Universidade do Minho

Escola de Ciências

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Use of Microbial Bioprotectants to protect olive trees from anthracnose

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Use of Microbial Bioprotectants to protect olive trees from anthracnose

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Trabalho efetuado sob a orientação do

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e da

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Use of Microbial Bioprotectants to protect olive trees from anthracnose

The olive tree (*Olea europaea* L.) is one of the most cultivated tree species in the world, as they produce olives and olive oil, both being known for their human health benefits. However, olive trees can be attacked by diseases that can damage olive production, such as anthracnose, which is caused by fungi of the genera *Colletotrichum*. Control of this disease is very hard to achieve, and the main methods for managing olive anthracnose are centered on the use of copper-based fungicides, which cause a negative impact on the environment and organisms. Therefore, biological control agents (BCAs) have gained popularity, to find eco-friendly options to manage this disease, since they can inhibit pathogens growth and frequently they can also promote plant growth. The main objective of this work was to determine the effect of a potential BCA in increasing the resistance or tolerance of olive trees to anthracnose. Hence, the antagonistic potential of the endophytic fungus was assessed by co-inoculating olives with both endophyte and pathogen. When the pathogen was inoculated alone, all olives exhibited severe anthracnose symptoms (orange masses of conidia or white/grayish mycelium), while when inoculated alongside the endophyte, only 12% of inoculated olives exhibited disease symptoms with low severity. Using RNA-seq, the transcriptome of olive trees inoculated with the same endophyte and pathogen was analyzed. Most differentially expressed genes (6799 DEGs) were found between treated plants (E, P, and EP) compared to control plants, in an early stage of infection, suggesting that olive plants were able to defend themselves against the pathogen. The most up-regulated genes found in both endophyte-treated and pathogen-infected plants were involved in plant defense response (production of secondary metabolites, and reduction of oxidative stress). The most down-regulated genes found in endophyte-treated plants were involved in plant cell growth and breakdown of cell wall components. No major differences were detected between treatments (endophyte, pathogen, or a combination of both), suggesting that the studied endophyte was not significantly changing gene expression of olive plants prior to pathogen inoculation. Further studies are required for evaluating the biocontrol potential of the studied endophyte.

Keywords: Biocontrol; *Olea europaea* (L.); Olive anthracnose; Plant defense; RNA-seq.

RESUMO

Uso de Bioprotetores Microbianos para proteger as oliveiras da antracnose

A oliveira (*Olea europaea* L.) é uma das espécies arbóreas mais cultivadas no mundo, pois produz azeitonas e azeite, ambos conhecidos pelos seus efeitos benéficos para a saúde humana. No entanto, as oliveiras podem ser atacadas por doenças que podem prejudicar a produção de azeitonas, como a antracnose, que é causada por fungos do gênero *Colletotrichum*. O controlo desta doença é muito difícil de se conseguir, e os principais métodos utilizados para tentar geri-la centram-se na utilização de fungicidas à base de cobre, que causam um impacto negativo no ambiente e nos organismos. Portanto, agentes de bio-controlo (BCAs) têm ganhado atenção, para encontrar opções amigas do ambiente para o controlo desta doença, uma vez que podem inibir o crescimento de patógenos, e, frequentemente, também podem promover o crescimento de plantas. O principal objetivo deste trabalho foi determinar o efeito de um potencial BCA no aumento da resistência ou tolerância das oliveiras à antracnose. Assim, o potencial antagonístico do fungo endófito foi avaliado ao inocular azeitonas com o endófito e o patógeno. Quando o patógeno foi inoculado sozinho, todas as azeitonas apresentaram sintomas graves de antracnose (massas alaranjadas de conídios ou micélio branco/acinzentado), enquanto quando inoculadas juntamente com o endófito, apenas 12% das azeitonas apresentaram sintomas da doença com baixa gravidade. Ao utilizar RNA-seq, o transcriptoma de oliveiras inoculadas com o mesmo endófito e patógeno foi analisado. A maioria dos genes expressos diferencialmente (6799 DEGs) foram encontrados entre plantas inoculadas (E, P e EP) em comparação com plantas de controlo num estado inicial de infeção, sugerindo que as oliveiras foram capazes de se defender contra o patógeno. Os genes mais expressos em plantas inoculadas com o endófito e plantas infetadas com o patógeno estavam envolvidos na resposta de defesa da planta (produção de metabolitos secundários e redução do stresse oxidativo). Os genes mais silenciados encontrados em plantas inoculadas com o endófito estavam envolvidos no crescimento de células vegetais e na quebra de componentes da parede celular. Não foram detetadas grandes diferenças entre os tratamentos (endófito, patógeno ou conjuntamente inoculados), sugerindo que o endófito estudado não alterou significativamente a expressão de genes das oliveiras antes da inoculação do patógeno. Mais estudos são necessários para avaliar o potencial de bio-controlo do endófito estudado.

Palavras-Chave: Antracnose; Bio-controlo; Defesa vegetal; *Olea europaea* (L.); RNA-seq.

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ABBREVIATIONS AND ACRONYMS

AMF Arbuscular Mycorrhizal Fungi

BCAs Biological Control Agents

BP Biological process

CC Cellular component

cDNA Complementary DNA

CHS chalcone synthase

CTAB Cetyl Trimethyl Ammonium Bromide

DEGs differential expressed genes

dpi days post inoculation

DTT Dithiothreitol

ERFs ethylene response factors

ETI effector-triggered immunity

EU European Union

FC Fold change

GST Glutathione S-transferase

hpi hours post inoculation

HR hypersensitive response

ISR Induced Systemic Resistance

MAMPs microbe-associated molecular patterns

MF Molecular Function

MB Microbial Bioprotectants

NBS-LRR nucleotide binding site-leucine-rich repeats

PAL phenylalanine ammonia lyase

PAMPs pathogen-associated molecular patterns

PCA Principal component analysis

PCD programmed cell death

PGPF Plant Growth Promoting Fungi

PGPR Plant Growth Promoting Rhizobacteria

POD Peroxidase

PPO Polyphenol oxidase

PR Pathogenesis-related

PRRs pattern recognition receptors

PTI pattern-triggered immunity

rcf relative centrifugal force

RIN RNA Integrating Number

RLKs receptor-like kinases

RLPs receptor-like proteins

ROS Reactive oxygen species

SAR Systemic Acquired Resistance

1. Introduction

1.1 The importance of olive tree

The olive tree (*Olea europaea* L.) is a woody plant from the Mediterranean region that has an important role in the economy of several countries (Bizos *et al.*, 2020; Guo *et al.*, 2018), being one of the most worldwide cultivated tree species. This evergreen tree can grow up to 15 meters and live for hundreds of years (Guo *et al.*, 2018). The olive tree species belongs to the *Oleaceae* family and is the only species from this family that produces edible fruits for human consumption (López-Escudero & Mercado-Blanco, 2011). The fruit of olive tree (olives) can be consumed as table olives or be used for the production of olive oil, as well as other olive by-products (Guo *et al.*, 2018). The composition of olives (and olive oil) makes them highly popular, due to the increasing awareness around the health properties of olive oil (Kolainis *et al.*, 2020). Furthermore, olive trees are well-adapted to climate change (Bizos *et al.*, 2020). Indeed, *Olea europaea* is extremely tolerant to drought and salt stress (Azevedo-Nogueira *et al.*, 2020), presenting a great adaptability to adverse soil conditions (Porras-Soriano *et al.*, 2009). These could be the reasons why the production of olives has been increasing since 2014, almost reaching 25 million tons of olives produced worldwide in 2018 (figure 1). These values make the olive tree a vital source of economic value. However, the production of olives decreased in 2019, which could be a result from the rising rates of *Xylella fastidiosa* (pathogenic bacteria), the lack of rainfall or poor agronomic practices (Dawson, 2020).

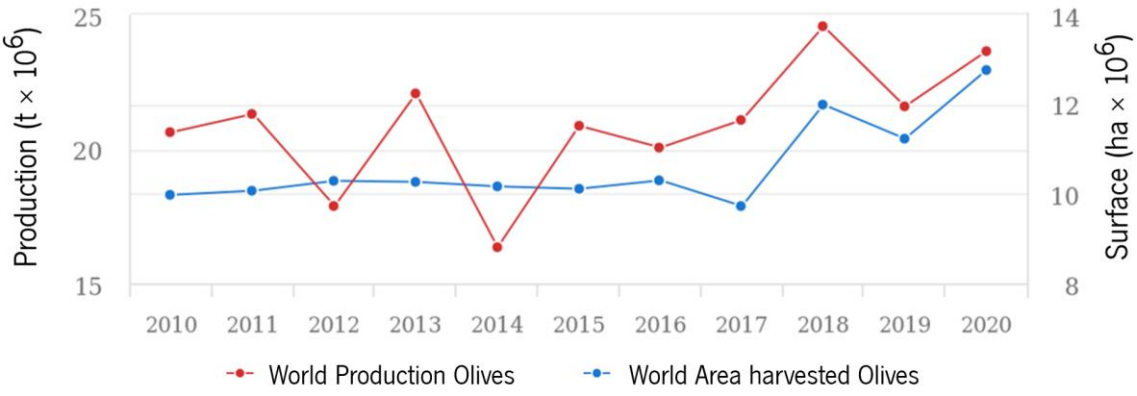

Figure 1. Total production of olives (red) and olive tree cultivation surface area harvested (blue) in the world from 2010 until 2020. Adapted from FAOSTAT (2021).

Due to the interest and high demand for olive oil and table olives, the olive cultivation currently occurs worldwide (figure 2), including on southern hemisphere countries (Torres *et al.*, 2017). However, this crop is particularly well adapted to the Mediterranean Basin conditions, where the most produced olives come from. Indeed, the olive tree is extensively cultivated by Mediterranean countries (figure 3). Since 2010, Europe has dominated the production of olives (61.8%), followed by Africa (19.3%) and Asia (15.2%). More specifically, the top 5 producers of

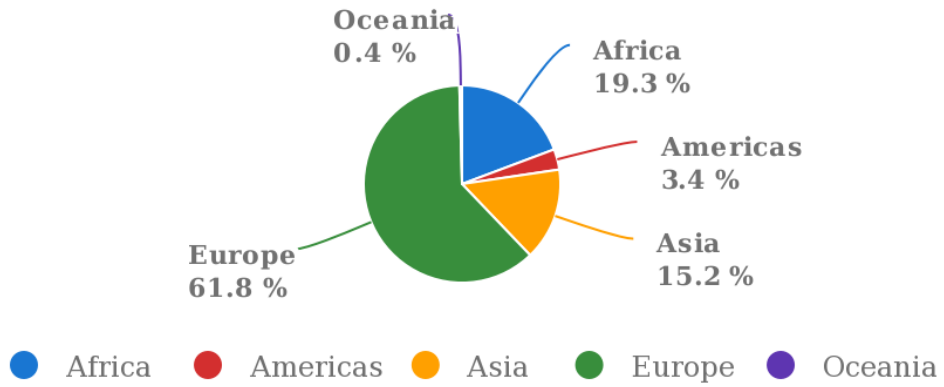

Figure 2. Production share of olives by region from 2010 until 2020 (FAOSTAT, 2021).

olives are Spain (72 million tons, cumulative production from 2010 until 2020), Italy (28 M tons), Greece (26 M tons), Turkey (18 M tons) and Morocco (15 M tons). Portugal is the tenth greatest producer of olives, producing 6.9 M tons of olives from 2010 until 2020 (figure 3) (FAOSTAT, 2021).

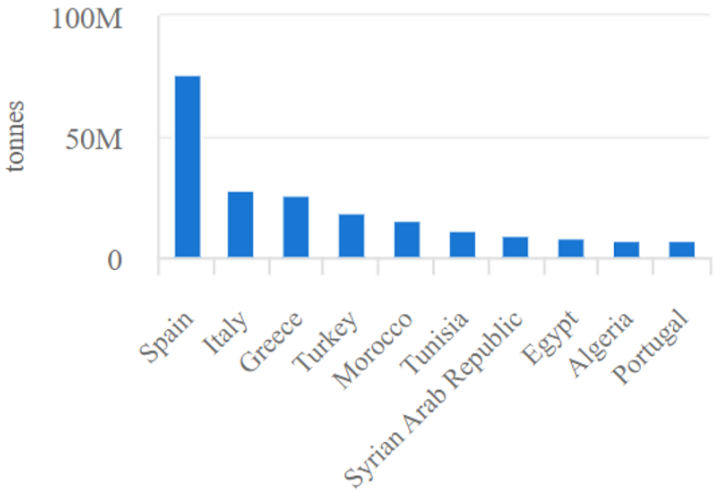

Figure 3. Top 10 olive producing countries. Results represent the cumulative olive production (in tons) from 2010 until 2020 (FAOSTAT, 2021).

According to the European Commission, the European Union (EU) is the largest producer of olive oil, accounting for 69% of the world production (EU News, 2020). Among members of EU, Spain is the main exporter (301,400 tons, in 2018) of olive oil (Eurostat, 2019). The other great exporter countries of olive oil are Italy (17%, 191,000 tons), Portugal (10%, 56,000 tons), and Greece (4%, 20,600 tons) (Eurostat, 2019). In total, members of EU exported over 1.6 M tons of olive oil in 2018, which was worth €5.7 billion (Eurostat, 2019), emphasizing the economic importance of olive tree in members of EU.

1.1.1 Olive fruit and olive oil properties

Olive trees produce small oval olive fruits that are green when unripe (although in some varieties ripen olives are still green) and black when fully ripen (Kiliçkan & Güner, 2008). They are rich in fatty acids and have a bitter flavor. Although in low quantities, olives also have phenolic compounds that are associated with human health benefits, the reason why they are highly consumed worldwide (Kiliçkan & Güner, 2008; Malheiro *et al.*, 2015; Romani *et al.*, 2019). Olives are also used to produce olive oil, which has been increasingly consumed in recent years, due to the health benefits that it brings, like reducing the incidence of cardiovascular diseases, preventing strokes and several types of cancers (Guo *et al.*, 2018; Unver *et al.*, 2017). The main reason for the bioactive properties of olive oils, like the cardioprotective effects, is due to the presence of high amounts of monounsaturated fatty acids, like oleic acid (Sánchez-Quesada *et al.*, 2013). Olive oil also have saturated fatty acids, polyunsaturated fatty acids, vitamins E (α-tocopherol) and K (phylloquinone), as well as minerals (such as sodium, calcium, potassium, and iron), which together gives the olive oil a high nutritional value. Hydroxytyrosol, oleuropein and tyrosol are the main phenolic compounds found in olive oils and provide stability against oxidation (Guo *et al.*, 2018; Tuck & Hayball, 2002).

1.1.2 Olive leaves properties

In the past few years, the society is keener to search for new functional foods with health benefits to improve the quality of life. The enrichment of olive oils with olive leaves, mainly because of the presence of benefic compounds (such as oleuropein), has been recently reported (Özcan & Matthäus, 2017). The effects of olive leaves in animal and human health have been extensively

studied. The medicinal properties of olive leaves are linked to the high contents of polyphenols and flavonoids present on them (Guo *et al.*, 2018). In these studies, several beneficial health effects of olive leaves have been reported, such as anti-inflammatory, antihypertension, cholesterol lowering and antimicrobial properties (Guo *et al.*, 2018; Özcan & Matthäus, 2017). Oleuropein is the most abundant phenolic compound present in leaves, followed by hydroxytyrosol (Özcan & Matthäus, 2017). The antioxidant and anti-inflammatory properties of oleuropein have been recognized, as well as its ability to inhibit inflammatory enzymes, such as lipoxygenases (Özcan & Matthäus, 2017).

1.2 The harm of anthracnose in the olive tree

The olive tree is a vital source of economic value and many beneficial health properties are associated to olive fruits and leaves, as well as to olive oil. However, olive production can be limited due to some diseases, like anthracnose (Azevedo-Nogueira *et al.*, 2020). Anthracnose is a disease that can cause significant yield losses, not only to olives, but also to other crops, such as strawberries (Guidarelli *et al.,* 2011), peppers (Ren *et al.*, 2020), açai (Peters *et al.*, 2020), mangoes (de los Santos-Villalobos *et al.*, 2013), and many others as well. Anthracnose is caused by fungi of the genera *Colletotrichum* (Talhinhas & Baroncelli, 2021). Anthracnose is so devastating that *Colletotrichum* spp. were ranked as the 8th most destructive plant pathogens (Dean *et al.*, 2012). So far, 248 *Colletotrichum* species have been reported, which are divided into 14 species complexes (Moral *et al.*, 2021). In olive trees, this disease is worldwide associated with at least 14 *Colletotrichum* species, belonging to three complexes, namely, *C. acutatum sensu lato* (*s.l.*), *C. gloeosporioides s.l.*, and *C. boninense s.l.* (Moral *et al.*, 2021). The 14 species known to be pathogenic to olive trees are listed in table 1, classified according to the *Colletotrichum* complex they belong (Azevedo-Nogueira *et al.*, 2020; Moral *et al.*, 2021). In Portugal, the predominant complex that causes anthracnose is *C. acutatum* (>97%), followed by *C. gloeosporioides* (<3%) (Kolainis *et al.*, 2020). The main species found are *C. acutatum sensu stricto* (*s.s.*), *C. nymphaeae*, and *C. godetiae* (Materatski *et al.*, 2018).

Table 1. *Colletotrichum* complexes (3) and respective species (14) known to be associated with olive anthracnose around the world. The most common in Portugal are underlined.

C. acutatum s.l.	***C. gloeosporioides s.l.***	***C. boninense s.l.***
C. acutatum s.s.	*C. gloeosporioides s.s.*	*C. boninense s.s.*
C. fioriniae	*C. theobromicola*	*C. karstii*
C. godetiae	*C. cigarro*	
C. nymphaeae	*C. siamense*	
C. rhombiforme	*C. queenslandicum*	
C. simmondsii	*C. alienum*	

In moist conditions, anthracnose symptoms on olive fruits include dark and necrotic sunken lesions, which are followed by a quick production of orange masses of conidia, causing fruit rot and eventual fruit drop (Materatski *et al.*, 2018). In drier conditions, symptoms on olive fruits include olive mummification (Materatski *et al.*, 2018). The olives mummification and premature drop, due to the olive anthracnose, are responsible for 80-100% olive yield losses in Portugal (Preto *et al.*, 2017). Although not as frequent as in olives, symptoms can also occur on flowers (infected flowers initially wilt and then dry), leaves and branches (Kolainis *et al.*, 2020; Talhinhas *et al.*, 2011). As a result, anthracnose can lead to necroses, defoliation and eventual death of branches. Furthermore, anthracnose symptoms can happen in pre-harvested but also in post-harvested olives, during storage, further enhancing the economic losses (Landum *et al.*, 2016). This fungal disease is particularly devastating in areas with high levels of relative humidity, where heavy infections occur, destroying completely any olive production (Kolainis *et al.*, 2020).

Besides the olive production, the olive oil quality is also affected by anthracnose. The olive oil, obtained from olive groves affected by this disease, display increased levels of acidity and peroxide content, as well as a reduction in oxidative stability, due to a decrease in phenolic compounds, such as hydroxytyrosol and tyrosol (Martins *et al.*, 2019; Preto *et al.*, 2017). The increased acidity is due to an increase in oleic acid, which will lead to an alteration in the organoleptic characteristics of olive oil, such as taste, smell, fluidity, and color (altered to red) (Azevedo-Nogueira *et al.*, 2020; Moral *et al.*, 2008).

1.2.1 Life cycle and infection process of *Colletotrichum* spp.

Colletotrichum spp. have sexual (rarely observed) and asexual reproductive stages, which occur on the host plant and in plant debris. Once conidia attaches to the host surface, germination occurs, which leads to the production and maturation of appressoria (figure 4). These structures are specialized for infection, as they enable the fungal penetration into plant tissues by puncturing host surfaces, while forcing fungal ingress using mechanical force and enzymatic degradation (De Silva *et al.*, 2017; O'Connell *et al.*, 2012). After penetration of the host cuticle and epidermal cell wall, the fungus starts to grow and colonize plant tissues, leading to the production of asexual reproduction strutures, such as acervuli (Wharton & Diéguez-Uribeondo, 2004). Conidia produced by acervuli are dispersed onto healthy leaves or fruits, due to rain splashing, irrigation and wind, leading to a new cycle of infection (da Silva *et al.*, 2020; De Silva *et al.*, 2017). Sporulating masses are generally observed in dead tissues after 7 days of initial infection (Wharton & Diéguez-Uribeondo, 2004).

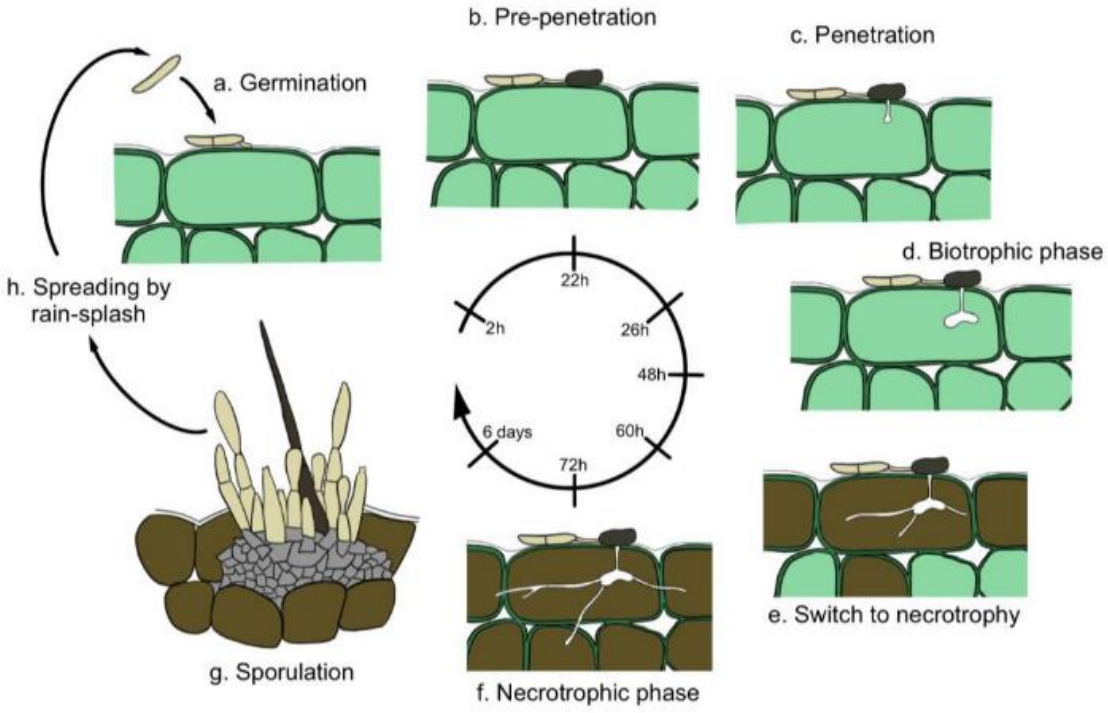

Figure 4. Asexual reproductive stages of *Colletotrichum* spp. Following conidia attachment to the host, conidia germinate and form appressoria to allow fungal penetration into the host. After fungal colonization, the pathogen produces acervuli, where conidia will be formed. Conidia dispersion restarts the infection cycle. Retrieved from Jayawardena *et al.* (2021).

Following colonization, *Colletotrichum* species differ on their post-infection strategies, ranging from necrotrophic, hemibiotrophic, and endophytic lifestyles. The most common is

hemibiotrophy, but most species can switch between lifestyles. The hemibiotrophic lifestyle consists of two sequential infection stages (De Silva *et al.*, 2017; Dean *et al.*, 2012). The fungus begins the infection process through a brief biotrophic phase that do not result in visible symptoms. The primary hyphae produce enlarged infection vesicles, inside epidermal and mesophyll cells, and obtain nutrients for fungal survival from living plant cells (De Silva *et al.*, 2017; Dean *et al.*, 2012). At this stage, the fungus does not kill plant cells and preserves host-cell integrity in order to keep receiving nutrients (da Silva *et al.*, 2020). In a second phase, the fungus switches to a necrotrophic phase, where thin secondary hyphae grow intracellularly and intercellularly (da Silva *et al.*, 2020; De Silva *et al.*, 2017; Dean *et al.*, 2012). These hyphae secrete toxins and lytic enzymes to degrade plant components (such as cell wall), causing plant cell disorganization and destruction (da Silva *et al.*, 2020; De Silva *et al.*, 2017; Dean *et al.*, 2012). Disease symptoms are associated with this second infection stage.

