New alternative solutions against *Colletotrichum fioriniae* and elucidation of their mode of action

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Abstract

Olive cultivation is a cornerstone of the European agricultural sector, particularly in Portugal; however, it suffers substantial losses due to anthracnose, a disease caused by various *Colletotrichum* species that negatively impacts both the yield and quality of olive oil and table olives. Characterized by fruit lesions, defoliation, and necrosis, anthracnose is traditionally controlled through copper-based fungicides, which now face increasing restrictions due to their high environmental toxicity, necessitating the urgent development of sustainable alternatives. Among these, Volatile Organic Compounds (VOCs) have emerged as promising solutions as they are naturally produced via plant secondary metabolic pathways and exhibit significant activity against phytopathogens. The present study evaluated the effect of six VOCs on mycelial growth, conidial production and germination of *Colletotrichum fioriniae*, and the half-maximal inhibitory concentration (IC_{50}) was calculated based on mycelial growth data. The results demonstrated that 3,7-dimethyl-3-octanol and dihydromyrcenol were the most effective VOCs, achieving 100% mycelial growth inhibition at 10 µL, while their 1:1 mixture exhibited superior efficacy by reaching 78.28% inhibition at a total volume of 1.95 µL (IC_{50}). Furthermore, these individual compounds directly inhibited conidial germination by 81.00% and 90.53%, respectively, whereas the mixture achieved complete (100%) inhibition. In detached fruit assays, although no compound prevented initial infection, all VOCs successfully reduced the disease progression, with the 1:1 mixture showing the highest efficacy by reducing disease severity by 21% and total fruit rot by 28%, compared with the positive control. Overall, these findings indicate that these volatiles limited fungal development and reduced disease impact, reinforcing their potential as sustainable alternatives to copper-based fungicides supporting further research on their physiological mechanisms of action and field-scale application strategies.

Keywords: Anthracnose, *Colletotrichum fioriniae*, *Olea europaea*, Sustainability, Volatile Organic Compounds.

Resumo

A olivicultura, é setor agrícola fundamental na Europa, Portugal em particular, sofre perdas substanciais devido à antracnose. Essa doença, causada por diversas espécies de *Colletotrichum,* impacta negativamente tanto a produção quanto a qualidade do azeite e das olivas de mesa. A antracnose manifesta-se por meio de lesões nos frutos, desfolhação e necrose. As estratégias de controle tradicionais são realizadas predominantemente com o uso de fungicidas à base de cobre. Embora eficazes, esses produtos enfrentam restrições crescentes devido à sua alta toxicidade ambiental. Nesse contexto, a necessidade de desenvolvimento de alternativas sustentáveis é, portanto, urgente. Soluções sustentáveis como compostos orgânicos Voláteis (COVs), tem demonstrado resultados promissores contra fitopatógenos além de serem produzidos naturalmente por vias metabólicas secundárias de plantas. O presente estudo focou em estudar o efeito de seis COVs, crescimento micelial, produção de conídios e germinação de *Colletotrichum fioriniae* e a concentração inibitória mínima sem-maxima (IC_{50}) foi calculada baseado nos dados de crescimento micelial. Os resultados demonstram que, 3,7-dimetil-3-octanol e dihidromircenol foram os COVs mais eficazes, exibindo forte inibição do crescimento micelial de 100% a 10 µL, além disso sua mistura 1:1 resultou em uma inibição superior atingindo 78,28% com volume total de 1,95 µL (IC_{50}). Esses mesmos compostos inibiram diretamente a germinação de conídios sadios em 81,00% e 90,53% respectivamente enquanto a mistura conseguiu inibir 100%. Também foi avaliado o impacto desses mesmos compostos na redução da antracnose em frutos destacados, porém nenhum COV impediu a infecção inicial, mas todos reduziram a progressão da doença. A mistura 1:1 apresentou a melhor eficácia, reduzindo a severidade da doença em 21% e diminuiu a podridão total dos frutos em 28%, comparado com o controle positivo. De forma global, os resultados indicam que estes voláteis atuam restringindo o desenvolvimento e reduzindo o impacto da doença, reforçando o potencial destes compostos como alternativas sustentáveis aos fungicidas cúpricos, dando suporte a pesquisas futuras sobre mecanismos fisiológicos envolvidos e estratégias de aplicação em condições de campo.

Palavras-chave: Antracnose, *Colletotrichum fioriniae*, Compostos Orgânicos Voláteis, *Olea europaea*, Sustentabilidade.

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List of Abbreviations

ANOVA - Analysis of variance

AUDPC (%) - Area under the disease progression curve

CAT - Catalase

CGI (%) - Conidial germination inhibition

CPI (%) - Conidial production inhibition

DS - Disease severity

DI - Disease incidence

GR - Growth rate

GRI (%) - Growth rate inhibition

HSD - Honestly significant difference

IC_{50} - Half maximal inhibitory concentration

IPM - Integrated Pest Management

MG - Mycelial growth

MGI (%) - Mycelial growth inhibition

PCA - Principal component analysis

PDA - Potato Dextrose Agar

RAUDPC (%) - Relative percentage area under the disease progression curve

RDS (%) - Relative disease severity

RH - Relative humidity

SDW - Sterile distilled water

SE - Standard error

SOD - Superoxide dismutase

VOCs - Volatile organic compounds

1. Introduction

1.1. Olives and their economic importance in Europe

The olive tree (*Olea europaea* L.) belongs to the Oleaceae family, which comprises approximately 25 genera and around 688 distinct species (Germano, 2025). These trees are highly tolerant to salt and drought stress and are well adapted to the subtropical climate, typical of the Mediterranean Basin (Bracci et al., 2011). Within the genus *Olea*, the cultivated olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) is the only species native to the Mediterranean Basin and is considered one of the first domesticated and cultivated trees (Green, 2002).

Olive trees are one of the oldest cultivated trees in the world, and their production practice dates back to more than 6,000 BC, with its supposed origin in Asia Minor, specifically in the regions of Syria and Palestine (Kaniewski et al., 2012). In Europe, it is believed that olives first arrived in countries such as Italy and Greece around 1,500 BC, however, they did not reach the Iberian Peninsula until the eleventh century (Terral et al., 2004). Olive trees were extremely important for the development of the ancient world, being represented in biblical, Greek, and Roman records. They played a fundamental role in traditions, religious cults, lifestyles, and economy. It is currently widespread in other regions of the world with a Mediterranean climate at latitudes between 30° and 45° in the north and south (Besnard et al., 2013; Barranco et al., 2017).

Commercially, it is estimated that 139 olive cultivars are responsible for 85% of total global production, with Picual, Arbequina, and Koroneiki varieties standing out; the first two are Spanish and the latter one Greek (Dawson, 2020). The market for both table olives and olive oil is valued at $15.61 billion in 2023, showing growth potential of up to 4.85% by 2029, reaching $19.78 billion. According to the Food and Agriculture Organization of the United Nations (FAO), Europe accounted for 59.8% of global olive production between 2013 and 2024 (Figure 1), suggesting that the European market is worth approximately $9.4 billion (FAO, 2024; Mordor Intelligence, 2024).

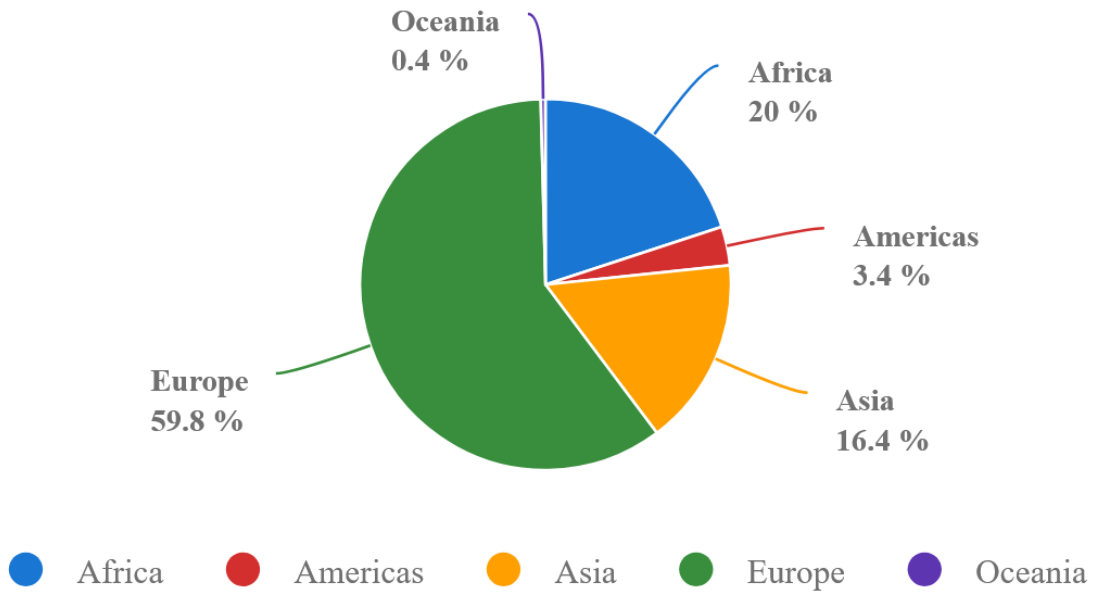

Figure 1. Participation of continents in olive culture worldwide (2013 - 2023). Source: FAO, 2024.

Olive production is one of the agroeconomic bases of Europe yielding around 23 million t of fruits in 2021. The main producers in this continent are Spain (32%), Greece (13%), Italy (10%), and Portugal (4%). In particular, Portugal grew its production around 183% between 2013 and 2023, achieving 1.194.990 t of olive in 2024, being the 4° largest producer in the European Union (EU) and the 9° on global production. It is estimated that Portugal exported 218.328 t in 2023/2024 resulting in €1,581 billion (FAO, 2024; IOC, 2025). The major producer regions are Alentejo, which accounts for 50% of the total olive growing area, followed by Trás-os-Montes (22%), Centro (18%); Ribatejo (7.7%) and Algarve (2.3%) (IOC, 2017).

Despite Europe's success in olive production and commercialization, this crop suffers from complex diseases capable of causing important losses such as olive leaf spot, olive knot and Verticillium wilt and pests such as olive fruit fly. Among all of them, anthracnose is one of the most devastating airborne diseases present in olive orchards, with a high contamination rate that spreads easily throughout plantations via wind and contaminated water, having a high destructive potential (Cacciola et al., 2012; Talhinhas et al., 2011). Its incidence is common in Western Europe, affecting countries such as Spain, Italy, and Portugal (Talhinhas et al., 2018).

1.2. Olive anthracnose

1.2.1. Symptomatology

Olive anthracnose (or “gafa” in Portuguese) primarily affects mature drupes, although under favorable environmental conditions, immature fruits from susceptible cultivars can also be severely impacted. It causes significant yield losses, as affected fruit cannot be used for table consumption or oil production. The infection in fruits is characterized by the appearance of dark brown, sunken and soft-textured lesions, and orange conidial masses produced by conidia (Figure 2). The manifestation of symptoms depends on the fruit's shape. In oblong drupes, the rot typically starts from the apical end, while in large, sub-spherical drupes, the initial symptoms appear as circular, sunken lesions with fungal structures (acervuli) forming in concentric rings from the center of the lesion (Cacciola et al., 2012). Under dry conditions, infected fruits undergo mummification and lose height due to severe dehydration. Affected drupes usually fall on ground prematurely, a process frequently associated with fungal infection in the olive peduncles (Cacciola et al., 2012). Although less frequently observed, this disease can also infect other plant organs, including leaves, shoots, and flowers (Figure 2). Symptoms on leaves and shoots include chlorosis, necrosis, severe defoliation, and dieback of twigs and branches (Moral et al., 2009). Infected flowers dry rapidly, resulting in premature drop (Moral et al., 2009). Outbreaks are more frequent during warmer and humid periods like autumn, resulting in seasonal epidemics (Talhinhas et al., 2018).

