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Résumé

Les pesticides de synthèses sont très critiqués pour leurs effets nocifs sur l'environnement. Pour les remplacer, les biopesticides sont de très bons concurrents et les huiles essentielles peuvent participer à ce changement. Pour permettre une application aisée de tels produits, une des solutions est d'utiliser ces huiles en tant qu'agent de priming sur les plantes. Ce travail étudie les effets potentiels de priming des huiles essentielles de *Satureja montana* L. et d' *Artemisia absinthium* L. var. Candial appliquées par enrobage des graines ou mixées avec le substrat sur les plants de *Solanum lycopersicum* L. var. Marmande pour combattre les nématodes *Meloidogyne javanica* (Treub) Chitwood. Les résultats montrent pour la première fois un effet de priming potentiel de l'huile essentielle de *S. montana* sur les plants de tomates contre les nématodes du sol en affichant une réduction significative du nombre de grosses galles par gramme de racine par rapport au contrôle infesté et une tendance à la réduction globale de l'infestation par les nématodes sur l'indice de nodulation. Les analyses métabolomiques par HPLC-MS, UPLC-MS/Qtof et GC-MS montrent une augmentation de la production d'une molécule de 770 m/z et du changement dans la composition relative d'une molécule identifiée comme étant l'acétylcitrate de tributyle qui pourraient être les preuves de l'effet de résistance conféré aux plantes par l'huile essentielle. Pour les autres traitements testés, aucune réduction significative de l'infestation de nématodes n'a été observée. Une biostimulation par l'effet synergique des deux huiles essentielles sur la production de biomasse des plantes a été observée et un effet de biostimulation plus faible a également été observé pour *A. absinthium* lorsqu'elle est appliquée sur les graines. Enfin, les analyses métabolomiques montrent l'induction d'une plus grande production d'α-tomatine et des changements dans la composition relative de l'acide palmitique et de l'hexatriacontane par les différents traitements. En conclusion, les huiles essentielles sont de bonnes candidates pour stimuler les défenses des plants de tomates contre les nématodes du sol, mais les mécanismes permettant aux molécules des huiles essentielles d'activer ses défenses sont encore inconnus. L'intensification de la recherche dans ce domaine pourrait améliorer les connaissances sur les molécules actives de priming et conduire à un choix adapté dans l'utilisation des huiles essentielles.

Mots clés: huile essentielle, priming, nématode, tomate, Satureja montana, Artemisia absinthium

Abstract

Synthetic pesticides are widely criticized for their harmful effects on the environment. To replace them, biopesticides are very good competitors, and essential oils, can be part of this change. To allow an easy utilization of such products, one of the solutions is to use these oils as priming agents on plants. This study investigates the potential priming effects of essential oils of *Satureja montana* L. and *Artemisia absinthium* L. var. Candial applied by seed coating or mixed with the substrate on *Solanum lycopersicum* L. var. Marmande plants to combat the nematodes *Meloidogyne javanica* (Treub) Chitwood. Results show for the first time a potential priming effect of *S. montana* essential oil on tomato plants against root knot nematode by displaying significative reduction of the number of big galls per gram of root compared to the infested control and a trend of global nematode infestation reduction on the nodulation index. Metabolomic analyses by HPLC-MS, UPLC-MS/Qtof and GC-MS show a production increase of a molecule with 770 m/z and a relative composition change of a molecule identified as tributyl acetylcitrate which could be proves of the resistant effect given to the plants by essential oils. As for the other tested treatments, no significative reduction of nematode infestation was observed. Bio stimulation by synergistic effect of both essential oils on plants' biomass production was observed and smaller bio stimulation effect was also observed for *A. absinthium* when applied on seeds. Finally, metabolomic analyses show the induction of a greater production of α-tomatine and relative composition changes of palmitic acid and hexatriacontane by the various treatments. In conclusion, essential oils are good candidates to prime tomato plants against root knot nematodes but the pathways allowing the active molecules of essential oils to prime tomato plants' defenses are still unknown. Increase research in this domain might improve the knowledge on active priming molecules and lead to tailored choice in essential oils use.

Key words: essential oil, priming, nematode, tomato, Satureja montana, Artemisia absinthium

List of abbreviations

Aa: *Artemisia absinthium* L. var. Candial

AChE: Acetylcholinesterase

ANOVA: Analysis of variance

AP: Aerial parts

BABA: β-aminobutyric acid

BABA-IR: BABA-induced resistance

CSIC: Consejo Superior de Investigaciones Cientificas

DCM: Dichloromethane

EO : Essential oil

ESI: Electrospray ionization

ETI: Effector-triggered immunity

FW: Fresh weight

GC-MS: Gas chromatography mass spectrometry

HPLC-MS: High performance liquid chromatography mass spectrometry

ICA: Instituto de Ciencias Agrarias

ISR: Induced systemic resistance

J2: Second-stage juvenile nematodes

MeOH: Methanol

NI: Nodulation index

PAMPs: Pathogen-associated molecular patterns

PPN: Plant parasitic nematodes

PTI: Pattern-triggered immunity

R: Roots

SAR: Systemic acquired resistance

SC: Seed coating treatment application method

Sm: *Satureja montana* L.

Sm:Aa: Mix of both essential oils

SMix: Soil mixing treatment application method

UPLC-MS/Qtof: Ultra performance liquid chromatography tandem mass spectrometry (quadrupole and time of flight)

VOCs: Volatile organic compounds

+Me: Infested plants

Table of content

List of tables

List of figures

List of supplementary tables

List of supplementary figures

1 Introduction

In the end of the year 2023, the authorization of glyphosate use came to its end. Nevertheless, the European Commission decided to extend its utilization for another ten years[1]. This decision rises a lot of debate as opinions of the Food Agriculture Organization of the United Nations and the World Health Organization[2], the European Food Safety Authority[3] and the European Chemicals Agency [4], Bayer[5] and of many scientific studies[6–9] over its effects on human health differ widely.

Glyphosate is not the only synthetic pesticide raising concerns. Multiple studies show that the use of biocides has multiple effects on human health inducing higher risks of cancer, diabetes, respiratory and neurological disorders and reproductive syndromes[10]. The environment is also a victim of pesticides use as it pollutes surface and groundwater, soil, fauna and flora[10]. Furthermore, it was found that synthetic pesticides and fertilizers are the second main drivers in the loss of insect biodiversity as they impact both targeted and non-targeted insect populations, the loss of the latter being harmful to the ecosystem [11].

With the synthetic pesticides use becoming more and more criticized and the growing of pest resistance cases [12,13], a reduction of the current reliance on such chemicals must be performed alongside with the study of new solutions for feeding human populations. In that continuity, the European farm to fork strategy aims to reduce the use of the more hazardous pesticides by half by 2030[14]. The need for new green plant protection products and a reshaping of current agricultural practices with more environmentally friendly practices is thus crucial to achieving a sustainable agriculture.

More environmentally friendly and sustainable pesticides, called biopesticides, are promising solutions to answer a part of this problem. With the implementation of integrated pest management practices, biopesticides support the reduction of the conventional pesticides use [15,16]. Those biopesticides are products based on viruses, bacteria, fungi or natural molecules derived from a plant source[17,18]. Mainly used in medicine, cosmetics and food industries, essential oils (EOs) have been increasingly studied these past few years for their application in the agronomic sector as biopesticides[19–21]. A part of those studies being their application as elicitors or primers of plant defenses. Examples of studies on essential oils as priming agents are gathered in section 2.1.7. Nevertheless, this study remains the only studies using essential oils as priming agents on tomato plants to fight root knot nematodes.

2 State of the art

2.1 Essential oils

2.1.1 Origin and definition

Aromatic plants have been used through ages in medicinal, religious and culinary practices but it is only in the beginning of the 16th century with the development of distillation techniques that EOs were obtained[22]. Today, more than 3000 EOs are identified but only about 300 are commercialized[13,15,23,24]. EOs are secondary metabolites extracted from aromatic plants[13,15,24–27]. They can be produced by all the plants' organs such as flowers, leaves, rhizomes, seeds, fruits, wood, bark, etc and stored in specialized plant structures including secretory cells, cavities, canals, epidermal cells or glandular trichomes[13,15,24,25,28]. As secondary metabolites, EOs are produced in response to a plethora of factors. Indeed, they can be secreted to repel unwanted herbivores or to attract pollinators. They also can be produced in response to pathogen attack or even to improve drought resistance[24–26].

2.1.2 Chemical characteristics

Those functions can be achieved thanks to their complex mixture of volatile compounds[16,28]. Indeed, EOs encompass teens or hundreds of substances but only two to three of them characterize the oil thanks to their high concentration while the others are present in trace mounts[24,29]. Multiple families of compounds cover this complex mixture; there are terpenoids containing mono and sesquiterpenes, phenylpropanoids and lastly, oxygenated compounds standing for alcohols, phenols, aldehydes and esters but also nitrogen and sulfur derivatives[13,15,19,24,25,29–31]. They are generally liquid at room temperature with a density lower than water[15,26,28]. These secondary metabolites can be colorless or with a brown to yellowish tone[15]. Furthermore, they are soluble in organic solvent but not in water[15,28].

2.1.3 Production processes

To recover these compounds from natural matrices and create EOs, various techniques exist. Indeed, they can be recovered either by distillation, mechanical means or by alternative extraction methods[13,15,25,32–34]. The most employed one is steam distillation where steam is passed through the plant material before being condensed and separated to isolate the EO from the extraction water[22,32–34]. Other techniques exist such as cold pressing for citrus, water and

dry distillation[13,15,22,32–34]. Much more extraction processes are known but they are not recognized for EO production (ISO 9235:2021). Maceration, solvent extraction and some new greener approaches also exist including CO_2 supercritical, microwave and ultrasound assisted extractions to name but a few[13,15,25,32–35]. Interesting studies also suggest the utilization of hydrolates (water residues from EOs steam distillation) as new biopesticides[36–38]. The valorization of such products can add a new dimension in the utilization of biopesticides by promoting a circular economy[37,38].

2.1.4 Biological effects

EOs are well known for their multiple biological effects. Antibacterial, antifungal, insecticidal, acaricidal, nematicidal and herbicidal properties allow EOs to have a strong biopesticide potential. They are also known for being cytotoxic and for their antioxidant, anti-inflammatory, cancer chemoprotective, antileishmanial, allelopathic and antimicrobial activities[13,16,28,29,33,34].

Researchers have been mainly focusing on antifungal, antibacterial and insecticidal activities of EOs[13], the latter being the most reviewed property[15]. The following reviews can be read to understand those bioactivities: Bakkali et al. (2008); Raveau et al. (2020); Werrie et al. (2020); Kesraoui et al. (2022); Gupta et al. (2023).