1.3 Control methods for olive anthracnose

Olive anthracnose is very hard to control and can be easily spread by rain and wind, also thriving in high humidity conditions. Currently, the management of olive groves is performed using a combination of methods, from cultural practices to chemical or biological control methods (Talhinhas *et al.*, 2018). In order to prevent olive anthracnose, diseased twigs could be pruned and removed from the olive grove (Martins *et al.*, 2019) or tolerant olive cultivars could be used, like cv. *Cobrançosa* (Gomes *et al.*, 2009). However, the cost for replacing an already established cultivar for a tolerant cultivar is very high (Landum *et al.*, 2016). Furthermore, in humid conditions, even the most tolerant cultivars can still get infected (Martins *et al.*, 2019). Therefore, the most used methods to control anthracnose are mainly based on agrochemicals, with which farmers spend a lot of their revenue (Bizos *et al.*, 2020). As disease eradication is very difficult, repeated applications of chemicals (mainly copper-based fungicides) are needed to maintain protection (twice a year, sometimes five times a year) (Kolainis *et al.*, 2020; Landum *et al.*, 2016; Poveda & Baptista, 2021). This constant application of fungicides can lead to increased resistance of pathogenic fungi to these chemicals and have a negative impact on beneficial organisms (Bizos *et al.*, 2020). Nevertheless, the use of fertilizers and pesticides bring many benefits to the olive culture, as they increase nutrients availability to the plants and reduce crop losses due to pests and diseases (Hossain *et al.*, 2017). Despite this, general public is becoming more aware and

concerned about the negative effects that chemicals have on the environment (soil and water pollution), in the human and animal health, on the microbiota, and other organisms biodiversity (Chenchouni *et al.*, 2020; Tripathi *et al.*, 2020). Therefore, researchers needed to find other suitable, eco-friendly options to control anthracnose, like for example the use of biocontrol agents.

1.3.1 Biological Control Agents

Biological Control Agents (or biocontrol agents, BCAs) are microorganisms that can inhibit the proliferation of pathogens or pests, restricting their effects on plants (Baker, 1987). Typically, BCAs consist of fungal or bacterial strains, obtained from the phyllosphere, endosphere or rhizosphere, which are able to antagonize pathogens. These microorganisms represent cheaper eco-friendly alternatives for plant disease management (Bizos *et al.*, 2020; Hossain *et al.*, 2017). BCAs can promote plant growth and offer new capacities to the plant for antagonizing pathogens, either through competition (where BCAs fight the pathogen for nutrients or space), or by the production of antimicrobial compounds, lytic enzymes (*e.g.* causing cell wall degradation) or toxins (Tripathi *et al.*, 2020), among others. Many microorganisms that interact with plants, namely Arbuscular Mycorrhizal Fungi (AMF), Plant Growth Promoting Rhizobacteria (PGPR), Plant Growth Promoting Fungi (PGPF), and endophytes, can act as biocontrol agents.

AMF participate in a mutualistic interaction that occurs in more than 80% of vascular plant families, including those from *Oleaceae* family. Mycorrhization with AMF is very important to plants regarding water uptake and mineral nutrition, playing also crucial roles during abiotic stresses, such as salt stress mitigation, and drought resistance (Chenchouni *et al.*, 2020). Recent research has shown that AMF colonization in olive trees under saline conditions, improves plant uptake of essential nutrients, which leads to a superior root surface area and higher biomass production (Porras-Soriano *et al.*, 2009). AMF also provide protection against pathogens (Castillo *et al.*, 2006). When a plant root system was colonized by AMF, a direct competition with soilborne pathogens for nutrients (such as phosphorous) and potential infection sites was detected (Castillo *et al.*, 2006). Other study revealed that AMF can induce olive tolerance to Verticillium wilt (disease caused by *Verticillium dahliae*, a soilborne fungus), while providing a better nutritional uptake (Boutaj *et al.*, 2020). These works suggested that AMF can not only provide protection against pathogens, but also enhance growth of olive plants. Although playing such key roles in plant ecology, few studies

have been done regarding their large-scale application in agricultural systems. Most studies regarding AMF inoculation are focused on their ability to stimulate plant growth and mainly reduce the impact of abiotic stress (Bizos *et al.*, 2020).

Many PGPR and PGPF that are found in the rhizosphere are capable of promoting plant growth or biological control (Hossain *et al.*, 2017; Vacheron *et al.*, 2013). The rhizosphere is the layer of soil in contact with roots and is the habitat of numerous microorganisms, such as bacteria, fungi, nematodes, arthropods, viruses, protozoa, algae, and archaea. These microorganisms benefit from the metabolites exudated by the plant roots that include many microbial nutrients (Lugtenberg & Kamilova, 2009). Recently, several reports suggested that plants selectively stimulate the presence of those microorganisms with beneficial traits for their growth and health (Hossain *et al.*, 2017). For example, many PGPR can increase plant nutrient availability or produce phytohormones, thus promoting plant growth (Verma *et al.*, 2019). In addition, many PGPF can improve plant growth by improving seed germination, enhancing photosynthetic ability, increasing biomass and yield of crop plants, and stimulating production of host secondary metabolites (Hossain *et al.*, 2017; Verma *et al.*, 2019). Both PGPR and PGPF, can have direct antagonistic effects against pathogens by producing antibiotics or by competing with pathogens for food and space. They can also induce plant defense mechanisms, such as the induced systemic resistance (ISR), which helps the plant to react more efficiently to invading pathogens (Hossain *et al.*, 2017; Kamle *et al.*, 2020). However, regarding induction of resistance against invading pathogens, PGPR have captivated more interest and have been more studied than PGPF (Naziya *et al.*, 2020).

The above-ground parts of plants (phyllosphere) are also inhabited by several microorganisms (bacteria and fungi) that do not cause any visible damage to the plant (Bezerra *et al.*, 2015). They are present either on the plant surface (epiphytes) or inside their tissues (endophytes). Recent studies reported that endophytic fungi are predominantly from the genera of *Aspergillus*, *Fusarium*, *Penicillium*, and *Piriformospora* (Rana *et al.*, 2019). But, in olive tree, the endophytic community is mainly composed by the genera *Pseudocercospora*, *Aureobasidium*, *Cladosporium*, *Vishniacozyma*, *Saccharomyces*, and by the following species *Alternaria alternata*, *Parastagonospora avenae*, and *Foliophoma fallens* (Costa *et al.*, 2021). Besides promoting plant growth, for example by producing phytohormones (such as, indole acetic acids, gibberellic acids, and cytokinin; Rana *et al.*, 2019), endophytes have gained a recent attention for the search of new biocontrol strategies (Bizos *et al.*, 2020). Indeed, by interacting with endophytes, plants benefit

from the bioactive secondary metabolites they produce, like flavonoids and volatile organic compounds, improving plants defense against biotic and abiotic stresses (Malhadas *et al.*, 2017; Nicoletti *et al.*, 2014). During a pathogen attack, the plant also benefits from endophytic cell wall-degrading enzymes (like chitinases and glucanases), which have the capacity to destroy the pathogens cell wall (Martins *et al.*, 2019). In addition, endophytes also produce mycotoxins, antimicrobial molecules and antibiotics that can decrease pathogen infection through mycoparasitism, competition (for space and nutrients) and antibiosis (Martins *et al.*, 2019). These compounds and processes are produced and occur both in the field and during fruits storage (Landum *et al.*, 2016). This is especially important for olive production because anthracnose symptoms can also happen in post-harvested olives, during their storage. Besides the direct effect, endophytes also induce indirect responses, like ISR for systemic protection against soilborne pathogenic fungi and bacteria, viruses, nematodes, insects and herbivores (Oukala *et al.*, 2021).

1.4 Plant defense against pathogens

During their lifetime, plants face various pathogen attacks that are harmful for their growth, reproduction, and development. With the aim to obtain tolerance or resistance, plants developed several mechanisms, including physical and biochemical processes, to cope with biotic stresses. While physical defense responses include aspects such as plant cell wall thickening and lignification (Trabelsi *et al.*, 2017), the main biochemical alterations include the synthesis of pathogenesis related proteins (PR), like chitinases or proteinase inhibitors, or the synthesis of different compounds (Ali *et al.*, 2020). In particular, pathogenesis-related (PR) proteins are a group of plant proteins highly induced during a pathogen attack that play important roles in plant disease resistance (Zaynab *et al.*, 2019). There are 17 PR protein families, presenting different roles during a pathogen attack (table 2).

These proteins accumulate locally in the infected and surrounding tissues, protecting plants from further infections (Zaynab *et al.*, 2019). Following a pathogenic attack, plants also increase the production of reactive oxygen species (ROS), which ultimately causes a toxic effect to the pathogen, delaying further colonization. ROS are oxygen-derivative molecules that are more reactive than oxygen molecules. They include radicals, such as superoxide $(O_2^{\bullet -})$ and hydroxyl $(OH^{\bullet})$, as well as their reaction products, but also many non-radical molecules, such as hydrogen peroxide

(H_2O_2) (Auten & Davis, 2009). As result of vital metabolic processes, like photosynthesis and respiration, plants are persistently producing several ROS. However, due to their toxic effect, plants possess several ROS detoxifying mechanisms, like antioxidant enzymes, to prevent plant cell oxidative stress. During stress situations, the production of ROS can outweigh their scavenging, causing damage to nucleic acids, lipids, and proteins (Auten & Davis, 2009; Khedia *et al.*, 2019). The main ROS produced upon a pathogen attack are superoxide anion and hydrogen peroxide, as their levels arise in and around the infected plant host cells. In these situations, plants can diminish ROS levels by producing, for example, glutathione S-transferases (GST) that are involved in reducing plant membranes damage promoted by ROS, by reducing lipid peroxidation (Pavlidi *et al.*, 2013). In addition, certain ROS can also be signaling molecules, important for inducing the expression of defense genes. Taken together, ROS are part of the plant defense by being involved in the direct killing of the pathogen, by activating expression of defense genes, or by promoting programmed cell death (PCD) of infected or nearby cells (Maffei *et al.*, 2006; Prasannath, 2017; Šnyrychová *et al.*, 2009; Štolfa Čamagajevac *et al.*, 2019). During PCD, known as a hypersensitive response (HR), the pathogen is prevented from infecting other sites and the activation of Systemic Acquired Resistance (SAR) occurs (Khedia *et al.*, 2019; Štolfa Čamagajevac *et al.*, 2019).

Table 2. Classification of pathogenesis-related proteins and their main roles during pathogenesis.

Families	Properties	Roles	References
PR-1	Antifungal activity	Multiple roles: antimicrobial function, defense signal amplification, potential sterol or effector recognition	(Breen *et al.*, 2017)
PR-2	β-1,3-glucanase	Hydrolytic enzymes that degrade β-1,3-glucan, structural component of cell walls of pathogenic fungi	(Gharbi *et al.*, 2017; Jain & Khurana, 2018)
PR-3	Chitinase (class I, II, IV, V, VI, VII)	Hydrolytic enzymes; degrade chitin and inhibit fungal growth	(Jain & Khurana, 2018; Nakazaki *et al.*, 2006)
PR-4	Chitinase (class I, II)	Hydrolytic enzymes; degrade chitin and inhibit fungal growth	(Bravo *et al.*, 2003; Jain & Khurana, 2018)
PR-5	Thaumatin-like	Antifungal activity (*e.g.* promoting pathogen plasma membrane disturbance and cell wall disorganization)	(Jiao *et al.*, 2018)
PR-6	Proteinase inhibitor	Regulate and balance protease activities in plant cell	(Habib & Fazili, 2007)

Table 2. *Continuation*.

Families	Properties	Roles	References
PR-7	Endoproteinase	Hydrolyze peptide bonds in the process of protein degradation (dissolution of microbial cell walls)	(Souza *et al.*, 2017)
PR-8	Chitinase (class III)	Bifunctional enzyme with lysozyme/chitinase activity (can damage gram-positive bacteria)	(Malik, 2019)
PR-9	Peroxidase	Reinforcement of cell wall by catalyzing lignification; detoxification of reactive oxygen species (ROS)	(Lüthje & Martinez-Cortes, 2018; Zaynab *et al.*, 2019)
PR-10	Ribonuclease-like	Multiple functions: antimicrobial activity, in vitro ribonuclease activity, enzymatic activities in plant secondary metabolisms	(Xie *et al.*, 2010)
PR-11	Chitinase, class I	Hydrolytic enzymes; chitin binding and inhibit fungal growth	(Malik, 2019; Taira *et al.*, 2002)
PR-12	Defensin	Antimicrobial peptides that induce the formation of pores in pathogen membranes; Protect vegetative tissue from pathogens attack by enhancing defensin constitutive expression	(Ali *et al.*, 2018; Souza *et al.*, 2017)
PR-13	Thionin	Induce the formation of pores in pathogen membranes, resulting in the release of calcium and potassium ions from the cell	(Zaynab *et al.*, 2019)
PR-14	Lipid transfer protein	Binds to lipid and sterol molecules; may interact with receptors at plant plasma membranes to trigger plant defense responses	(Cheng *et al.*, 2004)
PR-15	Oxalate oxidase	Generate H_2O_2 that is toxic to pathogens	(Souza *et al.*, 2017)
PR-16	Oxalate oxidase-like	Generate H_2O_2 that is toxic to pathogens	(Souza *et al.*, 2017)
PR-17	Unknown	Cell wall metabolism, or signal transduction, or antibiotic compounds that directly attack the pathogen.	(Christensen *et al.*, 2002)

As signaling molecules, ROS are able to induce plant systemic resistance, which comprise long-term resistance systems that protect the entire plant of further invasions (Gharbi *et al.*, 2017; Štolfa Čamagajevac *et al.*, 2019). There are two different systemic resistance mechanisms – the SAR and Induced Systemic Resistance (ISR). Both protect the whole plant from biotic stresses, even those parts unaffected by the attack (Kamle *et al.*, 2020). SAR is triggered when plants are challenged by biotrophic or hemi-biotrophic pathogens, leading to an accumulation of PR proteins, mediated by salicylic acid (SA) (Jain & Khurana, 2018; Kamle *et al.*, 2020; Vlot *et al.*, 2021). ISR is induced by (hemi-)biotrophic, necrotrophic pathogens and beneficial microorganisms, such as

PGPR and PGPF (Hossain *et al.*, 2017; Vacheron *et al.*, 2013; Vlot *et al.*, 2021). Studies showed that when ISR is induced, plants develop a faster and stronger cellular defense response than plants without induced ISR (Hossain *et al.*, 2017). While triggering SAR depends on the activation of SA, ISR depends on the activation of jasmonic acid (JA) and ethylene (ET) signaling pathways (Hossain *et al.*, 2017). Therefore, the activation of plant defense pathways are mediated by plant hormones (like SA, JA, and ET), whose production increase upon a pathogen attack (Gharbi *et al.,* 2017). Accordingly, some studies have shown that SA, JA, and ET may induce the expression of PR proteins and influence the antioxidant enzyme activation to prevent oxidative stress (Jain & Khurana, 2018; Peters *et al.*, 2017).

Another plant defense response is the production of secondary metabolites, which can prevent further oxidative deleterious effects or have antimicrobial activity (Trabelsi *et al.*, 2017). For this reason, following a pathogen attack, the plant activates several defense genes, including phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Jain & Khurana, 2018). PAL is a key enzyme in the metabolic pathway of phenylpropanoid compounds and catalyzes the removal of the amino group of phenylalanine to produce trans-cinnamic acid (Mayo *et al.*, 2015). This enzyme allows, for example, the production of important phenolic compounds, like lignin and phytoalexins. While lignin is an important component of plant cell wall, phytoalexins have inhibitory activity against various fungi (N'Guyen *et al.*, 2021; Zaynab *et al.*, 2018). Both are important players for plant resistance responses to pathogens. The gene expression of CHS, a key enzyme for flavonoid and anthocyanins biosynthesis, is also induced following the attack from microbial pathogens, resulting in the production and accumulation of compounds with antimicrobial activity, like flavonoid and isoflavonoid phytoalexins (Dao *et al.*, 2011).

1.5 Objectives/Aims of this study

In this project, we aim to determine the effect of Microbial Bioprotectants (MB) in increasing the resistance or tolerance of olive trees to anthracnose. As an olive endophyte (*Penicillium aff. commune*) has previously revealed potential to act as biocontrol agent against an anthracnose pathogen (*Colletotrichum acutatum*) using *in vitro* conditions (Castro, 2021), this work pretends to give a step forward in determining its biocontrol suitability. For this, the present work has the following specific objectives:

i) – to evaluate the protection levels conferred by MB (*P. aff. commune*) to olives challenged by *C. acutatum*;

ii) – to identify which genes are induced or repressed following the infection of olives with anthracnose causal agent (*C. acutatum*), in plants previously inoculated with MB (*P. aff. commune*) or not. Gene expression comparison is expected to elucidate the mechanisms underlying enhanced resistance of olive plants to anthracnose provided by MB.

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2. Detection of biocontrol potential of olive endophytes (*Penicillium commune*) against *Colletotrichum acutatum*

Olive anthracnose is a disease caused by fungi belonging to genera *Colletotrichum*, which severely damage olive production. The management of olive anthracnose is difficult because of the widespread of different *Colletotrichum* species and strains. This means that olive trees are exposed to distinct *Colletotrichum* strains that will lead to more damage to olive trees (Moral *et al.*, 2021; Preto *et al.*, 2017). Currently, the most common practice for managing olive anthracnose is the use of copper-based fungicides. The use of copper sulfate was reported to be very effective in inhbiting conidial germination of *C. godetiae* and *C. nymphaeae*, both belonging to the *C. acutatum* complex (Moral *et al.*, 2018). This practice however demands the constant application of fungicides, as they are washed-off by the rain. Also, the accumulation of copper on soils can have adverse environmental effects (Moral *et al.*, 2018). For these reasons, an environmental-friendly strategy to control olive anthracnose is in need.

Plants are constantly associated with endophytic microorganisms that can bring beneficial effects to them, either by improving their performance or by providing protection against biotic and abiotic stresses. In the past few years, more studies are being conducted with the aim to use endophytic fungi and bacteria as biological control agents (BCAs) against plants diseases and pests (Hong & Park, 2016; Mantzoukas & Eliopoulos, 2020). Since endophytes live inside plant tissues, they are a hopeful economic and environmental-friendly approach to manage olive anthracnose. Indeed, epiphytes and endophytes isolated from olive leaves were able to inhibit the growth of *Colletotrichum acutatum*. Both *Aspergillus niger* and *Nigrospora oryzae* isolates were able to inhibit the pathogen growth by 86.3% and 66.7%, respectively (Landum *et al.*, 2016). Also, epiphytes and endophytes isolated from olive fruits were able to inhibit *C. acutatum* growth (Preto *et al.*, 2017). In this work, the epiphyte *Chaetomium globosum* inhibited the growth of *C. acutatum* by 71.3%, followed by *Aspergillus westerdijkiae* (57.9%) and *Epicoccum nigrum* (57.2%), while the endophyte *Chondrostereum purpureum* only inhibited 30.9% of *C. acutatum* growth (Preto *et al.*, 2017). Both authors point out that *C. acutatum* growth was restricted through the competition for space and

food, since the epiphyte and endophyte isolates grew faster (Landum *et al.*, 2016; Preto *et al.*, 2017). In addition, Landum *et al.* (2016) revealed that all the studied 14 antagonistic isolates produced volatiles (like pyrazine and propanoic acid derivatives) with antifungal properties that could be responsible for reducing *C. acutatum* growth.

Although many microorganisms have been displaying antagonistic activity against olive anthracnose causal agents, very few studies have reported the effectiveness of such microorganisms (fungi or bacteria) under field conditions. Most studies have been performed using *in vitro* conditions, where conditions are controlled and do not mimic the environmental conditions (Martins *et al.*, 2019). For this reason, a BCA that was identified and studied using *in vitro* conditions does not necessarily give the same results using *in vivo* conditions (Martins *et al.*, 2019). Before using natural field conditions, where fungal isolates could be disseminated to the environment, the inoculation of harvested olives in controlled conditions could give some clues about BCA effectiveness under field conditions (Mina *et al.*, 2019).

When detached olives were simultaneously inoculated with *Colletotrichum acutatum* and microbial isolates obtained from leaves, a reduction of anthracnose severity symptoms was observed (Segura, 2003). Indeed, *Aureobasidium pullulans* (a yeast-like fungus), *Curtobacterium flaccumfaciens* (bacteria), and *Paenibacillus polymyxa* (bacteria) decreased the severity of anthracnose symptoms in 76.4%, 53.7%, and 51.6%, respectively (Segura, 2003). The antagonistic potential of *A. pullulans* against *Colletotrichum* spp. was also studied by Nigro *et al.* (2018) that revealed a 40% reduction of anthracnose severity after inoculating olive trees with *A. pullulans* in field trays (Nigro *et al.*, 2018). The antagonistic potential of yeasts isolated from vitivinicultural and olive environments against *Colletotrichum gloeosporioides* was also evaluated in detached olive fruits (Pesce *et al.*, 2018). *Candida tropicalis* Bo13b and *Wickerhamomyces anomalus* Bo156 were the two most promising antagonist isolates by reducing disease incidence by 90%. In addition, *W. anomalus* Bo156 was able to reduce disease severity by 70.5%. Altogether, these results suggest that different microorganisms could effectively control *Colletotrichum* spp. However, before using any microorganism as a BCA, several tests should be performed to ensure human and animal safety. When *C. tropicalis* Bo13b was tested for human pathogenicity, several parameters revealed that it could be pathogenic to humans (Pesce *et al.*, 2018). Further research revealed that *C. tropicalis* is indeed a well-known human pathogen and therefore would not be an option as a future biocontrol microorganism (Chai *et al.*, 2010).

In this chapter, the antagonistic potential of *Penicillium aff. commune* against *C. acutatum* is evaluated in detached olives. This *Penicillium* isolate was obtained from healthy leaves and branches of olive trees and previous studies revealed its ability to reduce the *in vitro* growth of *C. acutatum* pathogen (Castro, 2021). In the present work, detached olives were used to assess the effectiveness of the same isolate in reducing disease incidence and/or disease severity. This is the first step for assessing the suitability of this *Penicillium* isolate to be used as a BCA for controlling anthracnose in living tissues (olives).

2.1 Materials and methods

2.1.1 Plant material

Olive fruit samples were collected from the olive cultivar *Madural*, in *Vale de Telhas* (Northeast of Portugal, 41°36'28.4"N 7°13'27.0"W). This olive cultivar has been described as susceptible to anthracnose (Torres, 2007). Symptomless olive fruits, displaying most of the surface bright green (class 0, Guzmán *et al.*, 2015) were collected and stored at 4 °C until use. All detached olives were searched for visible damage, using a stereo microscope (Leica Zoom 2000). Only those olives showing completely undamaged epicarp were used.

2.1.2 Fungal isolates

Fungal isolates used for olive inoculation were previously obtained from *Olea europaea* L., at the Polytechnic Institute of Bragança (Martins, 2020). The *Penicillium aff. commune* isolate was collected from leaves and branches of apparently healthy olive trees, while *C. acutatum* isolate was collected from olives exhibiting anthracnose symptoms.

2.1.3 Inoculation of fungal isolates

Prior to olive inoculation, 200 healthy olives displaying either a black/reddish epicarp with some yellow spots (class 3, Guzmán *et al.*, 2015) or a completely black/purple epicarp (class 4, (Guzmán *et al.*, 2015) were rinsed with running water. Olives were then sterilized by a sequential immersion in 70% (v/v) ethanol for 2 min, 3-5% (v/v) sodium hypochlorite for 3 min, 70% (v/v)

ethanol for 1 min, and lastly rinsed three times (1 min each) with sterile distilled water. Sterile olives were placed in sterile flasks (5 olives/flask), containing a filter paper soaked in sterile distillated water (1 mL), for maintaining humidity to enhance fungal development.

