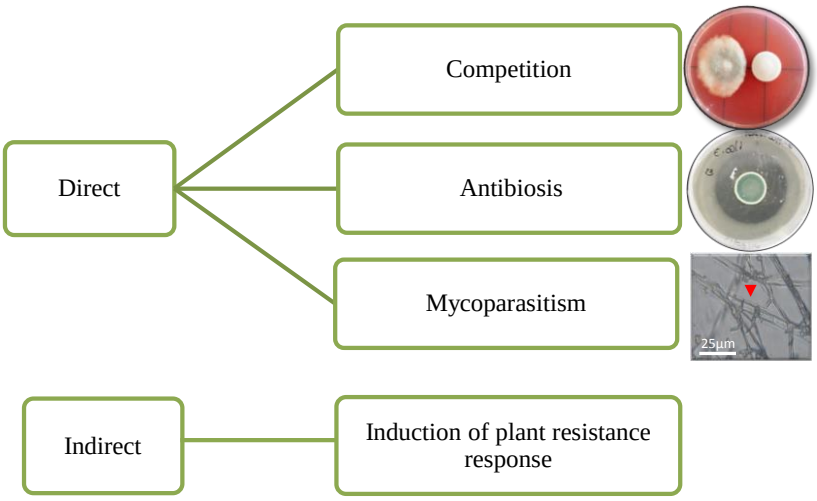


Figure 2.3. Mechanisms of plant disease suppression by endophytes. Several mechanisms may control diseases suppression, either directly on the pathogen by antibiosis, mycoparasitism and competition for resources, or indirectly by induction of plant resistance response.

Endophytes can also reduce herbivory and influence the dynamics of phytophagous insects and insect parasitoids (Lacava et al., 2014). Host plant resistance to herbivores via endophytes may involve both the production of toxic

compounds (*e.g.* alkaloids) and the induction/priming of resistance-related genes by the endophyte, or may be due to their function as entomopathogens and to enhance of both genetic and biochemical diversity of their host (reviewed by Partida-Martínez & Martin, 2011). These modes of action are not necessarily exclusive, as, for example, fungal endophytes can act as entomopathogenic, antagonistic of plant pathogens and might possibly even function as plant-growth promoting agent (Vega et al., 2009).

2.2.1. The Phyllosphere of olive tree

The microbiota of the olive tree phyllosphere have been only recently analyzed by using both culture-independent (Muller et al., 2015; Abdelfattah et al., 2015) and –dependent (Martins et al., 2016) approachs. Analysis of endophytic communities from olive leafs, allowed the detection of a high portion of bacteria taxa belonging to the phylum *Proteobacteria* (21.3–69.6%) and of archaeal phyla *Thaumarchaeota* (0.6–51.7%) and *Crenarchaeota* (1.9–28.6%) (Muller et al., 2015). Less abundant taxa that were detected belonged to *Firmicutes*, *Euryarchaeota*, and *Bacteroidetes*. Similarly, the fungal community associated with leaves, flowers and fruits of olive tree was revelead to be rich, being the Ascomycota the most dominating phyla representing more than 93% of the total number of detected sequences (reads) (Abdelfattah et al., 2015). In general, a higher level of fungal diversity was revealed for leaves compared to flowers and fruits. Incidence of Ascomycota was found to be generally higher in fruits compared to flowers and leaves. Basidiomycota accounted for 2.5–3.5% of the reads on leaves but were almost completely absent on flowers and fruits. Martins et al. (2016) observed similar endophytic structure associated with leaves and twigs of olive tree, with a dominance of fungi belonging to Ascomycota phylum. This study also outlined the effect of tissue type, time of the season and host plant location on fungal endophyte community structure of olive tree: endophyte community's similarity between above- and belowground organs, and between seasons (spring *vs*. autumn) was found to be very low. Fungal communities from proximate sites were more similar than those in more distant sites.

Overall, these studies highlight the existence of a complex microbial consortium including pathogenic and potentially antagonistic microorganisms that can have a significant impact on olive production. For instances, Abdelfattah et al. (2015) have detected the presence of several plant pathogens (e.g. *Colletotrichum* spp.,

Pseudocercospora spp.) on several olive tree organs (fruit, leaves and flowers). In addition, endophytic bacteria (Muller et al., 2015) and fungi (Landum et al., 2016) isolated from olive tree leaves were showed to inhibited the growth of olive-pathogenic fungus *Verticillium dahliae* and *C. acutatum*, respectively, under *in vitro* conditions.

However, all these studies have focused only on endophytic or epiphytic community, and none of them consider interactions among these two groups of microorganisms. We still have an incomplete understanding of how phyllosphere microorganisms interact among themselves and with their plant hosts. Similarly, phyllosphere microbiota functions and actual implications for plants, ecosystems, and agriculture, are mostly unknown. A more integrative approach to the biodiversity of the olive tree phyllosphere will facilitate the understanding of the complex interactions between the plant and the resident microflora, including potential pathogens and their antagonists. This information may be very usefull for the development of novel sustainable agricultural practices. For instances, phyllosphere microbiota could be managed to reduce the use of fertilizers and pesticides or to control plant growth (Newton et al., 2010). Therefore, accurate knowledge of the composition of olive tree phyllosphere microbiota may be essential to develop effective biological disease control strategies. There has been additionally considerable interest in screening of phyllosphere microorganisms for antagonisms to plant pathogens, in order to identified potentially biological control agents against diseases.

References

Abdelfattah A., Nicosia M.G.L.D., Cacciola S.O., Droby S., Schena L., 2015 Metabarcoding Analysis of Fungal Diversity in the Phyllosphere and Carposphere of Olive (*Olea europaea*). PLoS ONE 10(7): e0131069

Almeida M.J.V., 1899. La gaffa des olives en Portugal. Bulletin de la Société Mycologique de France. 15: 90-94

Andreote F.D., Gumiere T., Durrer A., 2014 Exploring interactions of plant microbiomes. Scientia Agricola 71:528-539

Bulgarelli D., Schlaeppi K., Spaepen S., Themaat E.V.L., Annu P.S.L. 2013.Structure and functions of the bacterial microbiota of plants. Plant Biology 64: 807-838

Cacciola S.O., Faedda R., Sinatra F., Agosteo G.E., Schena L., Frisullo S., Magnano di San Lio G., 2012. Olive Anthracnose. Journal of Plant Pathology. 94: 29-44.

Carvalho M.T., Simões-Lopes P., Monteiro da Silva M.J., 2008. Influence of Different Olive Infection Rates of *Colletotrichum acutatum* on Some Important Olive Oil Chemical Parameters. ActaHorticulturae. 791: 555-558.

Damm U., Cannon P.F., Woudenberg J.H., Crous P.W., 2012. The *Colletotrichum acutatum* species complex. Studies Mycology. 73:37–113.doi: 10.3114/sim0010.

Delmotte N., Knief C., Chaffron S., Innerebner G., Roschitzki B., Schlapbach R., von Mering C., Vorholt J.A., 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci U S A 106: 16428–16433.

Gao F.K., Dai C.C., Liu X.Z., 2010. Mechanisms of fungal endophytes in plant protection against pathogens. African Journal of Microbiology Research 4: 1346-1351.

Iannotta N., Perri E., Sirianni R., Tocci C., 1999. Influence of *Colletotrichum gloeosporioides* and *Camarosporium dalmatica* attacks on olive oil quality. ActaHorticulturae. 474: 573–576.

Kembel S.W., O'Connor T.K., Arnold H.K., Hubbelld S.P., Wright S.J., Green J.L., 2014. Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. PNAS. 111: 13715–13720.

Lacava P.T., Azevedo J.L., 2014 Biological Control of Insect-Pest and Diseases by Endophytes In Advances in Endophytic Research Part V. Verma V.C., Gange A.C. (Eds.) Springer India 231-256.

Landum M. C., Felix M. R., Alho J., Garcia R., Cabrita M. J., Rei F., Varanda C. M.R., 2016. Antagonistic activity of fungi of Olea europaea L.against *Colletotrichum acutatum*. Microbiological Research 183: 100-108

Lindow S.L., Brandl M.T., 2003. Microbiology of the Phyllosphere. Applied and Environmental Microbiology. 69(4): 1875–1883

Lo C.T., 1998. General mechanisms of action of microbial biocontrol agents. Plant Pathology Bulletin 7: 155-166

Malavolta C., Perdikis D., 2012. IOBC Technical Guidelines III. Guidelines for Integrated Production of Olives. IOBC/WPRS Bulletin. 77: 1-19

Martins F., Pereira J.A., Bota P., Bento A., Baptista P., 2016. Fungal endophyte communities in above- and belowground olive tree organs and the effect of season and geographic location on their structures. Fungal Ecology, 20: 193–201

Moral J., de Oliveira R., Trapero A., 2009. Elucidation of disease cycle of olive anthracnose caused by *Colletotrichum acutatum*. Phytopathology. 99: 548-556.

Moral J., Xaviér C., Roca L.F., Romero J., Moreda W., Trapero A., 2014. La Antracnosis del olivo y su efecto en la calidad del aceite. Grasas y Aceites 65 (2): e028

Muller T., Ruppel S., 2014. Progress in cultivation-independent phyllosphere microbiology FEMS Microbiology Ecology. 87: 2–17.

Muller H., Berg C., Landa B.B., Auerbach A., Moissl-Eichinger C., Berg G., 2015 Plant genotype-specific archael and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. Front Microbiol 6:138

Newton A.C., Gravouil C., Fountaine J.M., 2010. Managing the ecology of foliar pathogens: ecological tolerance in crops. Annals of applied biology. 157: 343–359.

Ownley B.H., Gwinn K.D., Vega F.E., 2010. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. BioControl 55: 113-128

Partida-Martínez L.P., Martin H., 2011. The microbe-free plant: factor artifact? Plant Science 2: 100.

Ren G., Zhang H., Lin X., Zhu J., Jia Z., 2015.Response of leaf endophytic bacterial community to elevated CO_2 at different growth stages of rice plant. Frontiers in Microbiology. 6: 1-13.

Schena L., Mosca S., Cacciola S.O., Faedda R., Sanzani S.M., Agosteo G.E., Sergeeva V., Magnano di San Lio G., 2014. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose. Plant Pathology. 63: 437–446.

Sergeeva V., 2011. What is effective against anthracnose? The Olive Press. 22-24

Sergeeva V., 2011. Anthracnose in olives: symptoms, disease cycle and management. 4th Olivebioteq International Conference for olive products. 1:269-274.

Soares M.E., Pereira J.A., Bastos M.L., 2006. Validation of a method to quantify copper and other metals in olive fruits by ETAAS. Application to the residual metal control after olive tree treatments with different copper formulations. Journal of Agricultural and Food Chemistry. 54: 3923-3928.

Sreenivasaprasad S., Talhinhas P., 2005.Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. Molecular Plant Pathology. 6:361–378.

Talhinhas P., Gonçalves E., Sreenivasaprasad S., Oliveira H., 2015. Virulence diversity of anthracnose pathogens (*Colletotrichumacutatum* and *C. gloeosporioides* species complexes) on eight olive cultivars commonly grown in Portugal. European Plant Pathology, 142: 73-83.

Talhinhas P., Mota-Capitão C., Martins S., Ramos A.P., Neves-Martins J., Guerra-Guimarães L., Várzea V., Silva M.C., Sreenivasaprasad S., Oliveira H., 2011. Epidemiology, histopathology and aetiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. PlantPathology. 60: 483–495.

