Evaluation of the biopesticidal properties of *Thymus mastichina* and *Trachyspermum ammi* essential oils against agricultural diseases and pests

Márcia Ferreira Peixoto
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Department of Geosciences, Environment and Spatial Planning
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Supervisor
Rose Marie Oliveira Ferreira de Sousa, Researcher, FCUP

Co-Supervisor
Ana Álvares Ribeiro Marques de Aguiar, Professor, FCUP
Manuel Fernandes Ferreira, Full Professor, FCUP

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

The search for alternatives to synthetic pesticides has led to an increased interest in natural products of botanical origin, namely essential oils (EOs). These plant-products are well-known for their biopesticidal properties against a variety of agricultural pests.

In this work, we aimed to investigate if *Thymus mastichina* and *Trachyspermum ammi* EOs have a relevant biopesticidal properties, specifically antibacterial activity against two phytopatogens *Pseudomonas syringae* pv. *actinidiae (*Psa) and *P. syringae* pv. *tomato* (Pst), antifungal activity against *Alternaria alternata*, herbicidal activity towards *Lolium multiflorum*, and anti-insect activity on *Tuta absoluta*.

Following a preliminary *in vitro* antibacterial assessment (disc diffusion assay) against Psa and Pst reference strains, *Thymus mastichina* and *Trachyspermum ammi* EOs were selected to assess minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) through the broth microdilution method. The results demonstrated that *T. ammi* EO has antibacterial activity against Pst DC3000 (MIC and MBC = 3.64 mg mL^{-1}). *In vitro* testing performed on *Alternaria alternata* to evaluate mycelium growth inhibition showed that *T. ammi* EO completely inhibited the fungal development throughout several days (12 days) at the concentration of 2 and 1 mg mL^{-1}. In addition, these two concentrations affected conidial germination after 24h (around 70% of not germinated conidia). Nevertheless, their antifungal activity was less pronounced when tested as preventive treatment against cherry tomatoes decay caused by this fungus. Besides, *T. ammi* EO also showed interesting allelopathic activities against the plant species tested (above 60 % of germination inhibition and 80% of radicle growth inhibition at 0.25 mg mL^{-1}). Besides, *T. ammi* EO exhibited deterrent activity against *Tuta absoluta* oviposition in tomato plants (> 60%), when assessed at a concentration of 5 mg mL^{-1}. On the other hand, *T. mastichina* EO, did not show relevant antibacterial effect nor antifungal activity on *A. alternata*. Nevertheless, some allelopathic activity on *L. multiflorum* radicle growth (80% of inhibition for concentration 1 mg mL^{-1}) and promising deterrent properties on *T. absoluta* oviposition (> 70%), were recorded.

Considering the range of target organisms used, we concluded that *T. ammi* EO showed a wide spectrum of bioactivity, which suggest a strong biopesticidal potential. This botanical product should be further investigated to ascertain if there is a real prospect for its application in crop protection.

Key words: Antifungal; Antimicrobial; Botanical-based pesticides; Essential oils; Natural herbicide; Oviposition deterrent

Resumo

A procura de alternativas aos pesticidas sintéticos levou a um aumento do interesse pelos produtos naturais de origem botânica, nomeadamente os óleos essenciais (OEs). Esses produtos naturais são conhecidos pelas suas propriedades biopesticidas contra uma variedade de pragas agrícolas.

Neste trabalho, o objetivo foi avaliar se os OEs de *Thymus mastichina* e *Trachyspermum ammi* possuem propriedades biopesticidas relevantes, em específico, atividade antibacteriana contra dois fitopatogéneos *Pseudomonas syringae* pv. *actinidiae* (Psa) e *P. syringae* pv. *tomato* (Pst), atividade antifúngica sobre *Alternaria alternata*, atividade herbicida sobre *Lolium multiflorum* e atividade anti-inseto sobre *Tuta absoluta*.

Após realizar um ensaio preliminar *in vitro* para avaliação da atividade antibacteriana de OEs (ensaio de difusão em disco) em estirpes de Psa e Pst de referência, *T. mastichina* e *T. ammi* OEs foram selecionados para avaliar a concentração mínima inibitória (CMI) e a concentração mínima bactericida (CMB) através do método de microdiluição. Os resultados demonstraram que o OE de *T. ammi* OE possui atividade antibacteriana contra a Pst DC3000 (MIC e MBC = 3,64 mg mL^{-1}). Os ensaios *in vitro* para avaliar efeitos inibitórios no crescimento micelial de *A. Alternata* mostraram que o OE *T. ammi* inibiu completamente o desenvolvimento fúngico ao longo de vários dias (12 dias) na concentração de 2 e 1 mg mL^{-1}. Além disso, essas duas concentrações afetaram a germinação de conídios após 24h (cerca de 70% de conídios não germinados). No entanto, a atividade antifúngica foi menos pronunciada aquando da avaliação dos seus efeitos contra a deterioração do tomate cherry causada por este fungo. Além disso, o OE *T. ammi* também apresentou atividades alelopáticas contra a espécie de planta testada (acima de 60% de inibição da germinação e 80% de inibição do crescimento da radícula a 0,25 mg mL^{-1}), e atividade dissuasora da oviposição de *T. absoluta* em tomateiro (> 60%), quando avaliado na concentração de 5 mg mL^{-1}. Por seu lado, o OE T*. mastichina*, não apresentou efeito antibacteriano relevante nem atividade antifúngica sobre *A. alternata*. No entanto, foi registada alguma atividade alelopática no crescimento da radícula de *L. multiflorum* (80% de inibição para concentração 1 mg mL^{-1}) e atividade promissora como deterrente da oviposição em *T. absoluta* (> 70%).

Considerando os organismos-alvo utilizados, concluímos que o OE *T. ammi* apresentou um amplo espectro de bioatividade, o que sugere um forte potencial

biopesticida. Este produto botânico deve ser investigado para se aferir se há uma perspetiva real da sua aplicação na proteção de culturas.

Palavras-chave: Antimicrobiano; Antifúngico; Biopesticidas de origem vegetal; Dissuasão da oviposição; Herbicida natural; Óleos essenciais

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Abbreviations and acronyms

BCK	Bacterial canker of kiwifruit
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
EO(s)	Essential oil(s)
EPPO	European and Mediterranean Plant Protection Organisation
ISO	International Standard Organisation
KB	King's B medium
MAPs	Medicinal and aromatic plants
MBC	Minimal bactericide concentration
MIC	Minimal inhibitory concentration
MFC	Minimal fungicidal concentration
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
PDA	Potato dextrose agar
PDB	Potato dextrose broth
Psa	*Pseudomonas syringae* pv. *actinidiae*
Pst	*Pseudomonas syringae* pv. *tomato*

1. Introduction

1.1. Pesticides use in agriculture

The agriculture sector has the continuous challenge of responding to the food demand of the world population. The evolution of the agricultural industry has led to increased yields and reductions in the cost of commodities which consequently allowed to establish a more abundant food supply. By 2050, food production is required to increase 60% due the growth of human population (Fischer et al., 2014). But the capacity to cover the food chain necessities can be negatively affected by crop yield losses due to agricultural diseases and pests. It is estimated 20 to 40% losses at preharvest and postharvest of global agricultural productivity because of pathogens, animals, and weeds (Sharma et al., 2017). So, to tackle this issue and minimize possible losses, it has been a regular strategy in crop management the application of pesticides. This term includes *any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying, or controlling any pest, or regulating plant growth* (FAO et al., 2014). In this definition, pest refers to *any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products, materials or environments and includes vectors of parasites or pathogens of human and animal disease and animals causing public health nuisance* (FAO et al., 2014).

There are many organisms that reduce quality and quantity of crop yield. So, producers rely in the application of different pesticides according to the target specie (e.g., bactericides, fungicides, herbicides, insecticides, nematicides) to minimize potential losses (Abubakar et al., 2020). In 2019, the global use of pesticides was 4 190 985 tonnes, which clrearly shows the dependence of modern agriculture on this kind of product (Figure 1). Though, pesticides use can vary from each region of the world because of the country's quantity of marketable production, pesticide's cost (most of them patented), and also the specific pests of each climatic/geographic region (Carvalho, 2017).

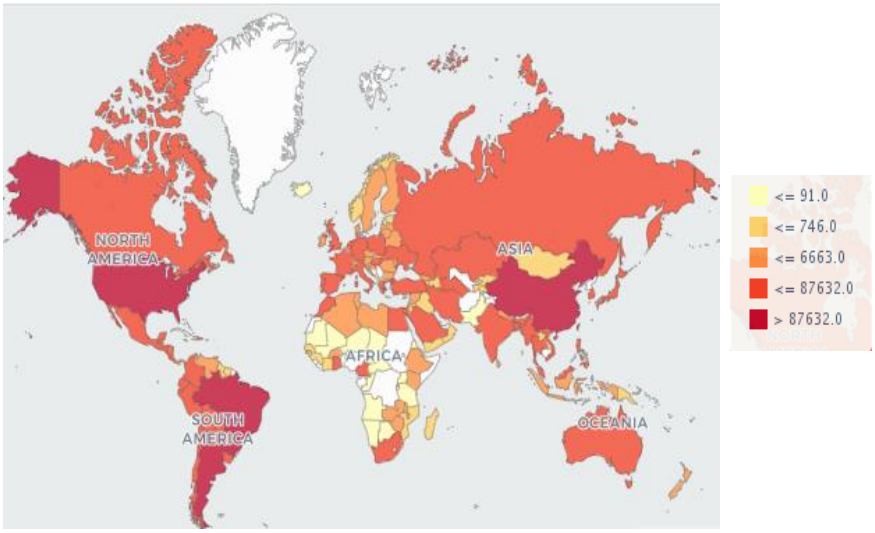


Figure 1: Quantities (in tonnes of active ingredients) of pesticides used in or sold to the agricultural sector for crops and seeds in 2019 (FAOSTAT, 2019).

Pesticides can be categorized depending on their origin: Organic source (natural - plant phytochemical, or synthetic – synthetic production); Inorganic source (mixture of inorganic salts); Biological (microbial pesticides - fungi, virus, bacteria) (Abubakar et al., 2020). Among these categories, synthetic pesticides had a key role in the development of agriculture after World War II (e.g. dicloro-difenil-tricloroetano (DDT), cyclodienes and organophosphorus insecticides) (Sparks et al., 2015). Since then, new products have been developed and it is a systematic agricultural tool across the world. However, side effects of those pesticidal residues have raised concerns regarding their use.

One of the problems associated with the incessant application of synthetic chemicals is the diminished soil quality. Particles remaining in the soil reduce beneficial soil microorganisms involved in the biodegradation of soil nutrients (Abubakar et al., 2020). Also, leaching processes can facilitate the movement of the hazardous substances to aquifers. That can expose aquatic life and threatening the sustainability of these ecosystems (Mfarrej et al., 2019). Besides, natural resources for human consumption, including groundwater and water for aquaculture activities, can be compromised (Sinha et al., 2009). Moreover, the application of broad-spectrum pesticides affects the target organisms and also the non-target species, like pollinators, essential to agriculture (Hashimi et al., 2020).

Another issue concerning these pesticides is the non-biodegradability that easily conducts to bioaccumulation in plants and animals (Hassaan et al., 2020) . It leads to their presence in the food chain and rises serious concerns about human health (Hashimi et al., 2020). It has associated problems like rashes, mellow sensitivities, chronic

diseases, neurotoxic and reproductive complications, among others. The exposure to the human body can happen through other routes, like soil, flora and fauna (Hashimi et al., 2020), via dermal, oral, eyes, or respiratory pathways (Kumar et al., 2019).

The repeated use of synthetic pesticides to control agricultural pests and crop diseases has led to the development of pesticide resistance, affecting different types of pesticide such as fungicides (Lucas et al., 2015), insecticides (Bass et al., 2015), and herbicides (Powles et al., 2010). Target-site resistance results from gene mutations or over-expression that reduces pesticide affinity (Matzrafi, 2019). Consequently, many of the existent pests (including weeds and diseases) became difficult to control.

In response to the issues mentioned above, the introduction of an integrated pest management (IPM) allowed the implementation of multiple tactics that decrease potential economic, health, and environmental risks. According to the General Directorate of Food and Veterinary Medicine (DGAV), it is defined as a rational, balanced and integrated use of all tools available (genetic, cultural, biological, biotechnical, and chemical) to maintain the crop's enemies population at levels that do not cause damage (DGAV, 2014b). This strategy has as its principal objective the reduction of the use of synthetic pesticides but, its total substitution for other products is still in discussion. So, the search for alternatives to ensure the sustainability of agricultural production systems have risen interest in natural pesticides. In this category**,** botanical extracts and EOs have been considered good candidates based on their several bioactivities against agricultural pathogens and pests.

1.2. Essential oils: their origin and nature

Essential oils (EOs) are natural mixtures of volatile compounds with a complex chemical composition (Bakkali et al., 2008). Plant secondary metabolism synthesizes volatile constituents for defence and communication (Pavela et al., 2016). They have a key role in plant thermotolerance through several mechanisms, as well as defence chemicals by conferring protection against bacteria, fungi, insects, viruses, and herbivorous animals (Bakkali et al., 2008). Plant volatile compounds are semi chemicals that can also display an attractive or repellent role in plant-animal interactions (Vokou, 2005). Some of these molecules act as chemical signal to attract insects responsible for seed and pollen dispersion, but also to call predators when herbivorous pests cause damages (Bakkali et al., 2008).

EOs are extracted from medicinal and aromatic plants (MAPs) native from tropical countries or temperate to warm countries like the Mediterranean (Bakkali et al., 2008). The synthesis and accumulation of volatile constituents can occur in different plant organs such as flowers (e.g., thyme, chamomile, and lavender), roots (e.g., saffron, ginger, and turmeric), fruits (e.g., parsley, laurel, and pepper), seeds (e.g., caraway, coriander, and fennel), leaves (e.g., eucalyptus, thyme, and bay), wood (e.g., sandalwood, rosewood, and amyris), and in other parts namely buds (e.g., clove) (B. et al., 2003; Dhifi et al., 2016). Plant volatile compounds are produced by secretory cells and stored in secretory structures like oil ducts, glandular trichomes or secretory cavities depending on the type of EO-bearing plant (Bakkali et al., 2008; Pavela et al., 2016).

EOs physic-chemical properties and composition are distinct from fixed oils which have a larger molecular size and contain glycerides of fatty acids (Zuzarte et al., 2015). At room temperatures, EOs are usually liquid and present different densities depending on their composition and botanical origin (Dhifi et al., 2016). As opposed to fixed oils, they evaporate when exposed to heat (Barbieri et al., 2018). Their coloration can vary from light yellow to brown or even colourless (Raveau et al., 2020). EOs are classified as liposoluble due to their high solubility in organic solvents and poor solubility in water (Raveau et al., 2020).

Concerning EOs, these are complex natural mixture, which may be constituted by 20 to 60 different compounds at different concentrations (Raveau et al., 2020). In general, EOs are constituted by a few major compounds found at higher concentrations (20–70%) and several minor compounds at lower percentages (Aziz et al., 2018). Terpenic compounds of low molecular weight, more precisely monoterpenoids (C_{10}) and sesquiterpenoids (C_{15}), are the most common class of metabolites, and terpene hydrocarbons represent a large fraction of EO composition, along with oxygenated compounds (namely aldehydes alcohols, esters, and phenols) (Raveau et al., 2020). Aromatic and oxygenated compounds are frequently found in EOs composition (Raveau et al., 2020) and related to their biological properties (Aziz et al., 2018). The separation, identification, and quantitative characterization of these complex and volatile substances can be done by analytic methods like gas chromatography (GC), gas chromatography-mass spectrometry (GC–MS), high-performance liquid chromatography with ultraviolet detection (HPLC-DAD), and liquid chromatography-tandem mass spectrometry (LC-MS^n) (Manion et al., 2017).

In general the extraction of fragrances from plants can be made by various methods depending on the botanical material utilized (Tongnuanchan et al., 2014) and

their final use. It can be considered two categories: conventional and innovative techniques (Aziz et al., 2018). The traditional methods are cold pressing (exclusively for the production of citrus EOs), solvent extraction, "enfleurage", and distillation (steam distillation or hydrodistillation) (Stratakos et al., 2016). On the other hand, supercritical extraction with gases, microwave-assisted extraction, controlled pressure drop process, and ultrasound-assisted extraction are relatively novel methods that can be used (Stratakos et al., 2016). The improvement of the traditional methods has led to optimization of the extraction procedure, which includes higher quality and production yield of botanical fragrances, besides efficiency in the energy dissipation and duration of the isolation (Aziz et al., 2018). Nevertheless, as it is defined by the International Organization for Standardization (ISO) *EOs are products obtained from natural raw materials of plant origin, which can be isolated by steam distillation, mechanical processes, or dry distillation after separation of the aqueous phase – if any – by physical processes* (ISO, 2021). Other processes can be used to prepare botanical products which may include fragrances and flavours in their composition along with other compounds of higher molecular weight (waxes, fats, gums, oleoresins, etc.), nevertheless, according to ISO, these are not labelled as EOs.

In all cases, a correct procedure is fundamental to ensure the quality of natural plant products by retaining the bioactivity and natural characteristics without modifying the chemical signature. Besides, plant physiology and growth conditions of the plant material have a significant impact on the yield, quality, and chemical composition of the final product (Angioni et al., 2006). There are several parameters that can influence the quality of the botanical products, such as: harvesting time, culture site (pollution, acidity, soil composition, mineral nutrition), plant age, climatic conditions (rainfall, temperature, light intensity, humidity), root colonization by symbiotic microorganisms (arbuscular mycorrhizal fungi), post-harvest drying and storage, and plant organ used in the extraction procedure (Angioni et al., 2006; Raut et al., 2014).

Of the 3000 EOs described, 300 are commercially valuable (Shankar et al., 2021). Over the years, the medicinal and pharmacological uses of EOs have raised interest, mainly because of their antimicrobial activity against different Gram-negative and Gram-positive bacteria and fungi, and their antioxidant, antiproliferative, antiviral, anti-inflammatory activities (Ayaz et al., 2017; Raut et al., 2014). The food industry also benefits from the biological properties of these botanical products. They can have applicability as food preservatives, flavouring agents, antimicrobials against foodborne pathogens, and prevent oxidation during food processing and/or storage (Falleh et al.,

2020). EOs and their isolated compounds have been traditionally used in cosmetic products and perfumery, not only for their pleasant attributes as fragrances, but also due to their beneficial effects to the body and skin, as natural or natural-like chemical preservatives (Sarkic et al., 2018). In the last years, aromatherapy has become a complementary therapy that uses EOs as therapeutic agents in holistic medicine (Ali et al., 2015).

1.3. EOs and their potential role in agriculture as alternative for the control of plant diseases and pests

Besides the diverse applications mentioned above, more recently EOs have found a potential use in agriculture. The urge of interest in EOs is related to advantages as compared with synthetic products in crop management. One of them is their low persistence in the environment by producing little or no toxic residues, which has less impact on the quality of soils, groundwater or surface water bodies (Essiedu et al., 2020). Besides, there are no evidences of residues in food products (Essiedu et al., 2020). Due to the low hazardous profile of most of their constituents against non-target organisms (e.g., aquatic life and mammals), these can be more compatible with biological control (Alonso-Gato et al., 2021). Additionally, the complex composition of EOs make these products less prone to induce resistance in target organisms (Alonso-Gato et al., 2021). Therefore, EOs or their constituents are considered more eco-friendly than synthetic chemicals, many of them being classified as low-risk biopesticides, and good alternatives as antimicrobial agents (Perczak et al., 2019), insecticides (Hennia et al., 2019), insect repellents (Nerio et al., 2010), nematicides (Andrés et al., 2012) and herbicides (Raveau et al., 2020).

Different natural products of botanical origin are considered useful as chemical compounds in products used in crop management (Table 1). Studies have demonstrated the potential of diverse MAPs species such as *Cymbopogon citratus* (lemon grass), *C. nardus* (citronella), *Eugenia caryophyllus* (clove), *Eucalyptus globulus*, *Melaleuca leucadendron*, *Ocimum basilicum* (basil), *Piper* species, *Rosmarinus officinalis* (rosemary) and *Thymus vulgaris* (thyme) (Dimetry, 2014; Koul et al., 2008). Among the plant families with a more abundant number of relevant MAPs species are Apiaceae, Asteraceae, Lamiaceae, and Myrtaceae families.

Table 1. Chemical substances with active registration in the European Chemicals Agency (ECHA).

Family	**Essential oil**
Asteraceae	*Artemisia herba-alba*
Asteraceae	*Matricaria recutita*
Anacardiaceae	*Schinus terebinthifolius*
Annonaceae	*Cananga odorata*
Burseraceae	*Boswellia carterii*
Cistaceae	*Cistus ladaniferus*
Cupressaceae	*Cupressus funebris*
Cupressaceae	*Juniperus mexicana*
Cupressaceae	*Juniperus mexicana*
Cupressaceae	*Platycladus orientalis*
Fabaceae	*Myroxylon balsamum* var. *pereirae*
Gramineae	*Cymbopogon flexuosus*
Lamiaceae	*Mentha spicata*
Lamiaceae	*Mentha cardiaca*
Lauraceae	*Cinnamomum camphora*
Lauraceae	*Cinnamomum zeylanicum*
Lauraceae	*Litsea cubeba*
Piperaceae	*Piper nigrum*
Rutaceae	*Citrus aurantium*
Rutaceae	*Citrus aurantium* var. *amara*
Rutaceae	*Citrus aurantium* var. *bigaradia*
Umballiferae	*Ferula galbaniflua*

EOs from MAPs belonging to *Lamiaceae* and *Apiaceae* families have shown positive results against agricultural pathogens and pests (Ebadollahi, 2013; Ebadollahi et al., 2020). With this perspective, it is relevant to evaluate EOs from plant species of these families and study their promising bioactivities. In this work, it was considered to study the bioactive potential of EOs from Lamiaceae and Apiaceae family.

As a preliminary analyse it was considered EOs from *Ammi visnaga*, *Satureja montana*, *Thymus mastichina*, and *Trachyspermum ammi*. Although, along with this work, it was studied the biopesticidal properties of *T. mastichina* and *T. ammi* EOs.