Before olive inoculation, spore suspensions were prepared from each fungal isolate (figure 5). The endophyte (*P. aff. commune*) and the pathogen (*C. acutatum*) were previously cultured on potato dextrose agar (PDA) for 16 and 21 days, respectively, at 20 °C with no photoperiod. The surface of the endophyte (or pathogen) culture was gently scraped with sterile toothpicks. Scraped

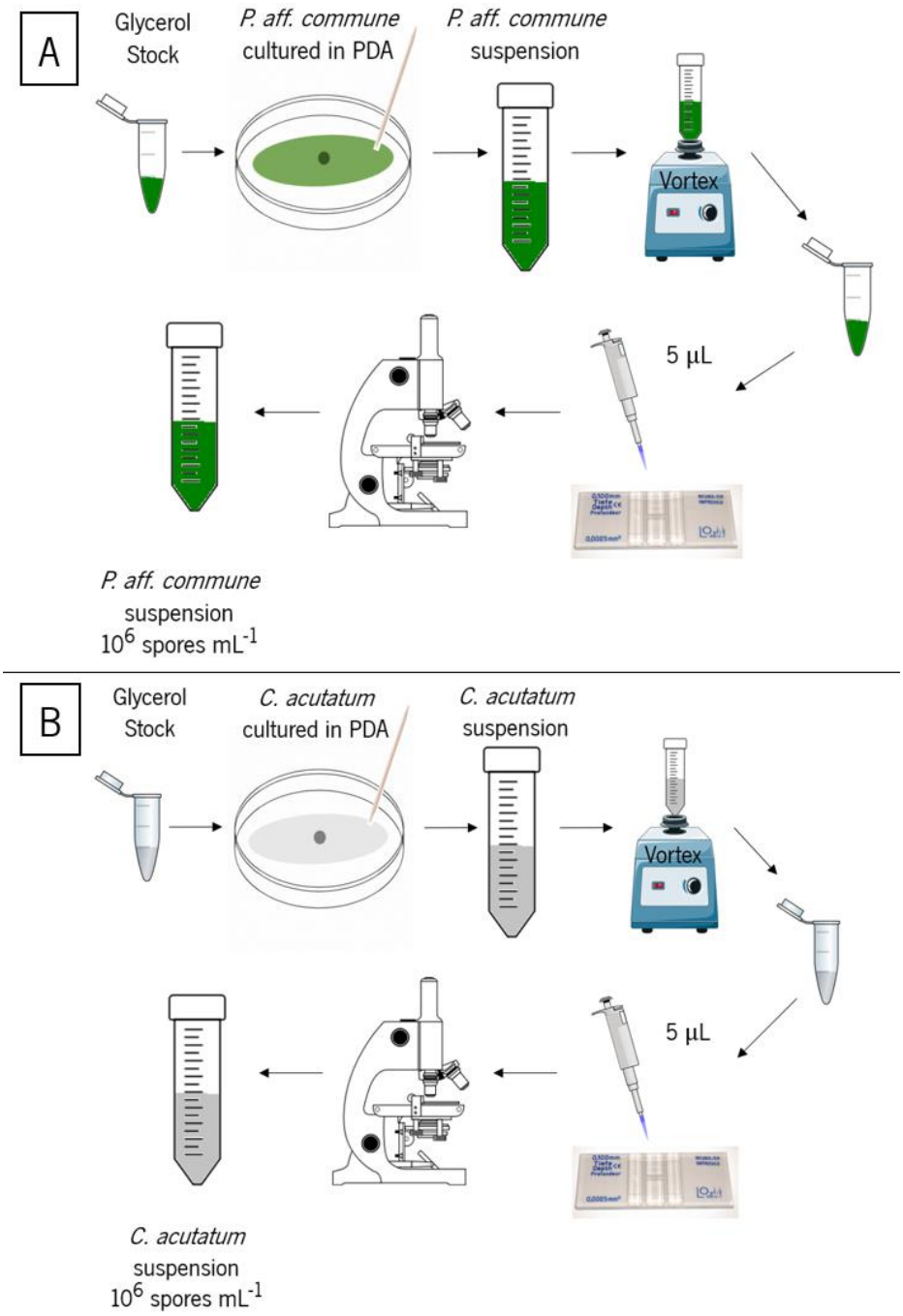

Figure 5. Experimental workflow for the preparation of spore suspensions of *Penicillium aff. commune* (A), and *Colletotrichum acutatum* (B). After scraping the fungal surface with toothpicks, a spore suspension was prepared. Following homogenization, suspension titer was determined using a Neübauer hemocytometer (see text for further details).

pieces were introduced into a tube, containing 30 mL of peptone water (1 g/L peptone, 8.5 g/L NaCl, pH 7.0) and 0.05% (v/v) Tween 20. The fungal spores were quantified using a Neübauer hemocytometer. The final spore concentration was adjusted with peptone water and 0.05% (v/v) Tween 20 up to 10^6 spores mL^{-1}.

To test the effect of the endophyte on restricting anthracnose symptoms, sterile olives were inoculated with both endophyte and pathogen spores, or with the endophyte or the pathogen individually (figure 6). Olives were firstly inoculated with 3 mL of *P. aff. commune* suspension

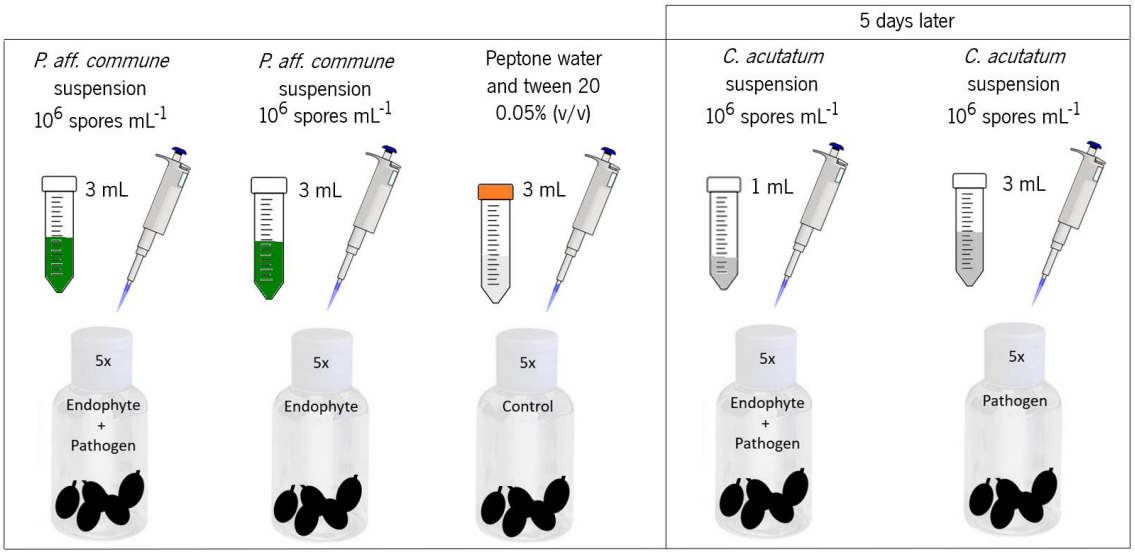

Figure 6. Experimental design used to evaluate the protection levels conferred by the endophyte *Penicillium aff. commune* to olives challenged with the pathogen *Colletotrichum acutatum*. Olives inoculated with both endophyte and pathogen, were inoculated first with 3 mL of *P. aff. commune* suspension followed 5 days later by 1 mL of *C. acutatum* suspension. Olives inoculated with the endophyte, were inoculated with 3 mL of *P. aff. commune suspension*. Olives inoculated with the pathogen, were inoculated 5 days later with 3 mL of *C. acutatum* suspension. Control olives were inoculated with 3 mL of peptone water and 0.05% (v/v) Tween 20.

(10^6 spores mL^{-1}), either for single inoculation (Endophyte) or for combined inoculation (Endophyte + Pathogen) treatments. A mock inoculation (Control), using only peptone water with 0.05% (v/v) Tween 20 was similarly prepared. The flasks were kept at 25 °C, with no photoperiod. After five days, 3 mL of *C. acutatum* spore suspension (10^6 spores mL^{-1}) were used for inoculating olives [single inoculation (Pathogen)]. For olives that have been treated with the endophyte, only 1 mL of *C. acutatum* suspension was used [combined inoculation (Endophyte + Pathogen)]. For each treatment, there were five replicates (flasks) and five olives per flask, as represented in figure 6.

Treated olives were kept at 25 °C with no photoperiod and anthracnose symptoms (dark/brown dead spots on the olive surface, orange masses of conidia, white-grayish mycelium) were evaluated for 18 days. The incidence (number of diseased olives/total number of treated olives) and the severity of the disease (percentage of the olive area affected by disease) was determined.

2.2 Results

Olive anthracnose is a devastating disease that is very hard to manage. Therefore, with the aim to control it, detached olive fruits were co-inoculated with an endophytic fungus (*P. aff. commune*) and a pathogen (*C. acutatum*). When olives were just inoculated with the pathogen, they soon revealed disease symptoms, like those reported by Moral *et al.* (2008) and by Talhinhas *et al.* (2018) (figure 7). Just 5 days post inoculation (dpi) with *C. acutatum*, primary symptoms of anthracnose were observed in 12% of treated olives. Dark/brown lesions appeared, along with an orange gelatinous matrix (figure 7.A).

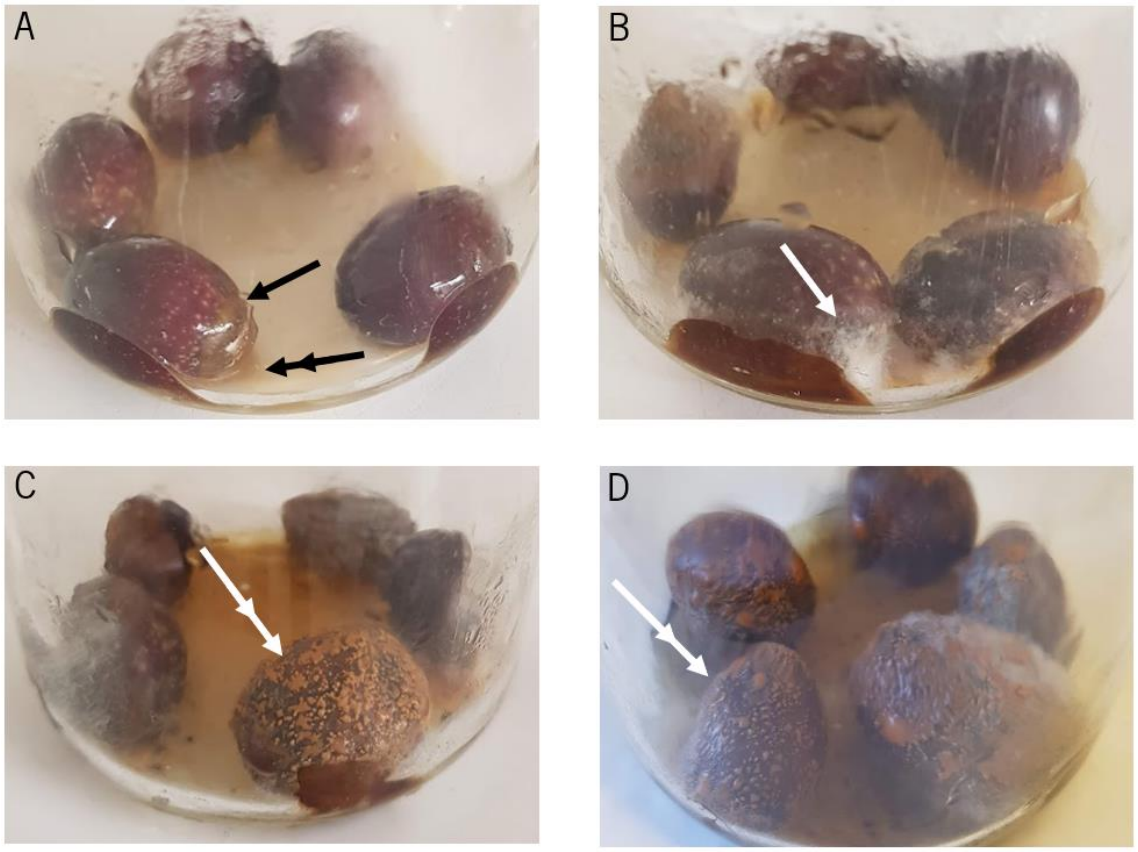

Figure 7. Representation of the symptoms that appeared on olives only inoculated with *Colletotrichum acutatum* after 5 days (A), 8 days (B), 11 days (C), and 18 days (D) post inoculation. After 5 days, a dark/brown lesion (black arrow) and orange gelatinous matrix (double black arrow) was detected in some olives. After 8 days, a white-grayish mycelium on the surface of a few olives was observed (white arrow). Upon 11 days, olives presented abundant production of orange masses of conidia (double white arrows). At the end of the assay (18 days) most olives exhibited severe symptoms as they were covered with oranges masses of conidia.

At 8 dpi, symptoms of anthracnose appeared in most olives (72%), which displayed expanded dark/brown lesions, orange masses of conidia and/or white-grayish mycelium on olives surface

(figure 7.B). At 11 dpi, fungal development intensified (figure 7.C) and at the end (18 dpi) 100% of olives exhibited severe anthracnose symptoms. Figure 7.D shows the severe attack of *C. acutatum* to these olives, as they have their whole surface covered with orange masses of conidia.

Meanwhile, in olives inoculated with both *P. aff. commune* and *C. acutatum*, no symptoms of anthracnose were observed at 5 dpi. However, the fungus *P. aff. commune* grew on the surface of the olives, as white and green masses of spores were observed (figure 8.A), which could be related with the high moisture in the flasks, creating a perfect environment for fungal development and/or the high concentration of spores suspension. Symptoms of anthracnose only appeared at 8 dpi, but only in 2% of olives, including orange masses of conidia and orange gelatinous matrix (results not shown).

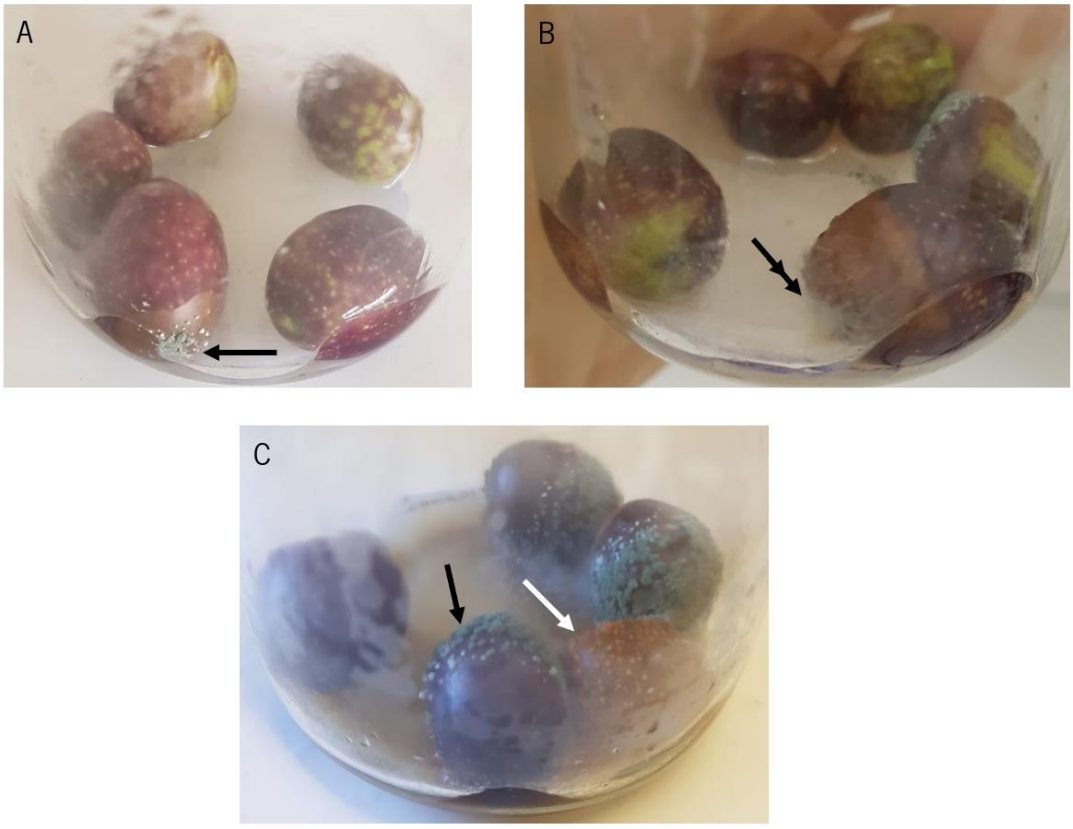

Figure 8. Representation of the symptoms that appeared on olives inoculated with both *Penicillium aff. commune* and *Colletotrichum acutatum* after 5 days (A), 14 days (B), and 18 days (C) post inoculation. After 5 days, no anthracnose symptoms were observed, but white and green masses of spores appeared in some olives (black arrow). After 14 days, there was a dark/brown lesion along with white-grayish mycelium on the surface of a few olives (double black arrow). At the end of the study (18 days) only a few olives exhibited symptoms (dark brown/lesions, orange masses of conidia (white arrow) and white-grayish mycelium).

At 14 dpi, only 8% of olives exhibited anthracnose symptoms (figure 8.B). Besides dark/brown lesions, some olives exhibited a small portion of their surface covered with orange masses of

conidia, while others had a white-grayish mycelium on the surface. At the end (18 dpi), only 12% of olives exhibited anthracnose symptoms (figure 8.C). The flasks that contained at least one infected olive exhibited an orange gelatinous matrix.

In olives only inoculated with the endophyte (*P. aff. commune*), white and green masses of spores appeared at 5 dpi and intensified during the whole assay (figure 9). Regarding anthracnose symptoms, just one single olive (0.02%) exhibited orange masses of conidia at 11 dpi. These symptoms suggest a possible cross-contamination with *C. acutatum*. Mock inoculations did not result in anthracnose symptoms.

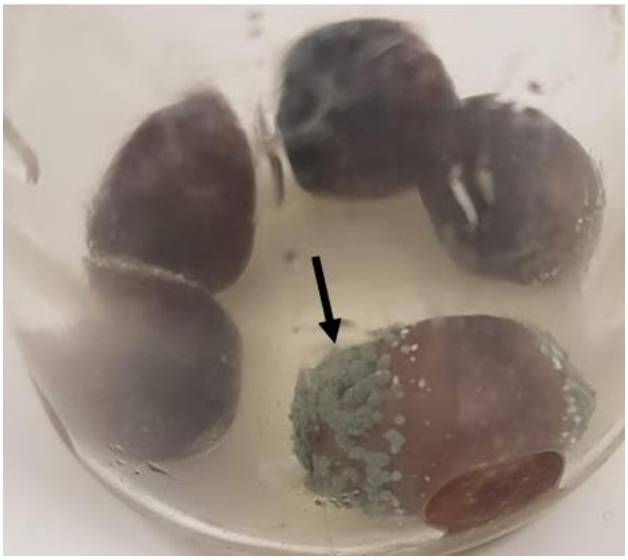

Figure 9. Representation of white and green masses of spores (black arrow) that appeared on olives only inoculated with *Penicillium aff. commune*, 14 dpi.

Altogether, these results reveal a progression of fungal infection during the whole assay, in particular when olives are only infected with the pathogen. When considering the number of olives that exhibited anthracnose symptoms (disease incidence), there was a significant increase in treatments with the pathogen compared with all other treatments (figure 10.A). This difference became evident just after 5 days and further intensified along time ($p < 0.005$ at 5 dpi; $p < 0.0001$ at following days). There was no significant difference among all other treatments. Only at 18 dpi a significant increase was detected for Endophyte + Pathogen treatment in relation to the control.

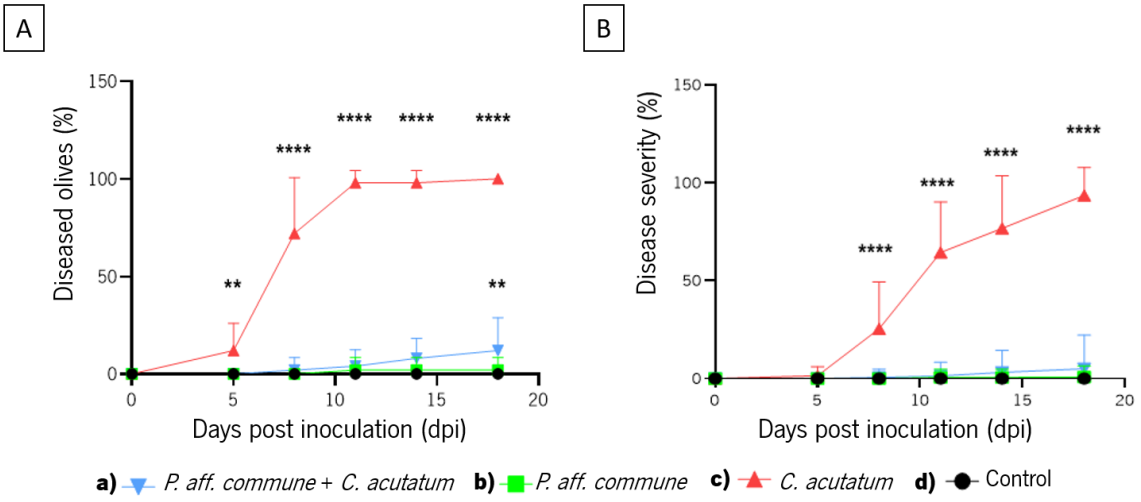

Figure 10. Evaluation of anthracnose symptoms on inoculated olives. The incidence (A) and disease severity (B) of treated olives in the following days post inoculation. Olive treatments included a) endophyte + pathogen (*Penicillium aff. commune*, *Colletotrichum acutatum*), b) only endophyte (*P. aff. commune*), c) only pathogen (*C. acutatum*), d) mock inoculation (control). Significant differences between treated olives and control olives are represented by ** (at $p < 0.005$) or **** (at $p < 0.0001$ (Dunnett's multiple comparisons test, following a two-way ANOVA).

The disease severity following the inoculation with *C. acutatum*, evaluated by the olive area affected by disease, increased steadily during the entire assay (figure 10.B). Indeed, comparing with control olives, a significant higher disease severity occurred from 8 dpi until 18 dpi ($p < 0.0001$). In comparison, those olives inoculated with both *P. aff. commune* and *C. acutatum* exhibited a reduced area of disease, not significantly different from the control or with the olives only treated with the endophyte.

Altogether, these results suggest that *P. aff. commune* was effective in reducing the incidence and the severity of the disease caused by *C. acutatum* on detached olives. The overall assay was repeated twice, with olives from class 3 and 4 (Guzmán *et al.*, 2015) and the same results were obtained.

2.3 Discussion

Olive anthracnose is a devastating disease that is spread worldwide, causing great economic losses. This disease, caused by *Colletotrichum* spp., affects olive fruits, flowers, leaves, and branches, which can lead to the destruction of the entire olive production (Moreira *et al.*, 2021). Anthracnose effects on olives are particularly serious as they alter olive oil quality, thus jeopardizing

the olive oil production. In this work, the antagonistic activity of a potential biocontrol agent (*Penicillium aff. commune*) was evaluated in detached olives. This isolate was previously described to reduce *C. acutatum* growth using *in vitro* conditions (Castro, 2021), but its effects on olives was never evaluated before. In the field, the most common anthracnose symptoms detected in olives is mummification, which did not happen in the performed assays, probably because of the high humidity. Under the assayed conditions, the most common disease signs were olive dark/brown lesions, an orange gelatinous matrix, orange masses of conidia and white/grayish mycelium in olives surface, as reported by Moral *et al.* (2008) and by Talhinhas *et al.* (2018). Treated olives were kept in a moisty environment at 25 °C throughout the assay with the intent to preserve the most advantageous conditions for anthracnose development. Indeed, these conditions have been reported to improve conidia germination, inoculum dissemination and fruit infection of anthracnose causal agents (Azevedo-Nogueira *et al.*, 2020; Kolainis *et al.*, 2020; Moral *et al.*, 2008; Msairi *et al.*, 2017; Talhinhas *et al.*, 2011). The presence of white/grayish mycelium is also probably due to the high moisture, since this symptom does not tipically happen in field conditions (Moral *et al.*, 2008).

The endophyte used in this work was a *P. aff. commune* isolate, collected from leaves and branches of healthy olive trees. This fungal species has been recognized for its high antimicrobial activity, as *P. commune* isolates from olive trees were recently suggested as an alternative and innovative source of antimicrobial agents (Malhadas *et al.*, 2017). Other *P. commune* isolates, obtained from foliar tissues of Cupressaceae family also revealed antifungal activity *in vitro* against *Pyricularia oryzae*, a rice blast pathogen (Hosseyni *et al.*, 2013). Also, the *P. aff. commune* isolate used in the present work was reported to reduce the microbial *in vitro* growth of *Colletotrichum* spp. pathogens (Castro, 2021). To test the effectivenness of the same isolate in restricting anthracnose causal agent (*C. acutatum*) in living tissues, olives were inoculated with both fungi and the arising of anthracnose symptoms was compared with olives only inoculated with the pathogen. At the end of the assay, while only 12% of all the olives inoculated with both fungi exhibited anthracnose symptoms, 100% of olives inoculated with the pathogen presented disease symptoms. The disease severity was also greater in olives only inoculated with *C. acutatum*, as the majority of olives surface exhibited dark/brown lesions and were also fully covered in orange masses of conidia. Disease severety in olives inoculated with both fungi was much lower. Based on these results, we can conclude that *P. aff. commune* has the capacity to inhibit *C. acutatum* or

at least delay the progression of anthracnose symptoms. This is the first report of the effective control of anthracnose in olives using *Penicillium comune* strains.

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3. Transcriptome profiling of olive leaves inoculated and non-inoculated by Microbial Bioprotectants

In the previous chapter, the antagonistic potential of the endophytic fungus *P. aff. commune* against *C. acutatum* pathogen was studied in detached fruits. *P. aff. commune* revealed to be able to inhibit *C. acutatum*, or at least delay the appearance of anthracnose symptoms, in olives inoculated with both fungi. In the present chapter, an *in vivo* assay (using greenhouse conditions) was conducted, with the aim to study transcriptional changes occurring in leaves of olive trees treated with the same endophyte and pathogen. An RNA-seq (RNA-sequencing) methodology was used for the transcriptome analysis. The transcriptome includes all coding (mRNA), and non-coding (transfer RNA, ribosomal RNA, and others small RNAs) ribonucleic acids present in the cell. Using RNA-seq, the overall gene expression (even the weakly expressed genes) can be evaluated and compared among biological samples, allowing also the detection of alternative splicing isoforms, discovery of new genes and gene fusions (Dillies *et al.*, 2013; Hrdlickova *et al.*, 2017). In the present work, differentially expressed genes in different plant treatments or plants collected at different time points were analyzed.