Talhinhas P., Neves-Martins J., Oliveira H., Sreenivasaprasad S., 2009. The distinctive population structure of *Colletotrichum* species associated with olive anthracnose in the Algarve region of Portugal reflects a host–pathogen diversity hot spot. FEMS Microbiology Letters. 296: 31–38.

Talhinhas P., Sreenivasaprasad S., Neves-Martins J., Oliveira H., 2005. Molecular and phenotypic analyses reveal the association of diverse *Colletotrichum*

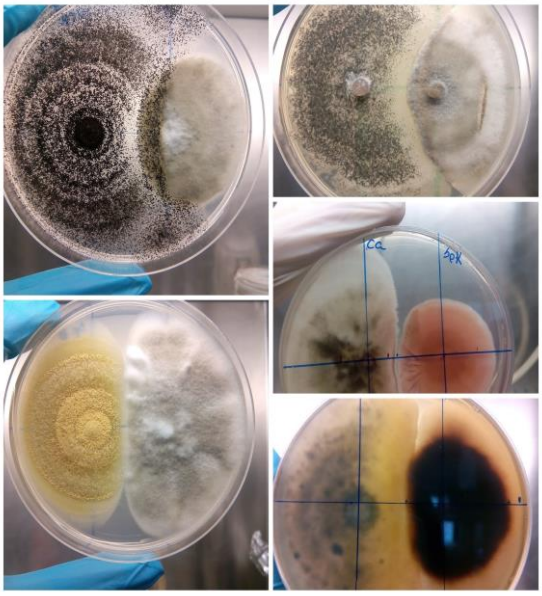
acutatum groups and a low level of *C. gloeosporioides* with olive anthracnose. Applied and Environmental Microbiology. 71: 2987–2998.

Vega F.E., Goettel M.S., Blackwell M., Chandler D., Jackson M.A., Keller S., Koike M., Maniania N.K., Rangel D.E.N., Roy H.E., 2009. Fungal entomopathogens: new insights on their ecology. Fungal Ecology 2: 149-159.

Weir B.J., Johnston P.R., Damm U., 2012. The *Colletotrichum gloeosporioides* species complex. Studies Mycology. 73: 115–180.

Whipps J.M., Hand P., Pink D., Bending G.D., 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. Journal of Applied Microbiology. 105: 1744-1755.

3. Fungal community of olives and their antagonistic activity against *Colletotrichum acutatum*



3.1. Introduction

Olive anthracnose, a disease of olive fruits mainly caused by diverse fungi clustering in the *Colletotrichum acutatum* species complex (Talhinhas et al., 2005, 2009), is one of the most serious constrains to the olive crop production worldwide (Cacciola et al., 2012). In several Mediterranean countries, such as Italy, Spain and Portugal, olive anthracnose was reported to cause 80-100% yield loss due to premature fruit drop (Talhinhas et al., 2011; Cacciola et al., 2012). Anthracnose is also responsible for quality degradation of olive oils by increasing the free acidity and the peroxide values, and by decreasing oxidative stability and the contents on phenolic compounds (Iannotta et al., 1999; Carvalho et al., 2008). The use of copper fungicides has been the main control strategy adopted against this disease (Cacciola et al., 2012). Besides not being completely effective on preventing olive anthracnose (Moral et al., 2014), there are numerous environmental risks and toxicity problems associated with it because of possible copper residues in olive fruits and consequently in oil (Soares et al., 2006). Chemical control measures are also not compatible with sustainable production systems (organic and integrated production) which are the pillars of the European Model for Agriculture.

Fruit-associated microorganisms may be explored, in an integrative perspective, for designing novel and environmental-friendly control strategies for management of olive anthracnose. The above-ground parts of plants, and called phyllosphere, are colonized by a diverse microbial community that can inhabit the interior of plant tissues (as endophytes) as well as plant's surface (as epiphytes) (Vorholt, 2012). Recent studies indicate that host plant genotype can influence the microbiome composition by acting on keystone species, which in turn affects plant colonization by many other microbes (Agler et al., 2016). In this changed microbial community, species with diverse functional types, form complex, interconnected microbial networks that could have important implications for plant health (Heijden & Hartmann, 2016). These findings give rise to novel ideas for sustainable management of plant diseases. For instances, genotype selection offers opportunities to select for certain keystone taxa, which in turn might recruit beneficial organisms or prevent invasion of pathogens (Heijden & Hartmann, 2016). However, knowledge about the diversity of those plant-associated microbes as well as of how phyllosphere microorganisms interact among themselves and with their plant hosts is still very

incomplete, which could jeopardize that strategy. This is especially true for the olive tree, being only recently analyzed the fungal community associated to their fruits (Abdelfattah et al., 2015). This study highlights the existence of a complex fungal consortium, including pathogenic (e.g. *Colletotrichum* spp.., *Pseudocercospora* spp..) and potentially antagonistic, that can have a significant impact on olive production. However, this study did not compare epiphytic and endophytic fungal communities on single olives. Stressors that challenge epiphytes (mainly external environmental factors such as temperature, humidity and solar radiation) are different from endophytes (mostly plant defense reactions), which might have important effects on both fungal communities composition (Rastogi et al., 2013).

In this work, we studied, for the first time, the culturable fungal epiphytes and endophytes inhabiting olives from two Portuguese cultivars with different susceptibilities to olive anthracnose: Madural cultivar is susceptible and Verdeal Transmontana is moderately tolerant to olive anthracnose. We wanted to determine whether some fungi can systemically colonize olives of the two cultivars or whether such cultivar eventually develops their own specific fungal communities. The inhibitory efficacy of several fungi isolated from both cultivars against the phytopathogenic *C. acutatum* was additionally evaluated, under *in vitro* conditions. We wanted to determine whether inhibitory efficacy displayed by fungi is dependent of their origin (*i.e.* olive cultivar) and to find new fungi with potential to be explored as biocontrol agents against *C. acutatum*.

3.2. Material and methods

3.2.1. Sample collection

Olive fruits were collected in three organic olive (*Olea europaea* L.) groves located in Mirandela (Northeast of Portugal, 41º33'29.6"N7º08'45.9"W), in November of 2015. Each one comprises two different Portuguese olive cultivars, Verdeal-Transmontana and Madural, with trees of medium size (ages ranging 60 years) and planted at 7 x 7m spacing. In each grove, seven olive trees of each cultivar were randomly selected. From each tree, five symptomless olive fruits, at a maturity index ranging from 0 to 3 (Hermoso et al., 1991), were aseptically handpicked all around the perimeter of the tree at the operator height, and placed directly into sterile

bags. The olives were transported to the laboratory in an icebox, and stored at 4ºC until isolation of epi- and endophytic fungi the following day.

3.2.2. Isolation of epiphytic and endophytic fungi from olives

Epiphytic fungi were obtained by imprinting the intact olive fruits samples gently on Potato Dextrose Agar (PDA, Difco) plates supplemented with 0.01% (w/v) chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). Olives were rolled over the agar surface (one fruit per plate), being used a total of 210 fruits (5 olives x 7 olive trees x 3 orchard x 2 different cultivars) for the isolation of epiphytes. Endophytic fungi were obtained from the same olives used to isolated epiphytes, after surface sterilization of fruit tissues. Surface sterilization was performed through sequential immersion of fruits in 70% (v/v) ethanol (1 min), 3% (v/v) sodium hypochlorite (2 min), 70% (v/v) ethanol (1 min), and further rinsed three times with sterile distilled water (one min each). After being dried on sterile filter paper, each fruit was cut into five segments (*ca*. 5 x 5 mm), and transferred into the same medium used to isolated epiphytes (PDA). Therefore a total of 1050 segments (5 olives x 5 segments per olive x 7 olive trees x 3 orchard x 2 different cultivars) were inoculated. Validation of the surface sterilization procedure was done by imprinting the surface sterilized fruit tissues onto PDA media. Plates were incubated at 25±2ºC in the dark and were daily observed for microbial growth and colonies counting. Single colonies were picked and cultivated on new PDA plates to obtain pure isolates for subsequent identification. Results of epiphytes were expressed as CFU/cm^2, *i.e.* the number of individual colonies of fungi adhered to fruit surface. For the approximate calculus of fruit surface area was used the prolate spheroid formula (Weisstein, 2013) from the longitudinal and transverse axes of fruits. For the Verdeal Transmontana and Madural fruits used in the present study, the average area was 11.61 cm^2 and 9.88 cm^2, respectively. Pure cultures of each isolate were preserved in tubes with glycerol kept at -20ºC, and deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

3.2.3. Identification of fungal isolates

Fungal isolates were identified by using morphological and molecular approaches. At first, groups of strains were formed according their morphological

similarity (culture colony, hyphae, spores and reproductive structures), and then one representative isolate of each morphotype was selected for molecular identification, using internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Total genomic DNA was extracted from mycelial/spores using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK). The ITS region (ITS1, 5.8S, ITS2) was amplified using the universal ITS1 and ITS4 primers (White et al., 1990), in a PCR protocol formerly described by Oliveira et al. (2012). The amplified products (~650 bp) were purified and sequenced using Macrogen Inc. (Seoul, South Korea) services, using the same primers to initiate the reaction. The obtained DNA sequences were analysed with DNASTAR v.2.58 software, and fungal identification was performed using both NCBI (http://www.ncbi.nlm.nih.gov) and UNITE (https://unite.ut.ee/) databases and BLAST algorithm. Blast results were sorted according to the higher identity score and the lowest E-value. For sequence identities >98%, the genus and species were accepted; for sequence identities between 95% and 97%, only the genus was accepted; and for sequence identities <95%, isolates were labelled as 'unknown' fungi. The sequences obtained are available at GenBank (Table S1). Each fungal taxon was taxonomically classified according to the Index Fungorum Database (www.indexfungorum.org).

3.2.4. Diversity and composition of fungal communities

Occurrence of fungal endophytes and epiphytes was measured by calculating the frequency of colonization (FC, %) and relative abundance (RA, %), respectively. The frequency of colonization was calculated as the total number of olive tissue segments colonized by each endophyte divided by the total number of olive segments surveyed. The relative abundance was determined as the total number of isolates of an epiphytic taxon divided by the total number of isolates of all taxa.

The diversity of fungal endophytes and epiphytes was evaluated at the level of their richness (total number of different taxa, and average number of taxa per tree), their abundance (total number of isolates, and average number of isolates per tree), and also by computing Shannon–Wiener (H) index species diversity, which accounts for both abundance and evenness of the species present (Magurran, 2004). This index was computed in Species Diversity and Richnessv. 4.0 software (Seaby & Henderson, 2006). Species-richness estimation was also performed by using species accumulation

curves, which were calculated using the sample-based rarefaction index (Mao Tau) and computed in EstimateS v. 9.1.0 (Colwell, 2013) using 1000 runs of bootstrapping with replacement. Results of diversity and abundance were presented as the mean of 21 independent experiments displaying the respective SE values or as the total number (the values for all samples lumped together). Differences among the means were determined by an analysis of variance (ANOVA) with SPSS v.21 software, 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 fungal's community among olive tree cultivars (Madural and Verdeal Transmontana). This analysis ranks fungal communities of each cultivar (represented by points) in ordination space in a way that the distance between two points is inversely proportional to their similarity (Kruskal, 1964). NMDS was performed by using Bray-Curtis similarities matrices (Bray & Curtis, 1957).. 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 species composition between-groups (olive tree cultivars) and generates an R-value that gives the degree of discrimination between groups. R-values range from 0 (completely similar) to 1 (completely dissimilar) (Clarke & Gorley, 2001). When a significant difference was observed, similarity percentage analyses (SIMPER) were performed to reveal which taxa contributed to the dissimilarity between olive tree cultivars. All multivariate analyses were done using the *Community Analysis Package*v. 4.0 (Henderson & Seaby, 2007).

