Application of peptides and endophytes for anthracnose control in olive trees

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“Somente aqueles que se arriscam a ir longe demais conseguem descobrir o quão longe podem chegar."

– T.S. Eliot

ABSTRACT

Anthracnose, caused by several fungal species of the *Colletotrichum* genus, is one of the most economically damaging diseases affecting olive cultivation worldwide. The induction of resistance in olive trees through resistance inducers may provide a sustainable approach to control this disease. This study aimed to evaluate the potential of the fungal endophyte *Penicillium commune* and the peptide systemin in conferring protection to olive trees against anthracnose and to elucidate the mechanisms underlying their protective effects. For this purpose, *Arbequina* olive plants were inoculated with *P. commune*, systemin, a combination of both, or sterile double distilled water (control). After a few days, plants from the different treatments were further inoculated with *Colletotrichum nymphaeae*. Up to 24 hours after inoculation with the pathogen, various biochemical parameters related to antioxidant defense were analyzed in the leaves. The results showed that treatment with *P. commune*, systemin, and their combination significantly reduced the incidence of anthracnose up to 2.8-fold compared to plants inoculated with the pathogen. This effect was related to the activation of antioxidant defense in plants, which was most pronounced in plants treated with *P. commune* + systemin. In this treatment, the levels of hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), and catalase (CAT) were significantly higher than in the other treatments, including in plants inoculated solely with the pathogen. When applied alone, both *P. commune* and systemin also activated defense mechanisms, but to a lesser extent. *P. commune* rapidly increases H_2O_2 levels, SOD and CAT activity, limiting oxidative damage and promoting pathogen detection. Systemin, initially reduces H_2O_2 levels to prevent early stress, then increases it later to strengthen defenses. Overall, the results demonstrate that both *P. commune* and systemin can be explored as resistance inducers against olive anthracnose, with a superior effect observed when used in combination, suggesting a synergistic effect.

Keywords: *Olea europaea*, *Colletotrichum*, systemin, *Penicillium commune*, plant defense system.

RESUMO

A antracnose, causada por várias espécies fúngicas do género *Colletotrichum*, é uma das doenças mais importantes da oliveira a nível mundial. A indução de resistência na oliveira, através de indutores de resistência, pode constituir uma abordagem sustentável para o controlo desta doença. O presente estudo teve como objetivo avaliar o potencial do endófito fúngico *Penicillium commune* e do péptido sistemina na proteção da oliveira contra a antracnose, bem como elucidar os mecanismos subjacentes aos seus efeitos protetores. Para tal, plantas de oliveira da variedade *Arbequina* foram inoculadas com *P. commune*, sistemina, uma combinação de ambos, ou com água bidestilada estéril (controlo). Após alguns dias, as plantas dos diferentes tratamentos foram posteriormente inoculadas com *Colletotrichum nymphaeae*. Até 24 horas após a inoculação com o patógeno, foram analisados nas folhas parâmetros bioquímicos relacionados com a defesa antioxidante. Os resultados demonstraram que o tratamento com *P. commune*, sistemina e a sua combinação reduziram significativamente a incidência da antracnose até 2,8 vezes face a plantas inoculadas apenas com o patógeno. Este efeito deveu-se à ativação da defesa antioxidante nas plantas, que foi mais evidente nas plantas tratadas com *P. commune* + sistemina. Neste tratamento, os níveis de peróxido de hidrogénio (H_2O_2), superóxido dismutase (SOD) e catalase (CAT) foram significativamente superiores face aos outros tratamentos, incluindo plantas inoculadas exclusivamente com o patógeno. Quando aplicados isoladamente, tanto *P. commune* como sistemina, também ativaram mecanismos de defesa, embora de forma menos acentuada. *P. commune* aumentou rapidamente os níveis de H_2O_2, bem como a atividade da SOD e CAT, limitando os danos oxidativos e promovendo a deteção do patógeno. Por sua vez, a sistemina inicialmente reduziu os níveis de H_2O_2 para prevenir stresse oxidativo, aumentando-os posteriormente para reforçar as defesas. De forma geral, os resultados demonstram que tanto o *P. commune* como a sistemina podem ser explorados como indutores de resistência contra a antracnose da oliveira, com um efeito superior observado quando utilizados em combinação, sugerindo um efeito sinérgico.

Palavras-chave: *Olea europaea*, *Colletotrichum*, sistemina, *Penicillium commune*, sistema de defesa vegetal.

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LIST OF ABBREVIATION

ANOVA – Analysis of variance

APX – Ascorbate peroxidase

BSA – Bovine serum albumin

CAT – Catalase

CIMO – Mountain Research Center

CIMO-CC –Mountain Research Center culture collection

DDW – Double distilled water

EDTA – Ethylenediaminetetraacetic acid

e.g. – *Exempli gratia* / for example

ET – Ethylene

EU – European Union

FDA – Food and Drug Administration

FW – Fresh weight

GC-MS – Gas chromatography-mass spectrometry

H_2O_2 – Hydrogen peroxide

HPLC – High-performance liquid chromatography

HR – Hypersensitive response

IOC – International Olive Council

IR – Induced resistance

ISR – Induced systemic resistance

ITS – Internal transcribed spacer

JA – Jasmonic acid

KI – Potassium iodide

LRR – Leucine-rich repeat

MDA – Malondialdehyde

MeJA – Methyl jasmonate

NADPH – Reduced nicotinamide adenine dinucleotide phosphate

NBT – Nitroblue tetrazolium chloride

•OH – Hydroxyl radical

O_2^- – Superoxide

PAST – Paleontological Statistics

PCD – Programmed cell death

PCR – Polymerase chain reaction

PDA – Potato dextrose agar

PR – Pathogeneis-relates

PRIs – Plant resistance inducers

ProSys – Prosystemin

rDNA– Ribosomal deoxyribonucleic acid

ROS – Reactive oxygen species

rRNA – Ribosomal ribonucleic acid

s.l. – *Sensu lato*

sp. – *Species*

s.s. – *Sensu stricto*

SA – Salicylic acid

SOD – Superoxide dismutase

TBA – 2-thiobarbituric acid

TCA – Trichloroacetic acid

U – One unit

VOCs – Volatile organic compounds

FRAMEWORK AND OBJETIVES

The cultivation of olive trees (*Olea europaea L.*) is one of the oldest and economically most significant agricultural activities in the Mediterranean region, particularly in countries such as Portugal, which accounts for a substantial share of global olive oil and table olive production (IOC, 2024a). However, production faces challenges due to biotic stressors, such as anthracnose, a disease caused by several fungal species of the genus *Colletotrichum*, which adversely affects both yield and product quality, including olive oil (Moral et al., 2014). Control of anthracnose relies on integrated practices that combine cultural, chemical, and biological methods. However, copper-based fungicides are still commonly used, despite their drawbacks related to environmental toxicity and soil accumulation (Bahri et al., 2021; Materatski et al., 2019).

In this context, sustainable strategies such as the use of biological control agents have gained relevance. Endophytes, microorganisms that naturally inhabit plant tissues, have shown great potential as biological control agents (Akram et al., 2023; Landum et al., 2016). One promising endophyte is the fungus *Penicillium commune*, which has been shown to induce the release of volatile compounds in olive trees capable of reducing the growth of *Colletotrichum nymphaeae in vitro* (Silva et al., 2023). Additionally, defense signaling peptides, such as systemin, have been studied for their role in activating plant defenses. Systemin, known primarily for its effect in solanaceous species, can induce the production of volatile compounds that aid in plant protection against pathogens and pests (Pastor-Fernández et al., 2023; Sánchez et al., 2023). Although preliminary studies indicate the potential of *P. commune* and systemin as resistance inducers, few studies have validated their efficacy and their modes of action in olive plantations. Moreover, the combined use of resistance inducers presents an interesting approach due to potential synergistic effects that could enhance efficacy. However, this strategy remains largely unexplored.

Therefore, this study aims to evaluate the efficacy of the fungal endophyte *P. commune* and the peptide systemin as resistance-inducers against anthracnose in olive plantlets. Specifically, it will examine the effects of the endophyte and the peptide, both individually and in combination, on the activation of the plant's antioxidant system under controlled greenhouse conditions, as an indicator of protection against olive anthracnose. This study introduces a novel approach by testing the combined use of the endophyte and

the peptide, offering a new perspective on their potential synergistic effects compared to their application individually. Based on previous results obtained in our lab, we hypothesized that *P. commune* and the systemin activate the plant immune system, leading to defense priming when exposed to the pathogen *Colletotrichum* spp.

1. INTRODUCTION

1.1. OLIVE TREE: TAXONOMY, GEOGRAPHIC RANGE, AND SOCIOECONOMIC SIGNIFICANCE IN PORTUGAL

The olive tree (*Olea europaea L.*) is from the *Oleaceae* family, which contains 25 genera and about 600 species (Azevedo-Nogueira et al., 2020). The *Olea* genus has more than 30 species and is the only representative genus of the *Oleaceae* family that produces edible fruit (Bizos et al., 2020). Today, it is estimated that there are more than 2,500 varieties of olive trees, but only a few of them are widely cultivated, with the *Olea europaea* subsp. *europaea* var. *europaea* being the cultivated domestic variety (Azevedo-Nogueira et al., 2020).

The domestication center of the olive occurred in the Western Mediterranean basin, with the first plantations supposed to have taken place between Turkey and Syria (Bahri et al., 2021). These trees became emblematic of the region, potentially cultivated for about 6000 years since the late Bronze Age (Barker, 2022; Dal Corso et al., 2019) and spread throughout the Mediterranean by the Phoenicians and other civilizations, although it is possible that secondary domestication events occurred in other regions, such as Spain or the Maghreb (Moral et al., 2014). The olive tree continued its expansion outside the Mediterranean, and nowadays, this culture is distributed across several continents, namely Europe, America, Asia, Oceania, and Africa (Azevedo-Nogueira et al., 2020; IOC, 2024b). Nevertheless, it is still in the Mediterranean region where 90% of world olive groves are concentrated, ensuring almost 80% of world production (Bahri et al., 2021; IOC, 2023a). The widespread adoption of intensive farming practices in developed countries has been marked by reduced cultivar diversity. For instance, a once-popular Portuguese cultivar, *Galega vulgar*, has been increasingly replaced by cultivars more favored by the industry, such as the Spanish varieties *Picual* and *Arbequina* (Azevedo-Nogueira et al., 2020). The *Arbequina* cultivar, characterized by its low vigor, high branching density, and significant fruiting capacity, produces high-quality oil with a content ranging from approximately 20% to 25%. It has been reported in the literature as moderately tolerant to salinity, demonstrating favorable performance in super-high-density olive groves, which have gained increasing interest in olive-growing regions in recent years (Caruso et al., 2021; Del Buono et al., 2021).

In European Union (EU), most of the total area of olive crop is devoted to olive oil production, being plantations producing table olives accounting a smaller area (IOC, 2023b). The southern Member States of the EU are the largest producers in terms of volume and planted area in the world (Figure 1), ensuring around 58.7% of the total olive oil production (IOC, 2023b). Spain is the largest producer ensuring 31.8% of world production, followed by Italy (12% of world production), Greece (8.1% of world production), and Portugal (6.2% of world production) (IOC, 2023b).

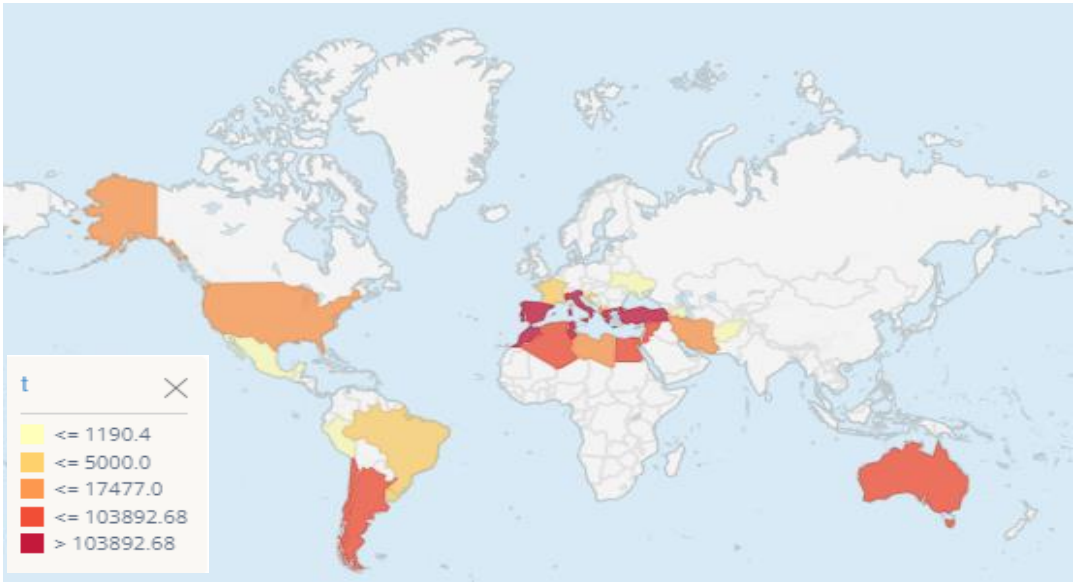

Figure 1: Production quantities (tonnes) of olive oil by country in 2021 – 2022. Source: FAO (2022).

