Study of synergy between essential oils through biocidal activity and priming role against the biotic stress meloidogyne javanica - the case study of satureja montana and artemisia absinthium

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TRAVAIL DE FIN D'ÉTUDES PRÉSENTÉ EN VUE DE L'OBTENTION DU DIPLÔME DE

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Summary

Meloidogyne spp sont des nématodes phytoparasites causant des pertes de rendement significatives à l'échelle mondiale. L'infection par ce nématode est reconnaissable à la formation de galles, lieu où ce parasite puise dans les nutriments de la plante au niveau des racines. Les plantes affaiblies par le parasite sont alors plus sensibles aux stress biotiques et abiotiques. Certaines pratiques agricoles comme le flooding, la jachère et la rotation des cultures peuvent être mises en place mais ne donnent pas des résultats immédiats et ne s'avèrent pas toujours rentables. L'utilisation excessive de nématicides chimiques peut nuire à l'équilibre du sol et de par leur effet non ciblé constitue un risque pour la faune et flore environnantes. C'est pourquoi cette étude vise à apporter de nouvelles données utiles au développement d'un moyen de control biologique de *Meloidogyne javanica* : les huiles essentielles. En effet, les huiles essentielles comportent des molécules dont les propriétés (insecticide, antifongique, antibactérienne, etc) peuvent être utilisées en agriculture. Deux approches ont été étudiées : l'action nématicide par contact direct et le priming des défenses de la plante. Des tests *in vitro* ont été menés pour tester l'effet sur la mobilité du nématode de six huiles essentielles en combinaison avec l'huile essentielle de *Satureja montana.* Une combinaison (1:1 w/w) entre l'huile essentielle de *S. montana* et *d'Artemisia absinthium a* été testée pour son effet sur l'éclosion des œufs de *M. javanica*. Cette même combinaison d'huiles essentielles a été mixée au substrat dans lequel des plants de tomates ont été semés. Les plantes ont été rempotées après 36 jours et inoculées avec 400 juvéniles de second stade de *M. javanica.* Après 63 jours d'expérimentation, les plantes ont été collectées, pesées et les métabolites extraits des racines et parties aériennes par macération dans du méthanol. Une analyse métabolomique non ciblée en HPLC-MS a été effectuée. La combinaison de l'huile essentielle de *S. montana* avec les huiles essentielles de *M. rotundifolia et T. zygis* ont montré un potentiel effet synergique tandis que les autres combinaisons d'huiles essentielles ont eu un effet antagoniste ou additif. Pour l'expérience *in vivo*, une différence de poids de la partie aérienne des plants infectés a été observé. Le poids des racines des plantes traitées par la combinaison d'huiles essentielles et infectées par *M. javanica* s'est révélé plus élevé que pour les plants traités mais non infectés, ce qui déroge à ce qui est communément relaté dans la littérature. Les résultats d'analyse HPLC-MS préliminaires ont montrés un changement dans le profil métabolique qui sera investigué par des analyses ciblées ou après optimisation de la méthode analytique non ciblée dans la continuité du projet.

Abstract

Meloidogyne spp are plant-parasitic nematodes that cause significant yield losses worldwide. Infection by this nematode can be recognized by the formation of galls, where the parasite draws nutrients from the plant roots. Plants weakened by the parasite are then more sensitive to biotic and abiotic stresses. Agricultural practices such as flooding, fallowing and crop rotation can be implemented, but do not produce immediate results and are not always profitable. Excessive use of chemical nematicides can disrupt soil balance and their non-targeted effect put the surrounding flora and fauna at risk. That's why this study aims to provide useful new data for the development of a biological control method to fight against *Meloidogyne javanica*: essential oils. Essential oils contain molecules whose properties (insecticide, antifungal, antibacterial, etc.) can be used in agriculture. Two approaches were studied: nematicidal action by direct contact and priming of plant defenses. In vitro tests were carried out to test the effect on nematode mobility of six essential oils in combination with *Satureja montana* essential oil. A combination (1:1 w/w) of *S. montana* and *Artemisia absinthium* essential oils was tested for its effect on the hatching of *M. javanica* egg masses. The same combination was mixed to substrate where tomato seeds were sown. The plants were transplanted after 36 days and inoculated with 4OO *M. javanica* second-stage juveniles. After 63 days of experimentation, the plants were harvested, weighed and the metabolites extracted from the roots and aerial parts by maceration in methanol. An untargeted metabolomic HPLC-MS analysis was carried out. The combination of *S. montana* essential oil with *M. rotundifolia* and *T. zygis* essential oils showed a potential synergistic effect, while the other essential oil combinations had an antagonistic or additive effect. Regarding the *in vivo* experiment, a difference in the aerial weight was observed for infected plants. Root weights of plants treated with the essential oil combination and infected with *M. javanica* were found to be higher than for treated but uninfected plants, in contrast to what is commonly found in the literature. Preliminary HPLC-MS results showed a change in the metabolic profile, which will be investigated by targeted analyses or after optimization of the non-targeted analytical method later in the project.

List of abbreviations

- AChE acetylcholinesterase
- AITC allyl isothiocyanate
- AGO1 argonaute 1
- AMF arbuscular mycorrhizal fungi
- APX ascorbate peroxidases
- BABA-IR BABA-induced resistance
- BTH 2,1,3-benzothiadiazole
- CAT catalases
- DAMPs damage-associated molecular patterns
- DCM dichloromethane
- DMSO dimethyl sulfoxide
- EOs essential oils
- ER reticulum endoplasmic
- ET ethylene
- ETI effector-triggered immunity
- G3P glycerol-3-phosphate
- GR growth rate
- HR hypersensitive response
- IBI1 aspartyl-tRNA synthetase
- ISR induced systemic resistance
- J2 second-stage juveniles
- JA jasmonate
- MDA malondialdehyde
- MeOH methanol
- NLRs nucleotide-binding leucine-rich repeat receptors
- ODR1 odorant response gene-1
- Pas polyamines
- PAMPs pathogen-associated molecular patterns
- PTI PAMP-triggered immunity
- Pip pipecolic acid
- PGPB plant growth promoting bacteria
- ROS reactive oxygen species
- RTs retention times
- SA salicylate
- SD steam distillation
- Sm:Aa mixture of *Satureja Montana* and *Artemisia absinthium* EOs at 1:1 ratio (w/w)
- SOD superoxide dismutases
- TEs transposable elements
- VOZ1/2 Vascular Plant One Zinc Finger 1/2

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1 STATE OF THE ART

1.1 Challenge

The world population is believed to reach 10 billion by 2057, an increase of 25%, which requires a crop productivity higher than the fields capacity nowadays (Lau et al., 2022 ; Worldometers, 2023). Pest occurrence represents a loss of 40% each year, which has been the driver for an intensive use of pesticides ([a]FAO, 2021).

For decades, farmers have relied on chemicals in an excessive way to improve crop yields that have led to environmental pollution and risks to human and animal health due to bioaccumulation process (Ali et al., 2021). Indeed, chemical pesticides aren't selective thus they have an impact on non-targeted species (Syromyatnikov et al., 2020). The European Green Deal with the contribution of the Common Agriculture policy has for objective the decrease of 50 % by 2030 in the use and risk of pesticides. The proposal for a new Regulation on the Sustainable Use of Plant Protection Products includes the transition to integrated pest management practices (EC, sd).

More and more attention is being paid to two alternative practices to improve crop yields: biopesticides (Khursheed et., 2022) and biostimulants (Van Oosten et al., 2017). Biopesticides are substances from biological origin as plants, microbes and some nanoparticles, applied in pest control (Kumar et al., 2021). Their main advantage is that their effects on the environment and non-targeted organisms are limited. However, biopesticides aren't persistent in the environment which also means that they could be less effective, requiring a contact with the target to operate (Ayilara et al., 2023).

Biostimulant define any substance other than a nutrient which is able to improve nutrient uptake and availability in the soil, crop quality or tolerance to abiotic stress (Du Jardin, 2015; EU, 2019). Seven types of biostimulants are widely investigated in the literature: humic and fulvic acids (Kłeczek, 2022), protein hydrolysates (Jolayemi et al., 2022), seaweed extracts (Nanda et al., 2022), chitosan and its derivatives (Stasińska-Jakubas et Hawrylak-Nowak, 2022), arbuscular mycorrhizal fungi (AMF), plant growth promoting bacteria (PGPB) (Fusco et al., 2022) and plant extracts (Zulfiqar et al., 2020). The increasing interest for these products is built on their ability to mitigate abiotic stress in a context of climate change and environmental concerns. Interestingly, some biostimulants have demonstrated an ability to improve tolerance to biotic stress (Rouphael et Colla, 2020). In addition, chitin and protein-based biostimulants can be formulated from waste products from respectively the seafood industry and dairy and livestock industries, contributing to the sustainability objective (Jolayemi et al., 2022; Stasińska-Jakubas et Hawrylak-Nowak, 2022). As biopesticides, biostimulants have the advantage of being biodegradable and less harmful for the environment and the wildlife (Ma et al., 2022). But biostimulant application raises challenges: the formulation may not be able to provide a proper application and the effect may not be guaranteed because of the complexity of plants physiological responses (Du Jardin, 2015; Ma et al., 2022)

1.2 Essential oils

1.2.1 Origin, chemistry and extraction processes of essential oils

Plant-derived essential oils (EOs) contain many secondary metabolites, such as terpenes, whose the odor related to their volatility is distinctive. As their name implies, they are distinguished by their oily appearance, partial vapour state at room temperature and their density generally lower than one. These properties are imparted by their main components, terpenoids or phenylpropanoids that are mostly low weight lipophilic metabolites, also responsible for their solubility in alcohol, organic solvent and oils (Assadpour et al., 2023; Khursheed et al., 2022). Essential oils are individually composed of up to hundred different components, generally from 20 to 60; one to three highly concentrated constituents characterize the oil (representing 20-70%) and the others appear as trace elements in the analysis (Jugreet et al., 2020)

Physiological and ecological functions in the plant are associated with the metabolites contained in essential oils: they can act as infochemicals, chemo-attractants, nutritional elements or internal messengers. Biotic or abiotic stress can lead to the production of these secondary metabolites as a part of a defense mechanism (Greff et al., 2023). Various plant organs host the synthesis, storage or secretion of compounds extracted as EOs such as buds, flowers, leaves, bark, seeds, zests, roots, twigs, fruits, wood, rhizomes, stems in their secretory cells, secretory cavities, secretory ducts, or glandular trichomes (Greff et al., 2023; Yingngam, 2022).

Three classes of organic chemicals constitute the EOs: terpenoids, phenylpropanoids and one “class” that regroups other chemical structures as furanocoumarins, amines, sulfur-containing molecules, carboxylic acids and fatty aldehydes. Some members of this former category can be associated with a fragrance such as the nitrogen-containing compound indole with floral scent. Terpenoids found in EOs can be subdivided in two groups: hemi-(a single isoprene unit), mono- (two isoprene units), di- (four isoprene units), sesquiterpenes (three isoprene units) and oxygenated structures as alcohols, phenols, aldehydes, oxides, lactones, etc (Yingngam,2022). Monoterpenes and sesquiterpenes generally form the major part of the EOs, monoterpenes can account for up to 90% of the molecules (Masyita et al., 2022).

The classic techniques for extracting EOs are steam distillation (SD) and hydro-distillation. But those require a huge energy consumption. For this reason, other techniques have emerged such as ohmic or microwave assisted extractions using tools from green chemistry, but they are not referenced as official methods. Even solar steam distillation has been reported. Hydro-diffusion that is distinguished from SD by the steam application from the bottom. Solvent extraction for EOs containing heat sensitive components can also be cited (Kant et Kumar, 2022).

1.2.2 Industrial applications of essential oils

Essential oils are now well incorporated in cosmetic, health care and food industries practices (Bolouri et al., 2022). Ancient civilizations used EOs as remedies paving the path nowadays at aromatherapy. Among their numerous applications, treatment of insomnia, depression and muscular pain can be cited. They have also been involved in bigger prospects such as immunity enhancement, fight against cancer, prevention of diabetes and cardiovascular diseases, etc (Liang et al., 2023; Jugreet et al., 2020). In an environmentally friendly and health aspect, the interest in natural cosmetics is growing. Essential oils are exploited in perfumery for their fragrance and in cosmetic products for their anti-inflammatory and antioxidant properties. However, their potential as antimicrobial in order to prevent microbial spoilage is less known. They constitute a natural way

to lengthen the shelf life of cosmetic products but also food products (Chang et al., 2022; Bolouri et al., 2022). As a matter of fact, EOs are exploited in the food industry as a preservative and an antioxidant. They address the consumer's concerns about the potential toxic effect of synthetic antioxidants and prevent lipid oxidation that has a negative impact on nutritional and organoleptic properties of the product (Falleh et al., 2020). EOs also tackle the issue of antibiotic resistant strains (Bolouri et al., 2022). Although their pungent aroma makes them a suitable spice, it can also be a barrier to their wider use in food products (Falleh et al., 2020).

1.2.3 Application of essential oils in agriculture

Essential oils can be an alternative to chemical pesticides as they show anti-bacterial, anti-fungal, anti-viral, anti-parasitic, nematicidal and insecticidal effects (Khursheed et al., 2022; Assadpour 2023). They display a broad spectrum of actions on insects: repellence, larvicidal, ovicidal and pupicidal activities, growth-regulating effects, synergism and antifeedant effects (Chang et al., 2022). Notably, EOs are a natural tool to preserve fruits and vegetables after the harvest. Their wide range of modes of action and components is a possible clue to avoid the development of pest resistance (De Clerck et al., 2021). An essential oil can be active against a large range of pests or to target some specific organisms, even essential oils from the same genus can have different activities (De Clerck et al., 2021; Chang et al., 2022). Biopesticides derived from essential oils have already been commercialized (Devrnja et al., 2022; cf. 1.2.5).

Nevertheless, more research needs to be conducted in order to overcome the obstacles to the wider use of essential oils in agriculture. The volatility of EOs make them slightly persistent in the environment which raises efficiency issues in the field (Kesraoui et al., 2022; De Clerck et al., 2021) Therefore progress has to be made on their stability during storage and transport and after their application (Chang et al., 2022). One of the main concerns regarding their use as biopesticides is their phytotoxicity, which gives them herbicidal properties that can harm the crops they are supposed to protect. In consequence, the right concentration to apply depending on the crop has to be found and mentioned to the users (Werrie et al., 2020). Lot of parameters influence the quality and composition of EOs as well as the yield. They can be divided into four major groups according to their nature: physiological, environmental, genetic and production parameters. Depending on the plant age, development stage, plant organ, the soil and the microbiota, the climate, the geographical location of the mother plant, the harvest time, pre-treatments, storage conditions among others, numerous variabilities can be observed (Ni et al., 2021; Jugreet et al., 2020). An option to address this issue is the domestication of the plants with a modification of the biological activities as a possible setback (Kesraoui et al., 2022).

