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Evaluation of essential oils from various medicinal and aromatic plants as biofungicides to control *Alternaria alternata* infection in tomato (*Lycopersicon esculentum*)

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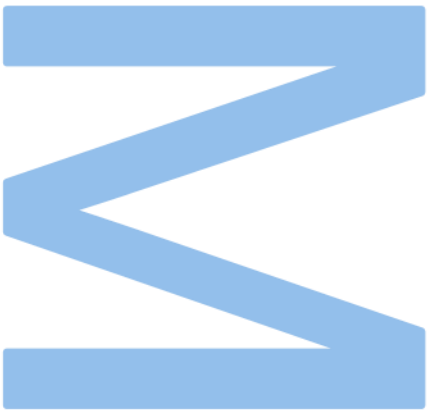

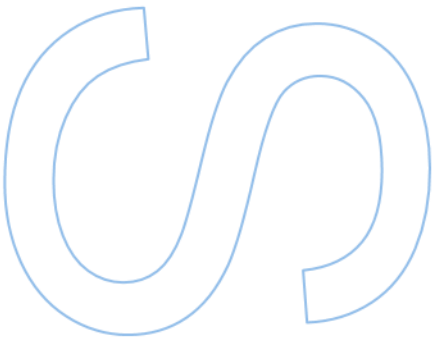

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Sworn Statement

I, Dionísio Meireles Coelho, enrolled in the Master Degree Applications in biotechnology and synthetic biology at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Dionísio Meireles Coelho

11/11/2022

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Resumo

A crescente consciencialização dos malefícios do uso de fungicidas sintéticos tem levado a um aumento da investigação incidente em produtos naturais de origem botânica, nomeadamente, óleos essenciais (EOS). Estes produtos são conhecidos pelas suas propriedades biopesticidas contra pestes e doenças.

No decorrer deste trabalho, um dos objetivos foi determiner se os EOs de *Anethum graveolens, Ammi visnaga, Cympogon flexuosus, Satureja montana* and *Salvia sclarea* possuem atividade biofungicida relevante, especificamente contra o patógeno *Alternaria alternata* (Fr.) Keissl.

Num ensaio *in vitro* preliminar a atividade antifúngica (ensaio de inibição de crescimento do micélio) contra *A. alternata*, o EO de *S. montana* foi selecionado para determinar a concentração mínima inibitória (MIC) e a concentração mínima fungicida (MFC) através de um ensaio de dose-resposta. Por outro lado, os restantes EOs testados não demonstraram atividade antifúngica relevante como inibidores do crescimento do micélio em ensaios *in vitro*. Estes resultados demonstraram que o EO de *S. montana* apresenta atividade antifúngica contra *A. alternata*. O Ensaio *in vitro* para avaliar a inibição de crescimento do micélio revelou que o EO de *S. montana* na concentração de 0.5 mg/mL e superior inibiu completamente o desenvolvimento do fungo ao longo de 12 dias para além de apresentar atividade fungicida. O valor da MIC e MFC após 12 dias foi de 0.5 mg/mL. Adicionalmente, a concentração 0.25 mg/mL inibiu completamente o crescimento do micélio em cultura líquida. Contudo, a atividade antifúngica foi reduzida quando testada como um tratamento preventivo contra apodrecimento em tomates cherry causada por *A. alternata* nas concentrações de 0.5 e 1 mg/mL.

O EO de *S. montana* demonstrou atividade promissora contra *A. alternata*, no entanto, a sua capacidade de protetora contra *A. alternata* deverá ser melhor investigada, assim como o seu efeito contra outras espécies de fungos, de forma a determinar o espectro de ação noutros patógenos fúngicos.

Palavras-chave: Antifúngico; Biofungicida; MIC; MFC; Óleo essencial; *Satureja montana*; *Cymbopogon flexuosus*; *Ammi visnaga*; *Anethum graveolens*; *Salvia sclarea*

Abstract

The increasing awareness of the disadvantages of the use of synthetic fungicides has led to an increase in research in natural products of botanical origin, namely essential oils (EOs). These products are well-known for their biopesticidal properties against various pests and diseases.

In this work we aimed to investigate if the EOs of *Anethum graveolens, Ammi visnaga, Cympogon flexuosus, Satureja montana* and *Salvia sclarea* have relevant biofungicidal properties, specifically against the pathogen *Alternaria alternata* (Fr.) Keissl.

Following a preliminary *in vitro* antifungal assessment (Mycelium growth inhibition assay) against *A. alternata*, *S. montana* EO was selected to determine minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) through a dose-response assay. On the other hand, all the other tested EOs did not show relevant antifungal activity as mycelial growth inhibitors in *in vitro* assays. The results demonstrated that *S. montana* has antifungal activity against *A. alternata*. *In vitro* testing performed to evaluate mycelium growth inhibition showed that *S. montana* EO completely inhibited the fungal development throughout 12 days at 0.5 mg/mL and above (fungistatic), besides displaying fungicidal effects. The MIC and MFC values after 12 days were 0.5 mg/mL for both. In addition, the concentration 0.25 mg/mL completely inhibited mycelial growth in a liquid culture. Nevertheless, the antifungal activity of *S. montana* EO at 0.5 and 1 mg/mL was reduced when tested as a preventive treatment against rotting of cherry tomato fruits caused by *A. alternata*.

S. montana EO showed some promising activity against *A. alternata*, however, its protective effects *in vivo* should be further investigated, as well as its antifungal activity against other fungi to determine its range biopesticidal activity on several fungal pathogens.

Keywords: Antifungal; Biofungicide; MIC; MFC; Essential oil; *Satureja montana*; *Cymbopogon flexuosus*; *Ammi Visnaga*; *Anethum graveolens*; *Salvia sclarea*

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3 List of Acronyms

DMAPP	DIMETHYLALLYL PYROPHOSPHATE
DXP	DEOXYXYLULOSE PHOSPHATE
EO	ESSENTIAL OIL
GC	GAS CHROMATOGRAPHY
GI	GROWTH INHIBITION
HMBPP	HYDROXYMETHYLBUTENYL PYROPHOSPHATE
HMG-COA	HYDROXYMETHYLGLUTARYL-COA
HST	HOST-SPECIFIC TOXINS
IGR	INHIBITION OF GROWTH RATE
IPP	ISOPENTENYL PYROPHOSPHATE
MAP	MEDICINAL AND AROMATIC PLANT
MECPP	METHYLERYTHRITOL CYCLOPHOSPHATE
MCS	METHYLERYTHRITOL CYCLOPHOSPHATE
MEP	METHYLERITHRYTOL PHOSPHATE
MIC	MINIMAL INHIBITORY CONCENTRATION
MFC	MINIMAL FUNGICIDAL CONCENTRATION
MVA	ACETATE/MEVALONATE
NHST	NON-HOST-SPECIFIC TOXINS
PDA	POTATO DEXTROSE AGAR
PDB	POTATO DEXTROSE BROTH

4 Introduction

4.1 The importance of agriculture

Agriculture is at the basis of society. The greater the population, the greater the number of people who must be fed and sustained by agriculture ((Mousavi & Eskandari, 2011)). In the past half-century, the world population more than doubled to just over 7.0 billion in 2012. Over the same period of time, total production of cereal had a faster growth rate than the population. This increase resulted mainly from unprecedented increases in crop yields (Beddow et al., 2014).

4.1.1 Challenges to agriculture

In conventional farming and monocropping systems, even though high yield per area unit has been able to sustain the nutritional needs of a growing population in some areas, these systems require direct and indirect high costs and energy from fossil fuels. In terms of ecology and environment, monocropping has been the cause of a series of serious problems. By excessive use of resources such as water, soil, pastures, forests, and other natural resources there is a serious risk of depleting them. Also, with the production of pollution caused by chemical fertilizers and pesticides there is a serious risk to the ecosystem. If farming activities are conducted based on ecological principles, not only it will prevent the destruction of natural ecosystems, it will also result in stable conditions. Agricultural systems must provide the need of the people, today and future generations, so it is essential to achieve sustainable agriculture (Mousavi & Eskandari, 2011).

Phytopathogens, with their easy dispersibility and adaptability in variable environments, end up overcoming all the active sources of disease management. Monoculturing and intense inputs of agrochemicals work as the selective pressures for pathogens' adaptation and evolution. Therefore, with the dynamic nature of phytopathogens, the outsmart management approach must be in line with environmental acceptability and specific context prevailing in the field and markets (P. P. Singh et al., 2021).

4.1.2 Plant Diseases

The Food and Agriculture Organization of the United Nations (FAO) estimates that crop diseases cost the global economy about $220 billion annually, with 20-40% of crop production lost to pests (Islam et al., 2019; 'Pathogens, Precipitation and Produce Prices', 2021). Plant pathogens and pests being responsible for up to 40% of maize, potato, rice, soybean, and wheat crop yield losses worldwide (Savary et al., 2019).

Post-harvest disease losses can be devastating, especially if farms are far from markets and supply chain infrastructure and practices are poor. Many post-harvest pathogens also produce toxins that cause serious health problems for consumers (Pedreschi & Lurie, 2015).

Postharvest stress physiology and technology has been key to preserve and extend the shelf-life of perishable foods and reducing losses. However, postharvest losses are still significant, and reducing them would be the most effective approach, in contrast to increasing food production. Postharvest strategies or technology implementation for which temperature reduction, modification of the atmosphere or chemical treatment are examples, serve to reduce the organism's respiration rate, retard ripening, decrease ethylene production and consequently retard senescence, prevent dehydration, and extend the shelf-life, preserving products´ quality. Several undesirable postharvest quality traits (like chilling injuries in tomatoes) are associated with or triggered by postharvest abiotic stress and harvested products may respond in a different way in those scenarios than a plant. The strategies mentioned above imply that the produces are submitted to abiotic stresses forcing them to utilize different metabolic pathways to deal with these stresses, avoiding undesirable quality trait that limit the produce's shelf-life (Pedreschi & Lurie, 2015).

Postharvest strategies can be used, among other things, to delay or prevent pathogen development and extend shelf life while preserving quality (Pedreschi & Lurie, 2015). Plants are affected by biotic factors, namely herbivores, plant pathogens, parasitic plants, and green algae. These biotic factors attack plants in search of nutrients and many of them are obligate parasites, which can only live on living plants. Pathogens enter plants through wounds or natural openings, utilizing enzymes that destroy cellular structures, or can be introduced by vectors such as insects. Pathogens also secrete other substances, including toxins and growth regulators that cause plant galling and overgrowth. Plants defend against pathogens through physical defences and by activating a range of chemical defences that attack pathogens to limit their progress or kill them (Islam et al., 2019; 'Pathogens, Precipitation and Produce Prices', 2021).

4.2 Fungal pathogens affecting crops

The importance of fungal infections of cultivated crops is rising due to the expansion of agricultural output to meet global food demand. Fungal pathogens are estimated to cause hundreds of billions of dollars in losses worldwide each year. Historically, humans have suffered the direct and indirect consequences of a fungal infestation.

Phytopathogenic fungi consist of a highly diverse group and a threat to food supply and security. Over nineteen thousand fungi are known to cause diseases in crop plants worldwide (Giraud et al., 2010; Jones & Aldwinckle, 1990; Waard et al., 1993). A survey proposed by (Dean et al., 2012). based on fungal pathologists' perception of the most scientifically/economical relevant fungal pathogens has ranked in a 'Top 10' the following species in their order of importance: (1) *Magnaporthe oryzae*; (2) *Botrytis cinerea*; (3) *Puccinia* spp.; (4) *Fusarium graminearum*; (5) *Fusarium oxysporum*; (6) *Blumeria graminis*; (7) *Mycosphaerella graminicola*; (8) *Colletotrichum* spp.; (9) *Ustilago maydis*; (10) *Melampsora lini*.

Fungi are a significant type of burden to plant production because under the right climatic conditions, fungal pathogens can grow exponentially and devastate crops. They may stay dormant but alive on living and dead tissue until conditions for their proliferation are met. Fungal spores are readily dispersed by wind, water, soil or insects, and other invertebrates. In this way, they may infect an entire crop (Jain et al., 2019). For example, the ubiquitous fungus called *Botrytis cinerea* can infect more than 1,400 plant species, including grapes, raspberries, blackberries, beans, lettuce, and flowers such as roses, gerberas, daisies, and orchids (Peter, 2006). This fungus can devastate more than 80% of strawberry flowers and fruits (Petrasch et al., 2019), and up to 70% of a tomato plants production, causing premature plant death (Peter, 2006). To protect crops from diseases, conventional and organic farming use adjusted agronomic practices to reduce the incidence of damaging diseases, for example, choosing a cultivar resistant or tolerant to diseases, crop rotation to minimize carryover of pathogens, among others. Resistance or tolerance to crop diseases, through natural traits or genetically modified organisms (GMO), is an area under intensive scientific study (Leadbeater, 2014).

4.3 The tomato plant (*Lycopersicon esculentum*)

4.3.1 Species taxonomy, origin, and morphological traits

The tomato plant, *Lycopersicon esculentum* Mill., from the *Solanaceae* (potato family) (Figure 1), is one of the most abundant crops at a global level. It has been consumed by the inhabitants of Central and South America since prehistoric times and it is considered native to the Peruvian and Mexican regions (Van Eck et al., 2006).