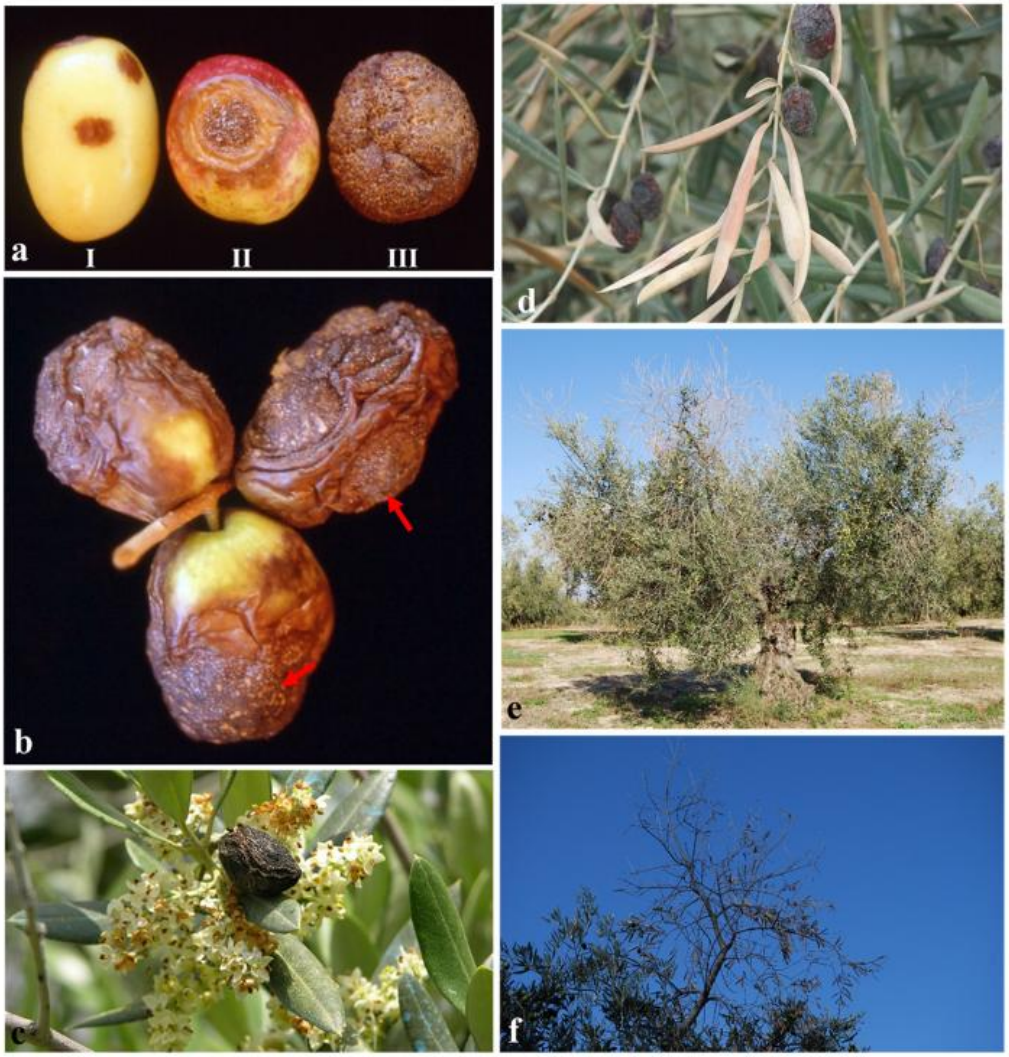

Figure 2. Typical symptoms of olive anthracnose. (a) Disease progression on naturally infected olive fruit (I: incipient symptoms; II: depressed, round, and ochre-brown lesions; III: rotted fruit with orange conidial masses produced by *Colletotrichum spp*.); (b) detail of orange conidial masses (red arrows) on infected olive fruit; (c,d) mummified fruit remaining in the tree canopy causing flower and leaf blight; (e,f) dieback of shoots and branches caused by *Colletotrichum spp.* in olive trees. Source: Moral et al. (2021).

1.2.2. Causal agents

Olive anthracnose has been associated with several species within the *Colletotrichum* genus (Talhinhas et al., 2018). The *Colletotrichum* genus is responsible for infecting a wide range of plant species worldwide and may even coexist with other species within the same genus, leading to mixed infections, affecting crops such as olives, apples, strawberries,

soybeans, and many other plants (Dowling et al., 2020; Talhinhas and Baroncelli, 2021). This pathogen has a broad global distribution, with variants adapted to tropical, subtropical, and temperate climates, reflecting its high species diversity and remarkable ability to adapt to different environmental conditions. According to the nomenclatural database Index Fungorum, there are currently 1,011 recorded species within the *Colletotrichum* genus (Index Fungorum Partnership, 2022). In the U.S. National Fungus Collection, there are 1,215 species cited for South America alone (Farr and Rossman, 2022).

Specifically, in olive tree, the disease is caused by species that belong to the species complexes *Colletotrichum acutatum* sensu lato (s.l.), *C. gloeosporioides* s.l. and *C. boninense* s.l. (Moral et al., 2021; Talhinhas et al., 2018). The *acutatum* species complex stands out as the most significant in terms of infection rate and severity in olive trees (Moral et al., 2021). Within this complex, *C. acutatum* sensu stricto (s.s.) and *C. nymphaeae* exhibit the highest pathogenicity, the latter species being the most predominant in Portugal (Moral et al., 2021). Other species belonging to this complex with intermediate severity include *C. godetiae* and *C. fioriniae*, which are also encountered in Portugal (Moral et al., 2021). Finally, within the *C. gloeosporioides* complex, *C. gloeosporioides* s.s. is associated with low severity (Schena et al., 2014).

1.2.3. Biological life cycle

The life cycle of *Colletotrichum* spp. in olive trees involves several stages. Typically, the pathogen overwinters in mummified olives, dead twigs (resulting from prior infection), and peduncles, allowing it to survive until climatic conditions become favorable for its proliferation (Cacciola et al., 2012; Dowling et al., 2020). During this inactive period, the fungus can be found in either its sexual or asexual forms (Talhinhas et al., 2011, 2018; Dowling et al., 2020). In spring, under favorable conditions like high humidity and optimal temperature, *Colletotrichum* spp. produces conidia (asexual spores) that are disseminated by wind and rain splashes to infect new tissues, including flowers, and developing fruits (Moral et al., 2009). After infection, the fungi enter a latent phase, remaining undetected until symptoms manifest during fruit ripening or after harvest, making them a silent threat. This hampers early detection, making preventive treatment before flowering the most effective approach (Børve and Stensvand, 2006). In Autumn, during the fruit ripening, the pathogen starts to produce visible

symptoms and new conidias. These conidias are then spread by rain splash and wind to newly fruits and other tree parts, giving rise to secondary infections. Ripe fruits are the most affected, turning the tree into a major source of contamination that can damage several hectares of olive orchards (Cacciola et al., 2012). Subsequent to the rainy season and with the arrival of winter, *Colletotrichum* spp. enters a dormant state or exhibits significantly reduced metabolic activity due to low temperatures (Figure 3).

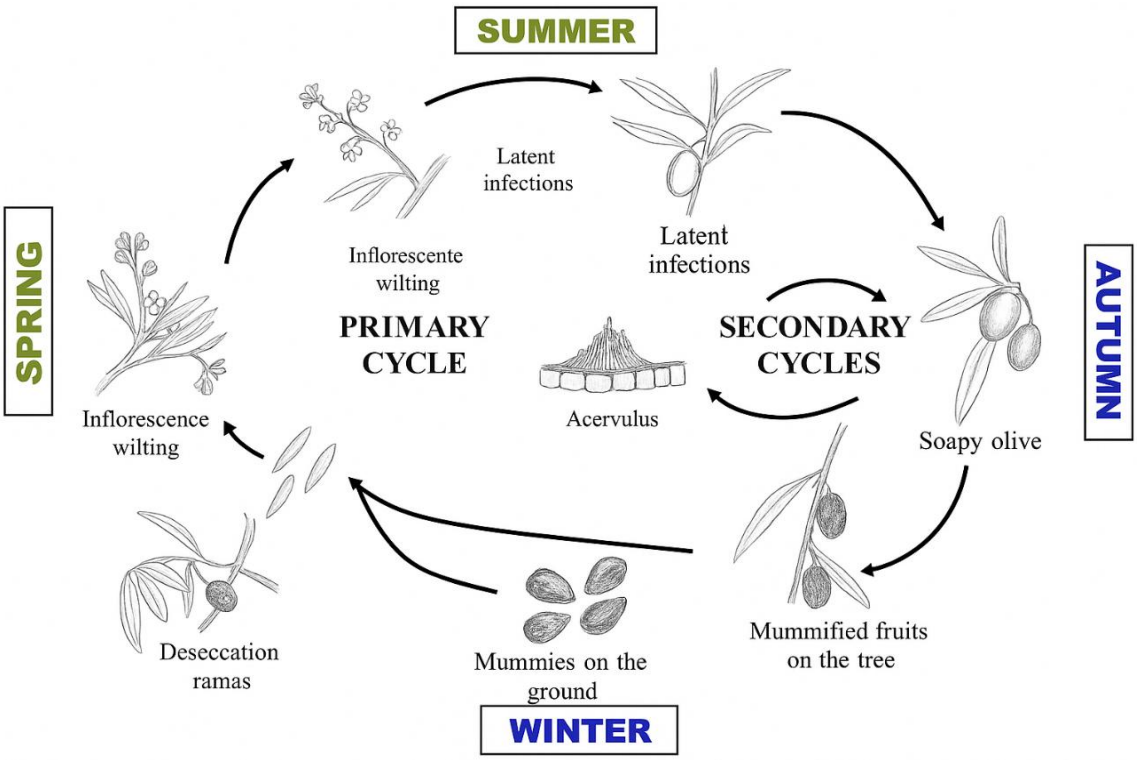

Figure 3. *Colletotrichum spp.* biological cycle of life in olive tree. Source: Cátedra UPL (2025).

1.2.4. Traditional control

The control of olive anthracnose can be challenging due to the complex nature of the disease and the various factors influencing its development. So far, traditional strategies used for the control of olive anthracnose are mainly based on the use of copper fungicides, which have a high environmental impact (Materatski et al., 2019; Moral et al., 2018). These fungicides are usually applied at the beginning of autumn to reduce infection or before the flowering period to act preventively and can be reapplied depending on the severity of the infection or rainfall. 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; Roca et al., 2007). However, these fungicides are increasingly restricted

because they not only control the pathogen, but also contaminate the soil, vegetation, water, and animals around (Roviello et al., 2021). Additionally, they present moderate toxicity to mammals, with a minimum lethal dose ranging from 300 to 2500 mg/kg of body weight in rats (Burandt et al., 2024; Neaman et al., 2024). Moreover, copper-based fungicides have low adherence to plant leaves and branches, making them easily washed away by rain, which requires frequent reapplications and intensifies their negative impact on the agricultural ecosystem (Neaman et al., 2024).

Cultural methods also play a substantial role in preventing *Colletotrichum* spp. infections. Among them, careful scheduling of harvest is essential to reduce production losses, with earlier harvesting recommended as olive susceptibility increases during ripening (Moral et al., 2014). In this sense, it is recommended to immediately harvest healthy fruits to prevent further contamination, since ripe olives are the most affected. Likewise, the selection of disease-resistant or tolerant olive cultivars for the establishment of new plantations, as well as appropriate pruning, fertilization, irrigation and pest management practices such as the olive fly, which could spread the pathogen, are necessary (Cacciola et al., 2012; Moral et al., 2014). Specifically, pruning enhances air circulation within the plant, reducing the density of leaves and branches and, consequently, decrease humidity and thereby the chances of infection (Cacciola et al., 2012).

1.3. Bioprotection as a control strategy

Integrated Pest Management (IPM) is a sustainable framework that combines multiple strategies to reduce reliance on chemical pesticides, enhance crop productivity, and promote ecosystem health. Recent advances in IPM, including biological and genetic control, targeted pesticide delivery, and eco-friendly tools, highlight its potential to conserve biodiversity and ensure food safety while addressing global agricultural challenges (Zhou et al., 2024). In this sense, bioprotection is positioned as an effective and sustainable strategy against olive anthracnose. The term ‘bioprotection’, proposed by Collinge et al. (2022) and Stenberg et al. (2021), encompasses the use of both living organisms (biocontrol) and non-living extracts and natural products, such as plant extracts, phenolic or volatile compounds. These agents can act directly through antimicrobial activity or competition with the pathogen, or indirectly by

triggering the plant's defense responses (Raza et al., 2016; Llorens et al., 2017; Poveda and Baptista, 2021).