3.2.5. Antagonistic tests

The isolated fungal strains were screened for their antagonistic activity against *C. acutatum* by dual culture method. This analysis was performed for seven fungal species: *Chondrostereum purpureum*, *Chaetomium globosum*, *Aspergillus westerdijkiae* and *Aspergillus* sp. 1 obtained from cv. Madural, and *Quambalaria cyanescens*, *Epicoccum nigrum* and *Aspergillus brasiliensis* isolated from cv. Verdeal Transmontana. Strains were selected based on their exclusively occurrence to one of the cultivars, and their capacity to growth on synthetic media after several subcultures. All these fungi were grown in PDA medium, for seven days at 25 ± 2ºC in the dark, in order to provide mycelium/spores for the establishment of dual

cultures. After that, mycelial discs (5 mm diameter) of each tested fungi and *C. acutatum* were removed aseptically from the colony margins and inoculated 3 cm apart on the surface of Petri dishes (9 cm diameter) containing 10 ml of PDA medium. Inoculation of fungi belonging to the genera *Aspergillus* and *Quambalaria*, was performed with 10 µL of a spore suspension (1×10^6 spore/mL, in 0.025% aq. Tween 80), due to their high capacity to sporulate. Controls consisted of PDA plates containing two inocula of the same taxa. Plates were incubated at 25 ± 2ºC in the dark, and each treatment was replicated five times. During interaction, the radial growth towards (internal radius) the interacting fungus and the distance between plant pathogen and tested fungi colonies were measured daily by using a graduated ruler, until the pathogen/fungi had reached the edge of the plate. With the data obtained was calculated the maximum radial growth rate of the pathogen (A, in cm day^{-1}). When the pathogen came into contact with the tested fungi, measurements of radial extension made after this time were used to determine pathogen growth rates in the zone-of-mixed culture (D). The inhibitory activity of each tested fungi was calculated according to the equation devised by Cray et al. (2015): Inhibition coefficient = [(100-B) x 0.4] + [(100-C) x 0.4] + [(100-E) x 0.2], where B is the growth-rate A as a percentage of radial extension of pathogen in control cultures, C is the distance travelled by the pathogen as a percentage of the distance between the sites of inoculation, and E is the growth-rate D as a percentage of radial extension of pathogen in control cultures. According to this equation, [(100- B) x 0.4] represents a potential 40% contribution of distal inhibition (prior to contact) of growth rate of the pathogen; [(100-C) x 0.4] represents a potential 40% contribution of prevention of development of the pathogen colony in the vicinity of the tested fungi; and [(100-E) x 0.2] represents a potential 20% contribution of ability to inhibit pathogen growth rate when in a zone-of-mixed culture (Cray et al., 2015). Significant differences on inhibition coefficient among the fungi tested were verified using one-way ANOVA with a Tukey test at *p*-value <0.05, conducted with SPSS v.21 software.

At the end of the dual culture assays, both sporulation and spore viability of *C. acutatum* in the interaction zone with the tested fungi were evaluated. For the assessment of sporulation, a spore suspension were prepared by vortexing three *C. acutatum* mycelial plugs removed from the interaction zone, in 1 ml of sterile 0.025% (v/v) Tween-80. The concentration of spores in this suspension (spores/mL) was then determined with a Neubauer counting chamber. Germination, as a measure of

viability, was assessed by inoculating 9-cm Petri dishes containing water agar (15g/L agar-agar) with the same spore suspension used to quantify sporulation. After incubation, at 25ºC ± 2°C in the dark for 12-24 hours, the percentage of germination was evaluated microscopically by counting the number of germinated spores. The ability of the tested fungi to reduced *C. acutatum* sporulation and viability was evaluated by computing the percentage of inhibition in relation to control. Percentage inhibition was calculated as difference between control and treatment, divided by the control value and, multiplied by 100.

3.2.6. Macroscopic and microscopic analysis of the dual-culture interactions

The interaction between fungi-phytopathogen was described according to the following categories: (1) contact inhibition, when growth of both species stops at the line of contact (no clear zone is formed); (2) inhibition at distance, when neither species can enter the area inhabited by the other (a clear zone is formed); (3) overgrowth of a mycelium over the other; (4) replacement, where mycelia of one fungus was replaced by its opponent; and (5) mutual replacement, where both fungi gained some of the territory of the other.

Hyphae morphology in the interaction zone was evaluated by light microscopy. Hyphal of the fungal colonies were removed from the interaction zone (bearing growing colonies) and transferred aseptically to sterile slides. The samples were examined under a light microscope (Leica DM 2000). Cultures of *C. acutatum* in PDA without epi-and endophytic fungi inoculation served as control.

3.3. Results

3.3.1. Description of the community

The isolation of fungi from all olive tissues allowed the identification of 27 operational taxonomic units (OTUs), 19 of which were identified up to the species level (Table S2). The ITS sequences of the remaining OTUs were not specified to the species level (7 OTUs) or even to the genus level (1 OTU). The identified species belonged to 17 genera and 14 families, all from the Ascomycota (22 taxa),

Basidiomycota (3 taxa) and Zygomycota (2 taxa) phyla. Species accumulation curves obtained for total endophytes and epiphytes reach an asymptote, suggesting that the sampling effort was sufficient to discover most of the fungal species inhabiting olives of both cultivars (Fig. 3.1).

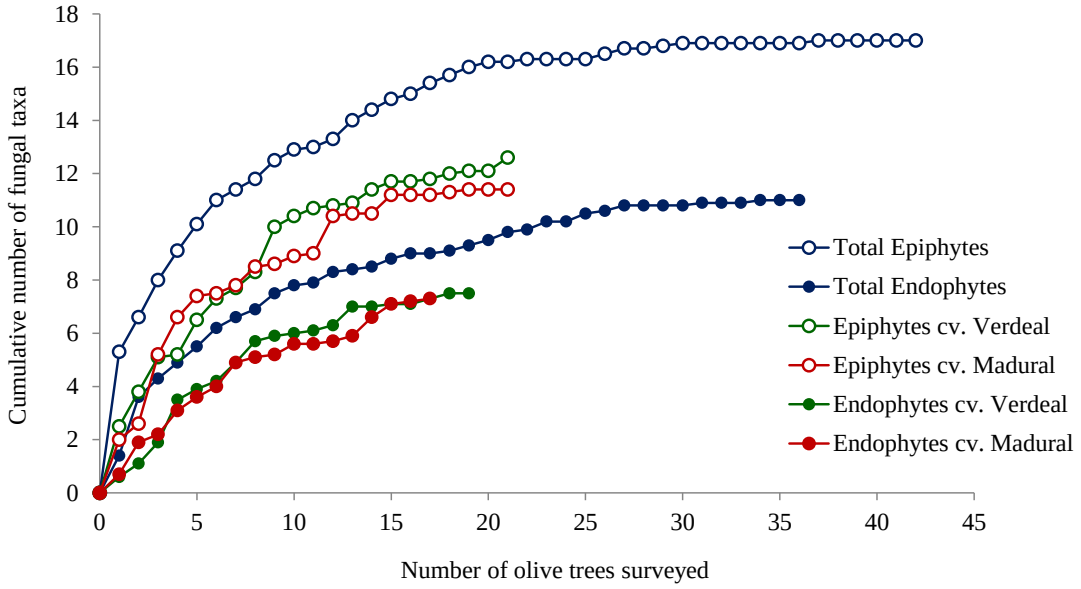


Figure 3.1. Species accumulation curves for all fungal endophytes and epiphytes isolated from olives collected from 42 olive trees (*Olea europaea* L.) of cultivars Verdeal Transmontana and Madural.

With regard to abundance, 1,100 fungal isolates were obtained from all olives surveyed (Table 3.1), with the greatest percentage of the total isolates being from Ascomycota (95.5%) and only 0.5% being from Zygomycota (Table S2). The most representative genera were *Cladosporium* (62.1% of the total isolates), followed by *Biscogniauxia* (8.1%) and *Alternaria* (6.9%) (data not shown). Among the species identified in all analyzed olive tissues, *Cladosporium cucumerinum* (N=659) and *Biscogniauxia mediterranea* (N=89) were the most frequently isolated, collectively representing 68.0% of the total isolates (data not shown). Many species were represented by few isolates (48.1% of the species were represented by only five or fewer isolates).

3.3.2. Comparison of epiphytic and endophytic fungal community

Overall, the diversity of fungal epiphytes was significantly ($p<0.001$) higher comparatively to endophytes, when considering either the total number of taxa (18 *vs.*

10 taxa) or Shannon-Winer diversity index (Table 3.1). Similarly, the overall average number of epiphytic taxa per tree was up to 3.1-fold significantly ($p<0.001$) higher than of endophytes. The average number of isolates per tree was calculated using the average number of all isolates in one tree, and with that average calculate the total average of the 21 trees of each cultivar

Table 3.1. Abundance and diversity of fungal endophytes and epiphytes isolated from olives of the two cultivars Verdeal Transmontana and Madural. Results of H index are present as the mean value ± SD (n=21) and as total number (in brackets). Different superscript lower case letters denote statistically significant differences ($p<0.05$) between cultivars.

	Parameters		Verdeal Transmontana	Madural	Total
Endophytes	Total nº of isolates		63	41	104
	Average nº of isolates per tree		3.00±0.51^{a}	1.95±0.38^{b}	2.48±0.35^{ab}
	Total nº of taxa		7	8	10
	Average nº of taxa per tree		1.62±0.25^{a}	1.29±0.21^{a}	1.45±0.17^{a}
	Frequency of colonization (%)		11.9	7.8	9.9
	Shannon-Wiener index (H)		0.6 ± 0.13^{a} (1.6)	0.4 ± 0.11^{a} (1.7)	0.5 ± 0.12^{a} (1.8)
Epiphytes	Total nº of isolates	(cfu)	503	493	996
		(cfu/cm^2)	8.67	9.97	18.64
	Average nº of isolates per tree	(cfu)	22.3±2.68^{a}	23.1±3.54^{a}	22.7±2.4^{a}
		(cfu/cm^2)	0.41±0.06^{a}	0.45±0.07^{a}	0.44±0.05^{a}
	Total nº of taxa		15	12	18
	Average nº of taxa per tree		5.00±0.42^{a}	3.95±0.30^{b}	4.48±0.24^{ab}
	Shannon-Wiener index (H)		1.1 ± 0.08^{a} (1.5)	0.8 ± 0.07^{b} (1.1)	0.9 ± 0.06^{ab} (1.4)

Epiphytes were also found to be more abundant than endophytes. Overall, either the total or average number of epiphytic isolates per tree was up to 9.1-fold significantly ($p<0.001$) higher than of endophytes (Table 3.1). Numbers of fungal epiphytes of both cultivars were between 0.21 and 0.97 cfu/cm^2 (avg. 0.44 cfu/cm^2). Percentages of olive fruit colonization by fungal endophytes was 9.9% (Table 3.1), when considering the global amount of samples assayed (1,050 segments).

Analysis of similarity (ANOSIM) showed that endophytic and epiphytic fungal communities, found in all olives surveyed, are significantly different in terms of

species compositions (R=0.850, p=0.001). In fact, each of the fungal communities was dominated by different taxa. The most frequently isolated endophytes were members of the genera *Gibberella* and *Neofabraea*, collectively representing 75.0% of the total isolates, whereas within epiphytic community, *Cladosporium*, *Biscogniauxia* and *Alternaria* were the genera most frequently found, accounting for 83.5% of the total epiphytic isolates (Fig. 3.2). According to SIMPER analyses, both *C. cucumerinum* and *B. mediterranea*, contributing more than 58% of the average dissimilarity found among fungal communities. These two species were exclusively found in olive epiphytic samples and were by far the most abundant (Table S2). The high number of exclusive fungal taxa associated to either endophytic (9) or epiphytic (17) fungal community could also account for the differences found. Only one taxa (*Cladosporium cladosporioides*), out of the 27 recovered in this study, was common to both fungal communities (Table S2). Non-metric multidimensional scaling produced a clear separation of the epiphytic and endophytic fungal communities (Fig. 3.3), which corroborates their low similarity in fungal species composition.