1.2.4 Modes of action of essential oils in agriculture applications

Essential oils exert different modes of action in function of their composition and their targets. For example, in microbes, EOs affect several functions: energy consumption by disrupting ATP synthesis and hydrolysis, electrochemistry balance by playing on membrane potential and proton pump, cell integrity by causing damage to the membrane and impairing permeability barrier and finally defense mechanisms by interfering with the metabolite production (Greff et al., 2023). EOs have been widely studied for their activities against insects, either by direct contact with the pest or fumigation (Khursheed et al., 2022). They act in the nervous system of insects by targeting enzymes and receptors. Indeed, EOs are able to act as a competitive or uncompetitive inhibitor of acetylcholinesterase (AChE), as an agonist of octopamine and tyramine and an antagonist of gamma-amminobutyric acid causing overexcitation or inhibition of nervous functions (Jankowska et

al., 2017). As an example, Rodríguez et al (2022) tested the potential of several phenols in the inhibition of AChE and obtained an EC_{50} of 0.019 mM for carvacrol. They suggested an influence of the lipophilicity and acidity of the components in their activity against *Sitophilus zeamais* and also assumed a role of hydroxyl groups. As a second example, *Melaleuca alternifolia* EO is able to disrupt the respiratory chain by playing on NAD+ /NADH dehydrogenase transcript regulation and to cause a vacuolization of the mitochondria (Liao et al., 2018), revealing another potential mode of action of EOs.

The use of EOs for nematode control has been studied in 348 plant species, half of which belong to Asteraceae, Fabaceae, Lamiaceae, Brassicaceae, Myrtaceae, Euphorbiaceae, and Apocynaceae families. EOs are known to possess nematicidal, hatching inhibition, nemastatic and root-galling reduction properties (Mwamula et al., 2022). Aromatic aldehydes might be able to inhibit the activity of V-ATPase which is involved in osmoregulation and detoxification of nematodes. Some targets of EO components observed in insects are shared by nematodes such as AChE and glutathione S-transferase in pinewood nematodes. Digestive enzymes as amylase and cellulase can also be affected by EO components (Chen et Song, 2021). El-Habashya et al (2022) tested six essential oils against *Meloidogyne incognita*. They observed particularly strong *in vitro* and *in vivo* hatching inhibition ability and nematicidal activity of *Artemisia judaica* L. The EO was also responsible for a reduction in the number of galls *in vivo*. *A. judaica* showed a similar nematicidal activity as oxamyl, a commercial nematicide and even three times greater activity in reducing eggs hatch. This latter was also effective for the three types of activities tested *in vivo* but not as much as the oxamyl. An increase in the expression of chitinase (PR3), thaumatin-like proteins (PR5) and polyphenol oxidase (PPO) genes identified as potential genes activated upon early plant defense response, was also shown (El-Habashy et al., 2022). Dutta et al (2021) assessed the bioactivity of the EO of black mustard seeds (MEO) against nematodes and reported nematicidal and temporary nemastatic activities as well as a reduction in their infectivity capacity after 1h exposure to the EO. The major component of MEO, allyl isothiocyanate (AITC) was submitted to a molecular docking analysis. This revealed a potential ability of AITC to affect neurotransmission and chemosensing functions of *M. incognita* via an interaction with AChE, odorant response gene-1(ODR1), and neuropeptide G-protein coupled receptor. The capacity of EO components to bind ODR1 was first put forward by Kundu et al (2021) who performed molecular docking on l-limonene, γ-terpinene, citronellal, β-terpineol and geraniol. Molecular docking on α-bulnesene, α-guaiene and patchoulol, major constituents of *Pogostemon cablin* EO supports the interaction between EO components and AChE and ODR1 as an EO mode of action against nematodes. An interaction between ODR3 and patchoulol was also demonstrated (Keerthiraj et al., 2021). Inhibition of the differentiation of *Meloidogyne javanica* eggs has been observed as an effect of carvacrol, a component of essential oils. This study also highlighted the potential of EO components as a nematicide against *M. javonica* (Nasiou et Giannakou, 2017). By testing the nematicidal activity of sixteen EOs, D'Addabbo and Avato (2021) demonstrated a potential structure-activity relationship, as shown by the high nematicidal activity of phenol-containing EOs, which is thought to be linked to their hydroxyl and spacer groups. The literature review of Catani et al (2023) provides more information on the extent of research into the nematicidal effect of EO and their efficiency.

Modes of action of EOs in plants have also been the subject of numerous studies. In fact, the phytotoxic effect of EOs depends on the product formulation and its mode of application as well as the plant itself (physiological state, phenological stage and organ targeted) (Werrie et al., 2020). Reactive oxygen species (ROS) are tightly involved in EOs phytotoxicity. An overproduction of ROS in *Avena fatua* roots caused by eugenol and resulting in oxidative damage affecting the membrane

and leading to electrolyte leakage was reported by Ahuja et al (2015). This finding was supported by the increase and decrease of respectively malondialdehyde (MDA) and conjugated dienes and the upregulation of antioxidant enzymes superoxide dismutases (SOD), ascorbate peroxidases (APX), catalases (CAT), guaiacol peroxidases and glutathione reductases. Li et al (2023) made the same observation for the use of *Artemisia argyi* EO on *Setaria viridis* L. concerning MDA, SOD, CAT and APX and also concluded to an oxidative stress with a burst of ROS. Besides, they noticed a decrease in photosynthetic pigments that suggests a disruption of the photosynthetic electron transport chain. This hypothesis was reinforced by the molecular docking that indicated a good binding ability of the major component of the EO with 4-hydroxyphenyl pyruvate dioxygenase (HPPD). The decreased content in this oxygenase enzyme (HPPD) involved in carotenoid formation complements the result from molecular docking. Araniti et al (2018) provided another explanation implying the ammonia assimilation pathway to the induced oxidative stress resulting from the application of EOs. They observed into *Arabidopsis thaliana* (L.) treated with *Origanum. vulgare* L. ssp. *hirtum* EO, an accumulation of inorganic nitrogen into leaves that might be the result of the glutamine synthetase enzyme which is essential in the incorporation of inorganic N into amino acids. In consequence, the function of PSII was altered leading also to the impairment of photorespiration confirmed by the reduction of the metabolites involved in this process. A more exhaustive overview of the phytotoxicity mechanisms is given by Werrie et al (2020).

1.2.5 Synergy between essential oils or their components

The bioactivity of EO is suspected to be the outcome of synergistic and additive effects between its components (Kesraoui et al., 2022; Isman, 2020) A synergistic effect is observed when the activity resulting from a mix of EOs is significatively superior to the sum of their individual activity (Ntalli et al., 2011). This concept has been broadened to the combination of components from different EOs to achieve an enhanced activity (Dassanayake et al., 2021). For example, Ntalli et al (2011) demonstrated the synergistic effect of terpenes from different essential oils in the fight against *Meloidogyne incognita*. Trans-anethole was highlighted for its recurring ability to synergism with geraniol, L-carvone, carvacrol, estragole, pulegone, thymol and eugenol.

Mix of EOs have also demonstrated a synergistic effect. The combination between *Cymbopogon citratus* and *Mentha piperita* was shown as increasing the nematicidal activity compared to the individual EO activities. Indeed, the EC_{50} decreased from 0,31 µL/mL to 0,10 µL/mL in an *in vitro* assay against the pinewood nematode (Gonçalves et al., 2022). The synergistic effect of essential oils can also be exploited to control insects. *Dysphania ambrosioides* L. EO was displayed as an efficient synergist in several EO combinations to control *Sitophilus zeamais*. In addition, Santana et al (2022) besides the insecticidal activity highlighted the repellent effect of the EO mixtures. A common way to assess the synergy between essential oils is to test the mix of EOs (1:1) at a concentration below 50% of their individual activity against the pest. Nevertheless, other techniques have arised. For example, Soulaimani et al (2022) worked with an augmented simplex-centroid design in order to find the best combinations of EO against *Stegobium paniceum* (L.) and *Tribolium confusum.* Based on fumigant toxicity bioassays, a special cubic model was used. It indicated as the most efficient combination against *T. confusum*: a mix of 48% of *S. alpina*, 31% of *R. officinalis* and 21% of *A. leucotrichus.* For *S. paniceum,* a mix of 57% of *A. leucotrichus* and 43% of *S. alpina* EOs was designated as the most efficient. This type of model was also used by Mahmud et al (2023) to find the most efficient combination of EOs and citrus extract as antimicrobial active against food-borne pathogens and spoilage bacteria. This study showed the potential of the use of EOs in combination to fight against microorganisms. Some insecticides and miticides based on EO mixtures

have already been commercialized as EcoTrol ™ , Eco-oil ® and Akabrown ® . However, the author didn't specify if their actions result in a synergistic effect (Isman, 2020). Mixtures of EOs can be formulated to be more efficient or suitable for application (Pandiyan et al., 2019). An adapted formulation might be a valuable tool to counter the high volatility of EOs incriminated for their loss of efficacy in fields.

1.3 Priming

Seed priming is not as new as many recent studies suggest. In fact, Darwin experienced priming by dipping the seeds from several plants into seawater and observed improvements in germination (Pagano et al., 2023). But to go into details in priming mechanisms, plant immunity needs to be understood as it is the starting point of priming.

1.3.1 Notions of plant immunity

Plants dispose of different immunity layers: PAMP-triggered immunity (PTI), effector-triggered immunity (ETI), systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Ngou et al., 2022; Vlot et al., 2021). PTI is the first one to be activated by the recognition of pathogens (PAMPs)-/damage(DAMPs)-/microbe-/herbivore-associated molecular patterns and phytocytokines through cell-surface pattern recognition receptors (PRRs). Interestingly, the perception of damaged or infected tissues respectively through DAMPS and phytocytokines (endogenous molecules) acts as a signal that amplifies the immune response. PRRs are either transmembrane receptor-like kinases or receptor-like proteins, most of them associated with co-receptors. The first is responsible for the activation of signalling molecules, calcium influx, ROS production, stomatal closure and callose deposition. While the activation of mitogen-activated protein kinases and calcium-dependent protein kinases in association with both types of receptors elicit transcriptional reprogramming and production of hormones involved in defense against pathogens (Ngou et al., 2022; Abdul Malik et al., 2020). Evolution led pathogens to secrete virulence factors. These effector molecules are released into the plant and suppress PTI (Abdul Malik et al., 2020). In response, plants developed nucleotide-binding leucine rich repeat receptors (NLRs) that by resistance (R) protein mediation activate ETI. This second layer of defense allows the plant to limit the spread of the infection to the initial infected tissue triggering hypersensitive response (HR) and finally programmed cell death. ETI is suspected to reinstitute PTI in order to build an efficient response against pathogens (Ngou et al., 2022).

The innate immunity is reinforced by long-lasting forms of immunity resulting from the phenotypic plasticity of plants: SAR and ISR which differ by their triggers: pathogens and non-pathogenic microorganisms respectively (Vlot et al., 2021). Both occur following an initial immune event and fortify the response. SAR particularly allows a wider protection of the plant by generating a systemic response through the phloem. Salicylate (SA) and ethylene/jasmonate (ET/JA)- mediated pathways are the defense mechanisms initiated following the attack of respectively biotrophic pathogens and necrotrophic and herbivorous pathogens (Abdul Malik et al., 2020). SAR is dependent on many signalling molecules that act both locally and systemically as SA and pipecolic acid (Pip) synthesized by the aminotransferase AGD2-like Defense Response Protein 1. But some might be considered as mobile SAR signals as methyl salicylate produced in infected tissue, monoterpenes, glycerol-3-phosphate (G3P), lipid-transfer proteins defective in induced resistance 1 and azelaic acid induced 1. Pip/NHP, di-carboxylic acid azelaic acid (AzA) and SA pathways intersect and might potentiate each other. AzA is dependent on SA, Pip/ *N*-hydroxy-Pip and the accumulation of G3P. G3P

participates in SAR by its accumulation but might also be involved in a feed-forward stimulation of Pip in coordination with AzA and AZI1. Regarding ISR, JA and ET are believed to be the main signalling molecules involved (Vlot et al., 2021).

1.3.2 Priming mechanisms

Priming is associated with the notion of immune memory related to stress (Pagano et al., 2023). A metabolic and transcriptional response is triggered by the priming-inducing stimuli which place the plant in a state of readiness (Vlot et al., 2021). This enables a subsequent response to the exposure to a biotic or an abiotic stress strengthened and prompt. Priming is closely related to induced resistance: it sets up SAR or ISR response but also β-aminobutyric acid -induced resistance (BABA-IR) which is extensively studied. BABA is a non-proteinogenic β-amino acid that can be found in minute amounts in plants following biotic or abiotic stresses. Innate immunity is involved in BABA-IR since PTI genes are activated by Vascular Plant One Zinc Finger 1/2 (VOZ1/2) transcription factors that interact with aspartyl-tRNA synthetase (IBI1), a BABA receptor. IBI1 is located into reticulum endoplasmic (ER) and might be translocated upon PTI- related ER stress to the cytoplasm where VOZ1/2 is localized (Cooper et Ton, 2022).

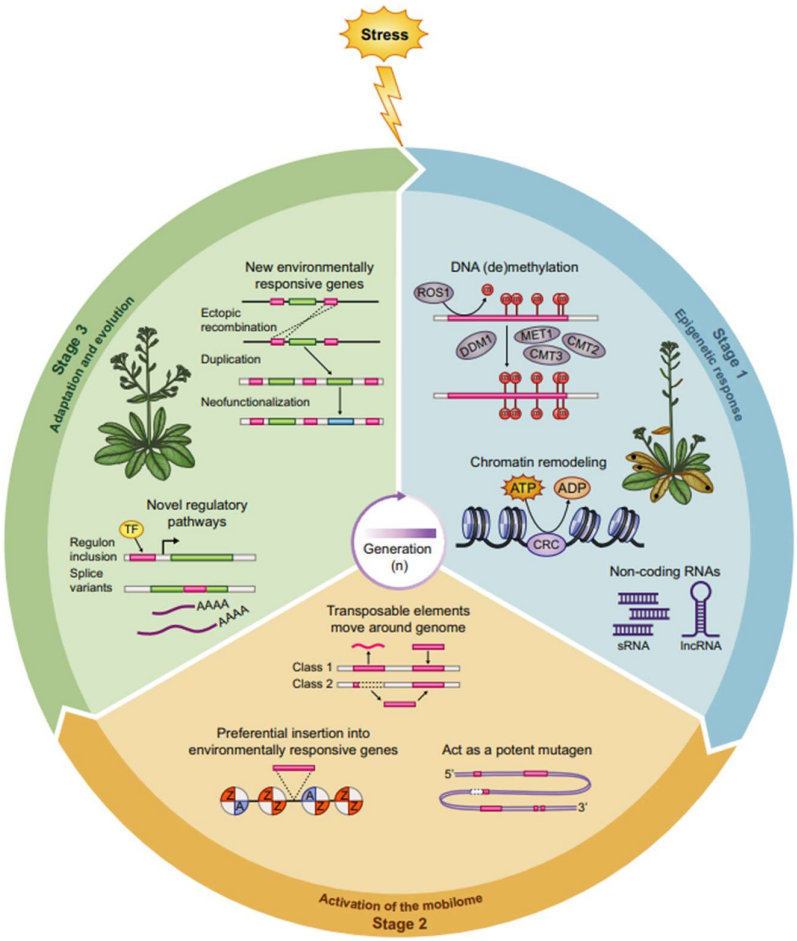

Figure 1. Summary of the three stages of priming from epigenetic reprogramming through metabolome activation to resistance transmission through evolution from the publication of Hannan Parker et al (2022).