Several studies have used RNA-seq to study plant-pathogen interactions, in order to understand pathogenicity mechanisms and defense response of plant hosts at different stages of infection. Plants possess cell surface-localized pattern recognition receptors [PRRs, which can be either receptor-like kinases (RLKs) or receptor-like proteins (RLPs)] that detect pathogens and other microorganisms through their pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs) (Kumar *et al.*, 2017; Otero-Blanca *et al.*, 2021; Zhang *et al.*, 2018). These recognition mechanisms trigger the pattern-triggered immunity (PTI). There is a second recognition mechanism, where intracellular immune receptors (e.g., nucleotide binding site-leucine-rich repeats, NBS-LRR proteins) detect pathogen virulence molecular effectors (Wang *et al.*, 2017; Zhang *et al.*, 2018). This recognition leads to the activation of effector-triggered immunity (ETI). PTI and ETI activate defense responses, such as hormone and ROS production, and transcriptional regulation of defense genes (Otero-Blanca *et al.*, 2021). An RNA-seq study of strawberries infected with *Colletotrichum fruticola* revealed that this plant species uses both PTI and ETI to prevent *C. fruticola* invasion (Zhang *et al.*, 2018). However, 15 of the top 100 up-regulated *C. fruticola* genes (after 24 hours post inoculation, hpi) encoded effectors, able to weaken hosts defenses by

suppressing PTI. Plants responded by up-regulating genes involved in lignin biosynthesis, in order to strengthen cell walls, although some genes involved in cell wall synthesis were also down-regulated at 72 and 96 hpi. Through the three stages of infection (24, 72 and 96 hpi), plants responded to *C. fruticola* inoculation by inducing up-regulation of salicylic acid (SA)-signaling genes (while jasmonic acid (JA) and ethylene (ET) signaling genes were inhibited). During the necrotrophic stage (96 hpi), an up-regulation of genes involved in ROS activation was detected, suggesting that the plant activated defense mechanisms to restrict further pathogen infection (Zhang *et al.*, 2018). The induction of a plant defense response was also detected when analyzing the transcriptome of mango fruits upon infection with *C. gloeosporioides* (Hong *et al.*, 2016). Genes encoding transcription factors that regulate defense responses against pathogens, such as ethylene response factors (ERFs), NBS-LRR proteins and PR proteins were up-regulated 3 days after infection (Hong *et al.*, 2016).

In order to control diseases caused by pathogens, researchers are using RNA-seq approaches to study the alteration of gene expression promoted by microorganisms that may be used as biocontrol agents. A recent study revealed that the fungal endophyte *Penicillium olsonii* ML37 was able to reduce Fusarium head blight (caused by *Fusarium graminearum*) in wheat (Rojas *et al.*, 2022). *P. olsonii* ML37 activated plant defense mechanisms in wheat, comprising the transcriptional activation of PR proteins and WRKY transcription factors, which are known to be involved in biotic and abiotic stress responses. The induction of defense responses were also detected when evaluating the biocontrol potential of the endophytic fungus *Chaetomium globosum* CEF-082 against *Verticillium dahliae* in cotton (Zhang *et al.*, 2020). In cotton plants inoculated with both fungi (endophyte and pathogen), 1209 differentially expressed genes were induced compared to plants only inoculated with *V. dahliae*. These genes were involved in ROS metabolic processes, including H_2O_2 metabolic process, superoxide dismutase activity, and antioxidant activity. Other identified genes included ERFs, RLPs, and glutathione S-transferases (GSTs), which may be related to cotton resistance to *V. dahliae*.

In contrast with RNA-seq, other gene expression studies, such as those using microarrays, can also be used to evaluate global gene expression. However, this method is not so suitable for studying the gene expression of low or very highly expressed genes (Segundo-Val & Sanz-Lozano, 2016). Also, microarrays require the existence of an available genome sequence, and the

expression results obtained via microarrays, need to be validated by qPCR (Segundo-Val & Sanz-Lozano, 2016). Using RNA-seq, the gene expression levels are accurately quantified and it requires a lesser amount of RNA (Wang *et al.*, 2009). The obtained results should be validated by using qPCR methods, although recent reports suggest that RNA-seq is robust enough to not always require validation by qPCR and/or other approaches (Coenye, 2021). These are the reasons why RNA-seq is replacing microarrays as the most used sequencing method for global gene expression analysis.

For studying changes in gene expression promoted by the endophyte (*P. aff. commune*) and the pathogen (*C. acutatum*) in treated olive plants, an RNA-seq approach was followed in the present work (figure 11). After extracting RNA from leaves collected from olive treated plants, libraries were prepared and sequenced using a high-throughput platform (Illumina), which generates millions of short nucleotide sequences (reads). After adequate filtering (Zhao *et al.*, 2016) to eliminate low-confidence nucleotide bases, the remaining reads were aligned to a reference genome. Even when using a plant species that does not have a sequenced genome available, this RNA-seq approach is still possible by performing *de novo* assembling of the transcriptome (Korpelainen *et al.*, 2014; Wang *et al.*, 2009). Due to their close proximity to olive plants under study (*Olea europaea* subsp. *europaea* var. *europaea*), the reference genome that was used was *Olea europaea* var. *sylvestris* (NCBI, BioProject PRJNA417827). This reference genome has 1.48 Gb, 23 chromosomes, and more than 50,000 genes (Unver *et al.*, 2017).

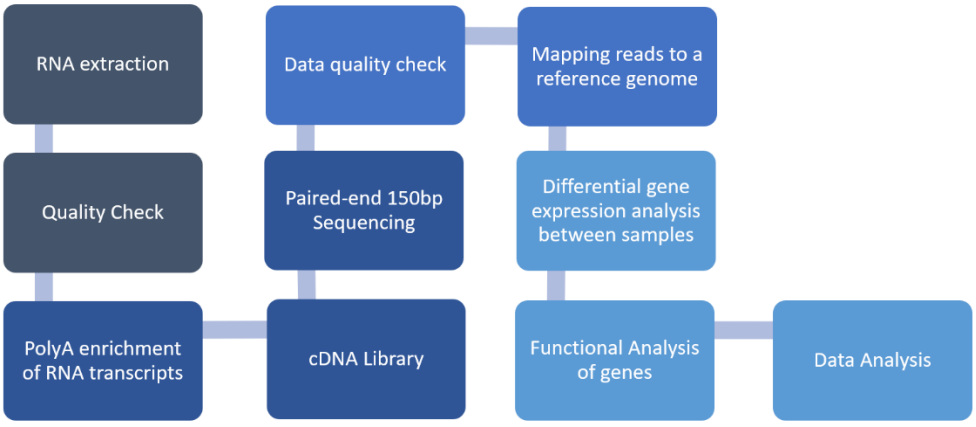

Figure 11. Overview of the experimental steps in an RNA sequencing study.

3.1 Materials and methods

3.1.1 Plant material

In this work, two-year-old olive plants (cv. *Cobrançosa*) were used for performing pot experiments. The used olive cultivar has been considered as moderately tolerant to anthracnose (Gomes *et al.*, 2009). Olive plantlets were produced as described by Mina *et al.* (2020) and were grown in a greenhouse with the following conditions: temperature of 25-30 °C, relative humidity of approximately 85%, and sprinkler watering for 10 seconds in 15 minutes intervals.

3.1.2 Fungal isolates

Fungal isolates used for olive tree inoculation were previously obtained from olive organs (Martins, 2020) as follows, *P. aff. commune* was collected from the endosphere of leaves and branches of apparently healthy olive trees, while *C. acutatum* was collected from olives that exhibited anthracnose symptoms. Fungal suspensions were prepared as described in section 2.1.3.

3.1.3 Inoculation of olive plants

In order to study the effects of the endophyte in controlling anthracnose symptoms, a pot experiment was performed. Olive plants were treated either: a) with the endophyte (*P. aff. commune*) and then with the pathogen (*C. acutatum*), b) with the endophyte alone, c) with the pathogen alone. As control, a buffer solution was used (mock inoculation). A total of 96 plants were used (24 plants for each treatment), as detailed in figure 12.

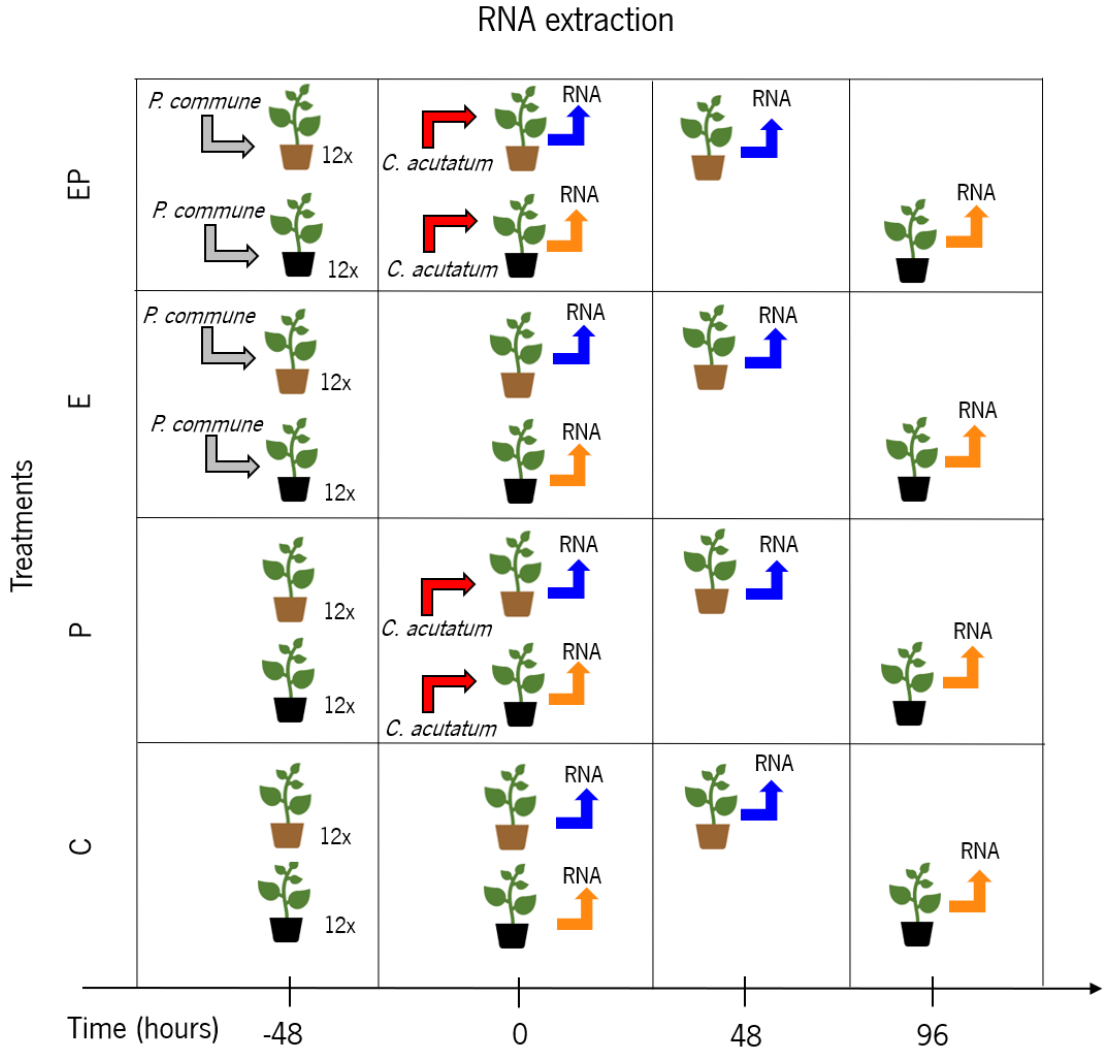

Figure 12. Experimental design used for studying the effects of the endophyte on infected plants with anthracnose causal agent. Plants treated with both endophyte and pathogen (EP), were first inoculated with a suspension of *Penicillium aff. commune* spores (-48h), and 48h later with a suspension of *Colletotrichum acutatum* spores (0h). Plants treated with the endophyte (E) were inoculated with a suspension of *P. aff. commune* spores (-48h) and sprayed with buffer solution at 0h. Plants treated with the pathogen (P) alone were mock inoculated at -48h and then inoculated with a suspension of *C. acutatum* spores at 0h. Control (C) plants were inoculated with a buffer solution (-48h) and then sprayed with buffer solution at 0h. Leaf samples were collected at 0, 48, and 96 hours post inoculation (hpi) of the pathogen, for RNA extraction. [Gray arrows – inoculation of endophyte; red arrows – inoculation of pathogen; blue arrows – collection of leaves from the same plants (48h); orange arrows - collection of leaves from the same plants (96h)].

Olive plants were subjected to four treatments and inoculated at two different occasions (-48 h and 0 h), as shown in table 3. Plants treated with both fungi and plants treated only with the endophyte were cut in the main stem and inoculated with 10 µL of a suspension of *P. aff. commune* spores (10^8 spores mL^{-1}). Similarly, plants that would be only treated with the pathogen and control plants were cut in the main stem and the wound was used to introduce 10 µL of buffer solution [0.025% (v/v) Tween 80 and 0.15% (w/v) agar], being completely wrapped with Parafilm (figure

13.A). Following 48 h, plants treated with both fungi and plants treated only with the pathogen were sprayed with 3 mL of *C. acutatum* spores (10^6 spores mL^{-1}). Plants treated only with the endophyte and control plants were sprayed with 3 mL of a buffer solution [0.025% (v/v) Tween 80 and 0.15% (w/v) agar] (figure 13.B).

Table 3. Inoculation process of olive plants treated with the endophyte (*Penicillium aff. commune*), the pathogen (*Colletotrichum acutatum*) or with buffer solution for control. Plants were cut in the main stem and inoculated with the endophyte spores (*P. aff. commune*) or with a buffer solution (Buffer sol.). Forty-eight hours later, plants were sprayed with pathogen spores (*C. acutatum*) or with a buffer solution.

Treatment	Control	Endophyte	Pathogen	Endophyte + Pathogen
P. aff. commune	-	10 µL (cut) (10^8 spores mL^{-1})	-	10 µL (cut) (10^8 spores mL^{-1})
C. acutatum	-	-	3 mL (spray) (10^6 spores mL^{-1})	3 mL (spray) (10^6 spores mL^{-1})
Buffer sol.	10 µL (cut) 3 mL (spray)	3 mL (spray)	10 µL (cut)	-

Figure 13. Inoculation of plants with a spore suspension of the endophyte (*Penicillium aff. commune*) by cutting the stem of the olive tree (A) and inoculation of plants with the pathogen (*Colletotrichum acutatum*) by spraying plants with a spore suspension (B). In A, the produced wound wrapped with Parafilm is observed.

Following inoculations, the plants were separately maintained at the same conditions (figure 14).

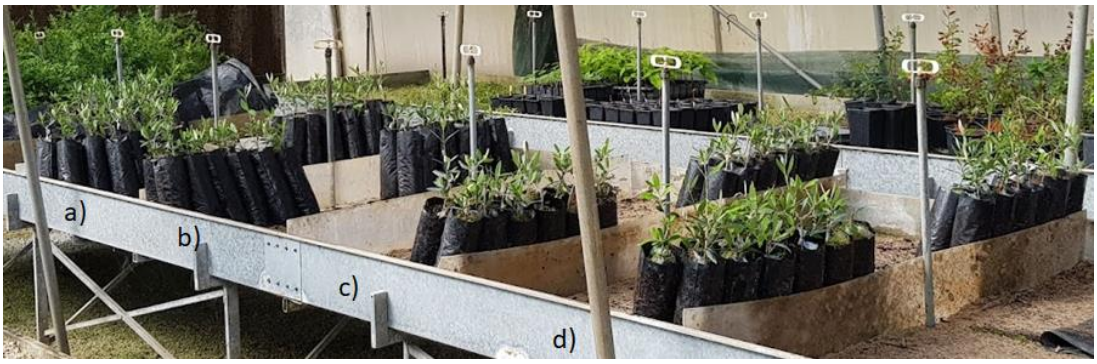

Figure 14. Olive plants after being inoculated. Control plants (a), plants treated with the endophyte (b), plants treated with the pathogen (c) and plants treated with both endophyte and pathogen (d).

Just after both inoculations, one leaf from three different plants (from the same treatment) were collected and combined. Each sample was immediately frozen in liquid nitrogen and stored at –80 °C until further use (labeled as 0h). Following 48 h, leaves from 48 plants (12 plants/treatment) were collected, combined and frozen as described above (labeled as 48h). Finally, 96 h post-inoculation, the remaining 48 plants (12 plants/treatment) were similarly frozen (labeled as 96h). This design resulted in the production of 64 samples, including 32 reference samples (samples collected at 0h) and corresponding 32 assayed samples [4 treatments (EP, E, P, C) x 2 collection times (48 h, 96 h) x 4 replicates].

3.1.4 RNA extraction

The obtained 64 samples were used for extracting RNA from olive leaves (figure 15).

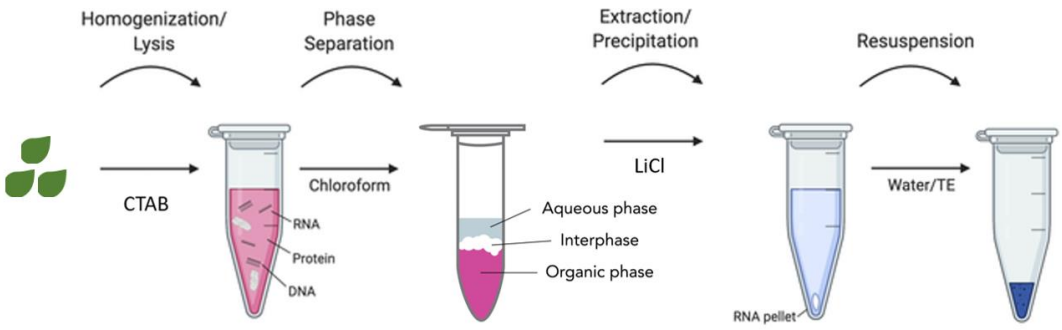

Figure 15. Schematic summary of the CTAB-based method used for extracting RNA from olive leaves. See text for further details. Adapted from (Addgene, 2020).

The protocol used for RNA extraction was based on Le Provost *et al.* (2007), using a CTAB-based extraction buffer [2% (w/v) CTAB (Cetyl Trimethyl Ammonium Bromide), 2% (w/v) PVP

(polyvinylpyrrolidone), 0.1 M Tris HCl pH 8.0, 30 mM EDTA, 2 M NaCl, 4% (w/v) DTT (added just before use)]. Olive leaves were firstly homogenized with liquid nitrogen. To help the breaking of plant cell walls and to maximize the separation of nucleic acids from polysaccharides, 900 μL of CTAB buffer were added. The resulting mixture was incubated at 65 °C in a thermomixer (Thermomixer comfort, Eppendorf), for 30 minutes and mixed gently by inverting tubes every 5 min. For RNA purification, 700 μL of chloroform: isoamyl acid (24:1) were added and the mixture was vigorously vortexed for 2 seconds. Following a centrifugation at 16,900 rcf (Centrifuge 5418 R, Eppendorf), for 10 min at 4 °C, the obtained supernatant was transferred to a clean microtube, where 700 μL of chloroform: isoamyl acid (24:1) were added, and the same process repeated. For RNA precipitation, ¼ volume of 8 M LiCl (lithium chloride) was added to the resulting supernatant and incubated at 4 °C for 1 hour. The mixture was then centrifuged at 16,900 rcf, for 25 min at 4 °C. The supernatant was discarded and the obtained pellet was washed by adding 700 μL of 2 M LiCl. Following a centrifugation at 16,900 rcf, for 25 min at 4 °C, the supernatant was discarded and the pellet was re-suspended in 30 μL of diethyl pyrocarbonate (DEPC)-treated water. The concentration of the RNA in the samples and its purity was evaluated using a *Nanodrop Spectrophotometer ND-1000* (Thermo Scientific). RNA integrity was evaluated after electrophoretic separation on a 0.8 % (w/v) agarose gel.

3.1.5 Library preparation and sequencing

RNA samples were shipped in dry ice to Novogene Co., Ltd (Cambridge, UK), where the library preparation and sequencing were performed. Before library preparation, quality control of samples was made with a bioanalyzer (Agilent 2100). RNA samples that did not have the required quality for library preparation/sequencing were discarded. Messenger RNAs were first purified from total RNAs, using poly-T oligo-attached magnetic beads. For preparing the libraries, mRNAs were fragmented, and the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. After ends repair and A-tailing reactions, the adapters (5´adapter: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT; 3´adapter: GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGATGACTATCTCGTATGCCGTCTTCTGCTTG) were ligated and a size selection was performed. After amplification and purification, the library

was checked with Qubit and real-time PCR for quantification and checked with bioanalyzer for size distribution detection. Sequencing was performed by using an Illumina NovaSeq6000 sequencer that generated at least 30 million paired-end reads (30M) with 150 base pair (bp) per library.

3.1.6 Bioinformatic analysis

The quality of sequenced reads (raw reads) was first controlled by the sequencing provider (Novogene Co., Ltd). Those reads containing N > 10%, reads with low quality base calling (when 50% of the read contains low quality nucleotides, Q score ≤ 5), and reads containing adapter sequences were removed before delivering. A QC report of raw data of each sequenced library/sample was produced by Novogene Co., Ltd (Cambridge, UK) (figure 16).

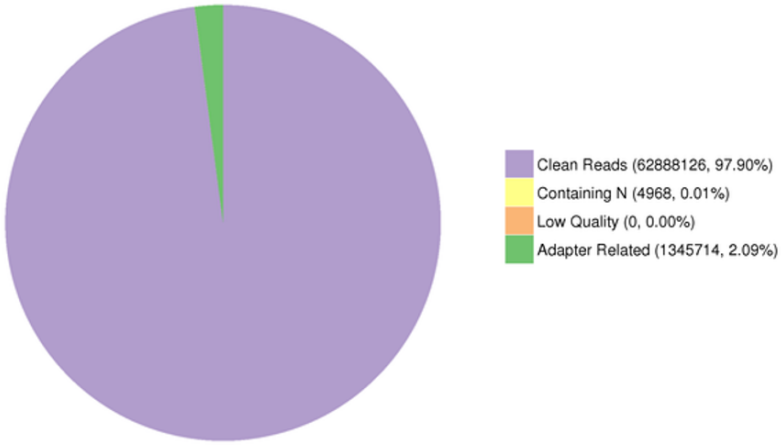

Figure 16. Example of a basic QC report delivered by the sequencing provider (Novogene Co., Ltd), representing the control sample C0h_1. Each color represents the percentage of filtered reads.

Filtered sequences from every sample were uploaded into *Galaxy* (https://usegalaxy.org/), which contains tools/programs used for data analysis (Afgan *et al.*, 2018). *Galaxy* was used for mapping filtered reads (in a FASTA format) to the olive tree reference genome, by using *Hierarchical Indexing for Spliced Alignment of Transcripts 2* [*HISAT2*, (Kim *et al.*, 2015)] tool. The reference genome was obtained from *National Center for Biotechnology Information* (NCBI BioProject PRJNA417827, https://www.ncbi.nlm.nih.gov/genome/10724?genome_assembly_id=352035). The output of *HISAT2* tool was a BAM file, where reads successfully mapped to the genome were identified. To count reads that aligned successfully to a gene, the tool *HTseq-count* was used

(Anders *et al.*, 2015), which uses the BAM file received from *HISAT2* tool and the GFF file of the olive tree reference genome. For obtaining the differentially expressed genes, the tool *DEseq2* (Love *et al.*, 2014) was used. In this tool, the files obtained from *HTseq-count* (the one that contains the number of reads that uniquely aligned to a gene) were inputted and two files were generated. The first file generates a table with the normalized counts for each gene and the fold change in $\log_2$. The second file includes principal component analysis (PCA) plots for results visualization. Genes with a significant change in gene expression ($|\log_2 FC| \geq 1$, where $\log_2 FC$ denotes $\log_2$(fold change); $p \leq 0.05$) were extracted using the tool *Filter data on any column using simple expressions* from Galaxy. To filter even more the results, we also used $|\log_2 FC| \geq 2.5$ cutoff. The web-based tool *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019) was used to discover genes molecular function and biological process. This tool maps the genes to known molecular functions, biological processes, and cellular components. The input of this tool is a BED file that contains the chromosome location of the gene (from the reference genome of *Olea europaea* var. *sylvestris*) and gene intervals (gene starts and where it ends). The output is a Manhattan plot where we can see the enriched terms obtained (and respective *p*-values), separated in three categories: molecular function (MF), biological process (BP) and cellular component (CC) (Raudvere *et al.*, 2019).