A. visnaga L., known as toothpick weed, visnaga, or khella, is a member of the *Apiaceae* family (Khadhri et al., 2011). It is a short annual or biennial herb indigenous to

the Mediterranean region of North Africa, Europe, and Asia (Khalil et al., 2020). The EO extracted from this MAP is known for its proprieties against coronary diseases and bronchial asthma (Khadhri et al., 2011). Besides, *A. visnaga* EO has been studied for its larvicidal, insecticidal and herbicidal activities (Lamiri et al., 2001; Travaini et al., 2016). The main components of this EO chemical profile are 2-methylbutyl-2- methylbutyrate and linalool (Keddad et al., 2016).

S. montana L., also known as mountain savory or winter savory, belongs to Lamiaceae family and it is a semi-shrub native from the Mediterranean region (de Oliveira et al., 2011). It has a strong aromatic flavour and is used in Mediterranean cooking mainly as food condiment (Silva et al., 2009). *S. montana* EO is reported for having pharmacological properties (Tepe et al., 2016), but also its insecticidal, fungicidal and nematicidal activity (Sánchez et al., 2016; Tepe, 2015). Its biological properties are related to the presence of two major chemical compounds, carvacrol and thymol (Silva et al., 2009).

1.3.1. *Thymus mastichina*

Thymus mastichina L. (Lamiales: Lamiaceae) is an endemic species of the Iberian Peninsula, also known as "Spanish marjoram", "Mastic thyme", "Tomilho Bela-Luz" and "Tomilho-alvadio-do-Algarve" (Rodrigues et al., 2020).

The species includes two subspecies: *T. mastichina* subsp. *mastichina*, found across the Iberian Peninsula, and *T. mastichina* subsp. *donyanae*, with specific growing areas such as Huelva and Seville (Spain) and Algarve (Portugal) (Rodrigues et al., 2020). This plant is a semi wood shrub that can reach up to 50 cm of height (Arantes et al., 2019) (Figure 2). It is characterized for its capitula or flower heads formed by small zygomorphic and bilabiate flowers, that blossom from April to June, and also for its opposite leaves (Cutillas et al., 2018; Miguel et al., 2004). *T. mastichina* grows in diverse sites like rupicolous, uncultivated and ruderal fields, and dry stony areas excluding calcareous regions (Miguel et al., 2004; UTAD, 2021).

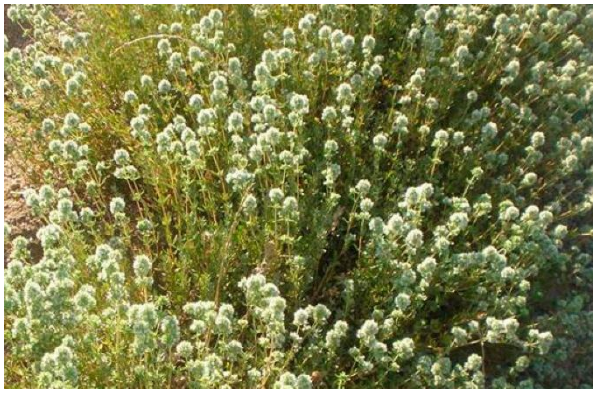


Figure 2: *T. mastichina* plant (*Rodrigues et al., 2020*).

This MAP has a traditional use in cuisine (Barros et al., 2011), in herbal infusions (Barros et al., 2010) and also as source of EOs for perfume and cosmetic industries (Rodrigues et al., 2020). Furthermore, extracts and EOs from the aerial parts of *T. mastichina* have been mentioned for its antioxidant, antiviral, antifungal and antibacterial activities (Rodrigues et al., 2020).

EO extraction from *T. mastichina* is usually done by hydrodistillation, and it has yield percentages from 0.40% to 6.90% (v/w) (Rodrigues et al., 2020). The analysis of *T. mastichina* EO chemical composition by GC-MS shows the existence of hydrocarbon and oxygenated monoterpenes and sesquiterpenes, which are susceptible to percentage variations (Rodrigues et al., 2020). The two main constituents of *T. mastichina* EO are linalool and 1,8-cineole (also referred as eucalyptol). Depending on their presence and ratio, three chemical subtypes (chemotypes) can be identified: the 1,8-cineole/linalool chemotype, the 1,8-cineole chemotype and the linalool chemotype (Rodrigues et al., 2020). Both compounds are reported to have antimicrobial activity (Sonboli et al., 2006). Some studies have been conducted to verify the potential uses of *T. mastichina* EO as a biopesticide (Table 2).

Table 2. Overview of the effective doses of *T. mastichina* activity against species with relevance to agroindustry.

Bioactivity	Plant part	Target organism tested	Effective dosage	Reference
Allelopathic	ns	*Cucumis sativus*	1 µL mL^{-1} (92% seed germination)	Ibáñez et al. (2020a)
		Solanum lycopersicum	1 µL mL^{-1} (42% seed germination)	
Allelopathic	ns	*Lolium multiflorum*	1 µL mL^{-1} (50% seed germination)	Ibáñez et al. (2017)

Table 2 (continued)

Antifungal	ns	*Alternaria brassicae*	MGI = 17% (30% v/v)	(2018)
Antifungal	ns	*Cladobotryum mycophilum*	MGI = 81% (30% v/v)	Diánez et al. (2018)
			ED_{50} = 627 mg L^{-1}	
Antifungal	Ap	*Fusarium avenaceum*	MIC = 1500 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2 mg mL^{-1}	
Antifungal	Ap	*Fusarium culmorum*	MIC = 1500 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2 mg mL^{-1}	
Antifungal	Ap	*Fusarium equiseti*	MIC = 2100 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2.4 mg mL^{-1}	
Antifungal	Ap	*Fusarium graminearum*	MIC =1 500 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2 mg mL^{-1}	
Antifungal	Ap	*Fusarium nivale*	MIC = 2000 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2.4 mg mL^{-1}	
Antifungal	Ap	*Fusarium oxysporum*	MGI = 43% (30% v/v)	Diánez et al. (2018)
Antifungal	Ap	*Fusarium poae*	MIC = 1500 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2 mg mL^{-1}	
Antifungal	Ap	*Fusarium semitectum*	MIC = 2000 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2.4 mg mL^{-1}	
Antifungal	Ap	*Fusarium sporotrichoides*	MIC = 2000 µg mL^{-1}	Fraternale et al. (2003)
			MFC = 2.4 mg mL^{-1}	
Antifungal	ns	*Phytophthora parasitica*	MGI = 79% (30% v/v)	Diánez et al. (2018)
Antifungal	ns	*Sclerotinia sclerotiorum*	MGI = 98% (30% v/v)	Diánez et al. (2018)
Insecticidal	ns	*Spodoptera littoralis*	LD_{50} = 19.3 mL m^3	Pavela (2005)
			LD_{50} = 0.058 µL/larvae	
Insecticidal	ns	*Frankliniella occidentalis*	LC_{50}= 3.56 mg L^{-1}	Stepanycheva et al. (2019)

Legend: Ap, aerial part; ns, not specified. ED_{50}, mean effective dose in inhibiting radial mycelial growth by 50%; MFC, minimal fungicidal concentration; MGI, Mycelium growth inhibition; MIC, minimal inhibitory concentration; LC_{50}, median lethal concentration; LD_{50}, median lethal dose.

1.3.2. *Trachyspermum ammi*

Trachyspermum ammi L. (Apiales: Apiaceae) is a native plant species from Egypt and it is commonly known as ajowan or ajwain, although other denominations can be found, depending on languages variations (Bairwa et al., 2012).

This MAPs grows in arid and semi-arid regions (Central Europe, Middle East, and Asia) and where soils have high salinity levels (Bairwa et al., 2012). It is an annual herb with 60-90 cm of height, characterized for its compound umbel with 4 to 12 umbellets, each containing 6 to 16 flowers (Chahal et al., 2017). The flowers are actinomorphic with white colouration, and its fruits are small and brownish (Figure 3).


Figure 3: *T. ammi* flowers (source: https://world-crops.com/ajowan/) and fruits (source: https://www.istockphoto.com/photo/macro-closeup-of-organic-ajwain-gm499209190-79971399).

Ajowan fruits are commercially valuable, with the harvest being made by the end of the Winter and during Spring (Chahal et al., 2017). Fruits are usually used as a spice and in traditional medicine to combat illnesses like asthma, colds, arthritis, hypertension, indigestion, etc., due its pharmacological properties (Chahal et al., 2017; Singh et al., 2021). Besides, *T. ammi* EO is a source for perfumery and food industries (Anwar et al., 2016; Bairwa et al., 2012).

EO extracted from Ajowan seed-like fruits by hydrodistillation have associated yields of 2% - 4% (Bairwa et al., 2012). From GC-MS analyses, *T. ammi* EO has as main compounds the oxygenated monoterpenes thymol and carvacrol (Pathak et al., 2010). Both monoterpenes are renowned as strong antifungal and antibacterial compounds (Anwar et al., 2016). Thymol is the principal active constituent found in *T. ammi* EO, and it can represent 30% to 60% of its composition (Chahal et al., 2017). Other monoterpenes like *p*-cymene, γ-terpinene and β-pinene are frequently found in high concentrations (Chahal et al., 2017; Subramaniyan et al., 2019).

Apart from its role in industries, *T. ammi* EO potential agricultural use in crop protection has been studied too. Some results showed significant effects against organisms causing damages to crop (Table 3).

Table 3. Overview of the effective doses *T. ammi* EO activity against species with relevance to agroindustry.

Bioactivity	Plant part	Target organism tested	Effective dosage	Reference
Allelopathic	Fr	*Lactuca sativa*	IC_{50} = 25 ppm	Mirmostafaee et al. (2020)
Antifungal	Fr	*Beauveria bassiana*	MGI = 100% GI = 93.1 -52.3% SI = 100% (235.2 µg L^{-1} air)	Jamali et al. (2021)

Table 3 (continued)

Antifungal	Fr	*Lecanicillium lecanii*	MGI = 100% GI = 96.2% SI = 100% (235.2 µg L^{-1} air)	Jamali et al. (2021)
Antifungal	Fr	*Purpureocillium lilacinum*	MGI = 100% GI = 79.4% SI = 100% (235.2 µg L^{-1} air)	Jamali et al. (2021)
Insecticidal	Fr	*Aethina tumida*	LD_{50} = 66.64 µg/adult	Bisrat et al. (2020)
			LC_{50} = 89.03 mg L^{-1} air	
Insecticidal	Fr	*Bruchus chinensis*	R (90%) = 4 µL L^{-1} air	Upadhyay et al. (2011)
Insecticidal	Fr	*Plodia interpunctella*	LC_{50} = 4.33 µL L^{-1} air	Soni et al. (2016)
Insecticidal	Fr	*Sitophilus oryzae*	LC_{50} (adults) = 0.37 µL cm^{-3}	Chaubey (2012)
Insecticidal	Fr	*Sitophilus zeamais*	LC_{50} (adult) = 0.323 µL cm^{-3} R (100%) = 0.8% (v/v)	Chaubey (2018)
Insecticidal	Fr	*Tribolium castaneum*	LC_{50} (adult) = 310 µL L^{-1} air	Jamali et al. (2021)
			LC_{50} (adult) = 13.48 µL LC_{50} (larval) = 11.62 µL	Chaubey (2007)
Insecticidal	Fr	*Trogoderma granarium*	1000 ppm (Adult mortality)	Kavallieratos et al. (2020)
Nematicidal	Fr	*Bursaphelenchus xylophilus*	LC_{50} = 0.431 mg mL^{-1}	Park et al. (2007)

Legend: Fr, Fruit. IC_{50}, median inhibitory concentration; GI, germination inhibition; MGI, mycelium growth inhibition; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; R, repellence; SI, sporulation inhibition.

1.4. Bacterial diseases of plants

Crops can be exposed to diverse bacterial pathogens that can severely affect their yield and growth. In crop fields it is often difficult to promptly detect the presence of those pathogens, mainly because visible symptoms of disease may take some time to develop. Consequently, early treatments are specially challenging to farmers. Bacterial diseases prejudice the production and economies of producers. Ultimately, in more severe cases such as those reported in African countries concerning the cassava bacterial pathogen and banana wilt pathogen, it might also originate hunger and despair

(Borkar et al., 2016). The *Pseudomonas syringae* species complex is among the most severe phytopathogens damaging food production.

1.4.1. *Pseudomonas syringae*

Pseudomonas syringae van Hall (Pseudomonadales: Pseudomonadaceae) is a Gram-negative bacterial species complex composed of different plant pathogens (Dye et al., 1980). This group of bacteria has been a model to unravel molecular mechanisms of plant-microorganism interactions and comprehend bacterial pathogenicity (Xin et al., 2018).

The *P. syringae* species complex incorporates ten species (Arnold et al., 2019). Additionally, it is divided into different intraspecific taxa where each one can infect a specific group of plant species (Arnold et al., 2019). Over 60 pathovars are described, and most of the significant economic crop species are affected by this bacterial group (Baltrus et al., 2017; Xin et al., 2018). This pathogen can colonize different plant tissues, including bark parenchyma, fruits, seeds, and seedlings (Preston, 2017). Even though *P. syringae* may exist in non-host environments, it is usually found in the epiphytic phase on plant species that can be infected by it (Helmann et al., 2019; Preston, 2017).

1.4.1.1. *Pseudomonas syringae* pathovar *actinidiae*

Pseudomonas syringae pathovar *actinidiae* Takikawa, Serizawa, Ichikawa, Tsuyumu & Goto (Psa) is the causal agent of bacterial canker of kiwifruit (BCK), responsible for significant economic losses in kiwifruit production (Donati et al., 2014; Pereira et al., 2021). Its presence was reported in Japan, Korea, China, and Italy before the devasting epidemic outbreak that occurred in central Italy during the year 2008 (Donati et al., 2014). A few years after this outbreak, the production losses were substantial across several countries with important kiwifruit industries (Spain, France, New Zealand, Italy, and other countries), including Portugal. According to EPPO (2021), since 2012, Psa is categorized in the European and Mediterranean Plant Protection Organization (EPPO) A2 list (quarantine pest and which is present in the EPPO region). In 2020, the European Union registered this disease in the emergency measures (EPPO, 2021).

This pathogen categorizes into 5 distinct biovars (biovars 1, 2, 3, 5, and 6) based on toxin production ability and genetic diversity (Sawada et al., 2019). Besides that, the severity of the disease associated with each Psa biovar can be different even though the symptoms do not differ from one biovar to another (Pereira et al., 2021). The one

responsible for the 2008 outbreak in Italy is named biovar 3, the most virulent of all biovar populations (Moura et al., 2015). The fast spread relates to the capacity of quick infection among vines of different ages and plants of different cultivars (Khandan et al., 2013).

BCK´s symptoms are easily observed on aerial parts, such as trunks, leaves, leaders, canes, flowers, fruits, and leaves (Figure 4). On leaves, the symptoms associated are necrotic foliar spots surrounded by a chlorotic halo (Figure 4A). This disease induces wilting of branches that can consequently provoke fruit dropping (Figure 4B). On trunks and leaders, it occurs exudation with ooze turning from dark white to a reddish-brown colour (Figure 4C). Also, it might occur colour change of the vascular tissues underlying the root cortex (EPPO, 2014).

Figure 4: Symptoms of BCK in kiwifruit. A-Necrotic spots surrounded by halos in a kiwifruit leaf, B-blossom necrosis, C-red-rusty exudates in a kiwifruit trunk (Kyeong Son et al., 2016).

During later winter or early spring, disease development and primary infection are facilitated by physical damage caused by rain, frost, and high winds (Donati et al., 2020). BCK symptoms can be unnoticeable until it reaches temperatures between 10 and 20 °C that urges invasive infection (Pereira et al., 2021). Psa cells spread through leaves and young shoots, and young plants are more susceptible (Pereira et al., 2021). Natural openings such as lenticels, stomata, trichomes, or hydathodes can contribute to the progression of Psa (Renzi et al., 2012). Also, infected bud flowers leads to the production of infected pollen which is rapidly spread by the pollinators (Donati et al., 2018). Temperatures above the ideal and a decrease of leaf wetness are responsible for the disease latency period during summer (Donati et al., 2020). When Autumn weather conditions are favourable, it leads to secondary symptoms, like withering and trunks cankers (Donati et al., 2020). Fruit and leaf abscissions scars are the principal re-entry or entry points of the pathogen at this season (Donati et al., 2020). The disease cycle of the bacterial canker of Actinidia has a stage of plant dormancy where Psa overwinters in roots and woody trunks (Pereira et al., 2021). The end of this phase happens to be when

xylem functionality reactivates. The root pressure development induce: swelling of dormant buds which preludes to bud break; reactivates cankers, or leads to the formation of new ones; provokes cracking and exudation of the trunk, leaders and canes (Donati et al., 2020).

Transmission factors of Psa can be related to contaminated soil and water, seeds and organic material, equipment used to manage kiwifruit orchards, operators, or wounds caused by animals (Pereira et al., 2021). Phytosanitary passports were created as a first measure of control to prevent potential transfers of contaminated biological material (Carvalho et al., 2017). In the case of Portugal, the official entities obligate the maintenance of a record of all plants purchased and the detailed history of the exits of plant materials (fruits and pruning wood) (DGAV, 2014a). Preventive measures have been implemented like: disinfection of agricultural tools, especially pruning tools; restrict access to orchards; monitoring the grafting process since it can be a source of contamination; installing protection systems against the wind, frost, and rain; providing water and nutrients in quantities suitable for healthy plant growth; limiting infection by lice, birds, cicadas, through the installation of mosquito screens (Mauri et al., 2016; Pereira et al., 2021). Also, breeding has become an option to acquire disease-resistant cultivars from kiwi cultivars less susceptible to a Psa infection (Kisaki et al., 2018; Vanneste, 2017).

The preventive practices mentioned above can be complemented with chemical treatments, but their effectiveness depends on when it is applied, so it is essential to act in the initial stage of the disease (Pucci et al., 2018). Copper products are often used against Psa, preventing the entrance of the bacterium in exposed lesions of the vines (Pereira et al., 2021). However, deleterious effects on ecosystems and the emergence of copper-resistant bacterial populations have been linked to excessive use of copper-based formulations (Simonetti et al., 2020). On the other hand, antibiotics can be effective, but these are not authorized in the European Union (Donati et al., 2014). The antibiotic treatments allow to slow down Psa progression by mitigating the multiplication of the pathogen and reducing the number of successful infections (Pucci et al., 2018), though, leaving residues on kiwifruits (Pereira et al., 2021) and potentiating acquired multiresistance (Mariz-Ponte et al., 2021). Apart from the ecotoxicological impact, the continuous application of those products decreases their efficiency, and no longer beneficiate kiwifruit producers in the management of KBC. Therefore, further investigation is needed to enlighten more effective control measures against *Pseudomonas syringae* pv. *actinidae* infection. Some *in vitro* assays have been carried

out to evaluate the potential of EOs as more sustainable alternatives for the control of BCK. EOs studied that show antibacterial activity against Psa are shown in Table 4.

Table 4. Antibacterial activity of EOs tested against *P. syringae* pv. *actinidiae*.

Plant species	Family plant	Plant part	Effective dosage	Reference
Cinnamomum cassia	Lauraceae	Bk	IZD = 12 – 22 mm (10µ/disc)	Song et al. (2016)
Cinnamomum zeylanicum	Lauraceae	ns	MIC_{90} = 912 ppm	Pucci et al. (2018)
Melaleuca cajuputi	Myrtaceae	Lf	IZD = 10 mm	Pucci et al. (2018)
Melaleuca linariifolia	Myrtaceae	Lf	IZD = 10 mm (10µ/disc)	Song et al. (2016)
Mentha suaveolens	Lamiaceae	Ap	MIC/MBC=6.25 gL^{-1}	Vavala et al. (2016)
Monarda didyma	Lamiaceae	Sd	MIC=50% (v/v)	(Mattarelli et al., 2017)
Monarda fistulosa	Lamiaceae	Sd	MIC=0.25-1% (v/v)	(Mattarelli et al., 2017)
Origanum vulgare	Lamiaceae	ns	MIC_{90} = 830 ppm	Pucci et al. (2018)
Pimenta dioica	Myrtaceae	Fr	5000 ppm (24h)	Song et al. (2016)
Pimenta racemose	Myrtaceae	Lf	5000 ppm (24h)	Song et al. (2016)
Syzygium aromaticum	Myrtaceae	ns	MIC_{90} = 875 ppm	Pucci et al. (2018)
Thymus vulgaris	Lamiaceae	ns	MIC_{90} = 855 ppm	Pucci et al. (2018)

Legend: Ap, aerial part; Bk, bark; Fr, fruit; Lf, leaf; ns, not specified; Sd, seeds. IZD; inhibition zone diameter. MBC, minimum bactericide concentration; MIC, minimum inhibitory concentration.

1.4.1.2. *Pseudomonas syringae* pathovar *tomato*

Pseudomonas syringae pv. *tomato* (Okabe) Young, Dye & Wilkie (Pst) is the etiological agent of bacterial speck of tomato, which is responsible for damages in tomato crops worldwide (Pietrarelli et al., 2006). Agricultural explorations affected by this bacterial pathovar have a reduction in yield and quality/marketability in fresh markets and processed tomatoes (Kravik, 2021). The first description of tomato plant symptoms

related to Pst was in 1933 in two different locations: the United States of America (Bryan, 1933) and Chinese Taipei (Okabe, 1933).

This pathovar preferentially survives on leaves around trichomes, sub-stomatal chambers, and between epidermal cells (Varvaro et al., 1993). Its survival is linked to the use of organic matter carried by the wind and also substances leached from the leaf (Schneider et al., 1977). Splashing waters play a role in providing favourable conditions for survival and growth of this microorganism and as well for lateral dissemination (Pietrarelli et al., 2006). The impact force of water on leaves' surface might eliminate part of the bacteria present, but populations located in a protected site can re-proliferate in the leaves (Pietrarelli et al., 2006). Low numbers of its population do not cause disease unless favorable conditions are assembled and lead to the production of visible damages (McGrath, 2021). Besides high relative humidity conditions, the multiplication of this pathogen can be beneficiated by crucial factors like temperatures under 24 °C, absence of antagonists and discharge of toxic substances (CropIPM, 2009; Upper et al., 2003). The bacterial proliferation ceases when higher temperatures are recorded (Kravik, 2021).