Priming is suggested to be the result of epigenetic reprogramming (Vlot et al., 2021). Hannan Parker et al (2022) defined epigenetics as "the study of changes in gene function that are mitotically and/or meiotically heritable, and that occur independently from changes in DNA sequence". The stress-induced hypomethylation of transposable elements (TEs) is one potential mechanism for the priming of defense genes. Euchromatisation is induced by cis-regulation under the control of intronic TEs and gives access to the transcription machinery to defense genes (Hannan Parker et al., 2022). Cis-regulation can also result in alternative splicing or polyadenylation of the defense genes. Trans-regulation may also be a mechanism involved in the priming process. This latter regulates genes distant from the genome location where the mechanism was triggered. It occurs during the re-silencing of the TEs where siRNAs are produced and activate argonaute 1 (AGO1) that in turn interacts with the SWI/SNF chromatin remodeling complex (Cooper et Ton, 2022). The transcription of hypomethylated TEs can also result in non-coding RNAs (lncRNAs) capable of target-mimicry that disrupts the action of defense-repressing miRNAs (Hannan Parker et al., 2022). Both pathways lead to the stimulation of distal defense genes either by a direct invigoration of the genes or a reduction of the silencing (Cooper et Ton, 2022).

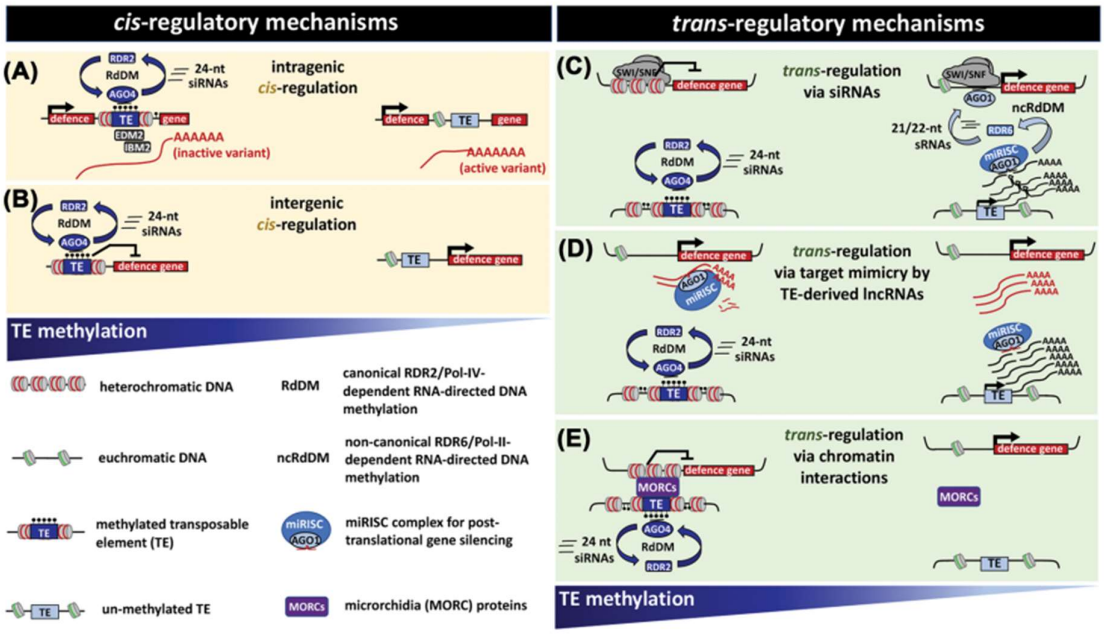

Figure 2. Summary of the mechanisms involved in cis- and trans-regulations from the publication of Cooper et Ton (2022).

The major interest in priming is its supposed long-lasting effect and by extension its potential transmission to the plant progeny (Cooper et Ton, 2022). Like any living being, the transfer of genes in plants should follow the Darwin principle. In fact, NRL genes and TEs are often found gathered in clusters. The hypomethylation generated by biotic stress at these clusters as seen above, is believed to promote Nonallelic homologous recombination (NAHR)-induced diversification. They might also have been found to increase the mutation rate of neighboring genes. This phenomenon can procure a selective advantage, a driver for Darwinian evolution (Hannan Parker et al., 2022). Taking into account that TEs seem to be particularly inserted in environmentally responsive genes involved in

plant defense response, these observations can be a piece of explanation for transgenerational induced resistance. Specific mechanisms relative to seed priming, information about the variety of techniques and their beneficial effects can be found in the reviews of Zulfiqar (2021), Paul et al (2022) and Pagano et al (2023).

1.3.3 Examples of priming

PGPR are beneficial microbes well known for their biostimulant and protective effects. But PGPRs have increasingly been studied for their role in priming. For example, Mhlongo et al (2021) reported a change in the metabolome of tomatoes treated with *Pseudomonas fluorescens* N04 or *Paenibacillus alvei* T22 and infected by *Phytophthora capsici* that might explain the reduced infection observed. Like PGPR, mycorrhizal symbiosis can also play a priming role. It is particularly interesting to see that they can act on herbivorous insects by modulating alkaloids, fatty acid derivatives and phenylpropanoid-polyamine conjugates metabolism (Rivero et al., 2021). Rivero et al (2021) also noticed an accumulation of deadly compounds into the plant that may explain the increase in larval mortality. A riveting example of priming is its induction in tomatoes by four molecular elicitors: polyamines (PAs), 2,1,3-benzothiadiazole (BTH), salicylate (SA) and chitosan. Lula et al (2022) suggested the activation of different immune mechanisms depending on the elicitors. PA, BTH and SA might initiate a SAR response as they are involved in SA related signalling. Whereas chitosan might rather trigger ISR response by inducing the production of glucosinolates regulated by JA.

Seed priming might become a precious tool to fight against nematodes. Several studies have already proved the efficacy of this process in *in vivo* experiments. JA has a priming role in tomatoes infected by *M. incognita.* Bali et al (2020) reported the upregulation of several antioxidative enzymes and the increase in protein content usually downregulated during infection. A clear distinction between treated but non infected plants and treated inoculated plants was made by the authors, suggesting that the metabolic response is activated by contact with the biotic stress. Leaf extracts were also reported as potential priming agent against root knot nematodes, reducing the number of galls, seconde-stage juveniles (J2), females and eggs (Arshad et al., 2022). Priming may also occur in tomatoes infected by *M. javanica*. Indeed, in presence of the fungus *Pochonia chlamydosporia,* the activities of polyphenoloxidases and peroxidases were enhanced. Peroxidases being involved in lignification of the tissues, they may prevent or interfere with the penetration of the nematodes in the roots (Medeirosa et al., 2015).

Plants are able to induce priming. An interesting example is the priming induced by exogenous volatile organic compounds demonstrated by Sukegawa et al (2018). The authors conducted a field experiment with soybean plants grown beforehand 50- or 100-cm apart from candy mint. A reduction of the damages caused by herbivores was observed. They perceived a relevant difference in the response of the plant in function of the distance between mint plant and the soybean plant. Grown 10 cm apart from mint, the transcript accumulation level was enhanced even before the perception of a biotic stress which contradicts the finding of Bali et al (2020). Using plant essential oils as priming agents has already been investigated. Rienth et al (2019) for example studied the effect of oregano essential oil vapour to strive against *Plasmopara viticola*, a major threat of grapevines. Aside from the potential priming effect, they observed an upregulation of chitinase pathogenesis-related genes usually transcribed when the plant is attacked by fungi containing chitin in their cell walls. Given that *P. viticola* is an oomycete, this could suggest that the priming response is not specific to the stress stimuli that triggered the immune response. In the study of Dalio et al (2020), only 12% of banana plants were reported infected by *Fusarium* wilt after the application 2

years earlier of essential tea tree oil on the mother plants. However, this incredible systemic protection caused by the EO may be specific to the physiology of banana plants. A last example is the priming role of thymus oil suggested by the upregulation of PR5 and PR8 in treated apples 48h after the inoculation of *Botrytis cinerea* (Banani et al., 2018).

2 FOCUS OF THIS STUDY

In 2020, tomatoes accounted for 16% of the world's vegetable production, with over 184 million tons produced. In the same year, production in Europe reached almost 23 million tons ([b]FAO, 2021). Tomato crops are threatened by phytonematodes, particularly root-knot nematodes. Talavera-Rubia et al (2022) reported a yield loss caused by *M. incognita* of up to 69% for tomatoes based on a series of field trials carried out between 2007 and 2021. *Meloidogyne* spp cause 5% loss in world crop production and was designated by members of Nematology Societies at the top of the list of plant-parasitic nematodes (Agrios, 2005; Jones et al., 2013).

Meloidogyne spp are obligate parasites of plants. Females can lay up to 1,000 eggs in a gelatinous envelope called an egg mass on the surface of roots or in plant tissues. Nematodes go through 4 moults, but only seconde-stage juveniles are infective and motile. Their stylet pierces the root epidermis and injects cellulolytic and pectolytic enzymes to disintegrate cell walls. Galls are formed by the creation of a feeding site of hypertrophied cells called giant cells following the penetration of J2 into the cortical tissues. The main symptom of infection by root-knot nematode is the swelling but a delay in plant growth, a reduced root system and wilting can also be observed. The aerial part of the plants can also show chlorosis and yellowing (Jones et al., 2013; Joshi et al.,2020; Azlay et al., 2022).

The main aim of this work was to make progress in the study of the use of essential oils to control a species of nematode, *Meloidogyne javanica*, with particular emphasis on the synergy between essential oils. Two approaches were adopted: Nematicidal activity through direct contact and plant defence priming.

First approach - Direct contact nematicidal activity

Objective 1 Evaluation of the synergistic potential of combinations of several essential oils with *Satureja montana* essential oil against *Meloidogyne javanica*

Objective 2 Evaluation of the effect of the combination between the essential oils of *Artemisia absinthium* and *Satureja montana* on second-stage juveniles of *Meloidogyne javanica* and egg mass hatching

Second approach – Plant defense priming

Objective 3 Evaluation of the effect of the combination of *Artemisia absinthium* and *Satureja montana* essential oils on the level of nematode infection on Solanum lycopersicum

Objective 4 Identification of changes resulting from the application of the combination of essential oils of *Artemisia absinthium* and *Satureja montana* on the metabolic profile of *Solanum lycopersicum*

3 MATERIAL & METHOD

3.1 Essential oils

3.1.1 Plant material and essential oil extraction

Satureja montana (Spain, 2020), *Artemisia absinthium* Linnaeus var. ®Candial (Spain, 2019), *Satureja montana* (Spain, 2018), *Salvia officinalis* (Spain, 2018), *Origanum vulgare* L. subsp. *virens* (Spain, 2018), *Lavandula lanata* (Spain, 2018), *Mentha rotundifolia* (Spain, 2018) and *Thymus zygis* (Spain, 2017) were grown in fields located in Teruel (Spain). The field conditions have been detailed by Burillo (2009). Plant material for each species was dried under shadow at ambient temperature. Then, the essential oil was extracted by steam distillation in two 3000 L vessels forming part of a semi-industrial stainless-steel plant and was decanted (Julio et al., 2017).

3.1.2 GC-MS characterization

An analysis by gas chromatography coupled with mass spectrometry was carried out for all the essential oils mentioned in 3.1.1 using a Shimadzu GC-2010 coupled to GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). The analytical conditions and method used for compound identification are described in Soudani et al (2022).

3.2 Meloidogyne javanica rearing

M. javanica were reared on *Solanum lycopersicum* L. (var. *Marmande*) in 1L pot in a growth chamber at 22 ± 1°C, 70% relative humidity with a photoperiod of 16:8 h (L:D). The 21-day plants were inoculated with nematodes by burying an infected root near the root of the tomato plant to be infected. After 2 months, the egg masses were handpicked under a binocular microscope and placed on filters immersed in distilled water for hatching in a closed opaque box kept in a growth chamber at 22 ± 1°C. The second-stage juveniles were collected every 3 days during the one-month hatching cycle.

3.3 Effect on nematodes: *in vitro* tests

3.3.1 Effect on second-stage juveniles

The activities of *Salvia officinalis* (Spain, 2018), *Origanum vulgare* L. subsp. *virens* (Spain, 2018), *Lavandula lanata* (Spain, 2018), *Mentha rotundifolia* (Spain, 2018) and *Thymus zygis* (Spain, 2017) EOs alone and in combination at 1:1 ratio (w/w) with *Satureja montana* (Spain, 2018) EO were tested against *M. javanica in vitro* at different concentrations specified in the table 1. At least two concentrations resulting in an activity greater than 50% and two concentrations resulting in an activity less than or equal to 50% were tested in order to calculate the EC_{50}. The activity of *Satureja montana* EO (Spain, 2018) alone was also tested. In particular, the activity of the mixture of *Satureja Montana* (Spain, 2020) and *Artemisia absinthium* Linnaeus var. ®*Candial* (Spain, 2019) EOs at 1:1 (w/w) ratio was tested as a complementary test to the *in vivo* experiment. All the EOs were diluted in dimethyl sulfoxide (DMSO) (99,7%, Fischer Scientific, UK) with 0,6% Tween® 20 (Calbiochem, EMD biosciences Inc.) as solvent to reach the tested concentrations.

This test was conducted according to the protocol of Navarro-Rocha et al. (2020) adapted from Andrés et al. (2012). For each assay, 5 µL of the test solution of EO(s) were filled into a well of a 96-well U-shaped-bottom Microtest plate (Becton Dickinson Labware Falcon®, USA). A 95 µL volume of a solution containing between 70 and 150 *M. javanica* per microliter of distilled water were added and mixed to the EO(s) solution. Each concentration of EO or mixture of EOs was tested in four replicates forming a square. The wells directly surrounding the square were filled with distilled water to avoid border effect and to keep the same level of humidity during the whole experiment. At the opposite corner of the Microtest plate, the same protocol was followed replacing the 5 µL of EO(s) by 5 µL of a control consisting of DMSO +0,6% Tween® 20. The Microtest plate was covered with aluminum foil and placed in the growth chamber at 22 ± 1 °C. After 72h, the number of paralyzed nematodes in the treated and control samples was counted under a binocular microscope (Olympus SZ51).