3.2 Results and Discussion

In order to identify genes that potentially may confer olive trees resistance/tolerance to anthracnose by microbial bioprotectants, a pot experiment was performed in which two-year-old olive trees were inoculated with the endophyte and then with the pathogen [Endophyte + Pathogen, EP], with the endophyte alone [Endophyte, E], or with the pathogen alone [Pathogen, P]. As controls, mock inoculations were performed [Control, C]. Leaves were collected at three different time points: 0 hpi, 48 hpi, 96 hpi. These time points were selected based on previous reports. In a study where cotton roots were inoculated with endophytic fungi and verticillium wilt causal agent (*V. dahliae*), a defense response was obtained just after 48 hpi (Yuan *et al.*, 2017). Specifically, three defensive genes (phenylalanine ammonia-lyase, *PAL*; polyphenol oxidase, *PPO*; peroxidase, *POD*) increased their expression in inoculated plants in comparison with control (non-inoculated

plants). When analyzing the mixed transcriptome of rice and *Magnaporthe oryzae* pathogen, the authors acknowledged that 24 hpi was an early stage of infection, as only 0.1–0.2% of preprocessed reads mapped to the fungal genome, and 61.5–62.4% mapped to the host rice genome (Kawahara *et al.*, 2012). Additionally, the mixed transcriptome of olive tree roots and *V. dahliae*, revealed a plant response just after two days of infection (Jiménez-Ruiz *et al.*, 2017). In this study, 918 DEGs were found at 2 days post inoculation (dpi) and 6871 DEGs at 7 dpi. Also, in *Colletotrichum fruticola* infection of strawberry plants, PTI and ETI responses were detected using three stages of infection (24, 72 and 96 hpi) (Zhang *et al.*, 2018). Therefore, in the present work, we collected leaves at 0, 48 and 96 hpi in order to evaluate gene expression at an early and late stages of *C. acutatum* infection.

After the collection of leaves, olive plants remained in the greenhouse for further two months, to evaluate visible anthracnose symptoms. Even in those olive plants only inoculated with *C. acutatum*, no symptoms of anthracnose were observed in the subsequent months, probably because anthracnose mainly manifests itself in fruits.

3.2.1 Preparation of RNA samples from olive leaves

For each treatment (EP, E, P, C) and time points (0h, 48h, 96h), four replicates were prepared (consisting of three leaves from three different plants from the same treatment). Following RNA extraction from all 64 leaf samples, RNA concentration and quality was checked by spectrophotometry (table 4). As nucleic acids mainly absorb at 260 nm, the ratios Abs260/Abs280 and Abs260/Abs230 give a clue about RNA purity. For RNA to be considered as pure, the ratios should be of approximately 2. For example, 16 (out of 64) samples resulted in low Abs260/Abs230 ratios, which could mean these samples were contaminated with carbohydrates or phenols that have absorbance near 230 nm. In any case, for RNA-seq purposes, RNA is acceptable if ratios are between 1.8 and 2.2 (for ratio Abs260/Abs280), or higher than 1.8 (for ratio Abs260/Abs230) (Novogene, 2021). According to these requirements, 14 samples were considered as not acceptable to proceed with RNA-seq (table 4). RNAs integrity was also checked by agarose gel electrophoresis. Undegraded RNA is distinguished by two rRNA bands (28S and 18S), whilst degraded RNA have a smeared appearance (representative gel in figure 17). From the 64 RNA samples, 22 had their RNA degraded (four of which had been previously considered as not

acceptable for RNA-seq). However, as the sequencing provider also determines the suitability of RNA samples for RNA-seq analysis, and for trying to sequence at least three replicates per treatment/time, 52 RNA samples were sent to the sequencing provider (Novogene). Following determination of RNA integrity number (RIN), only 17 samples presented a high quality and non-degraded RNA (RIN > 7). However, as Novogene considers that RIN values above 4 are acceptable for RNA-seq, 47 samples could be sequenced. Taking into consideration Novogene's evaluation (RIN values), 42 RNA samples proceeded to RNA-seq (table 4).

Table 4. Assessment of RNA concentration and quality (Abs260/Abs280 and Abs260/Abs230) determined by spectrophotometry and agarose gel electrophoresis. RNAs were described as Good if we could distinguish two rRNA bands (28S and 18S), acceptable (Acpt) if both bands were not so clear, and degraded (Degr) if a smear was observed. Samples that did not pass the requirements are represented in italics and those sequenced (Seq) in bold. C means control, E means Endophyte, P means Pathogen, and EP means Endophyte+Pathogen, at 0, 48 and 96 hours post inoculation.

Sample	Conc (ng/uL)	Abs260/Abs280	Abs260/Abs230	Gel	RIN	Seq
C0h_1	150.5	2.14	2.27	Acpt	7.70	**Yes**
C0h_2	325.6	1.94	*1.76*	*Degr*	-	No
C0h_3	341.2	*1.53*	*1.34*	*Degr*	-	No
C0h_4	101.3	2.06	2.01	Good	7.90	**Yes**
C48h_5	437.3	2.12	2.46	Acpt	6.00	**Yes**
C48h_6	304.6	2.13	2.37	*Degr*	5.80	**Yes**
C48h_7	145.1	2.17	2.37	Acpt	7.30	**Yes**
C48h_8	259.0	2.13	2.40	Good	7.80	**Yes**
C0h_9	-	-	-	*Degr*	-	No
C0h_10	-	-	-	*Degr*	-	No
C0h_11	63.3	2.01	*1.46*	Good	9.10	**Yes**
C0h_12	183.6	2.13	2.31	*Degr*	-	No
C96h_13	317.5	2.17	2.40	Acpt	6.90	**Yes**
C96h_14	258.1	2.11	2.29	Acpt	7.30	**Yes**
C96h_15	109.9	2.09	2.26	Good	6.70	**Yes**
C96h_16	129.4	2.15	2.46	Good	7.20	**Yes**
E0h_17	95.0	2.11	2.08	*Degr*	-	No
E0h_18	191.1	2.11	2.34	Acpt	5.90	**Yes**
E0h_19	87.6	2.10	2.32	*Degr*	-	No
E0h_20	249.2	2.06	2.07	Acpt	6.70	**Yes**
E48h_21	323.1	2.13	2.45	*Degr*	4.70	**Yes**
E48h_22	288.1	2.11	2.31	Good	6.60	**Yes**
E48h_23	187.0	2.20	2.53	*Degr*	-	No
E48h_24	301.6	2.14	2.48	Acpt	5.90	**Yes**
E0h_25	191.4	2.17	2.60	*Degr*	-	No
E0h_26	176.7	2.01	1.82	Good	7.30	**Yes**
E0h_27	139.5	2.06	1.96	*Degr*	-	No
E0h_28	157.9	2.14	2.29	Good	6.20	**Yes**
E96h_29	115.3	2.15	2.16	Acpt	5.10	**Yes**
E96h_30	137.0	2.03	*1.22*	Good	6.30	**Yes**
E96h_31	170.2	2.10	*1.67*	Acpt	4.80	**Yes**
E96h_32	98.9	2.06	*1.40*	Good	5.80	**Yes**
P0h_33	147.1	1.94	*1.46*	Good	7.30	No
P0h_34	161.9	1.99	*1.34*	Acpt	5.60	No
P0h_35	106.9	2.08	2.15	Good	6.60	**Yes**
P0h_36	72.2	1.94	1.89	Good	7.80	**Yes**
P48h_37	134.4	*2.23*	2.40	*Degr*	-	No
P48h_38	241.0	2.16	2.39	Acpt	4.20	**Yes**

Table 4. *Continuation.*

Sample	Conc (ng/uL)	Abs260/Abs280	Abs260/Abs230	Gel	RIN	Seq
P48h_39	155.5	2.16	2.38	*Degr*	-	No
P48h_40	37.2	2.18	2.59	Good	7.30	**Yes**
P0h_41	89.0	1.89	1.86	Good	7.10	No
P0h_42	118.3	2.10	2.38	Good	6.60	**Yes**
P0h_43	112.6	2.00	2.18	Good	5.40	**Yes**
P0h_44	160.7	1.97	2.02	*Degr*	4.20	No
P96h_45	221.4	2.17	2.49	*Degr*	5.00	**Yes**
P96h_46	186.2	2.06	*1.61*	Good	6.80	**Yes**
P96h_47	197.3	2.18	2.30	Acpt	5.70	**Yes**
P96h_48	162.8	2.13	2.30	Acpt	5.50	**Yes**
EP0h_49	112.8	2.14	2.51	*Degr*	3.20	No
EP0h_50	61.0	1.95	2.45	Good	7.30	**Yes**
EP0h_51	119.9	2.09	2.29	Good	6.40	**Yes**
EP0h_52	225.2	2.02	*1.64*	*Degr*	4.20	No
EP48h_53	165.4	2.13	2.38	Acpt	6.00	**Yes**
EP48h_54	138.9	2.17	1.98	*Degr*	2.50	No
EP48h_55	195.3	2.18	2.28	*Degr*	3.60	No
EP48h_56	212.5	2.13	2.73	Acpt	6.20	**Yes**
EP0h_57	166.4	2.06	1.89	*Degr*	4.30	No
EP0h_58	94.5	2.09	3.69	Acpt	5.50	No
EP0h_59	129.4	2.05	2.78	Good	7.70	**Yes**
EP0h_60	112.3	1.97	2.90	Good	6.50	**Yes**
EP96h_61	107.4	2.13	2.52	Good	7.30	**Yes**
EP96h_62	202.2	2.10	*1.67*	Good	7.70	**Yes**
EP96h_63	79.7	1.88	*1.37*	Good	9.00	**Yes**
EP96h_64	179.1	2.13	2.26	*Degr*	6.00	**Yes**

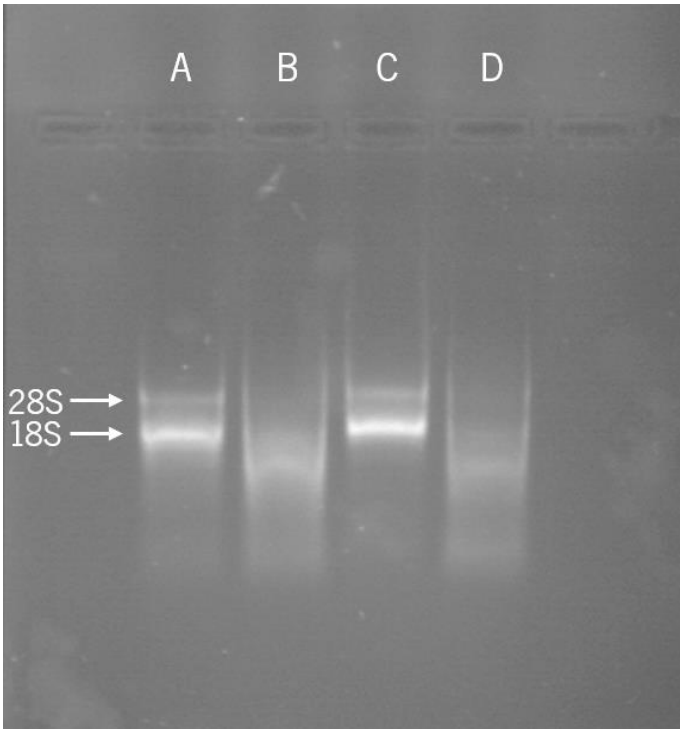

Figure 17. Example of RNA integrity assessment of extracted RNAs, by running an agarose gel electrophoresis (0.8% w/v). Representative samples correspond to: A - EP96h_61, B - EP96h_55, C - EP96h_63, D - EP96h_57. Undegraded RNA is distinguished by two rRNA bands (28S and 18S, white arrows). EP means Endophyte+Pathogen at 96 hours post inoculation.

The digestion of genomic DNA from RNA samples is usually recommended before and RNA-seq analysis (Zhao *et al.*, 2016). However, as RNA samples did not reveal contamination with genomic DNA, Novogene suggested there was no need for further purification.

3.2.2 Processing of Sequencing Data

The processing of sequencing data is summarized in table 5. The sequencing of the 42 RNA samples from treated olive plants resulted in 142,034,198 to 60,092,390 raw pair-ended reads per sample. The sequencing quality was very high, as Q20(%) scores ranged from 98.12% to 97.71%, and Q30(%) scores ranged from 94.43% to 93.52%.

Table 5. Processing of sequencing data. The total number of raw reads and percentage of clean reads obtained per sample is provided. The quality of sequencing is evidenced by the average base error rate (error %), the base quality scores [Q20 (error base of 1/100, 99%) and Q30 (error base of 1/1000, 99.9%)] and guanine-cytosine contents (GC %). C means control, E means Endophyte, P means Pathogen, and EP means Endophyte+Pathogen, at 0, 48 and 96 hours post inoculation.

Sample	Raw reads	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
C0h_1	64,238,808	97.90	0.03	97.90	93.92	43.85
C0h_4	60,092,390	98.52	0.02	98.02	94.21	44.45
C0h_11	60,735,794	97.73	0.03	97.71	93.52	44.63
C48h_5	65,187,870	97.23	0.03	97.95	94.02	43.13
C48h_6	64,628,610	96.94	0.03	97.90	93.91	43.43
C48h_7	64,057,944	98.67	0.03	98.01	94.14	43.60
C48h_8	65,488,590	98.68	0.03	97.82	93.73	43.16
C96h_13	62,937,604	97.72	0.02	98.07	94.35	43.34
C96h_14	105,224,844	98.10	0.03	97.92	94.00	48.12
C96h_15	109,694,910	98.12	0.03	97.95	94.13	48.60
C96h_16	63,327,204	97.98	0.03	97.98	94.12	43.36
E0h_18	140,504,872	97.19	0.03	97.93	94.21	50.55
E0h_20	63,630,964	98.94	0.03	97.97	94.17	44.74
E0h_26	63,466,484	99.09	0.03	97.86	93.90	44.19
E0h_28	63,430,250	99.07	0.02	98.04	94.19	45.18
E48h_21	62,851,182	99.18	0.03	97.83	93.76	44.19
E48h_22	64,999,858	98.94	0.03	97.92	93.98	43.66
E48h_24	63,915,434	99.24	0.03	97.96	94.03	44.02
E96h_29	64,875,636	98.68	0.02	98.06	94.31	43.69
E96h_30	138,061,942	97.80	0.03	97.87	94.06	48.59
E96h_31	63,909,026	99.05	0.02	98.08	94.32	44.16
E96h_32	63,562,170	98.74	0.02	98.12	94.38	44.23
P0h_35	84,452,466	98.62	0.02	98.00	94.20	46.79
P0h_36	101,014,424	98.35	0.02	98.07	94.43	48.28
P0h_42	92,449,514	98.72	0.02	98.03	94.31	47.34
P0h_43	131,366,826	98.25	0.03	97.86	94.04	49.67
P48h_38	73,662,082	98.97	0.02	98.00	94.18	44.49
P48h_40	66,057,646	98.89	0.02	98.10	94.36	44.51
P96h_45	62,160,360	98.95	0.03	97.88	93.93	44.40
P96h_46	64,366,420	99.02	0.03	97.98	94.12	44.39
P96h_47	63,994,098	99.17	0.03	97.95	93.98	44.52
P96h_48	63,045,112	99.10	0.03	97.79	93.76	44.35
EP0h_50	64,270,852	98.73	0.03	97.93	94.04	45.38
EP0h_51	62,871,964	98.84	0.03	97.98	94.17	44.54
EP0h_59	81,763,746	98.38	0.03	97.93	94.12	46.59
EP0h_60	142,034,198	98.17	0.03	97.90	94.15	53.62
EP48h_53	63,847,884	99.10	0.03	97.76	93.64	44.74
EP48h_56	64,296,902	99.03	0.03	97.94	94.06	44.78
EP96h_61	62,840,918	98.79	0.02	98.12	94.42	43.68
EP96h_62	66,753,768	98.39	0.03	97.99	94.13	43.16
EP96h_63	66,246,288	98.79	0.03	97.86	93.82	43.95
EP96h_64	64,898,274	98.81	0.03	97.91	94.00	44.75

For assigning a specific genome location, reads were mapped to the olive tree reference genome of *Olea europaea* var. *sylvestris* (NCBI, BioProject PRJNA417827). The percentage of mapped reads varied between 94.80% and 86% in different samples (table 6).

Table 6. Information about read mapping and alignment to olive annotated genes. The number and percentage of clean reads (after filtering), mapped reads (cleaned reads that successfully mapped with the olive tree genome) and aligned reads (mapped reads that aligned to olive annotated genes) is displayed. The percentage of mapped reads refers to the number of cleaned reads, while the percentage of aligned reads refers to the number of mapped reads. C means control, E means Endophyte, P means Pathogen, and EP means Endophyte+Pathogen, at 0, 48 and 96 hours post inoculation.

Sample	Cleaned reads	Mapped reads (%)	Aligned reads (%)
C0h_1	62,889,793	58,038,020 (87.2%)	46,120,518 (79.5%)
C0h_4	59,203,023	55,525,149 (88.5%)	43,929,191 (79.1%)
C0h_11	59,357,091	56,204,752 (88.1%)	42,710,766 (76%)
C48h_5	63,382,166	58,823,622 (87.1%)	47,167,951 (80.2%)
C48h_6	62,650,975	58,418,846 (87.3%)	47,082,101 (80.6%)
C48h_7	63,205,973	58,367,191 (88.2%)	46,817,287 (80.2%)
C48h_8	64,624,141	59,406,357 (87.9%)	47,391,800 (79.8%)
C96h_13	61,502,627	57,130,860 (87.2%)	45,511,649 (79.7%)
C96h_14	103,225,572	110,000,321 (89.8%)	67,396,366 (61.3%)
C96h_15	107,632,646	121,687,773 (92.2%)	74,957,097 (61.6%)
C96h_16	62,047,994	57,435,483 (87.4%)	46,168,998 (80.4%)
E0h_18	136,556,685	155,111,334 (89.7%)	85,346,488 (55.0%)
E0h_20	62,956,476	56,980524 (86%)	43,933,452 (77.1%)
E0h_26	62,888,939	59,342,204 (89%)	45,843,389 (77.3%)
E0h_28	62,840,349	58,447,526 (88.5%)	45,313,488 (77.5%)
E48h_21	62,335,802	56,888,683 (87.8%)	45,853,403 (80.6%)
E48h_22	64,310,860	59,703,504 (88.8%)	48,004,456 (80.4%)
E48h_24	63,429,677	58,114,519 (88.2%)	46,847,842 (80.6%)
E96h_29	64,019,278	58,392,091 (86.6%)	46,292,764 (79.3%)
E96h_30	135,024,579	152,213,385 (91.8%)	94,637,541 (62.2%)
E96h_31	63,301,890	57,432,629 (87.1%)	45,569,066 (79.3%)
E96h_32	62,761,287	57,766,560 (87.9%)	46,549,332 (80.5%)
P0h_35	83,287,022	81,183,816 (88.8%)	56,313,309 (69.4%)
P0h_36	99,347,686	100,442,377 (88.7%)	62,724,523 (62.4%)
P0h_42	91,266,160	94,170,747 (89.2%)	60,385,419 (64.1%)
P0h_43	129,067,907	137,929,337 (89.7%)	80,102,802 (58.1%)
P48h_38	72,903,363	67,915,699 (88.5%)	52,605,189 (77.5%)
P48h_40	65,324,406	61,512,870 (88.4%)	46,499,536 (75.6%)
P96h_45	61,507,676	56,164,281 (87%)	44,481,473 (79.2%)
P96h_46	63,735,629	58,996,929 (88.7%)	47,765,829 (81%)
P96h_47	63,462,947	58,952,051 (88.7%)	47,137,334 (80%)
P96h_48	62,477,706	57,269,015 (87.9%)	44,950,929 (78.5%)
EP0h_50	63,454,612	59,819,825 (87.8%)	43,961,846 (73.5%)
EP0h_51	62,142,649	58,318,212 (88%)	43,625,873 (74.8%)
EP0h_59	80,439,173	79,076,771 (88.5%)	54,715,511 (69.2%)
EP0h_60	139,434,972	183,946,606 (94.8%)	88,726,027 (48.2%)
EP48h_53	63,273,253	58,321,310 (88%)	45,734,032 (78.4%)
EP48h_56	63,673,222	59,772,193 (89.1%)	47,023,402 (78.7%)
EP96h_61	62,080,543	56,945,804 (86.9%)	44,875,098 (78.8%)
EP96h_62	65,679,032	61,232,601 (88.2%)	49,206,308 (80.4%)
EP96h_63	65,444,708	60,652,012 (88.3%)	47,819,240 (78.8%)
EP96h_64	64,125,985	59,715,245 (88.5%)	47,863,182 (80.2%)

After mapping, the number of reads that aligned to specific genes (table 6) was counted, using the tool *HTseq-count* that generates two files. The first file shows the number of reads that uniquely aligned to a specific gene (figure 18.A), while the second file shows the reads that did not aligned (figure 18.B). Detailed information of alignment process per sample is provided in Annex.

A

Geneid	Number of reads
gene-LOC111365227	10
gene-LOC111365228	853
gene-LOC111365229	0
gene-LOC111365230	297
gene-LOC111365231	0
gene-LOC111365232	0
gene-LOC111365233	26
gene-LOC111365234	461

B

Category	Number of reads
__no_feature	1189034
__ambiguous	442941
__too_low_aQual	2282171
__not_aligned	4257286
__alignment_not_unique	3746070

Figure 18. Representation of the output obtained from *HTseq-count* for C0h_1 sample. The number of reads that mapped to an annotated gene (A) and the number of reads with alignment problems (B) are presented. Non-aligned reads are divided in five categories: _no_feature (reads that could not be aligned to a gene); _ambiguous (reads that could align to more than one gene); _too_low_aQual (reads whose alignment quality was low); _not_aligned (reads from the BAM file that do not align); and _alignment_no_unique (reads with more than one reported alignment).

3.2.3 Gene expression level analysis

To evaluate the overall gene expression of different samples, a principal component analysis (PCA) was performed (figure 19). In this analysis, the samples obtained from 48 hpi were not included, as only two biological replicates from [E 48h] and [EP 48h] were obtained. The main detected difference was concerned with the samples collected in distinct time points (0h *vs.* 96h), in which almost all treatments after 96 hpi clustered together. This suggests that after 96h plants present more similar global gene expression than at the moment of pathogen spraying.

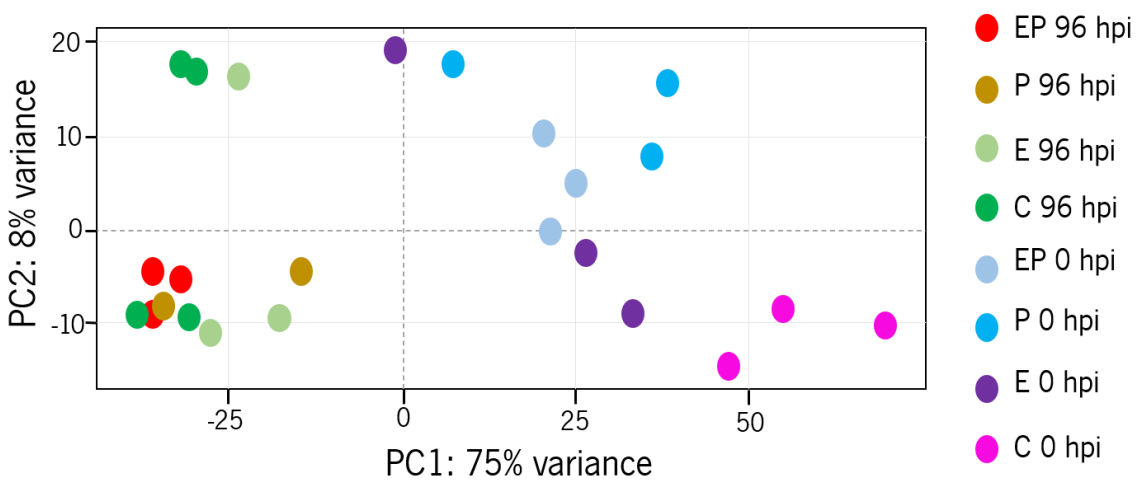

Figure 19. Principal component analysis (PCA) of gene expression levels in leaves from olive plants that had been treated with both Endophyte and Pathogen (EP), with the Endophyte (E), with the Pathogen (P), or with a buffer solution (C), after 0 hpi or 96 hpi. Symbol color indicates different treatments/collection times.

Significant differential expressed genes (DEGs) were obtained after filtering the results for genes displaying a $|\log_2 FC| \geq 1$ (with a *p*-value below 0.05). The number of down-regulated genes is almost always higher that the number of up-regulated ones (table 7).

Table 7. Number of DEGs with $|\log_2 FC| \geq 1$ and $|\log_2 FC| \geq 2.5$ (including up-regulated and down-regulated) between compared groups, where EP means olive plants treated with Endophyte and Pathogen, E treated with Endophyte, P treated with Pathogen, and C means Control.