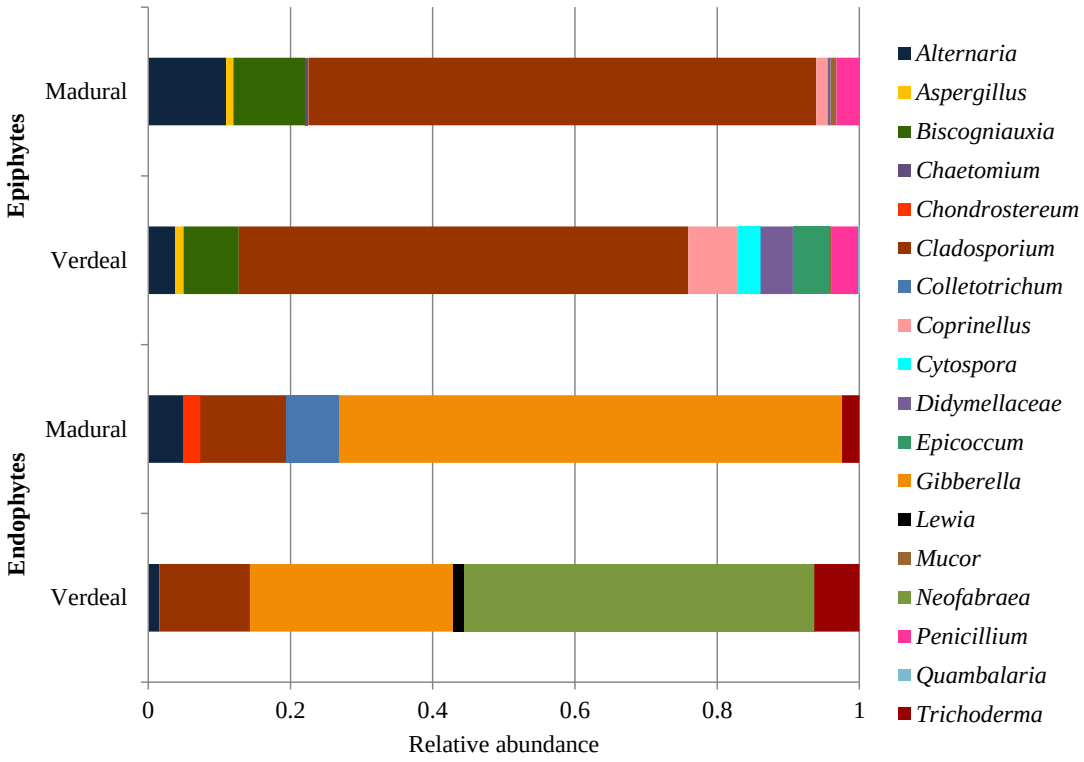


Figure 3.2. Relative abundance of endophytic and epiphytic fungal genera/family associated with olives from cvs. Verdeal Transmontana and Madural.

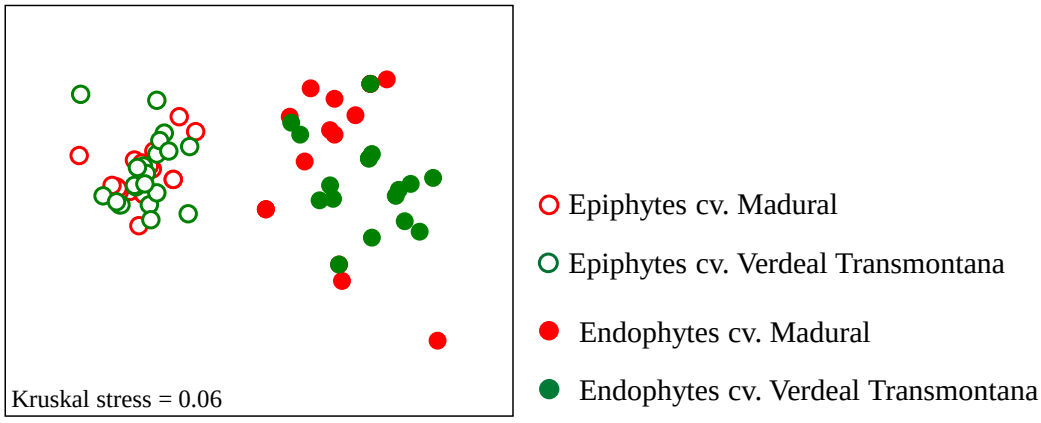


Figure 3.3. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of endophyte and epiphyte communities grouped by olive tree cultivar Madural and Verdeal Transmontana. Cluster analysis was performed with Bray-Curtis coefficient (raw abundance data). Kruskal's stress values less than 0.2 represent good ordination plots.

3.3.3. Comparison of microbial communities between olive tree cultivars

Species diversity differs significantly between olive tree cultivars only within epiphytic fungal community. This result was corroborated by corresponding species diversity index (H). Both the total number of epiphytic taxa as well as the average number of epiphytic taxa per tree was up to 1.3-fold significantly ($p<0.05$) higher on cv. Verdeal Transmontana than on cv. Madural (Table 3.1). Within endophytic fungal community, on the other hand, the species diversity did not differ significantly between olive tree cultivars. The total species number reached an asymptote (for epiphytes of cv. Madural and endophytes of cvs. Verdeal Transmontana and Madural) or was close to it (for epiphytes of cv. Verdeal Transmontana) indicating that the inventory was completed/near-completed, and that sampling has been sufficient to reveal the species present in olives of both cultivars (Fig. 3.1).

In what concerns abundance (expressed as total number of isolates and average number of isolates per tree) only within endophytic community was found significant differences ($p<0.05$) among cultivars: a 1.5-fold increase from cv. Madural to cv. Verdeal Transmontana (Table 3.1). The endophyte infection rates were similarly higher (up to 1.5-fold) in cv. Verdeal Transmontana than in cv Madural. Epiphytic fungal abundance did no differ significantly between olive tree cultivars, being ranging between 0.12 and 1.17 cfu/cm^2, or between 0.24 and 1.50 cfu/cm^2, in the cv. Verdeal Transmontana and cv. Madural, respectively.

The NMDS plot, based on Bray-Curtis index (Fig. 3.3), showed that only endophytic fungal communities composition differ significantly between olive tree cultivars (ANOSIM R=0.467, p=0.001), whereas epiphytic fungal communities composition of olives from cv. Madural was very similar to cv. Verdeal Transmontana (ANOSIM R=0.085, p=0.014). The differences in endophytic fungal communities were predominantly caused by the higher abundance of *Gibberella* genus (in particular of *Gibberella baccata*) in cv. Madural and the exclusive occurrence of *Neofabraea vagabunda* with high relative abundance in cv. Verdeal Transmontana (Fig. 3.2). Although epiphytic community inabithing olives of both cultivars overlapped, several fungal genera preferred either olives from cv. Madural (*e.g. Chaetomium*) or from cv. Verdeal Transmontana (*e.g. Cytospora*, *Epicoccum* and *Quambalaria*) (Fig. 3.2).

3.3.4. Interaction between C. acutatum *and fungal isolates*

Among the 27 OTUs obtained, four taxa from cv. Madural (*i.e. C. purpureum*, *C. globosum*, *A. westerdijkiae* and *Aspergillus* sp. 1) and three taxa from cv. Verdeal Transmontana (*i.e. Q. cyanescens, E. nigrum* and *A. brasiliensis*) were analysed to identify isolates with the greatest antagonistic efect against *C. acutatum*, by using dual-culture method. Antagonistic potency of fungal isolates was assessed by calculating an inhibition coefficient and additionally by evaluating fungi effect on both sporulation and germination of *C. acutatum*. This coefficient incorporated the potential contributions of distal inhibition of pathogen growth-rate; prevention of mycelium development in the vicinity of the tested fungi; and ability to inhibit pathogen growth-rate in the zone of mixed culture (in a ratio of 2:2:1). All the fungal tested were able to inhibited *C. acutatum* growth, with inhibition coefficients ranging from 30.9 for *C. purpureum* to 71.3 for *C. globosum*, out of a theoretical maximum value of 100 (Fig. 3.4). Inhibition displayed by the majority of the fungi was promoted mostly in the vicinity of *C. acutatum* colonies, as evidenced by the high contribution of this parameter for the calculated inhibition coefficient values. This was particularly noticed for *A. brasiliensis*, *Aspergillus* sp. 1 and *A. westerdijkiae*. Inhibition at distance and after contact between colonies had lower contribution. Among the tested fungi, *C. globosum* was the most potent inhibitor at a distance,

whereas *Q. cyanescens* was the most potent inhibitor following contact with pathogen.

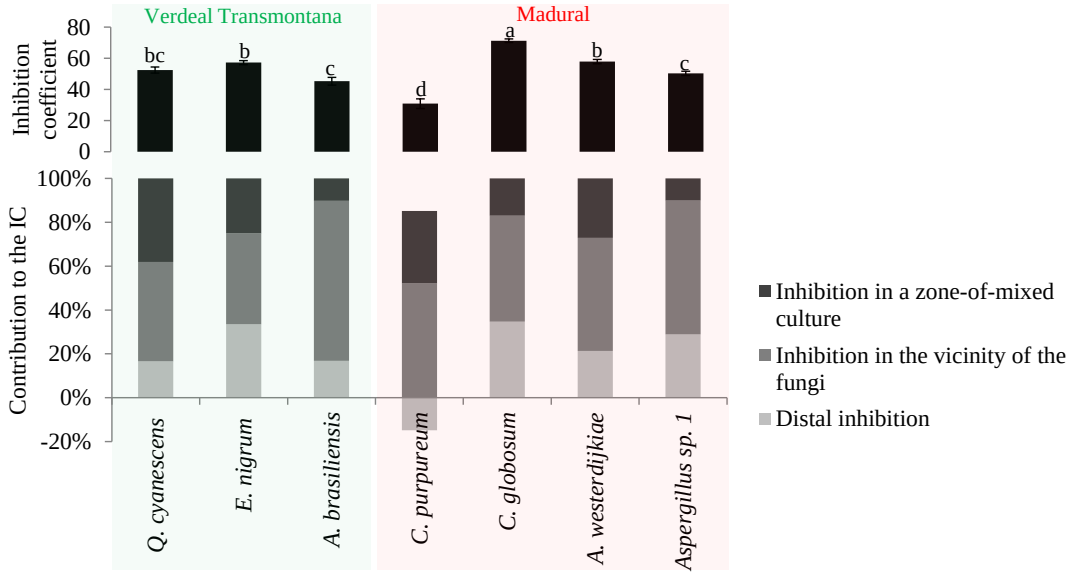


Figure 3.4. Inhibition coefficients (IC) of fungal isolates obtained from cvs. Verdeal Transmontana and Madural against *Colletotrichum acutatum* in dual culture assay and percentage contribution of each parameter to the isolate´s inhibitory efficacy. IC values are expressed as mean ± SE (n = 5). Negative values indicate that colony development of the pathogen was promoted. Bars with different lowercase letters indicate significant differences ($p<0.05$).