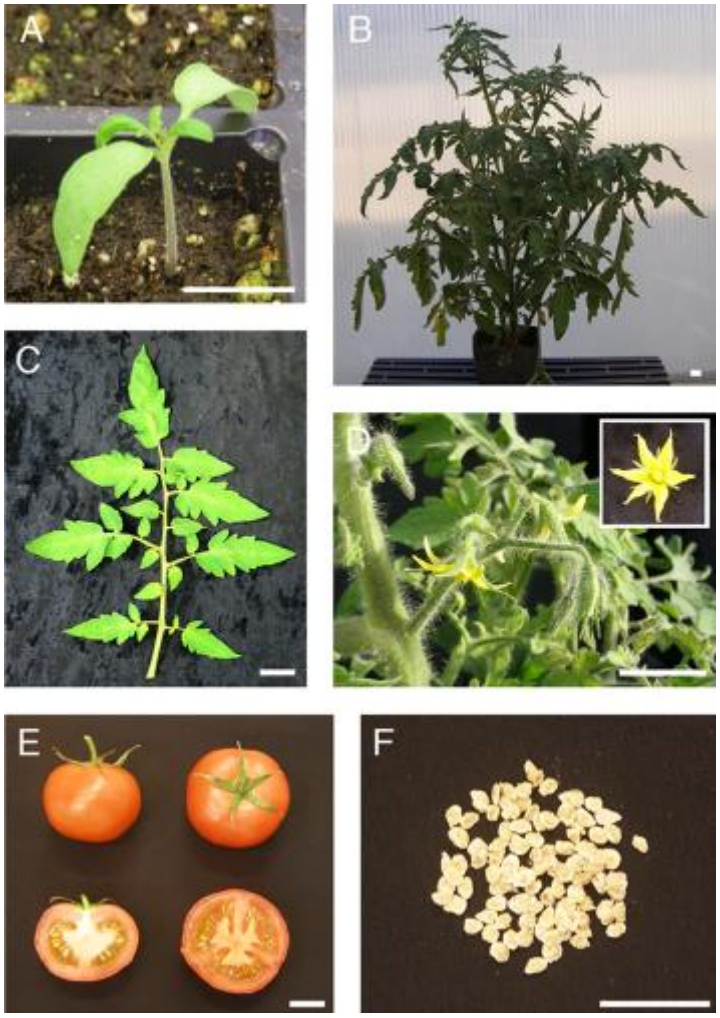

Figure 1: Tomato plants and parts: (A) seedling; (B) 40-d-old plant; (C) leaf; (D) flowers; (E) fruit; (F) seeds. Bar, 2 cm (Kimura & Sinha, 2008).

There are over 4000 tomato varieties differing in disease resistance, fruit characteristics, growth habitats, maturity date, and plant size (Heuvelink, 2018). The fruit is normally round, lobed, or pear-shaped and the diameter goes from 1 to 12 cm (Padmanabhan et al., 2016) (Figure 2).

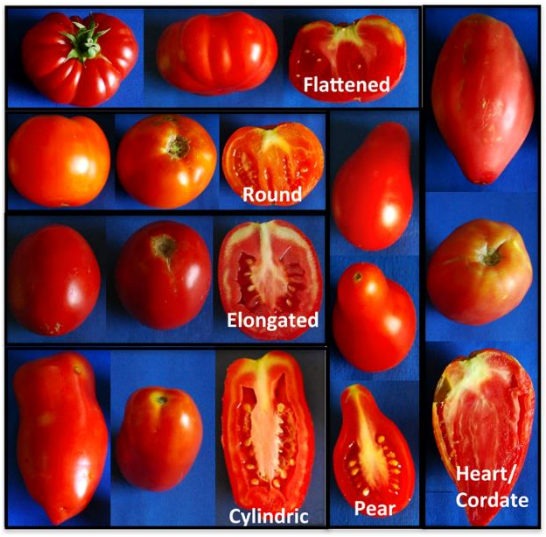

Figure 2: Diversity in tomato fruit shapes. Tomato fruit shape categories adapted from the IPGRI/Biodiversity protocol (1996) (Sacco et al., 2015).

New cultivars with differently shaped fruits have been developed to suit the mechanical harvesting and handling systems (Heuvelink, 2018). In addition, breeding for resistance/tolerance to abiotic and biotic factors has played an important role in improving crop sustainability and product toxicity profiles (Irikura, 1976).

4.3.2 Crop´s importance for the economy

Tomato is the most produced fruit worldwide (Panthee & Chen, 2010) , mostly as a processed finished product or as a tomato paste. Tomatoes serve as the main ingredient of salads and are the most important vegetable processed to make sauce, ketchup, purée, and incorporations in other processed foods, etc (Collins et al., 2022). It is the second most economically important vegetable in the world after potato, grown on an area of 5.0 Mha and generating an export value of $14.1 billion (both raw and processed) (Distefano et al., 2022). Tomato crops are mainly grown in Asia (2.6 Mha), followed by Africa (1.6 Mha), Europe (0.4 Mha), America (0.4 Mha) and Oceania (0.01 Mha) (Mauro et al., 2015; Wang et al., 2022). The wide distribution of the species is due to its adaptability to different growing conditions and the versatility of the product, which can be used for both fresh consumption and industrial processing (Waheed et al., 2020).

Tomatoes require warm conditions and are sensitive to frost, the optimum soil temperature for germination is 20 °C and the rate of germination bellow 16 °C is very low. Air temperatures between 25 and 35 °C are ideal for the growth of fruits and development is optimal with appropriate humidity. The maximum tolerable temperature for the plant can tolerate is 38 °C. When temperature goes bellow 20 or above 38 °C

then fruit development is compromised and subsequently, quality is severely reduced. Tomato plants also require 6 to 8 direct or bright sunlight hours per day, pH between 5.5 and 6.8 and requires water when the soil dries up to 3.05 cm in depth (Waheed et al., 2020). Also noteworthy, the current climate change situation can significantly influence the yield and costs of this water-demanding plants (Eyshi Rezaei & Webber, 2022).

Tomato fleshy berries are rich in minerals (mainly K, P, Mg), vitamins (ascorbic acid and niacin), carotenoids (lycopene, β-carotene, lutein) and polyphenols (chlorogenic acid, quercetin, naringenin), which makes it an adequate source for these nutrients (Chaudhary et al., 2018; Wang & Seymour, 2017).

4.3.3 Challenges in the production and post-harvest preservation of tomato

Shelf life is the length of time that a product can be stored from the time it is manufactured to the time it is consumed without losing its good quality and safety. It represents one of the most important quality characteristics of the pulp and can be influenced by various factors such as inadequate temperature and humidity, or pathogens that can promote overripening. Rapid overripening is a relevant challenge for the tomato industry as it leads to shortened shelf life (Conesa et al., 2014; Zhang et al., 2015).

Fruits and vegetables (and other harvested products) are separated from the mother plant and thus deprived of main nutrients and water. After being harvested they must use their carbon resources for respiration, water is lost from transpiration and are exposed to various abiotic stresses, after being utilized, these resources cannot be replenished (Pedreschi, 2017). The postharvest tomato fruit quality of long shelf-life Mediterranean landraces is substantially influenced by irrigation regimes (Wani et al., 2011).

4.3.3.1 Fungal pathogens and molds affecting tomato

The tomato plant and fruit are attacked by variety of pathogens, being the predominant type of fungal fruit rots (Wani et al., 2011). These fungal rots are responsible for causing serious production problems and become menace for successful cultivation of tomatoes. Fungal rots are world-wide in occurrence and have been reported almost in all parts of the world (Pandey et al., 2022). The principal fungal fruit rots reported all over the world with varying intensities on tomato includes *Alternaria* rot caused by *A. solani* and *A. tenuis*, *Anthracnose* ripe rot caused by *Colletotrichum phomoides*, *Phoma* rot (*Phoma destructive*) and *Fusarium* rot caused by *Fusarium* spp. (Pandey et al., 2022; Shakya & Aryal, 2020).

Tomato leaf mold is a common disease in tomato production. This disease usually manifests in high-temperature and humid environments, conditions that stimulate the

spread of the disease and rapid growth of the pathogen. Leaves are the main infected organ. In general, irregular chlorotic spots appear on the adaxial surface of lower tomato leaves, while a mold layer appears on the abaxial surface of the leaves. Tomato leaf mold is caused by the pathogen *Cladosporium fulvum*. A natural antagonist of the *Cf* gene in tomato mediates vertical resistance to tomato leaf mold caused by *C. fulvum*. Plant breaders introduced *Cf* resistance genes from wild cultivars into cultivated tomatoes to control this disease. The interaction between the *Cf* gene and the pathogens avirulence gene (*Avr*), which activates disease resistance signal transduction, is responsible for the tomato resistance to leaf mold (Zhao et al., 2022).

4.3.3.2 Strategies used to mitigate postharvest deterioration

One of the most commonly used approaches to extend the shelf life of tomatoes is to harvest the fruits at the ripe green stage, then store them at low temperatures and finally expose them to ethylene to initiate ripening (Kiferle et al., 2015; Zhang et al., 2015). Mature-green fruit can be stored for 2 weeks at 12.5–15°C, ripening fruit at 10–12.5°C for a week, and fully ripe fruit at 7–10°C for 3–5 days (McDonald et al., 1999). Mature-green fruit ripens best at 15–20°C, with ripening above 25°C producing soft, poorly colored fruit (Page et al., 2010; Tsaniklidis et al., 2014).

The atmosphere itself can often be controlled and modified to prevent deterioration. Reduced oxygen levels decrease the respiration rate, the production of and sensitivity to ethylene, and ripening (Kaynas & Çiftci, 2020) while high levels of carbon dioxide may cause discoloration, softening and uneven coloration. Injury occurs at oxygen levels below 2% and carbon dioxide concentrations above 5% (Cantwell & Saltveit, 2015).

Transgenic approaches, such as those exploiting mutations in genes involved in ripening, like *rin* (ripening inhibitor), *nor* (non-ripening), *Cnr* (Colorless non-ripening), *Nr* (Never-ripe), *Gr* (Green-ripe) and *alc* (alcobaca), are also utilized to slow-down ripening and thus extend shelf life (Ding et al., 2022; S. Li et al., 2022; Zhang et al., 2015). Genetic engineering approaches, such as transgenesis, have efficiently helped overcome these limitations by introducing only the genes/alleles of interest into an elite plant. This type of tool offers numerous important opportunities for improvement of existing elite cultivars and development of new cultivars. A major practical advantage of genetic engineering is that it allows breeders to rapidly develop new cultivars by introducing cloned genes into commercial cultivars without altering their existing genetic constitutions. With the advent of powerful genome engineering tools such as CRISPR-Cas9, the high-precision molecular breeding of tomato has become possible ((Vu et al., 2020)). Those have been

applied both to the tomato plant (R. Li et al., 2019; Ueta et al., 2017; L. Wang et al., 2017; T. Wang et al., 2019) and to the *Alternaria* fungus (Wenderoth et al., 2017).

Edible coating is an innovative method of food preservation, producing a physical barrier on the surface of fruits and vegetables that causes moisture and solute migration, gas exchanges and respiration and oxidative reaction rates reduction, extending the shelf-life and reducing the risk of pathogen growth on food surfaces. Chitosan, a partially deacetylated derivate of chitin that has antibacterial and antifungal properties, is known to inhibite a wide variety of bacteria and fungi. Chitosan has a variety of applications and can be utilized for developing various formulations. Chitosan-based edible coating can also be utilized as carriers of food ingredients to improve safety, quality and functionality of fruits and vegetables (Duan et al., 2019).

4.4 *Alternaria* sp.

4.4.1 Species taxonomy and mycotoxins production

The genus *Alternaria* (Phylum: *Ascomycota*; Order: *Pleosporales*; Family: *Pleosporaceae*) is ubiquitous in nature (Meena & Samal, 2019) and comprise widespread saprophytic species, which can grow on different type of substrates (food, textile, cereals, goods, paper, etc.), but also facultative phytopathogens that can be found in a variety of cultures, crops and manufactured products. The easily dispersible spores can attach themselves to leaves, stems, flowers, fruits, tubercules and roots on a field or in storage and the symptoms produced are as diversified as the organisms affected. Alternaria species produce spots or blights in leaves, spots and cankers in stems and twigs, damping off in seedlings and rots in fruits, tuber, roots and seeds(Lopez & Cabral, 1999). Therefore, *Alternaria* species are important fungi to study due to their saprophytic versus endophytic lifestyle (Akhtar et al., 2004).

These phytopathogens that causes early blight disease in many other crops. *Alternaria* rot has been considered as the most common disease of tomato fruits and causes heavy losses in quality of the fruits, thus rendering large quantity of tomato fruits unfit for consumption. As an example of common related diseases affecting the tomato plant, early blight consists of light to dark brown circular lesions that develop characteristic alternating light and dark concentric rings as the lesion expands over time. Yellow chlorotic tissue may surround the lesions and the entire leaf may turn yellow as lesions expand and coalesce. Infection under favourable conditions was found to cause severe defoliation, with considerable yield loss when it occurred prior to flowering (Akhtar et al., 2004). Traditionally this disease was attributed primarily to the fungus *A. solani*, but more

recent work has identified additional *Alternaria* spp. capable of causing early blight symptoms on tomato, including *A. tomatophila* (Rodrigues et al., 2010; Weir et al., 1998) and *A. grandis* (Rodrigues et al., 2010).

Many *Alternaria* species have been described based on morphological and/or host differences. However, the identification of *Alternaria* species may be confusing since environmental factors can cause considerable variations in field and laboratory cultures. This genus is characterized by dark colonies, grey to blackish-brown or black in color. Conidia are usually dictyosporic, sometimes with simple or branching, smooth or verrucose wall, arising on conidiophores which are usually geniculate and scared after detachment. Blast ontogeny is responsible for the formation of conidia, as outgrowths of protoplasms through a specific apical pore in the conidiogenous cell, that can be oval to subclavate, making the distal portion narrower (Lopez & Cabral, 1999).