Time	Compared Groups	$\|\log_2 FC\| \geq 1$	Up	Down	$\|\log_2 FC\| \geq 2.5$	Up	Down
0 hpi	E *vs.* C	2634	800	1834	125	46	79
	P *vs.* C	1815	621	1194	140	87	53
	EP *vs.* C	2350	633	1717	190	15	175
	E *vs.* P	20	2	18	0	0	0
	E *vs.* EP	12	2	10	0	0	0
	EP *vs.* P	46	1	45	0	0	0
96 hpi	E *vs.* C	239	106	133	0	0	0
	P *vs.* C	95	47	48	0	0	0
	EP *vs.* C	1	1	0	0	0	0
	E *vs.* P	120	20	100	0	0	0
	E *vs.* EP	112	62	50	0	0	0
	EP *vs.* P	4	2	2	0	0	0

The most intriguing result was the greater differences of gene expression detected between treatments/samples and controls (at 0 hpi) compared to 96 hpi. This was corroborated by the comparison on DEGs along time in each treatment (table 8), always revealing a high number of DEGs, even higher than differences among treatments. Altogether, the results point to an attenuation of global gene expression differences along time, as revealed by the reduced number of DEGs between treatments/samples at 96 hpi. Interestingly, the comparison among treatments (E, P and EP) did not result in such a great number of DEGs (table 7), even though the levels of gene expression in the same treatments/samples did not cluster together (figure 19). For better understanding gene expression changes during treatments with Endophyte, Pathogen and Endophyte+Pathogen, the obtained results will be separately analyzed.

Table 8. Number of DEGs with $|\log_2 FC| \geq 1$ and $|\log_2 FC| \geq 2.5$ (including up-regulated and down-regulated) between compared groups, where EP means Endophyte+Pathogen, E means Endophyte, P means Pathogen, and C means Control. For example, in the first group [EP 0-96h], the first treatment (EP0h) has 3308 up-regulated genes compared to the second treatment (EP96h), when $|\log_2 FC| \geq 1$.

Compared Groups	$\|\log_2 FC\| \geq 1$	Up	Down	$\|\log_2 FC\| \geq 2.5$	Up	Down
EP (0 *vs.* 96h)	4535	3308	1227	1028	1007	21
E (0 *vs.* 96h)	1873	1289	584	181	176	5
P (0 *vs.* 96h)	4072	3212	860	871	857	14
C (0 *vs.* 96h)	7917	5669	2248	3422	3179	243

3.2.3.1 Gene expression following endophyte treatment

Whenever the plants were treated with the endophyte (E and EP), there was a high difference in DEGs compared to the corresponding controls at 0 hpi. Since all plants were similarly cut at -48h, this difference in gene expression could be mainly related to the endophyte inoculation.

Up-regulation of genes induced by the endophyte

The most up-regulated genes ($\log_2 FC \geq 2.5$) induced by the endophyte, were detected by comparing [E0h *vs.* C0h] and [EP0h *vs.* C0h] samples and are listed in table 9.

Table 9. Up-regulated genes ($log_2FC \geq 2.5$) induced by the endophyte in both compared groups [E *vs.* C] and [EP *vs.* C], at 0h (corresponding to 48h following endophyte inoculations). The respective molecular function (MF) and biological process (BP) were obtained from *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019). Whenever *g:GOSt* tool was unable to find results for MF or BP, information obtained from UniProt was added (indicated by insertion of the accession number of searched protein).

Gene ID	Protein name	MF	BP	log_2FC	
				[E *vs.* C]	[EP *vs* C]
LOC111410277	terpene synthase 10-like, partial	terpene synthase activity; carbon-oxygen lyase activity, acting on phosphates	-	3.03	2.63
LOC111395219	(3S,6E)-nerolidol synthase 1-like	terpene synthase activity; carbon-oxygen lyase activity, acting on phosphates	diterpenoid biosynthetic process	2.94	2.62
LOC111392322	monothiol glutaredoxin-S2-like (UniProtKB - Q8L8Z8)	glutathione oxidoreductase activity	negative regulation of transcription by RNA polymerase II	2.90	3.25
LOC111384357	amino acid transporter AVT1H	-	amino acid transport	2.82	2.74
LOC111393432	adenylate isopentenyltransferase 5, chloroplastic-like (UniProtKB - Q94ID2)	ATP binding	cytokinin biosynthetic process	3.13	2.59
LOC111405392	uncharacterized protein	-	-	3.86	2.67

Up-regulation of genes related with plant defense responses

By using the web-based tool *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019), the molecular function and biological process of each gene was found. Two genes - *TERPENE SYNTHASE 10-LIKE, PARTIAL* and *(3S,6E)-NEROLIDOL SYNTHASE 1-LIKE* - present "terpene synthase activity" and "carbon-oxygen lyase activity, acting on phosphates" as molecular functions, suggesting that both are involved in terpenes synthesis. Therefore, the endophyte *P. aff. commune* seems to induce terpene production in olive plants, which is a common plant response upon abiotic or biotic stresses (Shrivastava *et al.*, 2015). For example, endophytic [*Beauveria bassiana*] and arbuscular [*Rhizophagus intraradices*] fungi, are reported to enhance monoterpenes and sesquiterpenes levels in tomato plants (Shrivastava *et al.*, 2015). Also, when

the larvae beet armyworm (*Spodoptera exigua* Hübner) fed on inoculated tomato plants, the larvae had less weight than larvae that fed on non-inoculated tomato plants, implying that inoculated tomato plants had a stronger defense response probably due to the higher levels of terpenoids (Shrivastava *et al.*, 2015).

The endophyte *P. aff. commune* also induced the expression of *MONOTHIOL GLUTAREDOXIN-S2-LIKE* gene (coding for an enzyme involved in reducing GSH-thiol disulfides) in olive plants, thus suggesting the activation of oxidative stress responses by the olive plant. Glutaredoxins are oxidoreductases known to be involved in defense against oxidative stress, being specifically implicated in the reduction of peroxides and dehydroascorbate (Rouhier *et al.*, 2008).

Up-regulation of genes related with signaling processes

The endophyte induced the expression of the *AMINO ACID TRANSPORTER AVT1H* gene in the host plant. Amino acids are an important source of nitrogen and are transported intra- and intercellularly through transporter proteins (Yang *et al.*, 2020). Once pathogens attack plants, they mobilize host nutrients and manipulate the host machinery for their own benefit. Therefore, fungi are suggested to induce plant genes for amino acid transport as a strategy for better exploiting host-derived amino acids (Sonawala *et al.*, 2018). Furthermore, changing the expression of amino acids transporters might influence the plant defense response. A study showed that overexpression of cationic amino acid transporter 1 (*CAT1*) enhanced the resistance of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae* through a constitutively activated SA pathway (Yang *et al.*, 2014). The perception of disturbances in the amino acid compartmentation, fluxes, or/and content are currently thought to trigger the onset of plant defense mechanisms (Guo *et al.*, 2021).

Up-regulation of genes related with plant development

P. aff. commune inoculation could also induce the production of cytokinin in olive plant, as there was an over-expression of *ADENYLATE ISOPENTENYLTRANSFERASE 5, CHLOROPLASTIC-LIKE* gene. Indeed, endophytic fungi can produce cytokinin and other plant hormones (ethylene and auxin) to enhance host plant growth and improve plant nitrogen use (Li *et al.*, 2018).

Down-regulation of genes induced by the endophyte

The most down-regulated genes ($log_2FC \leq -2.5$) after 48 h following endophyte inoculation resulted from the comparison among [E0h *vs.* C0h] and [EP0h *vs.* C0h] samples and are listed in table 10.

Table 10. Down-regulated genes ($log_2FC \leq -2.5$) in endophyte-treated plants in both compared groups [E0h *vs.* C0h] and [EP0h *vs.* C0h], at 0h (corresponding to 48h following endophyte inoculations). The respective molecular function (MF) and biological process (BP) were obtained from *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019). Whenever *g:GOSt* tool was unable to find results for MF or BP, information obtained from UniProt was added (indicated by insertion of the accession number of searched protein).

Gene ID	Protein name	MF	BP	log_2FC	
				[E *vs.* C]	[EP *vs.* C]
LOC111383433	metalloendoproteinase 4-MMP-like	metalloendopeptidase activity	collagen catabolic process	-3.63	-3.71
LOC111394214	endoglucanase 17 isoform X2 (UniProtKB - O81416)	hydrolase activity, hydrolyzing O-glycosyl compounds	cellulose catabolic process	-3.53	-2.95
LOC111379546	probable pectate lyase 1, partial (UniProtKB - Q940Q1)	pectate lyase activity	pectin catabolic process	-2.50	-3.95
LOC111398900	expansin-A1-like	-	plant-type cell wall organization or biogenesis	-2.75	-3.06
LOC111369928	fasciclin-like arabinogalactan protein 12 (UniProtKB - Q8LEE9)	-	plant-type secondary cell wall biogenesis	-2.58	-3.22
LOC111367834	CASP-like protein 3A1 (UniProtKB - Q5JM57)	4 iron, 4 sulfur cluster binding	-	-2.79	-2.64
LOC111367162	transcription factor MYB16-like (UniProtKB - Q9LXF1)	DNA binding	regulation of cutin biosynthetic process	-2.68	-3.17

Table 10. *Continuation*.

Gene ID	Protein name	MF	BP	log_2FC	
				[E *vs.* C]	[EP *vs.* C]
LOC111399217	disease resistance response protein 206-like (UniProtKB - P13240)	-	defense response	-5.19	-4.17
LOC111382402	protein SODIUM POTASSIUM ROOT DEFECTIVE 2-like (UniProtKB - Q58FZ0)	metal ion binding	-	-2.52	-2.75
LOC111386197	BTB/POZ domain-containing protein At3g19850-like, partial (UniProtKB - Q9LT24)	-	protein ubiquitination	-2.79	-2.64
LOC111373998	probable auxin efflux carrier component 1b (UniProtKB - P0C0X5)	auxin efflux transmembrane transporter activity	auxin-activated signaling pathway	-2.68	-2.82
LOC111376016	acid phosphatase 1-like (UniProtKB - P27061)	acid phosphatase activity	-	-2.72	-3.13
LOC111378100	probable acyl-activating enzyme 12, peroxisomal (UniProtKB - Q9SS00)	ligase activity	fatty acid metabolic process	-2.63	-3.17
LOC111369510	sucrose synthase 7-like	sucrose synthase activity	sucrose metabolic process	-2.91	-2.75
LOC111404909	uncharacterized protein	-	-	-2.80	-3.43
LOC111404850	uncharacterized protein	-	-	-2.62	-2.68

Down-regulation of genes related with plant cell wall modifications

Most down-regulated genes code proteins involved in plant cell wall and extracellular matrix modifications, namely *METALLOENDOPROTEINASE 4-MMP-LIKE*, *ENDOGLUCANASE 17 ISOFORM X2*, *PROBABLE PECTATE LYASE 1, PARTIAL*, *EXPANSIN-A1-LIKE*, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12*, *CASP-LIKE PROTEIN 3A1*, and *TRANSCRIPTION FACTOR MYB16*.

In *A. thaliana*, matrix metalloproteinases (At-MMPs) are active proteases involved in the extracellular matrix modification/degradation, as well as in extracellular and intracellular signaling pathways during plant growth (Marino *et al.*, 2014). The involvement of *At-MMPs* in *A. thaliana* defense against necrotrophic fungus *Botrytis cinerea* was tested, revealing that overexpression of *At2-MMP* enhanced disease resistance to the fungus (Zhao *et al.*, 2017). In addition, those plants exhibited higher level of callose deposition, which is characteristic of pattern-triggered immunity (PTI), therefore suggesting that *At-MMPs* contribute to PTI. Also, *A. thaliana* roots were inoculated with a beneficial root fungal endophyte (*Serendipita indica*), and there was no induction of *At-MMPs* (Zhao *et al.*, 2017). In the present work, *METALLOENDOPROTEINASE 4-MMP-LIKE* was down-regulated, suggesting that there was no callose deposition nor activation of PTI upon endophyte inoculation.

The product of *ENDOGLUCANASE 17 ISOFORM X2* gene is involved in the breakdown of cellulose. This enzyme, also known as *endo-1,4-β-glucanase 17* (UniProtKB - O81416) is involved in maintaining or degrading the shape of plant or fungal cell walls (Glass *et al.*, 2015). In the present work, *ENDOGLUCANASE 17 ISOFORM X2* is down-regulated, suggesting that there is no breakdown of plants cell wall cellulose, making the entry of the endophyte more difficult.

After inoculation with the endophyte, the olive plants showed a down-regulation of *PROBABLE PECTATE LYASE 1, PARTIAL*, suggesting that *P. aff. commune* did not induce the breaking of pectin on olive plants cell walls. Pectate lyases are enzymes that break pectin, which maintains plants cell wall integrity (Yang *et al.*, 2018). However, when wheat seeds were inoculated with the endophytic bacteria *Klebsiella oxytoca* VN13, there was an increase in pectate lyase levels, suggesting that bacteria colonization occurs via lysed pectin layers (Kovtunovych *et al.*, 1999).

Both *EXPANSIN-A1-LIKE* and *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12* are also involved in plant-type secondary cell wall biogenesis. A study where cotton seedling roots were inoculated with the endophytic bacterium *Bacillus amyloliquefaciens* showed an up-regulation of

genes encoding fasciclin-like arabinogalactan and expansin proteins, which are both involved in cell division, growth, and structure (Irizarry & White, 2018). More specifically, expansins are involved in the loosening of cell wall components, while fasciclin-like arabinogalactans are involved in cellular adhesion. However, in this work, both *EXPANSIN-A1-LIKE* and *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12* were down-regulated, suggesting that *P. aff. commune* restricts olive plants cell growth.

Similarly, a gene encoding a Casparian strip membrane protein (*CASP-LIKE PROTEIN 3A1*) that possesses a putative [Fe_4S_4] cluster-binding motif, was also down-regulated in olive plants during endophyte inoculation. These proteins are expressed in the root endodermis, in order to direct lignin deposition, but can also be involved in the plant response to pathogens by recruiting cell wall modifying enzymes (Lee *et al.*, 2019). In contrast with the present work, *Bacillus* PGPR induced the expression of *CASP-like 4D1* (gene that encodes a transmembrane protein involved in plant growth) in inoculated banana plants, and seems to be involved in the construction of the plant cell wall (Gamez *et al.*, 2019).

In addition to genes directly involved in plant cell wall modification, a transcription factor involved in cuticle formation was also down-regulated. Indeed, the *TRANSCRIPTION FACTOR MYB16* is involved in the biosynthesis of cutin, a polymer that alongside waxes forms the cuticle (Nawrath, 2006; Yeats & Rose, 2013). A study revealed that endophytic fungi inoculated in *Salvia miltiorrhiza*, induced the expression of three genes encoding MYB transcription factors, but three other genes encoding MYB transcription factors were down-regulated in the initial stage of interaction (Jiang *et al.*, 2019). Plant cuticles protects plants from abiotic and biotic stresses, and down-regulation of *TRANSCRIPTION FACTOR MYB16-like* could mean a reduction on cutin production.

Down-regulation of genes related with plant defense responses

Other down-regulated genes code proteins involved in plant defense processes, namely *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE*, *PROTEIN SODIUM POTASSIUM ROOT DEFECTIVE 2-LIKE*, and *BTB/POZ DOMAIN-CONTAINING PROTEIN At3g19850-LIKE, PARTIAL*. According to UniProt, *DISEASE RESISTANCE RESPONSE PROTEIN 206* seems to be involved in

the production of lignans, low molecular weight polyphenols implicated in plant defense protection against herbivores and microorganisms (Hano *et al.*, 2021). Accordingly, the induction of lignan-synthesis related genes have been described upon fungal exposure as a plant defense response against fungi (Seneviratne *et al.*, 2015). In the present work, *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE* was down-regulated in endophyte-treated plants, which could suggest that olive plants did not recognize *P. aff. commune* as a threat and did not increase lignan production.

The gene *PROTEIN SODIUM POTASSIUM ROOT DEFECTIVE 2* encodes a metal-binding protein, also called Heavy metal-associated plant protein 3. When exposed to toxic metals, *Arabidopsis arenosa* inoculated with the endophytic fungus *Mucor* sp. up-regulated the gene coding for this protein (Rozpądek *et al.*, 2018). This led to a reduction of metal accumulation. In the present work, *PROTEIN SODIUM POTASSIUM ROOT DEFECTIVE 2-LIKE* was down-regulated, which could suggest a lower tolerance to abiotic stressors (specifically to toxic metals).

BTB/POZ domain-containing proteins are components of E3 Ub ligases that mediate ubiquitination and degradation of immune regulators, thus acting as a negative regulator in plant defenses (Zhao *et al.*, 2022). There are no reports about endophyte induction or repression of this gene, but the ubiquitin system has been described as crucial for the regulation of plant responses to pathogens (Wang *et al.*, 2022). In the present work, down-regulation of *BTB/POZ DOMAIN-CONTAINING PROTEIN At3g19850-LIKE, PARTIAL* suggests an activation of plant defense responses.

Down-regulation of genes related with plant metabolism and development

Genes coding for proteins involved in plant metabolism and development were also down-regulated upon endophyte inoculation, namely *PROBABLE AUXIN EFFLUX CARRIER COMPONENT 1B*, *ACID PHOSPHATASE 1-LIKE*, *PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL,* and *SUCROSE SYNTHASE 7-LIKE*.

In the present work, a gene coding for a *PROBABLE AUXIN EFFLUX CARRIER COMPONENT 1B* was down-regulated. This protein is involved in the transmembrane transport of auxin (Ortiz *et al.*, 2019). A study revealed that the endophytic fungus *Chaetomium cupreum* inoculated in *Eucalyptus globulus* induced the overexpression of auxin production-related genes, which are involved in plant growth promotion (Ortiz *et al.*, 2019). One of those genes was an *AUXIN*

EFFLUX CARRIER COMPONENT 2, which transports auxin between different cells and tissues of the plant, promoting cell elongation and plant growth. In contrast, a *PROBABLE AUXIN EFFLUX CARRIER COMPONENT 1B* was down-regulated in the present work, suggesting a limitation of olive plant growth promoted by *P. aff. commune*.

ACID PHOSPHATASE 1-LIKE possesses acid phosphatase activity. Acid phosphatase enzymes are usually induced by plants upon inorganic phosphate limitation (Wang *et al.*, 2011). A study showed that *Achnatherum sibiricum* inoculated with endophytic fungi from *Neotyphodium* genus, significantly improved acid phosphatase activity in conditions of phosphorus deficiency (Li *et al.*, 2012). This led to higher concentration of phosphorus in roots, and a higher biomass of *A. sibiricum* inoculated with endophytes compared to non-inoculated. In the present work, *ACID PHOSPHATASE 1-LIKE* was down-regulated, suggesting that *P. aff. commune* did not induce phosphorus metabolism in olive plants.

PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL can act as a ligase by joining acid—thiol, activating carboxylic acids and forming acyl-CoAs. Acyl-activating enzymes are involved in the biosynthesis of amino acids and secondary metabolites, including compounds such as JA (Cheng *et al.*, 2018). There are no reports about endophytes inducing or repressing *acyl-activating enzymes*, but in this work *PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL* was down-regulated probably leading to a lesser production of specific compounds.

SUCROSE SYNTHASE 7-LIKE is involved in sucrose metabolic process. The endophytic fungus *Serendipita indica* alters sugar pools by changing the gene expression of sucrose synthases in *A. thaliana* (Opitz *et al.*, 2021). Through the activity of this enzyme, the fungus increases its own carbohydrate supply (fructose and UDP glucose). In the present work, *SUCROSE SYNTHASE 7-LIKE* is down-regulated, suggesting the inability of *P. aff. commune* to alter plant sucrose contents.

Considerations about gene expression upon endophyte inoculation

In summary, there were more down-regulated (16) DEGs in endophyte-treated plants than up-regulated (6). The up-regulated genes in plants, induced 48h after being inoculated by the endophyte (corresponding to the so-called “0 hpi”), seem to be involved in plant defense responses, either by producing secondary metabolites (terpenes), reducing oxidative stress (through the activity of glutaredoxins), and/or triggering plant defense mechanisms (disturbing

amino acid balance). The induction of plant defenses is also corroborated by the down-regulation of a negative regulator of defense responses. However, the possible production of plant signals for PTI (through metalloproteinases enzymes, such as *METALLOENDOPROTEINASE 4-MMP-LIKE*) could be affected after endophyte inoculation since the corresponding genes were down-regulated after endophyte inoculation. Other genes involved in plant stress responses (*DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE* and *PROTEIN SODIUM POTASSIUM ROOT DEFECTIVE 2-LIKE*) were also down-regulated after inoculation, suggesting that the endophyte does not seem to trigger plant stress responses.

The results also suggest that plant cell wall seems to be preserved after endophyte inoculation, as many genes for degrading cell wall enzymes are down-regulated (*ENDOGLUCANASE 17 ISOFORM X2* and *PROBABLE PECTATE LYASE 1, PARTIAL*). Also, the down-regulation of genes involved on plant cell wall synthesis (*EXPANSIN-A1-LIKE*, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12*, and *CASP-LIKE PROTEIN 3A1*) suggests the limitation in plant cell growth after endophyte inoculation. This is further corroborated by the down-regulation of an auxin transporter (*PROBABLE AUXIN EFFLUX CARRIER COMPONENT 1B*) and a MYB transcription factor involved in cuticle formation. Contrasting with these indications, cytokinin synthesis seem to be induced.

Finally, the host plant gene expression seems also to be manipulated by the endophyte for facilitating amino acid availability (activation of amino acid transporter). However, the endophyte does not seem to affect plant metabolism, including phosphorus metabolism (*ACID PHOSPHATASE 1-LIKE*), production of secondary metabolites (*PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL*), and sucrose levels (*SUCROSE SYNTHASE 7-LIKE*).

3.2.3.2 Gene expression following pathogen treatment

Concerning all plants infected with *C. acutatum* (P and EP), the difference in gene expression compared to the control allows to determine the impact of pathogen inoculation.

Up-regulation of genes induced by the pathogen

The genes induced ($log_2FC \geq 2.5$) in both compared groups [P0h *vs.* C0h] and [EP0h *vs.* C0h] are listed in table 11.

Table 11. Up-regulated genes ($log_2FC \geq 2.5$) induced by the pathogen in both compared groups [P0h *vs.* C0h] and [EP0h *vs.* C0h], at 0h. The respective molecular function (MF) and biological process (BP) obtained from *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019). Whenever *g:GOSt* tool was unable to find results for MF or BP, information obtained from UniProt was added (indicated by insertion of accession number of searched protein). Those genes that were similarly induced following endophyte inoculation (cf. Table 9) are underlined.

Gene ID	Protein name	MF	BP	log_2FC	
				[P *vs.* C]	[EP *vs.* C]
LOC111385774	endoglucanase-like (UniProtKB - Q0V7W1)	cellulase activity	cellulose catabolic process	2.62	3.05
LOC111410277	terpene synthase 10-like, partial	terpene synthase activity; carbon-oxygen lyase activity, acting on phosphates	-	2.89	2.63
LOC111392322	monothiol glutaredoxin-S2-like (UniProtKB - Q8L8Z8)	glutathione oxidoreductase activity	negative regulation of transcription by RNA polymerase II	2.73	3.25
LOC111368833	dehydration-responsive element-binding protein 1E-like	DNA-binding transcription factor activity	-	3.97	3.07
LOC111393432	adenylate isopentenyltransferase 5, chloroplastic-like (UniProtKB - Q94ID2)	ATP binding	cytokinin biosynthetic process	2.79	2.59
LOC111405392	uncharacterized protein	-	-	3.91	2.67
LOC111390994	uncharacterized protein	-	-	5.29	3.65
LOC111381653	uncharacterized protein	-	-	2.54	2.77

Up-regulation of genes related with plant cell wall modifications

The inoculation of olive plants with the pathogen induced the expression of an *ENDOGLUCANASE-LIKE* gene. As previously referred, endoglucanases are involved in the breakdown of cellulose, through the endohydrolysis of (1->4)-β-D-glucosidic linkages in cellulose. These enzymes (also called β-1,4-glucanases) have been related to plant pathogenicity by their role in weakening plant cell walls and allowing pathogen introgression (Zhu *et al.*, 2018). However, they may also play an important role in determining plant susceptibility, as their absence influences the responses of plants to different pathogens (Flors *et al.*, 2007). Furthermore, β-1,4-glucanases

can be secreted by plants into the extracellular space for preventing pathogens development that have cellulose in their cell wall (oomycetes). Accordingly, susceptible and resistant *Piper nigrum* L. (black pepper) plants to the pathogenic oomycete *Phytophthora capsici* revealed an increased activity of β-1,4-glucanases compared to uninoculated plants (Vandana *et al.*, 2014). However, the authors were unable to determine if this enzyme is of plant or pathogen origin. In the present work, the up-regulation of *ENDOGLUCANASE-LIKE* could be involved in the plant pathogenesis process (turning the plant more susceptible to the pathogen). Interestingly, another endoglucanase gene (*ENDOGLUCANASE 17 ISOFORM X2* gene) was detected as a down-regulated DEG, following endophyte inoculation. This result suggests the differential induction of genes in both situations.