Bacterial speck of tomato is associated with lesions of various sizes and shapes on leaves, fruit spurs, stems, petioles, and fruits (McGrath, 2021). It usually starts with symptoms appearing in younger leaves which are more susceptible, although it can appear first in older leaves (McGrath, 2021). When leaves in the early growing season are severely affected, it has a significant impact on the crop yield (McGrath, 2021). The foliage symptoms induced by Pst are generally black lesions surrounded by a yellow chlorotic halo twice the size of the necrotic region (Pernezny et al., 2017) (Figure 5A). It leads to more frequent damages near leaf margins which can cause marginal necrosis, and in case of a severe infection it can lead to defoliation (CropIPM, 2009). The speck lesions on green fruit are sunken, small, black spots or superficial flecks from dark brown to black colour in the case of ripe fruit (Pernezny et al., 2017) (Figure 5C). Stems petioles can show dark brown or black lesions with oval-shaped elongation (Pernezny et al.,

2017) (Figure 5B). The more intense is the plant infection, the more plant development slows down, leading to a delay in the harvest of tomato fruits (McGrath, 2021).

Figure 5: Symptoms of bacterial speck induced by *Pseudomonas syringae* pv. *tomato* on tomato leave (A), stems (B) and fruits (C) (*CropIPM, 2009*).

The spread of bacterial speck tomato has possibly different origins. The initial source of this pathogen can be infected transplants and seeds, contaminated tools, lateral contamination by infected plants in the surroundings, and incorrect practices of operators (McGrath, 2021). The pathogen can survive on stakes used to support the plants, plant debris in the soil, seeds, and weeds from the Solanaceae family (McGrath, 2021). Its ability to survive on the surface of non-host plants makes it a potential cause for damages in the following cropping season (McGrath, 2021). So, cultural control practices play a key role in mitigating the proliferation of this disease and its potential consequences. In crop management it is important to: destroy crop residues; disinfect pruning tools and stakes/trellises; eliminate alternative hosts; use pathogenic-free seeds, transplants and irrigation water (Gullino et al., 2020).

As an additional option, chemical control has been based on the application of copper-based formulations (Kravik, 2021). During the most susceptible stages of growth, the protectant activity of copper can prevent infection of the bacterium. Over the years, intensive use of copper compounds led to reports of resistance development of bacterial populations (Griffin et al., 2017). The presence of copper-resistant *Pseudomonas syringae* pv. *tomato* is extremely difficult to manage, therefore, it is essential to develop new approaches.

As an alternative, several natural products have been tested *in vitro* and have shown positive results, such as plants EOs when evaluated against *Pseudomonas syringae* pv. *tomato* (Table 5).

Table 5. Antibacterial activity of EOs tested against *P. syringae* pv. *tomato*.

Plant species	Family plant	Plant part	Effective dosage	Reference
Achillea cartilaginea	Asteraceae	Wp	IZD = 12-20 mm	Vasinauskiene et al. (2006)
Achillea filipendulina	Asteraceae	Fw or Lf	IZD = 6-12 mm	Vasinauskiene et al. (2006)
Acorus calamus	Acoraceae	Rz	IZD = 12-20 mm	Vasinauskiene et al. (2006)
Anethum graveolens	Apiaceae	St and Lf	IZD = 6.3 mm (10 µL/disc)	Soylu et al. (2009)
Carum carvi	Apiaceae	Fr	IZD >6 mm (4 µL/disc)	Cantore et al. (2003)
Cinnamomum verum	Lauraceae	Lf	IZD = 15 mm (5 µL/disc)	Ibáñez et al. (2020b)
Citrus sinensis	Rutaceae	Br	IZD = 31 mm (50 µL/well)	Sabir et al. (2017)
Coriandrum sativum	Apiaceae	Fr	IZD > 6mm (4 µL/disc)	Cantore et al. (2003)
Cuminum cyminum	Apiaceae	Fr	IZD >6 mm (8 µL/disc)	Cantore et al. (2003)
Dalea foliolosa	Fabaceae	Lf	MIC=155 µg mL^{-1}	Villa-Ruano et al. (2017)
Eucalyptus globulus	Myrtaceae	Lf and St	IZD = 12 mm (10 µL/disc)	Ibáñez et al. (2020b)
		Lf	IZD = 30 mm (50 µL/well)	Sabir et al. (2017)
Foeniculum vulgare	Apiaceae	Sd	IZD = 6.5 mm (10 µL/disc)	Soylu et al. (2009)
Gaultheria procumbens	Ericaceae	Lf	IZD = 14 mm (5 µL/disc)	Ibáñez et al. (2020b)
Lavandula angustifolia	Lamiaceae	Fw	IZD = 10 mm (10 µL/disc)	Ibáñez et al. (2020b)
Melaleuca alternifolia	Myrtaceae	Lf	IZD = 11 mm (5 µL/disc)	Ibáñez et al. (2020b)
Mentha pulegium	Lamiaceae	Lf	IZD = 45 mm (50 µL/well)	Sabir et al. (2017)
		Wp	IZD = 6-12 mm	Vasinauskiene et al. (2006)

Table 5 (continued)

Origanum majorana	Lamiaceae	LF and Fw	IZD = 14 mm (20 µL/disc)	Ibáñez et al. (2020b)
Origanum vulgare	Lamiaceae	Fw	IZD = 9 mm (1 µL/disc)	Ibáñez et al. (2020b)
		Lf	MIC= 23.1 mg mL^{-1}	Carezzano et al. (2017)
Rosmarinus officinalis	Lamiaceae	Ap	IZD = 14 mm (20 µL/disc)	Ibáñez et al. (2020b)
			IZD = 21 mm (50 µL/well)	Sabir et al. (2017)
Syzygium aromaticum	Myrtaceae	Lf	IZD = 15 mm (5 µL/disc)	Ibáñez et al. (2020b)
Thymus mastichina	Lamiaceae	Lf and Fw	IZD = 14 mm (20 µL/disc)	Ibáñez et al. (2020b)
Thymus vulgaris	Lamiaceae	Ap	IZD = 33 mm (50 µL/well)	Sabir et al. (2017)
		Lf	MIC = 11.5 mg mL^{-1}	Carezzano et al. (2017)
Zingiber officinale	Zingiberaceae	Rz	IZD = 12 mm (20 µL/disc)	Ibáñez et al. (2020b)

Legend: Ap, aerial part; Br, bark; Fw, flower; Fr, fruit; Lf, leaf; Rz, rhizome; Sd, Seed; St, stem; Wp, whole plant. IZD, inhibition zone diameters; MBC, minimum bactericide concentration; MIC, minimum inhibitory concentration.

1.5. Fungal diseases of plants

Annually, plant diseases have an economical negative impact in agriculture and 70 to 80% have as causal agent pathogenic fungi (Peng et al., 2021). Fungal diseases are responsible for deteriorating plants during growth or post-harvest storage (Singh et al., 2017). They are characterized for inducing scabs, wilting, mould, rotted tissues, rusts, and blotches (Pujari et al., 2014). Fungal infections may cause severe impairment of plant physiology, plant health, and plant homeostasis (Shuping et al., 2017).

Fungicide treatments have been an essential tool for maintaining healthy crops and high-quality yields. Different synthetic fungicides have been applied to control postharvest decay in vegetables and fruits, but its repetitive use ended in developed resistant strains, turning them gradually ineffective (Yang et al., 2019). In consequence, fungicide resistance is a widespread problem in global agriculture. Over the years, it

have been registered resistant pathogen populations related to a several agricultural crops (FRAC, 2020). For example, the intense use of several fungicides to manage fungal diseases in tomato (*Solanum lycospermum* L.), the world's second most consumed vegetable, is related to serious fungicide resistance problems (FRAC, 2020).

The control of these diseases is of utmost importance not only to reduce agricultural losses but also to ensure food security, since phytopathogenic fungi are responsible for synthesizing toxic secondary metabolites and induce high levels of mycotoxins in colonized plant tissue (Logrieco et al., 2003). Mycotoxins have been linked to human and animal health problems developed by ingestion, inhalation, or contact. The toxicity might cause prompt reactions (e.g., sore throat, dermatitis, cold, and flu symptoms), or perhaps long-term problems (Mamgain et al., 2013; Refai et al., 2013). Mycotoxigenic fungi are responsible for significant economic loss to producers worldwide, such as the plant pathogenic *Alternaria* species. The genus *Alternaria* is responsible for at least 20% of agricultural spoilage (Nowicki et al., 2012), and being the cause of decay and decomposition for over 400 host plants (Pegg et al., 2014). *Alternaria* spp. can live as saprophytes or weak parasites, and it is common to find in all kinds of environments (Mamgain et al., 2013). Usually, it is present in the soil or on decaying plants, but it can be found in agricultural commodities and seeds (Logrieco et al., 2003). *Alternaria* spp., typically named "field fungi", require high humidity in the substrate (>20%) to grow and synthesize mycotoxins (Logrieco et al., 2003). Some examples of those mycotoxins are altenuene, alternaric acid, alternariol, alternariol monomethyl ether, altertoxin I and II (Asam et al., 2013; Logrieco et al., 2003).

The species *Alternaria alternata* (Fries) Keissler (Pleosporales: Pleosporaceae) is a wide spectrum ubiquitous necrotrophic fungal pathogen, affecting a variety of agricultural crops such as tomato, which is responsible for causing post-harvest losses at high frequency (Feng et al., 2007). The range of crops affected by this pathogen can vary from fruit crops, vegetable crops, flower crops, and among others.

Fungal infection by *A. alternata* occurs when plants are debilitated by wounds, senescence, or different stresses (Mmbaga et al., 2011). It is an opportunistic pathogen that penetrates plant tissue through lesions on the plant surface or natural openings (e.g., stem ends, pedicels, stomata, or lenticels) and breaking through the host cuticle (Troncoso-Rojas et al., 2014). The *A. alternata* conidia after adhering to the cuticle, start to colonize the plant tissue. In case of this process happens in unripen tissue, it remains latent for weeks until the fruit ripens (Troncoso-Rojas et al., 2014). This is suggested to occur because of specific changes during fruit ripening. The aspects that are related to

this event are: alterations in the host pH; decrease of crop treatments with fungicide products; accessibility through cell walls due to fruit softening and ethylene induction; reduction in the capacity of host defence responses (Prusky et al., 2013). The infection pathway on fruits happens through chilling/sunburn damages or mechanical wounds, and it can occur before, during, or after harvest (Troncoso-Rojas et al., 2014). Its infection benefits from high moisture conditions and temperatures between 25 and 28°C.

On infected leaves, *A. alternata* infection appears as irregular and circular spots with a brown to dark brown color (Figure 6). The circular marks merge and lead to large patches known as leaf blight. Tender twigs and pods may show a dark spot in severe cases (Mamgain et al., 2013). Also, *A. alternata* is associated to heart rot (Aloi et al., 2021), leaf blotch and fruit spot (Gur et al., 2017), and post harvest fruit rot (Alam et al., 2019).

Figure 6: Tomato plant with symptoms of *A. alternata* infection (*Ontario, 2009*).

The dissemination of this pathogen can happen by water, wind, animals, and tools. Infected seeds with *A. alternata* spores or mycelium are another vehicle of potential infection into crop fields. This fungus can survive in perennial crops, susceptible weeds, or infected plants left on the ground (Mamgain et al., 2013). In order to diminish its propagation, it is important to properly store clean certified seeds, provide an adequate plant mineral nutrition, manage weeds which are potential reservoirs, and eliminate infected residues from a previous crop in the field (Mamgain et al., 2013).

Chemical control remains the main measure to reduce the incidence of postharvest diseases in various fruits and vegetables. But the issue of the development of fungicide resistant populations among crop pathogens leads to seek safer and eco-friendly alternatives to reduce postharvest losses. The synergic activity of EOs have been considered an option to overcome that problem. Considerable efforts have been

made to find natural products capable of controlling fungal infections. Among these, several EOs have been tested successfully *in vivo* and *in vitro* against *A. alternata* (Table 6).

Table 6. Antifungal activity of EOs tested against *A. alternata*.

Plant species	Family plant	Plant part	Effective dosage	Reference
Acacia karroo	Fabaceae	Lf	MGI = 400 ppm (>50%)	Sajid et al. (2020)
Baccharis ochracea	Asteraceae	Lf	MIC = 10 µL mL^{-1}	Tomazoni et al. (2019)
Baccharis trimera	Asteraceae	Lf	MIC = 5 µL mL^{-1}	Tomazoni et al. (2019)
Cinnamomum zeylanicum	Lauraceae	ns	IZD = 43.6 mm (2µL/disc)	Kishore et al. (2007)
Citrus reticulate	Rutaceae	Pl	MGI and SPI = 0.2 µL mL^{-1}	Chutia et al. (2009)
Foeniculum vulgare	Apiaceae	Wp	CGI and GTEI = 10 µg mL^{-1} air MGI = 240 µg ml^{-1} air	Soylu et al. (2015)
		Ap	CGI and MGI = 500 ppm	Mahmoudi (2017)
Laurus nobilis	Lauraceae	Wp	MGI = 560 µg ml^{-1} air CGI and GTEI = 160 µg ml^{-1} air	Soylu et al. (2015)
Lavandula stoechas	Lamiaceae	Wp	MGI = 640 µg ml^{-1} air CGI and GTEI = 120 µg ml^{-1} air	Soylu et al. (2015)
Moringa oleifera	Moringaceae	Lf	MGI = 200 ppm (>80%)	Sajid et al. (2020)
Ocimum basilicum	Lamiaceae	Lf	MIC = 1.25 µL mL^{-1}	Marco et al. (2020)
Origanum onites	Lamiaceae	Wp	MGI = 20 µg mL^{-1} air	Soylu et al. (2015)
Piper hispidinervum	Piperaceae	Lf	MGI = 1 mg mL^{-1}	Nascimento et al. (2008)
Syzygium aromaticum	Myrtaceae	ns	IZD = 36.6 mm (2 µL/disc)	Kishore et al. (2007)

Table 6 (continued)

Tetraclinis articulata	Cupressaceae	Ap	0.4 µL L^{-1} air (>50% infection inhibition)	Bouayad Alam et al. (2017)
Thymus capitatus	Lamiaceae	Ap	0.4 µL L^{-1} air (>70% infection inhibition)	Bouayad Alam et al. (2017)
Thymbra spicata	Lamiaceae	Wp	MGI = 80 µg mL^{-1} air	Soylu et al. (2015)

Legend: Ap, aerial part; Lf, leaf; ns, not specified; Wp, whole plant. CGI, conidial germination inhibition; GTEI, germ tube elongation inhibition; MIC, minimum inhibitory concentration; MGI, Mycelium growth inhibition; SPI, spore production inhibition.

1.6. Impacts of weeds in plant production

Agricultural production can face serious constraints due to uncontrolled growth of weeds. This biotic factor interferes with water management, competes with crops for resources, reduces yield and quality of food production, and shelters crop pests (Zimdahl, 2018). For example, early in the growing season, annual grasses might be a problem to corn fields because of their competition for nitrogen, an essential nutrient for growth and yield of this crop (Hartzler, 2009). Producers, in response to potential damage, rely on weed management to limit competition by minimizing weed numbers and allowing the crop to have advantage in capturing resources over weeds.

Lolium multiflorum Lam. (Poales: Poaceae), also named Italian ryegrass, is a weed species associated to winter- and spring-sown cereals, oilseed rape, flax, vegetable crops and orchards (CABI, 2021) (Figure 7). It is a highly competitive weed that can reduce yield from wheat and corn crops up to 60% (Pagnoncelli Junior et al., 2021).

Figure 7: *L. multiflorum* plant (source: https://jb.utad.pt/especie/Lolium_multiflorum#imagem-9969).

This species is a winter annual grass native from central and southern Europe, north-west Africa and south-west Asia that is currently dispersed throughout temperate

areas worldwide (Niinomi et al., 2013). It is an undesirable weed in agricultural fields, although it is common to cultivate it as a forage crop and cover crop (Brunharo et al., 2018). The successful competition of these plants towards plantation crops is due to its capacity of a high rate of germination, synchronization of germination, and seed longevity (Gundel et al., 2008).

According to Bagavathiannan et al. (2021), Italian ryegrass life cycle starts with seed germination when temperatures are relatively low during fall, although winter weather conditions decelerate its growth. With the increase of temperatures, *L. multiflorum* continues its physiological development until flowering from May to July. At the time seed maturation occurs, the plant usually dies. A sizable seed bank is produced to ensure its resurgence in the fall.

Over the years, weed management has relied upon the regular use of synthetic herbicides. However, the repeated use of the same class of herbicides has resulted in reports of herbicide-resistant biotypes (Gundel et al., 2008). One of the chemical substances most used for weed control in agricultural areas is the broad spectrum systemic herbicide glyphosate (N-(phosphonometyl) glycine) (Dill et al., 2008). The first report of a glyphosate-resistant *L. multiflorum* population occurred in orchards of Chile in 2003 (Perez et al., 2003). Since then, its resistance has rapidly spread. A possible explanation for this fact is related to the obligate-outcrossing and self-incompatible breeding system associated with this weed (Brunharo et al., 2018). Also, the wind has an active role in this dispersal, which can carry out pollen from *L. multiflorum* for at least 3 km (Niinomi et al., 2013). Besides, the ability of herbicide-resistant populations to increase the time of seed dormancy and postpone the seedling emergency as a result of adaptation to early-season controls was reported (Gundel et al., 2008). The latest generation of synthetic products produced as an alternative to glyphosate also have negative impacts, leading to similar environmental problems (Dumas et al., 2017).

The problems related to synthetic herbicides, such as environmental and health issues, have led to the interest in developing new approaches to manage weeds. An approach proposed by researchers is to take advantage of chemical interactions among plants. Studies related to the allelopathic properties of some MAPs species have demonstrated effectiveness of these allelochemicals in controlling the growth of other plants. Therefore, the use of such allelochemicals is considered a potential method in weed control before and after planting crops. EOs activities and their herbicidal potential over *L. multiflorum* are summarized in Table 7.

Table 7. Allelopathic activity of EOs tested against *L. multiflorum*.

Plant species	Family plant	Plant Part	Effective dosage	Reference
Eucalyptus citriodora	Myrtaceae	ns	0.25 µL mL^{-1} (Seedling growth)	Ibáñez et al. (2019b)
Lavandula angustifolia	Lamiaceae	ns	0.25 mg mL^{-1} (Seed germination) 0.5 µL mL^{-1} (Seedling growth)	Ibáñez et al. (2019b)
Mentha piperita	Lamiaceae	ns	0.125 µg mL^{-1} (Seed germination)	Ibáñez et al. (2018)
Origanum majorana	Lamiaceae	ns	1 µg mL^{-1} (Seedling growth)	Ibáñez et al. (2017)
Origanum vulgare	Lamiaceae	ns	0.125 µg mL^{-1} (Seed germination)	Ibáñez et al. (2017)
Pimpinella anisum	Apiaceae	ns	0.250 mg mL^{-1} (Seedling growth)	Ibáñez et al. (2018)
Satureja montana	Lamiaceae	ns	0.125 µg mL^{-1} (Seed germination)	Ibáñez et al. (2018)
Thymus mastichina	Lamiaceae	ns	1 µg mL^{-1} (Seedling growth)	Ibáñez et al. (2017)
Zingiber officinale	Zingiberaceae	ns	1 µg mL^{-1} (Seedling growth and Seed germination)	Ibáñez et al. (2019a)
		ns	0.1 mg mL^{-1} (Radical elongation)	Smeriglio et al. (2019)

Legend: ns, not specified.

1.7. Impacts of insect pests in plant production

Insect pests are one of the principal causes of damage in agricultural production and storage (Sharma et al., 2017). Two-thirds of known species from class Insecta are phytophagous that feed on living plant material (excluding pollen and nectar) (Douglas, 2018). Pest outbreaks are induced by a combination of factors: actions that inadvertently facilitate the population growth, seasonal pest biology, synchrony of host plant resources, and asynchrony of natural enemy species that otherwise limit pest population growth (Macfadyen et al., 2018). Other aspects can also facilitate dissemination

pathways, such as the purchase of non-free pest plants, and nursery stocks infected (Macfadyen et al., 2018).

Insecticides have been a powerful tool in controlling pests, principally the ones with synthetic origin. Their intensive and large-scale application has conducted to negative effects for the environment and human health. Also, the development of insect resistant populations and the incidence of multiresistance is frequently reported, which is a serious problem across the world with repercussions in crop yields and consequently economic losses (Hawkins et al., 2019).

Tuta absoluta Meyrick (Lepidoptera: Gelechiidae), also known as tomato leaf miner, tomato borer, South America tomato moth, or South America tomato pinworm, is an importante agricultural pest associated with tomato crops (Gebremariam, 2015). Although it feeds on numerous solanaceous plant species, it preferentially opts for tomato plants (Siqueira et al., 2000). This pest can quickly increase its population in suitable agro-ecological conditions, spreading rapidly in new areas and causing economically relevant damage. Its invasion occurs in both greenhouse and field conditions (Gebremariam, 2015).

From South America, it has spread to other regions of the world. In 2004, it was added to the EPPO A1 List of pests recommended for regulation (pests absent from the EPPO region), and, during 2006 it was first reported in Spain (Tropea Garzia et al., 2012). Soon after, it was present in Italy, France, Greece, Portugal, and other European countries (Tropea Garzia et al., 2012). Meanwhile, in 2009, it was transferred to the A2 list (pests locally present in the EPPO region) (Tropea Garzia et al., 2012). Also, damages caused by the tomato leaf miner are described from the Middle East (Baniameri et al., 2012), to North of Africa (Desneux et al., 2011), and other countries like Nepal, India, Burkina Faso (Bajracharya et al., 2016; Son et al., 2017; Swathi et al., 2017).

The life cycle of *Tuta absoluta* has four stages described in Figure 8.

Egg (4-6 days)

- Elliptic shape
- 0.2mm within and 0.4mm in length
- From white colour to yellow during embryonic development

Larvae (1-2 weeks)

- Fourth instar stages
- Black head with a yellow body, later acquire green colouration and at last stage pink shades
- 7-8 mm in length

Pupae (12 days)

- 4 mm in width and 10 mm in length
- Present on soil or remaining in silky cocoons on leaves
- Starts with a green colouration and it fades to brown

Adult

- Lifespan: 10-15 days for females and 6-7 days for males
- 1 cm long with a wingspan of about 1 cm
- Mottled grey colouration
- Females are bigger and more voluminous than the males

Figure 8: Life cycle of *T. absoluta* (Desneux et al., 2010; Tropea Garzia et al., 2012).

Adults activity is usually in the early morning and evening, while during the daytime they hidden in the vegetation (Illakwahhi et al., 2017). Fecund females tend to preferentially oviposit on leaves at any stage of tomato plants development (Proffit et al., 2011). *T. absoluta* has the capacity of overwinter as adults, pupae, or eggs, depending on environmental conditions (Illakwahhi et al., 2017). In the Mediterranean area, climatic conditions allow it to be a non-diapausing species during winter time (Cocco et al., 2015).