One factor for the success of *Alternaria* species might be the production of a diverse range of phytotoxins, which includes various host-specific toxins (HSTs) and non-host-specific toxins (nHSTs) that are dependent on physiological and morphological conditions. Pathogenicity of *Alternaria* species depends on host susceptibility or resistance and quantitative production of HSTs and nHSTs. The production of HSTs is essential for a successful infection since mutants without HSTs are incapable of infecting their designated hosts (Chung, 2012). Chemically, these toxins are low molecular weight secondary metabolites. Toxin effects mainly affect different parts of the cell such as mitochondria, chloroplasts, plasma membrane, Golgi complex and nucleus. *Alternaria* species produce several nHSTs such as tenuazonic acid, tentotoxin and ginniol. Acting at very low concentrations, HSTs affect only certain plant cultivars or genotypes and play a role in determining the host range of plant pathogen specificity. The well-known HSTs are the AAL, AK, AM, AF, ACR and ACT toxins, named for their host specificity, and they belong to different family groups. All these toxins have different modes of action, biochemical reactions, and disease-causing signaling mechanisms. Different species of *Alternaria* produce toxins that reveal biochemical and genetic effects on themselves as well as on host cell tissues (Akhtar et al., 2004).

4.4.2 *Alternaria alternata* role in post-harvest deterioration and human health issues

Alternaria alternata is mainly an outdoor fungus whose spores disseminate in warm, dry air. Thus, in temperate climates, their count peaks in the summers (Kustrzeba-Wójcicka et al., 2014). *Alternaria alternata* overwinters as mycelium in dead leaves on the ground,

in dormant buds and in mechanically injured twigs (Jones & Aldwinckle, 1990). Conidia are formed on lesions (Figure 3), in swollen and normal-looking lenticels, with latent infections on one-year-old shoots. The infection can then spread quickly under a combination of high humidity and high temperature (Kwon et al., 2011).

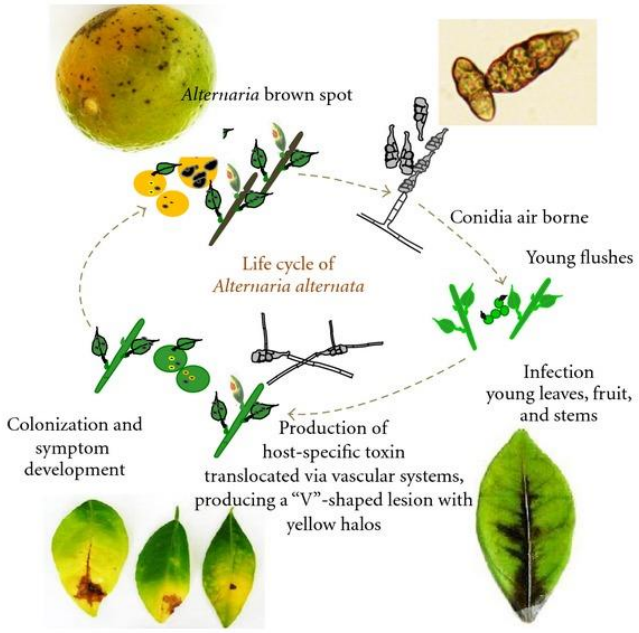

Figure 3: Life cycle of *Alternaria alternata*, the causal agent of citrus brown spot. ACT toxin produced by the tangerine pathotype of *A. alternata* is transported via the vascular system and formation of necrotic lesions on a detached calamondin leaf (bottom right) (Chung, 2012).

Alternaria alternata usually causes black spots but acts as a latent fungus which develops only during the cold storage of fruits, becoming visible during the marketing period thereby causing large postharvest losses. The fungus enters the fruit via wounds or natural openings, and remains quiescent until the fruit ripens, and the conditions are more favourable for disease development. This characteristic is of paramount importance because the fungus can sporulate and resist the postharvest treatments aimed at eliminating the microorganisms present in the produce and start infecting the fruit while on its way to the international markets. This species is most likely spread all over the world and able to proliferate in many different environments. Reports from many distant countries identify it as responsible for different symptoms and postharvest decay during the shelf-life of many different horticultural products (Bashar et al., 2012; Fatima et al., 2009; Kwon et al., 2011).

In addition to postharvest decay, *A. alternata* it is implicated in many negative effects on human health. It has been related to food poisoning due to the production of mycotoxins like include alternariol, altenuene, alternariol monomethyl ether, altertoxins and L-tenuazonic acid (Scott, 2001). These mycotoxins have various detrimental effects on human and animal health and are also involved in the occurrence of mutations, chromosomal aberrations, and DNA damage (Escrivá et al., 2017; Scott & Stoltz, 1980). For example, this fungus might play an important role in the etiology of human esophageal cancer (Liu et al., 1992).

Some of these toxins are dangerous and, indeed, alternariol and alternariol methyl ester can increase the rate of breaks in the DNA of human carcinoma cells by inhibiting DNA relaxation and stimulating the cleavage of DNA by topoisomerases I, IIα and IIβ (Fehr et al., 2009). It can also cause fungal keratitis in humans (Xu et al., 2013). Exposure to *A. alternata* have been associated to active asthma symptoms (Pulimood et al., 2007; Salo et al., 2006). To date, 16 allergens of *A. alternata* have been isolated (Kustrzeba-Wójcicka et al., 2014).

4.4.3 Host-pathogen interaction and induced plant defence response

This fungus has been reported to infect other parts of plants like seeds (Ska et al., 2012), leaves (Harteveld et al., 2013), stems (Choi et al., 2010) and flowers (Espinoza-Verduzco et al., 2012) reducing the agricultural production either directly by infecting the fruit or indirectly by impairing the physiology of plant photosynthesis (Lagopodi & Thanassoulopoulos, 1998). It can further induce changes in the host. For example, *Withania somnifera*, a high value medicinal plant, shows changes in leaf proteome profile after *Alternaria alternata* infection (V. Singh et al., 2017).

GABA (Gamma-Aminobutyric acid) can also induce resistance against *A. alternata*, by activating antioxidant enzymes, restricting the levels of cell death caused by reactive oxygen species, playing a vital role in the resistance mechanism underpinning GABA-induced plant immunity (Yang et al., 2017). The fungus itself, in response to stimulus from the plant host, can adapt from metabolic pathways down to the genomic level (Aradhya et al., 2001).

4.4.4 Strategies used for the management of pre- and postharvest damage caused by *A. alternata*

There is still no totally reliable protocol to identify and control postharvest diseases caused by *A. alternata*. Strategies usually include biological control, heat treatment, natural compounds (chitosan, isothiocyanates, EOs, elicitors of natural defence mechanism), etc. In addition, varietal resistance that protects the stems, and to a lesser extent the leaves, is present in some hybrid that has been transferred to fresh market (Ahmad et al., 2013; Alizadeh-Moghaddam et al., 2020).

Prompt storage and cooling of the fruit are essential to stop the development of the rot. However, these measures are not enough to control this disease. The application of fungicides before and after harvest has proven to be very effective in controlling the growth and development of *A. alternata* (Troncoso-Rojas & Tiznado-Hernández, 2014).

4.5 Fungicides

4.5.1 The importance of fungicides to agriculture

In a scenario of population growth, the control of fungal plant diseases becomes even more necessary to meet the demand for food. Growing plants and the resulting fruits and vegetables are highly susceptible to fungal spoilage, both in the field and during postharvest storage. The control of diseases with chemical fungicides are integral to the production of crops in many countries, resulting in increased yields and farmer revenue. Studies show undoubtedly that without fungicides to control plant pathogens, effective production of certain crops would be impossible in parts of the world (Ellis et al., 1998; Jørgensen et al., 2017; Wellman, 1977; Wiik & Rosenqvist, 2010).

Modern fungicides can be applied to crops using a range of technologies depending on the characteristics of the chemical and the most effective way to target the disease. Most fungicides used nowadays are applied using conventional spraying techniques to the foliage of plants (Toporek & Keinath, 2022; Z. Wang et al., 2021). These techniques have the advantage of being practical and convenient as well as being effective at targeting predominant foliar diseases with a range of fungicides with contact or systemic activity (Brandhorst & Klein, 2019).

4.5.2 Most common mechanisms of action

Fungicides are commonly classified based on their mode of action and chemical group but can also be classified by the moment in the disease cycle they are active: a) Protectant or preventive, if they are effective prior to infection or in the initial stages of

the disease cycle; b) Curative, effective against fungus growing on the leaf tissue after spore germination; c) Eradicant or antisporulant, fungicides that stop sporulation by the pathogen (Brandhorst & Klein, 2019; Kataoka et al., 2010).

Fungicides kill the fungi by damaging the cellular membrane, cleaving enzyme proteins like mucins which bind to membranes, or by interfering with key processes such as energy production or respiration, effectively reducing their survival and fertility. Fungicides such as asterones, clobazam, and betulin are used in fungicides for use against pathogenic fungi. Usually, all fungicides within a chemical group share a common mode of action and resistance mechanism regardless of differences in the precise chemical structure (Correia, 2016).

Synthetic organic fungicides can belong to many different classes which makes it hard to generalize their environmental fate. In soils, their behaviour is heavily influenced by the soil properties. Some fungicides are present in surface waters at higher levels than those allowed by the European union regulatory limits due to extensive use, which poses a potential environmental and human health risk. The fate of a fungicide in the soil are influenced by chemical and biological degradation, adsorption, diffusion, absorption by plants, runoff, erosion by the wind, volatilization, leeching and assimilation by microorganisms. Besides, roughly 25% of agrochemicals in use are chiral which, besides having the same physical and chemical properties, means stereoisomers will generally behave differently leading to different activities, toxicity, and degradation patterns (Correia, 2016).

As an example, difenoconazole has a mechanism of action involving the sterol synthesis. This compound inhibits the C-14 demethylation of lanosterol or 24-methylenedihydrolanosterol, this step occurs while converting lanosterol to ergosterol and subsequently, inhibiting the production of sterol in fungi (H. Wang et al., 2016).

4.5.3 Disadvantages of synthetic fungicides

Chemical control remains the main measure to reduce the incidence of postharvest diseases in various fruits and vegetables. However, due to the development of new physiological races of pathogens, many of these synthetic chemicals are gradually becoming ineffective (Guizzardi et al., 1995). Furthermore, the use of synthetic chemicals to control postharvest deterioration of food commodities is restricted, due to their possible carcinogenicity, teratogenicity, high and acute toxicity, long degradation periods, environmental pollution, and side effects on human beings (Panis et al., 2022).

Fungicides are the most effective means to minimize the fungal decay of fruits. Synthetic pesticides are factually a key component of crop protection programs, while the production becomes more intensive. The increasing use of pesticides has led to the emergence of pest resistance, pest resurgences, outbreaks of new pests, toxicity to non-target organism, and hazardous environmental impacts, jeopardizing the sustainability of ecosystems (Alagarmalai & Jesudasan, 2005). Also, there is the issue of pesticide resistance, which is recognized as a very serious threat to crop protection and vector control and is recognized by many stakeholders including industry, WHO, regulators and the public as a problem requiring a proactive approach (Dubey, 2011; Jones et al., 2022; Nauen, 2007; Nolden et al., 2021). Several countries have implemented a protective plan that can reduce around 50% of used pesticides (Macfadyen et al., 2014).

Although pesticides are beneficial from a crop production perspective, widespread use of pesticides can have serious consequences due to their biological amplification and persistence. They pollute air, water, soil, and entire ecosystems, posing serious health risks to living organisms. The extensive use of synthetic fungicides over the years has led to the increase of antimicrobial resistance, and environmental and animal health damage (Curl et al., 2020; Fantke et al., 2012; Z. Li, 2018). Therefore, it is increasingly urgent to explore new environmentally friendly fungicides that are low in toxicity and effective (Sharma et al., 2019).

4.6 Biopesticides

4.6.1 Definition

Among the existing alternative strategies for reducing pest populations, the use of plant-based products is currently one of the most promising since plants themselves have evolved a wide range of chemical and physical defenses against various invasive organisms (Adhikari et al., 2022; Alilou & Akssira, 2021; Ramzi et al., 2022; Ryan & Byrne, 1988). Allelochemicals, compounds derived from plants are more frequently explored for use in alternative or complementary approaches to synthetic insecticide treatments (Dubey, 2011).

4.6.2 Plant-based biofungicides: characteristics and properties

Plants contain a variety of volatile and non-volatile secondary metabolites displaying inhibitory activity against microorganisms, including fungal pathogens. Plants' own defence molecules, for instance phytoalexins, could provide a strong line of defence against fungi (Kazmi et al., 1995).

Garlic (*Allium sativum*) EO and neem (*Azadirachta indica*) oil were reported as effective bio-fungicides against different rot fungi (Kazmi et al., 1995; Ngadze et al., 2012). Besides, several EOs demonstrated antifungal activity against *Rhizoctonia solani*, for example, the EO of cinnamon (*Cinnamomum verum*), *Thymus vulgaris*, *Salvia fruticosa*, among others. EOs like those from *Trachyspermum ammi* L., *Nepata clarkei* and the oil from neem were active against *Macrophomina phaseolina* (Khaledi et al., 2015).