Nematicidal effect, a less studied yet interesting feature of EOs, would thus be more interesting to focus on as it is more than related to the subject of this study. The mechanisms at play are therefore important to understand.

Even if this matter will be dealt with later, it is important to first have in mind that the root knot nematodes, *Meloidogyne* spp, cause huge crop damage every year[13,19,29,39]. As depicted in the introduction of this work, the use of synthetic pesticides is not compatible with sustainable agriculture, so new green products to use against nematodes are needed.

The knowledge around the effect of EOs against nematodes is still scarce but thanks to the investigation of a wide number EOs and some of their components against *Meloidogyne* spp., it is possible to draw some hypotheses. Catani et al. (2023) gives a comprehensive overview of which EOs could be used in agriculture against nematodes thanks to a review of all scientific papers studying this subject[40].

The common way to assess this effect is to observe the degree of paralysis of the second-stage juvenile nematodes (J2) and the inhibition of egg differentiation and hatching several hours after implementing the EO based treatment. To understand the effects of EOs on those parameters, the molecular docking of the most nematicidal components of EOs to some proteins of interest is interesting to study[19,39,41–43]. Those methods of investigation allowed scientists to hypothesize a strong relationship between nematicidal and insecticidal effects of EOs[19,41] The review of Andrés et al. (2012) explains that EOs could interfere with the nematode's nervous system by disrupting cell membrane permeability[19]. Those effects could be due to the hindrance of the neuromodulator octopamine, the GABA-gated chloride channels and/or acetylcholinesterase activity[19,29,39,41]. Also, a study over carvacrol and thymol, two nematicidal compounds, revealed the possible triggering of SER-2 like receptors leading to a signaling cascade and the nematode death[19]. The affinities of geraniol, β-terpineol, citronellal, l-limonene and γ-terpinene for odorant response gene 1 was also uncovered, suggesting the disruption of chemosensory functions of nematodes by those components[42].

More studies about the modes of action of EOs on nematodes is still needed as no general rule of thumb has been uncovered yet. And for good reasons, a lot of factors must be taken into account. First, the chemical composition of the EO, which may vary depending on multiple factors that will be depicted later (see section 2.1.6), but also the combination of multiple EOs in various concentrations and ratios can lead to synergy or antagonism interactions. The position of some functional groups and the presence of double bounds also affect the activity of the mixture. Lastly, the nematode species and their life cycle are to be taken heed of since egg masses seem more resistant than J2[19].

2.1.5 Advantages of essential oils

EOs possess numerous advantages compared to conventional chemical pesticides. Indeed, their high volatility and biodegradability allow them to have low persistence in the environment or on foodstuff and low to no effects on groundwater and mammals making them eco-friendly pesticides[13,20]. Furthermore, the large diversity of constituents leads to synergies and thus to both specific or broad spectrum actions[20,21]. Also, this particularity permits a reduction in pest resistance by being able to act on many target sites[20,21,24,27].

2.1.6 Composition variability

Nevertheless, the use of EOs is really complicated as it raises numerous problems in terms of their production with a homogenous composition, their storage stability and their application in the field. The production is the first main concern because if EOs are to be used on a large scale, their composition needs to be homogeneous. Yet, a lot of factors are to be taken into account to achieve that. The plant cultivar is to be factored into this issue alongside with environmental conditions in which the plants grow and develop. This obviously includes climatic conditions with temperature, rainfall, humidity and light intensity but also the culture site with the soil composition, acidity, pollution and mineral availability which is directly linked with the geographical area where the plants are harvested. The harvesting time, the presence of root colonization by symbiotic microorganisms, the extraction method and the organ the EO is extracted from also play a role in the EO's composition[13,15,19,28,29].

Hopefully, plant domestication offers a solution to this situation. Indeed, it allows a standardization of many cultivation parameters thus inducing better control over harvested quantities. Desirable characteristics to ensure quality are also better managed based on phenotype selection. Furthermore, it plays a key role in species conservation while fulfilling the increasing demand for standardized raw material[21,29].

Even if the EO is produced and extracted correctly, the storage conditions might change their composition. Indeed, temperature, light exposure and oxygen availability are the main factors influencing EO storage stability. Even if the control over those parameters is more complicated, EO's composition, molecular structure of its constituents and the presence of contaminants can impact EO's stability[35,44].

2.1.7 Field application barriers

Their application in the field is undoubtedly challenging. Their high volatility and fast degradation in field conditions lead to the necessity of multiple vaporization steps to induce a resistance effect in the fields[13,15]. The nanoencapsulation method could be a solution to such problems by improving the overall stability of biopesticides and being able to control the release of active compounds. Chitosan, gum arabic or poly-DL-lactide-co-glycolide are examples of polymers used in those situations. Nonetheless, the reduced water solubility of EOs generates the use of organic solvent for such formulation that could be harmful for the environment[13]. As

EOs can be biocidal, their raw use could also have unwanted effects on untargeted plants while using it against pests[16].

To avoid such problems a very innovative solution is to apply EOs directly on seeds. Seed coating with EOs would thus prevent the use of new formulations or multiple vaporization steps. Moreover, this type of application could induce priming or elicitation in the plant so that it can protect itself from a future pest invasion. Using EOs for this purpose is possible as some experiments have shown the potential priming effects of EOs on plants against certain pests[45]. To cite some examples, Soudani et al. (2022) demonstrate the priming effect of EO *Artemisia absinthium* on tomato plants against *Fusarium oxysporum* with a seed coating application method of the EO[46]. Also, the results obtained by Banani et al. (2018) suggest that thyme EO induces resistance against *Botrytis cinerea* through the priming of defense responses in apple fruit[47]. Drought stress tolerance of bread wheat was increased when sage, rosemary and lavender essential oils were used as seed priming agents[48]. Furthermore, seed coating has proven some benefits on crop growth and yield[49,50] but also on protection against pathogens[49] and on germination[50]. A review of Sohail et al. (2022) gathers commercially available seeds coated products but also current research on the matter. The commercially available products use multiple treatments applied on seed to fight multiple threats, for example, through assisting seed germination, growth, and protection from pests[51]. Metal nanoparticles seem to have bright future as seed priming agents. However, sustainability concerns are raised upon the use of some capping and reducing agents for nanoparticles synthesis[51].

2.1.8 Essential oils in agriculture

EOs and their applications in agriculture have been under a lot of investigation with India, USA, Iran and Italy carrying out most of the research in that field[40]. EOs utilization in general seems to have a bright future as its industrial market is estimated to have a USD 13,94 billion market value in 2024[52]. As for the biopesticides market, it is estimated to reach a value of 15 billion USD by 2029 thanks to a compound annual growth rate of 14% over a 5-year period between 2017 and 2022[22,53]. Despite the amount of research being conducted and the growing market of biopesticides, the number of commercialized EO-based products is still scarce[13,40,54]. The cause of this is linked to the various drawbacks of EO applications as explained before but also to the European policy which requires these products to be approved before allowing their use[40]. The development of biopesticides based on EOs in the US is more important as no regulation for the ones usually used in the food industry is needed[15,54]. Furthermore, the

application of biopesticides has been occurring for 15 years in the US against less than 10 in Europe[54]. Nevertheless, some commercialized biopesticides exist with various compositions and targets. For example, bioinsecticides such as EcoTrol, TetraCURB or Prev-Am contain rosemary, peppermint, clove and/or orange EOs[54]. A mixed biofungicide and bioinsecticide product named LIMOCIDE also exists and contains sweet orange EO. Finally, Avenger Weed Killer is a bioherbicide that does not contain EO *per se* but d-Limonene which is an active component encountered in EOs[13].

2.2 Nematodes

Even if they are not the most known organisms on earth, nematodes are the most abundant multicellular organisms with more than 30 thousand species known today and over a million possibly existing scientists estimate[55,56]. Nematodes are non-segmented pseudocoelomate transparent round shaped worms with a bilateral symmetry[55,57].

Two types of nematodes exist: either free-living or parasitic to plants or animals[55,57]. With a length varying between 250 µm and 12 mm and a width between 15 and 35 µm[40], plant parasitic nematodes (PPN) are particularly damaging. In fact, they represent 10 to 15% of the world-wide crop yield lost or 125 billion dollars annually being the most destructive group of plant pathogens worldwide[19,29,39,57]. There are three types of plant parasites: ecto, semi-endo and endo-parasite, the latter inducing serious changes in the roots of the host they parasitize[19,57].

This study will focus on the fight against root-knot nematodes represented by the genera *Meloidogyne* spp. This endoparasite is the most damaging PPN in the world[19,58]. It causes galls on plants' roots allowing them to feed off the plant's water and nutrients causing the wilting of the last mentioned[57,59,60]. Synthetic pesticides such as oxamyl, fluazaindolizine, fluensulfone or fluopyram were thus used to try to stem their development but undesirable effects came with them as described previously[60,61]. Once again, natural products as EOs for example are the most likely to have a perennial use. The study of new environmentally friendly products against nematodes is thus more than needed.

2.3 Priming

Plants can undergo a lot of attacks coming from various types of pathogens[62]. Roughly, they developed a "multi-layer" immune system to counter these attacks. Indeed, the plant will first activate several mechanisms of defense by initiating the pattern-triggered immunity (PTI). This first barrier is set on when cell-surface pattern recognition receptors (PRRs) recognize pathogen-/ damage-/ microbial-associated molecular patterns (PAMPs/ DAMPs/ MAMPs). To counter PTI, pathogens have evolved and managed to secrete effector molecules which is called effector-triggered susceptibility (ETS). In response to this, plants have developed intracellular nucleotide-binding leucine rich repeat proteins (NB-LRRs). Those proteins, which are encoded by resistance genes, are able to detect those effectors and activate the effector-triggered immunity (ETI)[62].

Another way plants can fight against attackers is by priming its defenses[63–65]. This induced or systemic resistance is an immunological memory where plants fight against a particular stress with a stronger and faster activation of the defense response during a future challenge[63–66]. Plants can enter in a primed state when facing biotic or abiotic stresses[64,65]. The memory set by the plant can even be preserved through the life of the plant and be transferred to its progeny[63,64]. While priming allows plants to have a quicker and stronger response to a specific stress, it also allows them to have a higher fitness compared to non-primed individuals. Indeed, the loss of fitness caused by the priming stimulus is balanced by a far less fitness loss when exposed to an important stress thanks to a better resistance compared to non-primed individuals[67].