Reduction in crop growth and yield can be directly related to larval feeding due the decrease of plants photosynthetically active surface. The larvae feed on leaf parenchyma tissue, tender portions of the stems (especially axillary buds), and in developing and mature fruit, provokes a reduction in leaf area, fruit rot, bud drop, and fruit malformation (Proffit et al., 2011). Besides, these feeding activities induce multiple lesions that make the plants more vulnerable to pathogenic diseases (Tropea Garzia et al., 2012). This pest also causes also post-harvest losses because of the movement of larvae into the fruits (Figure 9). Accidental trades of infested fruits are assumed as presumably the main pathway for the international spread of this pest (Desneux et al., 2010).

Figure 9: Foliar and fruit damage caused by *T. absoluta* larvae (Aziz et al., 2018).

A set of strategies can be applied to mitigate the unwanted effects of *T. absoluta*. Cultural methods (e.g., crop rotation with non-solanaceous crops, destruction of infested plants and post-harvest plant debris), insect-proof screens, natural enemies, mass trapping control, and resistant varieties of tomato are used to control this pest (Illakwahhi et al., 2017). Nevertheless, the application of synthetic pesticides is the main method to control this pest worldwide (Tropea Garzia et al., 2012), and the repeated application of these products has enforced selection pressure, favouring the survival of resistant genotypes, and leading to the emergence of resistance (Lietti et al., 2005; Siqueira et al., 2000). Some chemical classes of insecticides are reported to have resistance problems such as indoxacarb, avermectins, milbemycins, diamides and others (IRAC, 2021).

The insecticide resistance reports show the strategy based on this kind of chemical control are not a sustainable solution. There is a need to develop other products to control insect crop pests that could be more eco-friendly and equally efficient. In this context, natural products with effective insecticidal activity, namely EOs, have been studied to evaluate their potential against *T. absoluta* (Table 8).

Table 8. Insecticidal activity of EOs tested against *T. absoluta*.

Plant species	Family plant	Plant Part	Effective dosage	Reference
Artemisia absinthium	Asteraceae	Ap	LD_{50} (24h) = 0.65 mg cm^{-2}	Umpierrez et al. (2012)
Citrus reticulata	Rutaceae	Pl	LC_{50} (ingestion) = 3.79 mg mL^{-1}	Campolo et al. (2017)
Citrus sinensis	Rutaceae	Pl	LC_{50} (ingestion) = 1.53 mg mL^{-1}	Campolo et al. (2017)
Elettaria cardamomum	Zingiberaceae	Sd	LC_{50} (24h) = 1.55 µL L^{-1} air LC_{50} (24h) = 1.87 µl L^{-1} air	Goudarzvand Chegini et al. (2017)
Eupatorium buniifolium	Asteraceae	Wp	LC_{50} (24h) = 0.50 mg cm^{-2}	Umpierrez et al. (2012)
Ocimum gratissimum	Lamiaceae	Ap	RI_{50} = 0.13 µL mL^{-1}	Essoung et al. (2020)
		Ap	LC_{50} = 0.24 µL mL^{-1} LT_{50} =17.39h	
		ns	OD = 5 mg mL^{-1}	Yarou et al. (2018)
Ocimum kilimandscharicum	Lamiaceae	Ap	RI_{50} = 0.5 µl mL^{-1} LC_{50} = 0.43 µL mL^{-1} LT_{50} = 15.49h	Essoung et al. (2020)
Satureja bachtiarica	Lamiaceae	Fwb	LC_{50} = 0.43 µL mL^{-1} LT_{50} = 15.49h	Rahmani et al. (2020)
Satureja khuzistanica	Lamiaceae	Fwb	LC_{50} = 17.51 µg L^{-1} air	Rahmani et al. (2020)
Satureja rechingeri	Lamiaceae	Fwb	LC_{50} = 34.33 µg L^{-1} air	Rahmani et al. (2020)
Tetraclinis articulata	Cupressaceae	Ap	0.04 µL ml^{-1} air (1^{st}, 2^{nd}, 3^{rd} larval mortality ≥80%, 1.5h) 0.2 µL mL^{-1} air (4^{th} larval mortality ≥80%, 1.5h)	Bouayad Alam et al. (2017)

Table 8 (continued)

Thymus capitatus	Lamiaceae	Ap	0.04 µL mL $^{-1}$ air (1st larval mortality ≥80%, 1.5h) / 0.2 µL mL^{-1} air (2^{nd}, 3^{rd}, and 4^{th} larval mortality ≥80%, 1.5h)	Bouayad Alam et al. (2017)

Legend: Ap, aerial part; Fwb, flower bud; ns, not specified; Pl, Peel; Sd, Seed; Wp, whole plant. LC_{50}, median lethal concentration; LT_{50}, median lethal time; OD, oviposition deterrence; RI_{50}, median repellent index.

2. Objectives

Essential oils have been considered an eco-friendlier option in comparison to synthetic pesticides. The need to search for solutions with lower environmental impact, urges the search for potential natural products with relevance in crop management.

Therefore, this thesis aimed to determine if *Thymus mastichina* and *Trachyspermum ammi* EOs have a relevant agricultural application concerning a large range of activity: antibacterial, antifungal, herbicidal, and insecticidal activity. To accomplish this major objective, we defined the following 4 specific goals:

- Assessment of the antibacterial activity *in vitro* against the two phytopathogens: *Pseudomonas syringae* pv. *actinidiae* and *Pseudomonas syringae* pv. *tomato*
- Assessment of the antifungal activity *in vitro* and *in vivo* against *Alternaria alternata*
- Assessment of the herbicidal activity *in vitro* towards *Lolium multiflorum*
- Assessment of the insecticidal activity through oviposition deterrence on *Tuta absoluta*

3. Materials and Methods

3.1. Media and reagents

During the assays it was required to prepare different media. For bacteria assays it was prepared King's B medium (KB), Mueller-Hinton Agar (MHA), and Mueller-Hinton Broth (MHB). Fungi assays required the preparation of Potato Dextrose Agar (PDA), and Potato Dextrose Broth (PDB).

Since EOs are poorly soluble in water, a solvent or emulsifier is frequently used to promote a homogeneous dispersion of EOs constituents in water or a water-based products (e.g., culture media). The choice of the solvent or emulsifier was made depending on the target organism to be tested and the type of bioassay (Table 9).

Table 9. Reagents used in the preparation of EOs' solutions/emulsions.

	Reagents	Assay
Emulsifier	Tween® 20 (0.1% w/v)	Broth microdilution Germination and seedling growth
Solvents	Acetone (99.8% purity, EMSURE®)	Oviposition deterrence
	DMSO (99.9 % purity, Fisher Chemicals)	Disc diffusion
	Ethanol (99.8% purity, ITW Reagents)	Fungal mycelium growth Fungal conidial germination *In vivo* tomato infection

3.2. Essential oils

Four EOs from plant species belonging to the Apiaceae (*Ammi visnaga* and *Trachyspermum ammi*), and Lamiaceae (*Satureja montana* and *Thymus mastichina)* families were first considered for a preliminary assessment of their antibacterial activity. Then, two EOs were selected to perform the several assays and tasks defined in the workplan.

In Table 10 are listed the EOs used, their origin and the respective chemical composition provided by the supplier or identified by the team of the EOIS-CropProt project. *Ammi visnaga, Satureja montana* and *Trachyspermum ammi* EOs were purchased from the company Florihana distillerie (Caussols, France). *Thymus mastichina* plant material (aerial parts) was acquired from the Portuguese company Ervital Plantas Aromáticas e Medicinais, Lda. Then it was submitted to a hydrodistillation

process using a Clevenger type apparatus to isolate the EO. All EOs were preserved from light and heat in amber vials.

Table 10. Description of the essential oils used in one or more assays.

Essential oils (EOs)	Supplier	Plants origin	Plant parts	Major compounds
Ammi visnaga	Florihana (EO)	Morroco	Fruits	Linalool (27%), 3-methylbutyl 2-methylbutanoate (16%), pentyl isobutyrate (9%), isoamyl isovalerate (7%)[1]
Satureja montana	Florihana (EO)	Albania/Spain	Flowering tops	Carvacrol (43%), *p*-cymene (17%), γ-terpinene (13%) and thymol (4%) [2]
Trachyspermum ammi	Florihana (EO)	India	Fruits	Thymol (50%), γ-terpinene (25%), *p*-cymene (23%) [1]
Thymus mastichina	Ervital (plants)	Portugal	Leaves and steams	Chemical caracterization in progress [2]

[1] Chemical composition provided by the supplier.

[2] Chemical compositions determined by the research team (Sousa RM and Fernandes-Ferreira M, Biology dept, FCUP)

3.3. *In vitro* evaluation of bacteria susceptibility to EOs

3.3.1. Bacteria strains and culture

Two pathovars of *Pseudomonas syringae*, pv. tomato (Pst) and pv. *actinidae* (Psa), were used in this work. The collection bacterial strains Pst DC3000 and Psa CFBP 7286 (Biovar 3 isolated in Italy in 2008) that were used in the antibacterial assays were gently provided by the Microbial Diversity and Evolution (MDE) group (Biology Department, FCUP, Portugal). The strain has been maintained cryopreserved (-80 ºC) in 30 % (v/v) glycerol in the MDE lab collection. The cultures were recovered from the frozen stocks and grown at 28 ºC on agarized King´s B medium (KB). Bacteria were subcultured on the same medium. Müeler Hinton (MH) solid (MHA) or liquid (MHB) medium were used for inoculum preparation and assays susceptibility tests. In the latter case, 20 mL of MHA was platted in Petri dishes using a 25±0.1 mL graduated sterile pipette. To prepare bacterial suspensions, one or two colonies were selected from 24h to 48h-old growing culture and resuspended in 5 mL of MHB. Bacterial suspensions were incubated (28 ºC, 180 rpm, 24h) before their use in the susceptibility tests. All manipulations and assays were performed on a class II cabinet with vertical laminar flow

at the MDE group lab (Biology Department, FCUP, Portugal), and following all biosafety recommendation for quarantine pathogenic bacteria.

3.3.2. Disc diffusion method

Initially we aimed to define which plant natural product would be used to perform the assays in the target bacteria. As a first approach, we selected four potential EOs from the plant species *Ammi visnaga*, *Satureja montana*, *Thymus mastichina* and *Trachyspermum ammi* (Table 10) to evaluate their potential activity toward both bacteria, using the disc diffusion as a screening method (Moujir et al., 2006). Then, two of the most active EOs were chosen for further evaluation by the microdilution method. For both assays we followed the CLSI standard procedures (CLSI, 2021).

3.3.2.1. Antibiograms

For the disc diffusion assay, 24h bacterial suspension of Pst DC3000 and Psa CFBP 7286 were prepared as described above. The bacteria culture used in bioassays were in the log phase of growth, which is a prerequisite for assay validation (Hudzicki, 2009). Suspension optical density at λ=600 nm (OD_{600}) was adjusted to 0.1 using a spectrophotometer (GENESYS 10UV, Thermo Scientific, EUA) to obtain an equivalent concentration of 2.0 x10^8 CFU mL^{-1}. While that value was not reached, it was necessary to perform dilutions using sterilised MHB. The inoculation of bacteria in MHA consisted of pipetting 1 mL of bacterial suspension per Petri plate, immediately followed by rotatory movements to uniformly spread bacteria onto the medium surface. The excess of suspension was removed with a micropipette.

A volume of 10 µL from each EOs stock solution or DMSO (5 mg $disc^{-1}$**,** negative control of the solvent) was pipetted onto sterile paper discs (6mm of diameter) (FiltraTECH, France), which were positioned as shown in the Figure 10. A positive control with the antibiotic kanamycin (30µg/disc) (antimicrobial discs, Oxoid) and a control plate for bacterial growth were also included in assays.

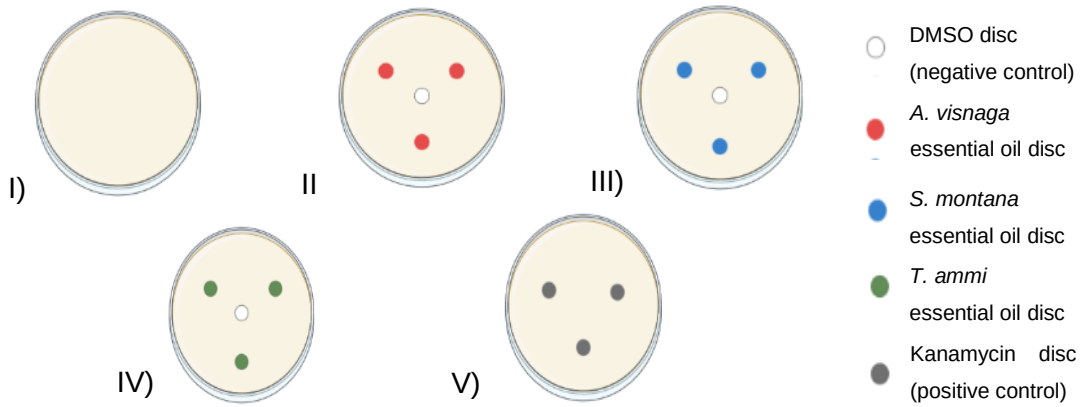


Figure 10: I) Representation of bacterial inoculum as negative control. II) Representation of *A. visnaga* EO antibiogram. III) Representation of *T. ammi* EO antibiogram. IV) Representation of *S. montana* EO antibiogram. V) Representation of kanamycin antibiogram.

The preparation of the EOs consisted in pipetting, in accordance with their densities, the respective volumes of each natural product and the solvent chosen, DMSO (99.9 % purity, Fisher Chemicals) (Table 11). EOs stock solutions were freshly prepared and maintained closed in sterile amber vials until their application in sterile paper discs.

Table 11. Essential oils and concentrations used in susceptibility tests performed by the disc diffusion method.

Essential Oil	**Density (g mL^{-1})**	**V_{EO} (µL)**	**V_{DMSO} (µL)**	**[EO] g mL^{-1}**	**EO_{mass} disc^{-1} (mg)**
A. visnaga	0.865	57.8	42.2	0.5	5
T. ammi	0.910	55	45		
T. mastichina	0.911	55	45		
S. montana	0.899	55.6	44.4		

Petri plates were incubated at 28ºC for 24h. The diameters of bacterial growth inhibition zones (mm) were measured after 24h of growth using a digital caliper, and photographic records were taken using the Gel Doc XR+ system (Bio-Rad USA) and processed by Image Lab software (Bio-Rad, USA). The validation of the assay was made by the existence of bacterial growth in the control plate, as well as the absence of inhibition zone around discs treated with DMSO (solvent negative control).

EOs and antibiotic were tested in triplicate against each bacterial strain and assays with EOs included three repetitions. The mean diameter of the bacterial inhibition zone from each EO and kanamycin were calculated and compared.

Accordingly, to the results obtained in the disc diffusion method, *T. ammi* EO was selected for further studies. Other antibacterial screening assays performed in colaboration with another researcher within the scope of the EOIS-CopProt project showed interesting results of antibacterial activity from *T. mastichina* EO (Fernandes, 2020), represented in Annex 7.1. Therefore, these two natural products were selected to perform the following bioassays.

3.3.3. Broth microdilution method

In this assay we aimed to assess the minimal inhibitory concentrations (MICs) of two EOs from *T. ammi* and *T. mastichina* on Pst DC3000 and Psa CFBP 7286 growth. To evaluate the growth inhibitory effect, we used 96-well microplates, which allows to perform serial dilutions of the natural products. Hence, EOs were tested in the final concentration of 3.64 to 0.23 mg mL^{-1}. EOs stock solutions were prepared in MHB at 4 mg mL^{-1}. For a better dispersion of the EOs, which are poorly soluble in aqueous medium, we use of an emulsifier, Tween® 20 (0.1% w/v). For the negative control, a solution of Tween® 20 with the same concentration was prepared in MHB. A positive control with the antibiotic kanamycin sulphate (PanReac, AppliChem, ITW reagents) was also included in the assays using a freshly prepared stock solution at 0.4 mg mL^{-1}. All solutions to be tested were sterilized through filtration using cellulose filters (0.2 mm) before their use.

To evaluate the growth inhibitory effects of kanamycin as a positive control against Past DC3000 and Psa CFBP 7286 we used the same plate layout (Figure 11). Kanamycin at the highest concentration (400 µg mL^{-1}) was applied in wells of column 2 to perform the serial dilution (180, 90, 45, 23, 11, 6, 2.8, 1.4 and 0.71 µg mL^{-1}).

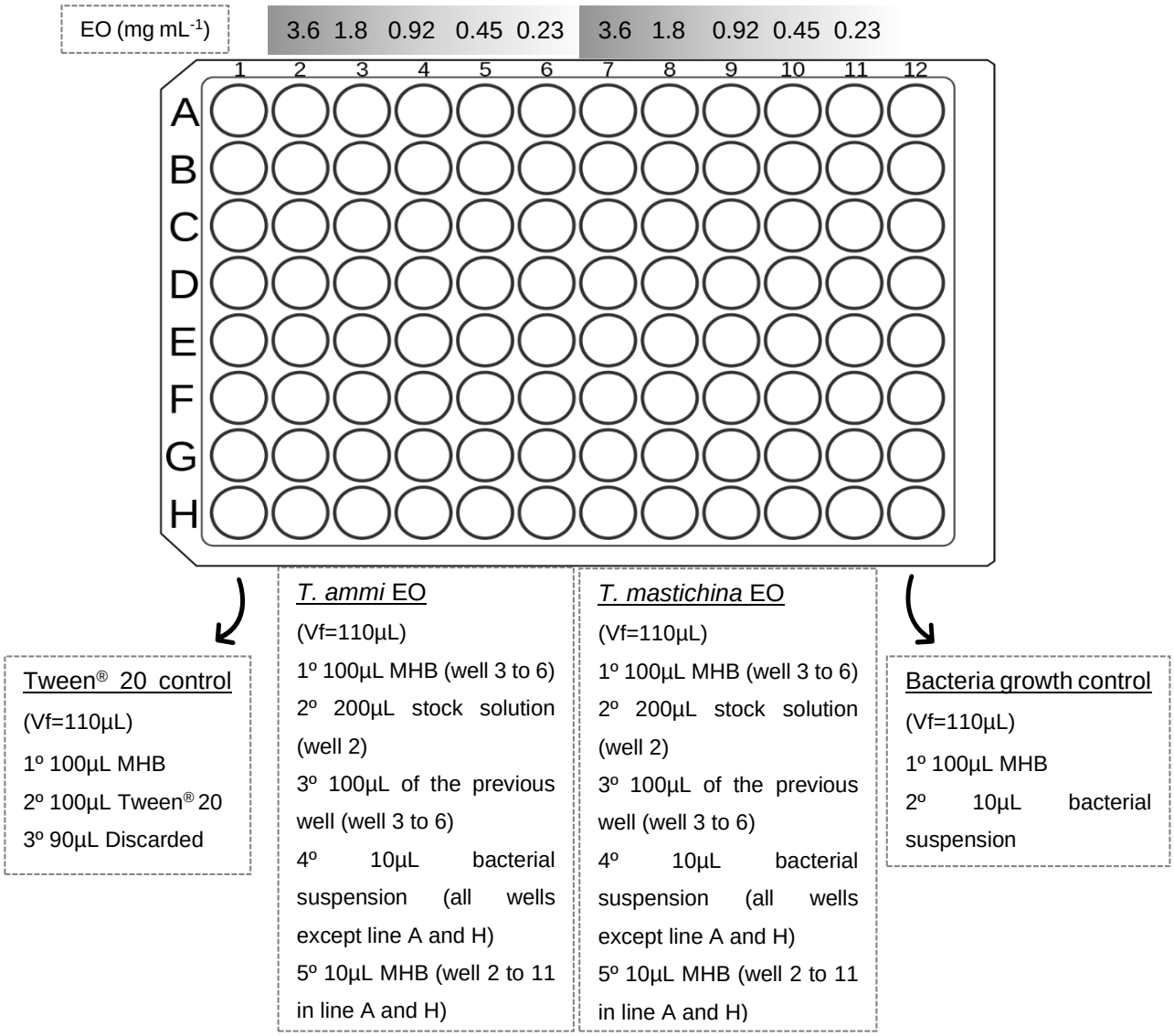


Figure 11: Schematic representation of the method and microplate layout used for the broth microdilution assay.

To evaluate the growth inhibitory effects of kanamycin as a positive control against Past DC3000 and Psa CFBP 7286 we used the same plate layout (Figure 11). Kanamycin at the highest concentration (0.4 mg/mL) was applied in wells of column 2 to perform the serial dilution (180, 90, 45, 23, 11, 6, 2.8, 1.4 and 0.71 µg mL^{-1}).

Wells of column 1 included only MHB, while the column 12 was chosen to be a control for bacterial growth in MHB. Lines A and H included EOs/antibiotic serial dilution in MHB without bacterial inoculum and were used as blanks for correction of absorbances in each respective column.

The bacterial inoculum of Pst DC3000 and Psa CFBP 7286 was prepared as already described and concentration adjusted to an equivalent of 5×10^5 cfu mL^{-1} (Moujir et al., 2006). Ten microliters of inoculum were pipetted into the wells from column 2 to

12 and from between line B to G. Each assay included EOs, and kanamycin tested in triplicate and assays were repeated twice in separate plates.

Microplates were incubated for 48h at 28 ºC and under orbital shaking at 180 rpm (Elmi SkyLine Digital Orbital Shaker S-3.16L). Absorbances were recorder at λ=600 nm using a microplate reader after 24h and 48h of incubation (Thermo Scientific™ Multiskan™ GO, spectrophotometer) with the SkanIt RE 6.1 software (Thermo Scientific™). To assess inhibitory effect towards bacterial growth at each EO and antibiotic concentration, it was applied the formula:

$$\%\ Bacterial\ growth\ inhibition = \frac{(\ Abs\ control\ \ growth - Abs\ treatment\ \ growth)}{Abs\ control\ \ growth} \times 100$$

The MIC values were determined whenever the lowest concentration of each natural botanical product and antibiotic showed a strong inhibition of bacterial growth (>90% bacterial growth inhibition).