On the other hand, some plant volatile compounds have documented antifungal activity. Carvacrol is a phenol monoterpene, which has been reported to inhibit fungal growth namely yeast, hyphae, and biofilm in *C. albicans*. Supposed mechanisms of action include the increase of membrane fluidity and the disruption of its integrity and ergosterol biosynthesis, leakage of protons and potassium ions which results in the loss of membrane potential and inhibition of adenosine triphosphate (ATP) synthesis, inducing stress in the endoplasmatic reticulum, unfolded protein response and perturbing the calcium ion homeostasis (Niu et al., 2020; Šimović et al., 2014).

p-Cymene (1-methyl-4-(1-methylethyl)-benzene), is another monoterpene with antifungal activity. It has been suggested that this compound blocks the biosynthesis of melanins by the fungus. There is evidence that the inhibition of melanin biosynthesis in pathogenic fungi can decrease their pathogenicity and increase sensitivity to biotic and abiotic stress. Treatment with *p*-cymene significantly decrease the ergosterol content, the downregulation of *erg28* expression induced by the treatment suggests an inhibition of ergosterol biosynthesis at the level of gene expression (F. Tian et al., 2018).

4.7 Essential oils

4.7.1 Definition

Commonly referred to as volatile oils, EOs are intricate blends of volatile chemicals produced by plants. These mostly consist of low-molecular-weight molecules like terpenes, terpenoids, aromatic and aliphatic chemicals (Bassolé & Juliani, 2012). EOs are obtained from plant material through steam distillation. These can be defined as products or mixtures of fragrant substances or as a mixture of fragrant and odorless substances (Cook & Lanaras, 2016).

Historically, the main method for isolating EOs was hydrodistillation, which consists in boiling the biological material in water. Vapours are then condensed by a cooling system to separate the EO from water. A variation of this method consists in the passage of steam through the plan material. During this process, volatile compounds are vaporized

and then condensed on cooling to produce an immiscible organic phase that is separated (Cook & Lanaras, 2016).

Cold pressing (expression) is a mechanical method almost exclusive to the production of citrus fruits EOs. Pressure is utilized to crush the pericarp by rolling the fruit against sharp objects, puncturing the EO glands and releasing the EO that is then washed away with water. The EO and water are separated by centrifugation, the whole process is caried out at low temperatures to avoid thermal degradation of the components (Cook & Lanaras, 2016).

4.7.2 EOs composition

Eos are mainly composed by mono- and sesquiterpene hydrocarbons and their oxygenated (hydroxyl and carbonyl) derivatives, along with aliphatic aldehydes, alcohols, and esters. Terpenes can be considered as the most structurally varied type of plant natural products, derived from the repetitive fusion of branched five-carbon units (isoprene units) (de Groot & Schmidt, 2016; Dosoky & Setzer, 2018; Teixeira et al., 2013; Yang et al., 2010; Zaouali et al., 2010).The components of an EO can mainly be classified into two groups: terpene hydrocarbons and their oxygenated derivatives, (acids, aliphatic aldehydes, ketones, alcohols, and esters) (de Souza et al., 2019; Rubiolo et al., 2010).

In this regard, analytical methods for characterizing EOs, most commonly Gas chromatography (GC) or GC coupled with mass spectroscopy (GC-MS) (Sadgrove et al., 2022), must consider a large number of molecular species. It is also very important to emphasize the importance of the chosen extraction method as the chemical profile of an EO is closely related to the extraction process used (Reyes-Jurado et al., 2015; Stratakos & Koidis, 2016).

Moreover, the same plant species from which the EO is derived is grown under a variety of conditions around the world, varying in geographic location, weather conditions, the type of soil, the age of the plants at the time of harvest and even the time of the day or year, resulting in differences in the chemical profile (Chamorro et al., 2012; Dhouioui et al., 2016; Nurzynska-Wierdak, 2013). All these factors add up to create wild strains with different chemical compositions (chemotypes) (Tisserand & Young, 2014).

4.7.3 EOs and their constituents as potential biofungicides for plant protection

The use of EOs or their constituents complements the holistic concept of nature because of their volatility, limited persistence in field conditions, and some of them being exempt from regulatory protocols. EOs and their components are gaining increasing interest

because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (el-mougy & Abdel-Kader, 2017; Matsumoto et al., 2016). The fact that EOs are generally non-toxic mammals makes them particularly appealing for use as fungicides or insecticides in general (Isman, 2000; Isman & Machial, 2006).

The application of EOs is a very attractive method for controlling postharvest diseases. The production of volatile compounds in plants is believed to be predominantly a defense mechanism against pathogens and pests (Gurav et al., 2022; Oxenham et al., 2005) and indeed the volatile fraction extracted from several MAPs have been shown to possess antimicrobial and antifungicidal properties (Cakir et al., 2005; Voda et al., 2003). For decades, they have been investigated for their insecticidal (Douda et al., 2010; Regnault-Roger, 1997), antimicrobial (Deba et al., 2008), antiviral (Bishop, 1995; Koul, 2008; Rao et al., 2010), antifungal (Bouchra et al., 2003; Daferera et al., 2003; Sokmen et al., 2004; Vilela et al., 2009) activities. It is rather important to explore and find the most effective EO that would be promising for the development of new botanical fungicides against a spectrum of fungal pathogens causing the most serious diseases, also considering their practical prices and availability of the MAPs used for producing those EOs (Shukla et al., 2017).

For all these reasons, EOs are one of the most promising products of natural origin, which could be used for the development of safer but effective antifungal agents (Shukla et al., 2017).

4.7.4 Relevant metabolic pathways

The most common chemical classes of constituents found in EOs are monoterpenoids, sesquiterpenoids, and phenylpropanoids, which are derived from the methyl-erythritol pathway (MEP), mevalonate pathway (MVA), and shikimic acid pathway (SA), respectively (Banerjee & Sharkey, 2014; Buhaescu & Izzedine, 2007; Shukla et al., 2017).

EOs can interfere with the membrane potential across fungi cell wall and disrupt ATP assembly, leading to cell wall damage, and they can also disintegrate mitochondrial membrane interfering with the electron transport system pathway (Tariq et al., 2019).

Terpenoids (or isoprenoids) are the biggest group of secondary metabolites, they play a role in various biological processes like defense mechanisms against biotic and abiotic stress agents, pollinator and seed disperser attraction and intracellular signal transduction. Isoprenoids are derived from two isomeric five-carbon units denominated

isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These two compounds are synthesized by two pathways, the acetate/mevalonate (MVA) pathway or the 2-C-methyl-D-erythritol (MEP) pathway (Banerjee & Sharkey, 2014).The MVA pathway starts with the synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) from acetyl-CoA and acetoacetyl-CoA. HMG-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonic acid, being the rate-limiting enzyme of the MVA pathway. Then, mevalonic acid is phosphorylated to mevalonate-5-phosphate by mevalonate kinase (MK), followed by phosphorylation of this compound by phosphomevalonate kinase (PMK) to produce mevalonate-5-pyrophosphate. In the last step, mevalonate-5-pyrophosphate is decarboxylated to form IPP by mevalonate-PP decarboxylase (ATP dependent). IPP may interact with isopentenyl pyrophosphate isomerase (IPI) to form DMAPP (Buhaescu & Izzedine, 2007).

Formerly known as the non-mevalonate pathway, the MEP pathway starts with the synthesis of 1-deoxy-D-xylulose-5-phosphate (DXP) from D-glyceraldehyde-3-phosphate and pyruvate by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS). DXP is then converted to MEP by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). Then the cyclic intermediate Methylerythritol-2,4-cyclopyrophosphate (MEcPP) is synthetized from MEP by performing three consecutive enzymatic steps, CTP-dependant cytidylation, ATP-dependent phosphorylation and cyclization. These reactions were performed by 4-diphosphacytidyl-2-C-methylerythritol synthase (CMS), 4-(cytidine-5'-pyrophospho)-2-C-methyl-D-erythritol kinase (CMK) and 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate synthase (MCS) respectively. Next, MEcPP is converted into hydroxymethylbutenyl pyrophosphate (HMBPP) by HMBPP synthase (HDS). Finally, HMBPP is reduced to IPP and DMAPP in a step catalyzed by HMBPP reductase (HDR). IPP and DMAPP can also be isomerized by IPI (Figure 4) (Banerjee & Sharkey, 2014; Rao et al., 2010).

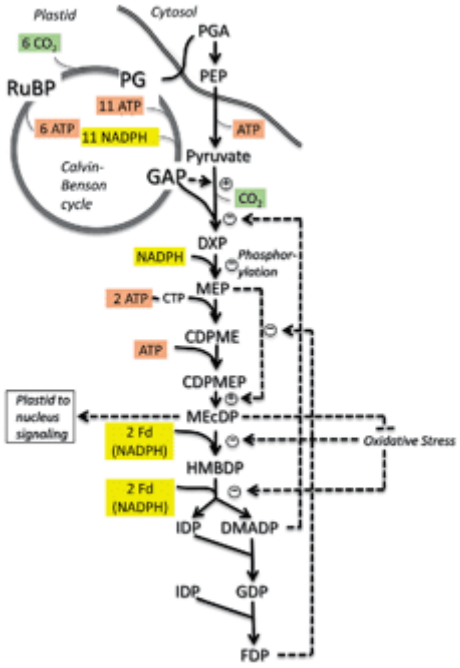

Figure 4: Methylerythritol phosphate (MEP) pathway and related metabolism showing the major metabolic regulatory points discussed. Abbreviations: GDP ¼ geranyl diphosphate, FDP ¼ farnesyl diphosphate, Fd ¼ ferredoxin (Banerjee & Sharkey, 2014).

4.8 EOs from Medicinal and Aromatic Plants (MAPs) showing antifungal activity

As an example, EOs and individual monoterpenoids from lavender (*Lavandula stoechas*), oregano (*Origanum vulgare ssp.*), sage (*Salvia fruticosa*) and spearmint (*Mentha spicata*) were found to prevent mycelial growth and conidial production of *Aspergillus terreus*, *F. oxysporum*, *Penicillium expansum* and *V. dahliae* (Duffy & Défago, 1999; Kadoglidou et al., 2011). Especifically against *A. alternata*, several EOs also inhibit mycotoxins synthesis (

EOs	Effect	Mechanism	Reference
Citral (from citrus fruit's peel EO)	MIC of 0.22 mg/mL; MFC of 0.89 mg/mL. Inhibition of spore germination and AOH and AME production.	Downregulation of gene expression related with the metabolism of carbohydrates, amino acids, sulfate, glutathione and xenobiotics, which leads to the disturbance of cell integrity.	(Mutlu-Ingok et al., 2020; L. Wang et al., 2019)
Brassica juncea	MIC and MFC of 0.25 mg/mL	-	(Lu et al., 2019)

Litsea cubeba	MIC of 0.5 mg/mL and MFC of 1 mg/mL	-	(Lu et al., 2019)
Ocimum basilicum	MIC of 0.5 mg/mL and MFC of 2 mg/mL	-	(Lu et al., 2019)
Mentha x piperita	IGR of 13.3 and 72.45% for concentrations 0.4 and 0.8%. MIC of 2.26%	-	(França et al., 2018)
Mentha spicata	IGRs of 30.4, 36.8, 44.8 and 66.1% for concentrations 1, 2, 4 and 8%	-	(Gadhi et al., 2020; Perveen & Bokhari, 2020)
Jasminum officinale	IGRs of 30.0, 45.9, 53.7 and 64.1% for concentrations 1, 2, 4 and 8%	-	(Gadhi et al., 2020)
Cinnamomum cassia	MIC at 0.3-0.5 mg/mL	-	(Feng & Zheng, 2007)

Table 1). In addition, several monoterpenes exhibited significant inhibitory activities against *A. alternata* (β-ionone, citral, γ-terpinene and myrcene (Mei et al., 2021), as well as cinnamaldehyde (Xu et al., 2018). Besides their antifungal activity, several EOs also inhibit mycotoxins synthesis (Khan et al., 2013; Sumalan et al., 2013; J. Tian et al., 2015).

EOs	Effect	Mechanism	Reference
Citral (from citrus fruit's peel EO)	MIC of 0.22 mg/mL; MFC of 0.89 mg/mL. Inhibition of spore germination and AOH and AME production.	Downregulation of gene expression related with the metabolism of carbohydrates, amino acids, sulfate, glutathione and xenobiotics, which leads to the disturbance of cell integrity.	(Mutlu-Ingok et al., 2020; L. Wang et al., 2019)
Brassica juncea	MIC and MFC of 0.25 mg/mL	-	(Lu et al., 2019)
Litsea cubeba	MIC of 0.5 mg/mL and MFC of 1 mg/mL	-	(Lu et al., 2019)
Ocimum basilicum	MIC of 0.5 mg/mL and MFC of 2 mg/mL	-	(Lu et al., 2019)

Mentha x piperita	IGR of 13.3 and 72.45% for concentrations 0.4 and 0.8%. MIC of 2.26%	-	(França et al., 2018)
Mentha spicata	IGRs of 30.4, 36.8, 44.8 and 66.1% for concentrations 1, 2, 4 and 8%	-	(Gadhi et al., 2020; Perveen & Bokhari, 2020)
Jasminum officinale	IGRs of 30.0, 45.9, 53.7 and 64.1% for concentrations 1, 2, 4 and 8%	-	(Gadhi et al., 2020)
Cinnamomum cassia	MIC at 0.3-0.5 mg/mL	-	(Feng & Zheng, 2007)

Table 1 - EOs which showed inhibitory effects against *Alternariaalternata*.as well as their inhibitory concentrations.