Priming is possible thanks to complex mechanisms of genome modification called epigenetic modifications[63–66]. Briefly, DNA methylation and histones modifications can change chromatin structure leading to the modification of DNA sequences for defense-related genes in heterochromatic and euchromatic regions, the last mentioned being a transcriptionally active region[46,65,68,69]. Non-coding RNA can also take part in this change[65,69]. Those mechanisms widely depend on the eliciting signals that will influence the controlling signaling pathways and effectiveness of the mechanisms[63].

As explained before, plants can defend themselves against pathogens by activating PTI or ETI. Those mechanisms will fight the infection by inducing various immune responses. These responses gather calcium ion signaling, nitric oxide and ROS production and a lot of other

mechanisms including perception of PAMPs and the production of defense phytohormones such as salicylic acid, jasmonic acid and ethylene. Those replies will affect epigenetic mechanisms and thus, the priming of the plant[69]. Those mechanisms are illustrated in Figure 1.

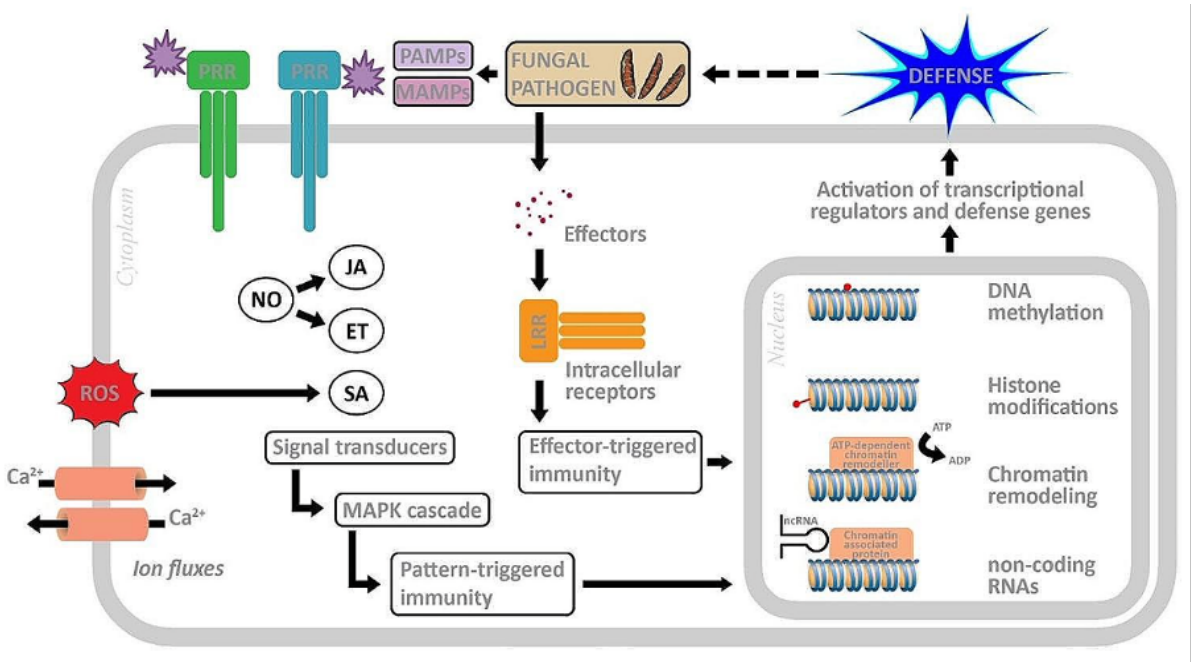

Figure 1: Inducers and pathways triggering epigenetic changes in plant defense.
JA, jasmonic acid; ET, ethylene; SA, salicylic acid; NO, nitric oxide; ROS, reactive oxygen species; LRR, receptors with a leucine-rich nucleotide binding site; MAPK, mitogen-activated protein kinase. Inspired from Mierziak et al. (2024)

The three most notorious priming responses arise from the production of defense phytohormones including the ones mentioned here above and called systemic acquired resistance (SAR), induced systemic resistance (ISR) and BABA-induced resistance (BABA-IR)[63,64]. SAR develops in response to local infection by pathogens and requires salicylic acid[63,64,66,69] or pipecolic acid[63,64,66] but could also be influenced by azelaic acid[63,64]. SAR is a long-lasting form of resistance against numerous pathogens[66]. ISR is caused by plant growth promoting rhizobacteria or fungi (PGPR or PGPF) that will interact with plant root[63,64,66] and stimulate plant growth and/or induce resistance responses against a broad range of pathogens. This immunological response depends on jasmonic acid and ethylene[63,64,69]. Finally, β-aminobutyric acid (BABA) can induce BABA-IR laying out protection against bio- and necro-trophic pathogens as well as against abiotic stresses through the priming of SA-dependent and -independent defenses[63].

Other stimuli can induce priming including volatile organic compounds (VOCs), such as terpenoids, small aliphatic alcohols or aldehydes, which can prime distal organs or eleven

neighboring plants[70]. Other stimuli are reviewed by Mauch-Mani et al. (2017). As for EOs, few studies have investigated how they can trigger priming in plants[46]. Yet, Banani et al. (2018) formulated an hypothesis which supports that thymol could stimulate pathogenesis-related genes through the enhancement of antioxidant levels, enzymatic and non-enzymatic systems[47]. This could be an initial focus point for further investigation.

2.4 Background of this study

This study is the fruit of the collaboration between the department of biopesticides of the "Instituto de Ciencias Agrarias", a branch of the "Consejo Superior de Investigaciones Cientificas" (CSIC-ICA) in Madrid, Spain, with the laboratory of chemistry of natural molecules of Gembloux Agro-Bio Tech in Belgium. Initially, the CSIC-ICA released a paper unraveling the priming effect of *Artemisia absinthium* L. EO on tomato plants (*Solanum lycopersicum* L.) against the fungus *Fusarium oxysporum* by seed coating[46]. This paper will then lead the way to new research on the priming effect of this EO but also to the collaboration between the two research groups.

The research project investigated the effect of this EO on tomato plants as Spain with its 3 651 940 t production of tomatoes in 2022 represents, with Italy and Portugal, 70% of Europe's production[71,72]. As depicted in section 1, the agricultural practices (with, for example, the greenhouses monocultures of Almeria, Spain) and the utilization of synthetic pesticides do not coincide with a sustainable agriculture. New biopesticides and new agricultural practices are thus needed to fight off the infestation of nematodes on tomato plants. The great advantage of working with tomato plants is the knowledge that the scientific community has built on the tomato metabolomics. Thus, leading to an easier comprehension of the mechanisms at play. The variety used in this experiment was *Solanum lycopersicm* L., var. Marmande as it is susceptible to root knot nematodes.

Firstly, in 2022 a part of the master thesis of Juliette Cassart studied the priming effect of *A. absinthium* EO on *S. lycopersicum* against the nematode *Meloidogyne javanica* by seed coating. This study showed no decrease in the global infestation of the roots by the nematodes. Even if some metabolic changes were seen thanks to the various chromatographic analyses, it seems that it was not enough to induce resistance in plants. Secondly, Emma Zoccolan in her master thesis in 2023, worked on the same subject but used a mix of *A. absinthium* and *Satureja montana* EOs by soil mixing. At the same time, Sabrina Kesraoui, as a part of her thesis in the CSIC-ICA worked alongside Emma Zoccolan with the same EOs and with the same application techniques but without mixing them and by applying them on different chosen seedlings. The results of this second study showed the same results as the previous study. The chromatographic analyses were not performed with the same parameters as in the first master

thesis. A part of this work was thus to re-analyze the samples with the proper parameters to compare the various experiments.

The first study led by Juliette Cassart studied the same parameters as the study of Soudani et al. (2022). This paper unraveled the priming effect of *Artemisia absinthium* L. essential oil applied by seed coating on tomato plants against *Fusarium oxysporum*. The first master thesis was thus based on the hypothesis that the same EO could also prime tomato plants against another biotic stress and in this case, against root knot nematodes. The choice of this essential oil is explained in the initial study[46]. Briefly, its use was based on an ongoing study of the CSIC on the domestication of thujone-free Spanish populations of *A. absinthium* to create a new variety with constant composition of its essential oil, the latter showing interesting properties to be used as a biopesticide[46,73].

The change of treatment method came from a proposed perspective of Julitte Cassart's work to add the EO directly in the vermiculite. Indeed, the hypothesis is that the inoculation of the biotic stress came after more time than the initial study of Soudani et al. (2022). The paper measured callose deposition and ROS production up to 12 days after the treatment, but no measurement was performed at larger time scale. In the case of root knot nematodes, the infestation occurred approximately a month after seeds treatment. The mixing of the EOs with the substrate might thus implement a progressive release of the chemical agents and thus, possibly a longer priming stimulus.

A second proposition of Juliette Cassart to test new EOs led to the utilization of *S. montana* EO to try to change the composition of the priming agent. Indeed, its composition differs from *A. absinthium* EO used in the initial study[46,74]. The use of a mix of these two EOs was based on the same idea by trying to test the possible synergistic effect of the two oils.

The use of *S. montana* EO was also investigated as it has been part of a study of the CSIC to domesticate this plant and valorize it to be used as a biopesticide. Indeed, the essential oil has multiple biological effects and is part of the native Spanish mountain flora[74] which also makes it interesting to be used as a new biopesticide. The use of the two studied essential oils implements the valorization of local resources to the study. Nevertheless, the essential oils were not initially selected based on a potential known priming effect of their constituents against root knot nematodes as those mechanisms are still unknown.

2.5 Objectives

As no previous research were carried out in this field of study, this work aims in unveiling potential effects of *Artemisia absinthium* and *Satureja montana* essential oils on tomato plants against root knot nematodes. The goal of this work is to study the last parameter that was not considered in the past, meaning the use of *S. montana* EO by seed coating. And in the same time, to summarize all the results of the previous works and of this work and to compare them in order to draw conclusions on this research journey. Results from *in vivo* measurements and from metabolomic analyses will thus be presented. Indeed, aerial parts and roots fresh weights will be studied along with the incidence of nematode infestations by evaluating global infestation thanks to a nodulation index but also by gall counting according to their size and corrected by the roots' fresh weight. Lastly, metabolomic analyses will be carried out to investigate the effects of the various treatments on tomato plants' metabolite production. Analyses by UPLC-MS/Qtof in the TERRA research center of selected secondary metabolites were also implemented to confirm or refute the identification of these molecules detected by the first analysis obtained by HPLC-MS in Madrid. These secondary metabolites were selected based on their intensities of production compared to the controls' ones.