3.3.4. Minimal bactericidal concentrations (MBC)

To verify if the EOs concentrations showing strong inhibitory effects on bacterial growth by the microdilution method exhibit a bactericidal or bacteriostatic effect, 10 µl of bacterial suspension were pipetted from wells where no bacterial growth was observed (% bacterial growth inhibition ≥ 90%) and inoculated on solid KB medium. After 24h of incubation at 28 ºC plates were observed, and photographic records were taken using the Gel Doc XR+ system (Bio-Rad) and processed by Image Lab software (Bio-Rad, USA). MBC was estimated whenever bacterial growth was absence.

3.4. Evaluation of EOs antifungal activity on *A. alternata*

3.4.1. Field isolate and subculturing

A fungal strain of *A. alternata* (code: QSDM-A-A_2') was gently provided by the Integrative Biology and Biotechnology (iB2) Laboratory (Biology Department, FCUP, Portugal). The isolates had origin in a field sample collected in 2019 from vine's leaf in Resende (Porto, Portugal).

The isolate was subculture from the preserved stock by placing 5mm × 5mm squares of fungal mycelium in PDA medium. The Petri dishes were sealed with parafilm

and incubated at room temperature. The maintenance of viable cultures was made by regularly subculturing 1cm×1cm squares into Petri dishes with PDA medium (Figure 12). The assays were performed on a class II cabinet with vertical laminar flow at a FCUP lab (Biology Department, FCUP, Portugal), and following all recommendations for manipulation of fungal subcultures.

3.4.2. *In vitro* mycelium growth inhibition assay

This assay evaluated the inhibitory effects on *A. alternata* mycelial growth by *T. ammi* and *T. mastichina* EOs. Different concentrations (2, 1, 0.5, 0.25 and 0.125 mg mL^{-1}) of both EOs were tested. Besides, the fungicide active substance, difeneconazole (Duaxo systemic fungicide, COMPO®) was used as a positive control and tested at 0.125, 0.25, 0.0625 and 0.03125 mg mL^{-1}. The solvent ethanol (99.8% purity, ITW Reagents, USA) previously sterilized by filtration (nylon membrane filters, 0.22 µm) was used to prepare stock solutions of the several EOs concentrations before their incorporation in the culture medium. For that reason, two negative controls were conducted: H_2O and ethanol (1.7% v/v). All treatments and controls were tested in quadruplicate.

Incorporation of the several treatments and solutions into the PDA medium was performed before its solidification by adding volumes to autoclaved PDA medium (~40ºC) (Bouayad Alam et al., 2017). A homogeneous mixing was promoted by gently stirring with a magnetic stirrer for a few minutes (H01 LBX instruments, USA). Then, volumes of 20 mL were promptly distributed in sterile Petri plates for plating and kept for 24h until use.

Discs of fungal mycelium (5 mm in diameter) were excised from one week old culture and transferred to the centre of the Petri plate (Bouayad Alam et al., 2017). Afterwards, all Petri dishes were sealed and incubated for 12 days at 27±1°C. Mycelial growth was recorded and measured with a digital caliper (150mm/6" Digital LCD Vernier Caliper with Fractions, Ketotek, China) for 12 days, until the mycelium growth in the negative control reached the border of the Petri plate.

Values of mycelium diameters were used to determine the mycelial growth inhibition and the index of mycelial growth rate, according to the formulae (1) (França et al., 2018) and (2) (Bouayad Alam et al., 2017):

1. $$\%\, Mycelial\ growth\ rate = \frac{(current\ mycelial\ growth - previous\ mycelial\ growth)}{number\ of\ days\ of\ incubation} \times 100$$

2. $$\%\, Mycelial\ growth\ inhibition = \frac{(negative\ control\ mycelial\ growth - treatment\ mycelial\ growth)}{negative\ control\ mycelial\ growth} \times 100$$

At the end of the evaluation of both EOs inhibitory activity on mycelium growth, it was assessed the viability of the fungal cultures that demonstrated absence of fungal growth. For this purpose, 5 mm plugs of fungal culture were replaced in a PDA plate. The Petri dishes were then incubated at 27±1°C for 7 days, and on the last day it was taken photographic recordings.

3.4.3. Fungicidal activity

3.4.3.1. *In vitro* conidial germination inhibition assay

In this assay we aimed to determine if the concentrations of EOs showing strong inhibitory activity against fungal growth (*T. ammi* EO at 2 and 1 mg mL^{-1}) also had inhibitory effects in conidial germination. The two negative control, H_2O and ethanol (1.6% v/v) were included, as well as the positive control difenoconazole (0.250 mg mL^{-1}). The assay was conducted in 50 mL falcon tubes containing autoclaved Potato Dextrose Broth (PDB), where the mentioned EO's, solvents or fungicide were incorporated. Ten-day old *A. alternata* mycelium was used to prepare a conidia suspension, from which 100 µL was inoculated in each falcon tube to achieve a final volume of 5 mL (Cf = 2×10^4 mL^{-1}) (Feng et al., 2007). Falcon tubes were incubated at 28 ºC on an orbital shaker-incubator (200 rpm) for 24 hours (Sajid et al., 2020). All treatments and controls were tested in quadruplicate.

After that period, conidia were observed under the microscope (ZEISS™, Primo Star) and counted using a haematocytometer. For each replicate, 100 conidia were counted, and the number of non-germinated conidia were recorded to calculate (Feng et al., 2007):

$$\%\ \text{Conidia not germinated} = \frac{\text{number of conidia not germinated}}{\text{total number conidia counted}} \times 100$$

At the end of incubation, 100µL of each replicate was inoculated in PDA plates to evaluated conidia viability. Fungal growth was observed and photographed after 24 and 48 hours of incubation at 28 ºC.

3.4.3.2. *In vivo* tomato infection

The purpose of this assay was to study if the fungistatic concentration of *T. ammi* EO (2 and 1 mg mL^{-1}), previously observed through *in vitro* assay, would be effective against *A. alternata* development on tomato. For that, commercial grown cherry tomatoes (Origin: Portugal), without external lesions and with similar size, were acquired. All fruits were submitted to a disinfection step before use. Tomatoes were immersed in a 0.1% sodium hypochlorite solution for 20 minutes, rinsed with sterilised distilled water and let to dry. Each tomato was placed in an individual Petri plate (Ø =55mm). A lesion (6 mm diameter, 2 mm deep) was made in the equatorial zone of tomato fruits, with the help of a sterile tweezer, where it was pipetted 40 µL of respective controls and treatments (Bouayad Alam et al., 2017; Feng et al., 2011). The experimental design consisted in testing the concentrations of 2 and 1 mg mL^{-1} of *T. ammi* EO, difenoconazole (0.250 mg mL^{-1}) and negative controls (distilled water and ethanol (2 % v/v)). After five minutes, 10 µL of *A. alternata* spore suspension (1×10^4 spores mL^{-1}) was inoculated (Xu et al., 2014). The 10 replicates of the same treatment were joined in a tray covered with cling film and kept at room temperature (19-22°C) (Figure 12). Humidified paper was placed in the bottom of each tray and wetted every day with distilled water to maintain the relative humidity at 50-60%. Condition inside each individual tray were daily monitored with a thermohygrometer and photographic recordings were done at the 2nd, 3rd and 6th day.

Figure 12: Antifungal bioassays performed in tomato fruits.

After 6 days, the number of infected fruits was counted for calculation of the percentage of decayed fruits (Bouayad Alam et al., 2017):

$$\%\ \text{Decayed fruits} = \frac{\text{decayed fruit number}}{\text{total fruit number}}$$

3.5. Evaluation of allelopathic activity of EOs on *L. multiflorum*

3.5.1. Germination test

Initially we aimed to evaluate the allelopathic activity of both EOs on large crabgrass *Digitaria sanguinalis* L. (Scop.) (Poaceae). This plant is a weed known to affect crops productivity, especially soybean, wheat, and maize (Zhou et al., 2013).The harvest of *D. sanguinalis* seeds was made in September 2020 in the field (Northern, Portugal). Seeds were collected and maintained in paper bags in the dark until the germination test was performed.

According to the EPA (2012), germination testing of chemicals could only be validated if the germination was at least 70%. Therefore, we tested the germination rate of *D. sanguinalis* seeds before using it. None of the three germination tests resulted in a satisfactory percentage. Seeds in petri dishes (Ø =85mm) containing one Whatman nº1 paper filter previously soaked with 4 mL of distilled water with light exposure had 43.6% germination and without light 5% germination. Also, seeds sowed in little pots had a rate of germination below the desirable (40%).

We concluded that seeds were not suitable to carry out the testing in accordance with standard protocol. As an alternative, we selected another grass species to test, *Lolium multiflorum* (Poaceae). Likewise, a previous germination test in Petri dishes with light exposure was performed to check seeds viability and estimate their germination percentage (87.5±3.5%) (Figure 13).

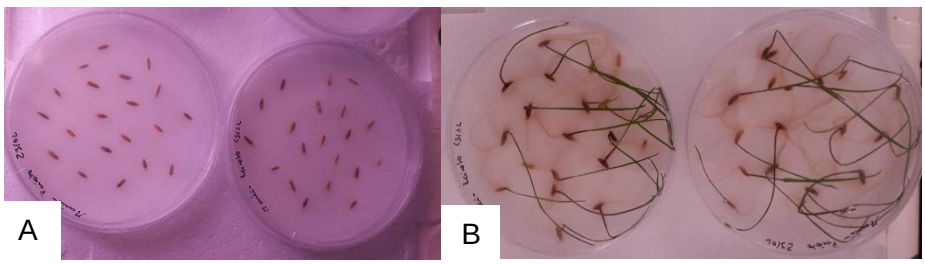


Figure 13: A - *L. multiflorum* seeds at the beggining of germination test. B - *L. multflorum* seeds germinated at day 10.

Therefore, *L. multiflorum* seeds were considered viable and suitable to be used in the following bioassay. They were preserved in the dark at room temperature until use.

3.5.2. Seed germination and seedling growth

T. ammi and *T. mastichina* EOs activity over *L. multiflorum* seed germination and seedling growth was assessed. Assays were performed in Petri plates (Ø=90mm) containing a Whatman No.1 filter paper and 20 seeds/plate (Fagodia et al., 2017). EOs emulsions at the several concentrations were prepared using Tween® 20 (0.1% w/v) as an emulsifier. Concentrations assessed were 1, 0.5, 0.25, 0.125, 0.375 and 0.1875 mg mL^{-1} for *T. ammi* EO and 1, 0.875, 0.75, 0.5, 0.25, 0.125 for *T. mastichina* EO. Distilled water and Tween® 20 (0.1% w/v) were included as negative controls and the herbicide RoundUp® UltraMax (Monsanto) with active substance glyphosate was used as the positive control at 0.125 mg mL^{-1} (within the range of recommended dosage by AgroManual (2020). Filter papers were soaked with 4 mL of the respective EO emulsion, or control solutions and each treatment was tested in quadruplicates (Ibáñez et al., 2020a). Plates were sealed and placed in a phytoclimatic chamber (23 - 26°C and 16:8 L:D photoperiod) for 6 days, until seeds germination in the negative controls attained at least 85%.

At the end of the assays, seeds with the primary root attaining a length of 5 mm were considered as being germinated (EPA, 1996). They were counted and seedlings fresh weight (mg) was determined using an analytical scale (M124Ai-M VWR™, e (g/ct) = 0.001g, USA). In addition, the radicle and coleoptile lengths (mm) were measured with a digital calliper (150mm/6" Digital LCD Vernier Caliper with Fractions, Ketotek, China). Effects on germination and seedling growth were determined by calculating the Germination inhibition (1) (Mirmostafaee et al., 2020), Radicle growth inhibition (2) (Atak et al., 2016) and Coleoptile growth inhibition (3) (Atak et al., 2016) with the following formulae:

(1) $$\%\ Germination\ inhibition = \frac{(negative\ control\ germination - treatment\ germination)}{negative\ control\ germination\ \%} \times 100$$

(2) $$\%\ Radicule\ growth\ inhibition = \frac{(negative\ control\ radicule\ lenght - treatment\ radicule\ lenght)}{negative\ control\ radicule\ lenght} \times 100$$

(3) $$\%\ Coleoptile\ growth\ inhibition = \frac{(negative\ control\ growth - treatment\ growth)}{negative\ control\ growth} \times 100$$

3.6. Evaluation of EOs anti-insect activity on *T. absoluta*

3.6.1. Plant material

Nursery trays filled with a commercial substrate (Profi-Substrat - GramoFlor) were prepared for tomato (*Solanum lycopersicum* L.) seedling growth. Two cultivars were sowed: *Coração de boi* SL/HS and *Rio Grande* VFO-1 (HortoSementes, Lda). The trays were disposed in a phytoclimatic chamber programmed with the following settings: 25 °C and photoperiod: 10:14 L:D. Additionally, it was set a monitor for relative humidity (RH: 70%-80%). Tomato seedlings were transplanted in pots filled with the same commercial substrate, and later staked at the appropriate growth phase. This procedure was repeated whenever more tomato plants were required to guarantee plant material for *T. absoluta* rearing. For the bioassay, tomato plants of the *Rio Grande* VFO-1 variety were used at an initial stage of development (2-3 leaves).

3.6.2. Insect rearing

T. absoluta's cages (1.5m x 0.5m x 1.2m) were installed in a phytoclimatic chamber with the same controlled conditions as describe above for tomato plant growth. A tulle fabric was used to confine *T. absoluta* adults, which were fed with a 5% (w/v) sugar solution. Tomato plants were placed in each cage to maintain *T. absoluta* population.

Field captures of *T. absoluta* occurred in two different places. The biological material collected was leaves with signs of infection of this pest. At first, those captures occurred in an agriculture exploration located in Vila Nova de Famalicão, Braga (Frescura Sublime - Unipessoal Lda., Portugal), where are produced two tomato varieties: Runner and Bigram (Figure 14A). To maintain the *T. absoluta* population, were also collected infested tomato leaves from greenhouse tomato plants from Vairão Campus, Porto (University of Porto, Portugal). The cultivar of those tomato plants is unknown (Figure 14B).

Figure 14: A - Greenhouse tomato plants from Frescura Sublime - Unipessoal Lda. B - Greenhouse tomato plants from Vairão Campus.

The collected leaves were observed in laboratory conditions and living larvae were selected. Then, they were released on tomato plants placed in the cage (Figure 15). This procedure was regularly made to maintain an exponential population.

Figure 15: A - Insect rearing in the phytoclimatic chamber; B - *T. absoluta* larvae from field sample leaves; C: *T. absoluta* larvae in a tomato leaf inside the insect rearing.

3.6.3. Bioassay of non-choice oviposition

This bioassay aimed to evaluate the effects of *T. ammi* and *T. mastichina* EOs on *T. absoluta* oviposition (adapted from Dehghani et al., 2013). Due the hydrophobic characteristic from EOs, an aqueous solution was prepared by using as solvent acetone (99.8% purity, EMSURE®). The natural botanical products were tested at a concentration of 5 mg mL^{-1}, in a single dose assay. In addition to a negative control for the solvent, H_2O was also included as negative control in the assays. The positive control chosen was the insecticide Coragen® (FMC), (active substance: chlorantraniliprole), which was tested at the concentration of 0.04 mg mL^{-1}, following the recommended doses to apply in tomato crops against this pest (AgroManual, 2021). Plastic carboys, cut in the first third and covered with tulle fabric, were used as containers to isolate each tomato plant (replicate) during the experiment (Figure 16).

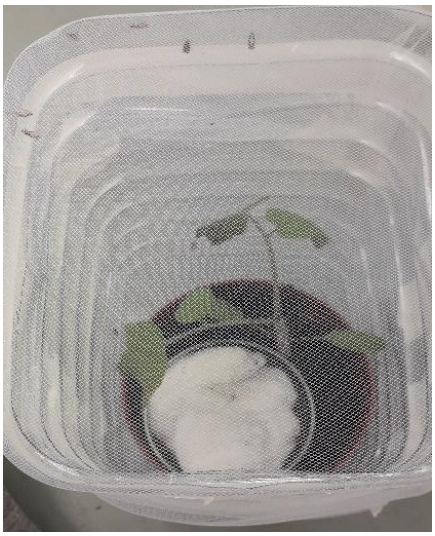


Figure 16: *T. absoluta* adults in contact with tomato plant replica sprayed with *T. ammi* EO solution.

Treatments and controls were freshly prepared and applied uniformly a volume of 3 mL via spray. Thirty minutes later, five *T. absoluta* adult couples captured from the insect rearing case were release in each plant. A 5% (w/v) sugar solution was placed inside each container to feed adults. The replicates were kept in a phytoclimatic chamber at 25°C with 10:14 L:D photoperiod and 70-80% RH. Each treatment was tested in triplicate. After 24h, *T. absoluta* adults were removed and the number of eggs oviposited in each replica was counted. In the assessment of oviposition deterrence was considered 6 areas in the tomato plant: Fa1, Fa2, P1, P2, C and S (Figure 17).

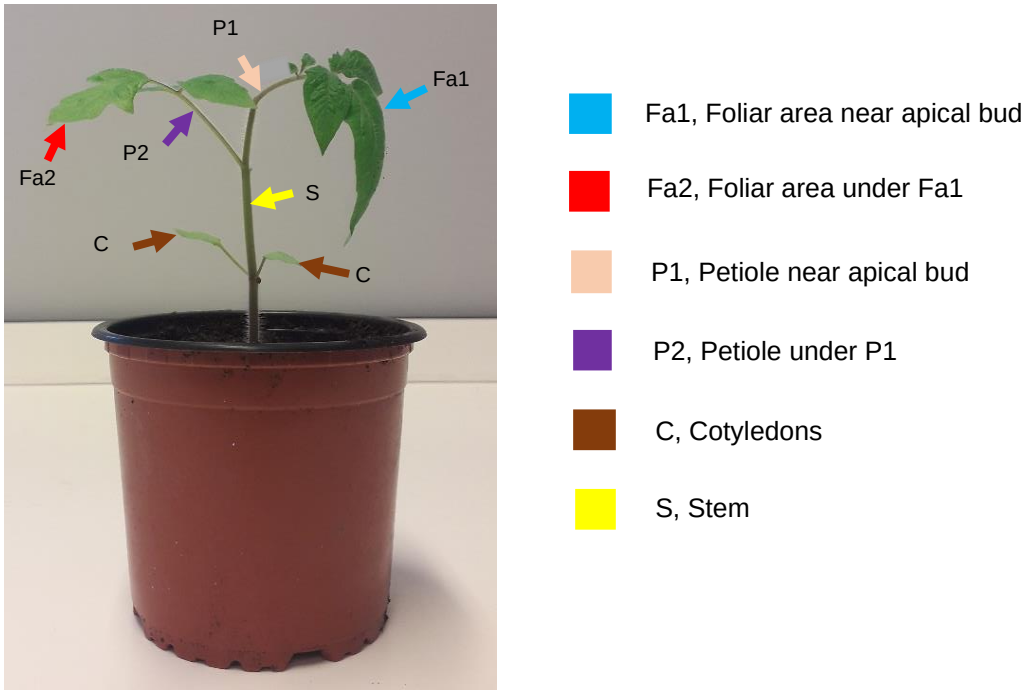


Figure 17: Representation of *S. lycopersicum* (Rio Grande VFO-1 variety) replica with the areas where eggs laid by *T. absoluta* females were assessed.

The oviposition was also analysed through the formula (Dehghani et al., 2013):

$$\%\ \text{Oviposition inhibition} = \frac{C - T}{C} \times 100$$

Where, C corresponds to the number of *T. absoluta* eggs counted on the leaflet sprayed with negative control and T means the number of *T. absoluta* eggs counted on the leaflet sprayed with EOs solution or insecticide.

Tomato plants were maintained in the phytoclimatic chamber for six days. Thereafter, photographic recordings of the leaves were done to later measure the area of leave's damage using the Bioleaf-Foliar Analysis™ application. Other visible symptoms of damage possibly related to treatments application were also recorded according to the following criteria: 0, no signal of foliar chloroses; 1, leaves with signal of chloroses; 2, leaves with signal of necrose; 3, leaves' death.

3.7. Statistical analysis

Analytical statistics was carried out in the statistical package IBM SPSS Statistics 27 for Windows. Data were analysed for treatment effects using one-way analysis of variance (ANOVA). Normality and homogeneity of the variances were checked using the Shapiro–Wilk and Levene tests, respectively, before ANOVA. The means were compared using the Tukey's HSD test ($p<0.05$). When data failed the ANOVA

assumptions, the non-parametric test Kruskal-Wallis was applied and determined significant differences between treatments by Duncan's multiple range test ($p<0.05$).

Statistical graphics were created using the program GraphPad Prism8 (GraphPad Software, Inc., USA).

4. Results and Discussion

4.1. Antibacterial activity of EOs

4.1.1. Inhibition zone diameters

The results of the bacterial susceptibility assessed *in vitro* by the Kirby-Bauer assay using disks impregnated with EOs and the antibiotic kanamycin are shown in Table 12. The inhibitory activity on Psa growth was significantly different among the tested OEs, as well as the antibiotic. Among the EOs tested, *T. ammi* EO caused a higher growth inhibition followed by the EOs from *S. montana* and *A. visnaga*. Concerning Pst susceptibility to EOs, mean values of inhibition halo were similar, with *T. ammi* EO showing a statistically significantly higher effect than *A. visnaga* EO.

Besides, Psa and Pst exhibited higher susceptibility to kanamycin with inhibition halo around 30 mm of diameter at the tested dose of 30 µg/disk. The antibiotic tested showed a more active antibacterial activity than both EOs.

Table 12. Growth inhibition zone (mm) on the pathovars *Pseudomonas syringae* pv. *actinidiae* (Psa CFBP7286) and *Pseudomonas syringae* pv. *tomato* (Past DC3000) after 24h of exposure to *A. visnaga*, *S. montana*, and *T. ammi* EOs (5 mg disc^{-1}), and the antibiotic kanamycin (30 µg disc^{-1}).

	Inhibition halo diameter at 24h (mm) (±SD)			
	A. visnaga EO	*S. montana* EO	*T. ammi* EO	Kanamycin
Psa CFBP7286	6.7 (±0.7)^{d}	8.8 (±0.4)^{c}	11.5 (±1.2)^{b}	29.4 (±0.4)^{a}
Pst DC3000	7.3 (±1.0)^{c}	8.2 (±0.4)^{bc}	8.8 (±1.2)^{b}	30.0 (±0.4)^{a}

Results presented for EOs are mean values (±SD) of triplicates and three repetitions (n=9). Kanamycin was tested in triplicates (n=3). Different lowercase letters indicate significant differences of the mean between EOs and kanamycin for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test.