Up-regulation of genes related with plant defense responses

The pathogen inoculation induced two genes (*TERPENE SYNTHASE 10-LIKE, PARTIAL* and *MONOTHIOL GLUTAREDOXIN-S2-LIKE*) that were similarly induced when plants were challenged by the endophyte. The *TERPENE SYNTHASE 10* gene was described to be induced upon the infection of *Medicago truncatula* roots by *Aphanomyces euteiches* pathogen (Yadav *et al.*, 2019). In addition, sesquiterpenes produced by *TERPENE SYNTHASE 10* inhibited the pathogen growth, reinforcing that *TERPENE SYNTHASE 10* was indeed involved in plant defense (Yadav *et al.*, 2019). After pathogen infection, an induction of glutaredoxins has been also described for maintaining cellular redox homeostasis (Li, 2014). Therefore, both the endophyte and pathogen seem to induce the synthesis of terpenes and reduce oxidative stress, processes that could be involved in olive plant defense responses against fungal infections.

A DNA-binding transcription factor, (*DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1E-LIKE*) was also up-regulated after pathogen inoculation. Although such transcription factors are commonly associated with abiotic stress conditions, the overexpression of dehydration-responsive element-binding 1 (*DREB1*) in *Solanum tuberosum* enhances the tolerance to *Fusarium solani* (Charfeddine *et al.*, 2015). Indeed, *DREB1* transcription factor targets genes from PR proteins, inducing the accumulation of PR proteins, like PR-2 (β-1,3-glucanase) (Charfeddine *et al.*, 2015). Therefore, the induction of *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1E-LIKE* could increase the plant defense response against the pathogen *C. acutatum*, inducing the accumulation of PR proteins.

Up-regulation of genes related with plant development

As observed after the endophyte inoculation, the *ADENYLATE ISOPENTENYLTRANSFERASE 5, CHLOROPLASTIC-LIKE* gene was also up-regulated in pathogen-infected olive plants. The overexpression of isopentenyltransferases led to an increase of cytokinins in *A. thaliana* plants, which in turn enhanced their resistance against *Pseudomonas syringae pv. tomato* pathogen (Choi *et al.*, 2010).

Down-regulation of genes induced by the pathogen

As detected for plant responses to endophyte, the pathogen inoculation down-regulated many genes ($log_2FC \leq -2.5$), which are listed in table 12.

Table 12. Down-regulated genes ($log_2FC \leq -2.5$) in plants inoculated with the pathogen in both compared groups [P0h *vs.* C0h] and [EP0h *vs.* C0h], at 0h. The respective molecular function (MF) and biological process (BP) were obtained from *g:GOSt* – functional enrichment analysis from *g:Profiler* (Raudvere *et al.*, 2019). Whenever *g:GOSt* tool was unable to find results for MF or BP, information obtained from UniProt was added (indicated by insertion of the accession number of searched protein). Those genes that were similarly down-regulated following endophyte inoculation (cf. Table 10) are underlined.

Gene ID	Protein name	MF	BP	log_2FC	
				[P *vs.* C]	[EP *vs.* C]
LOC111380271	glycerol-3-phosphate 2-O-acyltransferase 6-like (UniProtKB - O80437)	glycerol-3-phosphate 2-O-acyltransferase activity	cutin biosynthetic process	-2.54	-3.68
LOC111410995	dirigent protein 22-like (UniProtKB - Q66GI2)	-	phenylpropanoid biosynthetic process	-3.11	-3.10
LOC111397386	cellulose synthase A catalytic subunit 6 (UniProtKB - Q94JQ6)	cellulose synthase activity	cellulose biosynthetic process	-2.99	-2.78
LOC111367162	transcription factor MYB16-like (UniProtKB - Q9LXF1)	DNA binding	regulation of cutin biosynthetic process	-3.19	-3.17
LOC111394214	endoglucanase 17 isoform X2 (UniProtKB - O81416)	hydrolase activity, hydrolyzing O-glycosyl compounds	cellulose catabolic process	-2.97	-2.95
LOC111393999	endoglucanase 6-like (UniProtKB - Q42059)	cellulase activity	cellulose catabolic process	-4.20	-3.72

Table 12. *Continuation.*

Gene ID	Protein name	MF	BP	log_2FC	
				[P *vs.* C]	[EP *vs.* C]
LOC111379546	probable pectate lyase 1, partial (UniProtKB - Q940Q1)	pectate lyase activity	pectin catabolic process	-3.49	-3.94
LOC111367127	probable pectate lyase 18	pectate lyase activity	pectin catabolic process	-2.63	-3.53
LOC111384671	expansin-A8-like	-	plant-type cell wall organization	-2.64	-2.84
LOC111385342	expansin-A8-like	-	plant-type cell wall organization	-2.53	-3.10
LOC111369928	fasciclin-like arabinogalactan protein 12 (UniProtKB - Q8LEE9)	-	plant-type secondary cell wall biogenesis	-3.13	-3.22
LOC111383433	metalloendoproteinase 4-MMP-like	metalloendopeptidase activity	collagen catabolic process	-2.96	-3.71
LOC111399217	disease resistance response protein 206-like (UniProtKB - P13240)	-	defense response	-4.82	-4.17
LOC111371531	peroxidase 29 (UniProtKB - Q9LSP0)	peroxidase activity	hydrogen peroxide catabolic process	-2.65	-3.29
LOC111370815	basic leucine zipper 61-like (UniProtKB -Q9M2K4)	DNA-binding transcription factor activity	positive regulation of transcription, DNA-templated	-3.09	-2.57
LOC111377099	protein GAST1-like (UniProtKB - Q8L8X0)	-	gibberellic acid mediated signaling pathway	-3.33	-4.46
LOC111382674	protein GAST1-like (UniProtKB - Q8L8X0)	-	gibberellic acid mediated signaling pathway	-3.22	-4.22
LOC111400834	protein GAST1-like (UniProtKB - Q8L8X0)	-	gibberellic acid mediated signaling pathway	-2.55	-3.71
LOC111380164	subtilisin-like protease SBT1.1 (UniProtKB - Q84WS0)	serine-type endopeptidase activity	proteolysis	-3.18	-3.84
LOC111385779	trehalose-phosphate phosphatase A-like (UniProtKB - O64896)	trehalose-phosphatase activity	trehalose biosynthetic process	-2.63	-2.77
LOC111372052	plasma membrane ATPase 4-like (UniProtKB - Q03194)	ATP binding	proton export across plasma membrane	-3.33	-2.75
LOC111378100	probable acyl-activating enzyme 12, peroxisomal (UniProtKB - Q9SS00)	ligase activity	fatty acid metabolic process	-3.44	-3.17

Table 12. *Continuation*.

Gene ID	Protein name	MF	BP	log_2FC	
				[P *vs.* C]	[EP *vs.* C]
LOC111402673	coleoptile phototropism protein 1-like (UniProtKB - Q5KS50)	-	protein ubiquitination	-2.69	-2.76
LOC111377308	glycine-rich protein 5-like (UniProtKB - Q9LTP5)	structural constituent of cell wall	positive regulation of cell growth	-2.83	-4.52
LOC111374924	proline-rich protein 4-like isoform X2 (UniProtKB - Q9T0I5)	-	-	-2.77	-2.99
LOC111369169	pelargonidin 3-O-(6-caffeoylglucoside) 5-O-(6-O-malonylglucoside) *4'''*-malonyltransferase-like (UniProtKB - Q6TXD2)	acyltransferase activity, transferring groups other than amino-acyl groups	anthocyanin-containing compound biosynthetic process	-2.82	-2.71

Down-regulation of genes related with plant cell wall modifications

After inoculation of olive plants with *C. acutatum*, many genes coding for proteins involved in plant cell wall modifications were down-regulated, namely *GLYCEROL-3-PHOSPHATE 2-O-ACYLTRANSFERASE 6-LIKE*, *DIRIGENT PROTEIN 22-LIKE*, *CELLULOSE SYNTHASE A CATALYTIC SUBUNIT 6*, *TRANSCRIPTION FACTOR MYB16*, *ENDOGLUCANASE 17 ISOFORM X2*, *ENDOGLUCANASE 6-LIKE*, *PROBABLE PECTATE LYASE 1, PARTIAL*, *PROBABLE PECTATE LYASE 18*, *EXPANSIN-A8-LIKE*, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12* and *METALLOENDOPROTEINASE 4-MMP-LIKE*.

Many genes involved in the synthesis of cell wall components are repressed after *C. acutatum* inoculation, including those involved in cutin synthesis (*GLYCEROL-3-PHOSPHATE 2-O-ACYLTRANSFERASE 6-LIKE*), flavonoids and lignin synthesis (*DIRIGENT PROTEIN 22-LIKE*), and cellulose synthesis (*CELLULOSE SYNTHASE A CATALYTIC SUBUNIT 6*). Although not directly involved in plant cell wall synthesis the *TRANSCRIPTION FACTOR MYB16* (that regulates cuticle formation) was also repressed in olive plants inoculated with the pathogen. However, several reports have described the importance of the expression of such genes for plant defense against pathogens (Yeats & Rose, 2013). A study revealed that tobacco plants inoculated with *Fusarium*

solani overexpressing *PnDIR1* (gene that belongs to the dirigent family) were healthier compared to wild-type plants (Deng *et al.*, 2022). The overexpression of *PnDIR1* resulted in enhanced lignin biosynthesis and contributed for increased tobacco plants resistance to *F. solani*. The infection of *A. thaliana* roots by *Fusarium oxysporum* also resulted in a reduction on cellulose synthesis (Kesten *et al.*, 2019).

The results also revealed the down-regulation of many genes coding for proteins involved in the breakdown of plant cell wall components, namely cellulose (*ENDOGLUCANASE 17 ISOFORM X2* and *ENDOGLUCANASE 6-LIKE*) and pectin (*PROBABLE PECTATE LYASE 1, PARTIAL* and *PROBABLE PECTATE LYASE 18*). These genes have been reported to be induced during pathogenesis, in order to facilitate pathogen entry (Uluisik & Seymour, 2020). The observed down-regulation of these genes suggests that the entry of *C. acutatum* is not facilitated, as the cell wall integrity is maintained. Furthermore, two *EXPANSIN-A8-LIKE* genes were also down-regulated. These genes code for proteins involved in plant-type cell wall organization. Indeed, host expansins can be induced by pathogens, in order to change cell wall metabolism (by loosening of plant cell walls) upon infection (Bellincampi *et al.*, 2014). *EXPANSIN-A8-LIKE* down-regulation suggests that plants cell walls remain firm, instead of loosen, making the entry of the pathogen more difficult.

Fasciclin-like arabinogalactan proteins are involved in plant growth and cell wall biosynthesis, but they may play a role in plant response to pathogens (Wu *et al.*, 2020). Indeed, genes encoding fasciclin-like arabinogalactan proteins were down-regulated in *Nicotiana benthamiana* upon infection of turnip mosaic virus and *Pseudomonas syringae pv tomato* strain DC3000 (Wu *et al.*, 2020). The authors suggested that due to their role in cell adhesion, fasciclin-like arabinogalactan proteins may interact with receptor-like kinases (RLKs) and therefore may be involved in signal transduction. Also, metalloproteinases (involved in the extracellular matrix modification) could play a PTI triggering role in plant defense responses (Zhao *et al.*, 2017). Indeed, a gene coding for a metalloproteinase was up-regulated in soybean tissues infected either with the oomycete pathogen *Phytophthora sojae* or with the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Liu *et al.*, 2001). The authors suggested that this gene was involved in defense response of soybean against pathogens. However, in the present work, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12* and *METALLOENDOPROTEINASE 4-MMP-LIKE* genes were both down-regulated

following pathogen challenging, suggesting that the pathogen did not trigger the associated olive plants defense response.

Down-regulation of genes related with plant defense responses

In the present work, genes involved in plant defense processes, namely *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE*, *PEROXIDASE 29*, and *BASIC LEUCINE ZIPPER 61-LIKE* were also detected as being down-regulated DEGs. A similar repression of *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE* was detected following endophyte inoculation. These results contrast with the induction of peroxidase genes upon pathogen infection, in order to regulate ROS levels in plants (Lüthje & Martinez-Cortes, 2018). By being down-regulated in the present work, the scavenging of hydrogen peroxide does not seem to be needed upon *C. acutatum* infection, probably because ROS levels did not increase. A *BASIC LEUCINE ZIPPER 61-LIKE* transcription factor was also detected as a DEG following pathogen inoculation. Basic leucine zippers regulate many physiological processes and are involved in biotic stress responses (Alves *et al.*, 2013). A study showed that a basic leucine zipper transcription factor *CAbZIP1* is induced in pepper plants infected with either *Xanthomonas campestris* pv. *vesicatoria* or *Pseudomonas fluorescens* (Lee *et al.*, 2006). Also, transgenic *Arabidopsis* plants overexpressing *CAbZIP1* showed increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000. However, in this work, *BASIC LEUCINE ZIPPER 61-LIKE* was down-regulated, and therefore also not involved in plant defense against *C. acutatum*.

Down-regulation of genes related with signaling and transcription processes

Other down-regulated genes found code proteins involved in signaling and transcription processes, namely *PROTEIN GAST1-LIKE*, and *SUBTILISIN-LIKE PROTEASE SBT1.1*. Following pathogen inoculation, three DEGs encoding *PROTEIN GAST1-LIKE* were found, which could be involved in a gibberellic acid signaling pathway. A study showed that upon infection of *Candidatus* Liberibacter asiaticus, a tolerant citrus hybrid (US-897) revealed an up-regulation of *GA-responsive GAST1 protein homolog* (*GASA5*) (Albrecht & Bowman, 2012). The authors suggested that this gene could be involved in cell growth. As in the present work, the *PROTEIN GAST1-LIKE* genes

were down-regulated upon *C. acutatum* infection, suggesting that the signaling gibberellic acid pathway does not seem to be in place.

SUBTILISIN-LIKE PROTEASE SBT1.1 is involved in the cleavage of phytosulfokines, which are peptide hormones that promote plant cell differentiation. A study showed that when a subtilisin-like protease gene (*GbSBT1*) from *Gossypium babardense* was knockdown, the plant defenses were reduced against the fungus *Verticillium dahliae*, and the cotton plants exhibited a more severe wilting than control plants (Duan *et al.*, 2016). In addition, by ectopically expressing the gene *GbSBT1*, *A. thaliana* showed enhanced resistance against *Fusarium oxysporum* and *V. dahliae*, also activating the expression level of defense-related genes. However, in the present work, *SUBTILISIN-LIKE PROTEASE SBT1.1* was down-regulated, and therefore not involved in olive plants defense against *C. acutatum*.

Down-regulation of genes related with plant metabolism and development

Following pathogen inoculation, we also found DEGs coding for proteins involved in plant metabolism and development, namely *TREHALOSE-PHOSPHATE PHOSPHATASE A-LIKE*, *PLASMA MEMBRANE ATPase 4-LIKE*, and *PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL*.

TREHALOSE-PHOSPHATE PHOSPHATASE A-LIKE (or *TREHALOSE 6-PHOSPHATE PHOSPHATASE*) is involved in the formation of trehalose, which is a disaccharide involved in abiotic and biotic stresses. Indeed, trehalose 6-phosphate was suggested to act as an essential signaling molecule for growth, development, and plant stress responses through kinase activation (Fernandez *et al.*, 2010). However, *TREHALOSE-PHOSPHATE PHOSPHATASE A-LIKE* was down-regulated in this work, and therefore does not seem to be involved in olive plants defense against *C. acutatum*.

PLASMA MEMBRANE ATPase 4 is involved in the movement of hydrogen ions across plasma membrane, from the cytosol to the extracellular space. Since plasma membrane H^+-ATPases are largely involved in plant physiology, their presence could be manipulated by pathogens, either by inhibiting or stimulating their production (which can cause cell death) (Elmore & Coaker, 2011). For example, during pattern-triggered immunity (PTI) in guard cells, plasma membrane ATPases were described to be down-regulated, in order to cause loss of turgor and

therefore close stomata to prevent the entry of the pathogen (Elmore & Coaker, 2011). In this work, *PLASMA MEMBRANE ATPase 4-LIKE* was down-regulated, which could suggest that stomata were closed in response to the inoculation of the pathogen.

An acyl-activating enzyme, (*PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL*) was down-regulated after pathogen inoculation. This gene was similarly down-regulated upon endophyte inoculation. As previously discussed, the coded protein could be involved in a wide array of metabolic pathways, including amino acids biosynthesis or secondary metabolites production, such as JA and cutin biosynthesis (Cavaco *et al.*, 2021). By being down-regulated in this work, the production of such compounds could be compromised.

Considerations about gene expression upon pathogen inoculation

In summary, and just like in endophyte-treated plants, there were more down-regulated DEGs (26) in pathogen-infected plants than up-regulated (8). When olive plants were inoculated with the pathogen (*C. acutatum*), many induced genes seem to be involved in plant defenses against biotic/abiotic stresses, either by producing terpenes (*TERPENE SYNTHASE 10-LIKE, PARTIAL*), reducing oxidative stress (*MONOTHIOL GLUTAREDOXIN-S2-LIKE*), or by up-regulating transcription factors (*DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1E-LIKE*) related with PR proteins synthesis. In addition, plants inoculated with the pathogen seems also to induce cytokinin production (*ADENYLATE ISOPENTENYLTRANSFERASE 5, CHLOROPLASTIC-LIKE*) which can be involved in plant development and defense against pathogens. However, down-regulated DEGs also comprised genes that code other proteins related with plant defense processes, such as *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE*, and *PEROXIDASE 29.* Taken together, these results suggest the activation of certain defense pathways, but not others. Accordingly, *C. acutatum* seems to affect signaling pathways related with plant response to pathogens. For example, plant cell wall-related enzymes (*FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12* and *METALLOENDOPROTEINASE 4-MMP-LIKE*), which have been described to modulate plant defense responses were down-regulated, suggesting the inefficiency of olive plants to trigger plant defenses. Also, a leucine zipper transcription factor known to be involved in plant defenses was repressed.

As described for endophyte treatment, the pathogen inoculation repressed many genes related with cell wall synthesis (cutin, lignin, cellulose), suggesting a limitation on plant cell growth.

This was corroborated by the repression of expansin genes. Alongside the limitation in plant cell wall synthesis, a reduction on plant cell wall degradation seems to occur in pathogen-infected plants, as several genes coding for cell wall degradative processes were down-regulated following pathogen treatment. These included genes coding for proteins able to degrade cellulose (*ENDOGLUCANASE 17 ISOFORM X2* and *ENDOGLUCANASE 6-LIKE*) and pectin (*PROBABLE PECTATE LYASE 1, PARTIAL* and *PROBABLE PECTATE LYASE 18*). Interestingly, a different endoglucanase gene (LOC111385774) was detected as up-regulated, suggesting specificity of endoglucanase expression and potential differential activity.

Many up-regulated genes involved in plant defense were similarly induced after endophyte or pathogen treatment, by over-expressing secondary metabolites (*TERPENE SYNTHASE 10-LIKE, PARTIAL*), by reducing oxidative stress (*MONOTHIOL GLUTAREDOXIN-S2-LIKE*), or by producing hormones (*ADENYLATE ISOPENTENYLTRANSFERASE 5, CHLOROPLASTIC-LIKE*) besides the *UNCHARACTERIZED PROTEIN LOC111405392.* Also, seven down-regulated genes were similarly induced by the endophyte and pathogen, including *DISEASE RESISTANCE RESPONSE PROTEIN 206-LIKE*, *METALLOENDOPROTEINASE 4-MMP-LIKE*, *TRANSCRIPTION FACTOR MYB16-like*, *PROBABLE PECTATE LYASE 1, PARTIAL*, *PROBABLE ACYL-ACTIVATING ENZYME 12, PEROXISOMAL*, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 12*, and *ENDOGLUCANASE 17 ISOFORM X2*. Results suggest a common decrease on plant cell wall synthesis (cutin, cellulose, pectin) and plant cell wall loosening. Many genes that are usually induced upon a pathogen attack and are related to plant defense were down-regulated upon infection, suggesting that olive plants mainly responded to fungal inoculation through the down-regulation of genes related to cell wall modification.

When comparing the gene expression changes occurring after the endophyte or pathogen inoculation, the whole picture was very similar, suggesting that olive plants similarly responded to fungal inoculation. Indeed, when comparing endophyte-treated plants with pathogen-infected plants (either at 0 hpi and 96 hpi), there was no differences in gene expression (table 7). Also, when comparing plants inoculated with both fungi and plants treated only with the endophyte and only with the pathogen, the gene expression alterations were minimal. This means that olive plants

responded similarly to all treatments, and therefore the endophyte did not help the plant defend against the pathogen.

The most intriguing is that the main alterations in gene expression occurred just after pathogen inoculation (by spraying, at 0 hpi). At 96 hpi, both the endophyte and pathogen did not promote a large difference in plants gene expression compared to control plants, as only 239 DEGs were found between endophyte and control plants, and 95 DEGs between pathogen and control (table 7). Furthermore, when gene expression of plants treated with both fungi is compared with control plants, a single DEG (*ALPHA-FARNESENE SYNTHASE-LIKE*) was detected. According to other plant-microbe interaction reports, a higher number of DEGs was indeed expected at 96 hpi than at 0 hpi (especially between pathogen-infected plants and control plants at 0 hpi).

3.2.4 Defense related genes analysis

For getting a better picture of olive plant responses upon the application of the endophyte for controlling *Colletotrichum acutatum* disease, the defense related genes were specifically analyzed, including those coding for PR proteins, enzymes for the production of secondary metabolites and oxidative stress-related genes. For this analysis, all DEGs displaying a $|\log_2 FC| \geq 1$ were considered.

3.2.4.1 Expression of genes coding for PR proteins

Several differential expressed genes (DEGs) coding for PR proteins were found during RNA-seq analysis, including genes coding for chitinases (PR-3, PR-4, PR-8, and PR-11), thaumatin-like proteins (PR-5), proteinase inhibitors (PR-6), peroxidases (PR-9), defensins (PR-12), and lipid transfer proteins (PR-14) (table 13).

Plant chitinases are usually produced upon the attack of pathogens (Gharbi *et al.*, 2017; Jain & Khurana, 2018). As fungi contain chitin in their cell walls, the production of these enzymes can protect plant cells against fungal pathogens, as they degrade this cell wall crucial component (Ali *et al.*, 2020; Datta *et al.*, 2001; Jain & Khurana, 2018). Five DEGs encoding chitinases were found in the present work. Most chitinase genes were down-regulated when plants were challenged with endophyte and/or pathogen (comparing with control samples at 0 hpi, table 13). Genes coding

for plant chitinases were only up-regulated at 0 hpi in endophyte-treated plants, and at 96 hpi in endophyte-treated samples (compared to control) and in pathogen-infected plants (compared to endophyte-treated plants). This means that plant chitinases were induced upon both endophyte and pathogen inoculation, but not when inoculated together.

Table 13. Expression of PR proteins between sample groups. Down-regulation of genes between treatments (E, P, or EP) and corresponding controls (Cont, End, Pat) are denoted by a decreasing arrow (↘), while up-regulation by an increasing arrow (↗). Non-differentially expressed genes are denoted by (-). Results are shown for 0 hpi or 96 hpi. Endophyte (E or End), Pathogen (P or Pat), Endophyte+Pathogen (EP), Control (Cont).