As in other Mediterranean countries, the Portuguese olive sector has been gaining economic importance over the past two decades. In the 2022/2023 crop year, the exportation of olive oil in Portugal reached €911.3 million (IOC, 2024a). Alentejo followed by Trás-os-Montes are the main producing regions of olive oil in Portugal (Dawson & DeAndreis, 2023). Therefore, olive crops have particular economic importance in these rural regions, playing a major role in the development of local populations. The olive-oil tourism has also been an emerging activity in these rural regions and is viewed as a vital instrument for rural development (Pulido-Fernández et al., 2019). These aspects are very important, in particular for some regions of the Mediterranean basin that are expected to undergo important land abandonment processes (Castillo et al., 2020).

The economic importance of olive trees relies mainly on fruit usage as table olives and as extra virgin olive oil. Both are categorized as highly nutritional products. Research led by Dr. Ancel Keys in the 1950s and 1960s established a correlation between the Mediterranean diet, particularly olive oil consumption, and a reduced risk of heart disease, influencing the global popularization of olive oil, recognized in 2004 by the U.S. Food and Drug Administration (FDA) as a functional food with health benefits (Moral et al., 2014).

1.2. CONSTRAINTS TO OLIVE PRODUCTION: THE ANTHRACNOSE

Plants are constantly exposed to a multitude of challenges, including abiotic stresses such as drought and extreme temperatures, as well as biotic stresses including pathogenic infections and pests, impacting their growth and development (Zhang et al., 2022). Despite their resilience to abiotic stress, olive cultivation faces many challenges related to water scarcity, heat, and high irradiance, which make it vulnerable to climate change (Anguita-Maeso et al., 2023). Salinity poses an additional substantial threat to olive performance, disrupting growth, development and, ultimately, crop yield (Del Buono et al., 2021). In what concerns biotic stresses, both pests and diseases are the most damaging causing significant yield losses in olive production. Around 30% of the olive production in the Mediterranean area is estimated to be lost due to pests and diseases, with annual control costs surpassing 200 million euros (Fernandez-Escobar et al., 2013).

Olive orchards are susceptible to a range of diseases, with olive anthracnose being considered one of the most important, causing high losses in production as well as in the quality and value of olive products (Azevedo-Nogueira et al., 2020). First described in 1899, olive anthracnose affects olive trees worldwide, particularly in the Western Mediterranean Basin (Gomes et al., 2012). In Portugal, Spain, and Italy, the losses caused by this disease often attain 80 to 100% of the production (Bahri et al., 2021). In Spain, olive anthracnose causes approximately 85 million euros in economic losses each year. (Olive Oil Times, 2012). Subsequent studies in the Algarve region reported disease prevalence ranging from 65% to 100%, with up to 85% incidence in the rainiest year (Moral et al., 2014).

The disease affects mostly the fruits, causing fruit rot, but leaves, twigs and inflorescences may also be affected (Martins et al., 2019). Characteristic symptoms of anthracnose in olive fruits arise during the late ripening stage, and include dark necrotic

lesions and rot accompanied by the production of abundant orange masses of conidia. These symptoms often lead to premature fruit drop or mummification (Figure 2) (Talhinhas et al., 2018).

Figure 2: Typical symptoms and signs of olive anthracnose on fruits (a and b), leading to fruit mummification (c). Source: Ueno (2018).

Anthracnose-infected olives are often unsuitable for processing into high-quality olive oil or table olives (Azevedo-Nogueira et al., 2020). Olive oil produced from anthracnose infected fruits may exhibit distinct characteristics from those desired, such as a reduction in the content of phenolic compounds and tocopherols, as well as an increase in free acidity, which impairs the organoleptic characteristics of olive oil. Other characteristics, such as color, taste, and aroma, are also affected. There is a tolerance for infection rates below 16%, and the consumption of olive oil is discontinued when infection rates are above 46% (Azevedo-Nogueira et al., 2020; Moral et al., 2014).

Symptoms on leaves and shoots encompass chlorosis, necrosis, extensive defoliation, and the dieback of twigs and branches (Cacciola et al., 2012). Infected flowers undergo rapid drying, resulting in their premature drop (Sergeeva et al., 2008). The severity of infections is influenced by weather conditions, with relative humidity higher than 98% and air temperature warmest (10 to 30ºC) favorable for the development of the disease (Cacciola et al., 2012; Moreira et al., 2021a). Olive anthracnose incidence and severity also vary considerably depending on the susceptibility of olive cultivars, inoculum pressure, and agronomical practices, such as density of olive tree plantation, with higher severity observed in super high-density orchards (Moral et al., 2021; Talhinhas et al., 2018).

1.2.1. ANTHRACNOSE: CASUAL AGENTS AND LIFE CYCLE

Olive anthracnose has been associated with several species within the *Colletotrichum* genus (Talhinhas et al., 2018). The fungal genus *Colletotrichum* is an asexual fungus, with its sexual state traditionally classified under the genus *Glomerella* (Sordariomycetes; Hypocreomycetidae; Glomerellaceae; Glomerelales). It comprises nearly 200 species and exhibits various nutritional strategies such as necrotrophy, hemibiotrophy, and endophytism (Crouch et al., 2014). It is responsible for leaf anthracnose and rot diseases in most cultivated plants (Widiastuti et al., 2023). It manifests as various diseases with different names, such as bitter rot, ripe rot, and fruit rot (Dowling et al., 2020).

Anthracnose in olive trees is attributed to two fungal complexes of the genus *Colletotrichum*, namely *C. acutatum sensu lato* (s.l.) and *C. gloeosporioides* s.l. complexes (Talhinhas et al., 2018). Several species belonging to *C. boninense* s.l. complex has also been associated with olive anthracnose, but they do not appear to represent a serious threat when compared to the other two complexes (Schena et al., 2014). The most important pathogens associated with olive anthracnose worldwide within the *C. acutatum* s.l. complex, include *C. acutatum sensu stricto* (s.s.), *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. rhombiforme*, and *C. simmondsii* (Moreira et al., 2021b; Talhinhas et al., 2018). In the *C. gloeosporioides* s.l. complex, both *C. gloeosporioides* s.s. and *C. theobromicola*, were reported to be the most virulent on olives (Schena et al., 2014; Talhinhas et al., 2018). In the majority of olive-growing regions, *C. godetiae*, *C. acutatum* s.s., and *C. nymphaeae* are the most prevalent species (Talhinhas et al., 2018).

In Portugal, *C. nymphaeae* followed by *C. godetiae* and *C. acutatum* s.s., are the main causal agents of olive anthracnose, with *C. godetiae* causing major damage in the Trás-os-Montes region (Talhinhas et al., 2009).

Olive anthracnose has a complex life cycle which still remains incompletely understood, with uncertainty about the pathogen's ability to travel between organs. Some studies have shown that the pathogens can overwinter on infected leaves, branches and mummified fruits remaining in trees (Talhinhas et al., 2011). These infected tissues act as sources of primary inoculum for the disease in the next spring (Moral & Trapero, 2009; Talhinhas et al., 2018). During the spring, spores (conidia) are produced and spread by rain or wind, initiating infection on olive inflorescences and young fruit (Moral & Trapero, 2009). The pathogen normally remains latent on infected tissues until the fruit begins to ripen in autumn. During this period, the pathogen shifts to a necrotrophic stage, resulting in the manifestation of anthracnose symptoms by the production of spores on the surface of infected fruits (Moral & Trapero, 2009). These spores are spread through rain splash to newly formed fruits, initiating secondary disease cycles and leading to the occurrence of epidemic outbreaks (Talhinhas et al., 2018). Autumn is the most favorable season for anthracnose development when high humidity and warmer temperatures favor the germination of conidia, which were already present in the unripe fruits, and take advantage of fruit ripening to develop (Dowling et al., 2020).

The infection process of *Colletotrichum* spp. starts with the adhesion of conidia to the surface of inflorescence and olive fruits. Under favorable climatic conditions, the conidia germinate to form a germ tube that extends further and forms an appressorium (Smith et al., 1999). This structure can penetrate the plant's cuticle and epidermal cells to reach the intracellular space. Following penetration, *Colletotrichum* spp. undergoes a hemibiotrophic stage, where the mycelium spreads through the plant tissue without causing symptoms until the onset of fruit ripening in autumn. As the fruit ripens, the pathogen shifts to a necrotrophic stage, resulting in visible symptoms of anthracnose (Azevedo-Nogueira et al., 2020).

1.2.2. STRATEGIES CURRENTLY USED TO CONTROL OLIVE ANTHRACNOSE

The control of olive anthracnose can be challenging due to the complex nature of the disease and the various factors influencing its development. Therefore, its management relies on the combination of various approaches, including cultural, chemical, and biological control methods. Cultural methods play a substantial role in preventing *Colletotrichum* spp. infections, and may include (i) the use of cultivars tolerant/resistant to anthracnose in new plantations; (ii) pruning to enhance canopy aeration; (iii) optimizing drainage and irrigation; (iv) maintaining balanced fertilization; (v) and managing insect pests like the olive fruit fly that could potentially spread the pathogen and increase the risk of infections caused by ovipositor wounds (Cacciola et al., 2012; Moral et al., 2014; Sergeeva et al., 2008).

Chemical control, with the application of chemical fungicides, remains one of the most common methods; however, it presents limitations regarding effectiveness, safety for applicators, residues in food, and environmental contamination (Bahri et al., 2021). Copper-based fungicides, such as copper hydroxide or copper oxychloride, are the most widely used for the control of olive anthracnose due to their broad-spectrum antifungal properties (Cacciola et al., 2012). However, their effectiveness in controlling the disease is very limited. These copper-based products work primarily as protectant fungicides, forming a barrier on the plant surface and inhibiting the germination of fungal spores (Materatski et al., 2019).

The pressure for sustainable production methods emphasizes the use of living organisms as control agents against plant pests, diseases, and weeds. This includes the use of various entities such as predators, parasitoids, and microorganisms, including endophytes (Bahri et al., 2021; Martins et al., 2019). In the last decade, a number of studies have been performed to identify and optimize specific biological control agents, primarily microorganisms, tailored to combat olive anthracnose effectively. Certain microorganisms, such as *Aureobasidium pullulans*, *Curtobacterium flaccumfaciens* and *Paenibacillus polymyxa,* showed promising results by reducing up to 50% the severity of the symptoms produced by *C. acutatum* in inoculated detached fruits (Segura, 2003). The group of microorganisms that naturally inhabit the internal tissues of plants (called endophytes), show particularly interest as biological control agents due to the multiplicity of beneficial effects they confer to host plant, including protection to pathogens (Akram et al., 2023). Some endophytes isolated from olive tree leaves, such as *Alternaria* sp.,

Diaporthe sp., *Nigrospora oryzae,* and *Aureobasidium pullulans*, showed capacity to reduce significantly the growth, sporulation and germination of *C. acutatum* in dual culture assays, and *A. pullulans* in addition reduced the incidence and severity of anthracnose in detached fruits (Landum et al., 2016; Sdiri et al., 2022). Similarly, the endophytic fungi *Chondrostereum purpureum*, *Chaetomium globosum*, *Aspergillus westerdijkiae*, *Aspergillus* sp. 1, *Quambalaria cyanescens*, *Epicoccum nigrum,* and *Aspergillus brasiliensis*, isolated from olive fruits showed ability to reduced significantly the growth, sporulation and germination of *C. acutatum*, and to cause morphological alterations on pathogen hyphae in dual culture assays (Preto et al., 2017).