To the extent of our knowledge, there are no reports about the antibacterial effect of *T. ammi* EO against *Pseudomonas syringae* pv. *actinidiae* or *Pseudomonas syringae* pv. *tomato*. Therefore, the discussion of the results relies on antibacterial activity reported against other Gram-negative bacteria. *T. ammi* EO demonstrated a lower bacterial growth inhibition than reported in other studies against the species *Escherichia coli*, *Klebsiella pneumoniae, Proteus vulgaris* and *Salmonella typhimurium* (Hoferl et al., 2009; Rigane et al., 2016; Vitali et al., 2016). Although, strains from another

Pseudomonas species, *P. aeruginosa* (ATCC 27853 and LMG29), also showed low to moderate susceptibility to this EO with a halo diameter of 8.5 and 11 mm, respectively (Hoferl et al., 2009; Vitali et al., 2016).

Previous works against Gram-negative human pathogens, showed S*. montana* EO with a variable degree of antibacterial activity, in which inhibitory halos varied from 8 to 24 mm (Bezić et al., 2005; Ćavar et al., 2008; Marin et al., 2012). Antimicrobial activity has been associated to the presence of monoterpenes or related compounds such as cymene, carvacrol and thymol in *S. montana* chemical profile (Vitanza et al., 2019).

In this work, the mean inhibition halo obtained for *A. visnaga* EO on Psa was 6.7 (±0.7) mm (approximately the size of the disc), which shows a non-relevant antibacterial effect. However, previous reports, which have tested this natural product against other Gram-negative bacteria, demonstrated higher antibacterial activity (inhibitory zones ranging from 10 to 25 mm) (Al-Snafi, 2015; Rasooli et al., 2007).

As reported by Song et al. (2016), forty-nine EOs were assessed to verify antibacterial activity on other Psa strains. Among the three natural products that showed significant antibacterial activity, *Melaleuca linariifolia* and *M. cajuputii* EOs demonstrated a similar inhibition zone as *T. ammi* EO (inhibitioty halo < 11mm). Relatively to works evaluating Pst susceptibility to EOs, it was reported inhibitory effect in bacterial growth in accordance to the results obtained in this work, with inhibition zones varying from 6 to 12 mm (Vasinauskiene et al., 2006). But other natural products showed a stronger antibacterial activity when tested against Pst (inhibitory halo diameter>12 mm) (Ibáñez et al., 2020b; Sabir et al., 2017).

Overall, the preliminary screening performed by the disc diffusion assay evidenced the low *in vitro* antibacterial activity of the three EOs against the collection strains Psa CFBP7286 and Pst DC3000, except for *T. ammi* EO against Psa. Therefore, data obtained from disc diffusion assay performed under the scope of a current project and including the testing of another MAP species, *T. mastichina,* raised interest in assessing its bioactivity along this work (Annex I).

Susceptibility testing by the disc diffusion assay on agar medium, is one of the most used methods to obtain preliminary information about the susceptibility of bacteria. However, due to high hydrophobicity and volatility of the EOs, a diffusion assay may not be the most accurate to determine antibacterial activity. The low solubility of EOs components in water affects a uniform diffusion in agar and leads to a underestimation of the activity of the EOs (Griffin et al., 2000; Tariq et al., 2019). So, it was also

determined to evaluate the antibacterial activity by performing the broth microdilution method for the EOs of *T. ammi* and *T. mastichina*.

4.1.2. Determination of MIC and MBC values

MIC values estimated by the broth microdilution assay after 24h and 48h are presented in Table 13.

Table 13. Minimal inhibitory concentration and Minimal bactericidal concentration (mg mL^{-1}) of the selected EOs and kanamycin (µg mL^{-1}) against the tested bacterial strains of the pathovars *Pseudomonas syringae* pv. *actinidiae* (Psa) and *Pseudomonas syringae* pv. *tomato* (Pst).

	***T. ammi* EO (mg mL^{-1})**			***T. mastichina* EO (mg mL^{-1})**			**Kanamycin (µg mL^{-1})**		
	MIC (24h)	MIC (48h)	MBC	MIC (24h)	MIC (48h)	MBC	MIC (24h)	MIC (48h)	MBC
Psa CFBP7286	> 3.6	> 3.6	> 3.6	> 3.6	> 3.6	> 3.6	0.7	0.7	0.7
Pst DC3000	1.8	3.6	3.6	> 3.6	> 3.6	> 3.6	0.7	0.7	0.7

Results presented are six replicates and two repetitions (n=12).

T. ammi EO demonstrated growth inhibition for the range of concentration tested when assessed against both bacteria, however, it was only possible to estimate a MIC and MBC value for Pst. It is worth to note that the lowest concentration tested for *T. ammi* EO (0.23 mg mL^{-1}) had some growth inhibitory effect against Psa (14.4 ± 4.9 % at 24h and 26.3 ± 0.4% at 48h) and the inhibition attained for the highest concentration (3.6 mg mL^{-1}) was inferior to 100% (58.6 ± 10.5% at 24h and 52.1 ± 10.0 % at 48h, data not shown). These results suggests that MIC and MBC values would be possibly superior to 3.6 mg mL^{-1}. Overall, results obtained for *T. ammi* EO showed a bacteriostatic and bactericidal activity *in vitro* against Pst but not against Psa.

Regarding *T. mastichina* EO, it was not possible to determine MIC and MBC values for both bacteria. The highest concentration tested against Psa only reached 17.0 ± 8.7% and 17.6 ± 5.2% of growth inhibition at 24h and 48h, respectively. Likewise, none of the concentrations reveal inhibitory effect on Pst bacterial growth. Results obtained for *T. mastichina* EO indicate that this EO does not exhibit relevant bacteriostatic nor bactericidal effect at the tested concentration against both bacterial strains, despite the positive preliminary results obtained from the *in vitro* assay by the disc diffusion method performed by Fernandes (2020) (Annex I).

It is worth to mention that the estimated MIC (24h and 48h) and MBC values for the control kanamycin against both bacteria were the same (0.7 µg mL^{-1}), which demonstrated a higher antibacterial activity of the antibiotic against Psa and Pst when compared to EOs.

As far as we know, there are no reports regarding the *in vitro* antimicrobial activity in liquid medium for both EOs neither against the two bacterial strains in study nor other Gram-negative phytopathogenic bacteria.

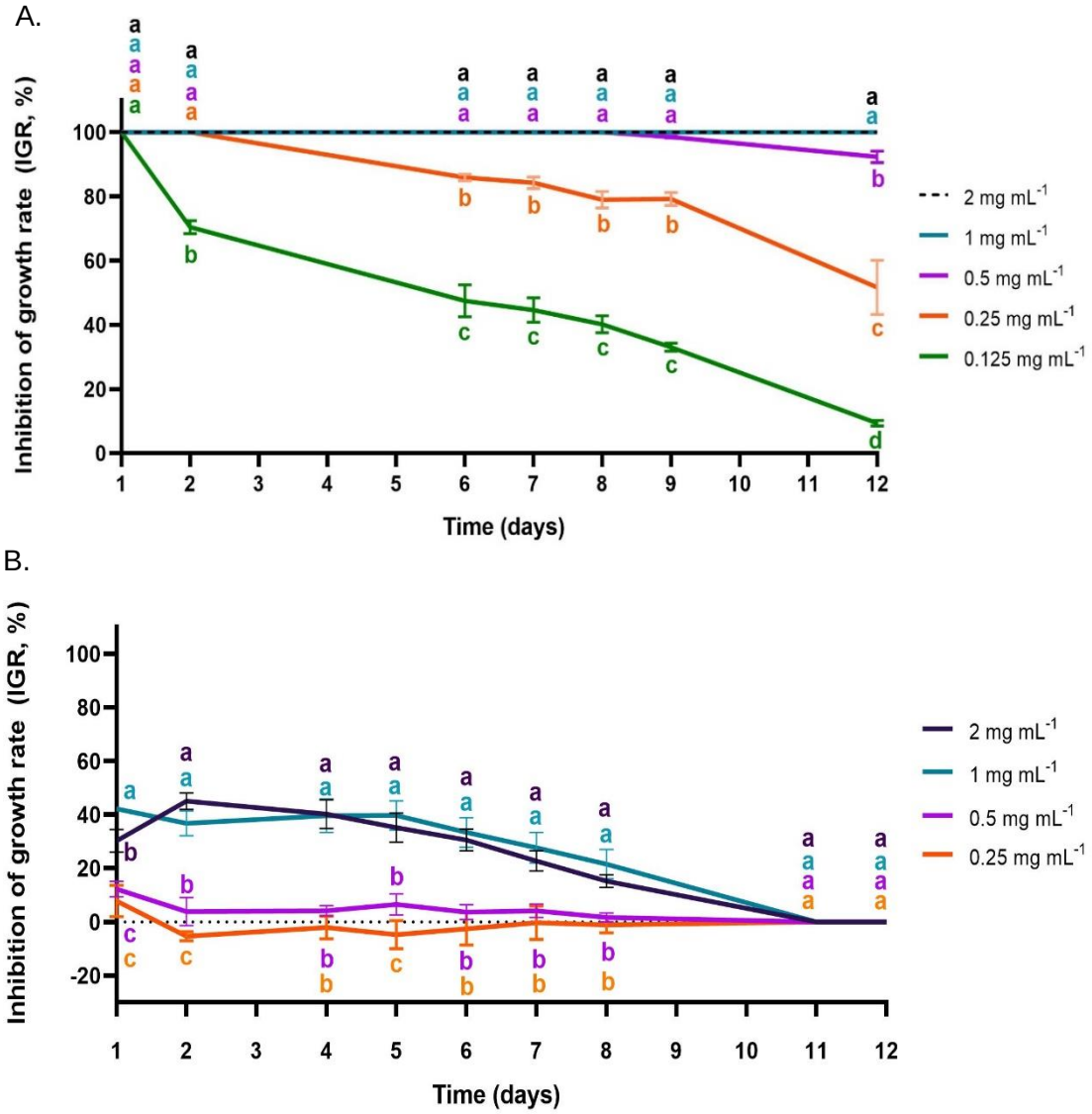
The MIC and MBC values estimated for *T. ammi* EO are in line with reports presented against other bacterial strains, in which values greater than 4 mg mL^{-1} are presented (Patil et al., 2016; Rigane et al., 2016). This natural product also has demonstrated antibacterial activity at lower concentrations, with exception to *P. aeruginosa* ATCC 27853 (MIC and MBC values > 4 mg mL^{-1}) (Moein et al., 2015; Moosavi-Nasab et al., 2016; Oliveira Ribeiro et al., 2020; Vitali et al., 2016). In these studies, it is reported a variation of the main constituent on *T. ammi* EO, thymol, ranging from 15 to 65%. The chemical composition of the *T. ammi* EO tested in this work showed to be constituted by 50% of thymol as already mentioned. According to Bakkali et al. (2008), it is well known that differences in EO chemical profile may determine its biological properties and consequent action against pathogens. Thymol is a terpene phenol associated with destabilization mechanisms of the bacterial membrane, which interferes with cell permeability and induces bacteria death (Xu et al., 2008).

MIC and MBC values for *T. mastichina* EO were not determined in the present study but these would probably be superior to 3.6 mg mL^{-1}, which is higher than values reported against other bacteria (Ballester-Costa et al., 2013; Fraternale et al., 2003). However, according to Cutillas et al. (2018), *T. mastichina* EO extracted from MAPs from different locations demonstrated a wide range of MIC and MBC values when tested against *E.coli*. Authors reported that, depending on the site where the plant was harvested, the antibacterial activity of *T. mastichina* EOs varied from 2.3 to 9.4 mg mL^{-1}, for MIC and MBC values, respectively. The authors hypothesized that this difference in *T. mastichina* EO antibacterial activity might be due to the variation in the content of one of its main constituents linalool (from 50 to 90%) (Cutillas et al., 2018).

4.2. Antifungal activity of EOs

4.2.1. Inhibitory effects on *A. alternata* mycelium growth

The inhibition (%) of *A. alternata* mycelium growth induced by *T. ammi* EO (A), *T. mastichina* EO (B) and difenoconazole (C) are illustrated in Figure 18 (A-C).



C.

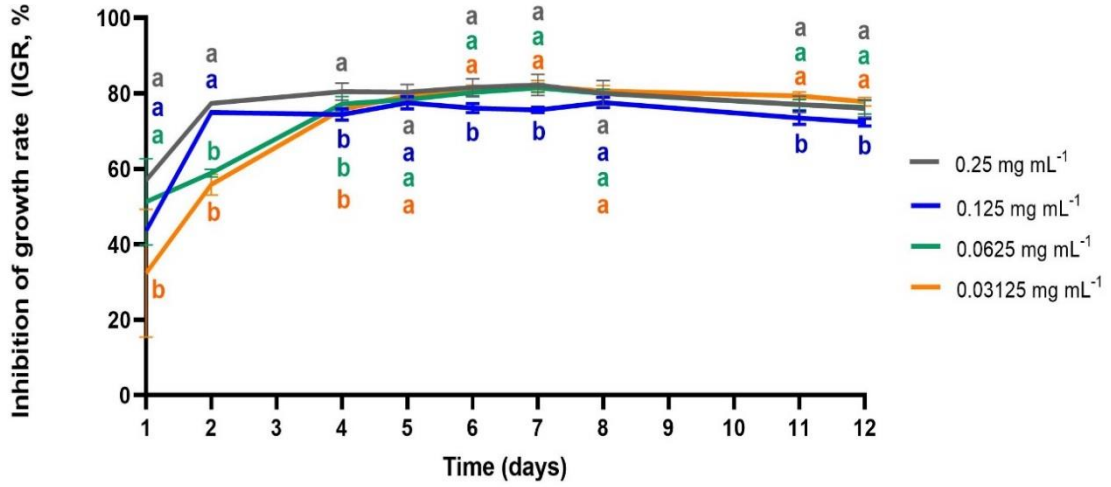


Figure 18: A- Evaluation of the inhibitory effect of *T. ammi* EO in *A. alternata* mycelium growth for 12 days. B- Evaluation of the inhibitory effect of *T. mastichina* EO in *A. alternata* mycelium growth for 12 days. C- Evaluation of the inhibitory effect of difenoconazole in *A. alternata* mycelium growth for 12 days. Different lowercase letters indicate significant differences of the means for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test.

The three higher concentrations of *T. ammi* EO (2, 1 and 0.5 mg mL^{-1}) showed similar antifungal activity against *A. alternata,* with 100 % of mycelium growth inhibition until day 8 (Figure 18 – A). Both concentrations of 2 and 1 mg mL^{-1} maintain their fungistatic activity until day 12. The lowest concentrations of 0.25 and 0.125 mg mL^{-1} exhibited some inhibitory effect on mycelium growth, but their effect decreased throughout time until the end of the assay (47.5 ±5.0% and 9.4 ±0.8%, respectively, at day 12).

The antifungal activity of *T. ammi* EO against isolates of *A. alternata* was previously reported by Khosravi et al. (2015). In their study, the authors used the broth micro dilution and macro dilution methods to estimate Minimal Fungicide Concentrations (MFC) which ranged between 0.19 and 0.78 mg mL^{-1} after 48h. Comparing with the present results at the same day, the MFC assessed for *A. alternata* was 0.25 mg mL^{-1}, which is between the range exhibited from *T. ammi* EO in the mentioned study. Also, EO from *T. ammi* showed an active antifungal activity against entomopathogenic fungi (*Beauveria bassiana*, *Lecanicillium lecanii* and *Purpureocillium lilacinum*) (Table 3). These fungi are used as a biological control, and it reveals the importance of further studies to analyse compatibility between EOs application and other biological controls.

The transfer to PDA Petri dishes of *A. alternata* mycelium plugs treated with *T. ammi* EO concentrations that showed fungistatic effect (2 and 1 mg mL^{-1}), revealed fungicidal activity at the concentration of 2 mg mL^{-1} (data not shown).

Concerning the results obtained with *T. mastichina* EO (Figure 18 - B), we found that the growth inhibition rate of the two higher concentrations was similar. At day 6, around 30% of inhibition was recorded for both (30.5±4.1% for the 2 mg mL^{-1} concentration and 33.4±5.5% for the 1 mg mL^{-1} concentration). However, at day 12 no inhibitory activity was recorded. The other two concentrations tested had negligible activity towards *A. alternata* mycelium growth (3.7±2.8 % for 0.5 mg mL^{-1}, and -2.6±6.0% for the 0.25 mg mL^{-1}). On the last day, no inhibitory effect in *A. alternata* mycelium growth was recorded for any of the concentrations.

As far as we know, *T. mastichina* EO antifungal activity against *A. alternata* was not reported yet. Concerning the genus *Alternaria*, *T. mastichina* EO demonstrated inhibitory effect on *A. brassicae* mycelium growth (17%) at a higher concentration than assessed in this study, 30 % (v/v) (Diánez et al., 2018). Assessments of its bioactivity against pathogenic fungi of vegetables and mushrooms has demonstrated radial growth inhibition values varying from 79 to 98% for concentrations much superior to the ones herein tested (30 % (v/v)) (Table 2). *T. mastichina* EO on other phytopathogenic fungus (*Fusarium* spp.) have demonstrated total mycelium growth inhibitory effects from concentrations between 2 and 2.4 mg mL^{-1} (Fraternale et al., 2003).

Mycelium growth inhibition induced by difenoconazole at different concentrations is represented in Figure 18 - C. The inhibitory effect at day 6 was similar (around 80%) for the concentrations of 0.25, 0.0625 and 0.03125 mg mL-1. In opposition to the time-dependent pattern observed for some EOs doses that showed a loss of activity, the fungicide had inhibitory effect above 70% until the end of the assay.

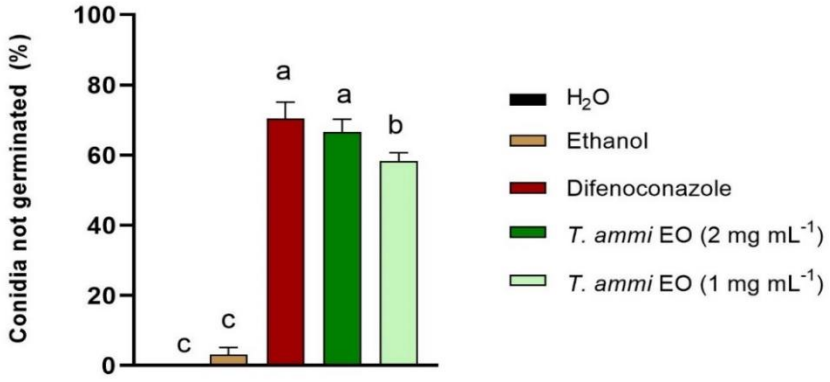
According to these finding, T. ammi EO showed higher activity than T. mastichina EO activity against fungal growth, but lower than the systemic antifungal. Based on these results, T. ammi EO was chosen for further assessments of its antifungal activity.

4.2.2. Inhibitory activity of *T. ammi* EO on *A. alternata* conidia germination

The results concerning the effect of *T. ammi* EO on *A. alternata* conidia germination are illustrated in Figure 19. The percentages of non-germinated conidia after 24h of exposure to EO solutions at 2 mg mL^{-1} and 1 mg mL^{-1} were significantly different (66.6±3.6 % and 58.3±2.3 %, respectively) (Figure 19A). Likewise, a high percentage of conidia did not germinate when exposed to the fungicide difenoconazole (70.4±4.8 %). at a lower concentration tested (0.25 mg mL^{-1}). Figure 19B exhibits the results concerning conidial viability at the end of the assay. As shown in the pictures (Figure

25B), no fungal growth was recorded after conidia exposure to both concentrations of *T. ammi* EO, as well as difenoconazole. These finding suggest that *T. ammi* EO (2 and 1 mg mL^{-1}) exhibit similar fungicidal activity against *A. alternaria* conidia, along with the fungicide at a lower concentration (0.125 mg mL^{-1}).

A.



B.

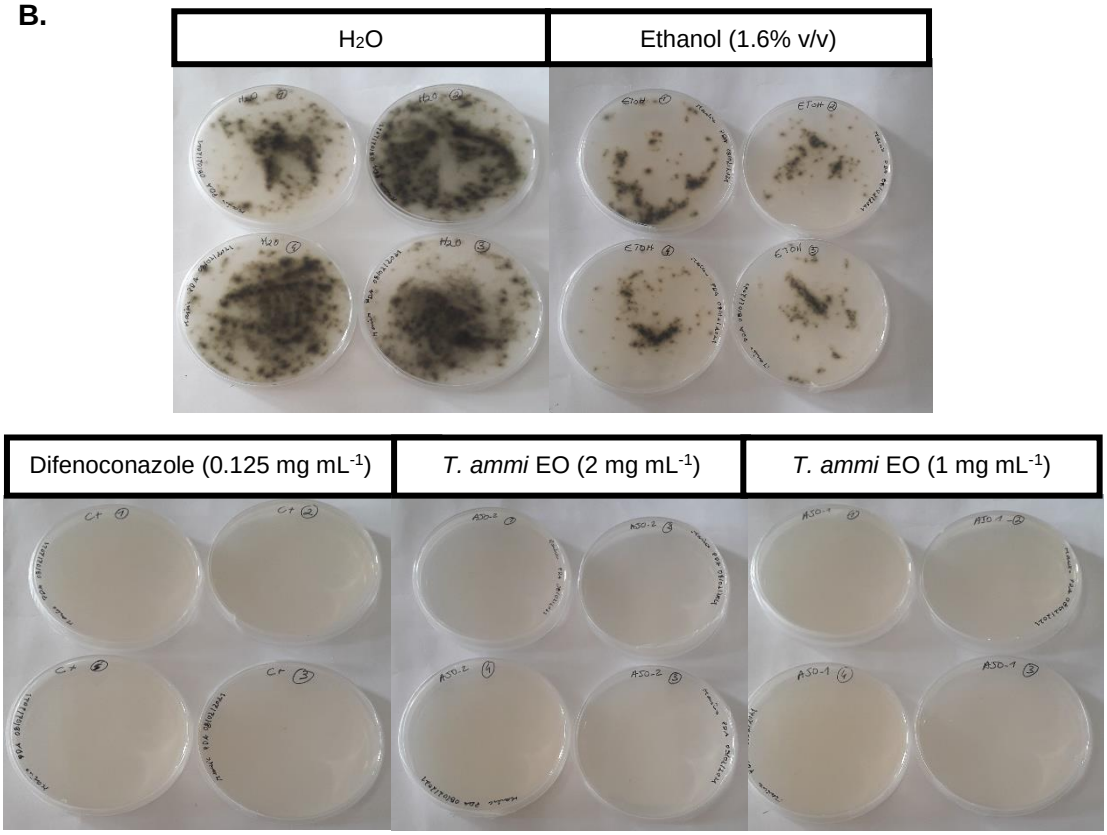


Figure 19: A - Average percentage of *A. alternata* germinated conidia after 24h of exposure to *T. ammi* EO and difenoconazole. The results are expressed as mean (±SD) (n=4). Different lowercase letters indicate significant differences of the means for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test. B - *A. alternata* growth in PDA medium for 48h to assess conidia viability.