Several studies have explored the use of bioprotection tools for controlling olive anthracnose, focusing on both microorganisms and natural products. Among microorganisms, those that inhabit the internal tissues of the olive tree (called endophytes) have emerged as the most extensively studied and explored group for their potential in managing olive anthracnose. A study screened a wide array of endophytic fungal species isolated from olive fruits, ultimately selecting seven microorganisms for their ability to inhibit *C. acutatum* under *in vitro* conditions (Preto et al., 2017). Among these, *Chaetomium globosum, Epicoccum nigrum* and *Quambalaria cyanescens* demonstrated significant fungal growth inhibition. Similarly, Angilé et al. (2025) confirmed the effectiveness of *E. nigrum* in the inhibition of *C. acutatum* mycelial growth. In another study conducted under more realistic field conditions, *Bacillus subtilis* and *Aureobasidium pullulans*, were shown to effectively reduce latent infection and the severity of *Colletotrichum* spp. in olive trees (Nigro et al., 2018). Despite these promising results, the precise mechanisms underlying this bioprotection remain unclear. Proposed mechanisms include the production of inhibitory compounds by these microorganisms and the stimulation of plant defense response (Raza et al., 2016; Llorens et al., 2017; Angilé et al., 2025). *A. pullulans*, an endophyte isolated from olive trees, significantly reduced the mycelial growth and olive anthracnose severity caused by *C. acutatum* in detached olive fruits, likely through changes in fruit volatile profiles, which appear to be associated with disease suppression (Sdiri et al., 2022). The application of the endophyte *Penicillium commune* in olive trees inoculated with *C. nymphaeae* under field conditions also induced the release of certain volatile organic compounds (VOCs), with ability to inhibit the pathogen (Silva et al., 2023). In particular, these VOCs inhibited *C. nymphaeae* mycelial growth by up to 1.4-fold and sporulation by up to 1.2-fold (Silva et al., 2023).

Crop bioprotection also includes the use of natural products derived from plant extracts, which typically contain bioactive compounds. These bioactive compounds are primarily secondary metabolites such as polyphenols, alkaloids, peptides, steroids, terpenoids, and VOCs (Greff et al., 2023; Gurjar et al., 2012; Pratyusha, 2022). These secondary metabolites contribute to plant protection against pathogens in multiple ways. Many of them, such as plant hormones, phytotoxins, and antimicrobial agents, act directly against the pathogen reducing

disease severity (Anjali et al., 2023). In addition, these metabolites can indirectly enhance plant resistance by inducing plant defense responses (Llorens et al., 2017; Poveda and Baptista, 2021). In this sense, Pangallo et al. (2022), demonstrated the efficacy of pomegranate peel extract against *C. acutatum* on olive plants of the cv. Arbosana and Ottobratica. Antón-Domínguez et al. (2025) demonstrated that carob and pomegranate extracts enhance plant defence mechanisms against olive anthracnose through antioxidant activity and phenolic compounds production. Similarly, Varveri et al. (2024) determined that five natural commercial products based on eugenol, thymol and geraniol; chito-oligosaccharides; and laminarin, significantly reduced *C. acutatum* conidial production and disease progression in detached olive fruits.

1.3.1. Volatile organic compounds

VOCs represent a promising alternative to conventional pesticides due to their low residue generation and their natural emission by all living organisms, forming complex mixtures known as volatilomes (Maffei et al., 2011). Although initially regarded as metabolically non-essential, recent studies have demonstrated that VOCs play critical ecological roles, mediating intra- and interspecific interactions among microorganisms, plants, and animals (Veselova et al., 2019). Accumulating evidence shows that these compounds can modulate microbial and plant growth (Hammerbacher et al., 2019 Elsherbiny et al., 2020), induce systemic resistance against biotic stresses (Razo-Belman and Ozuna, 2023), and function as insect attractants or repellents (Veselova et al., 2019). Therefore, developing efficient VOC-based formulations for agricultural use may contribute to sustainable strategies for disease and pest management, as well as productivity enhancement (Sharifi and Ryu, 2018).

However, the composition and quantity of VOC emissions vary widely depending on environmental and biological factors. In fungi and bacteria, emissions depend on taxon, life stage, growth phase, substrate availability, and temperature (Misztal et al., 2018). In plants, high temperatures, intense light, and herbivore attack tend to increase VOC release (Holopainen and Gershenzon, 2010). Although the use of pure volatiles may reduce such variability, their practical application faces challenges, as high vapor pressure and rapid diffusion make these compounds unstable, significantly shortening their effective half-life under normal environmental conditions (Bakry et al., 2016). This instability, combined with the need for

prolonged exposure to elicit beneficial effects, represents a major obstacle to the biotechnological application of VOCs (Xie et al., 2009). Beyond these practical limitations, VOCs are also involved in microbial interactions and fungal development, which supports their potential as bioprotective agents.

1.3.2. Fungal suppression by VOCs

In fungi, VOC production constitutes a multifunctional adaptive mechanism. Fungi frequently inhabit aerated environments where water is not an efficient medium for chemical diffusion, making volatile signaling advantageous for long-distance communication. These compounds regulate internal physiological processes, such as conidial germination, acting as autoinhibitors under high population density to avoid intracolonial competition. Furthermore, fungal VOCs play crucial roles in interspecific competition by inhibiting the growth of rival fungi and stimulating the production of mycotoxins associated with oxidative stress and competitive advantages (Pennerman et al., 2022). Due to these potent inhibitory and regulatory properties, VOCs are frequently reported as bioprotective agents against a wide range of pathogens. Their mode of action depends on the target pathogen and chemical composition. For instance, VOCs produced by *Pichia* spp. induce cellular DNA damage in *Monascus purpureus* (Zhang et al., 2021). Additionally, these volatiles can interfere with protein synthesis, disrupt mitochondrial metabolism, impair oxidative detoxification systems, and inhibit the radial growth of *Aspergillus carbonarius* mycelia (Tilocca et al., 2019).

Mycelial inhibition by VOCs involves mechanisms that compromise cellular integrity and hyphal elongation. These compounds can alter plasma membrane structure, disrupt metabolism, and cause structural damage in mycelia. Reported changes include cell wall thickening, membrane retraction, and the accumulation of abnormal vesicles, all of which indicate physiological stress (Tyagi et al., 2020). Phenethyl alcohol (PEA) exemplifies this mechanism by inducing marked morphological alterations in *Fusarium incarnatum*, thereby impairing normal hyphal development (Intana et al., 2021). VOCs also reduce radial growth and the ability of pathogens to colonize plant tissues, directly affecting fungal viability (Zhao et al., 2022). In *Botrytis cinerea*, trans-cinnamaldehyde as a fumigant resulted in complete inhibition of mycelial growth (Guo et al., 2019). The volatilome of *Trichoderma koningiopsis* PSU3-2 inhibits *Colletotrichum gloeosporioides* by enhancing chitinase and β-1,3-glucanase

activity, enzymes involved in fungal cell wall degradation (Ruangwong et al., 2021). Similar enzymatic mechanisms have been observed in *Trichoderma asperellum* T1 against *Corynespora cassiicola* and *Curvularia aeria* (Wonglom et al., 2020). *Daldinia cf. concentrica* also produces a volatilome capable of inhibiting *Aspergillus niger*, *Alternaria alternata*, *B. cinerea*, and *Fusarium oxysporum* (Razo-Belman and Ozuna, 2023).

The effect on conidia occurs mainly through inhibition of germination and structural deterioration of the propagules. These compounds reduce conidial viability, impair hyphal formation, and can induce visible deformities in conidia, blocking early stages of infection and preventing host colonization (Jaibangyang et al., 2021). Volatilomes produced by endophytic fungi such as *A. pullulans* also inhibit *C. acutatum* viability, reducing mycelial growth by 90% and conidial germination by 70%. Compounds such as benzyl alcohol and nonanal were strongly associated with reduced anthracnose severity in detached fruits, limiting disease progression during pathogen–host interaction (Sdiri et al., 2022). Likewise, *P. commune* released VOCs such as dihydromyrcenol and 3,7-dimethyl-3-octanol in contact with detached leaves and olives, reducing *C. nymphaeae* mycelial growth by 28.6% and sporulation by 16.7%, thereby slowing anthracnose progression (Silva et al., 2023).

Based on these findings, investigating VOCs as bioprotective agents is highly relevant for developing sustainable disease management strategies. Their broad activity against fungal pathogens, including effects on mycelial growth, conidial germination, and fruit infection, suggests that VOCs may serve as effective alternatives or complements to conventional fungicides. In the context of this study, these previous findings justify the evaluation of selected VOCs against *C. fioriniae*, since they were associated with reduced fungal development and lower disease severity in olive-related systems. Therefore, the present work focuses on assessing whether these compounds can similarly inhibit fungal growth, conidial germination, and disease progression in detached fruits under the experimental conditions tested.

2. Objectives

Olive anthracnose, caused by a wide diversity of *Colletotrichum* species, is a major constraint to olive production worldwide. Restrictions on copper-based fungicides and the lack of sustainable alternatives threaten the economic viability of olive orchards. For this reason, this study aimed to evaluate the antifungal potential of VOCs and their combinations against

Colletotrichum fioriniae and their efficacy in reducing disease progression in detached olive fruits.

The specific objectives are:

I) To identify VOCs with the ability to inhibit the mycelial growth, sporulation and conidial viability of *Colletotrichum fioriniae*.

II) To determine the half maximal inhibitory concentration (IC_{50}) and the best combinations of the most effective evaluated VOCs.

III) To evaluate the efficacy of the most effective VOCs and their combinations in reducing the disease progression in detached olive fruits.

3. Methodology

3.1. Fungal isolate

The pathogenic fungus *Colletotrichum fioriniae* (strain CIMO15FM001) belongs to the fungal culture collection of the Mountain Research Center (CIMO-CC). This strain was previously isolated from the inner tissues of naturally infected olives (Martins et al., 2021) and identified by sequencing the ITS region of rDNA by using ITS1-5.8S-ITS2 primers, as well as β-tubulin (Bt2a/Bt2b primers) and histone (CYLH3F/CYLH3R primers) genes. The fungal inoculum used in the experiments was prepared from frozen stocks by transferring mycelium to Potato Dextrose Agar (PDA; HiMedia, Mumbai, India) medium and subsequently incubating at 28 ± 2°C for 14 days.

3.2. *In vitro* inhibition of *Colletotrichum fioriniae* assay by VOCs

This assay evaluated the inhibitory effect of the following VOCs: (Z)-3-hexen-1-ol, 3,7-dimethyl-3-octanol, and nonanal (all three obtained from Thermo Fisher Scientific, Waltham, MA, USA); benzyl alcohol (TCI, Tokyo, Japan); dihydromyrcenol (Indagoo, Barcelona, Spain); and prenyl isobutyrate (Sigma-Aldrich, St. Louis, MO, USA).

3.2.1. Effect of VOCs on mycelial growth and IC_{50} calculation

Split Petri dishes with two sections were used to prevent direct contact between the VOC and the culture medium with the pathogen. In one section, 15 mL of PDA were poured. Once the medium was solidified, a mycelial plug (⌀ 6 mm) of *C. fioriniae* obtained from the margin of a 14-day-old actively growing colony was placed in the center of the Petri section. In the other section, a half-moon-shaped Whatman filter paper was positioned and impregnated with 10 µL of the VOC at five different doses (10µL, 5µL, 2.5µL, 1.25µL, and 0.625µL). All plates were then sealed with two layers of Parafilm® (Bemins, Wisconsin, USA) to minimize VOC loss to the environment (Figure 4).

Two VOC application protocols were compared to identify the most reproducible one. In the first protocol, each VOC was solubilized in sunflower oil to obtain the doses mentioned above. Thus, the doses corresponded to 10 µL of VOC alone, 5 µL of VOC + 5 µL of sunflower oil, 2.5 µL + 7.5 µL, 1.25 µL + 8.75 µL, and 0.625 µL + 9.375 µL. Petri dishes with filter paper impregnated with 10 µL of VOC-free sunflower oil were included as controls. In the second protocol, each VOC was applied directly without the diffusion-retarding agent. Petri dishes with unimpregnated filter paper were included as controls. This comparative design allowed us to assess not only the biological activity of the VOCs but also the influence of the release matrix on their performance.