3 Material and methods

3.1 Preliminary note

As the goal of this work is to continue what was investigated by former students but also to synthesize all the previous results generated by them, some results presented here were not produced by the author. The manipulations that were not carried out by the author will be explained in the supplementary materials. Furthermore, due to experimental setbacks and limitation in residence time in Madrid, all the manipulations realized at the end of the growth period were carried out by Ruben Muñoz, Felipe De la Peña and Maria Fé Andrés. The same manipulations but on different biological material was carried out by the author to gain laboratory experiment and to be aware of all the manipulations necessary for this work.

3.2 Biological material

Plants from *Satureja montana* L. (Sm) and *Artemisia absinthium* L. var. Candial (Aa) were harvested in 2017 and 2019, respectively, in Teruel (Spain) and their essential oil (EOs) extracted by steam distillation as described by Soudani et al. (2022).

Untreated seeds of *Solanum lycopersicum* L., var. Marmande from Ramiro Arnedo s.a. (Calahorra - La Rioja, Spain, lot 23/5020) were used in this experiment and were stored at 4°C until use.

Second-stage juvenile nematodes *Meloidogyne javanica* (Treub) Chitwood were used for *in vivo* tests. All information about the rearing of nematodes is depicted in section 8.1.

3.3 *In vivo* experiments

3.3.1 Seeds treatment

Fresh tomato seeds were previously stored at 4°C and dipped into distilled water for 24 hours before sowing to decrease germination time. After a first drying period on filter paper, seeds were dipped in their respective treatment one by one and air dried on aluminum foil. The treatments consisted in a 1,25 or a 0,7 mg/mL of Sm EO solution in 100% ethanol. Control and blank seeds were treated accordingly with 100% ethanol and distilled water, respectively. A

synthesis of the various treatments is gathered in Table 1. Two EO concentrations were use as the phytotoxicity effect of Sm EO on tomato seeds was not known when applied by seed coating. Depending on whether or not an inhibition effect would take place, one treatment would be preferred to the other. In the best-case scenario, if no inhibition occurs, the highest treatment concentration would be chosen as it could have a better priming effect on seeds. More explanations about the use of two EO concentrations are gathered in section 8.2.

Table 1: Synthesis of all tested treatments

Treatment number	Solution for seed coating	Nematode inoculation
1	Distilled water	No
2	Ethanol	Yes
3	Ethanol	No
4	Sm 1,25 mg/mL	Yes
5	Sm 1,25 mg/mL	No
6	Sm 0,7 mg/mL	/
7	Sm 0,7 mg/mL	/

As explained in section 2.4, various studies have already worked on the fight against root-knot nematodes. Table 2 gathers all the previously tested treatments that will also be discussed in this work. Also, section 8.3 presents the other tested treatment application method that was not used by the author.

Table 2: Previously tested treatments

Tested essential oils	Treatment application method	Essential oils concentration (mg/mL)
Artemisia Absinthium	Seed coating	5
Mixing of *Satureja montana* and *Artemisia absinthium*	Soil mixing	1,25 with both EOs in a 1:1 proportion (w/w)
Satureja montana	Soil mixing	1,25
Artemisia absinthium	Soil mixing	5

3.3.2 Plant growth conditions

Coated tomato seeds were germinated, and seedlings grown for 31 days in a growth chamber in jiffy® pots filled with humidified vermiculite at a density of two seeds per pot. The growth chamber was set on a 70% humidity at a temperature of 23,5 °C during the day and 20 °C during the night, with a 16 h light/8 h dark photoperiod. Plants were irrigated with regular water supply

and fertilizer (Miller, Nutri-chem NPK 20-20-20, lot 151237) diluted in water (± 4 g/L, m/V) was added once a week.

After the first growth period of thirty-one days, 1,25 mg/mL treated tomato plants were selected as no germination inhibition occurred. One plant per pot was selected and transplanted in pots filled with pre-sterilized humidified substate (mix of crumb sand with fine lavender sand, 1:1, V/V). Pots for treatments 2 and 4 were inoculated with approximately 1000 juveniles of stage 2 of *Meloidogyne javanica* before transplantation. A second thirty-two-day long growth period was needed before stopping the experiments. All plants were then weighed (aerial parts and roots separately) and the nematode infestation on roots of plants from treatments 2 and 4 evaluated.

Information about the plant growth periods used in the former studies can be found in section 8.4.

3.3.3 Nematode infestation evaluation

Two methods were used in this study to evaluate the nematode infestation. First, a nodulation index (NI) was created to assess the global infestation. This index based itself on the work of Hussey et al. (2002) and is presented in Table 3[75].

Table 3 : Nodulation index for global root-knot nematode infestation evaluation

Nodulation index (NI)	% of the root system possessing galls
0	0 (healthy, no infection)
1	1-25
2	26-50
3	51-75
4	76-90
5	>=91%

The second method evaluates the infestation in a more precise way. Indeed, it consists of counting the number of small, medium and big galls formed by the nematodes on the roots and divide those values by their respective roots' fresh weights. Sizes of galls are very important to analyze. Indeed, their size will increasingly impact infestation severity by having a higher disturbing effect on nutrient and water uptake by the roots[57,76,77] leading to limitations of plant growth and yield[57,76].

The study focusing on using Aa EO by seed coating only used the NI to evaluate nematode infestation. The research focusing on the soil mixing treatment application method (SMix), did not use that method but it evaluated the nematode infestation by counting the size of the galls. This work garters the two evaluation methods to compare its results with the ones of the former studies. Lastly, a NI based on the results of the SMix studies was calculated to compare all the results between them as it is suggested by Hussey et al. (2002). To do that, the total number of galls per g of roots of the infected controls were set as a NI of 5 as the control is supposed to have the least resistance capacity against nematodes. The NI of the EOs' treated plants were calculated proportionally to the NI of the controls. To try to avoid too much variability a single NI for the controls was calculated based on the gathered data of the last-mentioned.

All the infestation evaluations were carried out by Dr. Maria Fé Andres as it demands a certain experience.

3.3.4 Metabolomic analysis

3.3.4.1 Tomato extracts preparation

Only the leaves and the roots were kept for extraction. Leaves and roots of each treatment were separately macerated for 4 days in methanol (MeOH) for high performance liquid chromatography mass spectrometry (HPLC-MS) analysis and in dichloromethane (DCM) for gas chromatography mass spectrometry (GC-MS) analysis. Then, all solutions were sonicated for 15 minutes and vacuum filtered on a Büchner funnel with 2,5 µm paper filters. Extractions were achieved on all replicates before being combined to achieve higher analytes masses. The following results are thus less robust as only one analysis per modality was accomplished. The samples were then dried with a rotary evaporator and under air flow. Samples were then kept at 4°C until analysis. The extraction method for the plants treated by seed coating with Aa EO was slightly different and is presented in section 8.5.

3.3.4.2 HPLC-MS analysis of tomato extracts

MeOH extracts were analyzed by HPLC-MS. Dry MeOH extracts were re-dissolved in MeOH, filtered (reg. cellulose 0,2 µm, 17 mm, pk 100, Symta, Spain) and diluted to a concentration of 1 mg/mL in 100% MeOH prior to analysis. Samples were injected at a volume of 5 µL by an automatic injector (SIL-20A XR). The analyses were carried out with a Shimadzu apparatus equipped with an LC-20AD pump and a CTO- 10AS VP column oven, coupled to a triple quadrupole mass spectrometer as analyzer (LCMS-8040), with an electrospray ionization (ESI)

source. An ACE3 C18 column (150 mm × 4,6 mm, 3 µm particle size) with an ACE3 C18 analytical pre-column was used for separation. The elution gradient that was set as follow:

- Solution A: MeOH (LC-MS grade) with 0,1% acetic acid;
- Solution B: MiliQ water with 0,1% acetic acid;
- The solvent gradient started at 38% of solution A reaching 100% in 45 min, followed by 100 % during 10 min and then 38% of solution A for 7 min before the next injection, at a flow rate of 0,5 mL/min.

The nitrogen flow was 15 L/min. The electrospray capillary potential was set to + 4,50 kV and ESI was accomplished with the Full Scan in the positive mode (m/z = 110-850). Q3 quadrupole was used with a potential of -1,98kV and a capillary temperature of 250°C.

The samples from the study on SMix were re-analyzed with the above-described method as the first analysis were not performed in the same laboratory or with the same analytical parameters.

3.3.4.3 GC-MS analysis of essential oils and tomato extracts

EOs and DCM extracts were analyzed by GC-MS. DCM extracts were re-dissolved in DCM and filtered (reg. cellulose 0.2 µm, 17 mm, pk 100, Symta, Spain). EOs and DCM extracts were dissolved to a concentration of 4 mg/mL in 100% DCM prior to analysis. The apparatus and the parameters used for sample analyses are described in Soudani et al. (2022). NIST and Willey databases were used for compound identification.

3.3.4.4 UPLC-MS/Qtof analysis of tomato extracts

A supplementary analysis by ultra performance liquid chromatography tandem mass spectrometry (quadrupole and time of flight) (UPLC-MS/Qtof) was performed on selected compounds to verify the first identification of metabolites by HPLC-MS. Dry MeOH extracts were re-dissolved in MeOH, filtered and diluted to a concentration of 1 mg/mL in 100% MeOH prior to analysis. Samples were injected at a volume of 5 µL. In all experiments, a C18 Acquity UPLC ethylene bridged hybrid (BEH) column (2,1 mm × 50 mm × 1,7µm; Waters, Milford, MA, USA) was used at a flow rate of 0,6 mL/min and a temperature of 40°C. The elution gradient was set up as explained in Table 4 with H_2O and formic acid 0,1% as solvent A and acetonitrile and formic acid 0,1% as solvent B.

Table 4: Elution gradient used for UPLC analysis

Time (min)	Flux (mL/min)	% A	% B
0	0,6	100	0
3		100	0
11		0	100
14,5		0	100
15		100	0

All UPLC-MS analysis were performed using an Agilent 1290 Infinity II coupled with a mass detector (Jet Stream ESI-Q-TOF 6530) in positive mode with the parameters set up as follows: capillary voltage of 3,5kV, nebulizer pressure of 35 lb/in^2, drying gas of 8L/min, drying gas temperature of 300°C, flow rate of sheath gas of 11L/min, sheath gas temperature of 350°C, fragmentor voltage of 175V, skimmer voltage of 65V, and octopole radiofrequency of 750V. Accurate mass spectra were recorded in the m/z range of 100 to 1 700.