To the best of our knowledge, assays evaluating the effects of the studied EOs on conidia germination were not previously performed on *A. alternata*. At higher concentrations than 2 mg mL^{-1}, EOs from *Baccharis ochracea, Baccharis trimera*, *Mentha arvensis* and *Ocimum basilicum* showed an inferior inhibitory effect than *T. ammi* EO (a range between 30 to 60% of inhibition) (Perveen et al., 2020a; Perveen et al., 2020b; Tomazoni et al., 2019). The results in this assay support the potential use of *T. ammi* EO as biopesticide for *A. alternata* management.

4.2.3. Effects of *T. ammi* EO against tomato fruits decay caused by *A. alternata*

Table 14 represents the percentage of infected tomatoes 6 days after inoculation with *A. alternata* conidia suspension. Pictures of infected tomatoes are available in the Annex II (Figure 26-30). Results showed that the fungicide inhibited fungal colonization of tomatoes, in opposition to the negative control with H_2O or ethanol.

Table 14. Percentage of tomatoes that showed visible fungal growth after 6 days of the inoculation of *A. alternata* conidial suspension.

	Infected tomatoes (% ± SD)
H_2O	100.0 (± 0.0) [a]
Ethanol (2% v/v)	100.0 (± 0.0) [a]
Difenoconazole (0.250 mg mL^{-1})	16.7 (± 28.9) [b]
***T. ammi* EO (2 mg mL^{-1})**	46.7 (± 45.1) [b]
***T. ammi* EO (1 mg mL^{-1})**	60.0 (± 36.1) [ab]

Results presented are mean values (±SD) of ten replicates and three repetitions (n=30). Different lowercase letters indicate significant differences of the mean between controls and the concentrations of the EO tested for $p<0.05$, according to Kruskal-Wallis analysis followed by Dunn's multi comparisons test.

Despite the inhibitory effect of both concentrations of *T. ammi* EO in the fungal growth (fungal development was delayed in the first days of assays), both treatments were not as effective as the fungicide difenoconazole in preventing tomato decay. Nevertheless, more assays are needed to confirm these preliminary results and evaluated *T. ammi* EO as a potential biofungicide.

4.3. Allelopathic activity of EOs on *L. multiflorum* germination and seedling growth

The evaluation of both EOs on *L. multiflorum* seed germination, radicle growth and coleoptile growth are shown in Figure 20 (Annex III).

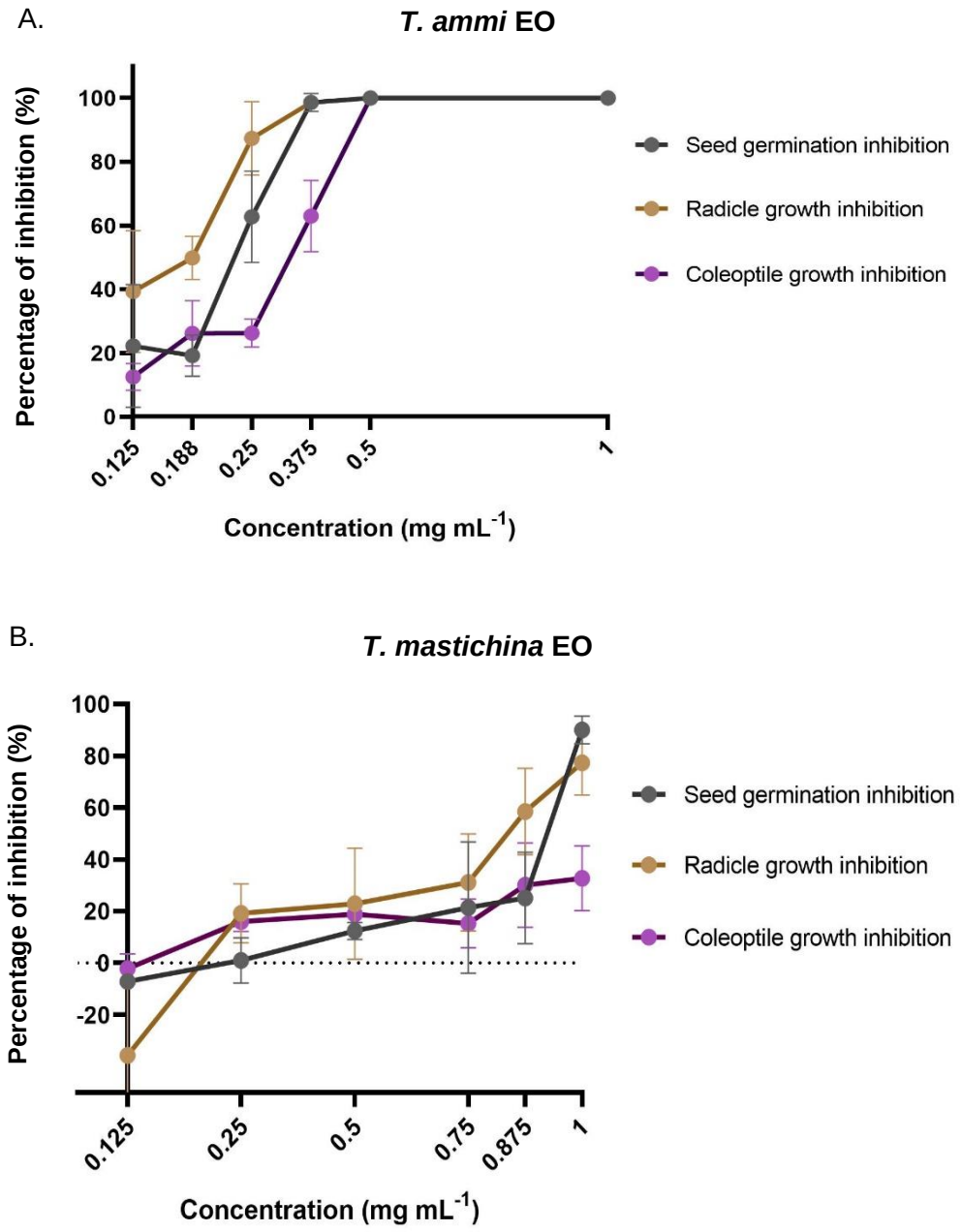


Figure 20: Percentage of inhibition of *L. multiflorum* seed germination, and growth of radicle and coleoptile when exposed to increasing concentration of *T. ammi* (A) and *T. mastichina* (B) EOs. The results are expressed as mean ± standard deviation (SD).

As it can be seen in graph A of Figure 20, the three highest concentrations of *T. ammi* EO (0.375, 0.50 and 1 mg mL^{-1}) induced the higher inhibition percentage of germination ($H(5) = 17.13$, $p<0.05$. Dunn's multiple comparisons test, $p>0.05$). In the case of *T. mastichina* EO (Figure 20B), it was found that only the concentration of 1 mg mL^{-1} had significant inhibitory effect on *L. multiflorum* seeds germination ($F(5) = 29.72$, $p<0.001$. Tukey post hoc test, $p<0.001$). The lowest concentration tested (0.125 mg mL^{-1}) did not demonstrate any inhibitory effect on the parameters analysed related to *T. mastichina* EO.

According to Mirmostafaee et al. (2020), the EO of *T. ammi* at a concentration of 1 mg mL^{-1} showed a germination inhibitory effect on *Lactuca sativa* around 95%. This value is in line with the results obtained in the allelopathic bioassay. *T. mastichina* EO inhibitory effect on seed germination was already evaluated on *L. multiflorum* with 1 mg mL^{-1} demonstrating 50% of reduction in seed germination (Ibáñez et al., 2017). Our results show a higher inhibitory effect. A main chemical class on EOs composition, monoterpenes, can affect the germination of seeds at low concentrations (Dudai et al., 2000). According to Ibáñez et al. (2017), the chemical profile of *T. mastichina* EO tested had β-Pinene as monoterpene hydrocarbon with major percentage (5.54%) and 1,8-Cineole as oxygenated monoterpene with the higher percentage (49.9%). Although it was not possible to determine the chemical composition of the EO from *T. mastichina* tested, a variation on the presence of monoterpenes might explain this difference in seed germination inhibition.

Comparisons of the results for EOs with those recorded for the herbicide glyphosate at the same concentration of 0.125 mg mL^{-1} were made (Figure 21).

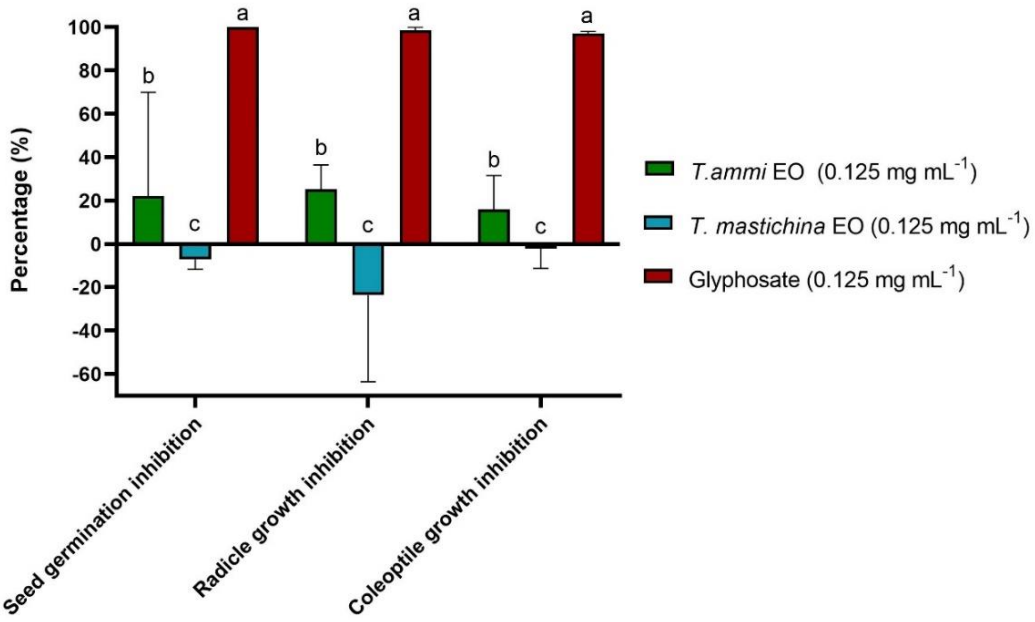


Figure 21: Comparison of the inhibition observed in all parameters assessed for both essential oils and glyphosate when tested at 0.125 mg mL^{-1} on *L. multiflorum* seeds. Results are expressed as mean ± standard deviation (SD). Different lowercase letters indicate the significant differences of the means for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post hoc test.

The herbicide exhibited a significantly higher inhibition of seed germination (100±0.0) % than both EOs. The effects on radicle growth observed indicate that *T. ammi* EO caused a reduction on *L. multiflorum* elongation (*T. ammi* EO = 25.3 ±7.3%) but it was significantly less effective than glyphosate at the same concentration. On contrary, *T. mastichina* EO had positive impact on radicle growth at this low concentration (-23.6±16%). Regarding the inhibitory effect on coleoptile growth, glyphosate was the most active with a percentage of 97.1 (±0.6) % of inhibition, followed by the EO of *T. ammi* (15.0±10.0%). *T. mastichina* EO did not show inhibitory activity.

Our findings suggest that *T. ammi* and *T. mastichina* EOs might show herbicidal activity, but further assessments of the inhibitory effect on germination and seedling growth of several important crops should be conducted to establish if these EOs could be more selective to some weed species than crops, avoiding jeopardize crops production.

4.4. Anti-insect potential of EOs on *T. absoluta*: oviposition deterrence

4.4.1. *T. absoluta* oviposition

Results concerning the total number of eggs oviposited by *T. absoluta* on the tomato plants are represented in Table 15 and oviposition in different plant parts are presented in the Annexe IV. The higher numbers of eggs counted correspond to the negative controls, as expected. The preferential area of *T. absoluta* females to oviposit in tomato plants showed to be in the leaves (Annex V).

Table 15. Mean number of eggs laid by *T. absoluta* females on tomato plants treated with EOs and controls.

	Total number of eggs
H_2O	53.3 (±4.6) [a]
Acetone (5 mg mL^{-1})	52.0 (±12.8) [a]
Chlorantraniliprole (0.4 mg mL^{-1})	14.3 (±6.8) [b]
***T. mastichina* EO (5 mg mL^{-1})**	15.0 (±1.0) [b]
***T. ammi* EO (5 mg mL^{-1})**	20.7 (±8.1) [b]

Results presented are mean values (±SD) of three replicates (n=3). Different lowercase letters indicate significant differences of the means for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test.

A more specific comparison was performed to evaluate the oviposition inhibition caused by Coragen® (Chlorantraniliprole, 0.4 mg mL^{-1}), *T. mastichina* EO (5 mg mL^{-1}) and *T. ammi* EO (5 mg mL^{-1}). Oviposition deterrence recorded for the three products were superior to 50% and no statistical differences were observed (Figure 22). These results demonstrated a similar deterrent activity of both natural products and the insecticide.

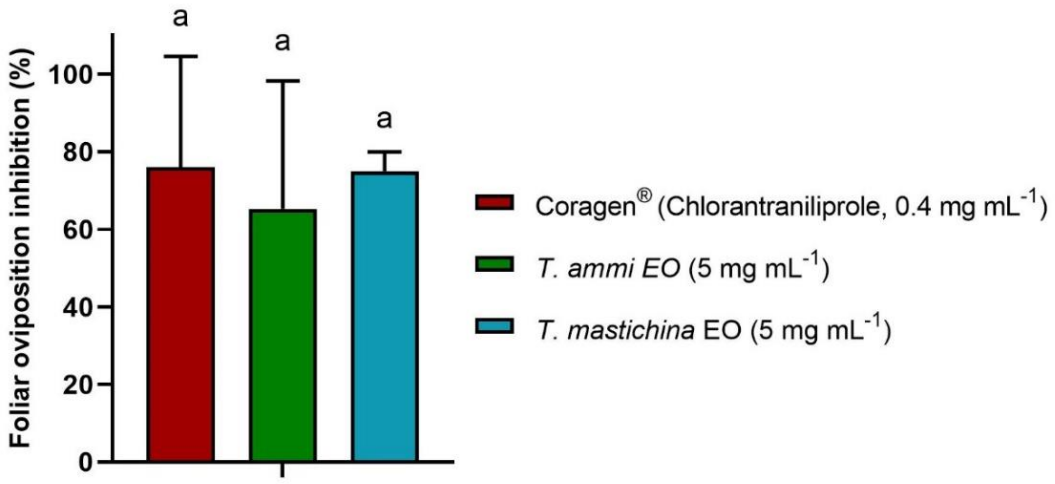


Figure 22: Inhibition of *T. absoluta* oviposition on tomato plants treated with the insecticide Coragen® and EOs. Different lowercase letters indicate significant differences of the means for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test.

4.4.2. Damage caused by *T. absoluta* larvae and assessment of phytotoxicity in tomato leaves

The estimated leaf area damaged by *T. absoluta* larvae feeding and processed photographic recordings are presented in Figure 25. The leaf area injured in plants treated with Coragen® and both EOs had lower percentage of damaged area when compared to the negative controls ($F(4) = 13.07$. Tukey post hoc test, $p<0.001$).

No phytotoxic symptoms were observed after the application of the different treatments on tomato leaves at 24h after or either at the end of the assay (Figure 23).

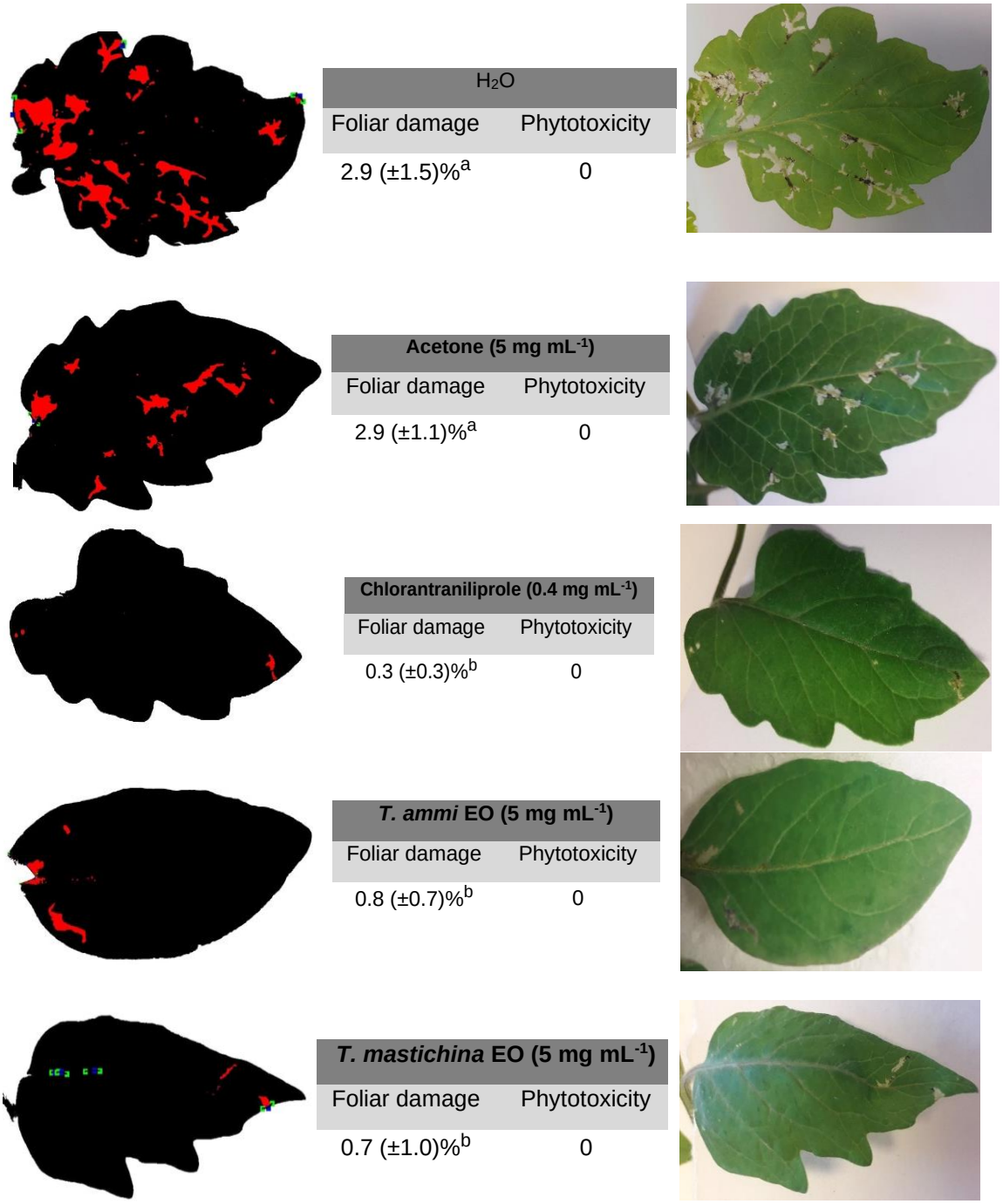


Figure 23: Percentage of foliar area damaged by *T. absoluta* larvae (mean ± sd, n=18) 6 days after oviposition and effects of treatments applied on tomato plants. Different lowercase letters indicate significant differences of the mean between EOs, negative and positive controls for $p<0.05$, according to a One-Way ANOVA analysis followed by Tukey post-hoc test.

Phytotoxicity: 0, no signal of foliar chloroses; 1, leaves with signal of chloroses; 2, leaves with signal of necrose; 3, leave's death.

Both natural botanical products seemed to have a similar effect on *T. absoluta* female behavior and leaf infestation. Insects' ability to search for host plants on which to lay eggs and feed are influenced by plant volatiles (Bruce et al., 2011). EOs compounds volatilization might play a role as non-host odor and reduce perception by females from recognizing tomato plants (Zhang et al., 2013). According to Chaudhari et al. (2021), deterrent activity of certain EOs compounds such as thymol and *p*-cymene (both constituents of *T. ammi* EO tested) and limonene and 1,8-cineole (identified to be present in *T. mastichina* EO) have been reported to act against several stored-products pests.

As far as we know, this is the first report of oviposition deterrent effects of *T. ammi* and *T. mastichina* EOs against *T. absoluta*. Effects of these two natural botanical products against Lepidoptera species are also scarce.

T. ammi EO showed larvicidal action against *T. absoluta* 4th instar larvae (Piri et al., 2020), but its oviposition deterrence was not described so far. Nevertheless, repellence on oviposition of *Musca domestica* was previously observed (100% of repellency at 5 mg mL^{-1} and 10 mg mL^{-1}) (Chantawee et al., 2018). Related to *Anopheles stephensi* oviposition, *T. ammi* EO demonstrated around 40% of oviposition deterrence at 0.05 mg mL^{-1} (Pandey et al., 2009). Besides, the insecticidal potential of *T. ammi* EO has already been reported by several researchers (Table 3).

On the other hand, to the best of our knowledge, there are no studies about *T. mastichina* EO oviposition deterrence towards *T. absoluta*. EOs from *Ocimum basilicum* and *Ocimum gratissimum* (Lamiaceae) have shown repellence of *T. absoluta* at concentrations of 5 to 10 mg mL^{-1}, respectively, when assessed by dual-choice behavioral assays (Yarou et al., 2018). Although the whole chemical profile reported for both natural products is different, *O. basilicum* EO also presented 1,8 cineol and linalool as major constituents, likewise *T. mastichina* EO.

It is worthy of note that none of the treatments seem to have negative impact on plants throughout the assay. However, since the number of experimental units included in this trial is small, interpretation of the results herein described should be careful. Further studies about the oviposition deterrent effect of *T. ammi* EO and *T. mastichina* EO using the dual-choice assay, as well as their ovicidal and larvicidal evaluation, are needed to conclude if these two OEs are, in fact, an option for the management of *T. absoluta*. The difference in the chemical profiles of these EOs (according to the relative proportions of their constituents) could be advantageous to prevent insect resistance/habituation and their alternated use might prove helpful.