Macroscopic examination of pairings showed that interactions can result in inhibition at mycelial contact, replacement and overgrowth of *C. acutatum* by endo- or epiphytic fungi (Fig. 3.5). Changes on mycelium pigmentation of *C. acutatum* also occurred during interspecific mycelial interactions, an effect that was not observed in the control. Margins of *C. acutatum* colonies become yellow or brown pigmented in the contact zone with *E. nigrum*, *A. brasiliensis*, *C. purpureum*, *C. globosum* and *Aspergillus* sp.1, which was best observed on the reverse side of the colony (Fig. 3.5).

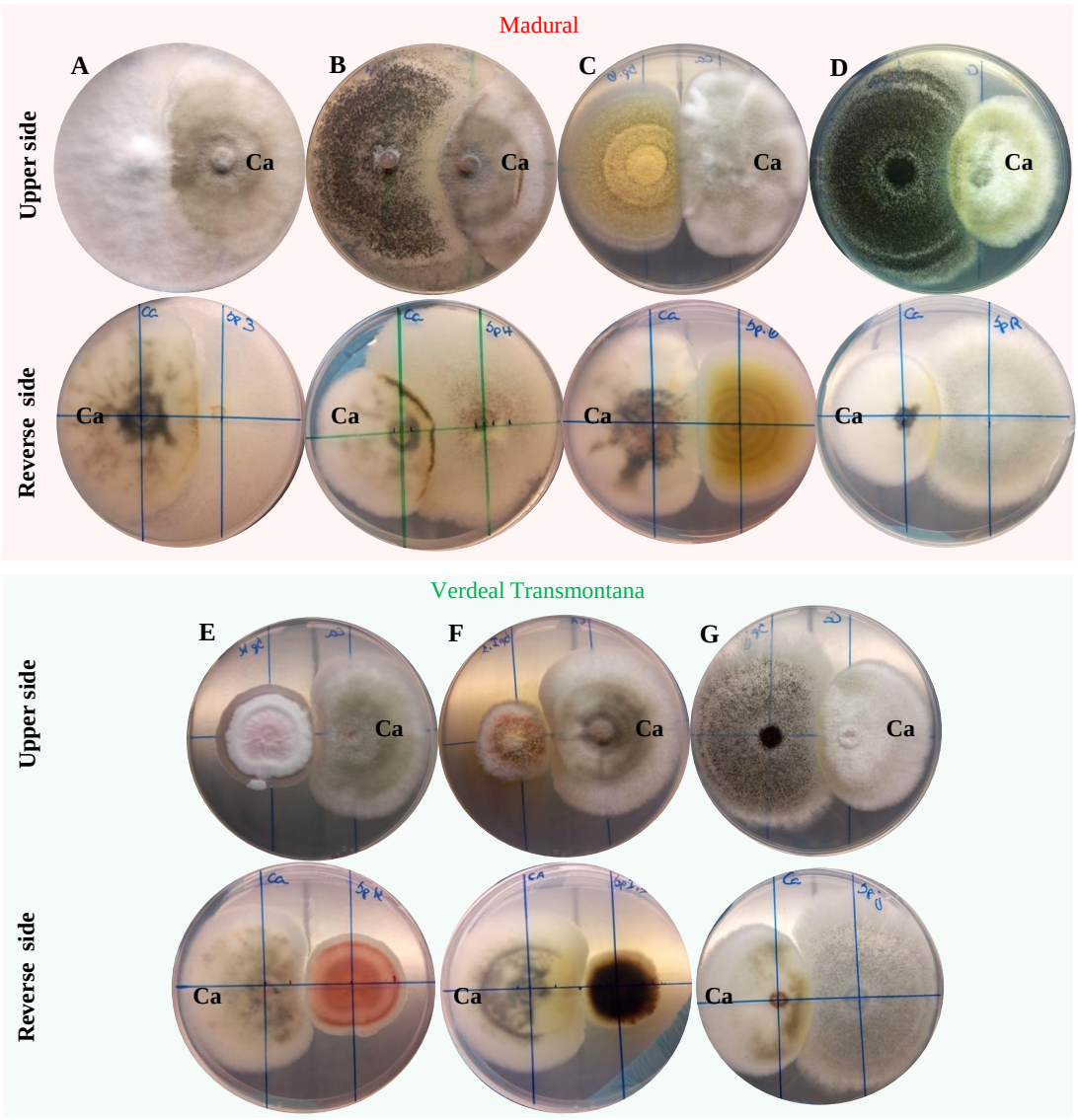


Figure 3.5. Interactions on potato dextrose agar between *Colletotrichum acutatum* (Ca) and fungal isolates obtained from cvs. Verdeal Transmontana and Madural, on the sixth day. In the **upper sides** of the dual-cultures (Ca, right of Petri dish; and fungal isolates, left of Petri dish) was noticed overgrowth of *C. acutatum* by *C. purpureum* (A) or *Aspergillus* sp.1 (D), replacement of *C. acutatum* by *A. brasiliensis* (G) or *C. globosum* (B), and inhibited the growth of *C. acutatum* through contact (contact inhibition) were *A. westerdijkiae* (C) and *Q. cyanescens* (E). In the **reverse sides** (Ca, left of Petri dish; and fungal isolates, right of Petri dish) margins of *C. acutatum* colonies become yellow or brown pigmented in the contact zone with *C. purpureum* (A), *C. globosum* (B), *Aspergillus* sp.1. (D), *E. nigrum* (F) and *A. brasiliensis* (G).

Observations made with a light microscope showed alterations on *C. acutatum* hyphae morphology in the interacting zones of all pairings (Fig. 3.6). In contrast to control (Fig. 3.6E), hyphae of *C. acutatum* become swollen and distended in co-culture with *C. globosum* (Fig. 3.6C) or collapsed and necrosed in dual cultures with *A. brasiliensis*, *C. purpureum*, *Aspergillus* sp.1 and *Q. cyanescens* (Fig. 3.6A and B).

Collapse and twisting of *C. acutatum* hyphae were also the most common alterations observed in the vicinity of *A. westerdijkiae* and *E. nigrum* (Fig. 3.6D).

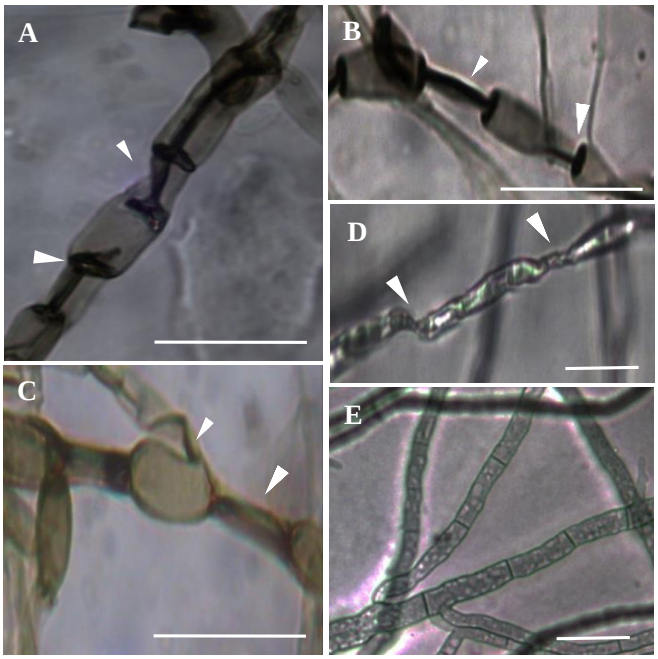


Figure 3.6. Hyphal morphology of *Colletotrichum acutatum* in dual-culture with fungal isolates obtained from cvs. Verdeal Transmontana and Madural. Collapse (small arrow) and necrosis (large arrow) on hyphae in the dual culture with *C. purpureum* (A) or *Aspergillus* sp.1 (B); Swilling (small arrow) and distention (large arrow) on hyphae in the dual culture with *C. globosum* (C); Collapse and twisting of hyphae in the dual culture with *A. westerdijkiae* (D); Normal hyphae (E) in control plates. Bar = 15 µm.

Effect of fungi isolates on *C. acutatum* sporulation and germination was also different depending on the species (Fig. 3.7). Three, out of the seven fungi tested (*i.e. Q. cyanescens*, *E. nigrum* and *Aspergillus* sp. 1) reduced significantly ($p<0.05$) the sporulation of *C. acutatum* by 46-86% in comparison to control. In contrast, both *C. globosum* and *A. westerdijkiae* promoted significantly ($p<0.05$) the sporulation of *C. acutatum* (up to 135% when compared to control). All the tested fungi, with exception of *E. nigrum* and *C. purpureum*, reduced significantly ($p<0.05$) the conidial germination of *C. acutatum* by more than 21% in relation to the control, being the highest inhibition displayed by *Q. cyanescens* (74%).

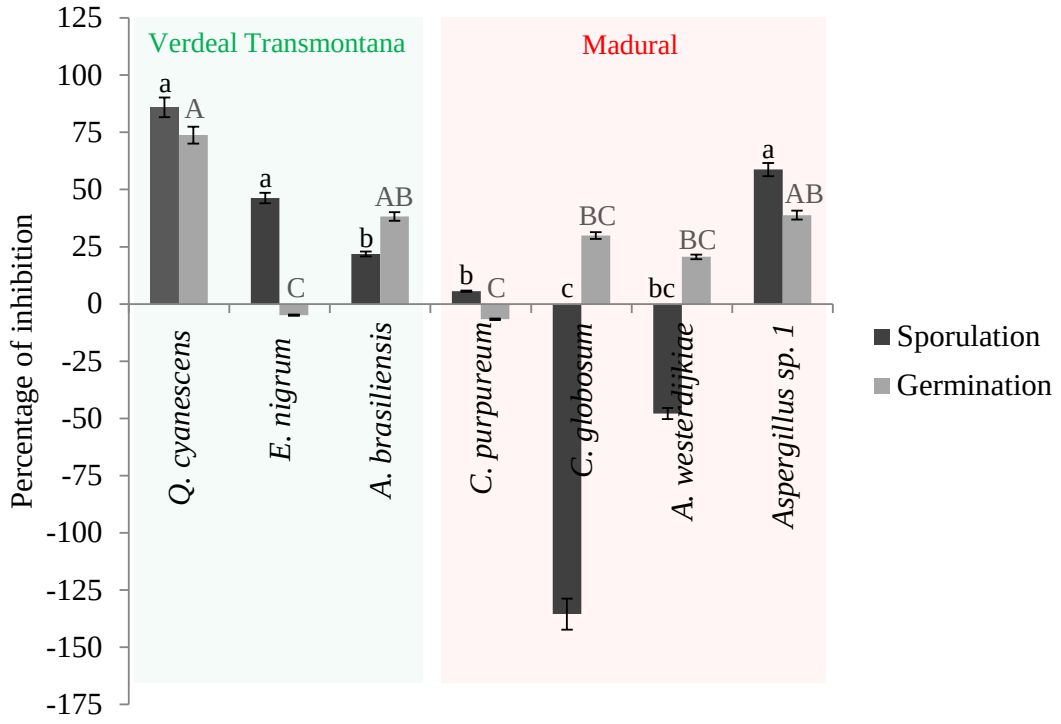


Figure 3.7. Percentage inhibition of sporulation and conidial germination of *Colletotrichum acutatum* in dual-culture with fungi isolated from cvs. Verdeal Transmontana and Madural. Negative values indicate that sporulation/germination of the pathogen was promoted. Each value is expressed as mean ± SE (n=5). Bars with different lowercase (sporulation) and uppercase (germination) letters indicate significant differences ($p<0.05$).