For untargeted MS/MS, the same MS1 parameters as described were used. MS2 untargeted acquisition mode was added with the parameters as follow: MS/MS range 50 to 1000 m/z, MS/MS scan rate 3 spectra/s, Isolation width MS/MS medium (approx. 4amu), Decision Engine Native, Fixed Collision Energies 10V, 20V and 40V, precursor selection: 3, threshold 10 000 (Abs), isotope model common, active exclusion after 3 spectra and released after 0,5 minute, sort precursors by charge state then abundance (charge state preference 1).

3.4 Statistical analysis

For all statistical tests, an analysis of the variability was first performed with the test of Levene. No normality check was performed as it was supposed to be verified as the number of replicates per treatment was below 10.

In the case of the acceptation of the variability hypothesis, an analysis of variance (ANOVA) was performed. T student test was used if a complementary test was needed. In the case of the rejection of the variability hypothesis, a Kruskal-Wallis test was used with the test of Dunn with Bonferroni correction for multiple mean comparison. In the case of qualitative data, a chi-square test was used. For all tests, p-value < 0,05 was considered as significant.

4 Results

4.1 GC-MS analysis of *Satureja montana* and *Artemisia absinthium* essential oils

The analysis of the composition of the essential oils (EOs) used in this study was carried out by gas chromatography mass spectrometry (GC-MS). Analysis of *Satureja montana* L. (Sm) EO shows that its major components are carvacrol, p-cymene, γ-terpinene and thymol at 40,43%, 25,28%, 9,13% and 5,32% respectively. The complete composition of Sm EO can be seen on Table 5.

Table 5 : Composition of *Satureja montana* L. essential oil analyzed by GC-MS

Compound identification	Retention time	% Area (>0,1%)
α-Thujene	3,69	1,61
α-Pipene	3,80	1,13
1-Octen-3-ol	4,34	1,07
β-Myrcene	4,52	1,26
α-Phellandrene	4,80	2,01
p-Cymene	5,10	25,28
γ-Terpinene	5,71	9,13
Linalool	5,88	1,61
trans-Sabinene hydrate	6,45	1,1
Borneol	7,88	1,82
Terpine-4-ol	8,14	1,26
Thymol	10,57	5,32
Carvacrol	10,80	40,43
trans-Caryophyllene	13,54	1,35

Artemisia absinthium (Aa) EO that was used in the previous experiments was an EO from the same chemotype and lot as the one used by Soudani et al. (2022). In their study, they characterized the EO by GC-MS. The oil contained cis-epoxyocimene (35%), cis-chrysanthenol (9,04 %), chrysanthenyl acetate (8,40 %), chamazulene (5,01 %) and t-caryophyllene (4,74 %) as main components. The detailed results of Aa EO can be found in section 8.6.

4.2 *In vivo* measurements

The aerial parts (AP) and roots (R) fresh weights (FW) but also an evaluation of the nematode infestation on all infested plants were measured to evaluate the effects of the various treatments on the phenotype of the treated tomato plants. The results have been obtained by different

operators at different times. The results shown here are thus always referred to their own control as variability between controls is high.

4.2.1 Effect of the treatments on the aerial parts and roots fresh weight

A comparison of the variation of AP and R FW of every treatment in fold change is presented here. Figure 2 displays the results obtained by soil mixing treatment application method (SMix) while Figure 3 displays the ones obtained by seed coating treatment application method (SC).

As it can be seen on Figure 2, the AP FW of plants treated with the mix of EOs (Sm:Aa) increases by 1,52 and 1,96 fold for non-infested and infested (+Me) treatments compared to the non-infested control (C1). For treatments where EOs were used alone, a decrease of their AP FW is noticeable compared to the controls with a higher diminution for non-infested treatments. Statistical analysis by Kruskal-Wallis test shows a significant variation of AP FW between healthy Sm:Aa treatments and Sm (p-value: 0,0172) or Aa (p-value: 0,0028) EOs used alone.

As for R FW results, they display an increase of Sm:Aa +Me of 1,83 fold compare to C1. The R FW obtained by this treatment is significantly higher than the other treatments. As for the results obtained for the treatments with EOs used alone, they follow the same trend as the one described for AP results.

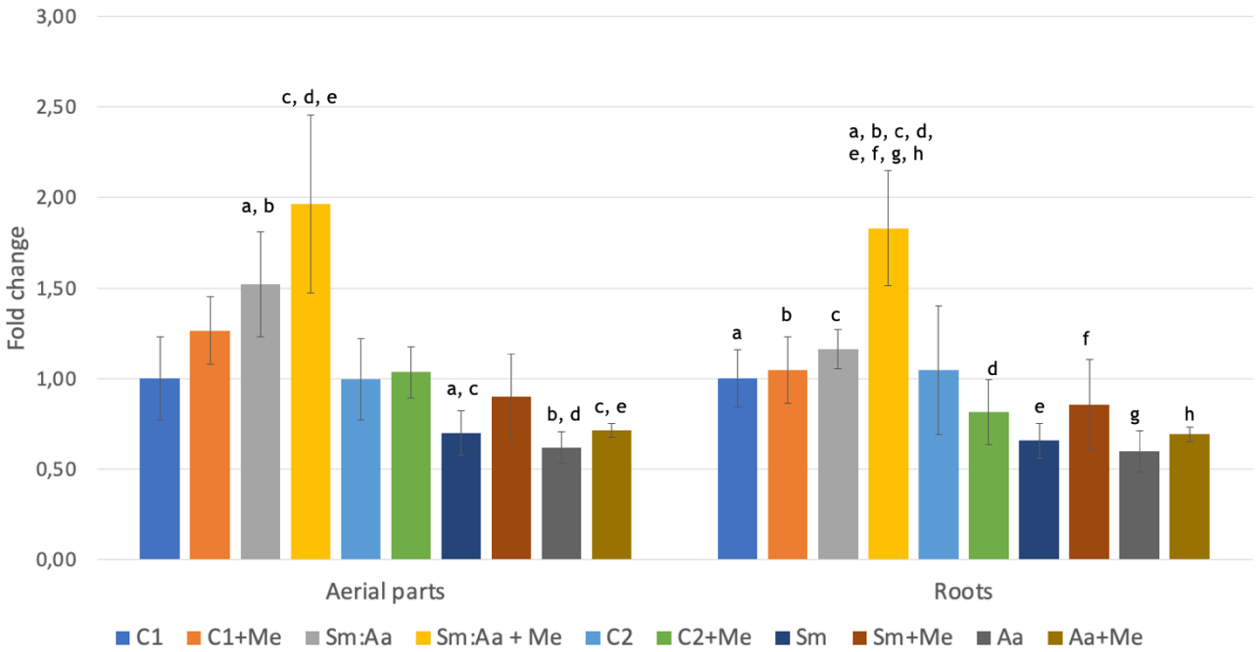

Figure 2: Effects of *Satureja montana* and *Artemisia absinthium* essential oils applied on their own or mixed on aerial parts and roots fresh weights in fold change when applied by soil mixing treatment application method.
The values shown here are means based on six replicates (or less if data was judged to be out of range) with the standard deviation. Each fold change of the replicates was calculated by dividing the weight of the plant by the mean of the control's weight. Bars showing the same letter show value with a significant difference according to Dunn test with Bonferroni correction (Kruskal-Wallis post hoc test) (p-value < 0,05).
C1: Control of the plants treated with the mix of essential oils; Sm:Aa: plants treated with *Satureja montana* and *Artemisia absinthium* essential oils mixed together; C2: Control of the plants treated with *Satureja montana* and *Artemisia absinthium* essential oils applied on their own; Sm: Plants treated with *Satureja montana* essential oil; Aa: Plants treated with *Artemisia absinthium* essential oil; +Me: Infested treatment with *Meloidogyne javanica*.

The AP FW of SC Sm +Me decreases by 17% compared to its control (C3) while no to very little modification occurs for C3+Me and Sm treatments. SC-Aa treated plants have the same trend as SMix Sm:Aa with an increase of the AP FW of 1,46 and 1,77 fold for non-infested and infested plants respectively compared to their control (C4).

SC Sm plants see their R FW decreasing by 24% compared to C3 while SC Sm +Me plants decline by 44% which is a significant drop compared to C3 and C3+Me. The effect of the Aa EO is quite different as R FW of Aa EO treated plants rises to 1,31 and 1,44-fold the control. The infested control also sees its root biomass expanding to 1,29 times C4.

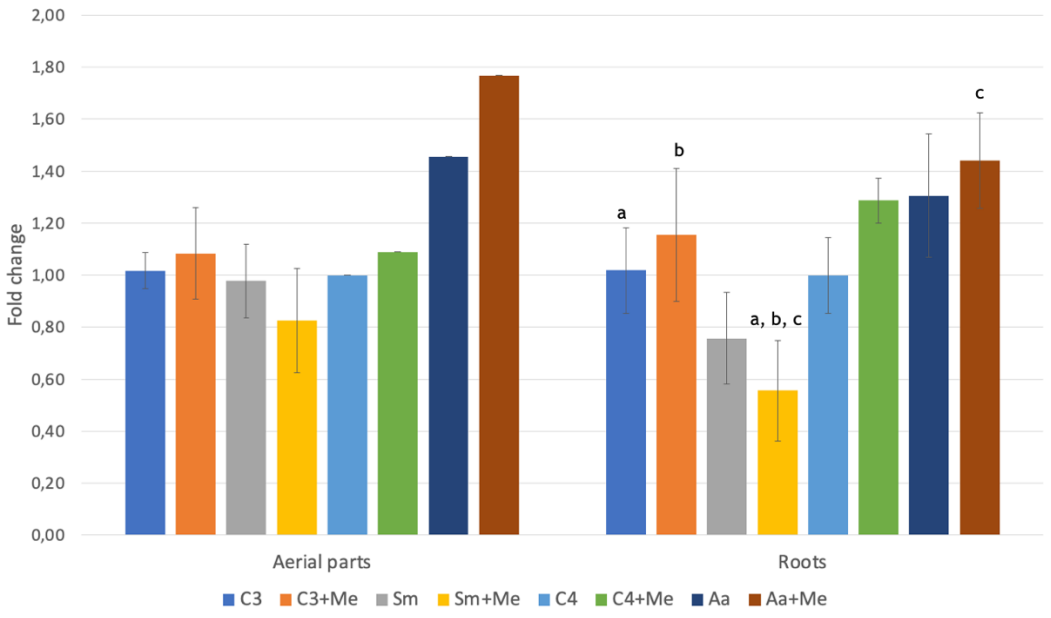

Figure 3: Effects of *Satureja montana* and *Artemisia absinthium* essential oils on aerial parts and roots fresh weights in fold change when applied by seed coating treatment application method.
The values shown here are means based on six replicates (or less if data was judged to be out of range) with the standard deviation. Standard deviation for aerial parts fresh weights of *Artemisia absinthium* essential oil results was not possible as only means' values were communicated. Each fold change of the replicates was calculated by dividing the weight of the plant by the mean of the control's weight. Bars showing the same letter show values with a significant difference according to t Student test (Anova post hoc test) (p-value < 0,05).
C3: Control of the plants treated *Satureja montana* essential oil; Sm: Plants treated with *Satureja montana* essential oil; C4: Control of the plants treated *Artemisia absinthium* essential oil; Aa: Plants treated with *Artemisia absinthium* essential oil; +Me: Infestation with *Meloidogyne javanica*.