	0 hpi						96 hpi					
	Cont			End		Pat	Cont			End		Pat
	E	P	EP	P	EP	EP	E	P	EP	P	EP	EP
endochitinase EP3-like (gene ID: LOC111393511)	↗	-	-	-	-	-	-	-	-	↗	-	-
chitinase 2-like (gene ID: LOC111369643)	↘	↘	↘	-	-	-	-	-	-	-	-	-
chitinase 2-like (gene ID: LOC111369641)	-	↘	↘	-	-	-	-	-	-	-	-	-
chitinase-like protein 1 (gene ID: LOC111382278)	↘	-	↘	-	-	-	↗	-	-	-	-	-
chitinase-like protein 1 (gene ID: LOC111392080)	-	-	↘	-	-	-	-	-	-	-	-	-
thaumatin-like protein (gene ID: LOC111380489)	-	-	↘	-	-	↘	-	-	-	↗	-	-
thaumatin-like protein 1 (gene ID: LOC111410113)	↘	-	-	-	-	-	-	-	-	-	-	-
thaumatin-like protein 1 (gene ID: LOC111390313)	↘	-	-	-	-	-	-	-	-	-	-	-
thaumatin-like protein 1 (gene ID: LOC111374883)	↘	-	-	-	-	-	-	-	-	-	-	-
thaumatin-like protein 1 (gene ID: LOC111401272)	↘	-	-	-	-	-	-	-	-	-	-	-
thaumatin-like protein (gene ID: LOC111372323)	↘	↘	↘	-	-	-	-	-	-	-	-	-
thaumatin-like protein 1b (gene ID: LOC111397378)	↗	-	-	-	-	-	-	-	-	-	-	-
proteinase inhibitor PSI-1.2-like (gene ID: LOC111394394)	-	↗	-	-	-	-	-	-	-	-	-	-
proteinase inhibitor PSI-1.2-like (gene ID: LOC111390049)	-	↗	-	-	-	-	-	-	-	-	-	-
proteinase inhibitor PSI-1.2-like (gene ID: LOC111373900)	-	↗	-	-	-	-	-	-	-	-	-	-
peroxidase 47-like (gene ID: LOC111379502)	↘	↘	↘	-	-	-	-	-	-	-	-	-
peroxidase 4-like (gene ID: LOC111388454)	↘	↘	↘	-	-	-	-	-	-	-	-	-
probable glutathione peroxidase 2 (gene ID: LOC111385527)	↘	-	↘	-	-	-	-	-	-	-	-	-
peroxidase 15-like (gene ID: LOC111386115)	↗	-	-	-	-	-	-	-	-	-	-	-

Table 13. *Continuation.*

	0 hpi						96 hpi					
	Cont			End		Pat	Cont			End		Pat
	E	P	EP	P	EP	EP	E	P	EP	P	EP	EP
peroxidase 25 (gene ID: LOC111382918)	-	-	-	-	-	-	↗	-	-	-	-	-
peroxidase 21-like isoform X2 (gene ID: LOC111399679)	↗	↗	↗	-	-	-	-	-	-	-	-	-
peroxidase 29 (gene ID: LOC111371531)	-	↘	↘	-	-	-	-	-	-	-	-	-
peroxidase 3-like (gene ID: LOC111368505)	↘	↘	↘	-	-	-	-	-	-	-	-	-
peroxidase 31-like (gene ID: LOC111368080)	↘	↘	↘	-	-	-	-	-	-	-	-	-
peroxidase 42-like (gene ID: LOC111395952)	↘	↘	↘	-	-	-	-	-	-	-	-	-
peroxidase 15-like (gene ID: LOC111399512)	↘	-	-	-	-	-	-	-	-	-	-	-
peroxidase 42-like (gene ID: LOC111402428)	↘	-	-	-	-	-	-	-	-	-	-	-
peroxidase 12-like (gene ID: LOC111372231)	↘	-	-	-	-	-	↗	-	-	-	↘	-
defensin Ec-AMP-D2-like (gene ID: LOC111402264)	-	-	↘	-	-	-	-	-	-	-	-	-
non-specific lipid transfer protein GPI-anchored 1-like (gene ID: LOC111406236)	↘	-	-	-	-	-	-	-	-	-	-	-
non-specific lipid transfer protein GPI-anchored 1-like (gene ID: LOC111403351)	↘	-	-	-	-	-	↗	↗	-	-	↘	-
non-specific lipid transfer protein GPI-anchored 2 (gene ID: LOC111388780)	↘	↘	↘	-	-	-	-	-	-	-	-	-

Seven genes coding for thaumatin-like proteins (PR-5 proteins) were also found as DEGs in this work. Many PR-5 proteins are known to be involved in plant defense responses and their genes are induced upon biotic and abiotic stresses (Léon-Kloosterziel *et al.*, 2005). In fact, upon colonization of *Arabidopsis* roots by *Pseudomonas* bacteria, a thaumatin-like protein gene (*AtTLP1*), coding for a protein with antimicrobial function, was activated (Léon-Kloosterziel *et al.*, 2005). In the present work, DEGs coding for thaumatin-like proteins were mainly down-regulated, especially in endophyte-treated plants (table 13), which means that *P. aff. commune* did not induce PR-5 proteins involved in plant defense. Despite this, thaumatin-like protein genes were also up-regulated in endophyte-treated plants at 0 hpi (LOC111397378) and in pathogen-infected plants (compared to endophyte-treated plants) at 96 hpi (LOC111380489), suggesting that the plant induced PR-5

proteins in response to both fungi. However, when both fungi (endophyte and pathogen) were inoculated together, thaumatin-like proteins were also down-regulated in comparison to control plants.

Proteinase inhibitors belong to the PR-6 proteins family. They are produced upon a pathogenic attack and are involved in plants defense mechanisms (Barta *et al.*, 2002). For example, proteinase inhibitors are known to induce the formation of an abscission zone, which sacrifices tissues to prevent further invasion of pathogens (Widana *et al.*, 2016). In the present work, three DEGs coding for proteinase inhibitors (*PROTEINASE INHIBITOR PSI-1.2-LIKE*) were found. The coded protein has been described as being involved in the inhibition of both trypsin and chymotrypsin, which are serine proteases that cleave peptide bonds in proteins (Antcheva *et al.*, 2008). A study showed an up-regulation of *PROTEINASE INHIBITOR PSI-1.2* gene in capsicum (*Capsicum annuum* x *C. chinense*) resistant to chlorosis virus comparing with susceptible plants (Widana *et al.*, 2016). Likewise, in the present work, the three *PROTEINASE INHIBITOR PSI-1.2-LIKE* were up-regulated in pathogen-infected olive plants (compared to control, table 13), which could suggest that plants were preventing further invasion of the pathogen *C. acutatum*.

In the present work, 13 DEGs encoding peroxidases (PR-9 proteins) were detected when plants were challenged with endophyte and/or pathogen. Several peroxidases are known to be involved in oxidative stress response and are induced upon increased levels of ROS for scavenging H_2O_2 (Lüthje & Martinez-Cortes, 2018). Surprisingly, in this work, most peroxidases were down-regulated just after fungal inoculation (compared to control, 0 hpi, table 13). Only *PEROXIDASE 21-LIKE ISOFORM X2* was induced after any fungal inoculation (at 0 hpi), while other DEGs were induced with only endophyte treatments. This result could suggest that olive plants generate higher amounts of ROS when treated with the endophyte, inducing peroxidase genes to cope with increased ROS levels.

Defensins (PR-12 proteins) are antimicrobial peptides important for plant defense system, by for example inhibiting proteases and protein synthesis (Odintsova *et al.*, 2008). A study showed that a defensin Ec-AMP-D2 isolated from *Echinochloa crusgalli* (L.) exhibited antifungal activity against the phytopathogenic fungus *Fusarium oxysporum* and the oomycete *Phytophthora infestans* (Odintsova *et al.*, 2008). However, in the present work, *DEFENSIN Ec-AMP-D2-like* was

down-regulated in plants inoculated with both the pathogen and the endophyte (compared to control), suggesting that defensins proteins could not be involved in olive plants defense.

Non-specific lipid transfer proteins (LTPs, PR-14 proteins) transport lipids between membranes and are involved in plant defense against pathogens ((Fahlberg *et al.*, 2019). Loss of glycophosphatidylinositol (GPI)-anchored LTPs in *A. thaliana*, increased the susceptibility to *Blumeria graminis* f. sp. *hordei* by allowing fungal penetration (Fahlberg *et al.*, 2019). Two proteins (LTPG1 and LTPG2) were described to be involved in the deposition and biosynthesis of cuticular waxes and cutin, explaining the higher penetration resistance to the *Blumeria graminis* f. sp. *hordei*. The involvement of LTPs in plant resistance is not in line with the results obtained in the present work, where the down-regulation of three LTP genes were observed. However, the up-regulation at 96 hpi of a single gene (LOC111403351) could suggest that there was a late response to both the endophyte and the pathogen. In any case, when both fungi were inoculated the expression levels of this gene were lower compared to endophyte-treated plants. This result could suggest that plants defended more aggressively towards the endophyte inoculation, since they were inoculated through a cut made in plants main stem.

3.2.4.2 Expression of genes coding for other described plant defense genes

Besides those genes coding for PR proteins, after challenging olive plants with endophyte and/or pathogen, several DEGs code for other defense related genes (table 14). Specifically, these DEGs are either involved in the production of secondary metabolites (those coding for alpha-farnesene synthase, phenylalanine ammonia-lyase, and chalcone synthases). or are related with oxidative stress (coding for polyphenol oxidase and catalase).

Table 14. Expression of defense related genes between sample groups. Down-regulation of genes between treatments (E, P, or EP) and corresponding controls (Cont, End, Pat) are denoted by a decreasing arrow (↘), while up-regulation by an increasing arrow (↗). Non-differentially expressed genes are denoted by (-). Results are shown for 0 hpi or 96 hpi. Endophyte (E or End), Pathogen (P or Pat), Endophyte+Pathogen (EP), Control (Cont).

	0 hpi						96 hpi					
	Cont			End		Pat	Cont			End		Pat
	E	P	EP	P	EP	EP	E	P	EP	P	EP	EP
alpha-farnesene synthase-like (gene ID: LOC111368536)	-	↘	↘	-	-	-	-	-	-	-	-	-
alpha-farnesene synthase-like (gene ID: LOC111368535)	-	-	-	-	-	-	-	-	↗	-	-	-
phenylalanine ammonia-lyase-like (gene ID: LOC111402181)	↗	↗	↗	-	-	-	-	-	-	-	-	-
probable chalcone–flavanone isomerase 3 (gene ID: LOC111387047)	↘	↘	↘	-	-	-	-	-	-	-	-	-
chalcone synthase-like (gene ID: LOC111374392)	↘	↘	↘	-	-	-	-	-	-	-	-	-
chalcone synthase (gene ID: LOC111374393)	-	↘	↘	-	-	-	-	-	-	-	-	-
chalcone synthase J-like (gene ID: LOC111379443)	-	↘	↘	-	-	-	-	-	-	-	-	-
chalcone synthase isoform X2 (gene ID: LOC111393361)	-	↘	↘	-	-	-	-	-	-	-	-	-
polyphenol oxidase, chloroplastic-like (gene ID: LOC111374154)	-	-	↘	-	-	-	-	-	-	-	↘	-
polyphenol oxidase, chloroplastic-like (gene ID: LOC111375158)	-	↘	↘	-	-	-	-	-	-	-	-	-
polyphenol oxidase I, chloroplastic-like (gene ID: LOC111375437)	-	↘	↘	-	-	-	-	-	-	-	-	-
polyphenol oxidase I, chloroplastic-like (gene ID: LOC111374156)	-	-	↘	-	-	-	↗	-	-	-	-	-
polyphenol oxidase I, chloroplastic-like (gene ID: LOC111370878)	↘	↘	-	-	-	-	-	-	-	-	-	-
catalase isozyme 3-like (gene ID: LOC111371766)	↗	-	-	-	-	-	-	-	-	-	-	-

The most significant molecular function of detected *ALPHA-FARNESENE SYNTHASE-LIKE* genes (*g:GOSt* results) was "terpene synthase activity", and the most significant biological process was "diterpenoid biosynthetic process". Most probably *ALPHA-FARNESENE SYNTHASE-LIKE* is involved in the synthesis of farnesese, a terpenoid that functions in plant defense (Li *et al.*, 2021). Indeed, the overexpression of alpha-farnesene synthase (*AFS*) in soybean resulted in an enhanced resistance to soybean cyst nematode, which is an important pathogen of soybean (Lin *et al.*, 2017). In the present work, the up-regulation of *ALPHA-FARNESENE SYNTHASE-LIKE* in samples treated with both endophyte and pathogen at 96 hpi (table 14), suggests that the endophyte could have induced a late response to the pathogen by producing terpenoids.

A DEG coding for an important enzyme of the phenylpropanoid pathway, *PHENYLALANINE AMMONIA-LYASE-LIKE*, was up-regulated in all three treatments (when compared to control at 0 hpi, table 14). Phenylalanine ammonia-lyase (*PAL*) results in the production of cinnamic acid, which subsequently forms flavonoids (like anthocyanidins) or lignin (Guerra *et al.*, 2015). These secondary metabolites play an important role in the plant defense against abiotic and biotic stresses. The accumulation of flavonoids can reduce ROS levels, as they have the capacity to act as antioxidants (Brunetti *et al.*, 2013), while the accumulation of lignin could halt the cell wall entry of the pathogen (Naziya *et al.*, 2020). Indeed, wounded plants and plants attacked by pathogens exhibit increased activity of *PAL* (Anand *et al.*, 2009; Ramanathan *et al.*, 2000). Taking this into consideration, the up-regulation of *PHENYLALANINE AMMONIA-LYASE-LIKE* in all three treatments (compared to control, 0 hpi) suggests that the plant reacted not only to the pathogen, but also to the endophyte by reinforcing their cell walls. In fact, endophyte-treated plants were also described to present an increase in phenylpropanoids, which shows that the plants respond to endophytes that do not usually cause disease (Rasmussen *et al.*, 2008). Despite *PAL-LIKE* gene up-regulation at 0 hpi, this gene was not differentially expressed at 96 hpi, in contrast with reports that describe enhanced expression following pathogen infection (Xu *et al.*, 2015).

After treating olive plants with endophyte and/or pathogen five genes encoding chalcones were detected as DEGs. Chalcone synthases are key enzymes involved in the flavonoid biosynthesis pathway and are responsible for producing many secondary products (Dao *et al.*, 2011). An induction of chalcone synthase genes have indeed been described as a defense response against pathogens or stress situations (Dao *et al.*, 2011). For example, the overexpression of a gene encoding a chalcone synthase (*VSAD1*) in *Arabidopsis* plants increased their resistance to *V. dahliae*, and also decreased ROS accumulation (Lei *et al.*, 2018). In contrast with reported results, in the present work, all genes encoding chalcones were down-regulated in treated plants (E, P, and EP, compared to control at 0 hpi), suggesting that flavonoids did not accumulate in olive plants in response to fungal inoculation. As *PAL* gene was indeed up-regulated, these results could suggest that olive plant defense could mainly reside on lignin production, and not flavonoid accumulation. In this case, *PAL* activity would play a stronger role in plant defense against fungal inoculation.

In addition to proteins related with secondary metabolites production, several DEGs were also involved in oxidative stress metabolism (peroxidases, catalases and polyphenol oxidases).

Several peroxidase genes (PR-9 proteins, discussed in section 3.3.2.) were mostly down-regulated following fungal inoculation (except *PEROXIDASE 21-LIKE ISOFORM X2*). In contrast, the only detected DEG for catalase (*CATALASE ISOZYME 3-LIKE*) was up-regulated in endophyte-treated plants (compared to control at 0 hpi), suggesting that the endophyte induced catalase expression to diminish hydrogen peroxide levels in leaves. Surprisingly, this gene was not up-regulated in the other olive plants treatments (pathogen alone or in combination with the endophyte), which is usually what happens upon plants infection (Anand *et al.*, 2009).

Following the inoculation of endophyte and/or pathogen, five DEGs encoding polyphenol oxidases were detected. These enzymes are known to be involved in the oxidation of catechols and in the formation of pigments. After a pathogen attack, plants catalyze the oxidation of catechol (o-diphenol) to form quinones, leading to an increase in ROS (Srideepthi *et al.*, 2017). For example, the inoculation of the pathogenic fungus *Phoma medicaginis* in alfafa (*Medicago sativa*), increased polyphenol oxidase (*PPO*) and peroxidase (*POD*) activity levels levels (Li *et al.*, 2021). In addition, the inoculation of this pathogen with the AMF *Rhizophagus intraradices* also increased *PPO* and *POD* levels (Li *et al.*, 2021). Also, the inoculation of the pathogens *Colletotrichum capsici* and *Alternaria alternata*, in chili (*Capsicum annuum* L.), increased *PPO* and *POD* levels comparing with healthy fruits (Anand *et al.*, 2009). In the present work, the olive plants inoculation with endophytes and/or pathogens mostly resulted in the down-regulation of *PPO* genes. Only one *PPO* gene was up-regulated in endophyte-treated plants (compared to control plants, 96 hpi). This suggests that the production of ROS is not enhanced in treated plants, at least as a consequence of catechol oxidation.

The analysis of defense related genes revealed that many DEGs were indeed related with plant defenses, such as PR proteins or oxidative stress-related proteins. However, their induction occurred just after inoculation of the pathogen, as detected for the analyzed DEGs in the previous section.

In summary, gene expression analysis of olive plants treated with endophyte, pathogen, and both (inoculated with endophyte and then with pathogen) revealed that the largest differences were detected at 0 hpi (6877 DEGs to 571 DEGs at 96 hpi). When restricting DEGs even more ($|\log_2 FC| \geq 2.5$), we found 455 DEGs at 0 hpi, and zero DEGs at 96 hpi. This means that after

96 hours, all treated and control plants present similar gene expression levels. This could suggest that the plant succeeded in preventing further infection of both the pathogen and the endophyte. In addition, the olive plants used in the present work were from the moderately tolerant cultivar to anthracnose (*Cobrançosa*). Also, minimal DEGs were found in endophyte-treated plants compared to pathogen-infected plants, which could suggest that olive plants responded similarly to the inoculation of both fungi and activated defense related genes in response.

The main differences between treated and control plants relied in the genes related with plant cell wall modification processes and plant cell defenses, which agrees with what is currently known about plant-microbe interactions. However, the results suggest a general repression on plant cell defenses, cell wall synthesis and cell wall degradation that are not in agreement with what is currently known. Furthermore, when genes involved in plant defenses were specifically analyzed, a general down-regulation of genes coding for PR proteins and oxidative stress-related proteins was detected. In addition to these intriguing results, there were more differences on global gene expression at 0 hpi (just after pathogen spraying) than at 96 hpi.

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4. Conclusion

Plants are the habitat of a great number of fungi that do not cause any harm to the plant. Indeed, they frequently provide essential nutrients for their growth and development, as well as they can improve plant defenses against pathogens. Olive trees can be attacked by several pests and diseases that can massively affect olive production. One of those diseases is olive anthracnose, which is caused by fungal species belonging to the genus *Colletotrichum*. In order to find a biological control agent to manage this disease, the antagonistic potential of the endophyte *Penicillium aff. commune* against the olive pathogen *C. acutatum* was studied. An assay using detached olives was performed by inoculating the endophyte or the pathogen, or a combination of both. *P. aff. commune* showed the capacity to inhibit *C. acutatum* infection, or at least delay the appearance of anthracnose symptoms, since only 12% of olives inoculated with both fungi exhibited symptoms (only after 8 dpi). The endophyte was also able to reduce the disease severity, as diseased olives inoculated with both fungi only had a small portion of the area affected with anthracnose symptoms.

For understanding the molecular processes behind the endophyte biocontrol trait, the transcriptome of olive leaves inoculated with both *P. aff. commune* and *C. acutatum* was studied through RNA-seq, at different post-inoculation periods. An induction in those processes related with plant defenses were expected to occur along time, whatever the fungal inoculation that took place (endophyte and/or pathogen inoculations). Indeed, a differential expression of many genes related with plant defences were singled out in this work, including genes coding for PR proteins, oxidative stress enzymes, or even plant cell wall modification proteins, all of which have been commonly reported in plant-microbe interactions. But the most intriguing result was that more DEGs were found when comparing treated samples (E, P, and EP) with controls at 0 hpi (6799 DEGs), than at 96 hpi (335 DEGs). This result suggested that more genes were affected in the earlier stages of infection, no matter what fungus was used. Alongtime, all the studied conditions displayed more similar levels of gene expression, suggesting that the plant succeeded in preventing further infection of the pathogen. This result was completely unexpected, as it goes against what has been described in the literature, and should be considered with caution.

In endophyte-treated samples (at 0 hpi), the most up-regulated genes were involved in plant defense responses, by inducing the biosynthesis of secondary metabolites, as well as inducing the reduction of oxidative stress. In addition, the endophyte seems to be involved in the promotion of plant growth by inducing the production of cytokinin and by facilitating amino acid availability. However, the most down-regulated genes in endophyte-treated samples were involved in plant or cell growth, participating for example in plant cell wall biosynthesis. The olive plant response to pathogen inoculation was almost similar, also promoting a plant defense response by inducing the production of secondary metabolites, enzymes that break pathogens cell wall, and genes involved in oxidative stress reduction. As detected for endophyte inoculation, down-regulated genes in pathogen-infected plants were involved in plant cell wall biosynthesis, and in the breakdown of plant cell wall components. Altogether these results suggest that olive plants mainly respond to inoculation by down-regulating genes involved in the breakdown of plants cell wall, making the penetration of the pathogen more difficult. Another common response in both endophyte-treated and pathogen-infected plants was the up-regulation of many PR proteins (for example, chitinases, and lipid transfer proteins), suggesting the activation of plant defense response. The plant responded to both fungi by attacking their cell walls (chitinases), and by making their penetration more difficult (lipid transfer proteins). In addition, plants responded to the pathogen by inducing proteins with antimicrobial functions (thaumatin-like proteins) and by inhibiting serine proteases (proteinase inhibitors). This information suggests that olives plants respond to the inoculation of both fungi by inducing genes related to plant defense, and that the plant could suceed in defending against the pathogen, whithout the help of the endophyte. Therefore, further assays should be done before considering *P. aff. commune* as a possible BCA that could be used to replace copper-based fungicides.

5. Annex

Annex 1. Information about alignment process for each sample. Number of reads that aligned and did not align to olive annotated genes: no feature (no feat) shows reads that could not be aligned to a gene; ambiguous (ambig) shows the reads that could align to more than one gene; too low aQual (low) shows the reads whose alignment quality was low; not aligned shows the reads from the BAM file that weren't aligned; alignment not unique shows the reads with more than one reported alignment.

Sample	Reads aligned	No feat	Ambig	Low	Not aligned	Align not unique
C0h_1	46,120,518	1,189,034	442,941	2,282,171	4,257,286	3,746,070
C0h_4	43,929,191	1,545,492	362,176	2,142,399	3,580,183	3,965,708
C0h_11	42,710,766	2,248,100	406,989	2,381,750	3,770,463	4,686,684
C48h_5	47,167,951	1,149,447	472,196	2,137,974	4,325,415	3,570,639
C48h_6	47,082,101	1,047,075	441,737	2,117,963	4,219,682	3,510,288
C48h_7	46,817,287	1,235,427	432,885	2,338,645	3,897,387	3,645,560
C48h_8	47,391,800	1,270,022	434,000	2,509,030	4,075,816	3,725,689
C96h_13	45,511,649	1,068,004	469,254	2,126,554	4,173,248	3,782,151
C96h_14	67,396,366	10,346,553	695,446	4,357,030	6,236,175	20,968,751
C96h_15	74,957,097	11,033,500	644,490	4,215,115	5,082,044	25,755,527
C96h_16	46,168,998	1,049,910	435,261	2,066,547	4,134,020	3,580,747
E0h_18	85,346,488	17,324,482	722,395	5,618,690	8,841,282	37,257,997
E0h_20	43,933,452	1,556,723	456,279	2,321,059	4,612,973	4,100,038
E0h_26	45,843,389	2,008,563	432,117	2,506,249	3,633,653	4,918,233
E0h_28	45,313,488	2,119,039	388,597	2,501,291	3,775,266	4,349,845
E48h_21	45,853,403	1,021,408	404,059	2,282,628	3,923,197	3,403,988
E48h_22	48,004,456	1,282,208	486,100	2,415,459	3,746,859	3,768,422
E48h_24	46,847,842	1,127,929	457,834	2,344,474	3,853,223	3,483,217
E96h_29	46,292,764	929,768	532,855	2,251,619	4,511,644	3,873,441
E96h_30	94,637,541	13,069,745	807,493	5,164,591	6,787,126	31,746,889
E96h_31	45,569,066	912,449	453,852	2,546,428	4,239,183	3,711,651
E96h_32	46,549,332	872,453	474,866	2,281,195	3,963,232	3,625,482
P0h_35	56,313,309	6,057,449	533,727	3,421,127	5,108,649	9,749,555
P0h_36	62,724,523	10,165,000	552,629	4,490,698	6,346,745	16,162,782
P0h_42	60,385,419	7,493,194	651,494	3,670,572	5,691,281	16,278,787
P0h_43	80,102,802	16,075,589	656,233	5,623,587	7,905,076	27,566,050
P48h_38	52,605,189	1,971,838	914,738	2,946,678	4,381,157	5,096,099
P48h_40	46,499,536	2,706,194	449,121	2,586,864	3,999112	5,272,043
P96h_45	44,481,473	929,593	532,593	2,274,649	4,167,738	3,778,235
P96h_46	47,765,829	963,304	438,108	2,390,091	3,741,714	3,697,883
P96h_47	47,137,334	1,158,780	466,845	2,383,476	3,750,643	4,054,973
P96h_48	44,950,929	1,151,677	583,311	2,674,233	3,924,092	3,984,773
EP0h_50	43,961,846	2,897,025	420,655	2,586,900	4,151,543	5,801,856
EP0h_51	43,625,873	2,527,973	446,495	2,553,599	3,945,267	5,219,005
EP0h_59	54,715,511	5,482,867	494,925	3,203,016	5,096,658	10,083,794
EP0h_60	88,726,027	26,447,304	855,438	5,974,566	5,020,510	56,922,761
EP48h_53	45,734,032	1,548,023	467,860	2,480,465	3,958,456	4,132,474
EP48h_56	47,023,402	1,686,406	452,706	2,471,607	3,640,813	4,497,259
EP96h_61	44,875,098	1,273,533	426,733	2,123,264	4,283,658	3,963,518
EP96h_62	49,206,308	1,186,904	534,043	2,269,174	4,071,212	3,964,960
EP96h_63	47,819,240	1,423,619	473,769	2,655,859	4,006,475	4,273,050
EP96h_64	47,863,182	1,133,249	480,186	2,359,828	3,845,563	4,033,237