3.4. Discussion

Most studies on phyllosphere microorganisms have focused only on endophytic or epiphytic group, and have been restricted mostly to leaves (Lindow & Brandl, 2003). In the present work, the diversity of fungi inhabiting the surface and the inner tissues of olives from two different Portuguese cultivars (*i.e.* Verdeal Transmontana and Madural) was evaluated for the first time. Following molecular identification, it was possible to identify a total of 27 OTUs, belonging to 17 genera and 14 families, from Ascomycota, Basidiomycota and Zygomycota phyla. *Cladosporium*, *Biscogniauxia* and *Alternaria* were the most abundantly detected genus, accounting for more than 77% of the total isolates. Most fungal taxa identified belong to genera comprising relevant pathogens of other plant species (*e.g. Alternaria*, *Gibberella*, *Lewia*, *Biscogniauxia* and *Cytospora*), but also of olive tree. For instances, *Neofabraea vagabunda*, the causal agent of leprosy of olive (Romero et al., 2016), was detected in high abundance in olives from cv. Verdeal Transmontana as endophyte. The abundant detection of *N. vagabunda* on asymptomatic fruits was quite surprisingly, suggesting a conspicuous colonization of olive tissues by this fungus before the appearance of symptoms. At lower abundance, other olive fungal pathogens represented by *Colletotrichum* spp.., a genus that contains different species associated with olive anthracnose (Schena et al., 2014), were also detected as endophyte in olives from cv. Madural. In this work was additionally found a number of nonpathogenic taxa, being some of them described in the literature as presenting the useful features of antagonism against phytopathogens, such as *Chaetomium globosum* (Awad et al., 2014), *Epicoccum nigrum* (Fávaro et al., 2012), *Penicillium brevicompactum* (Jackson et al., 1997) and *Trichoderma koningii* (Nikolajeva et al., 2012; Reddy et al., 2014), and/or entomopathogenecity (*e.g. Aspergillus westerdijkiae*; Baggio et al., 2016). There is very limited information on isolation and characterization of epiphytes and endophytes from fresh olives. As far as we known, only Abdelfattah et al. (2015) have recently evaluated the diversity of fungi associated to olive fruits of the Italian cultivar "Ottobratica" through metabarcoding. Although the difficult in comparing our results with this study, in which fungal diversity was assessed using culture-independent technique, both studies found that olive fruits were numerically dominated by Ascomycota and comprised a number of taxa belonging to the genera *Cladosporium*, *Alternaria* and *Colletotrichum*. Despite this,

the degree of fungal genera shared between the present and previous work was minimal. This suggests that, besides methodologic aspects, other factors may affect fungal community structure in olive fruits.

Overall, the total species number and fungal isolates was greater on surface than in the interior of olive of both cultivars (up to 1.8- and 9.1-fold higher, respectively). Very few studies have compared endophytic and epiphytic fungi, especially in fresh fruits. Comparison of epiphytic and endophytic fungal communities on single leaves of deciduous shrubs (Osono, 2007) or of woody plants, such as *Coffea arabica* (Santamariá & Bayman, 2005), *Camellia japonica* (Osono, 2008), *Alnus nepalensis*, *Castanopsis hystrix* and *Schima walichii* (Kayini & Pandey, 2010), supporting our findings. The higher number of epiphytes recovered suggests that the external environment suits the fungal population colonizing the surface of olive fruit, despite the harsh environmental conditions that typify this habitat (*e.g.* harmful UV light, high temperatures, low humidity, osmotic stress, and scarcity of nutrients) (Rastogi et al., 2013); whereas internal microenvironment of olive fruits appears to restricts the fungal population to a certain extent, probably due to the presence of antimicrobials in their tissues (*e.g*. phenolic compounds) and/or host plant defense reactions (Rastogi et al., 2013). In fact, several phenolic compounds present in olives, such as tannic acid, quercetin 3-methyl ether and gallic acid (Vinha et al., 2005), have been described to exhibited antimicrobial proprieties (Lacey & Mercadier, 1998; Parvez et al., 2004; Ahn et al., 2005). Similarly, the composition of fungal communities inhabiting the surface of olives was completed different from that found in internal olive tissues, a pattern which has also been noted in previous studies performed in leaves (Santamariá & Bayman, 2005; Osono, 2007, 2008). Only *Cladosporium cladosporioides*, out of the 27 fungal taxa recovered in this study, was common to both fungal floras. The most abundant endophytes were the phytopathogens *Gibberella baccata* and *Neofabraea vagabunda*. The former specie, which is associated with dieback, cankers and bud rots of woody trees and shrubs (Chen et al., 2016), has also been commonly encountered as endophytes of other woody plants, such as *Taxus* spp. (Xiong et al., 2013). The most abundant epiphytes were *Cladosporium cucumerinum* and *Biscogniauxia mediterranea*, both plant-pathogens that cause scab of cucurbits on cucumbers and charcoal canker in particular on the genus *Quercus*, respectively.

The diversity of epiphytic fungi in olives surface was found to be different in the two cultivars (up to 1.3-fold higher on cv. Verdeal Transmontana than on cv.

Madural), but their communities composition was not. In the three olive orchards surveyed, trees of the two cultivars are relatively close to each other, and thus exposed to the same aerial inoculum, which could account for the high similarity found in the epiphytic fungal communities between cultivars. Despite this high similarity, we have noticed that several epiphytes preferred either olives from cv. Madural (*Chaetomium* spp.) or from cv. Verdeal Transmontana (*Cytospora* spp., *Epicoccum* spp. and *Quambalaria* spp.). Thus, being exposed to the same fungal inoculum, olive tree cultivar seems also to shape the detected epiphytic fungal communities. A similar finding was reported for fungi colonizing the leaf surfaces of different cultivars of the same woody plant species (Kembel & Mueller, 2014) or berries of several grapevine varieties (Bokulich et al., 2013). In contrast, the composition of endophytic fungal communities harbored by olives was found to be different between cultivars, as reported in other studies (Gazzis & Chaverri., 2010). The exclusive presence of *N. vagabunda* with high relative abundance in cv. Verdeal Transmontana was the major contributor for the differences found in the endophytic fungal communities among cultivars. As mentioned earlier, the cultivars Verdeal Transmontana and Madural, have different susceptibilities towards diseases (in particular to olive anthracnose), which might be an important factor in shaping not only phytopathogen (e.g. *N. vagabunda* and *Colletotrichum* spp., were virtualy exclusively present in cv. Verdeal Transmontana and Madural, respectively), but also entire fungal communities. However, no clear correlation was found between known disease resistances in two cultivars and the endophytic fungal communities (data not shown). Similarly, Pan et al. (2008) found that resistance towards *Uromyces maydis* in genetically distinct maize recombinant inbred lines did not correlate with endophyte communities. Phenotypic characteristics exhibited by the cultivars may also influence fungal community composition of olives (Whipps et al., 2008). Overall, the results suggest that host genotype at cultivar level plays a somewhat more significant role in defining endophyte than epiphyte communities.

Dual-culture experiments showed that all the seven fungal tested were able to reduced *C. acutatum* growth, being the most potent inhibitors the epiphytes *C. globosum* (CI=71.3), *A. westerdijkiae* (CI=57.9) and *E. nigrum* (CI=57.2). Previous studies have also reported the potential of *C. globosum* and *E. nigrum* to reduce the growth of several phytopathogenic fungi (*e.g*. Larena et al., 2005; Lahlali & Hijri, 2010; Fávaro et al., 2012; Aggarwal, 2015), however, this is the first work showing an

effective antagonist activity of these two species against *C. acutatum*. As far as we known, previous works reported only the capacity of *E. nigrum* to inhibited other species belonging to *Colletotrichum* genus, such as C. *gloeosporioides* (Pandey et al., 1993), *C. kahawae* (Guerra-Guimarães et al., 2007) and *C. falcatum* (Fávaro et al., 2012). Similarly, studies examining antagonistic effect of *A. westerdijkiae* against phytopathogens have never been performed, being only tested its entomopathogenic properties against *Periplaneta americana* (Baggio et al., 2016).

In this work the suppression of *C. acutatum* growth by fungal isolates was mediated mostly at distance and/or in its vicinity, and in less extended following contact. Although initially all fungi formed an inhibition zone, they continued to grow at a higher rate than the *C. acutatum* leading a direct contact between colonies. Therefore, the investigated fungi might probably suppress *C. acutatum* growth either through general inhibition (*e.g.* involvement of inhibitory substances) and competition for space (due to their advantage of rapid growth).

Most of the fungal isolates (five, out of seven) also strongly reduced *C. acutatum* germination, and all of them induced morphological abnormalities in *C. acutatum* hyphae, highlighting the antagonist effectiveness of the isolates investigated. The observed effects were presumably the result of the lytic action of enzymes or inhibitory compounds, secreted by the antagonistic isolates, which seems to act synergistically. Some possible mechanisms behind antagonist activity of biocontrol agents included production of antibiotics, siderophores and a variety of enzymes, to supress phytopathogens (Peng & Ding, 2015; Spadaro & Droby, 2016). Therefore, it is likely that such bioactive compounds may act on *C. acutatum*. In fact, *C. globosum* has been known to produce many bioactive compounds, such as chaetoglobosins, chaetomin and chaetoviridins, that have revealed the capacity to inhibit colony growth of several phytopathogenic fungi (Biswas et al., 2012; Aggarwal, 2015). Similarly, biocontrol activity of *E. nigrum* against several phytopathogens has been associated with the production of antimicrobial compounds, such as flavipin, which prevents the germination of conidia (Madrigal et al., 1991; 1995). In our work, the antagonism displayed by *A. westerdijkiae* might be related to the release of toxic metabolite (*e.g*. ochratoxin A) by this fungus (Han et al., 2016). Both *C. globosum* (Aggarwal, 2015) and *E. nigrum* (Fávaro et al., 2012) are known to produce lytic enzymes (*e.g*. xylanase and β 1,3-glucanase).

During interaction the mycelium of *C. acutatum* become yellow/brown pigmented in the contact zone with *E. nigrum*, *A. brasiliensis*, *C. purpureum*, *C. globosum* and *Aspergillus* sp.1. Mycelium pigmentation may indicate the formation of melanin and melanin-like compounds. Many fungi produce melanins to protect cells against environmental factors such as visible and ultraviolet light, lytic enzymes, toxic metals and antagonistic microorganisms (Butler et al., 2009). Therefore the formation of pigments in *C. acutatum* mycelium may be a mechanism of the pathogen to protect hyphae from the antagonistic fungi by preventing access by cell wall degrading enzymes, a finding also reported by others (Larran et al., 2016; Begum et al., 2008). The lack of significant correlation between the sources of investigated fungal isolates (*i.e.* olive tree cultivars) and antagonist effectiveness (data not shown), suggested that the antagonistic activity of the isolates exhibited under *in vitro* conditions depended much more on the fungi than on their provenances.

In summary, host plant genotype at cultivar level seems to play a significant role in the selection of fungal communities associated to their fruits, particularly in inner tissues of olives. This selection might have important implications for olive tree health and growth since phyllosphere microorganisms are known to have a variety of positive or negative effects (Heijden & Hartmann, 2016). Olives of both cultivars harbour distinct fungal communities containing pathogenic (e.g. *Colletotrichum* spp.., *N. vagabunda*), and potentially antagonistics. In fact, all the seven fungal tested in our work exhibited antagonistic activity against *C. acutatum* by inducing inhibition of germination, lysis of fungal mycelia, or by exerting fungicidal/fungistatic effects. Further investigations are required to better ascertain the role of these fungi under natural conditions, and to assess their potential in the biocontrol of *C. acutatum*. The studies detailed here indicate that *Q. cyanescens*, *E. nigrum* and *C. globosum* holds great promise for this purpose.