5. Conclusions and future perspectives

Agricultural production is susceptible to a vast number of organisms, from pathogenic bacteria to insects. All target organisms used in this work are responsible for food production losses. Producers that face the challenge to manage these plant diseases and pests rely frequently on synthetic pesticides, but the risks associated to the continuous use of these products rise concerns about environmental and human health. Scientific reports and consumer awareness of these risks have urged the need to search for alternatives that provide sustainable practices for producers.

Sustainable agricultural practices concerning the application of EOs integrated with other pest management strategies could be an efficient alternative to conventional plant protection products. However, EOs performance and application in field conditions are limited due to their low molecular weight, hydrophobicity, and high volatility. To overcome these limitations, formulation have been an area of study to allow a controlled delivery of the natural products. A recent promising domain is the formulation of nano emulsion using bio-based surfactants, as well as other encapsulation techniques.

In addition, despite evidence of the biopesticidal potential of some EOs against agricultural pests, there is still a need to acknowledge the potential of different EOs towards different etiological agents of plant diseases. In this study, the results of *T. ammi* EO showed *in vitro* antimicrobial activity against Pst and strong antifungal effect on *A. alternata*. Complete assessment of its activity against these pathogens should be carried *in vivo* on tomato plants for a more comprehensive evaluation of their effectiveness as biopesticide for tomato protection. Furthermore, since both EOs tested are reported for having antibacterial activity against human pathogenic bacteria and food spoilage fungi, other species of agricultural relevance could be chosen to perform additional assays.

T. ammi EO also showed strong phytotoxic effect on *Lolium multiflorum* at concentration above 0.25 mg mL^{-1}. Additional tests of weeds germination inhibition and post-emergence effects on crops of interest would help to have a deeper understanding of its real herbicidal potential.

Preliminary results concerning EOs activity towards *T. absoluta* showed that both studied EOs may be good candidates for the control of this devastating pest, by dissuading female to lay their eggs on treated tomato plants. The work planned for assessing EOs activity towards *T. absoluta* was constrained by several setbacks related to climatic conditions, which delayed field captures of this pest, and to the difficulty in the establishment of the colony under laboratory conditions. Consequently, the evaluation

conducted against this pest is still limited and further assessments of the oviposition deterrence (namely by dual-choice assays) are necessary to draw specific conclusions about the effect of both EOs on the oviposition of this insect. Despite that, it is worthy of note that no negative impacts were observed on young tomato plants in consequence of EOs application, which is an encouraging result.

In conclusion, our finding evidence that *T. ammi* EO might have a large range of effectiveness and that it should be selected for further *in vivo* studies to demonstrate its biopesticidal potential against phytopathogens, weeds and insects.

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7. Annexes

7.1. Annex I - Inhibition zone diameter for *T. mastichina* EO against Psa CFPB 7286 and Pst DC3000

Table 16. Growth inhibition zone (mm) on the pathovars *Pseudomonas syringae* pv. *actinidiae* (Psa CFBP7286) and *Pseudomonas syringae* pv*. tomato* (Pst DC3000) after 24h of exposure to *T. mastichina* (*Fernandes, 2020*).

Essential oil	Psa CFBP7286	Pst DC3000
Thymus mastichina	7.6±0.5	8.1±2.5

Results presented for EOs are mean values (±SD) of triplicates and three repetitions (n=9).

7.2. Annex II - Photographic recordings of tomato infected with *A. alternata* conidial suspension

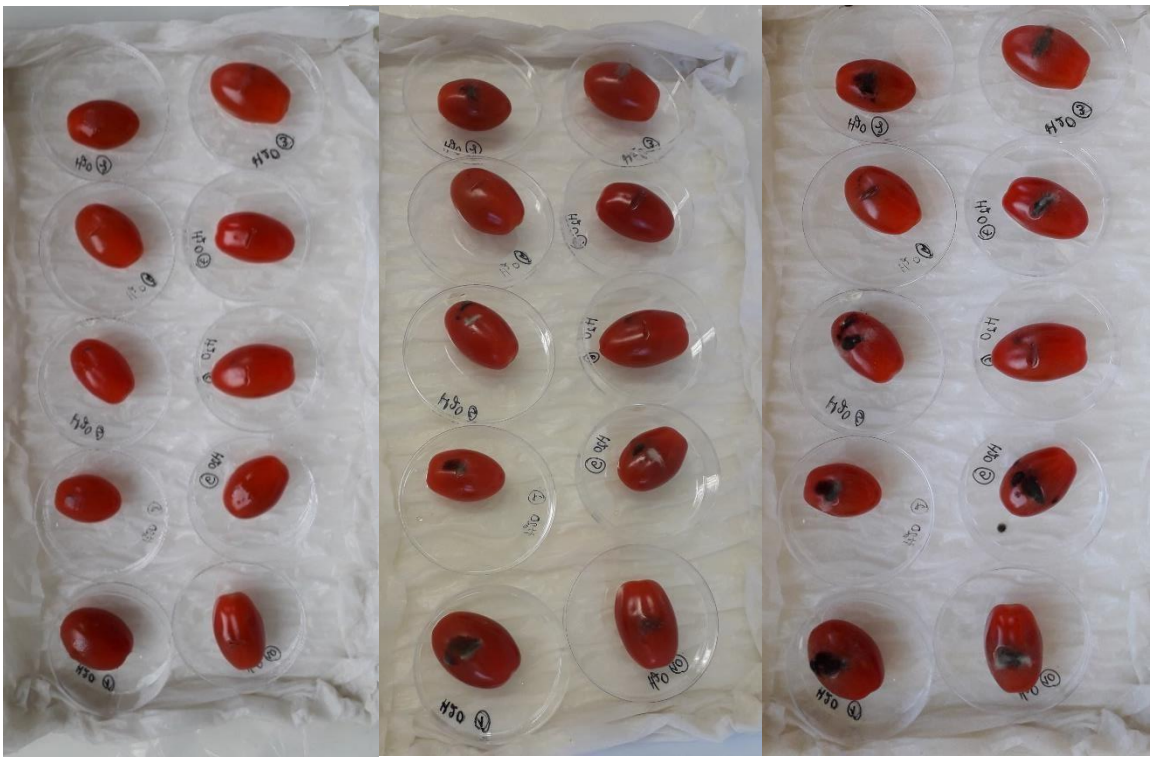


Figure 24. Photographic recording of tomatoes infected with *A. alternata* and treated with H_2O at 2nd (A), 3rd (B) and 6th (C) day of the assay.

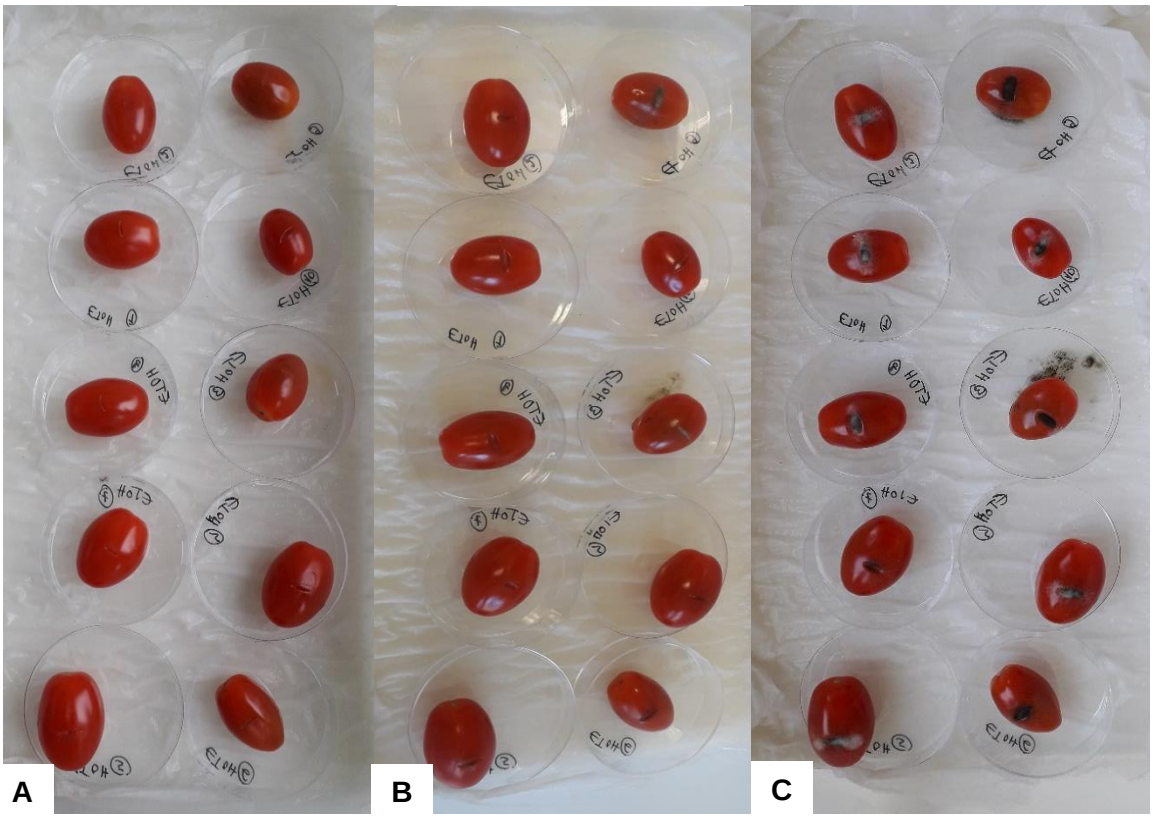


Figure 25. Photographic recording of tomatoes infected with *A. alternata* and treated with ethanol 2nd (A), 3rd (B) and 6th (C) day of the assay.

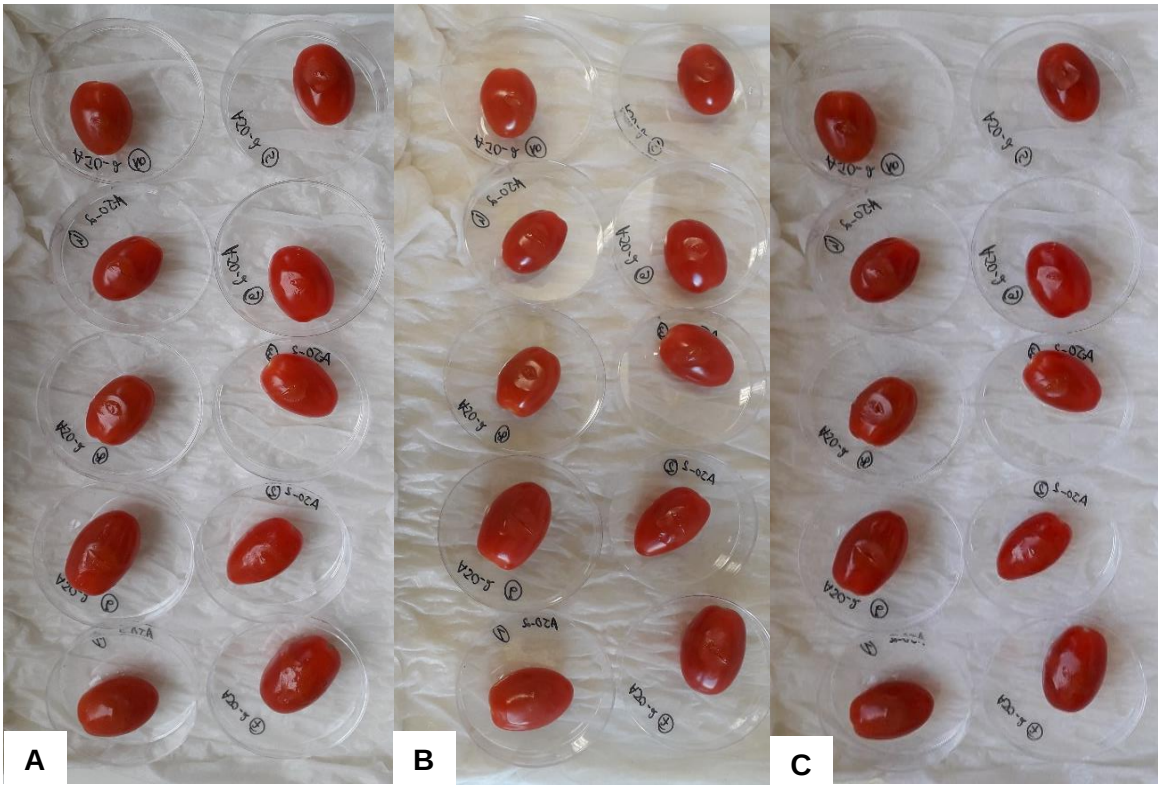


Figure 26: Photographic recording of tomatoes infected with *A. alternata* and treated with *T. ammi* EO (2 mg mL^{-1}) at the 2nd (A), 3rd (B) and 6th (C) day of the assay.

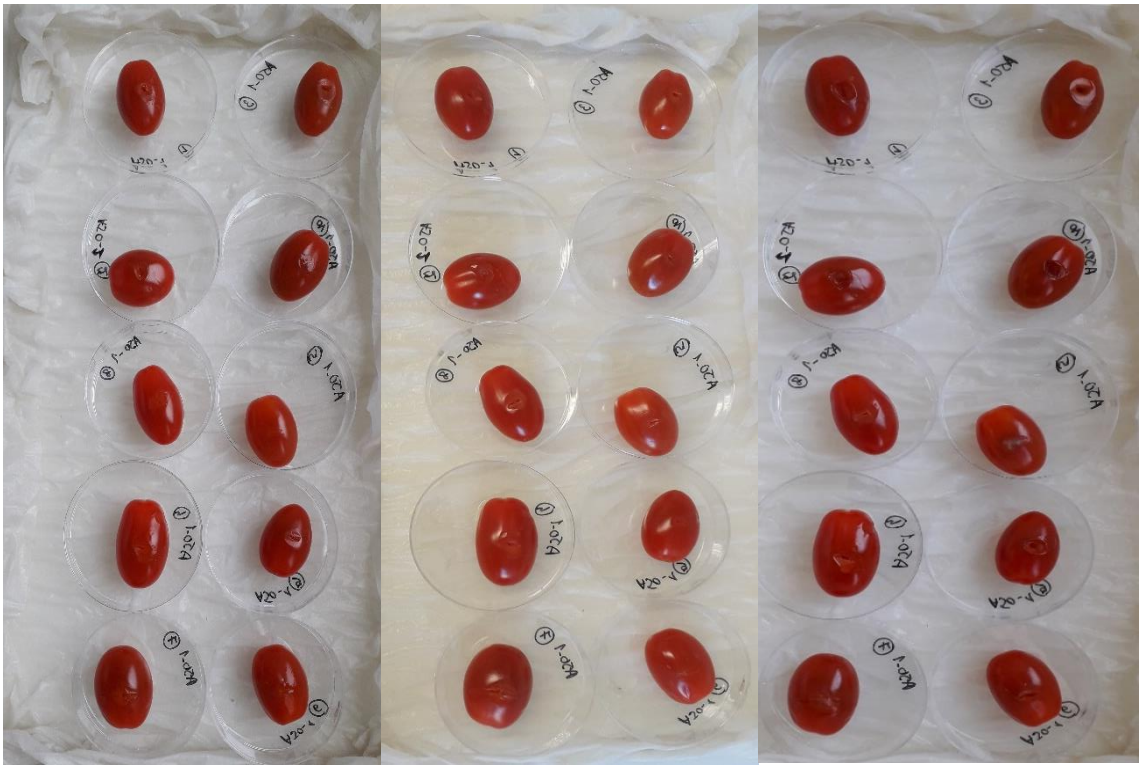
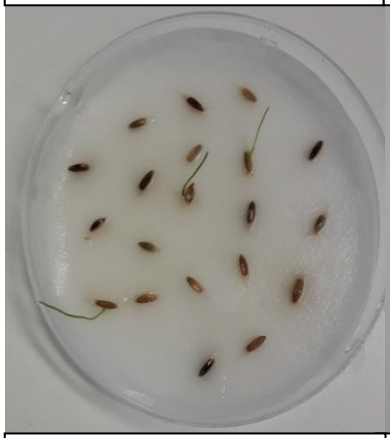


Figure 27: Photographic recording of tomatoes infected with *A. alternata* and treated with *T. ammi* EO (1 mg mL^{-1}) at the 2nd (A), 3rd (B) and 6th (C) day of the assay.

Figure 28: Photographic recording of tomatoes infected with *A. alternata* and treated with difenoconazole at the 2nd (A), 3rd (B) and 6th (C) day of the assay.

7.3. Annex III - Photographic recordings of the germination assay on *L. multiflorum* seeds

H_2O	Tween® 20	Glyphosate
		
T. mastichina EO (1 mg mL^{-1})	*T. mastichina* EO (0.875 mg mL^{-1})	*T. mastichina* EO (0.75 mg mL^{-1})
		
T. mastichina EO (0.5 mg mL^{-1})	*T. mastichina* EO (0.25 mg mL^{-1})	*T. mastichina* EO (0.125 mg mL^{-1})
		

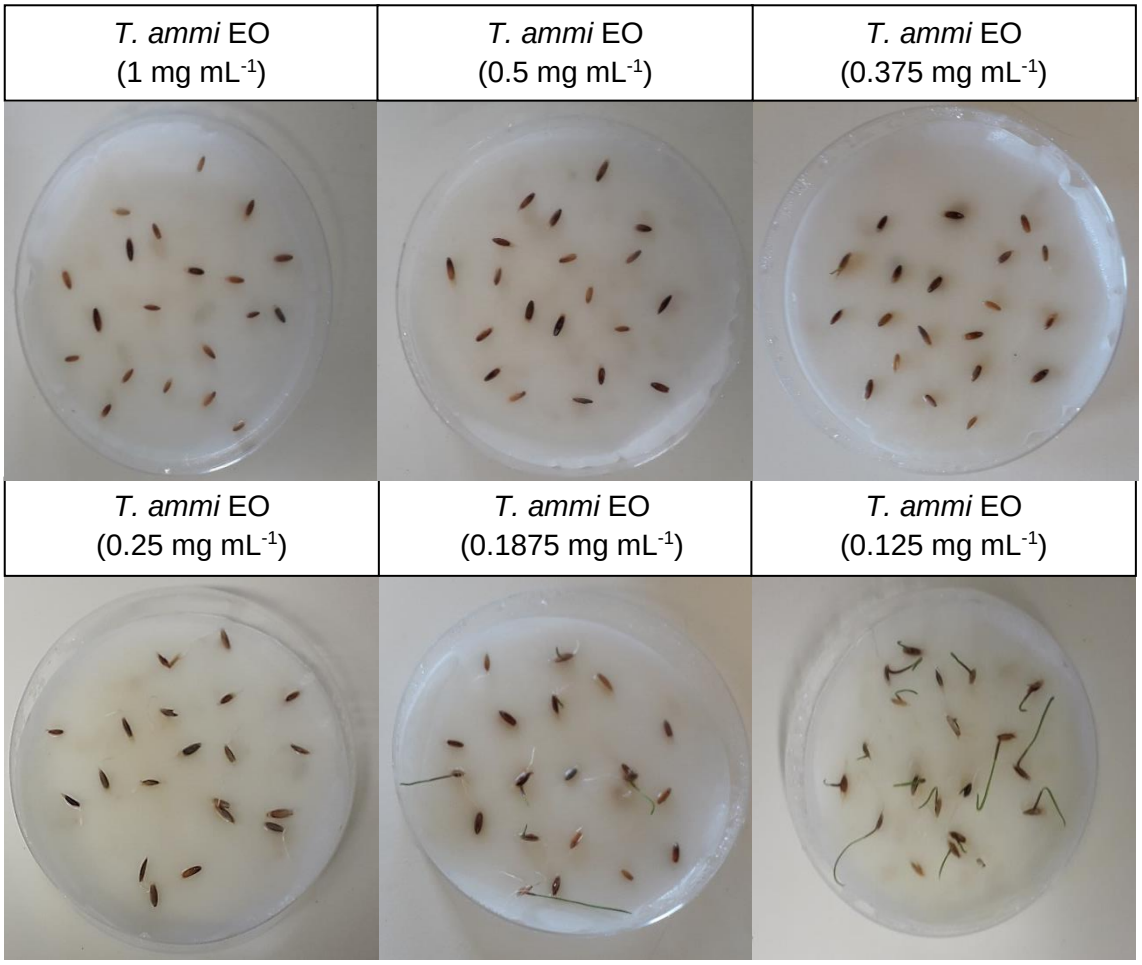


Figure 29: Photographic recordings of *L. multiflorum* seeds after 6 days of the treatment with H_2O, Tween 20, glyphosate and both EOs in study at different concentrations after 6 days.

7.4. Annex IV – Photographic recordings of *T. absoluta* eggs

Figure 30: Pictures of *T. absoluta* eggs oviposited on tomato plants.

7.5. Annex V - Assessment of the number of eggs oviposited by *T. absoluta*

Table 17. Oviposition of *T. absoluta* in the different zones defined for each tomato plants treated with the application of controls and *T. mastichina* and *T. ammi* EOs. Fa1, Foliar area near apical bud; Fa2, Foliar area under Fa1; P1, Petiole near apical bud; P2, Petiole under P1; C, Cotyledons; S, Stem.

	H2O	**Acetone**	**Chlorantraniliprole**	***T. mastichina* EO (5 mg mL^{-1})**	***T. ammi* EO (5 mg mL^{-1})**
Fa1	47.7 (±4.2)	39.3 (±14.2)	11.3 (±6.8)	10.3 (±2.1)	11.7(±5.9)
Fa2	5.7 (±1.2)	12.7 (±4.9)	3.0 (±0.0)	4.7 (±2.1)	9.0 (±6.2)
P1	4.7 (±2.5)	3.7 (±2.5)	0.7 (±1.2)	2.7 (±3.1)	0.3 (±0.6)
P2	1.7 (±1.2)	1.0 (±1.7)	1.0 (±1.7)	0.0 (±0.0)	0.7 (±1.2)
C	0.0 (±0.0)	1.5 (±3.7)	0.0 (±0.0)	0.2 (±0.8)	0.3(±0.8)
S	1.0 (±1.7)	2.0 (±3.5)	2.0 (±0.0)	0.7 (±0.6)	0.7 (±0.6)

Results presented for EOs are mean values (±SD) of triplicates and three repetitions (n=9).