Other general examples of plants whose EOs show antifungal properties against different fungi are *Ammi visnaga* (Toothpick-plant), this species EO shows total growth inhibion in *Candida* genus species and hyphal morphology alterations in *Fusarium oxysporum* (El-Sharkawy & Alshora, 2020; Ghareeb et al., 2011; Rashad et al., 2018; Thuwaini, 2021). *Anethum graveolens* (Dill) EO showed important antifungal activity against germination and sporulation spores in various fungal species including *A. alternata* (Khaldi et al., 2015; J. Tian et al., 2012). *Cymbopogon flexuosus* (Cochin grass) EO showed toxin production inhibitory activity against species in the *Candida* genus and mycelium growth inhibitory activity for *A. alternata* (Boukhatem et al., 2014; Martinazzo et al., 2019; Sawadogo et al., 2022). *Satureja montana* (Winter savory) EO showed activity against *C. albicans*, *S. cerevisiae* and *A. alternata* (Marin et al., 2012; Muñoz-Tébar et al., 2022). *Salvia sclarea* (clary sage) EO caused total growth inhibition and death in a variety of fungal species, including *A. alternata* (Džamić et al., 2008; Pitarokili et al., 2002; Sharopov & Setzer, 2012; Yuce et al., 2014), some of these EOs already have registered activity against *A. alternata* but their activity could be further investigated.

4.9 Aims of the work

Synthetic fungicides have played a central role in fungal disease control, however, their use poses a risk to human health and the environment. Therefore, more research is needed to catalyse the transition to the use of safer and more environmentally friendly alternatives such as plant-based products, namely EOs.

This study aims to evaluate the antifungal potential of several MAPs EOs and select the most promising ones to test their protective effect as biofungicides for the control of *A.*

alternata on *L. esculentum* under controlled environments (*in vitro* plants) and *in vivo*. For that, the following tasks were defined:

a) *In vitro* antifungal assessment of EOs from: *Ammi visnaga*, *Anethum graveolens*, *Cymbopogon flexuosus*, *Salvia sclarea* and *Satureja montana* against *Alternaria alternata*;
b) *In vitro* antifungal assessment to study the most promising EO and its dose-dependent activity to estimate antifungal parameters MIC (Mycelium growth inhibition assay) and MFC (dose-response assay);
c) Study the susceptibility of different tomato cultivars to *A. alternata* infection to test the protective effect of the most active EO in planta (*in vitro* and *in vivo*);
d) To test the protective effect of the most active EO on post-harvest rotting of cherry tomato fruits caused by *A. alternata* to evaluate its efficacy as a possible biofungicide able to prevent/delay postharvest deterioration of food commodities.

5 Materials and methods

5.1 Media and reagents

To perform the different assays and maintain cultures it was required to prepare different medium and solutions. The Potato Dextrose Agar (PDA) (ITW Reagents) was used for fungal growth and mycelium subculture of *A. alternata*. A sporulation medium constituted of 15g/L of calcium carbonate, 10 g/L of sucrose and 10 g/L of agar was used to stimulate conidia formation. Antifungal assays were performed in PDA and Potato dextrose Broth (PDB) (Liofilchem). Half strength Murashige and Skoog medium(Murashige & Skoog, 1962) (Duchefa, Biochemie, The Netherlands) (1/2 MS) was used for tomato *in vitro* culture. All mediums were autoclaved before use, during 15 min at 121°C.

Besides, the use of a surfactant solution was considered to facilitate the detachment of the conidia from the aerial mycelium and prepare conidia suspensions needed to perform several assays. The selected surfactant was Tween 20® (Alfa Aesar), which was mixed with previously autoclaved water (15 min at 121°C cycle).

On the other hand, EOs are highly hydrophobic natural products. Given their low solubility in water or aqueous solutions, an emulsifier or a solvent is frequently used to facilitate the homogenous dispersion of the EOs constituents in water solution or growth media. The solvent select in this case was ethanol (Table 2)

Table 2. List of reagents utilized in the preparation of the EOs solutions/emulsions and the extraction of conidia from the mycelium.

Reagents	Procedure/purpose	Assay
Tween 20® (prepared solution 0.01% v/v)	Surfactant for conidia separation	Pathogenicity testing *In vivo* cherry tomato infection Conidia extraction
Ethanol 99.9 % (Prepared solution 0.4% v/v)	Solvent for EOs dispersion	Fungal mycelium growth inhibition *In vivo* tomato infection Mycelium growth inhibition in liquid medium

5.2 Essential oils

Five EOs from five different plant species were considered for a preliminary assessment of their antifungal activity. Then, the EO showing the highest antifungal activity was selected to perform further assays.

In Table 3 are listed the EOs used and their origin. All EOs were preserved from light and heat in amber vials.

Table 3. The major components and origin of the plant EOs used in this work.

EOs	Supplier	Plant origin[1]	Plant parts	EO density[2]	**Major constituents[3]**
Ammi visnaga	J.E. International SARL/Florihana Destillerie	Morocco	Fruits	0.865±0.003	Linalool (27.16%) Isoamyl 2-methyl butyrate (15.94%) Amyl isobutyrate (9.48%)
Anethum graveolens		France/Hungary		0.893±0.009	Limonene (30.86%) α-Phellandrene (23.68%) Carvone (32.94%)
Cymbopogon flexuosus		India/Nepal	Leaves	0.873±0.009	Geranial (39.35%) Neral (30.82%) Geraniol (8.06%)
Satureja montana		Albania/Spain	Flowering tops	0.899±0.009	Carvacrol (43.61%) *p*-Cymene (17.14%) γ-Terpinene (13.11%)
Salvia sclarea		France/Hungary		0.879±0.007	Linalool (20.50%) Linalyl acetate (62.02%)

[1]According to products specifications provided by the supplier. [2] EOs density was determined from measurements performed in triplicates in the laboratory at a room temperature of 21°C.[3] EOs Summarized composition available from supplier´s chromatography sheet records.

5.3 Fungal isolate and culture

The fungal culture of *Alternaria alternata* (Fr.) Keissl. was kindly provided by the iB2 laboratory (Biology Department, FCUP, Portugal FCUP) (code: QSDM-A-A_2'). The culture was originated from a field isolate obtained in 2019 from vine leaves in a vineyard (Resende, Portugal). Its identification was first based on morphological characteristics and then confirmed by molecular analysis by Mariana Silva and Nuno Ponte (iB2 lab). The fungal culture has been maintained on a commercial PDA medium at room temperature and in the dark. For this work the culture was occasionally incubated at

28°C in the dark to optimize growth, or in different conditions of temperature and light to stimulate sporulation.

5.4 Antifungal assays

5.4.1 Mycelial growth inhibition in solid medium

The assay was conducted based on the method described by (Soliman & Badeaa, 2002). EOs obtained from *A. visnaga*, *A. graveolens*, *C. flexuosus*, *Salvia sclarea* and *S. montana* (0.25, 0.5 and 1 mg/mL) were used to assess their mycelial growth inhibitory activity. Pure ethanol (99.9%, ITW Reagents), previously filtered through a 0.2 µm nylon filter (Macherey-Nagel), was used as solvent to prepare EOs stock solutions. The freshly prepared EOs stock solutions were gently mixed with sterile molten PDA using a magnetic stirrer, for a proper dispersion of EOs. The five EOs were first tested using 3 doses: 0.25, 0.5 and 1 mg/mL Then, the most active EO was tested in a dose-response assay for a series of concentrations (from 0.0625 to 1 mg/mL). For all assays, the commercial systemic fungicide COMPO® Duaxo, containing difenoconazole as active ingredient (16.7 g/L), was used as a positive control and tested at 0.0625 and 0.125 mg/mL. Ethanol was used as the negative control at the final concentration of 0.4% v/v. The assay was conducted in 90 mm diameter Petri plates containing 15 mL of PDA medium with the respective control/EO solution. The medium was left overnight to properly solidify before being inoculated with a 5 mm circular plug from the active growth zone of a seven-day old *A. alternata* culture and four replicates were used for each treatment. Inoculated Petri dishes were kept in an inverted position and incubated in the dark for 12 days at 28°C, which was the time needed for mycelium in the control plates to reach the edge of the plate in these conditions. The mycelial diameter (cm) was measured from day 3 until day 12, with a ruler utilizing one or two diameters. MIC was determined by evaluating the lowest concentration at which there was no mycelium growth at a given day.

Inhibition percentage of mycelial growth with reference to the negative control was calculated for each time point and EO/fungicide concentration by applying the following formulae 1 (França et al., 2018) and 2 (Bouayad Alam et al., 2017):

1. $DGR_{ab} = \frac{D_x - D_0}{t_x - t_0}\ cm\ day^{-1}$

2. $\%I_r = \frac{DGR_c - DGR_t}{DGR_c} * 100$

Where:

D_x – Diameter at day *x* (cm)

D_0 – Diameter at the beginning (0.5 cm)

$t_x – t_0$ – exposure time interval (in days)

DGR_c – mean value for DGR in the control (cm day^{-1})

DGR_t – value for DGR in the treatment (cm day^{-1})

5.5 Transfer experiments to evaluate mycelia plugs viability

This assay was performed to distinguish the fungicidal and fungistatic effects of EOs concentrations being tested. Plugs from the replicates belonging to the groups that registered no growth were transferred to fresh PDA to test their viability. Their residual growth was determined by observation of the existence of mycelial growth (Thompson, 1989) after 4-day of incubation at the same conditions. This period was defined based on the mycelium normal growth on PDA, with an average diameter of 46.8 ± 2 mmThe MFC was determined by determination of the lowest concentration where no residual growth was observed.

5.6 Mycelial growth inhibition in liquid medium

A culture of *A. alternata* hyphae was started by submerging one large agar plug (from the growing edge of a mycelium) in 5 mL of autoclaved PDB medium. The fungus was allowed to grow during 3 days in an orbital shaker incubator (INNOVA® 40) (28°C; 180 rpm) (optimal conditions to achieve maximum growth) (Thompson, 1989). Afterwards, the mycelium was gently homogenized with a small Potter-Elvehjem glass tube, and 0.5 mL of the resulting homogenate was used to prepare the pre-inoculum, which was grown under the same conditions for 24h.

For assays, pre-inoculum was homogenized, and 1 mL of the resulting suspension was used to inoculate 9 mL of autoclaved PDB where small volumes of sterile testing solutions have been freshly incorporated. Experiments were performed in quadruplicates and included negative and positive controls (final concentrations of 0.0313 and 0.125 mg/mL, respectively) and *S. montana* EO tested at 0.0313, 0.0625, 0.125 and 0.25 mg/mL. After 48h, the mycelium was centrifuged at 3200G for 20 min at 4 ºC and then the liquid medium was discarded, and the mycelium was freeze-dried at -80°C. The wet and dry weights of each replicate were recorded to assess fungal growth. The GI(%) was calculated using the following formula:

$$GI(\%) = \frac{m_{treatment}}{m_{control}}$$

Where:

$m_{treatment}$ – average dry mass of the mycelium of the treatment group

$m_{control}$ – average dry mass of the mycelium of the control group

5.7 Tomato cultivars and culture

5.7.1 *In vitro* tomato culture: seed germination and shoots micropropagation

A *Lycopersicon esculentum* var. *cerasiforme* (cherry tomato) *in vitro* culture was established from commercial seeds (Vilmorin, France). Seeds were sterilized with commercial bleach (Pavão) containing 1.6% active chloride solution for 20 min with frequent agitation. The bleach was discarded, and the seeds were transferred to a sterile recipient and washed twice with sterile deionized water for 2 minutes. Then, the seeds were left in the water for 15 min under agitation to allow the seeds to get hydrated. Groups of 20 seeds were transferred to Magenta glass jars containing 1/2 MS medium (pH of 5.7) with 2% sucrose and placed on a phytotron chamber under controlled condition (16h of light: 8h of dark photoperiod; temperature of 25 °C) (Alatar et al., 2017). Germinated plantlets with at least 2-3 nodal segments were used for micropropagation. The established shoots culture was regularly micropropagated in 1/2 MS medium in the same controlled conditions and sterile environment.

5.8 Isolation of conidia

Sporulation induction was tested with the PDA medium in different condition: a) at 25°C total dark, b) at room temperature exposed and natural day-night cycle and c) at 25°C under constant light (Feng et al., 2011). Sporulation induction was also tested with the sporulation medium mentioned above (Masangkay et al., 2000), under the following conditions: d) room temperature and natural day-night cycle.

The conidia suspension was obtained from three-week-old mycelium grown in a sporulation medium (pH of 7.4) under the later condition d). A Tween 20® solution (0.01% v/v) was used to separate conidia from the cultures, by gently scraping the mycelium with a sterile blade. Then, the liquid was filtered with a sterile nylon net or gauze and collected in a falcon tube. The process was repeated using several plates to obtain a suspension containing a substantial number of conidia. The conidia suspension was filtered again before assessing its density. Conidia observations were made with a light microscope and counting were achieved with a haemocytometer (Carvalho et al., 2008).

The suspension was concentrated through centrifugation (20 min; 6000xg) whenever conidia density was lower than needed and diluted in sterile deionized water to adjust concentration.

5.9 Procedures for *A. alternata* inoculation on tomato

5.9.1 Micro Tom Leaves

In order to ascertain the susceptibility of Micro Tom tomato plants to the *A. alternata* isolate, Micro Tom tomato leaves from *in vivo* plants grown in culture chamber at the Department of Biology FCUP. Firstly, the leaves were washed with ethanol 70% solution for 2 min and then surface with an 0.8% active chloride solution for 1 min., Finally, leaves were washed several with sterilized distilled water. Then, each leaf was transferred to a sterile Petri plate containing 0.7% agar. Four inoculation spots were made on the adaxial surface of each leaf using 10 µL of conidia suspension ($1x10^4$ conidia/mL). The leaves infection state was monitored until 6 days after the inoculation.