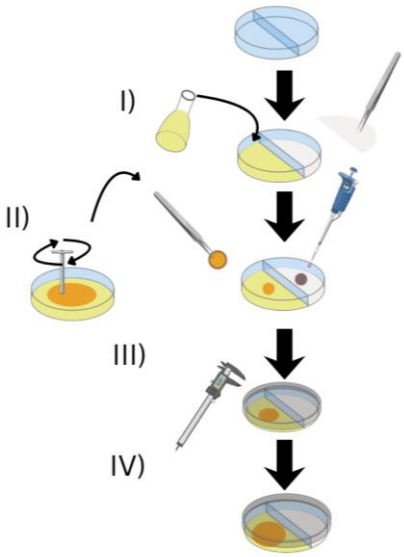

Figure 4. *In vitro* VOCs inhibition assay procedure: I) PDA pouring and halfmoon filter positioning, II) *C. fioriniae* mycelium disk cut and positioning on PDA, and VOC (with sunflower oil or without) pouring on halfmoon filter, III) plate closed with two Parafilm® layers and colony diameter measure, and IV) five days of incubation.

A factorial design was used with 30 treatments (six VOCs and five doses) and a control (no VOC added) as independent variables, and six replicate Petri dishes per combination or control (31 × 6 = 186 Petri dishes in total). The experiment was conducted twice at 28 ± 2°C. After five days of incubation under the previously established conditions, colony diameter was measured with a digital caliper and used to obtain the percentage of mycelial growth inhibition (MGI, %), with respect to the control using the following equation:

$$MGI\ (\%)\ =\ [1-(\frac{MG\ treatment}{MG\ control})]\times 100$$

where "MG treatment" is the mycelial growth (MG) diameter of *C. fioriniae* under each VOC treatment, and "MG control" is the MG diameter of *C. fioriniae* without VOC.

Then, the half maximal inhibitory concentration (IC_{50}), defined as the concentration that produces 50% inhibition, was calculated by regressing percentage mycelial growth inhibition against the logarithm of VOC dose, using the following equation:

$$LogIC50 = LogDose_{Low} + \frac{(50 - MGI_{Low}) \times (LogDose_{High} - LogDose_{Low})}{MGI_{High} - MGI_{Low}}$$

where "$LogDose_{Low}$" and "$LogDose_{High}$" are the logarithms of lowest and highest compound dose, respectively, and "MGI_{Low}" and "MGI_{High}" are the values of mycelial growth inhibition at lowest and highest compound dose, respectively.

Growth rate inhibition (GRI; %) represents the reduction in mycelial growth rate and was used to determine whether *C. fioriniae* grew more slowly under each VOC treatment. Higher GRI values indicate slower mycelium growth. For each experimental condition, "MG treatment" and "MG control" were recorded on the evaluation day "n" and used to calculate the normalized daily mycelial growth rates. This yielded the growth rates of treated "GR treatment" and the untreated control "GR control" colonies, expressed as:

$$GR_{treatment} = \frac{MG\ treatment}{n}$$

$$GR_{control} = \frac{MG\ control}{n}$$

The "GR treatment" and "GR control" values were then used to compare treated mycelium growth with the growth of the control. The arithmetic mean of all control values "$\overline{GR_{control}}$" was defined as 100% growth, and the treated values were expressed as a percentage of the control. The difference between these two values was then calculated as the percentage of growth inhibition caused by the treatment (GRI; %), and this parameter was only calculated for the optimized protocol:

$$\overline{GR_{control}} = \frac{1}{a} \times \sum_{i=1}^{a} GR_{control\ i}$$

$$GRI\ (\%) = 100 - \frac{GR_{treatment} \times 100}{\overline{GR_{control}}}$$

3.2.2. Effect of VOCs on conidial production

To determine the post-treatment effect of VOCs doses on *C. fioriniae* conidial production, plates from the previous assay were flooded with 3 –5 mL of sterile distilled water (SDW), and the conidia on the colony surface were gently scraped. The resulting suspension was filtered through a double layer of sterile gauze, and the filtrate was centrifuged at 13,000

× g for 5 minutes to pellet the conidia. After removing the supernatant, the pellet was resuspended in 100 µL of SDW. Then, conidial concentration was determined using a hemocytometer and the percentage of conidial production inhibition (CPI; %) with respect to the control was calculated using the previously adapted from MGI %, resulting in the following equation:

$$CPI\ (\%)\ =\ [1-(\frac{CP\ treatment}{CP\ \ control})]\times 100$$

where "CP treatment" is the number of conidia produced (CP) of *C. fioriniae* under each VOC treatment, and "CP control" is the number of conidia produced of *C. fioriniae* without VOC.

3.3. *In vitro* inhibition of *Colletotrichum fioriniae* using the most effective VOCs and their mixture

3.3.1. Effect of the most effective VOCs and their mixture on mycelial growth, and their influence on conidial production and viability

After identifying the VOCs (3,7-dimethyl-3-octanol and dihydromyrcenol) with the greatest impact on both the MGI (%) and the CPI (%) of *C. fioriniae*, an assay was performed to determine the IC_{50} effect of each VOC and of a 1:1 (v/v) mixture of them on MGI (%) and CPI (%) determined as previously described, both on a *C. fioriniae* mycelium disc and on a fungal conidial suspension, in order to ascertain whether there were differences depending on the type of inoculum. In addition, post-treatment viability of the conidia collected from the plates was evaluated by inoculating one 10 µL drop of the conidial suspension adjusted to 1 × 10^5 conidia/mL into the center of three microscope coverslips, which were placed in a Petri dish containing a thin layer of water agar [10 g of agar (HiMedia, Mumbai, India) per L]. The Petri dishes were sealed with a layer of Parafilm® to prevent agar dehydration and incubated at 28 ± 2°C for 14–16 h (Silva et al., 2023). After the incubation period, a 5 µL drop of 0.01% acid fuchsin in lactoglycerol was added to each inoculum drop to stop germination (Antón-Domínguez et al., 2025). Then, 100 conidia were randomly observed per slide and ungerminated and germinated conidia were counted using a microscope (Leica DM500) (Figure

5A), and conidial germination inhibition (CGI; %) was calculated, with respect to the control, following the above adapted equation, resulting in:

$$CGI\ (\%)\ =\ [1-(\frac{CG\ treatment}{CG\ control})]\times 100$$

"CG treatment" is the number of germinated conidia (CG) of *C. fioriniae* under each VOC treatment, and "CG control" is the CG of *C. fioriniae* without VOC.

3.3.2. Effect of the most effective VOCs and their mixture on conidial germination

A test analogous to the previous CGI (%) assay was designed to evaluate whether VOCs could directly inhibit conidial germination. Conidial suspensions were prepared from two-week-old untreated *C. fioriniae* colonies as described above. For this, split Petri dishes were used. In one section, three microscope coverslips were placed in water agar, while in the other section, a half-moon-shaped Whatman filter paper was positioned and impregnated with the IC_{50} concentration of the most effective VOCs (3,7-dimethyl-3-octanol and dihydromyrcenol), following the same conditions applied in the previous conidial germination assay (Figure 5B). This setup allowed assessment of whether direct exposure to the VOCs could inhibit conidial germination.

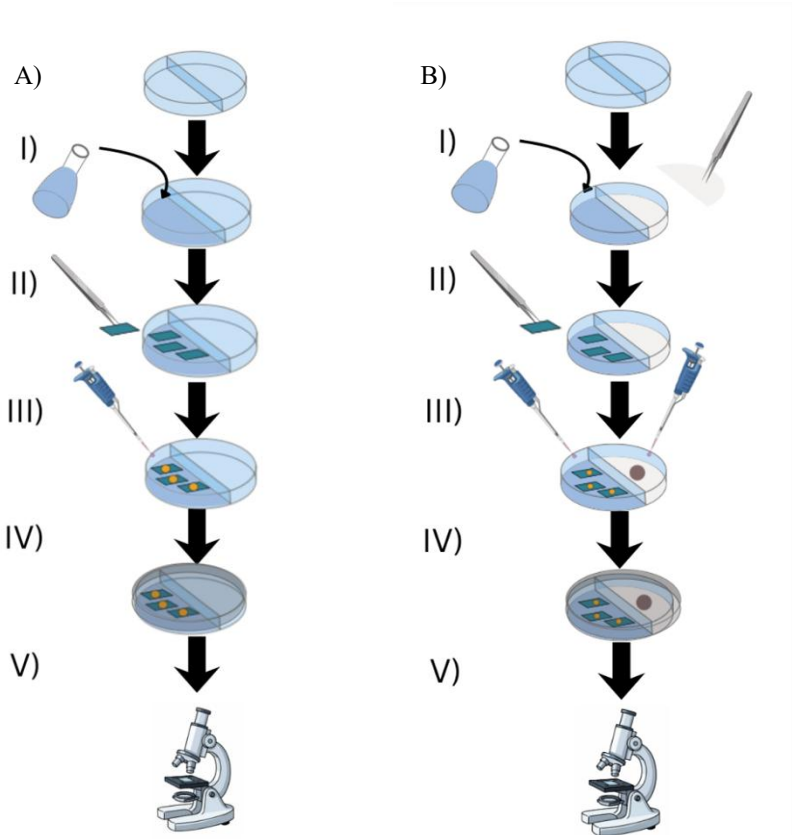

Figure 5. Conidial germination inhibition assay procedure. A) Previously treated conidia assay: I) thin layer of water agar, II) positioning of three microscope coverslips, III) post-treatment conidial suspension, IV) incubation and Parafilm® sealing, and V) germination stop and conidia evaluation. B) Direct VOCs exposure conidial germination inhibition assay: I) thin layer of water agar and halfmoon filter positioning, II) positioning of three microscope coverslips III) conidial suspension and pure volatile pouring on halfmoon filter, V) incubation and Parafilm® sealing, and V) germination stop and conidia evaluation.

3.4. Effect of VOCs on disease progression in detached olive fruits

Symptomless detached olive fruits of cv. Cobrançosa (moderately susceptible; Moral et al., 2017) were collected from an olive orchard in the Trás-os-Montes region and classified into color class 2-3 (onset of ripening) (Barranco et al., 2017). Once in the laboratory, fruits were disinfected by washing in a 0.02% (v/v) Tween 20 aqueous solution, and their surface was sterilized by immersing them first in a 70% (v/v) ethanol solution for 2 min and then in a 10% (v/v) solution of commercial bleach (Cl at 5 g/L) in SDW for 7 min. Then, the disinfected fruit was rinsed under running tap water and allowed to dry on sterile filter paper for 30 min, placed on plastic containers and stored at 4°C until used (less than 7 days) (Moral et al., 2008). Fruits were placed in glass vessels used as humidity chambers at 100% relative humidity (RH), each

vessel containing a thin layer of sterile perlite (around 6.4 g) hydrated with 5 mL of SDW, on which 5 olives were deposited. Fruits were inoculated by spraying 200 µL of a *C. fioriniae* conidial suspension adjusted at 10^5 conidia/mL with a hemocytometer. Whatman filter disk (22 mm ⌀) was placed under the chamber lid and soaked with the selected VOCs (3,7-dimethyl-3-octanol and dihydromyrcenol) at IC_{50}. Glass vessels with fruits sprayed with only SDW or with *C. fioriniae* with SDW soaked under the chamber lid were included as negative or positive control, respectively. The fruits were incubated at 24 ± 2 ºC with 100% RH for 25 days after pathogen inoculation.

A randomized complete block design was used with the two best VOCs and their 1:1 mixture and two controls (negative and positive) as independent variables, with five replicated humidity chambers (blocks) and five replicate olive fruits per block (5 × 5 × 5 = 125 olives in total). Disease severity (DS) was assessed every three days using a rating 0-4 scale adapted from Moral et al. (2021): 0 = olives without symptoms, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% affected olive surface. The area under the disease progression curve (AUDPC) was calculated using trapezoidal integration (Campbell and Madden, 1990). Subsequently, AUDPC and DS were expressed as relative percentages (RAUDPC; RDS; %) with respect to the inoculated and untreated fruits (positive control). At the end of the experiment, the disease incidence (DI; number of total symptomatic fruits) and the number of total rotten fruit were counted and expressed as percentage (%).