References

Abdelfattah A., Li Destri Nicosia M.G., Cacciola S.O., Droby S., Schena L., 2015. Metabarcoding Analysis of Fungal Diversity in the Phyllosphere and Carposphere of Olive (Olea europaea). PLoS ONE 10(7): e0131069

Abdelfattah A., Nicosia M.G.L.D., Cacciola S.O., Droby S., Schena L.,2015 Metabarcoding Analysis of Fungal Diversity in the Phyllosphere and Carposphere of Olive (*Olea europaea*). PLoS ONE 10(7): e0131069

Aggarwal R., 2015. *Chaetomium globosum*: A potential biocontrol agent and its mechanism of action. Indian Phytopath, 68(1): 8-24

Agler T.A., Ruhe J., Kroll S., Morhenn C., Kim S.T., Weigel D., 2016. Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLoS Biology, 14(1): 1-31

Ahn Y., Lee H., Oh H., Kim H., Lee Y., 2005. Antifungal activity and mode of action of Galla rhois-derived phenolics against phytopathogenic fungi. Pestic Biochem Physiol, 81: 105–112

Awad N.E., Kassem H.A., Hamed M.A., El-Naggar M.A.A., El-Feky A.M.M., 2014. Bioassays guided isolation of compounds from *Chaetomium globosum* Isolation des composés de *Chaetomium globosum* basée sur des tests biologiques. Journal de Mycologie Médicale/Journal of Medical Mycology, 24(2): e35-e42

Baggio M. V., Ferreira M. C., Monteiro A. C., Junior W. M., Lemos M. V. F., 2016. Pathogenicity of *Aspergillus westerdijkiae* to females and oothecae of *Periplaneta Americana*. Ciência Rural, 46(1): 20-25

Begum M. M., Sariah M., Zainal Abidin M. A., Puteh A. B., Rahman M. A., 2008. Antagonistic Potential of Selected Fungal and Bacterial Biocontrol Agents against *Colletotrichum truncatum* of Soybean Seeds. Pertanika Journal of Tropical Agricultural Sience, 31(1): 45-53

Biswas S. K., Aggarwal R., Srivastava K. D., Gupta S., Dureja P., 2012. Characterization of antifungal metabolites of C*haetomium globosum* Kunze and their antagonism against fungal plant pathogens. Journal of Biological Control 26(1): 70–74

Bokulich N., Thorngate J.H., Richardson P.M., Mills D.A., 2013. Microbiol biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proceedings of National Academy of Sciences, 111(1): e139-e148

Bray, J.R., and Curtis, J.T., 1957. An ordination of the upland forest communities of Southern Wisconsin. Ecological Monographies. 27:325-349

Butler M. J., Gardiner R. B., Day A. W., 2009. Melanin synthesis by *Sclerotinia sclerotiorum*. Mycologia, 101(3): 296-304

Cacciola S.O., Faedda R., Sinatra F., Agosteo G.E., Schena L., Frisullo S., Magnano di San Lio G., 2012. Olive Anthracnose. Journal of Plant Pathology. 94: 29-44

Carvalho M.T., Simões-Lopes P., Monteiro da Silva M.J., 2008. Influence of Different Olive Infection Rates of *Colletotrichum acutatum* on Some Important Olive Oil Chemical Parameters. Acta Horticulturae, 791: 555-558

Chen C., Gerard J. M. V., Sun G., Groenewald J. Z., Crous P.W., 2016. Redefining common endophytes and plant pathogens in Neofabraea, Pezicula, and related genera. *Integrative Taxonomy - Uncovering Fungal Diversity*, Fungal Biology, 120(11):1291-1322

Clarke, K.R. and Gorley, R.N., 2001. Primer v5: user manual/tutorial. PRIMER-E, Plymouth

Colwell, R.K., 2013. EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. Version 9. User's Guide and Application

Cray, J. A., Houghton, J.D.R., Cooke, L.R., Hallsworth J.E., 2015. A simple inhibition coeficiente for quantifying potency of biocontrol agentes against plant-pathogenic fungi. Biological Control 81: 93-100

Fávaro L. C. L., Sebastianes F. L. S., Araújo W. L., 2012. Epicoccum nigrum P16, a Sugarcane Endophyte, Produces Antifungal Compounds and Induces Root Growth. PLoS One, 7(6): e36826

Gazis, R., Chaverri, P., 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecology 3: 240- 254

Guerra-Guimarães L., Azinheira H. G., Martins A. C., Silva M. C., Gichuru E. K., Varzea V., Bertrand B., 2007. Antagonistic interaction between *Epicoccum nigrum* and *Colletotrichum kahawae*, the Causal Agent of Coffee Berry Disease. 21st International Conference on Coffee Science: 1284- 1290

Han X., Chakrabortti A., Zhu J., Liang Z. X., Li J., 2016. Sequencing and functional annotation of the whole genome of the filamentous fungus *Aspergillus westerdijkiae*. BMC Genomics 17: 633

Heijden, M.G.A., Hartmann, M., 2016. Networking in the Plant Microbiome. PLOS Biology 14(2): e1002378

Henderson, P.A. and Seaby, R.M.H., 2007. Community Analysis Package 4.0 Pisces Conservation Ltd, Lymington, UK

Hermoso, M., Uceda, M., García, A., Morales, B., Frías, M., and Fernández, A., 1991 Elaboracion de aceite de calidad. Consejeria de Agricultura y Pesca, Serie Apuntes, 5/92, Sevilla

Iannotta N., Perri E., Sirianni R., Tocci C., 1999. Influence of *Colletotrichum gloeosporioides* and *Camarosporum dalmatica* attacks on olive oil quality. Acta Horticulturae, 474: 573–576

Jackson A.J., Walters D.R., Marshall G., 1997. Antagonistic interactions between the foliar pathogen *Botrytis fabae* and isolates of *Penicillium brevicompactum* and *Cladosporium cladosporioides* on faba beans. Biological control: theory and applications in pest management 8(2): 97-106

Kayini A., and Pandey, R.R., 2010. Phyllosphere Fungi of Alnus nepalensis, Castanopsis hystrix and Schima walichii in a Subtropical Forest of North East India. Journal of American Science, 6(3): 118-124

Kembel S. W., Mueller R.C., 2014. Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities. Botany, 92(4): 303-311

Kruskal, J., 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika. 29: 1–27

Lacey L., Mercadier G., 1998. The effect of selected allelochemicals on germination of conidia and blastospores and mycelial growth of the entomopathogenic fungus, *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes). Mycopathologia, 142: 17–25

Lahlali R., Hijri M., 2010. Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. FEMS Microbiology Letters, 311: 152-159

Larena I, Torres R, De Cal A, Linan M, Melgarejo P, et al. (2005) Biological control of postharvest brown rot (*Monilinia* spp.) of peaches by field applications of *Epicoccum nigrum*. Biological Control 32: 305–310

Larran S., Simón M. R., Moreno M. V., Santamarina Siurana M. P., Perelló A., 2016. Endophytes from wheat as biocontrol agents against tan spot disease. Biological Control, 92: 17-23

Lindow S.L., Brandl M.T., 2003. Microbiology of the Phyllosphere. Applied and Environmental Microbiology. 69(4): 1875–1883

Madrigal C., Melgarejo P., 1995. Morphological effects of *Epiccocum nigrum* and its antibiotic flavipin on *Monilinia laxa*. Canadian Journal of Botany 73: 425–431

Madrigal C., Tadeo J. L., Melgarejo P., 1991. Relationship between flavipin production by *Epicoccum nigrum* and antagonism against *Monilinia laxa*. Mycological Research 95: 1375–1381

Moral J., Xaviér C., Roca L.F., Romero J., Moreda W., Trapero A. 2014. La Antracnosis del olivo y su efecto en la calidad del aceite. Grasas y Aceites, 65(2): e028

Nikolajeva V**.,** Petrina Z., Vulfa L., Alksne L., Eze D., Grantina L., Gaitnieks T., Lielpetere A., 2012. Growth and Antagonism of *Trichoderma* spp. and Conifer Pathogen *Heterobasidion annosum* s.l. *in Vitro* at Different Temperatures. Advances in Microbiology, 2: 295-302

Oliveira, I., Pereira, J.A., Lino-Neto, T., Bento, A., and Baptista, P., 2012. Fungal diversity associated to the olive moth, *Prays oleae* Bernard: a survey for potential entomopathogenic fungi. Microbial Ecology. 63: 964-974

Osono T., 2008. Endophytric and epiphytic phyllosphere fungi of *Camellia japonica*: seasonal and leaf age-dependent variations. Mycologia, 100(3): 387-391

Osono, T., 2007. Ecology of ligninolytic fungi associated with leaf litter decomposition. Ecological Research 22 (6): 955- 974

Pan J.J., Baumgarten A.M., May G., 2008. Effects of host plant environment and *Ustilago maydis* infection on the fungal endophyte community of mayze (*Zea mays*). New Phytologist, 178: 147-156

Pandey R. R., Arora D. K. Bubey R. C., 1993. Antagonistic interactions between fungal pathogens and phylloplane fungi of guava. Mycopathologia, 124:31–39

Parvez M., Tomita-Yokotani K., Fujii Y., Konishi T., Iwashina T., 2004. Effects of quercetin and its seven derivatives on the growth of *Arabidopsis thaliana* and *Neurospora crassa*. Biochemical Systematics Ecology, 32: 631–635

Peng W., Ding F., 2015. Biomolecular recognition of antagonists by $\alpha 7$ nicotinic acetylcholine receptor: Antagonistic mechanism and structure–activity relationships studies. European Journal of Pharmaceutical Sciences, 76: 119-132

Rastogi G.,Coaker G.L., Leveau J.H.J., 2013. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiology Letters 348(1): 1-10

Reddy B. N., Saritha K. V., Hindumathi A., 2014. *In vitro* Screening for Antagonistic Potential of Seven Species of *Trichoderma* against Differente Plant Pathogenic Fungi. Research Journal of Biology, 2: 29-36

Romero J., Raya M. C., Roca L. F., Moral J., Trapero A., 2016. First Report of *Neofabraea vagabunda* Causing Branch Cankers on Olives in Spain. Plant Desease 100: 2-527

Santamariá J., Bayman P., 2005. Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). Microbial Ecology, 50: 1–8

Schena L., Mosca S., Cacciola S.O., Faedda R., Sanzani S.M., Agosteo G.E., Sergeeva V., Magnano di San Lio G., 2014. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose. Plant Pathology. 63: 437–446

Seaby, R.M., and Henderson, P.A., 2006. Species Diversity and Richness Version 4. Lymington, UK: Pisces Conservation

Soares M.E., Pereira J.A., Bastos M.L. 2006. Validation of a method to quantify copper and other metals in olive fruits by ETAAS. Application to the residual metal control after olive tree treatments with different copper formulations. Journal of Agricultural and Food Chemistry, 54: 3923-3928

Spadaro D., Droby S., 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends in Food Science & Technology, 47: 39-49

Talhinhas P., Mota-Capitão C., Martins S., Ramos A.P., Neves-Martins J., Guerra-Guimarães L., Várzea V., Silva M.C., Sreenivasaprasad S., Oliveira H., 2011. Epidemiology, histopathology and aetiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. Plant Pathology, 60: 483–495

Talhinhas P., Neves-Martins J., Oliveira H., Sreenivasaprasad S., 2009. The distinctive population structure of *Colletotrichum* species associated with olive anthracnose in the Algarve region of Portugal reflects a host–pathogen diversity hot spot. FEMS Microbiology Letters. 296: 31–38

Talhinhas P., Sreenivasaprasad S., Neves-Martins J., Oliveira H., 2005. Molecular and phenotypic analyses reveal the association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. Applied and Environmental Microbiology. 71: 2987–2998.