Table 1. Concentrations tested for each essential oil and mixture of essential oils

	Concentration (mg/mL)								
Essential oils	**1**	**0,85**	**0,75**	**0,60**	**0,50**	**0,35**	**0,25**	**0,125**	**0,0625**
Satureja montana (Spain, 2018)	X				X		X	X	X
Salvia officinalis	X								
Origanum virens	X								
Lavandula lanata	X								
Mentha rotundifolia	X				X	X	X	X	
Thymus zygis	X		X		X		X		
Salvia officinalis : Satureja montana (Spain, 2018)	X		X		X		X		
Origanum virens :Satureja montana (Spain, 2018)	X		X		X		X		
Lavandula lanata :Satureja montana (Spain, 2018)	X	X	X	X	X		X		
Mentha rotundifolia :Satureja montana (Spain, 2018)	X		X		X		X		
Thymus zygis :Satureja montana (Spain, 2018)	X				X	X	X	X	
Artemisia absinthium :Satureja montana (Spain, 2020)	X				X	X	X	X	

The corrected percentage of paralyzed nematodes was calculated using the formula of Schneider-Orelli (1947):

$$\%P_{Tcorr} = \frac{\%P_T - \%P_C}{100 - \%P_C} \times 100$$

Where $\%P_T$ is the average percentage of paralyzed nematodes in the treated samples
$\%P_C$ is the average percentage of paralyzed nematodes in the control samples
$\%P_{Tcorr}$ is the corrected average percentage of paralyzed nematodes in the treated sample

The effective doses (EC_{50} and EC_{90}) were calculated by Probit analysis using Statgraphics Centurion® version 19.5.01 and version 16.1.18 (Stat Point Technologies, Inc., Warrenton, VA, USA) software. EC_{50} and EC_{90} represent the concentration at which 50% and 90% of the nematode population, respectively, is paralyzed.

The type of interaction between the essential oils active against *M. javanica* tested in combination was determined on the basis of their EC_{50} according to Widley's formula (Tak et al., 2016):

$$Expected\ EC_{50} = \frac{a + b}{\frac{a}{EC_{50(a)}} + \frac{b}{EC_{50(b)}}}$$

Where a is the proportion of the first essential oil in the combination
b is the proportion of the second essential oil in the combination
$EC_{50(a)}$ is the median effective dose of the first essential oil alone
$EC_{50(b)}$ is the median effective dose of the second essential oil alone

The expected EC_{50} was compared to the EC_{50} obtained by testing the combination of essential oils:

$$R = \frac{expected\ EC_{50}}{observed\ EC_{50}}$$

The interaction between essential oils was considered to be either synergistic (when R>1.5), additive (when 1.5 ≥R >0.5) or antagonistic (R< 0.5).

For the combinations between *S. montana* EO and an EO that was not active against *M. javanica*, the interaction between the EOs was determined by comparing the dose response graphs of *S. montana* alone and the combination (cf. 5.1).

3.3.2 Effect on egg mass hatching

In a 24-cell plate, the two central lines of wells were filled with either 400 µL of the mixture of *Satureja Montana* (Spain, 2020) and *Artemisia absinthium* Linnaeus var. ®*Candial* (Spain, 2019) EOs at 1:1 (w/w) ratio diluted in DMSO +0,6% Tween® 20 (0,5 mg/ mL) or distilled water as a control, each line containing 4 replicates of the same treatment. Three egg masses as uniform as possible were placed in a filter immersed in the solution filled in each well. The plate was wrapped in aluminum foil and placed in the growth chamber at 22 ± 1 °C. After 5 days, (a) the solution of each well was removed and the number of newly hatched nematodes in the solution was counted under a binocular microscope (Nikon SMZ 745). (b) The filters containing treated and control egg masses

were placed in 400 µL of distilled water in a new plate following the same disposition as for the beginning of the experiment and wrapped in aluminum foil. The two last steps (a,b) were repeated every seven days during 4 weeks and each time the plate was returned to the growth chamber at 22 ± 1 °C.

The relative suppression rate was calculated based on this formula:

Relative suppression rate:

$$(\%) = \frac{\overline{H_C} - H_T}{\overline{H_C}} \text{ x100}$$

Where $\overline{H_C}$ is the average number of J2 hatched in the control sample
H_T is the number of J2 hatched in the treated sample

3.4 *In vivo* experiment: Testing the priming potential of a mixture of *S.montana* and *A.absinthium* EOs

For the germination test and the *in vivo* experiment, *Solanum lycopersicum* L. var. *Marmande* seeds purchased from Ramiro Arnedo s.a. (Calahorra, Spain) were used.

3.4.1 *In vitro* preliminary test: Germination test

The phytotoxic effect on germination of the mixture of *Satureja Montana* (Spain, 2020) and *Artemisia absinthium* Linnaeus var. ®*Candial* (Spain, 2019) EOs at 1:1 (w/w) ratio was compared with that of the two EOs alone. The assay was conducted at 10, 5, 2.5, 1.25 mg/mL for the mixture and both EOs and the supplementary concentration of 0,625 mg/mL was also tested for *Artemisia absinthium* EO. The EOs were diluted in ethanol (99,5%, PanReac AppliChem ITW Reagents, Barcelona Spain) to reach the concentration tested.

The protocol set up by Martín et al. (2011) was used for this assay. The tomato seeds were hydrated 6 hours. In a 12-cell culture plate (SPL Life Science Co., Ltd, Korea), was placed in each well a 2 cm diameter Whatman® paper filter (Scharlab S.L, Barcelona Spain) soaked in the EO or mixture of EOs at the concentration tested. Ten seeds were added by well with 500 µL of distilled water. This test was conducted in 4 x 10-seeds replicates. A second culture plate containing four paper filters soaked in ethanol was made as a control. Both plates were wrapped in clear paper to avoid desiccation and placed in the growth chamber at 25°C with a photoperiod of 16:8 h (L:D) for 7 days. The number of germinated seeds was counted every day from day 3. The last day, 25 germinated seeds from each treatment randomly selected were stuck on a sheet of paper. The hypocotyl length of these seeds was measured by ImageJ program (https://imagej.nih.gov/ij/download.html).

The percentage of germinated treated seeds corrected by the percentage of germinated control seeds was calculated using the following formula:

$$\%G_T = \frac{\overline{G_T}}{\overline{G_C}} \times 100$$

Where $\overline{G_T}$ is the average of germinated treated seeds
$\overline{G_C}$ is the average of germinated control seeds
$\%G_T$ is the corrected percentage of germinated treated seeds

The growth rate of treated seeds corrected by the growth rate of control seeds was also determined using the same formula but by replacing the number of germinated seeds by the length of the hypocotyl.

3.4.2 Vermiculite treatment and plant growth

Based on the germination test, a concentration of 1.25 mg/mL was chosen for the *in vivo* experiment.

One hundred milligrams of the mixture of *Satureja Montana* (Spain, 2020) and *Artemisia absinthium* Linnaeus var. ®*Candial* (Spain, 2019) EOs at 1:1 (w/w) ratio (Sm:Aa) were dissolved in 80 mL of ethanol and mixed with 80 g of vermiculite. A volume of 80 mL of ethanol was also mixed with 80 g of vermiculite as control. For both batches of vermiculite, the solvent was air-dried until complete evaporation (one day). The vermiculite batches were then divided in 20 pots each. Two tomato seeds were sown per pot (80 seeds in total). The plants were watered every other day. After 36 days, 20 tomato plants per treatment were transplanted in a mixture (1:1 w/w) of different sand sizes (fine arena de rio and arena de miga) into clay pots.

3.4.3 Inoculation and plant harvesting

One week after transplanting, six pots per treatment were each inoculated with 400 nematodes suspended in distilled water by injection near the root as positive controls and treatments. Six other plants per treatment were kept uninfected as negative controls and treatments.

The remaining eight tomato plants per treatment were harvested the day of the inoculation for chromatographic analysis.

Twenty days after inoculating, the 24 tomato plants were harvested and the roots were cleaned under water. The root systems were dried as much as possible with paper. The aerial parts and the root systems were weighted separately. The number of galls were counted on the infected roots from the control and treated plants. The galls were sorted by size (small, medium and big) to take into account the different nematode quantities depending on gall size.

3.4.4 Extraction

To extract the metabolites produced in the aerial part and the root system, both parts of the plants were macerated in methanol (MEOH; 99,8%, HPLC grade, Fisher Scientific, UK) and dichloromethane (DCM; 99,8%, Analytical reagent grade, Fisher Scientific, UK) for at least 4 days. The replicates were pooled and the half of the plant material was used for MeOH maceration and the other half for DCM maceration. Twenty-four samples listed in table 2 were obtained for chromatographic analysis. Before removing the roots or aerial parts, the macerates were placed in a sonication bath for 15 min. Then, they were filtered on cotton. The filtrates were evaporated on a rotary evaporator with a bath at 40°C to flask volume. The remaining volumes were transferred to a pre-weighed flask and evaporated to dryness by air flow.

Table 2. List of samples for chromatographic analysis

Samples
43-day plants
Aerial part control plants macerated in MeOH Aerial part control plants macerated in DCM Root system control plants macerated in MeOH Root system control plants macerated in DCM Aerial part treated plants macerated in MeOH Aerial part treated plants macerated in DCM Root system treated plants macerated in MeOH
63-day plants
Control
Aerial part of infected plants macerated in MeOH Aerial part of infected plants macerated in DCM Aerial part of uninfected plants macerated in MeOH Aerial part of uninfected plants macerated in DCM Root system of infected plants macerated in MeOH Root system of infected plants macerated in DCM Root system of uninfected plants macerated in MeOH Root system of uninfected plants macerated in DCM
Treatment
Aerial part of infected plants macerated in MeOH Aerial part of infected plants macerated in DCM Aerial part of uninfected plants macerated in MeOH Aerial part of uninfected plants macerated in DCM Root system of infected plants macerated in MeOH Root system of infected plants macerated in DCM Root system of uninfected plants macerated in MeOH Root system of uninfected plants macerated in DCM

3.4.5 HPLC-MS analysis

The methanolic plant extracts (table 2) were analyzed by liquid chromatography coupled to mass spectrometry (HPLC-MS) in a Waters apparatus equipped with ACQUITY UPLC pump coupled to a quadrupole time-of-flight mass spectrometer as analyzer (SYNAPT-G2) and an electrospray ionization source (ESI). ESI was performed in the Full Scan positive mode (m/z = 50-1200). All extracts (0,8-1,5 mg) were dissolved in 100% ACN for injection (stock solutions). A 10 µL volume of the stock solutions diluted to 0.1 mg/ml was injected with an automatic injector (ACQUITY AutoSampler).

The data obtained was the result of one analysis per sample, with no repetition.

Table 3. Liquid chromatography conditions for the analysis of methanolic plant extracts

	Liquid chromatography conditions
Column parameters	1.7 µm[1], 2.1 x 150 mm
Flow rate	0.4 mL/min
Stationary phase	C18 AccQ-TagT Ultra 1.7µm
Mobile phase	ACN (LC-MS grade) (B): MiliQ water with 0.5% acetic acid
Solvent gradient	5%-95% B in 15 min; 100% for 0.10 min; 5% B for 3 min before next injection

3.4.6 Statistical analysis

Statgraphics Centurion® version 19.5.01 (Stat Point Technologies, Inc., Warrenton, VA, USA) and Rstudio were used for all the statistical analysis. The homogeneity of the sample variances was first checked with a Levene test. The comparison between infected and uninfected plants for each treatment was performed with a t student test with a 95.0 % confidence level. For the comparison between *Satureja montana*, *Artemisia absinthum*, Sm:Aa and both controls, a one-way analysis of variance (ANOVA) was performed. The normality of the samples was verified by testing the normality of the residuals from the statistical tests with the Shapiro-Wilk W test. Data that didn't meet the normality condition were normalized by a logarithmic transformation ($\log_{10}(x)$). When the assumption of homogeneity of variances was not respected, a Welch's t-test or a Welch's ANOVA, with a 95.0 % confidence level were carried out.

4 RESULTS

4.1 Chemical composition of essential oils

The main constituents of the eight EOs are summarised in Table 4 and classified by chemical groups. The GC-MS analysis showed that the chemical profiles of EOs are diversified. One to three compounds prevailed in most EOs (over 20% of the EO composition) except for *S. officinalis*, which showed a large number of terpenic compounds in moderate proportions (5%-13%). Particularly, thymol was found in moderate to large amount, 7.25 %, 9,94%, 21.17% and 20.90% respectively in *S. montana (Spain, 2020), S. montana(2018)*, *T. zygis* and *O. virens* EO*s*. Besides, *S. montana* (Spain, 2020) and *S. montana* (Spain, 2018) EOs mainly contained carvacrol (respectively 37.14% and 38.05%), ρ-cymene (25.48% and 18.56%) and γ-terpinene (7.13% and 11.17%).The composition of this EO varied slightly depending on the year. Piperitenone (27.55%) and piperitenone oxide (37.73%) were the most prevalent secondary metabolites in *M. rotundifolia* EO while lavandulol (29.79%) and β-bisabolene (18.21%) were the main compounds in *L. lanata* EO. Cis-epoxyocimene (34.85%) was identified as the main constituent of *A. absinthium* EO which also contained in smaller amount, cis-chrysanthenol (9.04%) and cis-chrysanthenyl acetate (8.40%). The results also showed the significant presence of linalool (11.61%) and linalyl acetate (17.92%) in *T. zygis* EO and trans- α -necrodyl acetate (10.86%) and γ-terpinene (15.46%) in *O. virens* EO*.*

Table 4. Chemical composition of tested essential oils and percentage contribution[a] of components

Compound name	*S. montana (Spain, 2018)*	*S. montana (Spain, 2020)*	*A. absinthium (Spain, 2019)*	*S. officinalis (Spain, 2018)*	*O. virens (Spain, 2018)*	*L. lanata (Spain, 2018)*	*M. rotundifolia (Spain, 2018)*	*T. zygis (Spain, 2017)*
Monoterpene hydrocarbons								
Camphene				2,97				
ρ-Cymene	18,56	25,48			3,74			8,04
Limonene							5,09	
β-Myrcene				2,26				
trans-Necrodol					1,65			
trans- α -Necrodyl acetate					10,86			
α-Pinene				7,72				
β-Pinene				8,27				
α-Terpinene	2,26	1,94			1,88			
γ-Terpinene	11,17	7,13		1,95	15,46			1,88
Monoterpene oxygenated								
Borneol				5,19				2,50
Camphor			1,97	5,76		17,83		2,68
Carvacrol	38,05	37,14						
cis-Chrysanthenol			9,04					
cis-Chrysanthenyl acetate			8,40					
1,8-Cineole				10,64		4,15		1,51
cis-Epoxyocimene			34,85					
trans-Epoxyocimene			2,37					
Geranyl acetate					2,98			

Compound name	*S. montana (Spain, 2018)*	*S. montana (Spain, 2020)*	*A. absinthium (Spain, 2019)*	*S. officinalis (Spain, 2018)*	*O. virens (Spain, 2018)*	*L. lanata (Spain, 2018)*	*M. rotundifolia (Spain, 2018)*	*T. zygis (Spain, 2017)*
Lavandulol						29,79		
Linalool						7,44		11,61
Linalyl acetate								17,92
l-Pinocarveol								
Piperitenone							27,55	
Piperitenone oxide							35,73	
α-Thujone				6,08				
β-Thujone				12,87				
Thymol	*9,94*	7,25			20,90			21,17
Diterpene								
Geranyl-α-terpinene			3,24					
Manool				1,88				
Sesquiterpene								
trans- β-Bergamotene						1,55		
Bicyclogermacrene					1,81			
α-Bisabolene						2,80		
β-Bisabolene	2,15	3,36				18,21		
δ-Cadinene					1,96			3,01
Caryophyllene			4,74				5,61	4,00
l-Caryophyllene				9,26	3,49	4,76		
Caryophillene	4,14	3,90						
β-Farnesene							1,64	2,89
Germacrene-D			2,41		3,87		8,19	

Compound name	*S. montana (Spain, 2018)*	*S. montana (Spain, 2020)*	*A. absinthium (Spain, 2019)*	*S. officinalis (Spain, 2018)*	*O. virens (Spain, 2018)*	*L. lanata (Spain, 2018)*	*M. rotundifolia (Spain, 2018)*	*T. zygis (Spain, 2017)*
α-Humulene				5,43				
Selina-3,7(11)-diene					1,92			
Sesquiterpene oxygenated								
α-Bisabolol								5,45
γ- 1-Cadinene aldehyde					2,03			
α-Cadinol					1,69			
Caryophyllene oxide						1,55		
Viridiflorol				7,03	2,31			
Others								
Chamazulene			5,01					
3,6-Dihydrochamazulene			3,37					
Isothymol methyl ether					1,80			
Methyl thymylether					1,66			

[a] Only components that contributed to more than 1,5% of the composition are listed.