More recently, the endophytic fungus *Penicillium commune* (strain CIMO 14FM009) isolated from olive tree twigs, showed the capacity to induce the release of volatile compounds on the olive trees, leading to a significant reduction in the growth and sporulation of *C. nymphaeae* (Silva et al., 2023). However, these promising results have been reported in studies using *in vitro* and *in vivo* tests on detached fruits under controlled conditions. Only a few studies were conducted in field conditions (Silva et al., 2023). Therefore, ongoing research is essential to further understand the mode of action of the endophytes in conferring host plant protection against pathogens for their effective use as biological control agents.

1.3. MECHANISMS OF FUNGAL ENDOPHYTES IN PLANT PROTECTION AGAINST PATHOGENS

Fungal endophytes play a pivotal role in defending host plants against pathogens by employing a combination of direct and indirect mechanisms to enhance plant health and vigor. Direct mechanisms typically involve the production of a diverse array of secondary metabolites and compounds such as peptides, flavonoids, phenols, quinones, terpenes, alkaloids, volatile organic compounds, and polyketides, which directly suppress pathogens through antibiosis, hyperparasitism, and competition for nutrients (Fadiji & Babalola, 2020). In addition to secondary metabolites, lytic enzymes, including β-1,3-glucanases, chitinases, and cellulases, are produced by endophytes to hydrolyze the cell wall of pathogens, further contributing to the protection of host plants (Akram et al., 2023; Grabka et al., 2022).

Concurrently, indirect mechanisms focus primarily on inducing systemic resistance (ISR) in host plants by endophytes, priming them to mount a faster and more effective defense response against pathogens (Fontana et al., 2021; Mauch-Mani et al., 2017). This defense priming offers advantages by reducing energy costs associated with constant defense activation, while providing long-lasting protection that endures throughout the plant's life and can even be inherited by future generations (Tiwari et al., 2022). The mediation of host disease resistance by endophytes occurs through the jasmonic acid (JA) and ethylene (ET) signaling pathways, leading to the upregulation of genes associated with defense and stress responses, including those encoding pathogenesis-related (PR) proteins, components of the phenylpropanoid pathway, and enzymes responsible for cell wall reinforcement and oxidative reactions, such as oxidases and peroxidases (Akram et al., 2023).

Indeed, upon the recognition of the pathogen by plant receptors, a signaling cascade is activated leading to the generation of reactive oxygen species (ROS) (Mansoor et al., 2022). This ROS burst, occurring as part of the plant's first line of defense, play several roles, depending on their cellular concentration. At higher levels at the infection site, the ROS act as antimicrobial agents by creating toxic conditions for the pathogen, leading to the oxidation of their proteins, lipids, and deoxyribonucleic acid (DNA) (Lamb & Dixon, 1997). This rapid accumulation of ROS can also lead to localized cell death in plants, a phenomenon called hypersensitive response (HR), creating a barrier that prevents pathogen spread (Heath, 2000). In small concentrations, ROS act as signaling molecules, activating downstream defense pathways and preparing distant cells to respond to potential infections, thereby contributing to the whole-plant resistance (Grant & Loake, 2000). The most abundant and stable ROS in plants include radical superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) (Mansoor et al., 2022). Their levels in plant cells are regulated through a combination of production and scavenging mechanisms (Mansoor et al., 2022). This balance is essential to manage the beneficial roles of ROS in defense and signaling without allowing harmful accumulation. The plasma membrane NADPH oxidases are the primary source of ROS during pathogen defense (Rahikainen et al., 2016).

In turns, antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), scavenge ROS by converting them into less

harmful molecules (Zandi & Schnug, 2022). The primary function of superoxide dismutase (SOD, EC 1.15.1.1) is to convert superoxide radicals into hydrogen peroxide and oxygen, thereby preventing the formation of highly reactive hydroxyl radicals through the Haber-Weiss reaction. This regulates the levels of substrates that could lead to the production of hydroxyl radicals, reducing the risk of cellular damage (Das & Roychoudhury, 2014; Zandi & Schnug, 2022). Catalase (CAT, EC 1.11.1.6) plays a crucial role in detoxifying hydrogen peroxide (H_2O_2), converting it into water and oxygen. This enzyme acts mainly in peroxisomes, being essential in processes such as photorespiration and β-oxidation of fatty acids, where H_2O_2 is frequently produced. The energetic efficiency of this reaction highlights the importance of CAT in protecting cells against oxidative stress (Tuzet et al., 2019; Zandi & Schnug, 2022). Ascorbate peroxidases (APX, EC 1.1.11.1) are fundamental for the control of H_2O_2 toxicity, especially in chloroplasts and cytosol. APX enzymes catalyze the reduction of H_2O_2 into water using ascorbate as a reducing factor (Zandi & Schnug, 2022).

1.4. *PENICILLIUM COMMUNE*

The ascomycete genus *Penicillium* encompasses contrasting worlds: a ubiquitous agent of spoilage in food and indoor environments, and a biotechnological asset, both in the biological control of agricultural pests and in the production of pharmaceuticals and food products (Diblasi et al., 2015; Nicoletti et al., 2014). Its metabolic versatility and filamentous growth make it an excellent candidate for cellular factories, capable of efficiently producing various secondary metabolites (Diblasi et al., 2015). *Penicillium* strains have been recognized as integral components of the 'extended phenotype' or symbiotic community of plant species, showcasing their close association with hosts and participation in complex ecological relationships. Ranging from a balanced antagonism to potential mutualism, these microbes demonstrate versatile ecological and trophic interactions within plant tissues (Nicoletti et al., 2014).

Penicillium is primarily known for its production of secondary metabolites with diverse biological properties, such as antibiotics, enzymes, and toxins. The discovery of penicillin by Alexander Fleming in 1929 marked a milestone in modern medicine. Since then, other *Penicillium* species have been explored for the production of bioactive compounds with antimicrobial, anticancer, antiviral, and antioxidant properties(Fleming,

1929; Toghueo & Boyom, 2020). These compounds play crucial roles in biotechnology, pharmacology, and agriculture, where they are used to combat plant pathogens and promote plant growth (Tacconelli et al., 2018).

In agriculture, several *Penicillium* species have been investigated for their ability to promote plant growth. The mode of action of these species includes the production of plant hormones, such as indole acetic acid, and the ability to solubilize soil nutrients, such as phosphate, which are essential for plant growth (Hassan, 2017; Waqas et al., 2012). Additionally, endophytic *Penicillium* species have demonstrated the ability to reduce both biotic and abiotic stress in plants, protecting them from pathogens and enhancing nutrient uptake (Toghueo & Boyom, 2020). *Penicillium* has shown great potential as a biocontrol agent. Species, such as *Penicillium chrysogenum* and *Penicillium commune*, produce antifungal and antibacterial compounds that inhibit the growth of agricultural pathogens, including those responsible for diseases like anthracnose (Gashgari et al., 2016; Mishra et al., 2016). Specifically, *Penicillium commune* has been effective against *Colletotrichum acutatum*, the pathogen responsible for anthracnose in olive trees, by producing antifungal compounds and competing for nutrients (Malhadas et al., 2017). Besides plant disease control, *Penicillium* has also been studied for its ability to produce compounds with insecticidal activity, which can contribute to the control of agricultural pests. Compounds such as hamisonine, isolated from *Penicillium oxalicum*, have demonstrated efficacy in mosquito larval control studies (Seetharaman et al., 2017). Studies on the interaction of *Penicillium* with plants indicate that these fungi can colonize plant tissues, promoting plant growth, helping cultivated plants in the phytostabilization of heavy metals and enhancing induced systemic resistance (Ikram et al., 2018). The production of plant hormones and nutrient solubilization are key mechanisms through which *Penicillium* positively impacts plant development.

Although many studies have demonstrated the potential of *Penicillium* in controlling anthracnose and other agricultural pathogens, significant gaps remain in understanding the exact mechanisms of action, especially under field conditions. Further research is needed to explore the role of *Penicillium* in plant-microorganism interactions and its application in sustainable agricultural systems (Toghueo & Boyom, 2020). The use of *Penicillium commune* as a biocontrol agent in olive plantations presents great potential, particularly considering its effectiveness against pathogens and their

environmental safety. However, its efficacy under field conditions, the stability of the compounds produced, and interactions with other microorganisms require further investigation to ensure its practical application in a safe and sustainable manner (Malhadas et al., 2017).

1.5. SYSTEMIN AS A PLANT RESISTANCE INDUCER

Plant Resistance Inducers (PRIs) are substances that enhance protection against pathogen attacks by activating the innate defense mechanisms of plants, a process known as Induced Resistance (IR). These agents are also referred to as resistance activators, plant defense activators, and elicitors (Alexandersson et al., 2016). PRIs can be chemical compounds, microbial extracts, or plant extracts. However, they rarely provide complete control over pathogens, and the success of this approach is influenced by multiple factors such as plant genotype, developmental stage, environmental conditions, as well as the timing and method of PRI application (Walter & Fountaine, 2009; Walters et al., 2013).

A number of peptides with defensive signaling functions against biotic stressors have been identified recently in various plant species, including tomato, potato, *Arabidopsis*, and soybean (Pastor-Fernández et al., 2023). These peptides are released in response to pest or pathogen attacks and trigger a series of plant defenses, strengthening the plants' immune response (Pastor-Fernández et al., 2023). From these peptides, systemin, an 18-amino acid peptide, was reported to be essential for both direct and indirect plant defenses. This peptide was showed to play a key role in plant defense by directly enhancing resistance against herbivores and pathogens (Coppola et al., 2015; Molisso et al., 2020; Pastor-Fernández et al., 2022). It also supports indirect defenses by enhancing the production of volatile compounds that attract natural enemies of pests and alert neighboring plants, thus contributing to the plant's indirect systemic defense (Coppola et al., 2017; Sánchez et al., 2023). The systemin is formed from a larger 200-amino acid protein called Prosystemin (ProSys), which plants produce when tissues are damaged or in response to other signals (Degenhardt et al., 2010). Once formed, systemin binds to a receptor on the plant cell membrane, the SR160 receptor, which is part of the

leucine-rich repeat (LRR) kinase receptor family (Ryan & Pearce, 1998; Scheer & Ryan, 2002). Its recognition leads to a cascade of signals and defense responses, including the movement of signaling molecules like jasmonic acid (JA) and volatile organic compounds, which help activate defense throughout the plant (Zhang et al., 2020). Indeed, the absence of JA in certain tomato mutants has demonstrated its essential role in the effectiveness of systemin-based defenses (Ryan & Pearce, 1998; Schaller & Ryan, 1996). Further investigations, including gas chromatography-mass spectrometry (GC-MS), have reinforced systemin's importance in triggering the release of defense-related compounds, notably the increase of certain monoterpenes, which are key to the plant's response to herbivore damage (Corrado et al., 2007). Molecular studies, such as real-time PCR, have confirmed the link between systemin and the activation of specific genes involved in defense and volatile production, emphasizing the potential of these findings for agricultural applications (Corrado et al., 2007). Because of its well-known ability to prime plant defenses, systemin application could be a valuable tool for sustainable pest and disease control.

2. MATERIAL AND METHODS

2.1. MICROBIAL ISOLATES AND PREPARATION OF INOCULA

The endophytic fungus strain *Penicillium commune* (CIMO 14FM009) (Figure 3a) and the pathogenic fungus strain *Colletotrichum nymphaeae* (CIMO 15FM003) (Figure 3b) were obtained from the fungal culture collection of the Mountain Research Center (CIMO-CC). The *P. commune* strain was isolated from asymptomatic twigs of the anthracnose-tolerant cultivar 'Cobrançosa', and its identity was confirmed by sequencing the internal transcribed spacer (ITS) region of ribosomal DNA (ITS1, 5.8S rRNA, and ITS2 regions), β-tubulin (Bt2a/Bt2b primers) and calmodulin (cmd5/cmd6 primers) genes (Silva et al., 2023). The obtained sequence of the ITS region is available in the GeneBank database under the accession number KM519651.1. *Colletotrichum nymphaeae* (CIMO 15FM003), a primary causal agent of olive anthracnose in Portugal (Talhinhas et al., 2018), was isolated from the inner tissues of naturally infected olives. The identification of this strain was performed by sequencing the ITS region of rDNA using both ITS1/ITS4 primers and degenerate primers Coll1F/Coll3Rb, and in addition the β-tubulin (Bt2a/Bt2b primers) and histone (CYLH3F/CYLH3R primers) genes (Silva et al., 2023). These two strains have been maintaining in the CIMO-CC, in an aqueous glycerol solution (30%, v/v) at -80ºC.