4.2.2 Nematode infestation

Even if comparing means of qualitative data is not statistically correct, it allows to roughly visualize the global severity of nematode infestation on plants according to their treatment. Figure 4 displays those means. The two treatments that show a decrease in global nematode infestation are the ones treated with Sm EO by SMix or by SC. It is to be noted that the control of the SC Sm treatment does not have a nodulation value of 5 but of 4,4. The difference between the two decreases is thus the same.

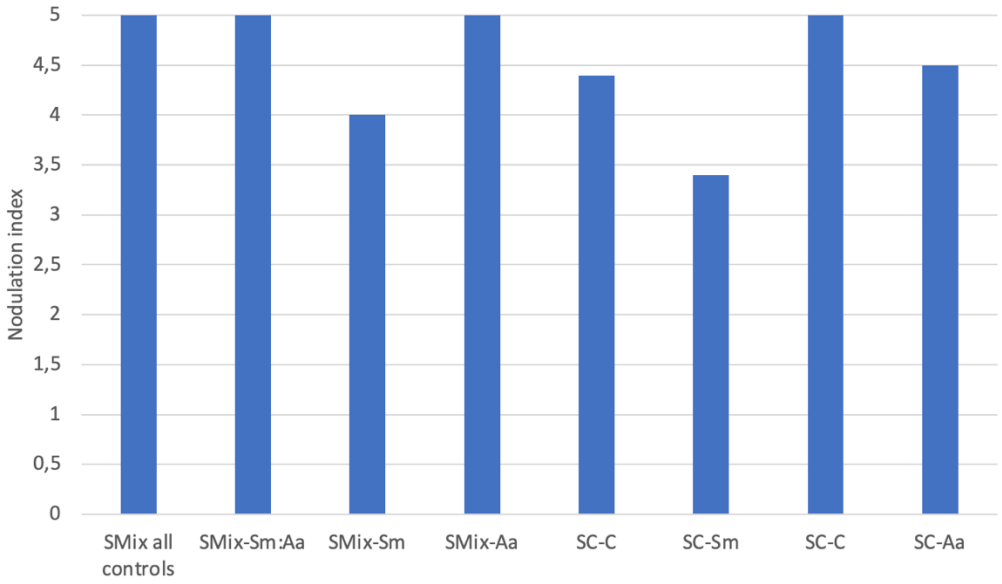

Figure 4 : Nodulation values comparison for all infested treatments.
Soil mixing (SMix) treatment application method values were calculated based on number of galls of a certain size per g of root as explained in section 3.3.3. Plants treated by seed coating (SC) treatment application method with Aa and Sm EOs values are means of observed replicates, data of each replicate for SC-Aa were not available.

For Sm EO treatments by SC, a chi-square statistical test was conducted as it was the only treatment for which complete data was available but no significancy was shown. In such circumstances, Figure 5 was created to show the trend of plant resistance when treated with Sm EO. Indeed, the proportions of nodulation percentages decrease by a value of one between the control and the Sm EO treated plant.

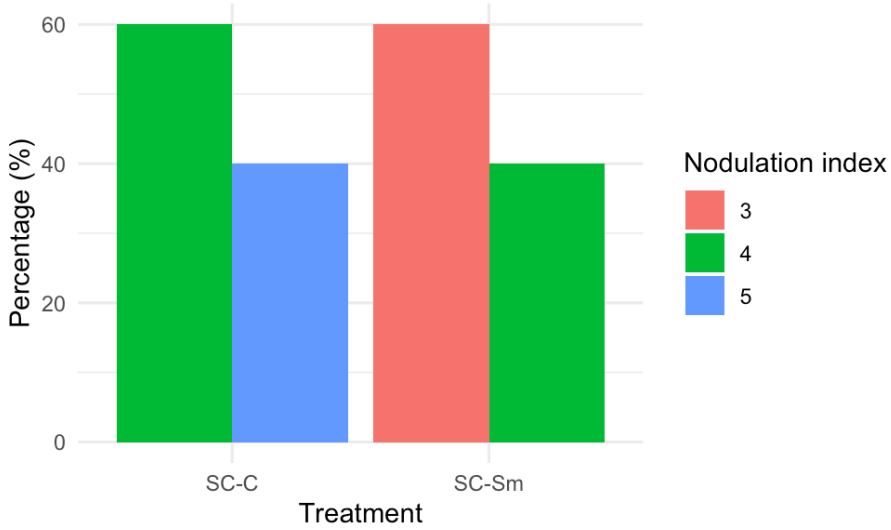

Figure 5 : Percentages of nodulation index values for *Satureja montana* essential oil treated plants and control for seed coating treatment application method.

To report the number of galls of a certain size to the root fresh weight is another way to analyze the degree of nematode infestation and thus the potential resistance of a certain plant to the

root-knot nematode *Meloidogyne javanica*. Figure 6 presents those results and clearly displays a trend for each category. Indeed, the number of small galls per g of root is always higher when the plant is treated with EOs. The increase is even larger for SMix Aa than for SMix Sm treated plants. Medium galls show very little change compared to controls and big galls per g of root is always lower when plants are treated with EOs. As for the total number of galls, it increases when plants are subjected to EOs. The only change that is significant is the diminution of the number of big galls per g of root for the SC Sm treatment with a p-value of 0,022.

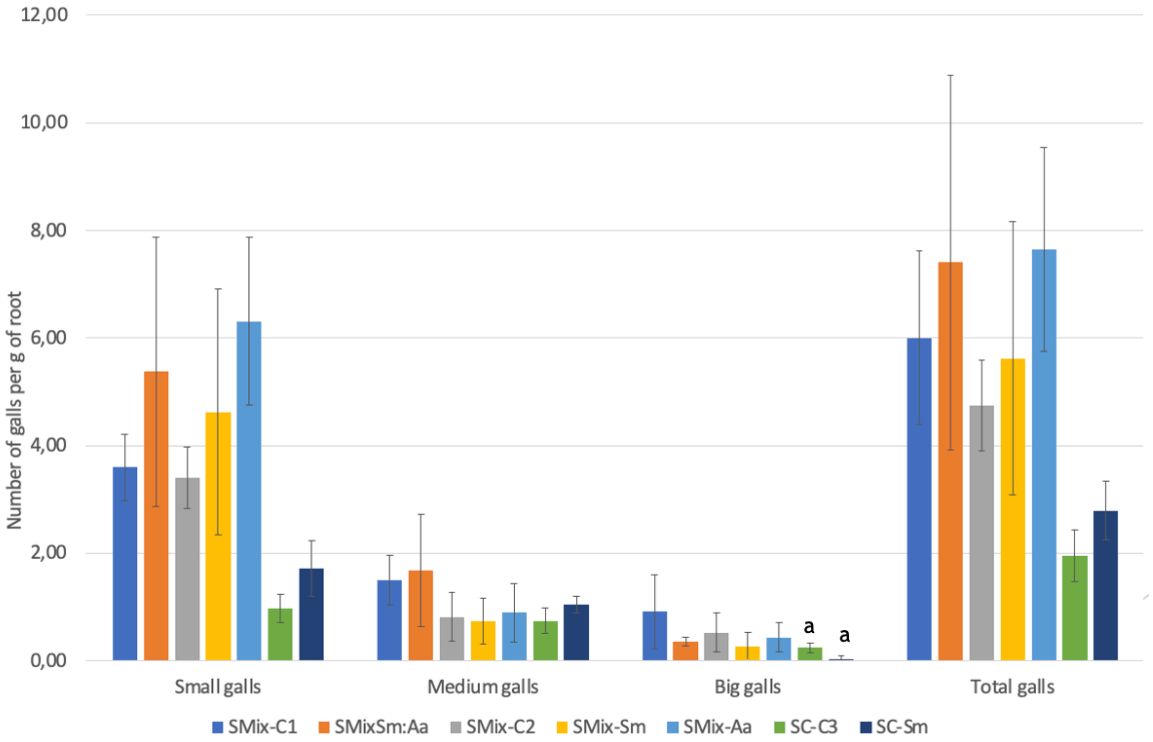

Figure 6 : Number of galls of each size and total number of galls per gram of root fresh weight. The values shown here are means based on six replicates (or less if data was judged to be out of range) with the standard deviation. Bars showing the same letter show values with a significant difference according to t Student test (Anova post hoc test) (p-value < 0,05).

4.3 Metabolomic analysis

Metabolomic analyses were performed on leaves and roots of methanol (MeOH) and dichloromethane (DCM) extracts for high performance liquid chromatography mass spectrometry (HPLC-MS) and gas chromatography mass spectrometry (GC-MS) analysis respectively. Those analysis were carried out to study potential changes in the metabolomic profile of the plants.

The results are showed as corrected area counts or percentages. Indeed, the area under the response curve, for HPLC, or the area percentage, for GC, of each peak was measured and the value was subtracted by the value obtained for the same peak of the related control (control infested or non-infested). This technique was used to present the results as clearly as possible as a fold change calculation was not possible due to the absence of production of certain metabolites by controls.

As an example of HPLC-MS chromatogram obtained after analysis, the chromatogram of leaves extracts of Sm EO treated plants by SC is presented in Figure 7.

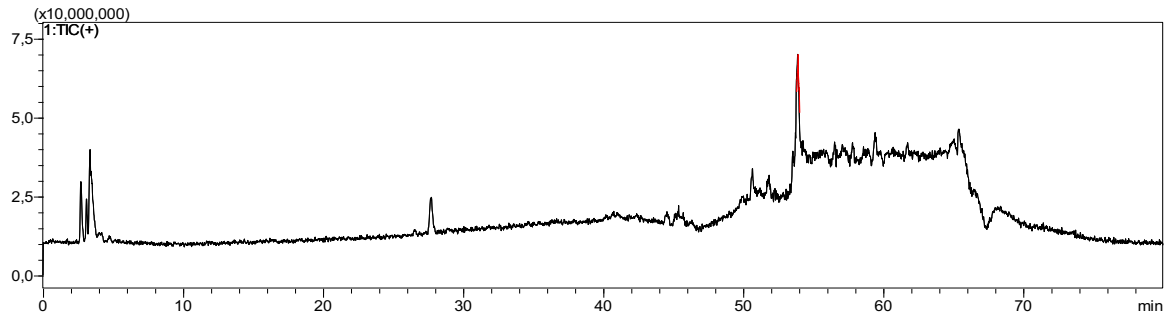

Figure 7 : HPLC-MS chromatogram of leaves extracts of plants treated with *Satureja montana* essential oil when applied by seed coating treatment application method.