3.5. Data analysis

The data from the two replicates of each experiment were combined after confirming that there were no significant differences between them ($p \geq 0.05$). The homogeneity of variances and normality of the data were checked before performing analysis of variance (ANOVA). A factorial ANOVA was performed with compound, dose and their interaction as independent variables and MGI (%), CPI (%), CGI (%), total rotten fruit (%) and RAUDPC (%) as dependent variables. Logarithmic transformations of the data were performed when necessary. MGI (%), CPI (%), CGI (%) were compared according to the Scott-Knott cluster analysis test at $p = 0.05$ (Scott and Knott, 1974). Data analyses were performed using the function “SK” from the package (Jelihovschi et al., 2014) R v.4.5.2 and Rstudio 2026.01.2+418 (R Core Team, 2026). To identify the VOCs that best discriminate the different

treatments ((Z)-3-hexen-1-ol; 3,7-dimethyl-3-octanol; nonanal; benzyl alcohol; dihydromyrcenol and prenyl isobutyrate) in *C. fioriniae* cultures at each dose (10µL, 5µL, 2.5µL, 1.25µL, and 0.625µL), a principal component analysis (PCA) was performed. R v.4.5.2 and Rstudio 2026.01.2+418 (R Core Team, 2026) software was used to perform this analysis using the PCA function of the 'FactoMineR' package (Lê et al., 2008). The PCA biplot was plotted using the fviz_pca_biplot function from the 'factoextra' package and some auxiliary functions from "ggplot2" (Wickham., 2016; Kassambara and Mundt, 2020). The PCA arrows represent the contribution of each variable (MGI, GRI, and CPI) to the principal components (arrow length), and the color gradient indicates their contribution to explaining the variance in the data set. Data of MGI (%) and CPI (%) were plotted using Veusz v.4.2 (Sanders, 2026). Variables from detached olive fruits (RDS (%), DI (%), total rotten fruit (%) and RAUDPC (%)) were made using the function "TukeyHSD" native from R v.4.5.2 and Rstudio 2026.01.2+418 (R Core Team, 2026).

4. Results and Discussion

4.1. *In vitro* VOCs inhibition assay

The initial screening indicated that several VOCs dissolved in sunflower oil were able to inhibit mycelial growth. In Figure 6, the compounds dihydromyrcenol, 3,7-dimethyl-3-octanol, and nonanal demonstrated a pronounced ability to inhibit MG, achieving more than 90% of MGI at the highest tested dose. Their effects remained high at 5 µL, where dihydromyrcenol reached 96.70%, 3,7-dimethyl-3-octanol 82.52%, and nonanal 77.72% of MGI. The inhibitory effect decreased at 2.5 µL, with 70.33%, 47.63%, and 59.36%, respectively. Dihydromyrcenol was the most consistent VOC because it showed a significant difference compared to the control down to 1.25 µL with 29.33% of MGI. (Z)-3-hexen-1-ol and benzyl alcohol reached 25.59% and 23.58% of MGI, respectively, at 10 µL, while prenyl isobutyrate remained consistent at 10 µL, 5 µL, and 2.5 µL with 22.23%, 18.57%, and 18.34% of MGI, respectively. Substantial variability showed up in the dataset requiring multiple assay reproductions. This inconsistency was likely associated with the use of sunflower oil as a VOC

carrier, which initially compromised statistical reliability and prevented hypothesis testing. Consequently, IC_{50} calculations were not performed using these data.

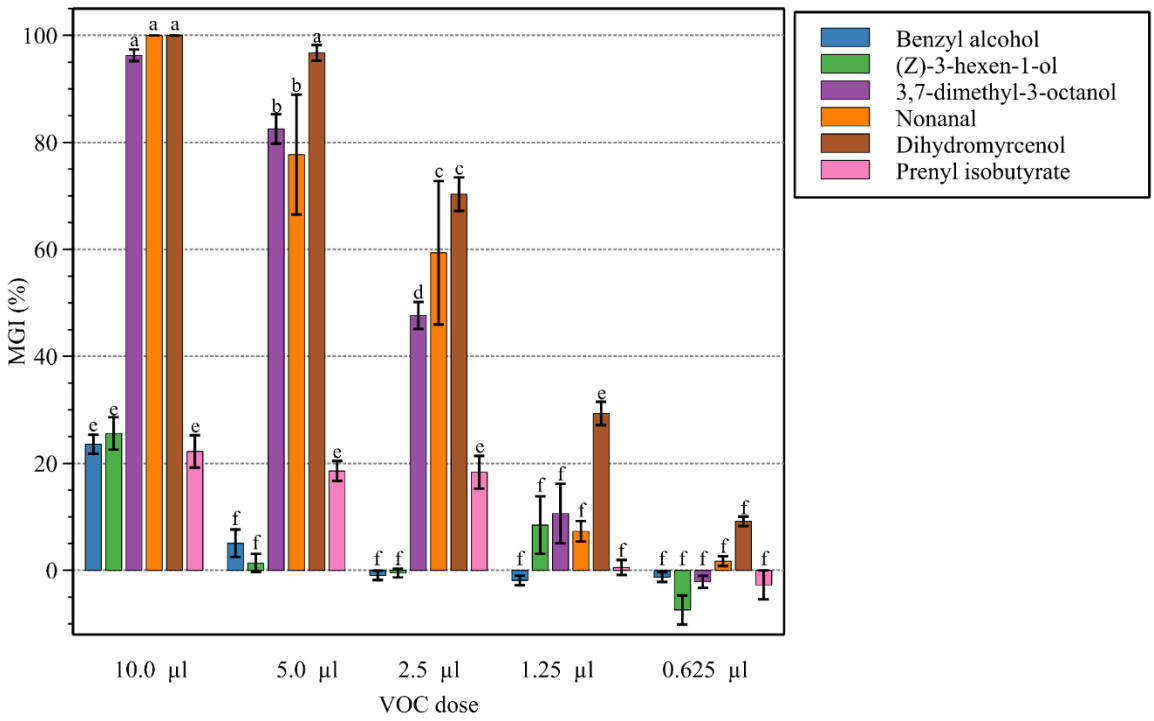

Figure 6. Effect of VOCs solubilized in sunflower oil on mycelial growth inhibition (%) of *Colletotrichum fioriniae*. Results are expressed as mean ± standard error (SE), with N = 6. Columns with different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments, considering compound type and applied volume

The initial use of sunflower oil was intended to mimic the behavior of hydrophobic natural mixtures, such as essential oils, which can protect volatile compounds from degradation and slow their diffusion, thereby modulating their release (De Groot and Schmidt, 2016). However, this approach resulted in high variability in the dataset, suggesting that the carrier matrix interfered with VOC volatilization and release. For this reason, the assay was repeated without sunflower oil, which removed an unnecessary experimental variable and yielded more consistent results (Lalanne et al., 2008).

The second assay without using sunflower oil as the solvent showed a reduced variability and lower SE error. There were significant differences between MGI among VOCs, doses, and their interaction ($p < 0.05$). The VOCs 3,7-dimethyl-3-octanol and dihydromyrcenol were able to completely inhibit the mycelial growth of *C. fioriniae* when 10 µL were applied, after 5 days of analysis no change in colony diameter was observed (Figure 7, 8). Both VOCs were able to inhibit by 73.64 ± 8.54% and 92.30 ± 1.74%, respectively, when 5 µL were applied, also achieve more than 50% of inhibition at 2.5 µL, and significantly inhibition were even observed at the lowest doses of 0.625 µL (Figure 7). The remaining VOCs analyzed also displayed antifungal activity, although to a lesser extent (Figure 8).

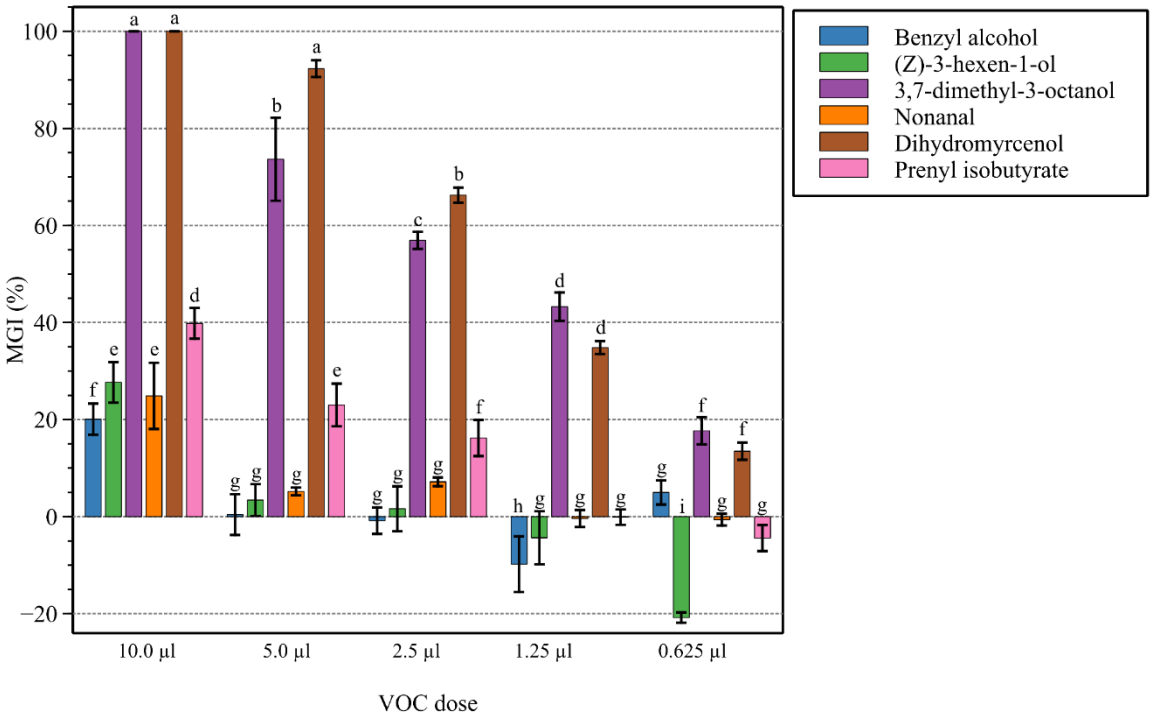

Figure 7. Effect of VOCs nonsolubilized in sunflower oil on mycelium growth inhibition (%) of *Colletotrichum fioriniae*. Results are expressed as mean ± standard error (SE), with N = 6. Columns with different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments, considering compound type and applied volume.

The high efficacy previously observed for nonanal was no longer evident in the oil-free assay. Although the overall inhibition trends remained broadly consistent between both trials (with and without sunflower oil), nonanal showed a marked reduction in activity when sunflower oil was not used. This observation may be explained by the autoxidation behavior of nonanal, leading to degradation catalyzed by light and oxygen. Under the oil-based protocol, the sunflower oil may have acted as a physical barrier, limiting oxygen exposure and moderating VOC release, thereby helping to sustain antifungal activity. In fact, nonanal has previously been reported as an effective fungicide against *B. cinerea* by damaging conidial membranes and increasing oxidative stress through the modulation of superoxide dismutase (SOD) and catalase (CAT) enzymes (Choe and Min, 2006; Zhang et al., 2017). Despite this, the present results indicate that these VOCs exert direct antagonistic effects on the pathogen. These findings are particularly significant because they demonstrate that these VOCs are not only active against *C. nymphaeae* and *C. acutatum*, as previously reported, but are also capable of inhibiting another *Colletotrichum* species (Sdiri et al., 2022; Silva et al., 2023).