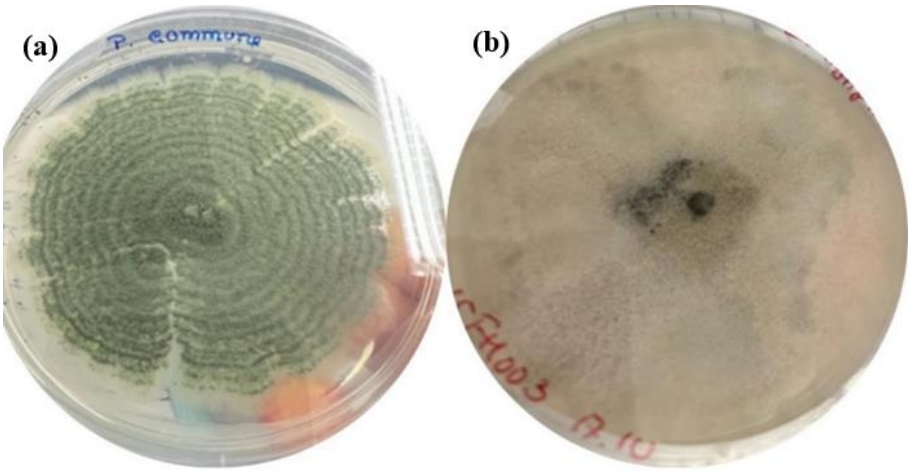

Figure 3: Macroscopic aspects of (a) *Penicillium commune* (CIMO 14FM009) and (b) *Colletotrichum nymphaeae* (CIMO 15FM003) colonies growing on potato dextrose agar medium, after 15 days.

The inocula of both fungi were prepared by transferring spores from frozen stocks to plates containing potato dextrose agar (PDA; HiMedia Laboratories) and incubating

them for 15 days at room temperature. The spores were scraped from the PDA plates using a sterile loop and suspended in a 0.025% (v/v) sterile Tween 80 aqueous solution. Spore counts were performed using a Neübauer chamber under a Leica DM500 light microscope. The spore suspensions were adjusted to a concentration of $1x10^8$ conidia/mL with the same Tween 80 aqueous solution for *P. commune* and *C. nymphaeae* and used for the inoculation of olive plantlets.

2.2. PREPARATION OF SYSTEMIN

The systemin peptide used in this research is a synthetic compound of high purity (>90% by HPLC), with the sequence AVQSKPPSKRDPPKMQTD (N-C terminal), custom-produced by Biomatik Corporation, Canada. The lyophilized systemin was reconstituted in double distilled water (DDW) to obtain a stock solution of 500 nM. From the stock solution, a working solution with a final concentration of 10 nM was prepared. This concentration was used for the inoculation of the plants.

2.3. OLIVE TREE INOCULATION AND SAMPLING

The efficacy of the fungal endophyte *P. commune* (strain CIMO 14FM009) and the peptide systemin as resistance-inducers against olive anthracnose was tested in greenhouse pot experiments conducted at the School of Agriculture, Instituto Politécnico de Bragança (Figure 4). During the whole assay, the plants were maintained at 26°C, 8 h light/16 h dark photoperiod and 70% relative humidity, and automatic watering scheduled twice daily via sprinkler irrigation for 1 minute each time. Accordingly, one-year-old olive plants of cv. *Arbequina* with fruits were obtained from a commercial nursery. The assay was designed in order to evaluate the effect of the endophyte and the peptide, both individually and in combination, on the activation of the plant's antioxidant system.

Figure 4: Olive plants of cv. *Arbequina* in the greenhouse.

To assess the effect of *P. commune* alone, 25 olive plants were inoculated by applying 50 µL of the endophyte spore suspension ($1x10^8$ conidia/mL) into a V-shaped wound (1 cm long) made on the main stem with a sterile scalpel 5 cm above the substrate level (Figure 5). One week after inoculation, 13 plants were further inoculated with the pathogen *C. nymphaeae* and the remaining 12 plants were inoculated with sterile double distilled water (DDW, negative control), as illustrated in Figure 6. Both *C. nymphaeae* ($1x10^8$ conidia/mL) and the DDW control were inoculated by spraying, at a rate of 3 ml per plant.

Figure 5: Inoculation of olive plants with a spore suspension of *P. commune* into a wound made in the main stem.

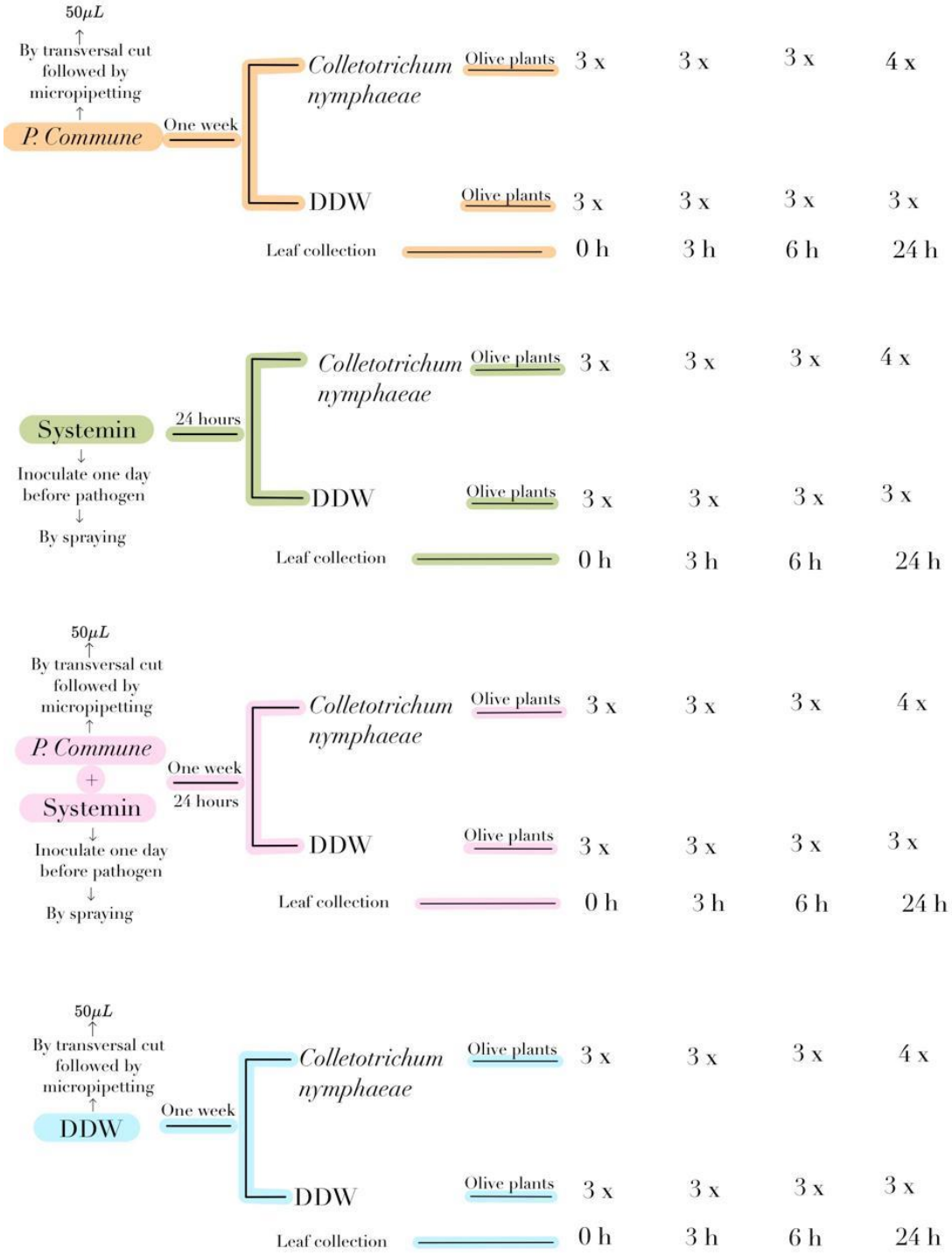

Figure 6: Schematic representation of the different treatments performed, showing the inoculation of the plants and the collection of leaves for analysis. DDW – Double distilled water.

For the systemin treatment, 25 olive plants were sprayed with an aqueous solution of systemin at a concentration of 10 nM, applying 3 mL per plant from a height of 5 cm

above the substrate level. After 24 hours of the inoculation, 13 plants were further inoculated with the pathogen *C. nymphaeae* ($1x10^8$ conidia/mL) and the remaining 12 plants were inoculated with sterile double distilled water (DDW, negative control), by spraying at a rate of 3 mL per plant (Figure 6).

In the combined *P. commune* + systemin treatment, 25 olive plants were first inoculated with *P. commune* by applying a spore suspension ($1x10^8$ conidia/mL) to a stem wound as previously described, followed six days later by a systemin spray application (10 nM) at a rate of 3 mL per plant. One day later of the systemin inoculation, 13 olive plants were further inoculated with the pathogen *C. nymphaeae* ($1x10^8$ conidia/mL) and the remaining 12 plants were inoculated with sterile double distilled water (DDW, negative control), by spray application of 3 mL per plant (Figure 6).

For the control treatment, 25 olive plants were first inoculated with DDW by applying 50 µL DDW in a wound made in the main stem as previously described for the endophyte treatment. One week later, 13 olive plants were further inoculated with *C. nymphaeae* ($1x10^8$ conidia/mL) and the remaining 12 plants were inoculated with double distilled water (DDW, negative control), by spray application of 3 mL per plant (Figure 6).

The greenhouse experiment was conducted in a completely randomized experimental design with 4 treatments (*P. commune*, systemin, *P. commune* + systemin, and a control). The experiment included 13 replications per treatment with *C. nymphaeae* inoculation and 12 replications per treatment with DDW inoculation, totalizing 100 experimental units. Each plant was treated individually.

After 0, 3, 6, and 24 hours of pathogen inoculation, 21 leaves from three different plants (from the same treatment) were collected and combined (to obtain 3 replicates per treatment and hour). Each sample was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. The collected leaves were then used for the evaluation of biochemical parameters involved in plant resistance, including H_2O_2 content and lipid peroxidation, as well as antioxidant enzymes (SOD and CAT). Incidence of olive anthracnose were also evaluated visually every week. At the end of the three months after pathogen inoculation, the disease incidence (%) was estimated by dividing the number of fruits showing symptoms by the total number of fruits per olive plant, in each treatment.

2.4. EVALUATION OF ROS (H_2O_2) AND LIPID PEROXIDATION

The contents of H_2O_2 in leaves was estimated following the procedure described in Baptista et al. (2007). Briefly, 0.07 g of fresh leaf previously grinded in liquid nitrogen were homogenized with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C, and supernatant was collected. Aliquots of the supernatants were added to a reaction medium composed of 10 mM potassium phosphate buffer at pH 7.0 and 1.0 M potassium iodide (KI). The H_2O_2 content was estimated by spectrophotometric readings at 390nm in an UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), considering a calibration curve using solutions with known H_2O_2 concentrations (0.0979 mM – 0.00653 mM). Absorbance readings obtained in the leaf samples were converted to H_2O_2 concentration using the linear regression equation from the calibration curve ($y=0.3469x+0.0088$, with a $R^2 = 0.99$). The results were expressed as μmol g^{-1} fresh weight (FW). Three combined biological replicates, each with three technical replicates, were analyzed for each treatment and time point.