HPLC-MS of leaves extracts results for plants treated by SMix are displayed by Figure 8 and SC by Figure 9. The plants treated with the mix of EOs show interesting production of compound number 4 and 5 as infested treatment produce more of these compounds than the un-infested ones compared to their controls. For SMix Sm plants, compounds 1, 3 and 9 display the same trend with a higher production for Sm+Me than for Sm. Lastly, Aa plants produce more of the compound 1 when infested. For plants treated by SC, Sm +Me only produces compound 11 in higher amounts than Sm but for Aa, it is the case for compound 1, 3 and 9 but also for compound 2, 5, 6 and 8 but in lesser amounts. HPLC-MS analyses of roots extracts were also assessed but no interesting variation in compounds production were detected.

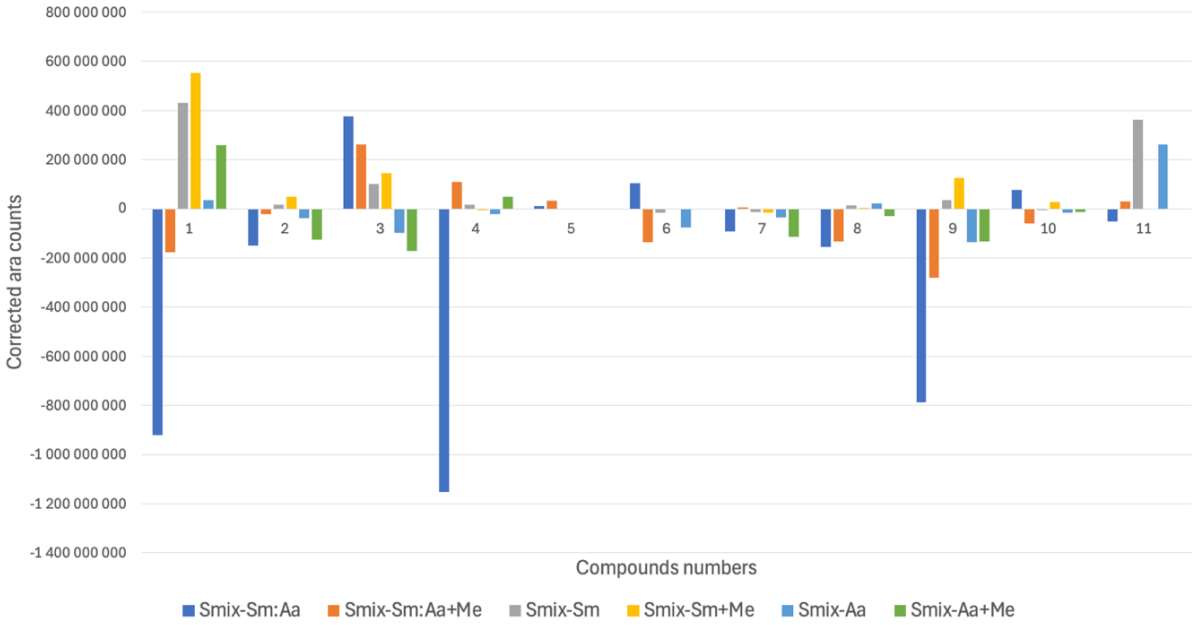

Figure 8 : HPLC-MS analyses results of leaves extracts of tomato plants treated with *Satureja montana* and *Artemisia absinthium* essential oils applied on their own or mixed by soil mixing treatment application method.
Results are presented as corrected area counts meaning that area under the curve of metabolite for EO treated plant infested or not was subtracted with area under the curve of the same molecule but of the infested or non-infested control. Compounds numbers are based on retention times.

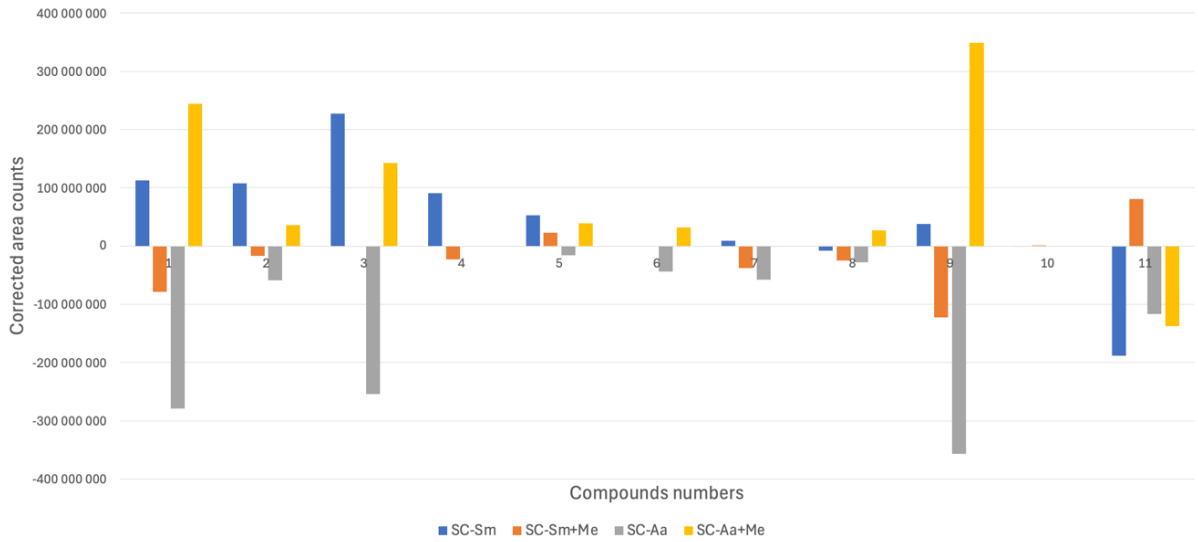

Figure 9 : HPLC-MS analyses results of leaves extracts of tomato plants treated with *Satureja montana* and *Artemisia absinthium* essential oils by seed coating treatment application method.
Results are presented as corrected area counts meaning that area under the curve of metabolite for EO treated plant infested or not was subtracted with area under the curve of the same molecule but of the infested or non-infested control. Compounds numbers are based on retention times.

A first identification of the compounds was assessed after HPLC-MS analysis. Nonetheless, ultra performance liquid chromatography tandem mass spectrometry (quadrupole and time of

flight) (UPLC-MS/Qtof) was used to verify those identifications. The tandem mass spectrometry analysis focused on compounds number 1, 3 and 9. The choice of the analyzed compounds was based on variations of corrected area counts. Analysis of the leaves extracts of SMix Sm:Aa+Me sample was analyzed for compound identification confirmation as it contained the most amount of the studied compounds. The first identification believed that the pin-pointed peaks with number 1, 3 and 9 were caffeic acid, 4-hydroxybenzoic acid and a lycopene related compound respectively. 4-hydroxybenzoic acid was not detected in the samples after UPLC analysis despite the use of a standard to verify the analytical method. The masses that first led to the identification of caffeic acid were not detected either and the retention time of the analyzed peak suggests that those masses could be in the injection peak. The peak that was believed to be a potential lycopene related compound was actually α-tomatine.

The total ion chromatogram (TIC) of the analyzed sample and the extracted ion chromatogram (EIC) of α-tomatine with m/z 1034,553 are presented in Figure 10.

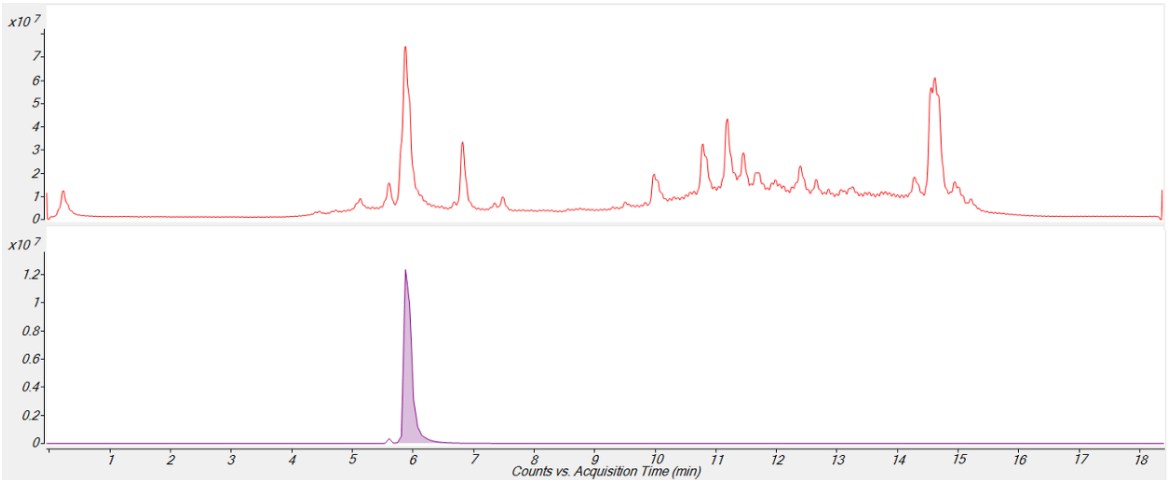

Figure 10 : Total ion chromatogram of leaves extracts of infested plants treated with the mix of *Satureja montana* and *Artemisia absinthium* by soil mixing treatment application method and extracted ion chromatogram of the selected mass of α-tomatine.

The molecules obtained through MS/MS analysis are α-tomatine with m/z 1034,553 and molecules coming from its fragmentation pattern with tomatidine galactoside (m/z 578,402)[78,79] which was the molecule initially thought to be related to lycopene and, finally, tomatidine (m/z 416,351)[78].

Firsthand identification believes that compound 6 is chlorogenic acid but no identification was found for compound 11. The mass of compound 11 was even not found in the results of the UPLC analysis.

Preliminary results for GC-MS analysis were also investigated. The changes of production of the most produced compounds of each treatment were studied and are displayed by Figure 11.