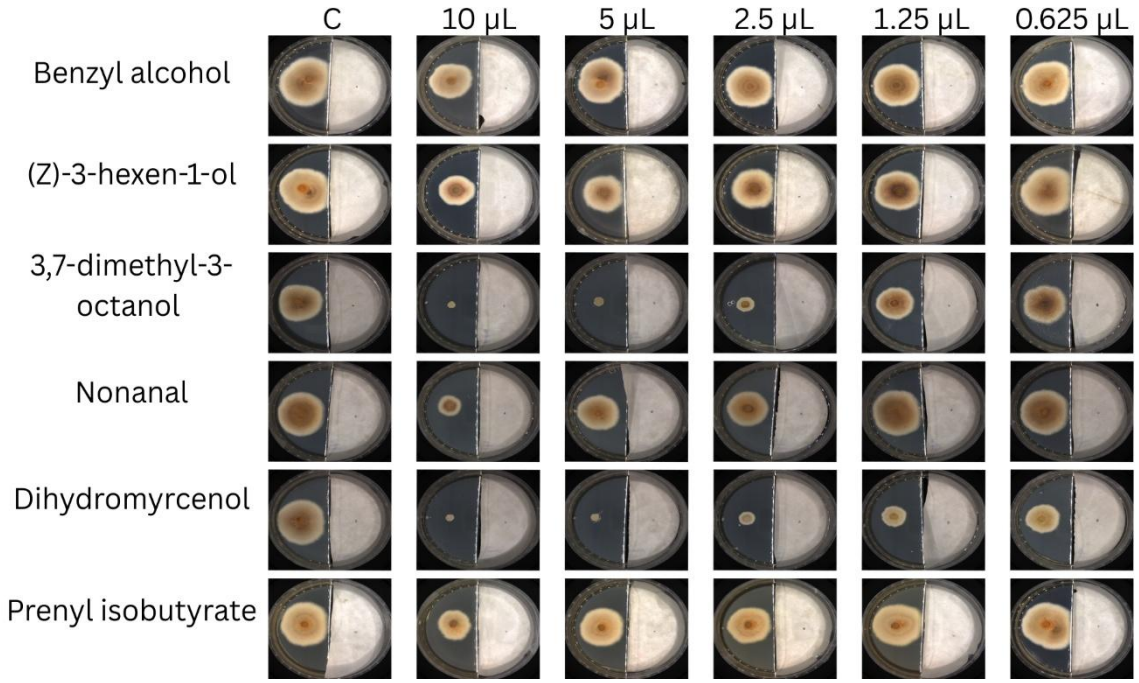

Figure 8. Macroscopic aspect of *Colletotrichum fioriniae* colonies showing mycelium growth inhibition by the analyzed VOCs nonsolubilized in sunflower oil at different doses (from 0.625 µL to 10 µL). VOC-free cultures were used as control (C).

About the effect of VOCs solubilized in sunflower oil on *C. fioriniae* conidial production inhibition, this assay demonstrated a pronounced suppressive effect (Figure 9). The control treatment yielded an average of 8.17×10^7 conidia/mL, whereas the treatments with dihydromyrcenol and nonanal at 10 µL resulted in no detectable conidia. In contrast, 3,7-dimethyl-3-octanol caused a 55.51% inhibition, with a mean production of 7.11×10^3 conidia/mL, representing a decrease of four orders of magnitude. This pattern is consistent with the MGI results observed in Figure 6, where the same VOCs also displayed the strongest activity, suggesting their high potential in reducing the growth of the pathogen.

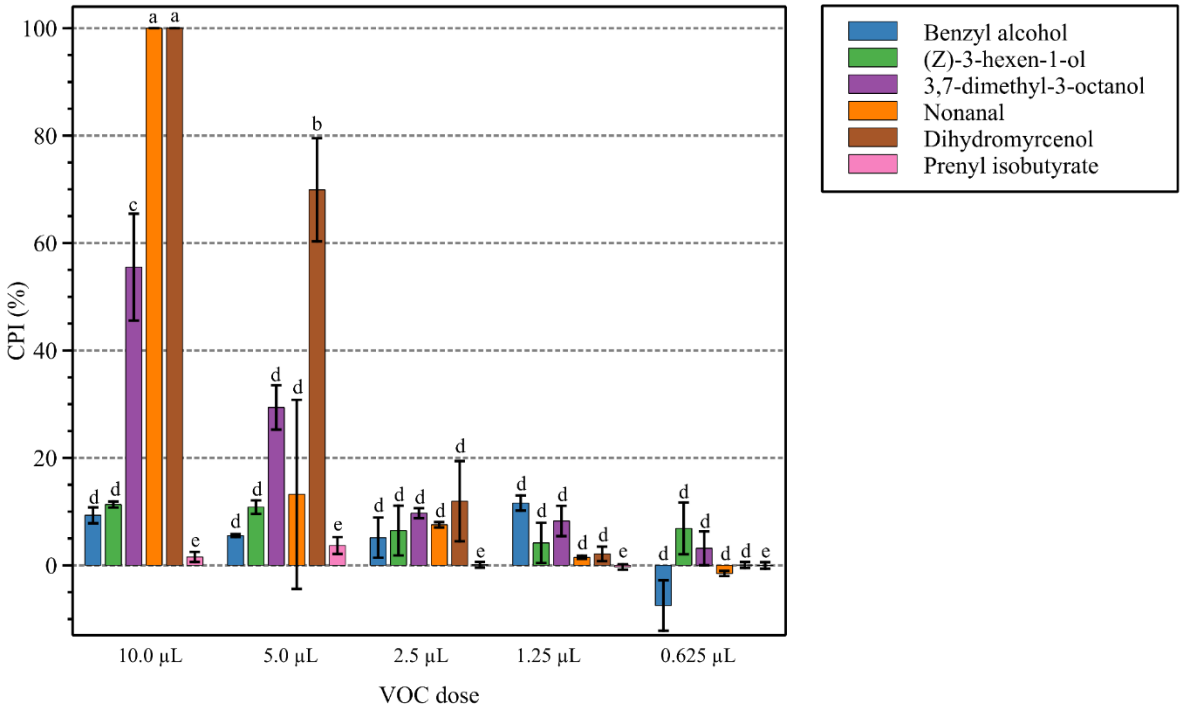

Figure 9. Effect of VOCs solubilized in sunflower oil on conidial production inhibition (%) of *Colletotrichum fioriniae*. Results are expressed as mean ± standard error (SE), with N = 6. Columns with different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments, considering compound type and applied volume.

With respect to the oil-free CPI assay, the control treatment yielded 4.18×10^8, whereas no conidia were detected for dihydromyrcenol and 3,7-dimethyl-3-octanol at 10 µL. At 5 µL, only 3,7-dimethyl-3-octanol still showed a noticeable inhibitory effect, with a 27.57% inhibition, corresponding to 1.76×10^6 conidia/ml (Figure 10). In both assays, these reductions

are particularly relevant given that olive orchard contamination is primarily driven by these infectious propagules.

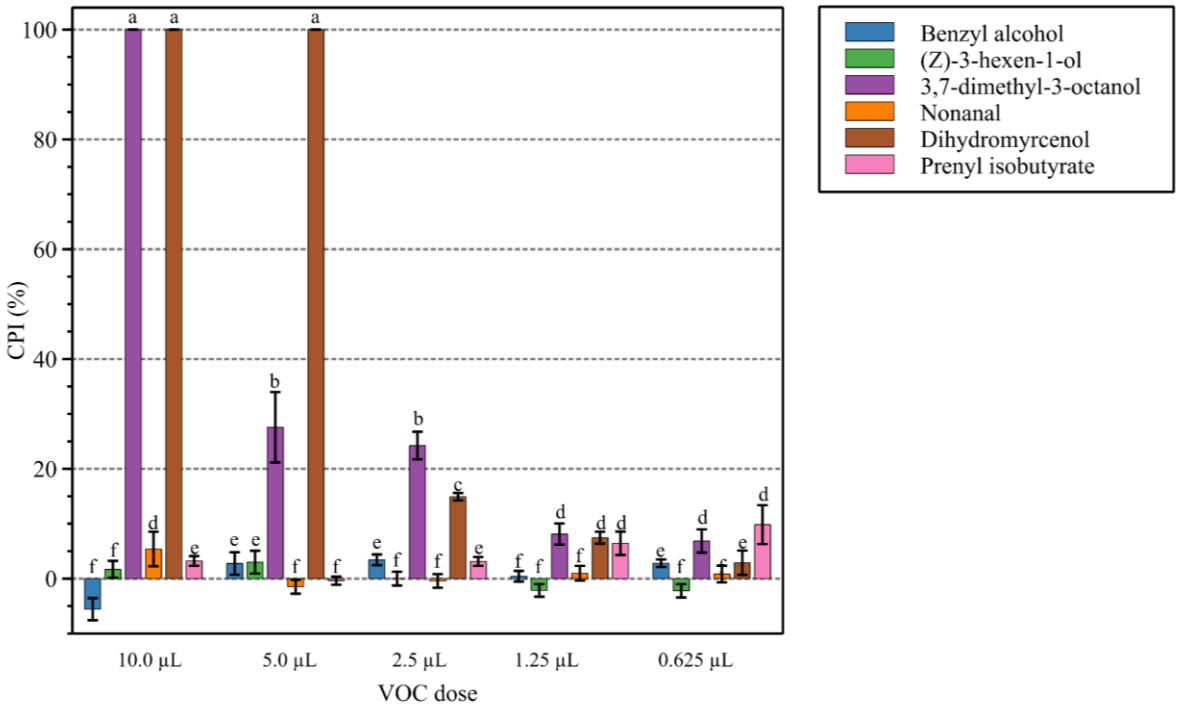

Figure 10. Effect of VOCs nonsolubilized in sunflower oil on conidial production inhibition (%) of *Colletotrichum fioriniae*. Results are expressed as mean ± standard error (SE), with N = 6. Columns with different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments, considering compound type and applied volume.

Based on the consistent MGI data obtained in the oil-free assay, IC_{50} values were determined for the most effective VOCs. 3,7-dimethyl-3-octanol exhibited the highest efficacy with an IC_{50} of 1.85 µL, followed closely by dihydromyrcenol at 2.05 µL. For all other evaluated compounds, the values found were overly high, indicating insufficient antifungal potential to *C. fioriniae*. Consequently, due to this lack of significant inhibitory activity, these substances were not selected for subsequent trials.

4.2. Principal component analysis

Principal component analysis (PCA) was used to evaluate the multivariate response of *C. fioriniae* to VOC exposure at five different doses. The analysis included GRI (%), MGI (%) and CPI (%), calculated relative to the untreated control (negative control) derived from the assay without sunflower oil. Separate PCAs were performed for each VOC dose to assess dose-dependent changes in the overall response pattern (Figure 11). The PCA plots revealed very high explained variances of 99.7%, 99.4%, 99.1%, 96.6%, and 79.5% for the doses from highest to lowest, respectively. Considering the two primary dimensions (Dim1 and Dim2), the plots showed clear clustering according to treatment, especially at the higher doses.

Notably, down to a dose of 2.5 µL, the multivariate pattern remained highly consistent, with two compounds standing out: 3,7-dimethyl-3-octanol and dihydromyrcenol. These two compounds exhibited the highest MGI and CPI values, reaching complete inhibition at the highest doses, 10.0 and 5.0 µL. This pattern suggests that both compounds maintained a strong inhibitory effect even at lower doses. In contrast, as the VOC dose decreased to 1.25 µL and 0.625 µL, the variance captured by Dim1 declined and the separation between treatments and negative control became less pronounced. Treatments such as nonanal and benzyl alcohol moved closer to the negative control cluster, indicating a loss of inhibitory activity at lower doses. Prenyl isobutyrate and (Z)-3-hexen-1-ol displayed intermediate behavior and notably, the first maintained moderate CPI even at low doses, suggesting that CPI can persist even when MGI is reduced. At 1.25 µL, 3,7-dimethyl-3-octanol and dihydromyrcenol still maintained measurable inhibitory activity. However, at 0.625 µL, a marked reduction in efficacy was observed, consistent with reduced separation in the PCA and weaker contrast between treatments. Overall, PCA suggests a dose-dependent reduction in antifungal activity across the tested VOCs, with the strongest effects observed for 3,7-dimethyl-3-octanol and dihydromyrcenol.