Lipid peroxidation was estimated by quantifying the malondialdehyde (MDA) content, following the method of Heath & Packer (1968). For this, 0.16 g of fresh leaf, previously grinded in liquid nitrogen, were homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath. The homogenates were then centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was carefully transferred to a new tube kept on ice. Subsequently, 500 µL of the supernatant was mixed with 500 µL of 2-thiobarbituric acid [TBA reagent: 0.5% (w/v) TBA in 20% (w/v) TCA]. This mixture was heated at 95 °C for 30 minutes in a water bath and then quickly cooled in an ice bath to room temperature. The absorbance of the colored supernatant was measured at 532 nm and at 600 nm to correct for non-specific turbidity, in an UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The MDA content was calculated using its molar extinction coefficient (0.155×10^{-3} M^{-1} cm^{-1}) and expressed as μmol g^{-1} FW. Three combined biological replicates, each with three technical replicates, were analyzed for each treatment and time point.

2.5. ANTIOXIDANT ENZYMES (SOD AND CAT)

The activities of the ROS-scavenging enzymes, namely CAT and SOD, was estimated following the procedure described in Baptista et al. (2007). To do this, the protein must first be extracted from the leaves and quantified. Protein quantification was performed using the Coomassie Blue microassay, following the method described by Bradford (1976). Approximately 0.15 g of fresh leaf previously grinded in liquid nitrogen was homogenized in 1.5 ml of 80 mM phosphate buffer (pH 7.0), 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone at 4°C. After the incubation at 4 °C for 15 min, the homogenates were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were recovered to a new tube. The protein content was then determined using the Coomassie Blue microassay, with Bovine Serum Albumin (BSA) as the standard, according to the method of Bradford (1976). The absorvance readings were performed at 595 nm in an UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and further converted to protein concentration (mg/g FW) using the linear regression equation from the calibration curve ($y=0.0313x+0.011$, with a $R^2=0.9966$).

The SOD (EC 1.15.1.1) activity was determined according to the Beyer & Beyer & Fridovich (1987) method, which was based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium chloride (NBT) by the superoxide radicals generated photochemically. The reaction mixture consisted of 100 mM phosphate buffer (pH 7.8), 0.2 mM EDTA, 19.8 mM l-methionine, 0.05% (v/v) Triton X-100, 570 µM NBT, 9 µM riboflavin, and 5-100 µL protein extract. After 6 min of incubation at 30°C under continuous light, absorbance was read at 560 nm in a Genesys 10 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under the above assay conditions, and the results were expressed as U μg^{-1} protein.

CAT (EC 1.11.1.6) activity was determined according to Aebi (1984) by following the decomposition of H_2O_2, via monitoring the decrease in absorbance at 240 nm due to H_2O_2 breakdown (ε = 39.4 mM^{-1} cm^{-1}) in an UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Reactions were performed in 80 mM phosphate buffer (pH 7.0) containing 1 mM H_2O_2 and initiated by adding 10 µL of protein extract.

One unit (U) of CAT activity is defined as the amount of enzyme necessary to decompose 1 μmol min^{-1} H_2O_2 under the above assay conditions. The CAT activity was expressed as U μg^{-1} protein. For all antioxidant enzymes, three combined biological replicates were analyzed for each treatment and time point.

2.6. STATISTICAL ANALYSIS

The results obtained from the evaluation of the biochemical parameters involved in plant resistance, including ROS (H_2O_2), lipid peroxidation (MDA) and antioxidant enzyme activity (SOD and CAT), as well as the incidence results, were statistically analyzed to determine significant differences between treatments and time points. Before conducting the analysis to determine differences among the means, the normality of the data was assessed using the Shapiro-Wilk test. If the data were normally distributed ($p > 0.05$), a one-way analysis of variance (ANOVA) was performed to compare group means, followed by Tukey's post-hoc test to identify pairwise differences between treatments. For data that did not follow a normal distribution ($p < 0.05$), the non-parametric Kruskal-Wallis test was applied to evaluate differences between groups. Following the Kruskal-Wallis test, Dunn's post-hoc test was performed to identify significant pairwise differences between treatments.

All statistical analyses were conducted using PAST v4.03 software, with a significance level set at $p < 0.05$ for all tests. Graphs illustrating the results were created using Microsoft Excel (Office 16).

3. RESULTS AND DISCUSSION

3.1. INCIDENCE OF ANTHRACNOSE

In this study, the effectiveness of the endophyte *P. commune* and the peptide systemin, either when used alone or in combination, to control anthracnose in olive plants was evaluated under greenhouse conditions. Accordingly, olive plants cv. *Arbequina* were inoculated with *P. commune*, systemin, with the two, or with sterile double distilled water (control). A few days after inoculation (up to 1 week), plants from the different treatments were inoculated with the pathogen *C. nymphaeae*, and the incidence of anthracnose disease was evaluated three months later. The results demonstrated that *P. commune* and systemin, applied individually or in combination, effectively protected olive plants against *C. nymphaeae* infection (Figure 7). The inoculation of plants with *P. commune*, whether alone or combined with systemin, reduced significantly ($p < 0.05$) the incidence of anthracnose up to 2.8-fold compared to plants inoculated with the pathogen. The inoculation with systemin, when applied alone to olive plants, also significantly reduced anthracnose incidence by up to 2.21-fold. Overall, this result suggests that the exogenous application of *P. commune* and systemin significantly reduced olive susceptibility to infection, whether applied individually or together, offering a novel perspective on the combined use of endophytes and peptides for protecting olive crops.

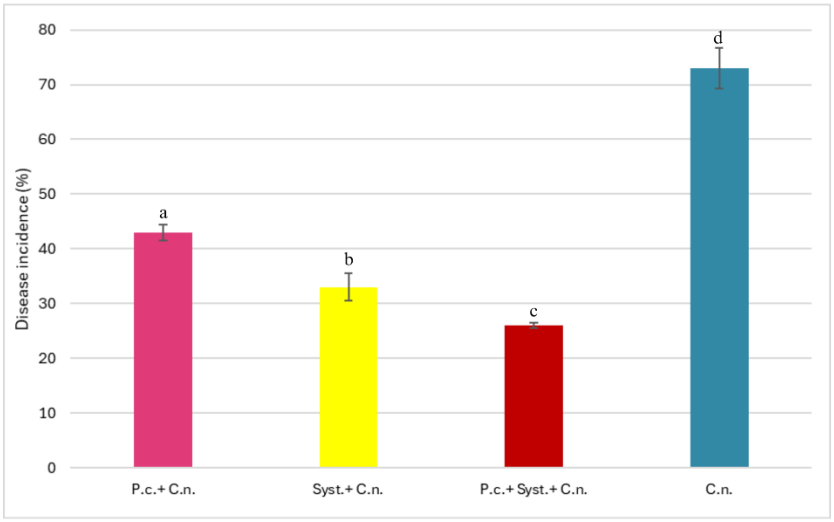

Figure 7: Incidence of anthracnose in olives plants inoculated with *Penicillium commune* and *Colletotrichum nymphaeae* (P.c.+C.n.), systemin and *C. nymphaeae* (Syst.+C.n.), *P. commune* and systemin and *C. nymphaeae* (P.c.+Syst.+C.n.), and *C. nymphaeae* (C.n.). Values are presented as percentages ± SD (n = 13), with statistically significant differences between treatments indicated by different letters ($p < 0.05$).

Previous studies have similarly showed the ability of *P. commune* to protect olives from *Colletotrichum* spp. infection. For example, in artificially inoculated olive fruits, Amaral (2022) observed a significant reduction on both incidence and severity of anthracnose by up to 70% in olives treated with *P. commune*. This endophyte was similarly reported to induced the release of volatile compounds on fruits and leaves of the olive trees, with the capacity to reduce significantly the growth and sporulation of *C. nymphaeae* (Silva et al., 2023). In bioassays with olive fruits, Martins et al. (2013) also demonstrated that the strain *P. commune* significantly reduced the growth, sporulation, and germination of *Colletotrichum acutatum*. To the best of our knowledge, this was the first study reporting the protective effect of systemin against *Colletotrichum* spp. infection. The application of systemin have been showed to enhance plant tolerance to other pathogens, such as *Botrytis cinerea* and *Plectosphaerella cucumerina* across various plant species. For example, the application of systemin in eggplant (*Solanum melongena*) and grapevine (*Vitis vinifera*) showed to confer protection against *B. cinerea* by activating multiple defense mechanisms. These include the accumulation of phenolic compounds, the enhancement of antioxidant enzyme activity, and the upregulation of defense-related genes (Molisso et al., 2020). Similarly, the application of systemin in *Arabidopsis* was reported to confer protection against the pathogen *P. cucumerina* by activating early signaling events and enhancing defense-related metabolites (Pastor-Fernández et al., 2022). In tomato plants, Aprile et al. (2022) demonstrated that the treatment with *Trichoderma afroharzianum* and systemin, confers significant protection against the fungal pathogens *Fusarium oxysporum* and *Botrytis cinerea*, as well as the insect pest *Tuta absoluta*. This defensive response was notably correlated with an increased accumulation of jasmonic acid and its related metabolites, alongside a reduction in salicylic acid levels. These findings reinforce the potential of biocontrol agents and peptides in reducing disease severity, aligning with the protective effects observed in this study using *P. commune* and systemin against anthracnose in olives.

The mechanisms of plant protection conferred by *P. commune* and systemin were further elucidated through assessments of plant oxidative stress responses, trough the evaluation of oxidative stress markers, like MDA, and ROS dynamics.

3.2. ANTIOXIDANT DEFENSE RESPONSE BY APPLICATION OF *PENICILLIUM COMMUNE*

It is widely recognized that ROS play a crucial role in plant defense against pathogens (Mansoor et al., 2022). However, plants need to maintain an optimal balance between the production of ROS and the mechanisms that scavenge them (Neill, 2002; Yang & Poovaiah, 2002). This balance is crucial, as excessive ROS can damage critical cellular components, such as lipids, proteins, and DNA, leading to cell death. Conversely, controlled ROS levels are vital for triggering defense signaling pathways and supporting the plant's adaptation to stress (Grant & Loake, 2000; Lamb & Dixon, 1997).

As shown in Figure 8, the inoculation of olive plants with *P. commune* induced a rapid oxidative response upon *C. nymphaeae* infection. Indeed, in the treatment P.c.+C.n., a significant increase in H_2O_2 levels was recorded 3 hours after pathogen infection reaching a maximum value at 6 hours (2.71-fold), followed by a decrease at 24 hours, compared to the control. As expected, in plants treated solely with *C. nymphaea,* H_2O_2 levels increased significantly immediately upon pathogen infection (1.67-fold), then decreased at 6 hours, before rising again at 24 hours, compared to the control plants. This result suggests that the plant is attempting to induce oxidative responses to limit infection. However, since *C. nymphaeae* is a necrotrophic pathogen, this prolonged increase in H_2O_2 could favor infection by causing tissue death, which provides a nutrient source that the pathogen can exploit and thus accelerating the infection process (Glazebrook, 2005; Nikraftar et al., 2013; Taheri et al., 2014). The inoculation of olive plants with only the endophyte *P. commune* did not trigger an oxidative defense response, with only a slight increase in H_2O_2 levels observed at 24 hours compared to control plants (Figure 8).