5.9.2 *In vitro* cherry tomato leaves

Shoots with at least four leaves were excised from well-developed *in vitro* cherry tomato cultures. Every leaf was inoculated with 10 µL drops of a conidia suspension ($6.25x10^3$ spores/mL). Inoculated shoots were maintained in separated Magenta glass jars containing fresh 1/2 MS medium. The infection state was monitored until 8 days after the inoculation.

5.9.3 *In vivo* cherry tomato

Cherry tomato plants originated from seeds as described above were allowed to grow for 7 weeks and were watered when the soil was dry at the surface. After this time, four plants were sprinkled with a $6.25x10^3$ spores/mL suspension and were isolated with the help of Plastic bottles and the development of the disease was monitored for 12 days.

5.9.4 *In vivo* tomato fruits infection

This assay aimed to study if the fungistatic concentrations 0.5 and 1 mg/mL, previously verified in *in vitro* assays for *S. montana* EO would be effective in preventing *A. Alternaria* infection on tomato and fruit decay. For that, commercially available mature cherry tomatoes with homogenous dimensions and oblong shape were acquired. All fruits were disinfected before use by immersing in 0.1% active chloride for 10 min and then rinsed with sterilized distilled water and let to dry. Each tomato was individually placed in a Petri plate (55 mm diameter). A lesion (6 mm) was made in the equatorial zone, with the help of a sterile scalpel (Bouayad Alam et al., 2017; Feng et al., 2011). The experimental

design consisted of 10 replicates per concentrations of *S. montana* EO (0.5 and 1 mg/mL), difenoconazole (0.125 mg/mL) and negative control (0.4% v/v ethanol). Five minutes after treatments/controls application through spraying, a volume of 10 µL of *A. alternata* conidia suspension ($1x10^4$ conidia/mL) was inoculated in the lesion. The 10 replicates of the same treatment were joined in a tray covered with cling film and kept at room temperature. Humidified paper was placed on the bottom of each tray and wetted regularly to maintain the relative humidity at 50-60%. Temperature and humidity inside each tray were daily registered with a thermohygrometer and infection stage of each tomato were monitored at the 2nd, 4th, 6th and 8th day. After 8 days, the number of infected fruits was counted for calculation of the infection rate (Bouayad Alam et al., 2017):

$$\%\ Infected\ fruits = 100 \times \frac{Infected\ fruit\ number}{total\ fruit\ number}$$

5.10 Statistical analysis

Analytical statistics was caried out in the statistical package IBM SPSS Statistics 28 for Windows. Data were analysed for treatment effects using one-way analysis of variance (ANOVA). Normality and homogeneity of variance were checked using the Levene test, prior to one-way ANOVA analysis. The means were compared using the Tuckey HSD ($p<0.05$). When data failed the ANOVA assumptions, the non-parametric test Kruskal-Wallis was applied and pairwise comparisons were performed by the Dunn´s test ($p<0.05$) to compare variables (treatments, EOs, doses) effects. Dose-response curves were established for different time of exposure by non-linear regression method to relate *S. montana* EO inhibitory effects with dose and estimate the concentration that inhibited 50% of mycelial growth (EC_{50}). The variable slope (four parameters) model was applied to compute inhibitory dose-response curves using the Graphpad Prism 8 (Graphpad Software, Inc., USA). Goodness of fit was evaluated using R squared and sum of squares and the replicates test was performed to assess the model adequacy. This model followed the following equation:

Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope))

where x is equal to the base 10 logarithms of *S. montana* EO concentration and y corresponding to the mycelium growth inhibition.

All graphics and regressions were created using the software Graphpad Prism 8 (Graphpad Software, Inc., USA).

6 Results and Discussion

6.1 In vitro antifungal activity against *A. alternata*

6.1.1 Mycelial growth inhibition in solid medium

The results concerning EOs´ activity against *A. alternata* mycelial growth are presented in Figure 5 and Figure 6. As shown in Figure 5, the tested concentrations of *A. visnaga*, *A. graveolens*, *C. flexuosus* and *S. sclarea* EOs had moderate (*C. flexuosus* at 1 mg/mL, Figure 4C) to low activity (*A. visnaga*, *A. graveolens*, *Salvia sclarea*, Figure 4 A,B,D, respectively) for the tested period. The one-way ANOVA statistical analyses of EOs inhibitory effects throughout time considering the area under the curve (AUC), show that within *S. sclarea*, *A. visnaga* and *A. graveolens* EOs the concentrations 0.25 and 0.5 mg/mL are not statistically different. In the case of *C. flexuosus* the three concentrations were all significantly different from each other.

As regards *S. montana* EO, the concentrations of 0.5 and 1 mg/mL achieved total inhibition of the mycelial growth for the entire duration of the assay. However, when tested at a lower concentration of 0.25 mg/mL, this EO´s activity was significantly reduced over time (statistically different from the highest concentrations), and the mycelial growth inhibition at the end of the tested period remained negligible.

In the case of the positive control, difenoconazole, the tested concentrations 0.0625 and 0.125 mg/mL maintained their effectiveness throughout the entire assay, compared to the tested EOs (Figure 5).

After analysing the data from Figure 5 and acknowledging the loss of effect through time, it was considered important the comparison of the different EOs effects in one selected day, and the selected day was day 7. The comparison of EOs activity at day 7 (Figure 6), showed that, for all the tested concentrations, *S. sclarea*, *A. visnaga* and *A. graveolens* EOs are not significantly different from each other, according to the Kruskal-Wallis non-parametric analysis and Dunn´s post-test. However, *C. flexuosus* and *S. montana* EOs are significantly different from the other EOs for all the tested concentrations. Within the EO of *S. sclarea*, *A. visnaga*, *A. graveolens* and *C. flexuosus*, the concentrations 0.25 and 0.5 mg/mL are similar, whereas concentration 1 mg/mL is significantly different from the other concentrations. Concerning *S. montana* EO, the concentration 0.25 mg/mL is significantly different from the other two concentrations. Overall, these results line up with the One-way ANOVA performed on the AUC data mentioned above.

Overall, the results obtained through this assay aren't in line with what can be seen in the literature. *A. visnaga* demonstrated an inhibition average of 22.17% of the tested concentrations 15, 20, 30 and 40%. The demonstrated antifungal activity seems to be in line with the very weak antifungal activity of the EO, from the same species, determined in this study (Sabry et al., 2014). *A. graveolens* EO obtained the following IGR ranges with the same concentrations used in this study, at day 9 post inoculation: 21.5–28.6%, 37.4–57.9%, 49.1–77.2% (0.25, 0.5 and 1 mg/mL respectively) (J. Tian et al., 2011), which are better results than the ones herein reported. This difference might be attributed to differences of EO composition, since the employed method was similar. In the mentioned study were carvone (41.5%), limonene (32.6%) and apiol (16.8%) (J. Tian et al., 2011) and in this work were limonene (30.86%), α-phellandrene (23.68%) and carvone (32.94%). With this comparison, it can be speculated that the synergy between limonene and carvone with apiol results in a stronger antifungal effect than with α-phellandrene. In a different study, *C. flexuosus* EO achieved 100% IGR with 1 mg/mL after 7 days (Castro et al., 2017), which can be explained by EO composition variations and genetic variability within the *A. alternata* species. The main components in this work were geranial (39.35%), neral (30.82%) and geraniol (8.06%) and in the mentioned study were geranial (42.6%), neral (35.1%) and geraniol (5.21%). With this data, the stronger antifungal activity can be attributed to the higher concentrations of geranial and neral present in (Castro et al., 2017).

Sm EO was previously tested on *Alternaria alternata* and the following IGRs of 68, 80 and 100%, for concentrations 0.1, 0.2 and 0.3 mg/mL, respectively, were obtained after 7 days. Therefore, the present results, together with evidence found in the literature (Verdeguer et al., 2020), demonstrate that this plant EO has a strong antifungal activity.

Salvia sclarea EO exhibited reduced antifungal activity against a related fungal species, *A. solani* (29.5, 51.8, 62.9% of inhibition rates for the concentrations 0.2, 0.4, 0.8, respectively) (Giamperi et al., 2005). However, results presented in the later study are more promising than the one herein described for *A. alternata*. Such difference in effect can be justified by the genetic differences between the species, as well as EO composition, therefore the same assay should be made with *A. alternata*. *S. sclarea* EO also had 2.2 mg/mL as both its MIC and MFC, also, it is suggested that the moderate antifungal activity is associated with the linalyl acetate and linalool content in the EO (Dzamic et al., 2008).

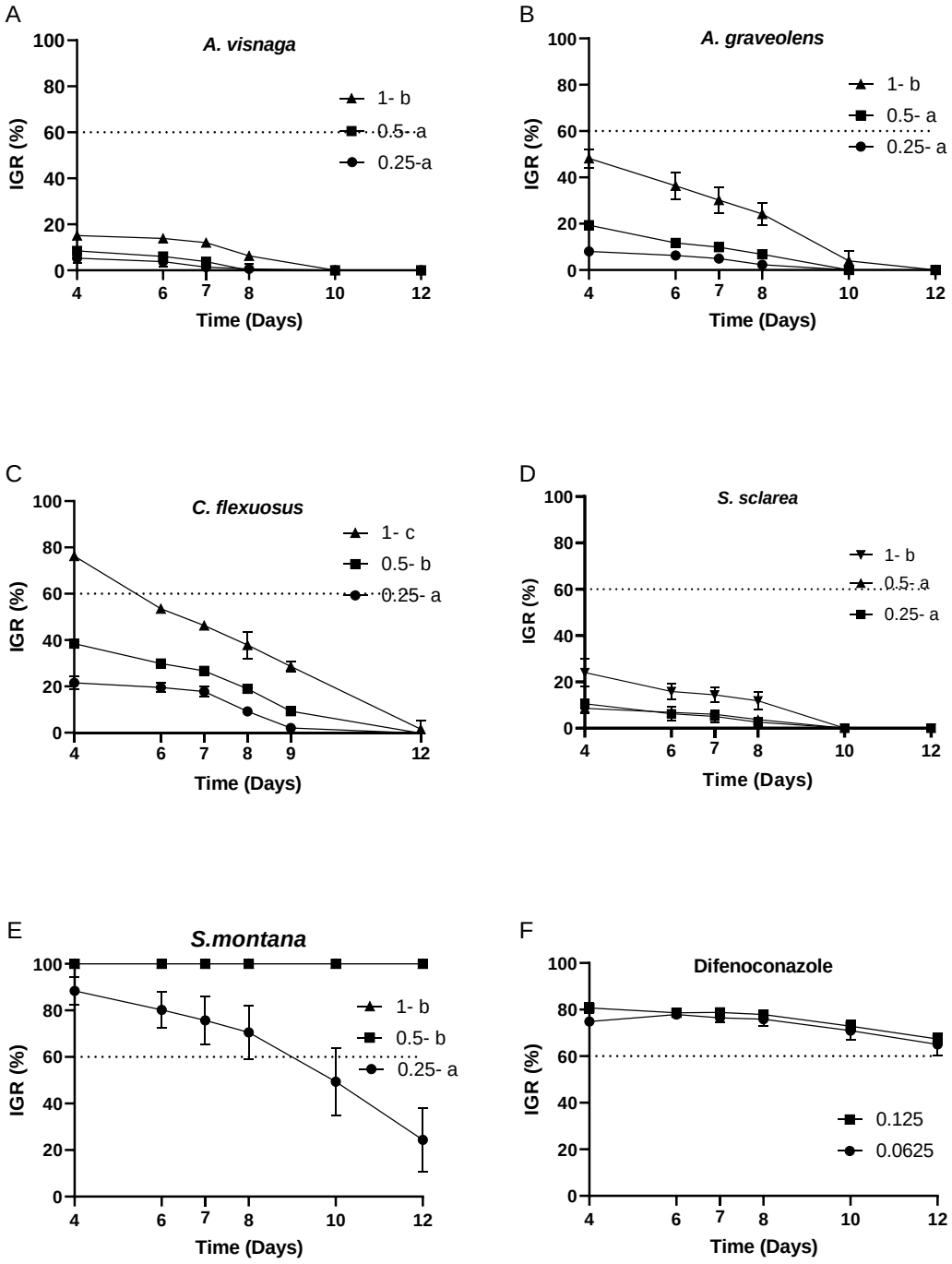

Figure 5: Inhibitory effect of the concentrations of EO, 0.25, 0.5 and 1 mg/mL and a synthetic fungicide (mg/mL) over *Alternaria alternata* mycelium growth for 12 days. A- *Ammi visnaga*; B- *Anethum graveolens*; C- *Cymbopogon flexuosus*; D- *Salvia sclarea*; E- *Satureja montana*; F- Fungicide difenconazole. Average values and the respective standard deviation were obtained from 4 replicates. In the legend, different lowercase letters after each tested concentration indicate significant differences of the means area under the curve (AUC) for p=0.05, according to a One-Way ANOVA followed by Tukey post-hoc test.