In conclusion, the PCA plots suggested that 3,7-dimethyl-3-octanol and dihydromyrcenol were the compounds with the most significant influence on *C. fioriniae* development, particularly at higher doses. This high biological efficacy may be related to the chemical nature and structural similarities of these compounds. A detailed molecular examination of both compounds (Figure 12) shows that they are C10 monoterpenoid alcohols

derived from isoprenoid biosynthesis (Spitzer, 2004; Bakkali et al., 2008). In literature, monoterpenoids are frequently described as major constituents and fundamental bioactive components of essential oils, being widely associated with potent antimicrobial activity (Spitzer, 2004). The behavior observed for *C. fioriniae* is consistent with previous studies showing that related VOCs can inhibit the growth of other *Colletotrichum* species. In this regard, citral and carvacrol demonstrated efficacy against *C. musae* and *C. gloeosporioides* (Garcia et al., 2008). In this context, 3,7-dimethyl-3-octanol and dihydromyrcenol appear to be promising candidates for the control of this pathogen.

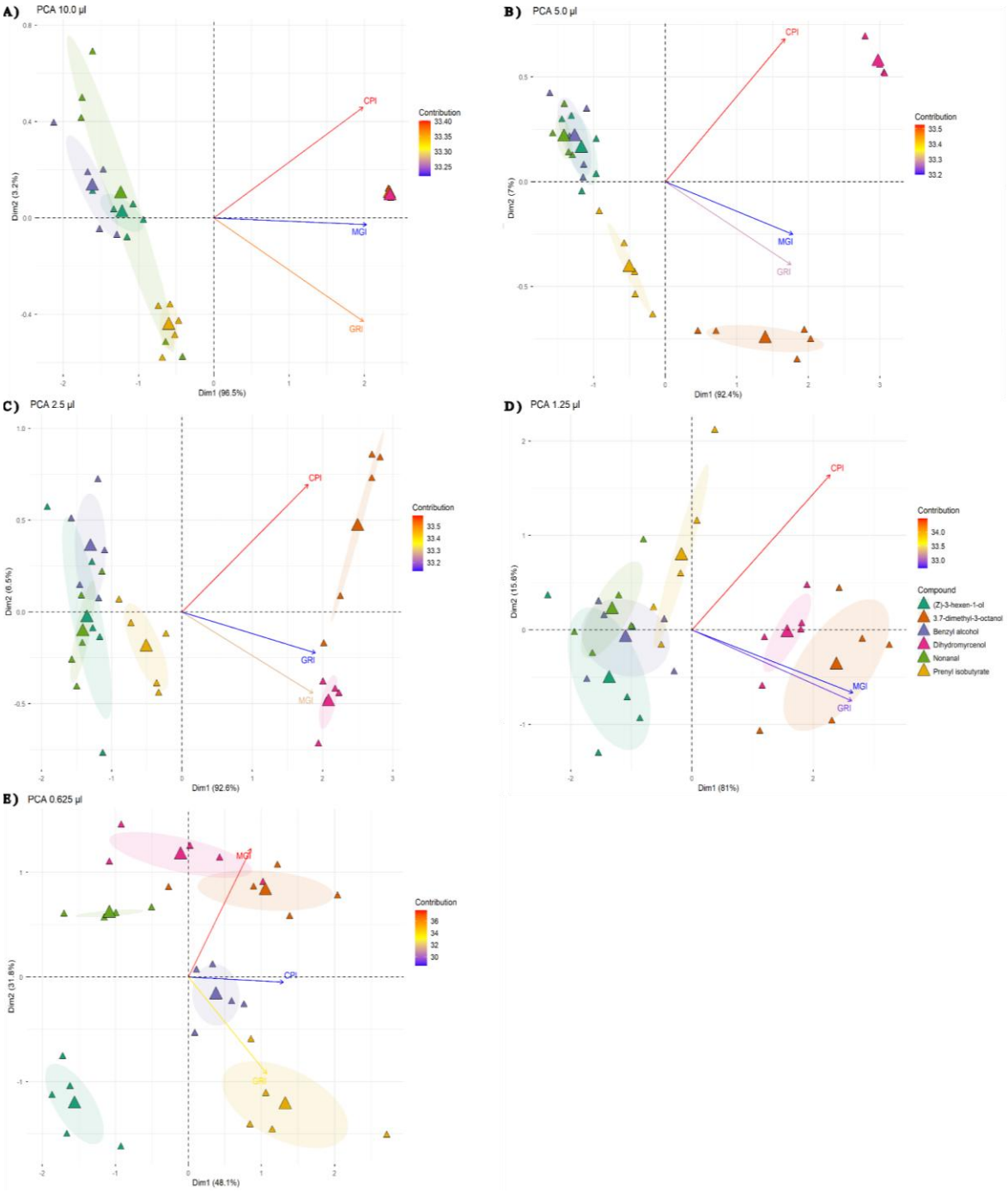

Figure 11. Principal Component Analysis (PCA) biplots of the interaction between VOCs and *Colletotrichum fioriniae* at different doses: A) 10.0 µL, B) 5.0 µL, C) 2.5 µL, D) 1.25 µL, and E) 0.625 µL. Individual samples are represented by triangles, where colors identify the specific treatments; larger triangles indicate the centroid (mean) of each group. Confidence ellipses (95%) illustrate the dispersion within each treatment. Triangles of the same color represent the same treatment, with the largest triangle being the average. The color of the gradient represents the contribution of each VOC to the explanation of the largest variance in the data set.

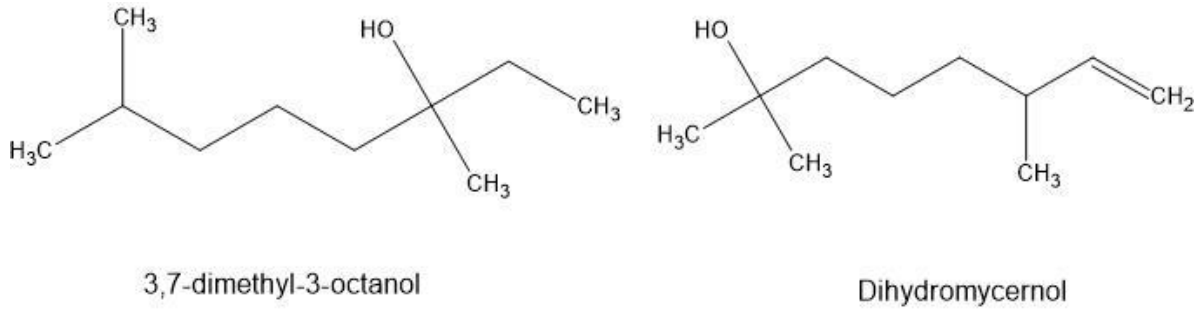

Figure 12. Molecules of 3,7-dimethyl-3-octanol and dihydromyrcenol.

The effectiveness of monoterpenoid alcohols in phytosanitary control has been linked to their lipophilic nature, which allows for accumulation within the lipid bilayer and the consequent disruption of cellular permeability. Compounds such as citral, citronellal, and L-carvone demonstrate a fungal growth inhibition of pathogens like *Colletotrichum* and *Fusarium* in a dose-dependent manner, causing hyphal collapse and severe damage to the plasma membrane (Cowan, 1999; Garcia et al., 2008). Beyond physical damage, these molecules can trigger progressive metabolic dysfunction. Specifically, citral interferes with oxidative reactions and the Krebs cycle (Luo et al., 2004), reducing energy capacity and favoring the accumulation of cytoplasmic protein aggregates. This interference extends to ATPase activity and organelle integrity, resulting in a functional collapse that explains the fungicidal nature of these agents (Parveen et al., 2004). The chemical architecture of these compounds, including oxygenated functional groups and hydrophobic character, may enhance their interaction with fungal cells and help explain their inhibitory effects (Naigre et al., 1996). This may be particularly relevant for reproductive structures, since conidial germination and early development are metabolically demanding processes and can be sensitive to VOC exposure (Luo et al., 2004; Souza et al., 2005). Nevertheless, additional targeted assays would be needed to confirm the specific mechanism of action of these specific VOCs.

4.3. *In vitro* inhibition using most effective VOCs and their mixture

No significant differences were observed between inoculum types (mycelial disc vs. conidial suspension) for MGI. For CPI, differences among treatments were only detected in assays using mycelial discs. Although the IC_{50} model targets a 50% inhibition threshold, 3,7-dimethyl-3-octanol and dihydromyrcenol exhibited values slightly exceeding this baseline. Such discrepancies are inherent to mathematical modeling and typical of biological variation

in confirmatory assays (Ishii et al., 2022). Overall, both VOCs demonstrated potent antifungal activity against *C. fioriniae* (Table 1, Figure 13). Notably, the 1:1 VOC mixture achieved inhibition levels exceeding 70% for MGI across both inoculum types, significantly outperforming the individual compounds. Regarding CPI, the mixture reached 40.89% inhibition when using mycelial discs, whereas the effect on conidial suspensions was markedly lower at 20.18%. These findings indicate that the VOC mixture is the most effective treatment, regardless of the inoculum source (Table 1). This outcome may suggest synergism or at least an additive interaction, a phenomenon widely reported in studies involving volatile organic compounds and secondary metabolites used in the control of phytopathogens (Chávez-Avilés et al., 2024). The literature indicates that VOC mixtures may act complementarily, targeting multiple cellular pathways and thereby reducing the adaptive capacity of the pathogen (Chávez-Avilés et al., 2024). Accordingly, the enhanced efficacy observed in the combined treatment may be attributed to simultaneous interference with distinct metabolic routes, oxidative stress mechanisms, or structural processes essential for fungal development.

Table 1. Effect of 3,7-dimethyl-3-octanol, dihydromyrcenol, or their 1:1 mixture at IC_{50} on mycelial growth (MGI; %) and conidial production (CPI; %) inhibition of *Colletotrichum fioriniae*, with respect to the control. Results are expressed as mean ± standard error (SE), with N = 6. Different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments.

VOC	Volume (µL)	Mycelial Growth Inhibition (MGI; %)		Conidial Production Inhibition (CPI; %)	
		Mycelial disc	Conidial suspension	Mycelial disc	Conidial suspension
3,7-dimethyl-3-octanol	1.85	53.73 ± 2.34 b	53.61 ± 5.89 b	15.86 ± 2.70 b	13.00 ± 2.12 a
Dihydromyrcenol	2.05	62.82 ± 5.56 b	54.54 ± 2.50 b	18.32 ± 4.38 b	13.00 ± 1.42 a
Mixture 1:1	1.95	78.28 ± 1.46 a	74.19 ± 3.58 a	40.89 ± 15.27 a	20.18 ± 4.21 a

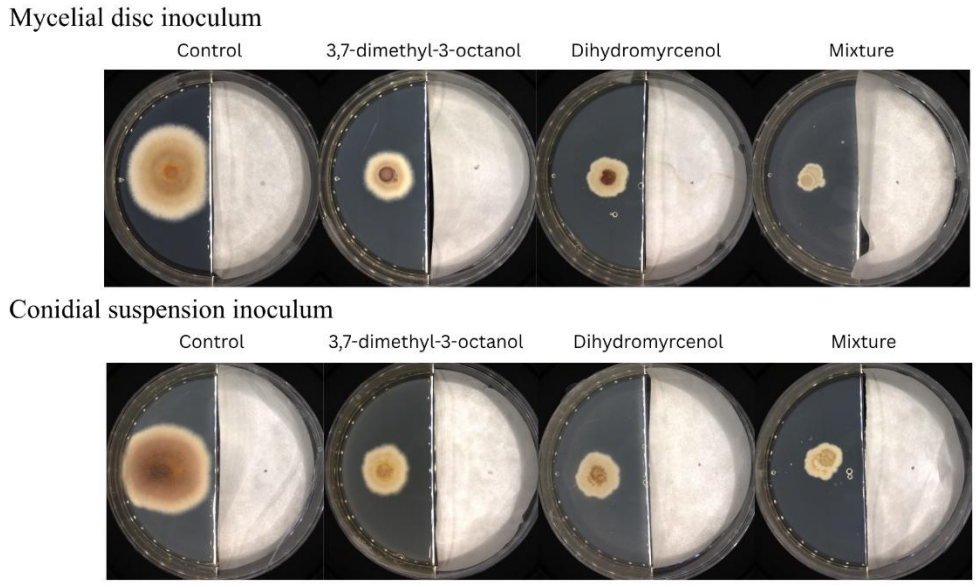

Figure 13. Effect of 3,7-dimethyl-3-octanol, dihydromyrcenol, or their 1:1 mixture at IC50 on mycelial growth inhibition (MGI; %) of *Colletotrichum fioriniae*, with respect to the control.