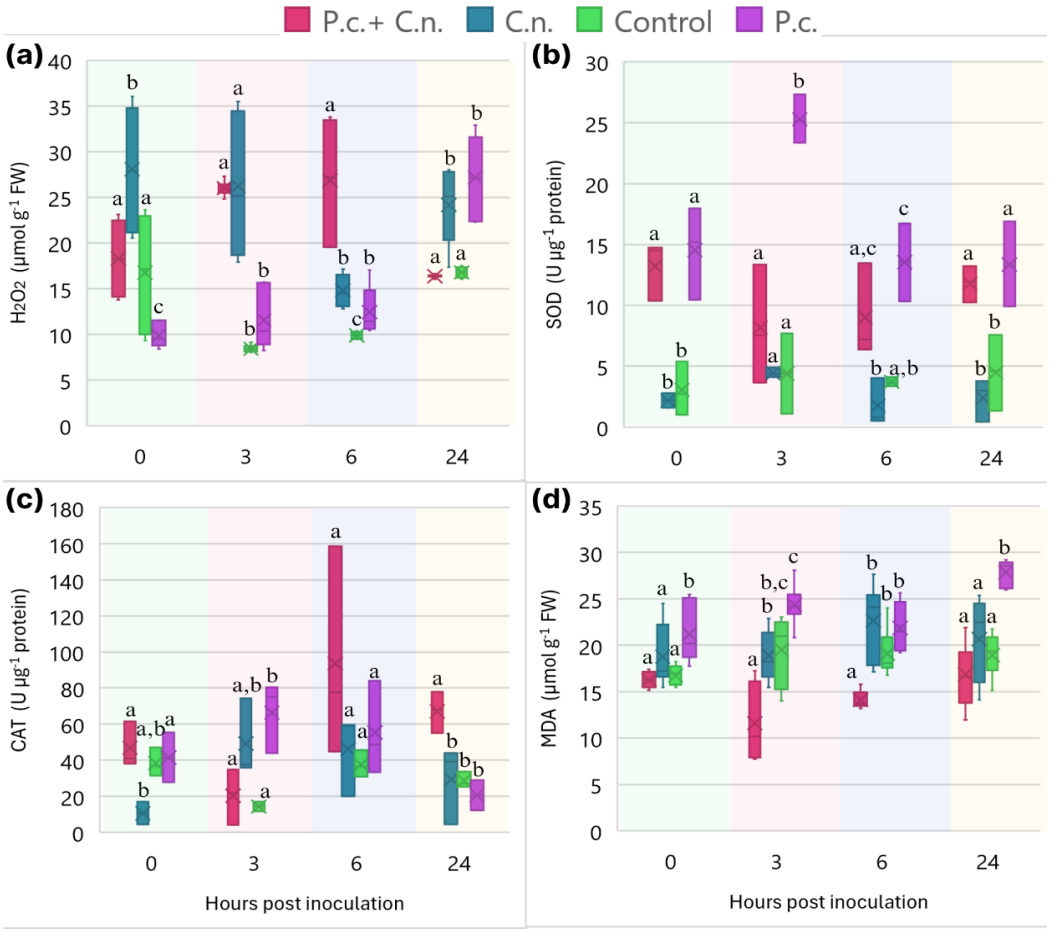

Figure 8: H_2O_2 levels (a), SOD (b) and CAT (c) activities, as well as MDA (d) contents in olive leaves from plants inoculated with *Penicillium commune* and *Colletotrichum nymphaeae* (P.c.+C.n.), *C. nymphaeae* (C.n.), *P. commune* (P.c.) or double distilled water (Control), at several time points (0 to 24 hours) after pathogen inoculation. Distinct letters indicate statistically significant differences between treatments for each time point ($p < 0.05$; $n = 3$ or 9).

The antioxidant enzymes SOD and CAT play critical roles in mitigating oxidative stress caused by ROS. Indeed, SOD catalyzes the dismutation of superoxide radicals into H_2O_2, while CAT further converts H_2O_2 into water and oxygen, thereby reducing potential oxidative damage (Neill, 2002; Yang & Poovaiah, 2002). As shown in Figure 8, the inoculation of plants with *P. commune* significantly increased the activities of both SOD and CAT enzymes after 0 and 6 hours upon pathogen infection, respectively, followed by a second increase at 24 hours, when compared to control plants. This very early increase in SOD activity indicates that the plant, under the influence of the endophyte *P. commune*, was better prepared to cope with oxidative stress upon pathogen infection by rapidly

converting superoxide (O_2^-), a highly toxic reactive oxygen species, into H_2O_2 (Rai, 2023). At 0 hours, CAT activity was also significantly higher in the treatment P.c.+C.n., than in plants inoculated only with the pathogen, suggesting that the plant was proactively regulating H_2O_2 levels by decomposing it into water and oxygen to prevent oxidative damage. The further significant increase in CAT activity observed at 6 hours (up to 2.51-fold) in the P.c.+C.n. treatment, along with the elevated activities of both CAT and SOD at 24 hours (up to 2.33- and 2.62-fold, respectively) compared to control plants, supports the idea that the endophyte *P. commune* helps the plant in modulating ROS levels. Overall, the results suggest that *P. commune* was assisting the plant in modulating oxidative stress, initially promoting an increase in H_2O_2 for signaling purposes, followed by its degradation to prevent long-term damage. This process prevents the pathogen from exploiting dead plant tissues and helps to maintain a strong and sustained defense response.

In contrast, in olive plants inoculated only with the pathogen *C. nymphaeae,* no significant changes were observed in the activities of SOD and CAT compared to control plants (Figure 8). The absence of a regulatory increase in these two enzymes can lead to cellular damage and increased susceptibility to stress, potentially resulting in cell death if ROS levels become critically high. In plants inoculated solely with the endophyte *P. commune*, the levels of SOD activity were consistently significantly higher than the control plants, during the whole period surveyed (Figure 8). This treatment also exhibited a significant peak in CAT activity at 3 hours post-inoculation relative to the control plants. These results suggest that the action of *P. commune* extends beyond biotic stress caused by pathogen infection, confirming the fungus's role as an active inducer of the plant's antioxidant defenses.

The profile of malondialdehyde (MDA) levels, an indicator of lipid peroxidation and cellular damage due to ROS, reinforces this dynamic of oxidative stress enzymes. Indeed, in the treatment P.c.+C.n., MDA levels are significantly lower at 3 and 6 hours (up to 1.67-fold and up to 1.35-fold, respectively) compared to the control (Figure 8), suggesting that the presence of *P. commune* moderates oxidative damage by promoting a more efficient antioxidant response. The modulation of oxidative stress by *P. commune* appears to contribute to keeping ROS at non-damaging levels, thus preventing the excessive formation of MDA upon pathogen infection, as MDA levels were consistently

lower during the whole experiment. In contrast, in olive plants inoculated with *P. commune* alone, MDA levels were significantly elevated at both 0 and 24 hours compared to the control, suggesting some oxidative stress on cellular membranes. This increase may reflect an initial stress response due to endophyte colonization.

Overall, the temporal profile of H_2O_2, SOD, CAT, and MDA levels reveals that the exogenous application of *P. commune* induces an antioxidant defense response against *C. nymphaeae*. In the early time points, a significant increase in SOD activity and H_2O_2 levels was observed, indicating an immediate response to oxidative stress. This initial increase is crucial for signaling the presence of the pathogen and activating both local and systemic defenses. However, the subsequent control of H_2O_2 levels by CAT is essential to prevent excessive accumulation and oxidative damage, as evidenced by the elevated CAT activity at the 6-hour mark. The sustained high levels of CAT activity up to 24 hours suggest that *P. commune* promotes a prolonged and regulated response, ensuring that the initially produced H_2O_2 in response to infection is effectively degraded when it is no longer needed as a signaling molecule. The *P. commune* seems to aid olive plants in maintaining a balance between antioxidant defense and ROS regulation, promoting an effective and pathogen-specific defense response.

Numerous studies support the potential of endophytes in modulating antioxidant responses and promoting plant resistance. Chen et al. (2022) observed that *Trichoderma citrinoviride* increased SOD and CAT activity in *Rheum palmatum* infected with *Fusarium oxysporum*, enhancing the antioxidant response and assisting the plant in regulating H_2O_2 under oxidative stress. Nassimi & Taheri (2017) reported that *Piriformospora indica* helped rice resist sheath blight caused by *Rhizoctonia solani* by balancing H_2O_2 levels and reducing oxidative damage. Similarly, Daroodi et al. (2022) demonstrated that *Acrophialophora jodhpurensis* boosted antioxidant enzyme activity in tomato, priming the plants for more effective defense responses. Dehghanpour-Farashah et al. (2019) also noted the action of *P. indica* in wheat against *Fusarium pseudograminearum*, where the combination of the endophyte with polyamines and nitric oxide increased CAT activity, suggesting enhanced antioxidant resistance. Finally, Bagy et al. (2019) showed that *Epicoccum nigrum* and arbuscular mycorrhizal fungi reduced oxidative stress in potatoes infected by *Pectobacterium carotovora*, increasing SOD and CAT levels while lowering MDA.

To enhance the results obtained in this study and expand knowledge on the role of *P. commune* in antioxidant resistance against *C. nymphaeae*, future research could pursue several important directions. Firstly, gene expression studies and molecular signaling analysis would be relevant; by examining the expression of defense pathway genes, particularly those related to the antioxidant system and reactive oxygen species (ROS) production, it would be possible to identify the molecular mechanisms activated by the endophyte and their variations in the presence or absence of the pathogen. It is necessary to conduct ROS and antioxidant quantification assays over longer periods with more frequent intervals, which would help capture the full dynamics of the antioxidant response throughout the infection cycle, allowing for the identification of key moments in ROS regulation and the role of the endophyte in this modulation. Lastly, long-term and field studies are essential to assess whether the resistance induced by the endophyte is maintained under natural and more complex environmental conditions across different climatic seasons.

3.3. ANTIOXIDANT DEFENSE RESPONSE BY APPLICATION OF SYSTEMIN

Studies involving exogenous application of systemin in plant species have successfully demonstrated its role in promoting pathogen resistance (Coppola et al., 2015; Molisso et al., 2020). However, most studies focus on solanaceous species (Pastor-Fernández et al., 2023), highlighting a knowledge gap regarding the peptide's effects on non-solanaceous species, such as olive trees. In a study by Sánchez et al. (2023), it was demonstrated for the first time that the exogenous application of systemin can also confer protection to olive trees against pests like the olive fruit fly (*Bactrocera oleae*) through the production of repellent volatile organic compounds (VOCs). In the present work was demonstrated that systemin can also confer protection to olive plants against olive anthracnose caused by the fungus *C. nymphaeae*.

The protection conferred by systemin against this disease seems to be related to the activation of antioxidant defense response in olive plants. Indeed, in the early time points (0 and 3 hours), H_2O_2 levels were significantly reduced ($p < 0.05$) up to 2-fold in the treatment systemin + pathogen (Figure 9), indicating that the peptide helps suppress the initial accumulation of ROS, preventing premature oxidative damage and cellular collapse. This initial suppression phase is thought to allow the plant time to mobilize its

defenses without incurring significant damage (Fichman & Mittler, 2020). However, at 6 and 24 hours after pathogen infection, H_2O_2 levels increased significantly up to 1.91-fold and 2.11-fold, respectively, in the Syst.+C.n. treatment compared to the control. This response pattern—a preliminary suppression of ROS followed by a significant increase in later stages—suggests that systemin, when combined with the pathogen, modulates ROS levels to avoid initial oxidative damage and subsequently activates the plant's antioxidant defenses intensively (Pastor-Fernández et al., 2023). This behavior is consistent with an induced resistance mechanism, where the plant adaptively primes its defenses to combat the pathogen more effectively during critical phases of infection.

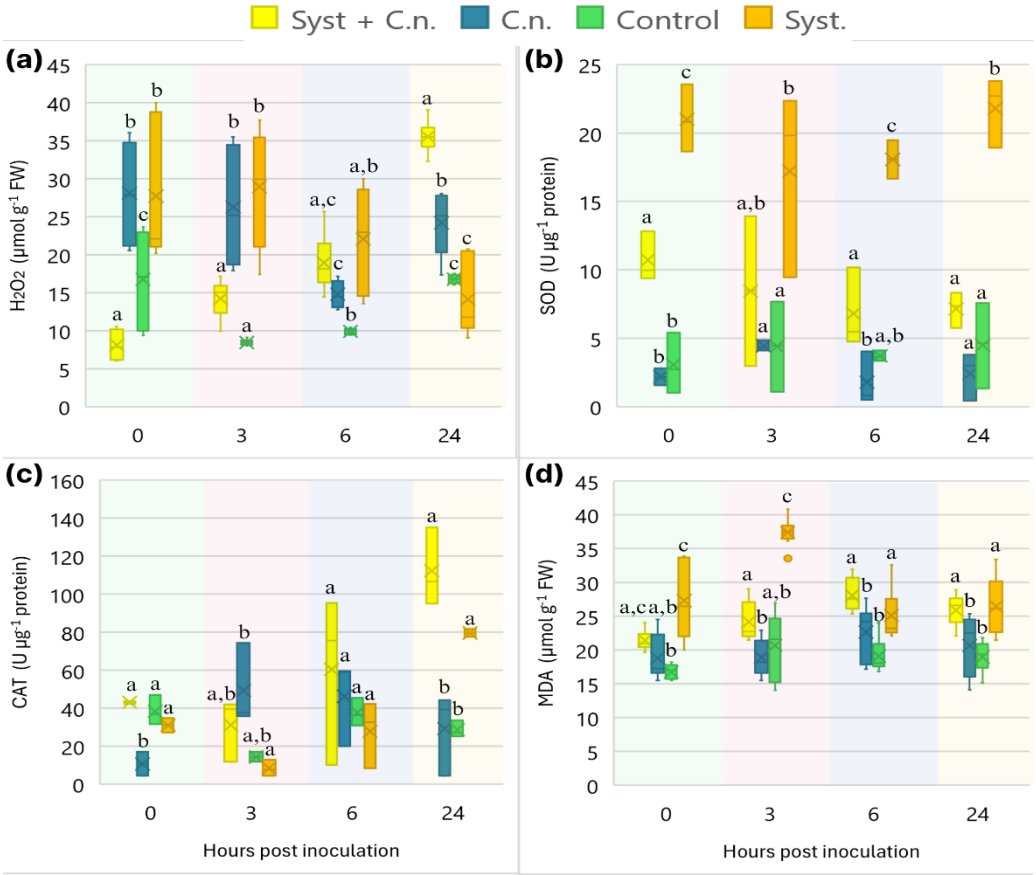

Figure 9: H_2O_2 levels (a), SOD (b) and CAT (c) activities, as well as MDA (d) contents in olive leaves from plants inoculated with systemin and *Colletotrichum nymphaeae* (Syst.+C.n.), *C. nymphaeae* (C.n.), systemin (Syst.) or double distilled water (Control), at several time points (0 to 24 hours) after pathogen inoculation. Distinct letters indicate statistically significant differences between treatments for each time point ($p < 0.05$; n = 3 or 9).