4.2 In vitro experiments: effect of essential oils on *M. javanica*

4.2.1 Effect of essential oils against second-stage juveniles

The essential oils that paralyzed at least 70% of the nematode population after the correction by the control were considered as active against *M. javanica*. All the mixtures of EOs showed a toxic effect on J2 while half of the EOs tested independently were not active. *S. officinialis*, *O. virens* and *L. lanata* EOs were found not active against *M. javanica.* These EOs reduced J2 population by respectively only 15.41%, 24.32% and 14.96 % (Table 5). On the contrary, *S. montana* (Spain, 2018), *M. rotundifolia* and *T. zygis* EOs exhibited a respective activity of 99.34%, 100% and 84.38% at the same concentration. EO from *M. rotundifolia* and EO from *S. montana* combined with those from *S. officinalis* and *O. virens* showed a constant activity close to 100%, which dropped at concentrations below 0.75 mg/mL, while the same phenomenon was observed for EO from *S. montana* alone and in combination with EO from *M. rotundifolia* and EO from *T. zygis*, with a decline at concentrations below 0.5 mg/mL. The activity of *L. lanata* EO plunged between 0.6 mg/mL and 0.5 mg/mL (figure 4). A median effective concentration (EC_{50}) was calculated based on the dose-response curve of the EOs (Table 5). The EO from *S. montana* exhibited the highest toxicity against J2 followed by *M. rotundifolia* EO and the mixture of *S. montana* and *T. zygis* EOs. This latter is the only mixture that exerted an activity (EC_{50}= 0.36 mg/mL) that comes close to the activity of *S. montana* EO (EC_{50}=0,25 mg/mL). Interestingly, the lowest toxicity was observed for *T. zygis* EO alone that declined at concentrations below 1.mg/mL. The combinations between *S. montana* EO and the EOs of *L. lanata*, *M. rotundifolia*, *O. virens*, *S. officinalis* and *A. absinthium* displayed a moderate activity with an EC_{50} between 0.45 and 0.51 mg/mL.

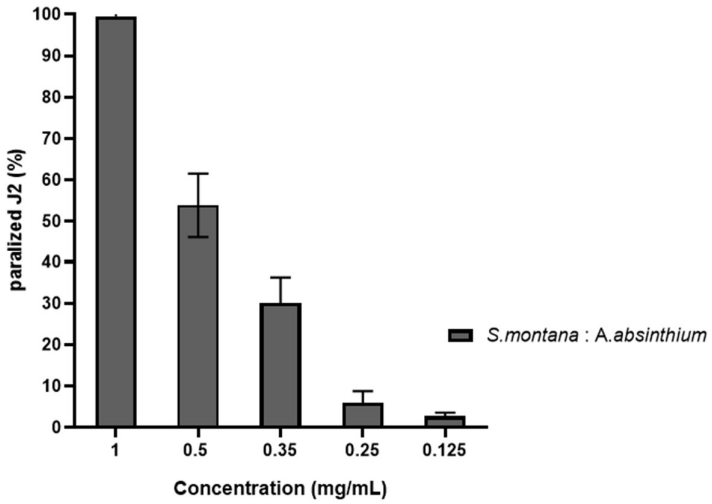

Figure 3. Percentage of paralyzed M. javanica second-stage juveniles after 72h exposure to mixture of A. absinthium and S. montana EOs in a concentration range of 0.125 -1 mg mL^{-1}. Data are means of four replicates and are shown as the mean ± SE corrected by the control according to Schneider-Orelli formula.

4.2.2 Effect of the combination between *A. absinthium* and *S. montana* essential oils on egg mass hatchability of *M. javanica*

The mixture of *S. montana* and *A. absinthium* EOs reduced the total number of nematodes hatched from egg masses by 70.53 ± 9.93%. The reduction in egg mass hatching reached a peak after 21 days with a suppression rate of 88.67 ± 4.84%. The hatching of J2 in control and treated samples showed a similar evolution with the highest rate on day 14 (Figure 5). From this day, a significative difference between control and treatment was obtained (p-value < 0,05).

Table 5. EC_{50} and EC_{90} values (mg ml^{-1}) of the essential oils and mixture of essential oils tested and parameters of Probit analysis

Essential oil	% immobility at 1 mg/mL	EC_{50} (mg/mL)	95% confidence limit (mg/mL)		EC_{90} (mg/mL)	95% confidence limit (mg/mL)		Slope± SE	Intercept ± SE	Interaction
			Lower	Upper		Lower	Upper			
S. montana (Spain, 2018)	99.34 ± 0.23	0,25	0,24	0,26	0,42	0,40	0,43	7,76 ± 0,25	-1,95 ± 0,06	-
S. officinalis (Spain, 2018)	15.41 ± 2.41	-	-	-	-	-	-	-	-	-
O. virens (Spain, 2018)	24.32 ± 3.35	-	-	-	-	-	-	-	-	-
L. lanata (Spain, 2018)	14.96 ± 1.43	-	-	-	-	-	-	-	-	-
M. rotundifolia (Spain, 2018)	99.47 ± 0.36	0,31	0,30	0,33	0,50	0,48	0,52	7,01 ± 0,31	-2,21 ± 0,10	-
T. zygis (Spain, 2017)	84.37 ± 2.19	0,68	0,64	0,72	1,35	1,26	1,46	1,90 ± 0,11	-1,29 ± 0,07	-
A. absinthium (Spain, 2019)	0.74 ± 0.81[a]	-	-	-	-	-	-	-	-	-
S. officinalis (Spain, 2018) *:S. montana* (Spain, 2018)	99.16 ± 0.31	0,51	0,49	0,52	0,75	0,73	0,78	5,17 ± 0,20	-2,62 ± 0,11	antagonistic
O. virens (Spain, 2018) *:S. montana* (Spain, 2018)	98.53 ± 0.86	0,45	0,44	0,47	0,71	0,69	0,74	4,90 ± 0,19	-2,22 ± 0,10	additive
L. lanata (Spain, 2018) :S. montana (Spain, 2018)	99.87 ± 0.13	0,48	0,47	0,49	0,68	0,67	0,70	6,37 ± 0,21	-3,06 ± 0,12	antagonistic
M. rotundifolia (Spain, 2018) *:S. montana* (Spain, 2018)	100 ± 0.0	0,47	0,45	0,48	0,70	0,67	0,72	5,58 ± 0,23	-2,60 ± 0,12	additive/ synergistic
T. zygis (Spain, 2017) *:S. montana* (Spain, 2018)	99.82 ± 0.18	0,36	0,36	0,37	0,57	0,55	0,59	6,21 ± 0,22	-2,27 ± 0,08	additive/ synergistic
A. absinthium (Spain, 2019) *:S. montana* (Spain, 2020)	99,54 ± 0,28	0,48	0,46	0,49	0,72	0,69	0,75	5,36 ± 0,22	-2,56 ± 0,09	additive

[a] This value is the result of the test carried out last year in the lab (Instituto de Ciencias Agrarias) with *A. absinthium* (Spain, 2019) EO. The same protocol as for this study was used.

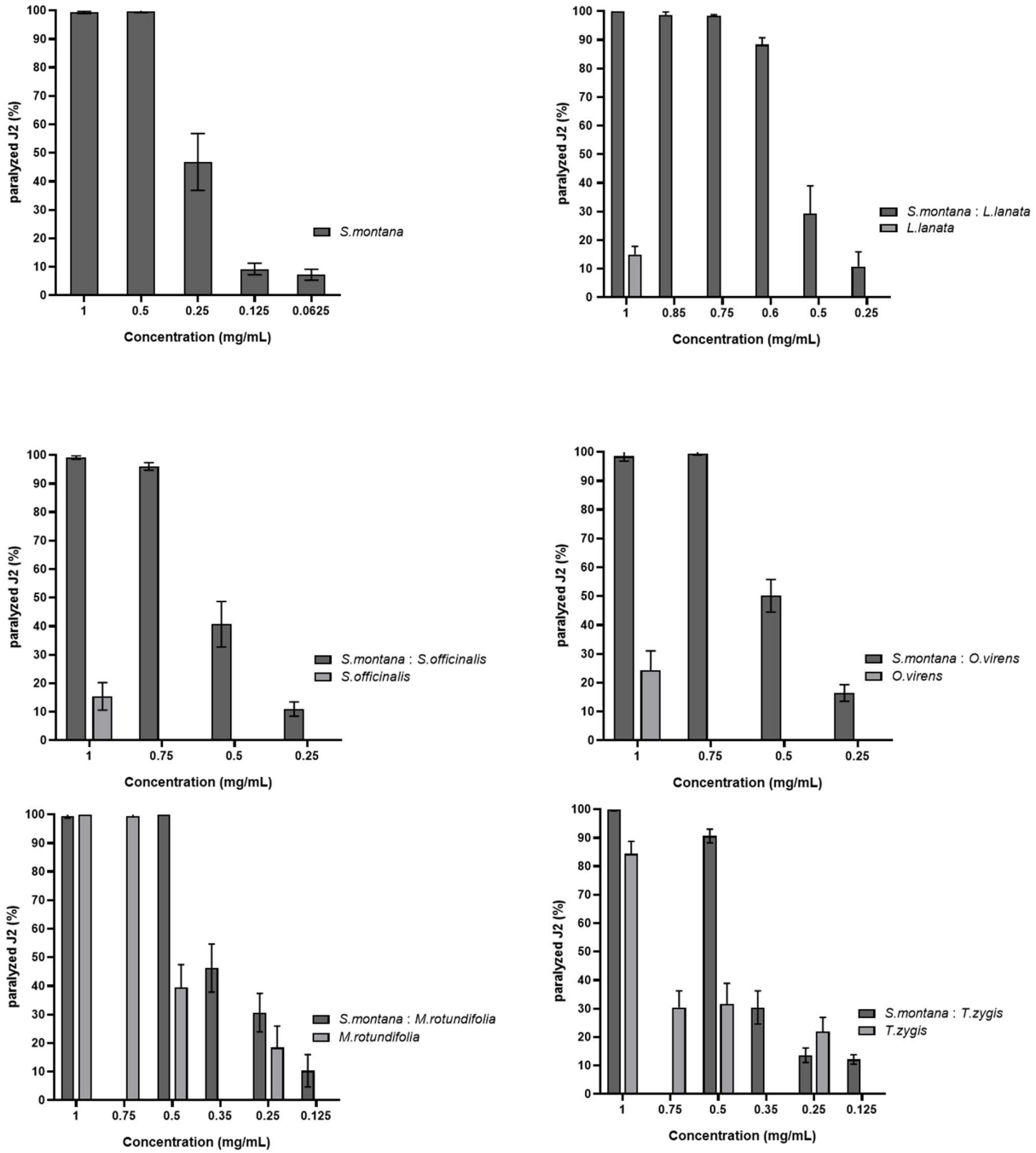

Figure 4. Percentage of paralyzed M. javanica second-stage juveniles after 72h exposure to the EOs in concentration range of 0.0625-1 mg mL^{-1}. Each bar chart except for S. montana EO shows the effect of the essential oil tested independently and in combination with S. montana EO. Data are means of four replicates and are shown as the mean ± SE corrected by the control according to Schneider-Orelli formula.

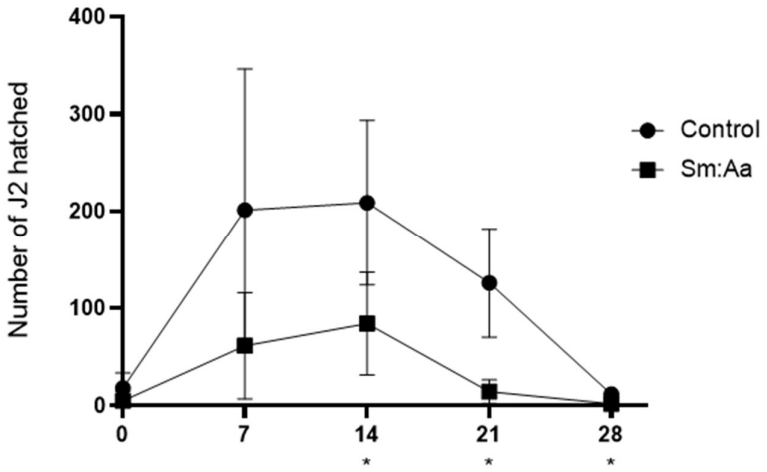

Figure 5. Number of M. javanica J2 hatched from control and treated egg masses. Time 0 : 5 days of continuous exposure to the tested solutions (distilled water and Sm:Aa); Time 7, 14, 21 and 28 : respectively 7, 14, 21 and 28 days after the time 0. Data are means of four replicates and are presented as the mean ± SE. An asterisk (*) below the time indicates a significative difference in hatching between the treated and control egg masses at this time (Welch's ANOVAs, 95,0% confidence level).

4.3 *In vivo* experiment

4.3.1 Preliminary *in vitro* phytotoxicity test on germination

Germination and hypocotyl growth rates were observed to determine a concentration of Sm:Aa that is not phytotoxic for the tomato seeds. These parameters were also compared between *S. montana* EO, *A. absinthium* EO and Sm:Aa treatments with ANOVA or Welch's ANOVA analysis, at confidence level of 95%. The phytotoxicity was assessed by comparison with the control with the same statistical analysis.