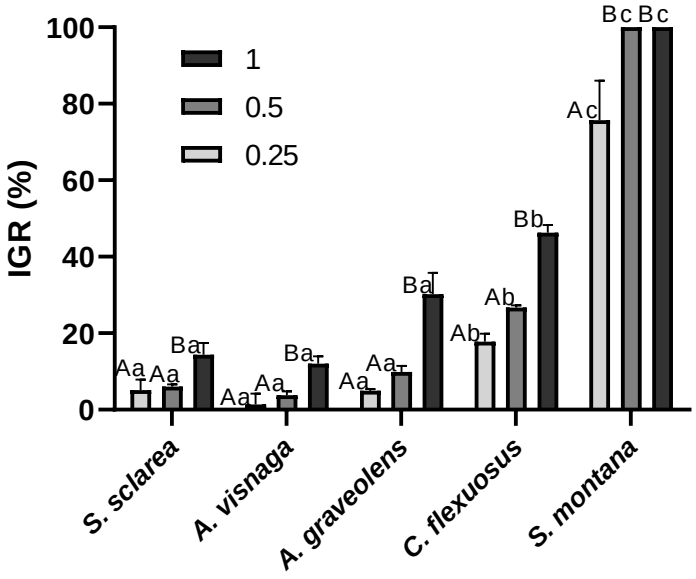

Figure 6: Mycelial growth inhibition rate of *Alternaria alternata*, 7 days after the plugs inoculation in PDA containing EO of: *Salvia sclarea; Ammi visnaga*; *Anethum graveolens*; *Cymbopogon flexuosus* and *Satureja montana*. Different uppercase letters indicate significant differences among concentrations within each EO for p=0.05 according to a Kruskal-Wallis analysis followed by Dunn´s post-hoc test. Different lowercase letters indicate significant differences among EOs for the same concentration for p=0.05 according to a Kruskal-Wallis analysis followed by Dunn´s post-hoc test.

Through observation of Figure 5 and Figure 7, it is noticeable the decay of the *Satureja montana* EO effect throughout time for concentration below 0.5 mg/mL. Comparisons of the AUC mean values, through the Tukey post-hoc test, indicate statistical differences between doses, except for 0.5, 0.75 and 1 mg/mL. Results suggest that the inhibitory activity on *A. alternata* growth was time- and dose-dependent. The effects observed for 0.0625 and 0.125 mg/mL were significantly lower than other concentrations (below 60% from day 4) and IGR decrease was recorded earlier.

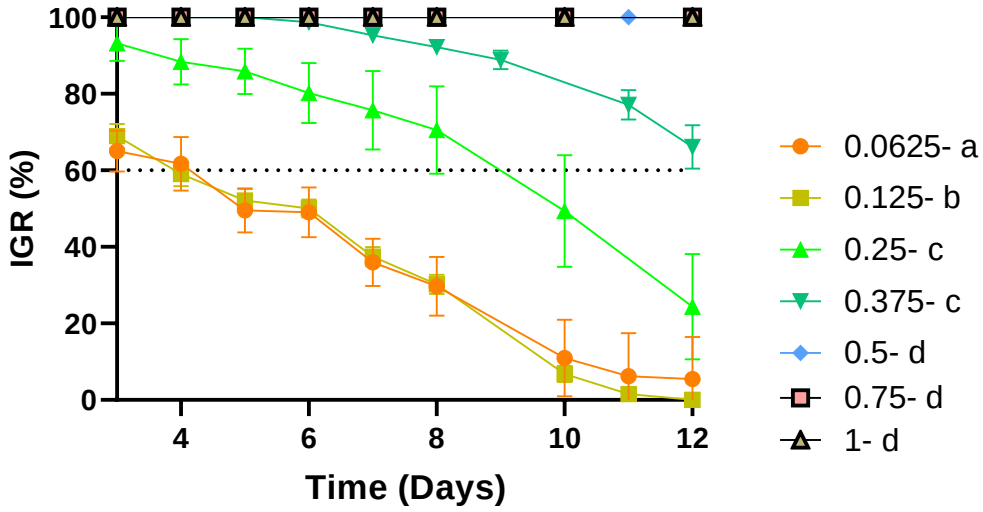

Figure 7: Inhibitory effect of several *Satureja montana* EO concentration on *Alternaria alternata* mycelium growth for 12 days. In the legend, different lowercase letters indicate significant differences of the area under the curve mean values for p=0.05, according to a One-Way ANOVA analysis followed by Tukey post-hoc tests.

The observable loss of EOs inhibitory effect for some concentrations can be explained by the volatilization of the EO active compounds from the medium matrix during the assay, or the fungus ability to metabolize some volatile compounds. In the case of the fungicide, difenoconazole, the results obtained can be interpreted as the fungicide degrading over time and experimental design and errors. Difenoconazole is a sterol biosynthesis inhibitor, suppressing the C-14 demethylation of lanosterol or 24-methylenedihydrolanosterol, a step in the conversion of lanosterol into ergosterol (the main sterol in this species), inhibiting the production of fungal sterols(H. Wang et al., 2016). Based on the results obtained and results obtained previously by the group, there is a “ceiling” of inhibitory activity at approximately 80%. This can be explained by the fact that alternative sterols can be produced by variations in the ergosterol/cholesterol pathway, especially in the later steps of the pathway and the production of alternative final products (Sayari et al., 2021). These sterols, allow the fungal cells to avoid death by partially replacing ergosterol in its vital functions.

6.1.2 Transfer experiment to evaluate mycelia plug viability

The concentrations 0.5, 0.75 and 1 mg/mL of the *S. montana* EO totally inhibited the mycelial growth of the plugs inserted in the medium, as can be seen in Figure 7. To clarify if these concentrations had a fungistatic or fungicidal effect transfer experiments

were performed. After four days, no mycelial growth was observed in any plug of the tested concentrations (

Figure 8), which indicated that all three concentrations have a fungicidal effect.

Figure 8: *A. alternata* inoculum after 4 days of incubation in fresh PDA medium, following a 12 days exposure to *Satureja montana* EO at 0.5, 0.75 and 1 mg/mL(from left to right) .

6.1.3 Dose-response curves, MIC and MFC of *Satureja montana* EO

Dose-response curves disposing the IGR in function of the concentration of *S. montana* EO at the days 4, 6, 8 and 12 are represented in Figure 9. Sm EO´s effectiveness increased considerably for concentrations above 0.125 mg/mL. 100% IGR were reached for concentration between 0.375 and 0.5 mg/mL, depending on the day that is being analyzed. In addition, the estimated concentration for which the IGR value is 50% increases considerably through time from day 6 to 12 (Table 4). These values were obtained using a non-linear regression log(inhibitor) vs. response which throughout all the selected days presented high R^2 values (from 0.9752 to 0.9867), indicating that the input data is well represented by the model. The Sum of squares metric, indicate that the data variation increases throughout time, suggesting that the regression model might be less adequate for data collected for later timepoints (day 8 and 12).

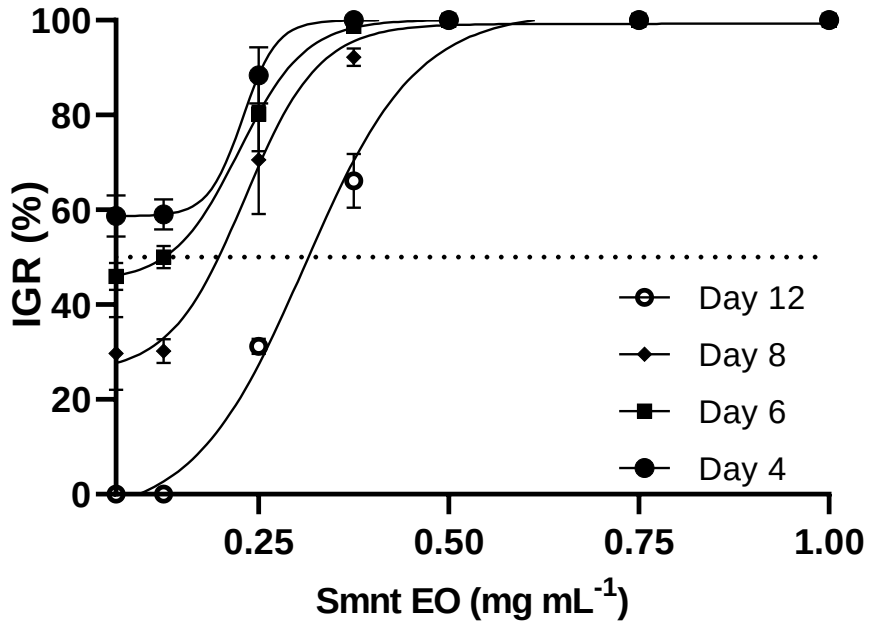

Figure 9: Non-linear regressions curves for different time points (4, 6, 8 and 12 days) showing the dose-dependent response of *Alternaria alternata* growth inhibition to increasing concentrations of *Satureja montana* EO.

Table 4: Equation of non-linear regression curves fitting the sigmoidal dose-response model and the estimated parameter for the different timepoints.

Days	4	6	8	12
Equation	$y = 58.89 + \frac{41.11}{1 + 10^{(-0.6242 - log(x)) * 18.21)}}$	$y = 47.60 + \frac{52.40}{1 + 10^{(-0.6382 - log(x)) * 6.317)}}$	$y = 28.60 + \frac{71.40}{1 + 10^{(-0.6295 - log(x)) * 5.052)}}$	$y = 0.1604 + \frac{99.84}{1 + 10^{(-0.5142 - log(x)) * 4.784)}}$
R^2	0.9794	0.9818	0.9752	0.9867
Sum of squares	173.5	239.3	633.8	627.7
IGR=50%[a]	N/A	0.1422	0.1984	0.3058

[a] Estimated dose of Sm EO causing 50% of *Alternaria alternata* growth inhibition *in vitro*.

N/A- Not estimated since experimental data comprised values above 50%.

The observed MIC at day 4 was 0.375 mg/mL, while this parameter increased to 0.5 mg/mL for all the other timepoints. It is worth to note that this EO presents a minimal fungicidal concentration (MFC) of 0.5 mg/mL at day 12, since the tested concentrations equal and above 0.5 mg/mL are lethal (

Figure 8). In a previous study, *S. montana* EO had a MIC equivalent to 0.3 mg/mL after 7 days (Verdeguer et al., 2020), which is close to the one obtained in this study. In

addition, *S. montana* EO also registered an MIC of 0.3 mg/mL against *A. solani*, in accordance with the study on *A. alternata* (Fraternale et al., 2007). In this study, a concentration of 0.45 mg/mL wasn't tested, however, since the concentration 0.5 mg/mL was tested and had a fungicidal effect, it can be suggested that the MFC for *A. alternata* is lower than was determined (Fraternale et al., 2007).

S. montana EO had an MIC of 0.3 mg/mL (Verdeguer et al., 2020) which is a better result than the one obtained in this study, this difference can be explained by composition differences from the EO used in the assays presented here and the one used in the study. It is possible that these drastic differences in effect could be partially explained by the method difference. Also, the study suggests that the strong antifungal activity of this EO is on a major part caused by the synergized effect of three of its major components: carvacrol, p-cymene and γ-terpinene. This idea is reinforced by the also strong antifungal activity of EOs with similar major compositions (Verdeguer et al., 2020). On another study, Sm EO, registered an MIC of 0.3 mg/mL on *A. solani*, in accordance with the previous study on *A. alternata*. In this study, an MFC of 0.45 mg/mL, this concentration wasn't tested, however, since the concentration 0.5 mg/mL was tested and had a fungicidal effect, it can be suggested that the actual MFC for *A. alternata* is lower than was determined (Fraternale et al., 2007).

Differences in effect could be partially explained by slight variations in the methods. Besides, many authors have discussed the possible influence of EOs composition and variations in the ratio of their major constituents to explain major differences observed among studies, organisms' susceptibility and/or EOs toxicity. According to (Verdeguer et al., 2020). the strong antifungal activity of this EO is mostly caused by the synergized effect of three of its major components: carvacrol, p-cymene and γ-terpinene. This idea is reinforced by the strong antifungal activity of similar EOs containing the same major compounds.

6.1.4 Mycelial growth inhibition in liquid medium

To study the Growth inhibition (GI) in liquid cultures the following *S. montana* EO concentrations were tested: 0.0313; 0.0625; 0.125 and 0.25 mg/mL. The first time this assay was performed, the tested concentrations were 0.0625, 0.125 and 0.25 with the positive control 0.125 mg/mL of difenoconazole. All the treatments, except for the concentration 0.0625 mg/mL produced insufficient biomass to perform weight measurements. In fact, it was observed that the concentration 0.25 mg/mL of *S. montana* EO inhibited the growth of the mycelium completely as shown in Figure 11. With this new

knowledge, the assay was repeated with lower concentrations of *S. montana* EO 0.0313 and 0.0625 mg/mL and the positive control, 0.0313 mg/mL, yielding the results present in Figure 10. According to the Kruskal-Wallis non-parametrical analysis and treatments comparison through the Dunn´s test differences in growth inhibition of the two EO doses and the positive control were not statistically different.

Even though there is a high discrepancy in the date for the *S. montana* EO concentration 0.0313 mg/mL, it was possible to verify, based on the decrease of the mycelium dry weight compared to the negative control, that both concentrations have some inhibitory activity. Nevertheless, it would be necessary to repeat this assay to consolidate these preliminary results. For the data in Figure 10 a Kruskal-Wallis non-parametrical test with Dunn post-test was performed, from the data obtained from this statistical analysis, the three treatments have no significant differences.