The activity of the antioxidant enzyme SOD was significantly increased in response to systemin treatment, particularly when applied alone and in less extent when applied in combination with the pathogen (Figure 9). In the early time points (0 hours), SOD exhibited a significant increase ($p < 0.05$), being up to 6.88-fold higher in plants treated solely with systemin and up to 3.51-fold higher in plants treated with systemin in combination with the pathogen, compared to the control. This early increase in SOD may reflects systemin's capacity to prime the plant to rapidly manage oxidative stress by converting superoxide into H_2O_2 and maintaining redox balance (Rai, 2023). Afterwards, SOD activity in the Syst.+C.n. treatment returned to those in control plants (Figure 9). This result suggests that, right after pathogen infection, systemin helps trigger an initial oxidative burst for defense signaling, then regulates oxidative stress to protect cell components from damage. In contrast, in plants treated only with systemin, SOD activity was consistently and significantly higher than in control plants until the end of the assay.

A significant increase in the activity of CAT was observed at later stages (24 hours) in plants treated with systemin alone (up to 2.77-fold) and in combination with the pathogen (up to 3.91-fold) compared to the control (Figure 9). This increase in CAT activity highlights systemin's role in regulating H_2O_2 by decomposing it into water and oxygen, thus preventing excessive cellular damage. The significantly elevated CAT levels ($p < 0.05$) sustained for up to 24 hours indicate that systemin promote a prolonged antioxidant response, essential for protecting plant cells against long-term damage.

Although systemin demonstrates a robust antioxidant effect, an increase in MDA levels was observed in Syst.+C.n. or systemin treatments, particularly within the first 3 hours, indicating lipid peroxidation and potential damage to cellular membranes (Figure 9). This finding suggests moderate oxidative stress, possibly related to the activation of localized defense responses, such as programmed cell death (PCD). Studies indicate that extracellular ROS can function as signaling molecules, triggering PCD in infected cells to limit pathogen spread (Zurbriggen et al., 2010). The production of extracellular ROS has been linked not only to defense signaling but also to direct lipid peroxidation and alkalinization of the apoplast, which propagates oxidative signals through alkali-responsive peroxidases (Mur et al., 2008; Rai, 2023). Additionally, ROS act as secondary messengers in Induced Resistance (IR), where signaling molecules, along with ROS, activates defense mechanisms in tissues distant from the initial site of infection (Corpas

et al., 2020; Rai, 2023). The elevated MDA levels at 24 hours in plants treated with systemin alone or combined with the pathogen indicates prolonged oxidative stress, supporting the hypothesis that systemin may be associated with the induction of resistance. While this process may cause slight damage to cellular membranes, it likely triggers the mobilization of antioxidant defenses, keeping the plant in a heightened state of alert and enhancing its responsiveness to future oxidative stresses.

Overall, the results suggest that systemin primed the plant, exhibiting a biphasic response in the management of ROS. Initially, there is a modulation or suppression phase of ROS, followed by a controlled increase in these compounds in later stages. This behavior aligns with the concept of priming described by Mauch-Mani et al. (2017), where the plant, upon exposure to the inducer—in this case, systemin—prepares its defense system to respond more effectively to the pathogen by reducing the initial ROS accumulation and preventing premature oxidative damage. In the critical phases of infection, the late increase in ROS promoted by systemin strengthens the antioxidant response, adaptively intensifying defenses. The role of systemin as an inducer of resistance, with the ability to amplify the plant's immune response against pathogens, has been similarly documented in previous studies (e.g., Pastor-Fernández et al., 2023).

Further studies on the exogenous application of systemin, such as the one conducted by Molisso et al. (2020), highlight the potential of this peptide to enhance plant resistance to pathogens by activating antioxidant defenses in eggplant and grapevine against *Botrytis cinerea*. The application of systemin resulted in a significant increase in the enzymes CAT and ascorbate peroxidase (APX), promoting the elimination of ROS. Similarly, Pastor-Fernández et al. (2020) observed that systemin and other related peptides, can induce resistance against *P. cucumerina*, triggering protection at very low doses. Pastor-Fernández et al. (2022) observed that systemin originally found in tomatoes, can trigger defense responses in other plant species that lack specific receptors for it, like *Arabidopsis*, eggplant, and grapevine. This suggests that in olive plants, systemin may similarly activate antioxidant and defense pathways by engaging general defense-related receptors that detect common signaling patterns in plant defense peptides.

To confirm these effects in olive trees, further analyses are necessary to deepen the understanding of mechanisms induced by systemin. Gene expression analyses (RT-qPCR) would help identify the activation of specific defense-related genes, such as those

linked to the antioxidant system and systemic response, thereby corroborating systemin's potential to induce resistance in olive trees.

3.4. ANTIOXIDANT DEFENSE RESPONSE BY *PENICILLIUM COMMUNE* COMBINED WITH SYSTEMIN

The combined effect of the endophyte *P. commune* and peptide systemin on the antioxidant defense of olive trees against *C. nymphaeae* was also tested. This analysis aimed to clarify if their joint application enhances plant defense mechanisms, leading to a stronger and more sustained antioxidant response. Evaluating the impact of this combination may reveal synergistic or additive interactions, ultimately improving the effectiveness of the plants' defenses against the pathogen *C. nymphaeae*.

The results indicated that H_2O_2 levels in plants treated with the combination of *P. commune*, systemin, and *C. nymphaeae* were always significantly higher than in control plants across all time points, with particularly significant increases observed at 0 and 3 hours (Figure 10). This consistently elevated H_2O_2 levels indicates that the combination of *P. commune* and systemin induced a robust antioxidant response in olive plants. This response can be attributed to the synergistic role of the fungus and peptide. *P. commune* may stimulate the production of bioactive compounds that enhance the plant's ability to detect and respond to the pathogen, while systemin amplifies defense signaling, promoting the production of ROS, such as H_2O_2, which serves as both a signaling and defensive molecule. Similar results were obtained by Zehra et al. (2017), who observed an oxidative burst in tomato plants treated with *Trichoderma harzianum*, salicylic acid (SA), and methyl jasmonate (MeJA) in response to *Fusarium oxysporum* infection. In that study, SA treatment, especially in combination with the pathogen, also significantly increased H_2O_2 production within the first 24 to 48 hours, followed by a gradual decrease, aligning with the peaks and stabilization of H_2O_2 levels observed in the present study. The combination of *P. commune* and systemin, even in the absence of the pathogen, led to an increase in H_2O_2 levels compared to control plants at 3 hours (Figure 10). This suggests that *P. commune* and systemin may initiate a baseline defense response, priming the plant's defenses and readying it to respond more effectively to future pathogenic threats.

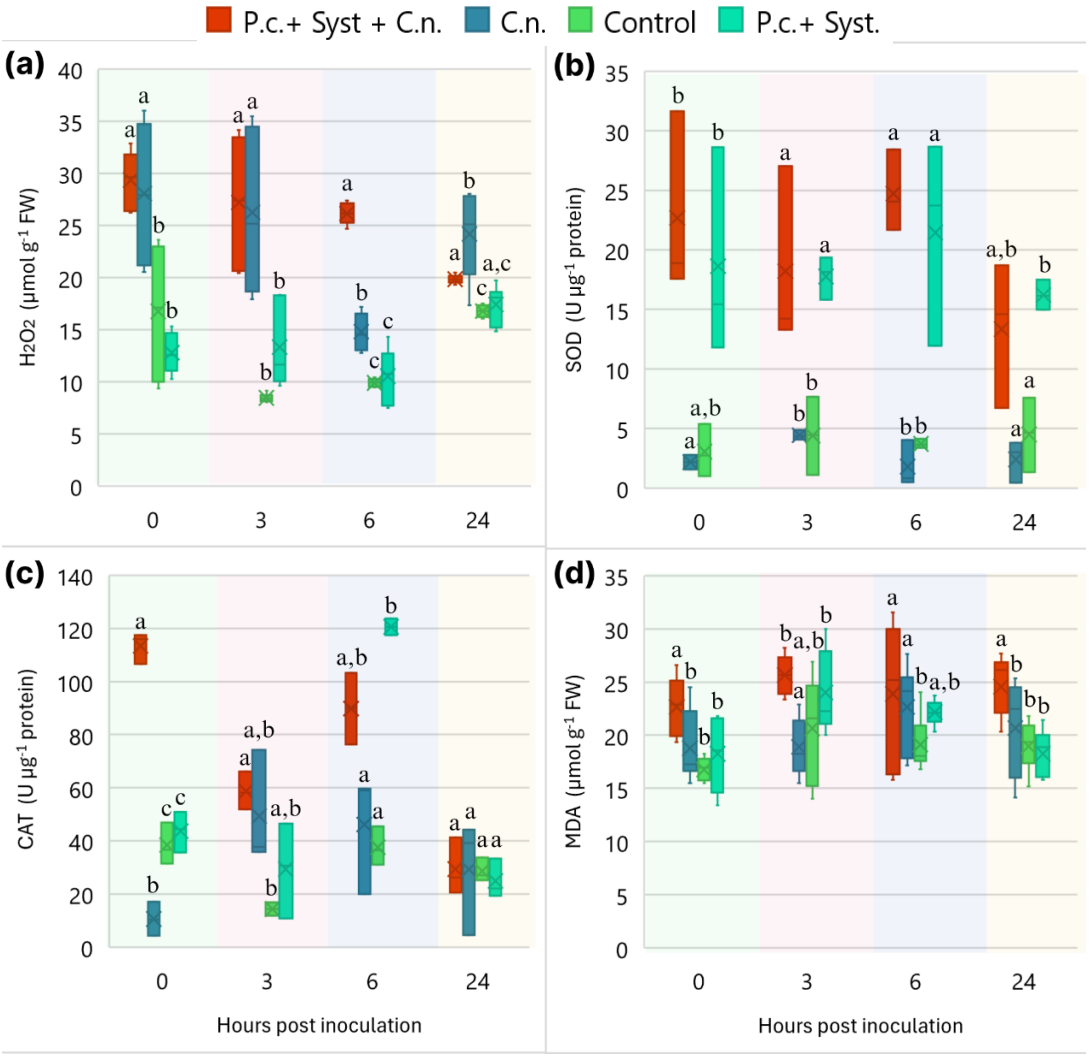

Figure 10: H_2O_2 levels (a), SOD (b) and CAT (c) activities, as well as MDA (d) contents in olive leaves from plants inoculated with *Penicillium commune* and systemin and *Colletotrichum nymphaeae* (P.c.+Syst.+C.n.), *C. nymphaeae* (C.n.), *P. commune* and systemin (P.c.+ Syst.) or double distilled water (Control), at several time points (0 to 24 hours) after pathogen inoculation. Distinct letters indicate statistically significant differences between treatments for each time point ($p < 0.05$; $n = 3$ or 9).