At a concentration of 10 mg/mL, the germination rate of the tomato seeds treated with *S. montana* EO, *A. absinthium* EO and the mixture of both essential oils was reduced compared to the control by respectively 82.5%, 17.5% and 40 % as observed 6 days after the treatment. But the seventh day, the germination rate of seeds treated with *S. montana* and *A. absinthium* EOs increased at respectively 65 ± 12.6 % and 97.5 ± 2.5 %. This data was not collected for the combination of EOs. Below this concentration, the germination rate of treated seeds no longer differed from that of the control.

The hypocotyl growth rate (GR) of the seeds treated with the EOs mentioned above is depicted on the figure 6. At 10 mg/mL, *A. absinthium* EO and Sm:Aa showed a unequivocal effect on the growth rate of tomato hypocotyl with respective reductions of 73.83% and 79.66% . Regarding *S. montana* EO treatment, too many seeds didn't germinate to have a representative sample for the growth rate calculation which indicates a strong phytotoxicity. At 5 mg/mL and 2.5 mg/mL, the three treatments exerted phytotoxicity with a significant stronger effect of *S. montana* EO and Sm:Aa compared to *A. absinthium* EO (at least a p-value < 0,05). A Sm:Aa concentration of 1.25mg/mL was no longer phytotoxic (GR=91.63 ± 7,5 %; p-value > 0,05) for tomato seeds while a significant reduction of the growth rate (RGR) was still observed with *A. absinthium* (RGR = 21,82%; p-value <0,01) and *S. montana* (RGR = 47,97% ; p-value < 0,001) treatments compared with the control. At 0.625 mg/mL, no treatments were phytotoxic for the seeds.

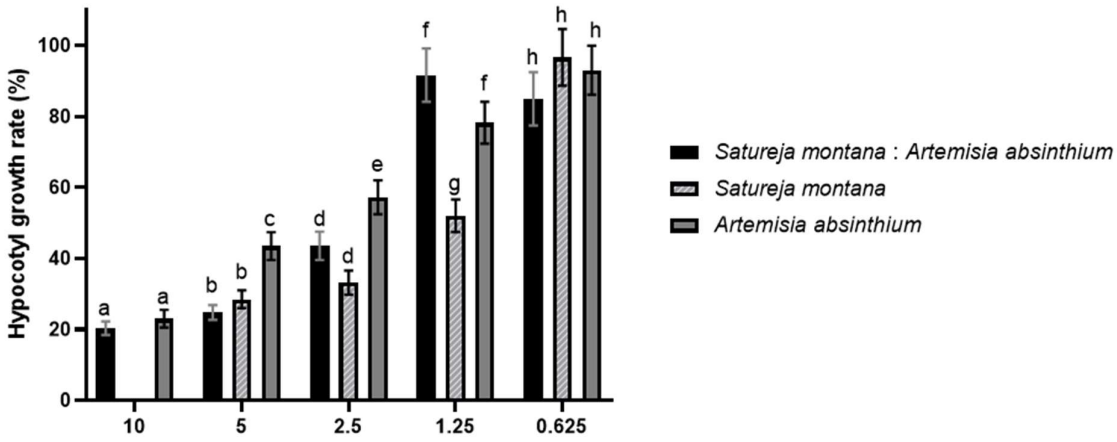

Figure 6. Percentage growth rate of hypocotyl from Solanum lycopersicum L. seeds treated with S.montana EO, A.absinthium EO and the mixture of the two EOs (1:1 w/w) at 0,625; 1,25; 2,5; 5; and 10 mg/ml. The hypocotyl lengths were measured after 7 days of experimentation and the growth rate is the relative length in relation to the control. Data are means of 25 replicates and are expressed as the mean ± SE. For each concentration, bars that show different letters represent values that are significantly different according to Tukey's HSD test (p-value < 0,05)

4.3.2 Effect on gall infestation and growth parameters of tomato plants

In this section are compared the results of Sm:Aa treatment with *S. absinthium* and *S. montana* EO treatments. The *in vivo* experiments linked to these latter two treatments were carried out by a PhD student, Sabrina Kesraoui over the same period of time, according to the same protocol and in the same conditions.

ANOVAs or Welch's ANOVAs with a confidence level of 95%, were carried out to compare the degree of infestation of plants treated by *S. montana* EO, *A. absinthium* EO and Sm:Aa. These analyses also compared the treatments with the controls to evaluate their potential efficiency. As shown in figure 7, there is no difference regarding the total number of galls per root gram between the three treatments and between each treatment and the controls. To accurately assess the degree of infestation, the galls were sorted by size (small, medium and big). Because this ranking was arbitrary and depends on the operator, the number of small, medium and big galls per root gram was only compared between *Sm:Aa* treatment and the second control and between the treatments based on *S. montana* and *A. absinthium* EO and the first control. No difference was observed for any size of gall for both comparisons. It should be noted that the number of galls per root gram as shown in figure 7, is highly variable between replicates.

Statistical tests showed no difference in the weight of uninfected tomato roots between treatments and compared with the two controls. For the aerial part weight of uninfected plants, no difference was found between Sm:Aa treatment and its associated control (2) or *S. montana* and *A. absinthium* treatments and their associated control (1). In contrast, a significant difference was observed between control 1 and Sm:Aa treatment, this latter showing an higher aerial part weight. A significant lower weight compared with control 2, for the aerial part of plants treated with *A. absinthium* EO was observed for infected and uninfected plants. Regarding infected tomato plants, Sm:Aa treatment showed a significant higher root weight than *A. absinthium* treatment and control 2. No difference was observed between the other treatments including the controls. The infected plants treated with Sm:Aa showed the highest weight for the aerial part. This observation was

confirmed by a Welch's ANOVA analysis. Interestingly, a difference was found in the root weight between infected and uninfected tomato plants treated with Sm:Aa. The infected plants presented a higher root weight.

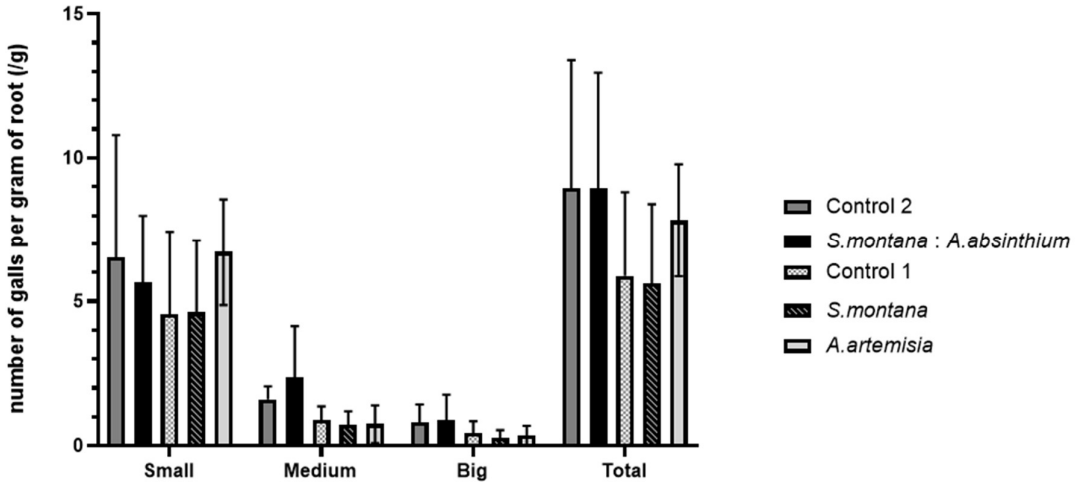

Figure 7. Number of galls of M. javanica per root gram from infected tomato plants grown for 36 days in vermiculite treated with S. montana EO, A. absinthium EO and the mixture of the two EOs. Both controls are infected plants grown in vermiculite treated with ethanol. Plants were harvested 20 days after the inoculation. Galls were sorted by size (small, medium and big). Data are means of five replicates for control 2 and Sm:Aa treatement and six replicates for control 1, A. absinthium EO and S. montana EO treatments. Each value is expressed as the mean ± SE.

Table 6. Root and aerial part fresh weights of uninfected and infected tomato plants grown for 36 days in vermiculite treated with *S. montana* EO*, A. absinthium* EO and the mixture of the two EOs

Treatments	Conc (mg mL)	Uninfected		Infected	
		Root fresh weight (g)± SE	Aerial part fresh weight (g) ± SE	Root fresh weight (g) ± SE	Aerial part fresh weight (g) ± SE
Control 1*		5.39 ± 0.85$^{d.A}$	11.59 ± 1.30$^{a.b.B}$	4.30 ± 0.56$^{e.A}$	12.23 ± 0.94$^{g.k.B}$
Control 2*		4.63 ± 0.37$^{d.C}$	13.27 ± 1.51$^{b.c.D}$	4.84 ± 0.42$^{e.f.C}$	16.78 ± 1.25$^{g.D}$
Satureja montana	1.25	3.78 ± 0.25$^{d.E}$	8.67 ± 0.70$^{a.b.F}$	4.92 ± 0.63$^{e.f.E}$	11.17 ± 1.30$^{g.j.F}$
Artemisia absinthium	5	3.18 ± 0.36$^{d.G}$	6.97 ± 0.83$^{a.H}$	3.89 ± 0.13$^{e.G}$	8.24 ± 0.63$^{h.j.k.H}$
***S.montana* : *A.absinthium* (1:1)**	1.25	4.34± 0.69$^{d.I}$	18.67 ± 2.30$^{c.K}$	7.57 ± 1.12$^{f.J}$	24.92 ± 3.14$^{i.K}$

Different lower case letters within column indicate a significative difference between values according to Tukey HSD test (p-value < 0,05)
Different capital letters within a treatment for the same parameter indicate a significant difference between infected and uninfected plants for this parameter according to at t-student test (p-value <0,05)
*Both controls are tomato plants grown for 36 days in vermiculite treated with ethanol

4.3.3 Metabolic profiles of tomato plants treated with the essential oils of *A. absinthium* and *S. montana* and the combination between the two essential oils

The analytical conditions were not optimized. These results are therefore considered preliminary. Further analyses, in particular GC-MS analysis and compound identification, are currently underway. The 43-day plant extracts will be analyzed at a later date to optimize the duration of the experiment according to the priming response, which may be time-dependent.

Various compounds were detected by liquid chromatography at retention times (RTs) of 0.54; 0.65; 2.59; 5.45; 7.62; 7.74; 7.88; 8.13; 8.49 minutes in aerial part extracts from plants treated with *A. absinthium* EO, *S. montana* EO; Sm:Aa and ethanol (controls). Particularly, a metabolite with a retention time of 9.85 was found only in aerial part extracts from uninfected plants treated with Sm:Aa. The compound with a retention time of 7.74 min was not detected in aerial part extracts from infected plants treated with *A. absinthium* EO. For all the treatments, the metabolite with a retention time of 2.59 min was detected in uninfected plants. But it was found only for control 2 and Sm:Aa treatment in aerial part extracts from infected plants.

Regarding the root extracts, for all the treatments, compounds were eluted at retention times of 0.55; 0.65; 7.66; 7.73; 7.89; 8.15 and 14.41 minutes. A metabolite with a retention time of 14.35 min was found only for Sm:Aa treatment and for control 2, for this latter only in uninfected plant. The molecule with a RT of 7.92 min was found neither in extracts from plants treated with *A. absinthium* EO nor in one of the controls (1). The compound with a RT of 11.11 min was detected for all the treatments and control 2 but not for control 1. Conversely, metabolites with RTs of 9.47, 9.74 and 9.84 min were found in control 1 but not in control 2. These same molecules were not detected in extracts from plants treated with Sm:Aa (9.47 and 9.74) or *S. montana* EO (9.84).

No details will be given on the relative areas of the compounds because of their poor reliability due to the low resolution. As shown in Figure 8 used as example, the compounds are coeluted at 0.65, 7.70, 9.52 and 9.77 min. Therefore, analysis of the mass spectra was not possible, as the ions and fragments belonging to each metabolite could not be distinguished. In addition, the chromatograms showed baseline drift.

Three-dimensional bar charts for the analysis of root and aerial extracts are nevertheless presented to observe the differences in metabolic profiles resulting from the different treatments (figure 8 and 9). Some intriguing results have been retained as leads for future analysis (cf. 5.2).

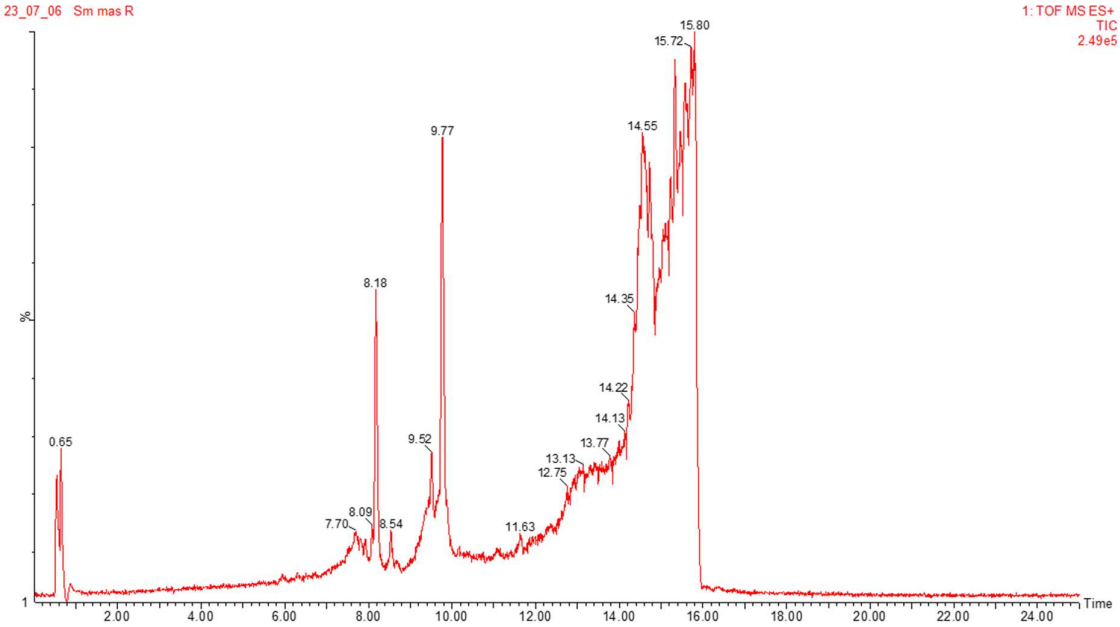

Figure 8. Chromatogram resulting from high-performance reverse-phase liquid chromatographic analysis of root extract from plants treated with S. montana EO and infected with Meloidogyne javanica