Vinha A., Ferreres F., Silva B., Valentão P., Gonçalves A., Pereira J., Oliveira M., Sebra R., Andrade P., 2005. Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): Influences of cultivar and geographical origin. Food Chem, 89: 561–568

Weisstein, E.W. (2013). "Prolate Spheroid." From MathWorld - A Wolfram Web Resource. Available at: http://mathworld.wolfram.com/ProlateSpheroid.html [accessed on june 14, 2016]

Whipps J.M., Hand P., Pink D., Bending G.D., 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. Journal of applied microbiology, 105(6): 1744-1755

White, J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Shinsky, J., and White, T. J. (ed) PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, pp. 315-322.
wild rubber trees (Hevea brasiliensis) in Peru. Fungal Ecology 3: 240- 254

Xiong Z.Q., Yang Y.Y., Zhao N., Wang Y., 2013. Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglojap yew, *Taxus x media*. BMC Microbiology, 13(1): 1-10

Supporting information

Table S1 – Fungal taxa isolated in this study and the summary of BLAST results, showing the coverage of the sequences and sequence similarities with the most closely related organisms.

Isolated (GenBank Acc. no. of the ITS sequence)	Query coverage	Sequence similarity	E-value*	Organism with the highest sequence identity, GenBank Acc. no.
Alternaria alternata KY001590	100	99	0.0	KX179489.1
Alternaria brassicae KY001589	66	99	0.0	KF871433.1
Alternaria sp. 1 KY001588	100	100	0.0	KX156938.1
Alternaria sp. 2 KY001599	66	99	0.0	KF871433.1
Aspergillus brasiliensis KY001612	100	100	0.0	KU325087.1
Aspergillus sp. 1 KY001603	100	100	0.0	KU681408.1
Aspergillus westerdijkiae KY001601	100	100	0.0	NR_135389.1
Biscogniauxia mediterranea KY034428	100	100	0.0	KU325025.1
Chaetomium globosum KY001609	100	100	0.0	KT832070.1
Chondrostereum purpureum KY001582	100	93	0.0	GQ411519.1
Cladosporium cladosporioides KY001584	91	98	0.0	FJ932747.1
Cladosporium cucumerinum KY001597	100	99	0.0	KR912311.1
Cladosporium sp.1 KY001610	100	99	0.0	KX082931.1
Colletotrichum sp. 1 KY001583	100	100	0.0	KU612886.1
Coprinellus micaceus KY001591	100	100	0.0	JX160060.1
Cytospora sp. 1 KY034429	100	100	0.0	KJ739458.1
Didymellaceae sp. 1 KY001596	98	100	0.0	JX243762.1

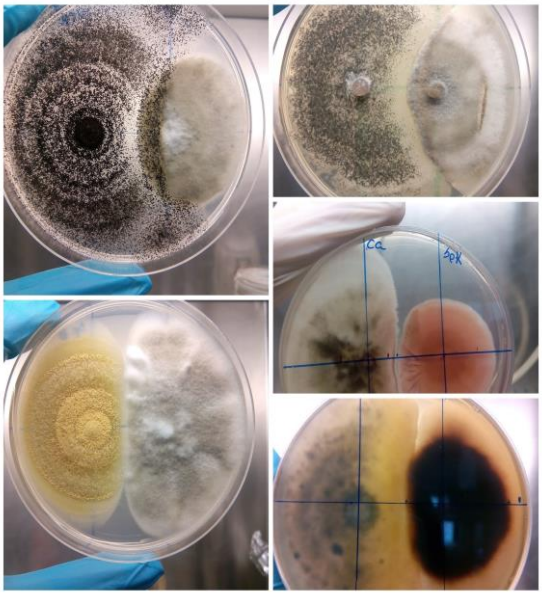
Epicoccum nigrum	99	100	0.0	KT192200.1
KY001595				
Gibberella avenacea	86	99	0.0	KT804130.1
KY001585				
Gibberella baccata	100	99	0.0	KU214542.1
KY001592				
Lewia sp. 1	97	99	0.0	EF432297.1
KY001594				
Mucor fragilis	99	99	0.0	FJ904925.1
KY001587				
Mucor hiemalis	100	100	0.0	KT896656.1
KY034430				
Neofabraea vagabunda	100	100	0.0	KU325344.1
KY001598				
Penicillium brevicompactum	100	100	0.0	KM265115.1
KY001611				
Quambalaria cyanescens	100	100	0.0	KU663655.1
KY001602				
Trichoderma koningii	100	100	0.0	Z95495
KY001593				

Table S2 –Frequency of colonization (FC, %) of each endophyte (End) and relative abundance (RA, %) of each epiphyte (Epi) isolated from olives of the cultivars Verdeal Transmontana and Madural.

Family, genera and species	Verdeal Transmontana		Madural		Total	
	End FC %	Epi RA %	End FC %	Epi RA %	End FC %	Epi RA %
Chaetomiaceae						
Chaetomium						
C. globosum Kunze	0.00	0.00	0.00	0.41	0.00	0.20
Cladosporiaceae						
Cladosporium						
*Cladosporium*sp. 1	0.00	0.80	0.00	0.00	0.00	0.40
C. cladosporioides (Fresen.) G.A. de Vries	1.52	0.80	0.95	0.61	1.24	0.70
C. cucumerinum Ellis & Arthur	0.00	61.63	0.00	70.70	0.00	66.16
Cyphellaceae						
Chondrostereum						
C. purpureum (Pers.) Pouzar	0.00	0.00	0.19	0.00	0.10	0.00
Dermateaceae						
Neofabraea						
N. vagabunda (Desm.) Rossman	5.90	0.00	0.00	0.00	2.95	0.00
Didymellaceae						
Didymellaceae sp. 1	0.00	4.57	0.00	0.41	0.00	2.51
Epicoccum						
E. nigrum Link	0.00	5.17	0.00	0.00	0.00	2.61
Glomerellaceae						
Colletotrichum sp. 1	0.00	0.00	0.57	0.00	0.29	0.00
Hypocreaceae						
Trichoderma						
T. koningii Oudem	0.76	0.00	0.19	0.00	0.48	0.00
Mucoraceae						
Mucor						
M. fragilis Bainier	0.00	0.20	0.00	0.81	0.00	0.50

M. hiemalis Wehmer	0.00	5.26	0.00	0.00	0.00	0.20
Nectriaceae						
Gibberella						
G.avenacea R.J. Cook	0.76	0.00	1.33	0.00	1.05	0.00
G. baccata (Wallr.) Sacc	2.67	0.00	4.19	0.00	3.42	0.00
Pleosporaceae						
Alternaria						
Alternaria sp. 1	0.19	0.00	0.19	0.00	0.19	0.00
Alternaria sp. 2	0.00	2.58	0.00	4.87	0.00	3.71
A. alternata (Fr.) Keissl	0.00	1.19	0.00	6.09	0.00	3.61
A. brassicae (Berk.) Sacc	0.00	0.00	0.19	0.00	0.10	0.00
Lewia						
Lewia sp. 1	0.19	0.00	0.00	0.00	0.10	0.00
Psathyrellaceae						
Coprinellus						
C. micaceus (Bull.) Vilgalys, Hopple & Jacq. Johnson	0.00	6.67	0.00	1.52	0.00	4.10
Quambalariaceae						
Quambalaria						
Q. cyanescens (de Hoog& G.A. de Vries) Z.W. de Beer, Begerow & R. Bauer	0.00	0.20	0.00	0.00	0.00	0.10
Trichocomaceae						
Aspergillus						
Aspergillus sp. 1	0.00	0.00	0.00	0.61	0.00	0.30
A. brasiliensis Varga, Frisvad & Samson	0.00	0.19	0.00	0.00	0.00	0.60
A. westerdijkiae Frisvad & Samson	0.00	0.00	0.00	0.41	0.00	0.20
Penicillium						
P. brevicompactum Dierckx	0.00	3.78	0.00	3.25	0.00	3.51
Valsaceae						
*Cytospora*s p. 1	0.00	3.18	0.00	0.00	0.00	1.61
Xylariaceae						
Biscogniauxia						
B. mediterranea (De Not.) Kuntze	0.00	7.75	0.00	10.14	0.00	8.94

4. Conclusions and future perspectives



4.1. Conclusions and future perspectives

The olive tree is an extremely important crop for the Mediterranean countries, including Portugal. The olive anthracnose, mainly caused by the fungus *Colletotrichum acutatum*, is one of the most serious constrains to the olive crop production worldwide. Their control has relied extensively on the use of chemical pesticides. This poses significant risks to the environment and non-target organisms, including humans. Both epiphytic and endophytic fungal community associated with olive fruits may be explored in an integrative perspective in order to design new strategies for the biological control of this disease.

In this work we intend to explore microbiome mediated beneficial effects on olive crop protection against anthracnose disease, aiming at finding an alternative to chemical pesticide use. For this, both epiphytic and endophytic fungi were assessed by culture-dependent method, in olives of two cultivars with different susceptibilities to anthracnose: Madural (susceptible) and Verdeal Transmontana (relative resistant). The capacity of native isolates to antagonize *C. acutatum* was further evaluated by using the dual-culture method.

The results indicate that olives from both cultivars supported great species richness and diversity. A total of 1,100 fungal isolates (104 endophytes and 996 epiphytes) belonging to 27 OTUs, 19 of which were identified up to the species level (17 genera, 14 families) were isolated from cv. Madural and cv. Verdeal Transmontana. Ascomycota was the most dominating phyla, representing more than 95% of the total isolates. Fungal community found on olives of both cultivars encompassed a complex species consortium including both phytopathogenic (of olive tree as well as of other plant species) and potentially antagonistic microorganisms.

Fungal communities were also found to differ in size and in composition between the surface and the interior tissues of olives of both cultivars. These variations could be caused by differences in the physical and chemical nature of the two habitats, and the type and survival strategies of colonizing fungal populations. Host genotype, at cultivar level, was found to affect endophytic fungal community composition, but not of epiphytic. This suggests that cultivar plays a somewhat more significant role in defining endophyte than epiphyte fungal communities. Overall, the results suggest that the survival and grow of fungal populations in olive fruits are dependent on the environment, and on physicochemical and genetic features of the

cultivar, which together determine the structure and diversity of the fungal community. This finding could offer opportunities to select specific beneficial microbiomes by selecting particular cultivar. In fact, the manipulation of the plant microbiome is seen as vital for sustaining plant production in a changing world, because has the potential to reduce the incidence of plant disease and increase agricultural production.

All the seven fungal isolates tested showed potentialities in the biocontrol of *C. acutatum*, being the most proeminent candidates *Chaetomium globosum* (isolated from cv. Madural), *Epicoccum nigrum* and *Quambalaria cyanescens* (both isolated from cv. Verdeal Transmontana). In general, the antagonistic activity displayed by the fungal tested was mediated at a distance and following contact, leading an inhibition of *C. acutatum* growth, germination, sporulation and exerthing abnormalities in fungal mycelia. The antagonistic activity of the isolates exhibited seems to depend much more on the fungi than on their provenances (*i.e.* type of cultivar).

In addition to providing insights into fungal endophyte and epiphyte community structure, our survey provides candidates for further evaluation as potential management tools against olive anthracnose disease. More studies including field trials must be performed to better ascertain the role of these fungi under natural conditions, and to assess their potential in the biocontrol of *C. acutatum*.