It is worthy of note that the inhibition obtained from the treatment 0.25 mg/mL of *S. montana* EO is not coincident with the results obtained for that same concentration in the previous assessment performed on solid medium. This apparent stronger inhibitory effect might be explained by the fact that the liquid medium was contained in a recipient less susceptible to allow gas leaks, maintaining the real concentration of the EO for longer. Further research should be conducted to estimate the MIC and MFC values using *A. alternata* liquid culture, but we hypothesize that the effect of *S. montana* EO incorporated in liquid medium might be stronger.

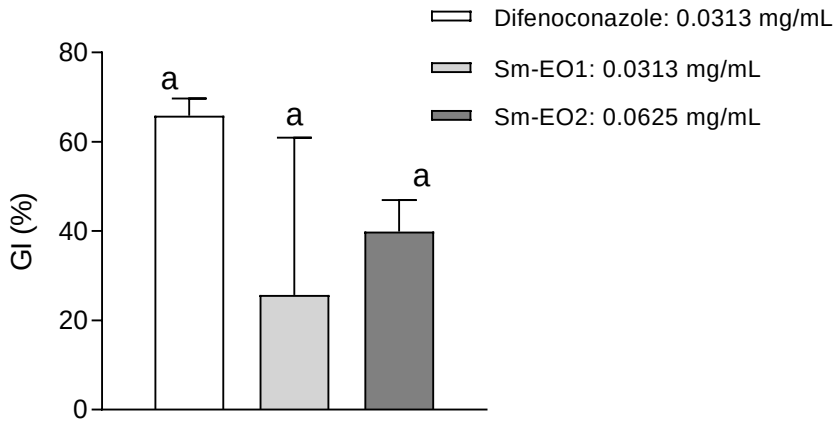

Figure 10: *Alternaria alternata* Growth inhibition (GI) after 48h of incubation incubated in PDB medium containing a reference fungicide and two concentrations of *Satureja montana* essential oil (Sm-EO). The same lowercase letter indicates that there are no significant differences among treatments for p=0.05, according to a Kruskal-Wallis analysis followed by Dunn´s post-hoc tests.

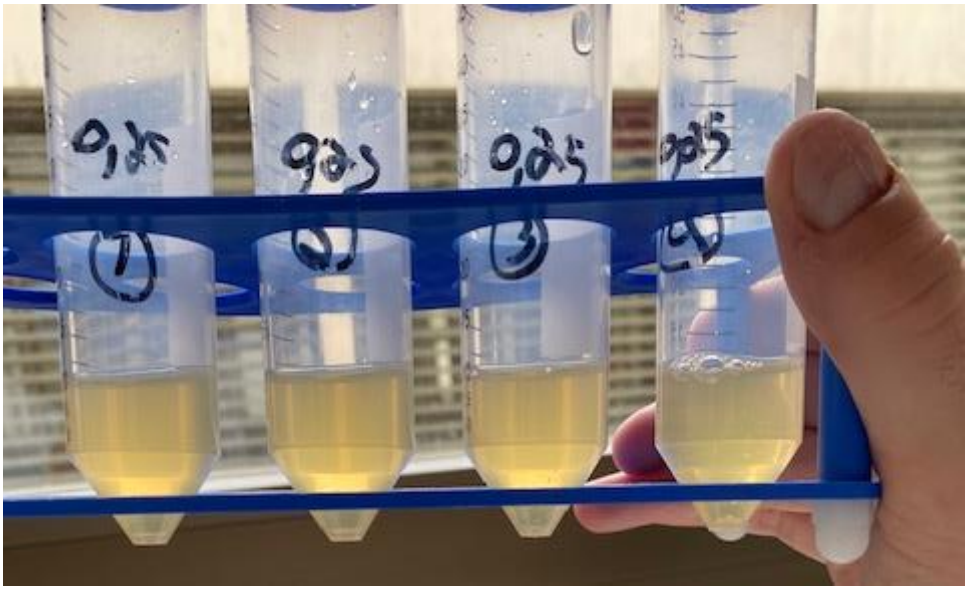

Figure 11: Absence of *Alternaria alternata* mycelial growth after 48h incubation in PDB medium containing 0.25 mg/mL of *Satureja montana* EO.

6.2 Susceptibility of cherry and MicroTom tomato leaves to *A. alternata*

6.2.1 *In vitro* susceptibility of cherry and MicroTom tomato leaves to *A. alternata*

In Figure 12 the MicroTom leaves don´t show signs of *A. alternata* infection, just signs of natural decay. In the case of Figure 13, there are signs of infection, necrosis in the leaf and growth of a mycelium on top of the leaves. This response suggests that cherry tomato plants could be more susceptible to the *A. alternata* isolate.

Figure 12: MicroTom tomato leaves 6 days post inoculation with *Alternaria alternata* conidia ($1x10^4$ conidia/mL).

Figure 13: Cherry tomato shoot in 1/2 MS medium showing symptoms of fungal infection 6 days after inoculation with *Alternaria alternata* conidia ($6.25x10^3$ conidia/mL).

6.2.2 *In vivo* susceptibility of cherry tomato leaves to *A. alternata*

Since the MicroTom leaves demonstrated no susceptibility to the *A. alternata* isolate being studied, we considered only the cherry tomato cultivar for further tests *in vivo*. In Figure 14 it can be observed that the leaves present lesions caused by the pathogen 12 days post inoculation, with evident mycelium growth on top of some leaves. These results suggest that cherry tomatoes plants might be susceptible to this field isolate of *A. alternata* and would be a good plant model for testing Eos´ efficacy as preventive or curative treatments to control *A. alternata* colonization of growing tomato plants.

Figure 14: *In vivo* Cherry tomato leaves infected with *Alternaria alternata* conidia ($6.25x10^3$ conidia/mL) 12 days post inoculation.

6.3 *In vivo* tomato fruit infection

The *in vivo* assay with cherry tomatoes was performed to assess Sm EO to prevent *A. alternata* development and post-harvest decay of tomato fruits. In Figure 15, it can be observed that 8 days after *A. alternata* inoculation and EO treatment, only the fungicide had considerable preventive property. This can be explained by the loss of EO volatile components through time. It is also possible that some slight decay of a fruit could be misjudged as an early-stage infection, influencing the data.

In Figure 16, it is possible to see the evolution of the number of infected fruits through time, for each treatment. It wasn't possible to run statistical tests for these data sets because the assay was only performed once when it should have been performed a minimal of three times. On the other hand, to optimize this assay we should consider testing higher *S. montana* EO concentrations, or a distinct application method to ensure a better dispersal of the treatment solution on tomato fruits surface. Nevertheless, with

this data set it is possible to speculate that the EO might have a conidia germination retarding property but might not prevent entirely the infection. A conidia germination assay should be conducted to confirm this theory.

The results presented don't line up with a similar study where the fruits were coated in a *S. montana* EO solution, Sm EO 600 µg/mL in a 0.1% Tween 20 solution and 0.25% agar, creating a film, and achieving a mycelial growth inhibition of 100% with a dose as low as 300 µg/mL (0.3 mg/mL). Also, for up to 20 days, only 10% of the cherry tomatoes coated with the EO presented damage caused by *A. alternata*, showing a promising conservation property (Verdeguer et al., 2020), contrarily to what was reported in the preversent preliminary study. This difference in results shows that there is a possibility that the method selected for this assay wasn't suited, and if the described new method was replicated it would yield results more in line with those presented in Figure 7 and Figure 11.

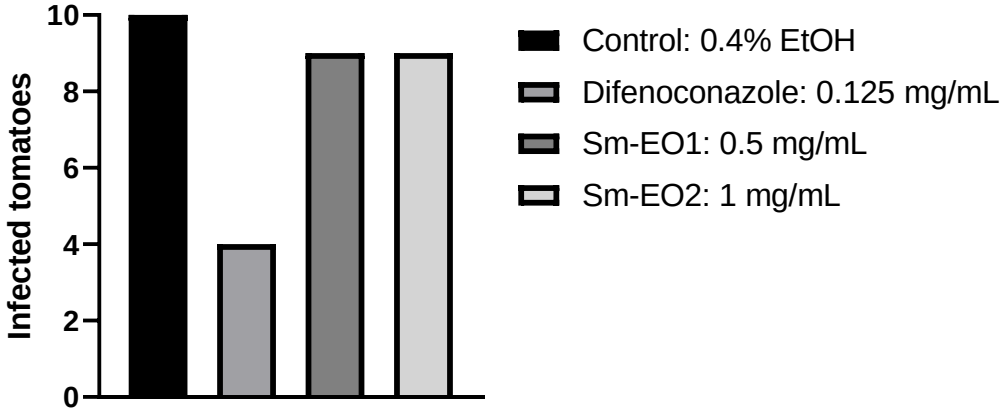

Figure 15: Number of cherry tomatoes showing fungal colonization eight days after inoculation with *Alternaria alternata* conidia.

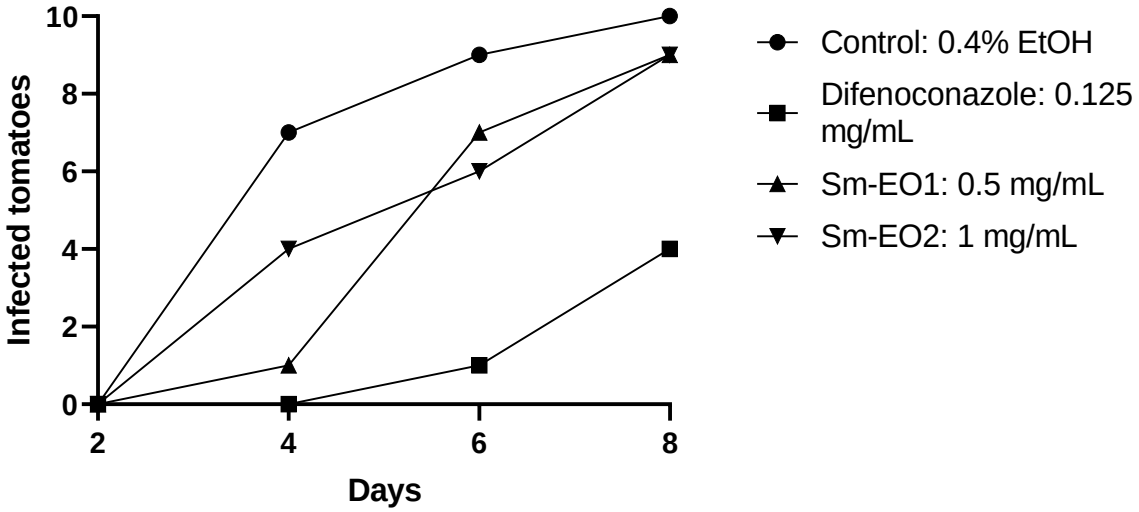

Figure 16: Number of cherry tomatoes showing fungal colonization and effects of *Satureja montana* EO and fungicide on delaying *Alternaria alternata* infection in cherry tomato for 8 days.

7 Conclusions and future perspectives

Tomato plants are susceptible to a variety of pathogenic organisms, including fungi. The fungal pathogen used in this work, *A. alternata*, is responsible for losses in food production and post-harvest deterioration of food commodities. Agricultures that produce Tomatoes and other affected crops must manage these diseases and frequently rely on synthetic pesticides, the continuous use of these substances rises concerns regarding the environment and human health. Scientific evidence and consumer awareness of these risks have raised the need to find alternatives that allow for sustainable practices.

Despite evidence of the biofungicide potential of some EOs against *A. alternata*, there is still a need to acknowledge the potential of different EOs toward treating *A. alternata* infection *in vivo*. In this study, the results of *S. montana* EO showed strong *in vitro* antifungal activity against *A. alternata* and *A. visnaga*, *C. flexuosus*, *A. graveolens* and *S. sclarea* showed weak inhibitory effects.

The work planned for assessing EOs activity towards *A. alternata* was constrained by several setbacks related to spore production, which made impossible the execution of a spore germination assay on time. On the other hand, A *S. montana* EO nanofornulation utilizing chitosan nanoparticles to encapsulate the EO was tested once through the mycelium inhibition assay. Results were inconclusive due to contamination issues. Additional assays, which were planned in collaboration with researchers from another

institution, suffered setbacks related to the timing of the preparation of the nanoparticles and the age of the mother cultures.

Complete assessment of its activity against these pathogens should be performed *in vivo* on tomato plants for a more thorough evaluation of their effectiveness as biopesticides for tomato protection. As confirmed by the literature review, all the tested EOs possess antifungal and antibacterial activity, thus additional assays should be performed to evaluate activity against other food-spoilage fungi, and bacteria like *Pseudomonas syringae* and human pathogenic bacteria.

Satureja genus EOs have shown considerable allelopathic properties, inhibiting germination and seedling growth, however, at lower concentrations some EOs stimulated germination in tomato and in the case of rye seedling growth was also stimulated. Testing the allelopathic properties of the *S. montana* EO in tomato plants would help to have a deeper understanding of its herbicidal potential and the range of usable concentrations.

In conclusion, our finding evidence that *S. montana* EO might be a good candidate to be selected to *in vitro* and *in vivo* studies (including the nano chitosan- *S. montana* EO formulations) to demonstrate its biopesticidal potential against phytopathogens. However, the other tested EOs, are weak options to treat *A. alternata* infections but might be good candidates to treat other phytopathogens.

Sustainable practices concerning the application of EOs incorporated in nanoformulations could be an efficient alternative to conventional crop protection products since EOs´ performance and application in field conditions are limited due to their high volatility and hydrophobicity and low molecular weight. Formulations have been studied to overcome these limitations and allow for a controlled delivery; biomolecule-based nanoparticles are a promising domain. Chitosan-based nanoparticles appear to be a suitable option to encapsulate EOs.

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