Moreover, several authors have emphasized that isolated VOCs tend to be less effective than complex mixtures, reinforcing the hypothesis that chemical interactions among compounds can potentiate antifungal activity (Jayakumar et al., 2021). Therefore, the findings of this study support the notion that combined approaches represent a promising strategy for controlling *Colletotrichum*, particularly in scenarios where pathogen genetic variability may facilitate rapid adaptation to individual compounds (Ishii et al., 2022).

4.3.1. Effect on germination

Building upon the IC_{50} findings, the effect of VOCs on CGI was evaluated. No significant differences in CGI (%) were observed between the two inoculum types (mycelial disc and conidial suspension) for conidia obtained from colonies previously treated (pre-treated colonies) with IC_{50} concentrations, nor in comparison with the control ($p > 0.05$). Given this consistency, data were pooled for subsequent analysis. These results confirm that, although VOCs strongly inhibit germination upon direct exposure, conidia produced by the pre-treated mycelium remain viable, regardless of the initial inoculum type used. While MG and CP were affected, conidial viability itself was not compromised in the surviving population.

However, this does not imply a lack of activity of VOCs against conidial germination. When conidia from untreated colonies were directly exposed to VOCs, CGI reached 100.00 ± 0.00% for the 1:1 mixture, followed by 90.53 ± 3.28% for dihydromyrcenol, and 81.00 ± 8.07% for 3,7-dimethyl-3-octanol. All treatments were significantly different from the control ($p < 0.05$), showing near-total inhibition of conidial germination (Table 2). This result suggests that, in the absence of an adequate nutritional environment, these compounds may act as strong germination inhibitors.

Table 2 Effect of 3,7-dimethyl-3-octanol, dihydromyrcenol, or their 1:1 mixture at IC50 on conidia germination inhibition (CGI; %) of *Colletotrichum fioriniae*, with respect to the control. Results are expressed as mean ± standard error (SE), with N = 9. Different letters indicate statistically significant differences according to Scott-Knott cluster analysis ($p < 0.05$) among treatments.

VOC	Volume (µL)	Conidia Germination Inhibition (CGI; %)	
		Conidial suspension derived from pre-treated colonies	Conidial suspension derived from untreated colonies
3,7-dimethyl-3-octanol	1.85	0.33 ± 3.28 a	81.00 ± 8.07 b
Dihydromyrcenol	2.05	2.52 ± 2.10 a	90.53 ± 3.28 a
Mixture 1:1	1.95	-0.73 ± 1.57 a	100.00 ± 0.00 a

4.4. Effect of VOCs on disease progression in detached olive fruits

Although there were significant differences in RAUDPC and DI between the treatments and the negative control, no significant differences were observed among the evaluated treatments, indicating that neither compound alone or combined was able to prevent the initial infection of the fruits. RAUDPC ranged from 71.50 ± 7.96 to 87.23 ± 19.40% for the mixture and dihydromyrcenol, respectively. A reduction of 28.50% in RAUDPC was observed for the mixture compared with the positive control (Table 3). Although not statistically significant, this trend suggests a potential effect on the temporal dynamics of disease development.

In contrast, significant differences were detected for total rotten fruit and RDS among treatments ($p \leq 0.05$). When applied individually, both 3,7-dimethyl-3-octanol and dihydromyrcenol did not differ from the positive control, indicating that neither compound alone was effective in reducing DS or fruit decay. However, when the two compounds were

applied as a 1:1 mixture, a clear significant effect was observed. The combination resulted in a 32% reduction in total rotten fruit and an approximately 21% reduction in RDS compared with the positive control (Table 3). These findings indicate a potential synergistic interaction between the compounds, enhancing their effectiveness against the disease, even though this synergy was not strong enough to produce significant differences in RAUDPC.

Overall, although the isolated compounds did not exhibit meaningful effects on DS or fruit decay, their combination shows promising potential by reducing both final damage and disease intensity. The lack of statistical significance in RAUDPC does not negate the observed trend, which suggests that the mixture may also moderately slow disease progression over time (Figure 14).

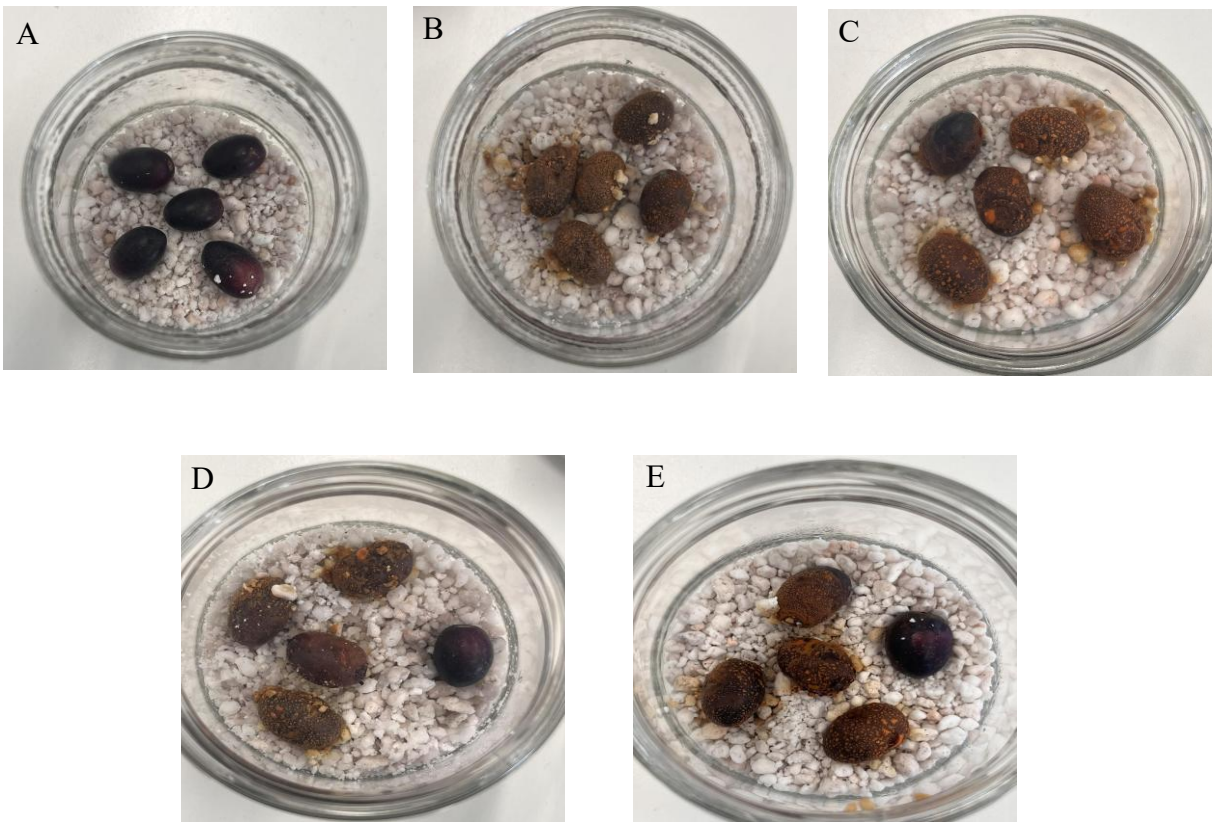

Figure 14. Disease symptoms on detached olive fruits of cv. Cobrançosa at 25 days after inoculation with conidial suspensions of *Colletotrichum fioriniae* and treated with 3,7-dimethyl-3-octanol, dihydromyrcenol or 1:1 mixture at IC50. A) Negative control, B) positive control, C) 3,7-dimethyl-3-octanol, D) dihydromyrcenol, and E) mixture 1:1.

Table 3. Disease-related parameters evaluated on detached olive fruits of cv. Cobrançosa inoculated with conidial suspensions of *Colletotrichum fioriniae* and treated with 3,7-dimethyl-3-octanol, dihydromyrcenol, or mixture 1:1 at IC50, with respect to the positive control. Results are expressed as mean ± standard error (SE), with N = 5. Different letters indicate statistically significant differences according to Tukey HSD comparison analysis ($p < 0.05$) among treatments.

VOC	DI (%)	Total rotten fruit (%)	Final RDS (%)	RAUDPC (%)
3,7-dimethyl-3-octanol	100.00 ± 0.00 a	80.00 ± 0.06 ab	91.41 ± 3.49 ab	80.76 ± 13.47 a
Dihydromyrcenol	95.00 ± 0.04 a	80.00 ± 0.11 ab	88.07 ± 8.47 ab	87.23 ± 19.40 a
Mixture 1:1	100.00 ± 0.00 a	64.00 ± 0.04 b	79.29 ± 4.96 b	71.50 ± 7.96 a
Positive control	100.00 ± 0.00 a	92.00 ± 4.90 a	100.00 ± 0.62 a	100.00 ± 6.84 a
Negative control	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 b

A relevant aspect emerging from the detailed analysis of the raw data is the disparity in the stability of the control exerted by the treatments. While the isolated use of dihydromyrcenol resulted in greater variability in temporal and severity parameters evidenced by a standard error (SE) of 19.40 in RAUDPC and 8.47 in RDS, the 1:1 mixture showed a more homogeneous and predictable response across replicates, with SE values of 7.96 and 4.96, respectively. This reduction in variability suggests that the combination of volatiles not only enhances inhibition but also compensates for the erratic dispersion that individual monoterpenes may exhibit under saturated humidity conditions.

Statistical difference was observed in total rotten fruit, the volatile mixture reduced final fruit decay to 64.00%, while maintaining an initial incidence of 100.00%, that strongly reinforces the hypothesis of a mechanism of action centered on limiting tissue colonization rather than preventing primary infection. This pattern is also consistent with the inhibition of conidial germination observed in previous assays, which suggests that these VOCs may interfere with early infection events and restrict subsequent pathogen development. In such a scenario, the treatment may contribute to delaying disease progression once infection has already been established.

Although direct biochemical measurements were not performed in this assay, this pattern could be consistent with a possible impact on fungal stress responses or on fruit–pathogen interactions during colonization. However, elucidating these mechanisms will require further physiological analysis, including measurements of oxidative stress markers and

defense-related enzymes such as superoxide dismutase (SOD) and catalase (CAT), to be confirmed. Specifically, these enzymes are essential for neutralizing superoxide radicals and hydrogen peroxide, respectively, preventing the oxidative damage that facilitates fungal exploitation (McCord and Fridovich, 1969; Santos-Sánchez et al., 2019).

5. Conclusions

The main finding of this study was the superior antifungal efficacy of C10 monoterpenoid alcohols, specifically 3,7-dimethyl-3-octanol and dihydromyrcenol. These VOCs demonstrated a remarkable ability to inhibit mycelial growth and conidial production, even at low doses where other VOCs were less effective. A synergistic 1:1 mixture of these two compounds was also evaluated at IC_{50}. *In vitro*, this combination achieved inhibition levels above 70%, significantly outperforming the individual VOCs.

The practical efficacy of the VOC treatments was further validated through detached fruit assays on olive fruits cv. Cobrançosa, where the 1:1 mixture reduced total fruit rot by 28% and relative disease severity by 21%, compared with the positive control. These findings suggest that the VOCs do not prevent initial infection, but actively constrain the ability of the pathogen to colonize detached fruits. Although the underlying mechanisms were not directly investigated in this study, the observed effects are consistent with previous reports on antifungal monoterpenes, which are known to induce metabolic oxidative stress and structural hyphal and conidial damage.

Regarding practical applications, these VOCs offer a viable path toward reducing the agricultural sector's reliance on copper-based fungicides, which face increasing environmental restrictions. Field application remains challenging under orchard conditions; therefore, future work should focus on controlled-release formulations, improved volatilization strategies, and determining the effective range of action and exposure time of VOCs. Additionally, assessing oxidative stress markers such as SOD and CAT could help explore a possible priming effect in fruit and plant defenses. Developing slow-release formulations for field use could further strengthen integrated pest management in olive groves, supporting both economic viability and environmental sustainability for the olive oil industry.

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