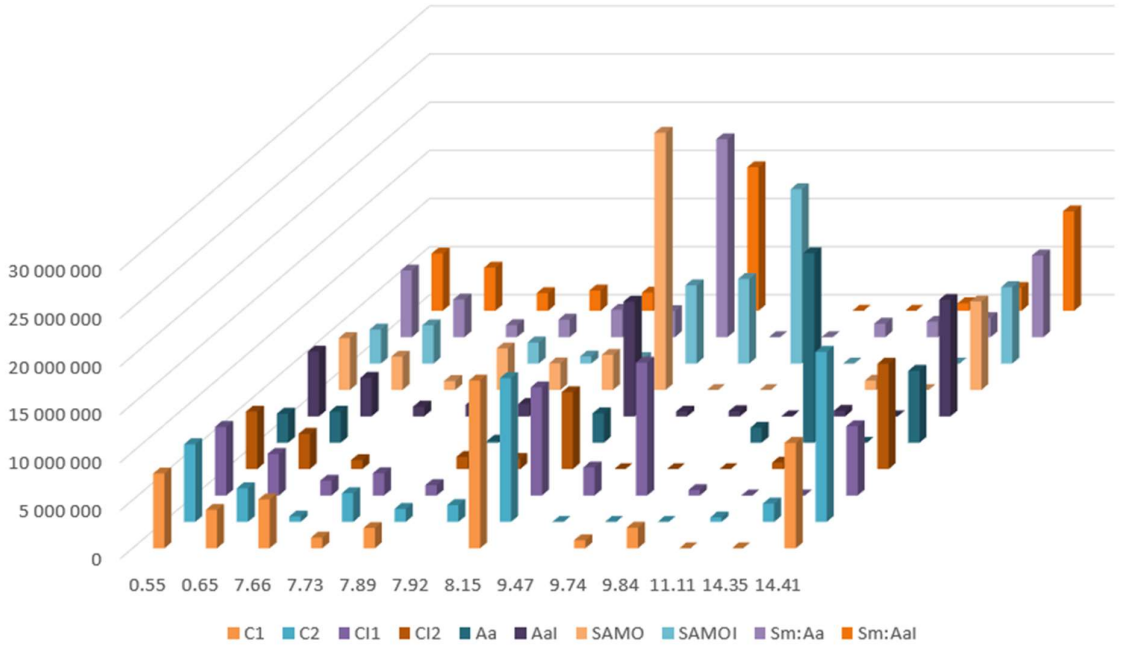

Figure 9. Relative area of the compounds detected by the reverse phase liquid chromatographic analysis of the methanolic root extracts from tomato plants infected (I) or uninfected with M. javanica and treated with ethanol (C1 and C2), A. absinthium EO (Aa), S. montana EO (SAMO) and the combination between S. montana and A. absinthium EOs (Sm:Aa). The relative areas are represented on the vertical axis and the retention times (min) on the horizontal axis.

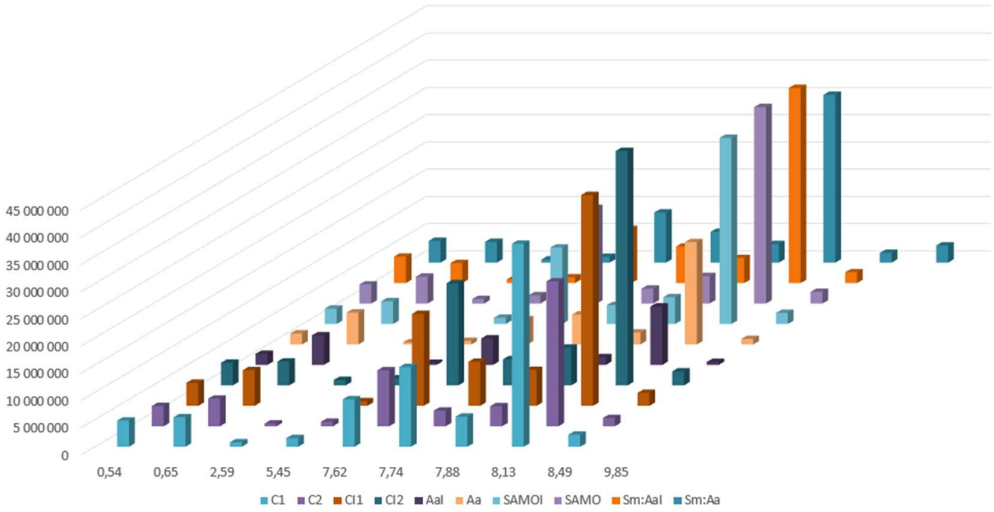

Figure 10. Relative area of the compounds detected by the reverse phase liquid chromatographic analysis of the methanolic extracts from aerial parts of tomato plants infected (I) or uninfected with M. javanica and treated with ethanol (C1 and C2), A. absinthium EO (Aa), S. montana EO (SAMO) and the combination between S. montana and A. absinthium EOs (Sm:Aa). The relative areas are represented on the vertical axis and the retention times (min) on the horizontal axis.

5 DISCUSSION & PROSPECTS

5.1 *In vitro* experiments

One of the aims of this study was to assess whether combining essential oils would increase the nematicidal effect via synergistic mechanisms. To this end, several essential oils were tested alone and then in combination with *Satureja montana* EO. In particular, the effect on *Maloidogyne javanica* second-stage juveniles and egg mass hatching of the combination of *S. montana* and *A. absinthium* EOs was tested in addition to the *in vivo* experiment.

The results indicate that *Mentha rotundifolia*, *Thymus zygis* and *Satureja montana* EOs were toxic to *M. javanica* J2. Their nematicidal property was already recorded by Oka et al (2000), Andrés et al (2012) and Navarro-Rocha et al (2019). Nevertheless, the activity reported in these studies was higher for the three essential oils with respective EC_{50} values of 0.226 mg/mL and 0.204 mg/mL for *T. zygis* and *M. rotundifolia* EOs and an EC_{50} value below 0.1 mg/mL for *S. montana* EO. This could be explained except for *M. rotundifolia* EO, by a richer composition of their respective main component precisely known to affect the mobility of J2 (Andrés et al., 2012; Nasiou et Giannakou, 2017; Nasiou et Giannakou, 2023). Indeed, Andrés et al (2012) reported that thymol represented 74% of *T. zygis* EO and carvacrol as 76% of *S. montana* EO compared with respectively 21.17% and 38.05% in the tested EOs. The composition of *M. rotundifolia* EO differs from that used by Oka et al (2000), whose major compound was an isomer of 1,2-epoxymenthyl acetate. The EO tested in this project, contained piperitenone (27.55%) and piperitenone oxide (35.73%) as main components. Piperitenone was found active against *M. javanica* with a median lethal concentration (LC_{50}) value of 0.15 mg/mL in the study of Kimbaris et al (2017). Given the moderate LC_{50} value of this secondary metabolite, the activity of *M. rotundifolia* EO could result from a synergistic effect of its components or an additive effect. However, in the absence of data on the nematicidal activity of its other components, notably piperitenone oxide, it is impossible to make any firm assumptions. *Artemisia absinthium* EO didn't show an activity against J2. Amora et al (2017) reported a potent nematicidal activity of this EO against *M. javanica.* The composition of EO used in that study differs from the EO tested in this experiment with a high content in β-thujone. On the contrary, García-Rodríguez et al (2015) tested an EO of *Artemisia absinthium* with similar composition to that used for the project and showed no activity. This difference of activity may indicate a nematicidal activity of β-thujone. *O. virens* EO was not active against *M. javanica* despite its thymol (20.90%) and γ-terpinene (15.46%) content. This result was unexpected knowing that El-Habashy et al (2020) reported a potent activity of γ-terpinene on *M. javanica* with an LC_{50} value of 36.22 mg/L. This result may suggest an antagonistic interaction between the two compounds, or even with other lower content components of the EO. This type of interaction between terpenes and terpenoids has been studied against *Meloidogyne incognita* by Ntalli et al (2020). *Lavandula lanata* EO showed the lowest activity (14.96%) against J2 at the highest concentration tested, 1 mg/mL. The biocidal activity of *L. lanata* EO and its major components lavandulol and β-bisabolene is poorly documented. But the essential oil of several species of the same genus have been tested against pinewood nematode *Bursaphelenchus xylophilus* without showing any activity either (Barbosa et al., 2010). Interestingly, Andrés et al (2017) revealed a nematicidal activity of hydrolates from *Lavandula × intermedia* var. super and *Lavandula luisieri*, the by-products of the EO production. *Salvia officinalis* EO didn't exert a significant activity against *M. javanica* in agreement with the result of Oka et al (2000). No significant activity of β-pinene, l-caryophyllene and 1,8-cineole which account for 28.17% of the EO content, has been reported in the literature. In contrast, the study of El-Habashy et al (2020) showed a strong nematicidal activity of α-pinene against *M. javanica* with a LC_{50} value of 43.28 mg/L. In

addition, as already mentioned thujone could have nematicidal property. All this information and the composition of the EO suggest that the content of nematicidal compounds is low and so that the components do not interact synergistically. This analysis is mainly based on the main components of the essential oils and the individual activity of those. However, Jiang et al (2009) compared the effect on *Trichoplusia ni* of *Litsea pungens* and *Litsea cubeba* EOs and a blend containing all the main compounds of these oils. They also tested blends not containing one of the major compounds to assess its toxicity in the mixture. A greater toxicity was found for limonene, carvone and 1,8-cineol in the blend than their individual activity against cabbage looper. These observations, and the fact that an activity similar to that of the EOs was only achieved for mixtures also containing inactive EO components, underline the importance of synergy between constituents (Jiang et al.,2009).

A potential synergistic effect was found for the combination between *S. montana* EO and EOs individually active against *M. javanica* (*M. rotundifolia* and *T. zygis*). EO. Wadley's formula indicated an additive effect of the combinations between these EOs. However, the dose-response relationship observed on the graphs of *M. rotundifolia* EO, *S. montana* EO and the combination suggest a synergistic effect. A concentration of 0.5 mg/L for the combination means that the EOs are individually concentrated at 0.25 mg/mL. But at this concentration, the percentage of paralyzed nematode reached only 18.42% and 46.82 % for respectively *M. rotundifolia and S. montana* EOs. The sum of the activity of the two EOs would be 65.2% so a value below the value obtained by testing the combination (100%) at 0.5 mg/mL which corresponds to the synergy definition. This observation highlights the limitation of the model used for Probit analysis. In fact, the EC_{50} value of 0.47 mg/mL obtained for *M. rotundifolia* EO combined with *S. montana* EO is probably overestimated, knowing that at 0.35 mg/mL, the percentage of paralyzed nematodes reached 46.24%. Likewise, according to Wadley's formula, the percentage of paralyzed nematodes obtained by the application of *T. zygis* and *S. montana* EOs in combination is the result of an additive effect. This is not consistent with the graphs representing the dose-response relationship of *T. zygis* and *S. montana* EOs and the combination. Indeed, the combination at 0.5 mg/mL caused the paralysis of 90.64% of the nematodes. The graphs indicate that a percentage of paralyzed nematodes of 46.82 was found at 0.25 mg/mL for *S. montana*. The percentage of paralyzed nematodes caused by *T. zygis* EO application is 21.90% at this concentration. Following the same method as above, the sum of the paralyzed nematode percentage caused by each EO is 68.72% which is lower than the percentage obtained for the combination. However, the significant error values for the activity values do not allow this type of interaction to be firmly confirmed. An antagonistic effect was observed for *L. lanata* and *S. officinalis* EOs combined individually to *S. montana* EO following the same method based on the graphs. The percentage of paralyzed nematodes found for the combination (40.68%) between *S. officinalis* and *S. montana* EOs at 0.5 mg/L is even lower than the percentage for *S.montana* EO alone (46.82%) at 0.25 mg/mL. A lower percentage than *S. montana* EO alone at 0.25 mg/mL was also observed for the combination with *L.lanata* EO (29.29%) at 0.5 mg/mL. The mechanisms governing synergies are not yet well understood. 50.12 % of paralyzed nematodes were found for the combination with *O. virens* EO at 0.5 mg/ml. The sum of the percentages of paralyzed nematodes caused by O. virens EO (below 24.32%) and *S.montana* EO (46,82%) at 0.25 mg/mL would be between 46.82% and 71.14%. So the percentage of paralyzed nematode obtained by application of the combination could be the result of an additive or an antagonistic effect. O. virens EO should be tested at 0.25 mg/mL to determine with certainty the type of interaction. The combination between *A. absinthium* and *S. montana* EOs resulted in an additive effect on second-stage juveniles. Indeed, the percentage of paralyzed nematodes caused by the application of *S. montana* EO at 0.25 mg/mL is similar to that of the combination at 0.5 mg/mL. This observation is

consistent with the fact that *A. absinthium* EO showed zero activity. A line of research to understand the interactions between EOs is the mode of action of their constituents. Liu et al (2022) mentioned that synergy may result from the multiplication of modes of action, increased penetration or even the disruption of the detoxification system.

As mentioned above, the EC_{50} values found with Probit analyses are not always accurate and so should be taken as an indication. Particularly, an EC_{50} value of 0.48 mg/mL was obtained for the combination with *L. lanata* EO whereas at 0.50 mg/mL, only 29.29% of the nematodes were paralyzed. The *T. zygis* EO at 0.75 mg/mL caused only 30.40%, although an EC_{50} value of 0.68 mg/mL was found.

The experiments conducted in this study assessed the effect of the EOs on the mobility of *M. javanica* J2. In the future, it could be interesting to confirm the death of the paralyzed nematode by a revival test. For example, D'Addabbo et al (2020) immerged the nematodes in distilled water for couple of days after the end of the exposition time to the EO. But a common test is exposure to a few drops of 1 M sodium hydroxide (NaOH) after transferring the nematodes to a Petri dish with distilled water to observe their ability to move (Dutta et al., 2021).

Regarding the activity of Sm:Aa on egg mass hatching, a significative reduction was observed on days 14, 21 and 28. Comparing with *S. montana* EO studied by Andrés et al (2012), the reduction is more important for the combination with *A. absinthium* EO. The concentration of *S. montana* EO used was higher (1 mg/mL) than the tested concentration of Sm:Aa which could indicate a synergistic effect between the two EOs. Interestingly, the evolution over time also differs. *S. montana* EO sharply reduced the hatching of egg masses on day 0 and day 2 and then caused a reduction between 50-60% until the end of the experiment (Andrés et al., 2012). The opposite was observed for the combination of EOs. Although 72% reduction was obtained at day 0, the relative suppression rate was reduced on day 7 and 14 and finally reached its highest value on day 21 and 28. No data was found for *A. absinthium* EO alone.

5.2 *In vivo* experiment

Studying the plant metabolome is necessary to understand its defense response. A time-dependent reprogramming of metabolic pathways takes place, resulting in a change in the metabolic profile with the accumulation or depletion of specific primary and secondary metabolites. For this reason, a non-targeted metabolomic analysis of tomato root and aerial extracts was carried out. This type of analysis deals with a large number of metabolites and is generally used to compare metabolic profiles. Two technical approaches are commonly use: nuclear magnetic resonance and mass spectrometry (MS) (Alonso et al., 2015). The latter was coupled with reverse phase liquid chromatography in this project to assess the influence of the application of *Satureja montana* EO, *Artemisia absinthium* EO and their combination on tomato plants, while also evaluating the effect of the nematode infestation. A paper reporting on the priming of tomato plants by *Artemisia absinthium* EO against *Fusarium oxysporum* was published in 2022 (Soudani et al., 2022). A first *in vivo* experiment on tomato plants using *A. absinthium* EO was carried out last year with no significant results. In this experiment, tomato seeds were dipped in the EO solution for one second. However, the results presented here are preliminary as this is the first test with the combination between *S. montana* and *A. absinthium* EOs.