SOD and CAT activity also displayed significant variations between treatments. Immediately upon pathogen infection, plants treated with both *P. commune* and systemin showed a significant increase in SOD and CAT activities compared to control plants (Figure 10). This indicates an early activation of the antioxidant enzyme, which remained significantly elevated over 3 hours (for CAT) and 6 hours (for SOD), reflecting a sustained antioxidant response. Similarly, Zehra et al. (2017), observed that combining the chemical inducer MeJA with *Trichoderma harzianum* lead to the highest activity in

CAT and APX in tomato plants infected by *Fusarium oxysporum* f. sp. *Lycopersici.* Plants treated only with *P. commune* and systemin also showed a significant increase in SOD activity at 3, 6, and 24 hours, and of CAT activity at 6 hours, compared to control plants (Figure 10). This result suggests that when combined, *P. commune* and the systemin act as resistance inducers that can prime the plant's defense system, even in the absence of pathogen infection. In line with these observations, Abdelaziz et al. (2024) also reported that treatments combining beneficial fungi (*Aspergillus oryzae* and *Aspergillus tubingensis*) and salicylic acid (SA), resulted in increased enzymes activities in tomato plants, not only in *Fusarium*-infected plants but also in healthy ones, supporting the concept of induced resistance.

MDA levels, an indicator of lipid peroxidation, were also significantly greater in the P.c.+Syst.+C.n. treatment compared to control, at all time points analyzed, except at 3 hours (Figure 10). This persistent elevation of MDA indicates that although the combination of *P. commune* and systemin induces an antioxidant response, it also results in greater ROS production, reflecting an intense defense against the pathogen. The elevated MDA levels observed in the P.c.+Syst.+C.n. treatment may also be attributed to a potential hypersensitive response (HR), characterized by localized cell death that helps contain the pathogen but also temporarily increases oxidative damage. This increase in MDA is consistent with an HR, which often involves a rapid ROS burst, overwhelming antioxidant systems and generating higher lipid peroxidation (Fernandes et al., 2009).

Overall, the combination of *P. commune* and systemin in plant defense against *C. nymphaeae* reveals a well-coordinated defense response in three distinct phases. In the early activation phase (0–3 hours), H_2O_2, SOD, and CAT levels rise sharply, initiating protection through ROS signaling while preventing oxidative damage via SOD and CAT detoxification. The sustained response phase (3–6 hours) maintains high SOD and CAT activity to balance ROS at manageable levels, supporting ongoing defense without excess damage. At 24 hours, SOD and CAT activities decline as ROS stabilize, signaling a transition to homeostasis.

For a more comprehensive understanding of the potential synergistic effect between *P. commune* and systemin, future research could include detailed molecular analyses, such as the activation of defense pathways related to jasmonic acid, ethylene, salicylic acid and calcium signaling, which are crucial in the induction of plant defense

responses as indirect mechanisms through which endophytes inhibit necrotrophic pathogens (Chaudhary et al., 2022). Further analyses to support the activation of these pathways are also necessary, including flavonoids, polyphenols, phytoalexins, and signaling transduction pathways activated by jasmonate/SA or ethylene.

3.5. COMPARISON BETWEEN TREATMENTS

Figure 11 provides a comparative analysis of the different treatments, in order to identify which of them contributed most to the activation of the plant's antioxidant defenses against *C. nymphaeae*. Among the treatments evaluated, *P. commune* + systemin + *C. nymphaeae* (P.c.+Syst.+C.n.) was shown to trigger the most potent defense response. This treatment consistently showed significantly higher levels of H_2O_2 along with elevated SOD and CAT activities compared to the other treatments, thereby suggesting an immediate and sustained antioxidant response critical for defending olive plants against *C. nymphaeae*. The combination *P. commune* + systemin was most effective at initiating a rapid oxidative burst, essential for early pathogen detection, and maintaining high antioxidant activity, which maximizes plant defense.

On the other hand, the treatment systemin + *C. nymphaeae* (Syst.+C.n.) exhibited a more moderate H_2O_2 dynamic over time, with a delayed increase in both H_2O_2 levels and CAT activity, suggesting a more gradual response aimed at protecting cells from prolonged damage. It is likely that systemin modulates oxidative stress over a prolonged period, potentially helping the plant to cope with long-term stress without depleting its defenses. The treatment *P. commune* + *C. nymphaeae* (P.c.+C.n.) triggered a moderate antioxidant response, marked by an early defense activation with high H_2O_2 levels and increased SOD and CAT activities within the first 6 hours post-infection compared to the control. This early defence activation with lower oxidative stress levels over time, indicated by MDA analysis, suggests *P. commune* potential for controlling oxidative damage without overwhelming the plant's defense. Overall, P.c.+Syst.+C.n. proved superior in both rapid defense activation and sustained ROS management, making it the most effective at enhancing plant resistance against *C. nymphaeae*.

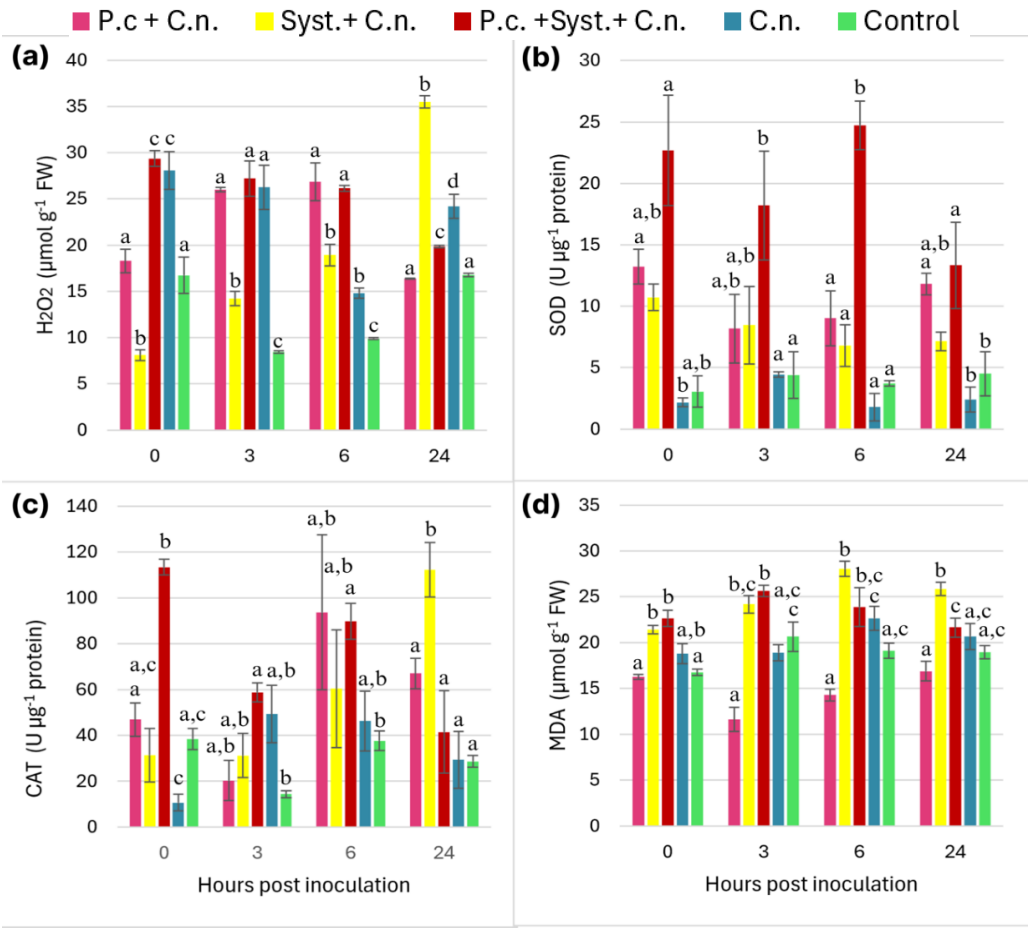

Figure 11: H_2O_2 levels (a), SOD (b) and CAT (c) activities, as well as MDA (d) contents in olive leaves from plants inoculated with *Penicillium commune* and *Colletotrichum nymphaeae* (P.c.+C.n.), systemin and *Colletotrichum nymphaeae* (Syst.+C.n.), *P. commune* and systemin and *Colletotrichum nymphaeae* (P.c.+Syst.+C.n.), *C. nymphaeae* (C.n.), or double distilled water (Control), at several time points (0 to 24 hours) after pathogen inoculation. Means ± SE are shown (n = 3 or 9). Distinct letters indicate statistically significant differences between treatments for each time point ($p < 0.05$).

The next steps of this research aim to deepen the understanding of the antioxidant defense mechanisms induced by *P. commune* and systemin, as well as to explore the feasibility of their practical application. First, molecular studies focused on the gene expression of antioxidant defense-related genes and signaling pathways, such as those associated with the defense hormones jasmonic acid and salicylic acid, are recommended. This type of analysis could clarify how *P. commune* and systemin influence defense responses at the molecular level, contributing to a detailed understanding of their efficacy. Additionally, proteomic and metabolomic profiling studies of the treatments will provide

a comprehensive view of the compounds involved in the antioxidant response. Such analyses will identify specific proteins and metabolites associated with oxidative stress, allowing for a more complete mapping of the cellular processes activated by *P. commune* and systemin. These profiles may help uncover new compounds with potential defensive properties and provide a better understanding of how the treatments alter cellular metabolism to protect against pathogens.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

This study aimed to investigate the effectiveness of the endophyte *P. commune* and peptide systemin as inducers of plant resistance against the causal agent of olive anthracnose, *C. nymphaeae*. The results demonstrated that both *P. commune* and systemin, when applied individually or in combination, significantly reduced the incidence of anthracnose in olive plants, by up to 2.8-fold compared to plants inoculated with only the pathogen. These findings validate the hypothesis that both *P. commune* and systemin are effective resistance inducers and could serve as a sustainable approach in olive tree protection against anthracnose.

The induction of plant immunity triggered by exogenous application of *P. commune* and systemin, either alone or combined, was analyzed in relation to the plant's antioxidant defense system. Overall, the results showed that:

1) The endophyte *P. commune* significantly enhanced the antioxidant response in olive plants against *C. nymphaeae* infection, by rapidly increasing the H_2O_2 levels within the first 6 hours, triggering a defensive oxidative burst crucial for early detection of the pathogen. Concurrently, both SOD and CAT activities were increased, helping to regulate H_2O_2 levels, minimizing potential oxidative damage. MDA levels, a marker for cellular damage, were lower suggesting that *P. commune* supports the plant in balancing ROS production and degradation to avoid tissue damage.
2) Systemin enhances olive plant defenses against *C. nymphaeae* by modulating ROS levels in two phases. Initially, it reduces early H_2O_2 accumulation, preventing oxidative damage and allowing the plant to mobilize its defense. Later, it increases H_2O_2 levels and activates the antioxidant enzymes SOD and CAT, strengthening the plant's response to infection. However, moderate increases in MDA levels suggest some oxidative stress, which is potentially linked to programmed cell death as a defense strategy to contain the pathogen.
3) The combined application of *P. commune* and systemin in olive plants provides a more robust and prolonged antioxidant defense against *C. nymphaeae* than when used alone. This combination rapidly increased H_2O_2, SOD, and CAT activity, signaling an immediate and sustained antioxidant response critical against the pathogen. Over time, high levels of MDA

indicated increased lipid peroxidation, suggesting that the treatment may trigger a hypersensitive response to contain the pathogen while managing ROS levels.

These results advance agricultural biotechnology by expanding our knowledge on the use of fungal endophytes and peptides as defense inducers, in crop protection. The potential to reduce dependence on chemical fungicides through the use of resistance inducers presents a sustainable and eco-friendly alternative for agricultural disease management. Moreover, this study represents an innovation by exploring the combined use of *P. commune* and systemin to protect plants against anthracnose. The results suggest a potential synergistic effect between them, where the endophyte seems to prime the plant's immune response while systemin is likely to amplify the defense signaling.

While this study elucidated promising mechanisms under controlled greenhouse conditions, field application requires further investigation, considering environmental variables and interactions with other organisms. Additionally, the long-term response of plants under varied climatic conditions needs to be studied to assess the feasibility of combined use of *P. commune* and systemin.

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