Chromatograms showed low resolution, with most of the metabolites eluting over a short period of time (1-2 min). Several parameters could be optimized. The first step would be the sample preparation. Other extraction techniques as Soxhlet and solid phase extraction would enable the

concentration of the metabolites of interest and, above all, reduce the interferences that are common in this type of complex matrix. Parameters such as temperature, pressure and pH also influence extraction. Purification techniques as column preparation can follow the extraction step. The column parameters could be revised as the type and brand of stationary phase, the pore size, diameter and length. The injector system impacts also the width of the peaks. The mobile phase could be optimized by testing different combinations of solvents in different ratios. For example, the linear gradient could be replaced with a stepwise gradient, which may allow a longer interaction with the stationary phase, resulting in improved resolution. Another option is the use of two-dimensional chromatography as LC-LC and LCXLC that could increase the selectivity and facilitate coupling to the mass spectrometer. An interesting tool for untargeted metabolomic analysis featuring a complex data set is chemometric analysis including principal component analysis and hierarchical cluster analysis. This method orders the data in clusters, highlighting some general patterns (Mhlongo et al., 2020; Mhlongo et al., 2021). However, developing a chromatographic method is time-consuming and highly technical. Another approach would be to target priming marker metabolites. Indeed, some metabolites are specifically involved in plant defense and priming as some amino acids (tyrosine, (acetyl) tryptophane, phenylalanine, etc), phenylpropanoid pathway metabolites (flavonoids, benzoic acids and hydroxycinnamic acid derivatives), organic acids from tricarboxylic acid cycle and glycoalkaloids (Pastor et al., 2014; Mhlongo et al., 2020; Mhlongo et al., 2021; Zeiss et al., 2022; Soudani et al., 2022).

Given the low resolution of chromatographic analysis, the results discussed below can only be considered as leads for further research.

Different metabolites may be shared by root and aerial part as they are observed at the same retention time. It may concern the metabolites at 9.84, 8.15, 7.89, 7.73 and 0.55 minutes but should be confirm with a better chromatographic separation coupled to mass spectra.

Regarding the chromatographic analysis of the aerial parts, the main observation is a lower content of most of the metabolites found in plants treated with *Artemisia absinthium* EO compared to the other treatments and the controls (RT of 5.45, 7.62, 7.88, 8.13, 8.49 min). Overall the Sm:Aa treatment and *S. montana* EO showed a metabolite level in aerial part slightly lower than or equal to the controls. It could be interesting to investigate the metabolites appearing at 7.62 min. The rate of this metabolite increased with infection but not for the plants treated with the combination of *S. montana* and *A. absinthium* EOs. Slightly lower metabolite level was observed and remained stable after infection. For the metabolite at 2.59 min, the opposite was observed. Metabolite level dipped to zero with infection for the control 1 and all the treatments, but not for Sm:Aa treatment where the level stayed almost constant.

Regarding the roots, the results are particularly difficult to analyze, as the values between the two controls are very different, with, in some cases, the presence of a metabolite in one control, even in large quantities, but not in the other. To ensure reliable results, several replicates should be analyzed. However, it is interesting to note that metabolite level between infected and uninfected plants treated with *A. absinthium* EO tended to vary in the opposite direction to other treatments and controls (RT of 14.41, 8.15, 7.89 and 0.55 min). Three metabolites could be interesting to analyze: the metabolites at 8.15, 14.35 and 14.41 min. About the first mentioned, the level of this metabolite was boosted for *S. montana* EO and Sm:Aa treatment but decreased after infection as for the controls. The metabolite at 14.35 min was only found in tomato plants treated with Sm:Aa, exception of uninfected control 2 plants. It would be interesting to identify this metabolite and investigate its properties. The variation in metabolite content (RT of 14.41 min) between uninfected and infected plants should be closely analyzed. The metabolite level in plants treated with *Artemisia*

absinthium EO and Sm:Aa increased with infection. But the opposite was observed for the controls (1 and 2) and plants treated with *S. montana* EO.

The biochemical modulation of plant metabolism can influence their phenotype and be observed by a morphological study but also by measuring certain growth parameters. In this project, the fresh weight of the aerial part and root system of the plant was measured, as it can be an indicator of nematode infestation.

Two main observations are worth discussing. The plants treated with *A. absinthium* EO showed the lowest weight for the aerial part and root system. In contrast, the plants treated with Sm:Aa showed the highest weight for the aerial part and root system, except for the uninfected roots. The two statements combined explain the significant differences in aerial and root weights observed between the two treatments. A significant difference of aerial weight was observed between plants treated with *A. absinthium* EO and control 2. However, this observation is not repeated with control 1, although the weight averages are different. Two factors may explain the insignificance of this difference. On one hand, although there is no significative difference of aerial weight between both controls infected or uninfected, the aerial average weight of control 2 is higher than that of control 1. On the other hand, the replicates of control plants are quite variable, which weakens the statistical analysis. To confirm a significant effect of the *A. absinthium* EO on the aerial weight of tomato plants, the experiment should be repeated with a larger number of replicates. This explanation also applies to root weight of infected plants that are significantly different between Sm:Aa treatment and control 1 but not in comparison with control 2.

The experiment revealed an effect of Sm:Aa treatment on the aerial weight. Regarding the uninfected plants, the above-ground weight of the plants treated with Sm:Aa is significantly higher than for the plants treated with another treatment, exception of the control 2. This result may indicate a biostimulant effect of the combination of EOs but needs to be confirmed with a larger number of replicates. A biostimulant effect of essential oil has already been observed on tomato plants. Foliar application of 1000 ppm rosemary EO increased shoot and root fresh weights while soil application increased root fresh weight only (Souri et Bakhtiarizade, 2019). However, this result was quite unexpected because as already mentioned, EOs and in particular *A. absinthium* EO are known to be phytotoxic in many instances (Werrie et al., 2020). Even more interestingly, the effect was found significant compared to all the treatments for the aerial weight of the infected tomato plants with an average weight three times higher than with *A. absinthium* EO as treatment. But there is no significant difference with the aerial weight of uninfected plants.

The most interesting finding was the increase of the root weight for plants treated with Sm:Aa after infection compared to uninfected plants that could indicate a stimulation triggered by nematodes infestation. This observation is the opposite of what is generally reported in the literature. In fact, nematode infestation reduces water and nutrient uptake due to the formation of giant cells, which are the nematodes' feeding sites (Azlay et al., 2022). This phenomenon leads to a lower root weight (Elsharkawy et al., 2022).

Treated plants showed no reduction in nematode infestation, with the number of galls per root not significantly different from controls. However, combining this information with the increase in root weight after infection, it could be interesting to carry out a confocal morphological analysis to assess whether there has been an effect on gall size (Díaz-Manzano et al., 2023). Although the galls were sorted by size during counting, the size distinction was arbitrary and not precise. Morphological analysis of the root system could also be used to assess whether there is adaptation of the roots as a defense response.

6 CONCLUSION

The *in vitro* tests revealed a potential synergistic effect of the combinations of the essential oils of *Satureja montana* with *Mentha rotundifolia* and *Thymus zygis* essential oils and significative reduction of egg mass hatching of *Meloidogyne javanica* caused by the combination between the essential oils of *Satureja montana* and *Artemisia absinthium*. The combination of *Satureja montana* and *Artemisia absinthium* essential oils did not reduce *Meloidogyne javanica* infestation on tomato plants. However, a difference in fresh root weight between infected and uninfected tomato plants treated with the combination of essential oils was observed. The above-ground weight of infected plant treated with the combination was significantly higher than for the other treatments. The experiment should be repeated with more plicates to confirm the results. Some interesting changes in the metabolic profile of tomato plants have been highlighted, but targeted metabolomic analysis or optimized untargeted metabolomic analysis is needed to identify the metabolites involved in the tomato defense response against *Meloidogyne javanica*.

7 SOME CONSIDERATIONS

Working with biological material is no simple matter, as each individual has intrinsic variability and is sensitive to external conditions that cannot be totally controlled. It is therefore impossible to obtain exactly the same response for each individual, or even for each sample of individuals. This underlines the importance of having a sufficient number of replicates, and of testing inter-day variability by repeating the whole experiment at different points in time.

Due to the existence of numerous chemotypes, using essential oils as biopesticide is challenging and still require more investigations for industrial-scale production. First, the results obtained under controlled conditions must be confirmed under agronomic conditions which is poorly studied. Second, A well thought-out formulation must be developed to maintain a standard, effective and long-lasting activity for field application.

Essential oils are complex mixtures of compounds whose individual modes of action are still poorly understood. Synergism between these molecules, or more broadly several essential oils, is a promising means of achieving high efficacy, and is economically attractive since smaller quantities could be used. However, to understand phenomena such as antagonism, additivity and synergy, in-depth knowledge of the modes of action of each compound in each essential oil is required.

Non-targeted metabolic analyses and, more generally, "omics", are powerful tools for understanding the modes of action of essential oils and their constituents, providing a wealth of information. However, advanced knowledge of analytical chemistry is required to optimize these techniques and obtain robust, reliable results that can be exploited from a scientific point of view.

This work, although providing promising results, is a small contribution to the mass of knowledge that remains to be acquired to be able to rationally advance in the formulation of biocontrol agents based on essential oils.

Personal contribution

This study is part of a project to develop the use of essential oils as priming agents. The guidelines were established by Azucena González Coloma with the approval of Professor Marie-Laure Fauconnier. This work is based on a publication from this lab (at the Instituto de Ciencias Agrarias) revealing the priming role of *Artemisia absinthium* essential oil against *Fusarium oxysporum*. This study also forms part of a project to investigate the nematicidal activity resulting from a synergistic effect of essential oil combinations. I contributed to the acquisition of new data concerning synergy between essential oils by carrying out nematicidal activity tests. For the first time, this work made it possible to evaluate the effect of the combination of essential oils of *Satureja montana* and *Artemisia absinthium* on the hatching of *M. javanica* eggs and on nematode infection of tomatoes *in vivo*. I carried out the nematode egg mass experiment, set up and monitored the *in vivo* experiment, extracted metabolites from tomato plants, collected plants and prepared samples for analysis, some of whose results are not included in this work. At my suggestion, data were collected at mi-experimentation in order to optimize the protocol and will be analyzed. I also carried out all the statistical analyses for nematicidal activity and the *in vivo* experiment, including comparisons between the essential oils of *S. montana* and *A. absinthium* and the combination of *S. montana* and *A. absinthium*. This work was carried out under the supervision and guidance of Azucena and Maria Fe Andrés Yeves throughout its development. The preliminary results generated by the analysis of

my extracts have for the first time highlighted changes in the metabolic profile of treated tomatoes, opening the door to new investigations.

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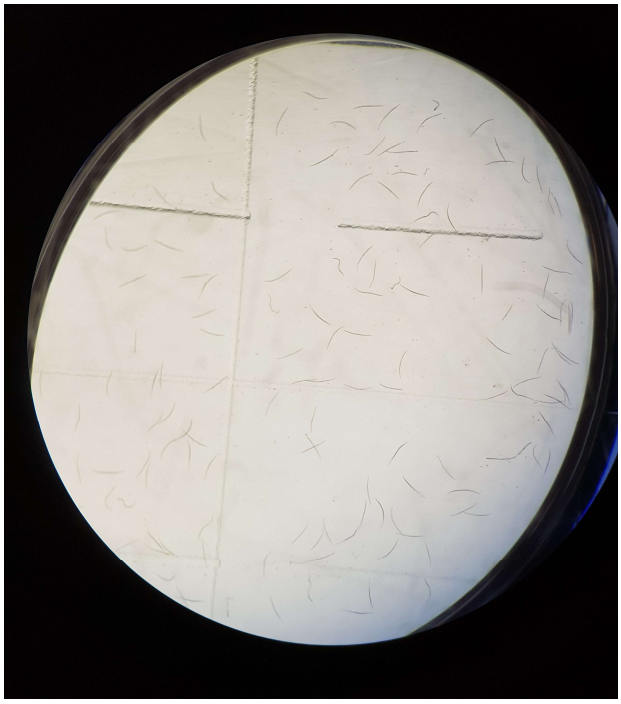
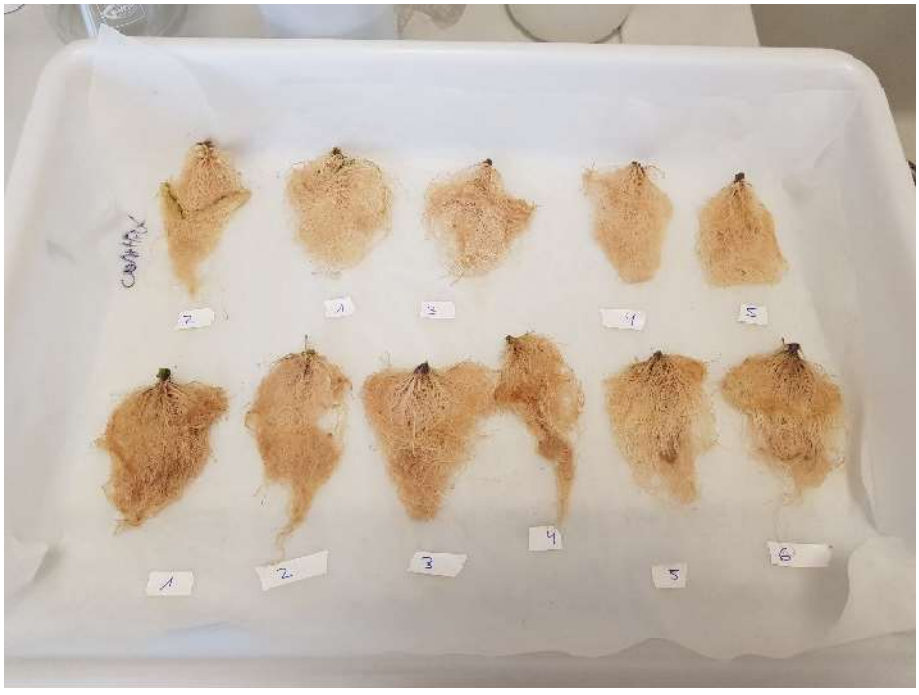
9 APPENDIXES



Appendix 1. M.javanica under microscope

Appendix 2. Egg mass hatching test

Appendix 3. Root systems of control plant (top row and Sm :Aa treated tomato plants (bottom row) after 43 days of experimentation



Appendix 4. Root systems of control plants (top row) and Sm :Aa treated tomato plants (bottom row) after 63 days of experimentation infected by M.javanica

Appendix 5. *Root systems of control plants (top row) and Sm :Aa treated tomato plants (bottom row) after 63 days of experimentation not infected by M.javanica*

Appendix 6. *Galls observed on the roots of the tomato plants after the in vivo experience*