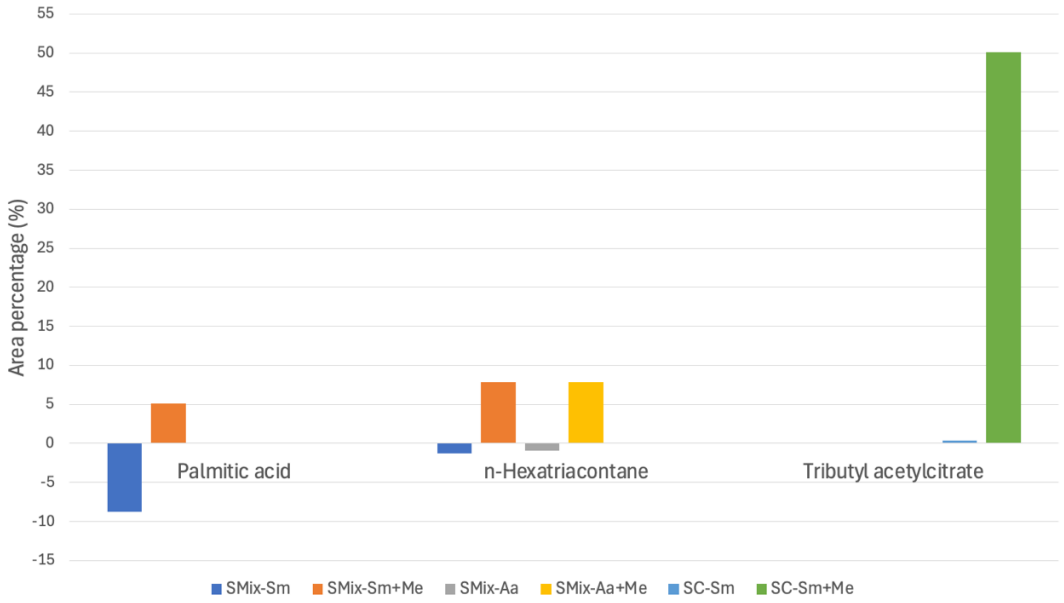

Figure 11 : GC-MS analyses results of leaves extracts of tomato plants for all treatments.
Results are presented as corrected area percentages meaning that the area percentage of a metabolite produced by an infested or not EO treated plant was subtracted with area percentage of the same molecule but produced by the infested or non-infested control.

Plants treated with the mix of EO showed no interesting modification in the relative composition of any of their major components. The SMix-Sm+Me treatment enabled the plants to produce palmitic acid, which represents 5% more of the total production of metabolites than the percentage of production of this same molecule in the total production of the infested control. The same change is visible for hexatriacontane but this time with an increase of nearly 8% induced by both Sm EO and Aa EO treatments.

As for SC treatment application method, Sm+Me treatment led the plants to produce tributyl acetyl citrate which represents 50% more of the total production of metabolites than the percentage of production of this same molecule in the total production of the infested control. For the same molecule, the difference between un-infested Sm EO plants and un-infested control is close to 0. At last, no interesting modification in the relative composition of any of the major components for SC-Aa EO treatments were measured. GC-MS analyses for roots extracts were not carried out for Aa EO treated plants by SC and roots extracts of SC-Sm plants are still under analysis at the CSIC-ICA.

5 Discussion

5.1 GC-MS analysis of *Satureja montana* and *Artemisia absinthium* essential oils

The GC-MS analysis of the two oils witness what was already stated in section 2.4. Indeed, it shows that their major components differ from one another justifying their selection to study two essential oils (EO) with different composition. For *Satureja montana* (Sm) EO, thymol and carvacrol are known to confer to the oil nematicidal and insecticidal properties even if the oil has much more biological effects than those two[74]. As for *Artemisia absinthium* (Aa) EO, cis-epoxyocimene and its sesquiterpenes are known to have insecticidal effects even if the oil is also antifungal and antiparasitic[73]. The EO was however not found to be nematicidal[42].

5.2 *In vivo* measurements

Results of fresh weights (FW) of plants treated by soil mixing treatment application method (SMix) might witness a synergistic effect of Sm and Aa EOs when mixed together (Sm:Aa). Indeed, on the one hand, the aerial parts (AP) and roots (R) FW of plants treated with the EOs alone decreased compared to their control. On the other hand, AP FW of Sm:Aa treated plants were 1,52 and 1,96-fold superior to the control for non-infested and infested plants respectively. It is thus possible that EOs used alone by SMix might have an inhibition effect on tomato biomass production and that, when mixed, those same EOs have a biostimulation effect on AP biomass production by synergistic effect of the active molecules contained in those EOs. Statistical results even support this hypothesis by showing significant differences in AP FW between plants treated with the mix of EOs or applied alone. This effect might remain even when plants are subjected to nematode infestation, but statistical results only support this hypothesis for Sm:Aa compared to Aa EO used alone but not for Sm EO. Biostimulation effects on tomato plants thanks to EOs treatments have already been studied but for direct foliar application. Indeed, Souri et al. (2019) tested rosemary EO on tomato plants which showed an increase in plant FW [80]. The same effect has also been reported by Chrysargyris et al. (2020) for a mix of rosemary and eucalyptus EOs [81]. However, it is not possible to stipulate that the effect of the oils is synergistic or additive as EOs were not tested separately. Plant FW production elicitation is also possible by seed priming as it is explained by Waqas Mazhar et

al. (2022). Although this time, the effect was observed when iron oxide nanoparticles were applied on flax seeds and not on tomato seeds[82].

The trend of FW increasing for treated and infested plants compared to treated but un-infested plants by SMix is unclear. For treatments with EOs used alone, this variation of FW does not witness the behavior of plants in a primed state. Indeed, primed plants would prefer the allocation of their resources towards defense activation in preference of plant growth [83,84]. Once submitted to the stress, primed plants would have a defensive response that limits weight loss[83,85]. Thus, under stress free condition, primed plant displays smaller weights compared to non-primed plant while when plants face a stress, primed plant shows higher weight than non-primed plant[83]. Nevertheless, primed plants under stress free condition will have a higher weight than in stress condition as showed by van Hulten et al. (2006). In the case of the plants treated by SMix with EOs used alone, this behavior is not respected and the variation in FW might be due to phytotoxicity of the essential oil. As for Sm:Aa treatments, the promoting effect on biomass production of this treatment could be over-expressed when EOs treated plants face a stress.

The behavior of the Sm:Aa plants seems to be different for its roots' biomass production. Indeed, only the EO treated and infested plants show an increase compared to the control. The mix of EOs might thus have no effect on un-infested roots while, when plants face a stress, roots' biomass production soars. The behavior of Sm:Aa treated plants remains, to the best of my knowledge, not observed in the literature. Moreover, nematode infestation should have a negative impact on plants' weight[86] which was not observed on inoculated controls. The changes described in this paragraph, apart from the change in the roots of the mixed EOs treatments, are simply observed trends. No statistical test has been able to confirm them. Discrepancies with the literature are therefore normal.

Potential effects of the seed coating treatment application method (SC) on AP and R FW were also investigated and Sm EO does not seem to have any effect on AP FW as opposed to when it was applied by SMix. The decrease in AP FW of SC Sm+Me plants might not have any meaning as no statistical result shows a potential relevancy to that change, but the diminution of that mean could witness the suffering of the plant when subjected to Sm EO and nematodes even if no effect were measured when the two parameters were applied separately. As for R FW for Sm EO treatment, it seems the EO still has an inhibiting effect on roots' biomass

production and that the combination of EO treatment and nematode infestation increased this effect. A second hypothesis would be that SC Sm EO treatment could prime the defenses of the plant but not against nematodes. The infestation could thus impair roots' biomass production of a plant already weakened by priming. As a reminder, under stress free condition, primed plant have lower weights than unprimed plants[83]. This hypothesis will be discussed further as nematode infestation is evaluated. On the contrary, Aa EO might improve the AP and the R FW of tomato plants when applied by SC as a trend of this effect is visible. This hypothesis could be supported by the paper of Souri et al. (2019), Chrysargyris et al. (2020) and Waqas Mazhar et al. (2022) that were already mentioned earlier.

The changes in FW, discussed here above, might be the cause of an inhibition effect by the EOs when applied on their own and by SMix. Indeed, results display the inhibition effect of EOs on tomato plants when applied alone by SMix while, when applied by SC at the same concentration, Sm EO shows no effect on AP biomass production and Aa EO displays an elicitation of biomass production. This might be due to the duration of the contact between the seeds and the active molecules contained in treatments. As these molecules are in fewer amounts in the mixed treatment, the inhibition effect cannot occur, and a synergistic effect takes place. When applied by seed coating, active molecules stay much less time in contact with the seeds, preventing biomass inhibition effects during plant development. While when applied alone by SMix, EOs are at higher concentrations and remains more time in contact with the seeds leading to phytotoxicity. Finally, some comparison between the SMix treatments were possible as, even if they were carried on by different operators, they were assessed at the same time and respect the same growth period. Variation between results is thus lower than for SC treatments as the manipulations were directed by different operators, with different growth periods and two years apart from each other's.

The comparison of the means of the nodulation index (NI) values of all treatments only showed a global reduction of the nematode infestation. This reduction of one category on the NI occurred on plants treated with Sm EO by SMix or by SC compared to their control. As the complete set of NI results was only available for plants treated with Sm EO by SC, only one statistical test was possible. The chi-square test showed no significancy of a possible resistance effect against root-knot nematodes, however, the amount of data required to obtain a reliable result was not respected. To have reliable results on a chi-square test, a minimum of five data per category must be respected, which was not the case here. The result of this test may thus be

incorrect. A graph showing the proportions of the various index values was thus created. It is possible to see on Figure 5 a trend were plants treated with Sm EO by SC were able to better counter nematode infestation compared to the control.

Although the NI is a great mean for global nematode infestation evaluation, the comparison of the various means is not statistically correct and thus not sufficient to stipulate on the better treatment inducing tomato plants priming against nematodes. Counting the number of galls of each size and dividing that count by the roots fresh weight allows a better evaluation of plants resistance. The only result showing a significant difference with its control is for Sm EO treated plants by SC. The treatment may thus have affected the plants to allow them to resist against nematodes. The plants prevented the nematodes to develop to the same stage as the nematodes of the control which led to the decrease of big sized galls. The same behavior is visible when comparing the gall sizes on resistant and susceptible plants[87,88]. The weights variations measured for this treatment might support the hypothesis of plant priming as, apart from the increase in FW for infected control which remains unexplained, the decrease of FW for SC Sm+Me compared to C4 and Sm coincide with what is described by Singh et al. (2021) and by Walters et al. (2008)[89].

As stated here above, SC Sm treated plants showed a trend of a decrease in global nematode infestation. The index evaluates global infestation and so the spread of the galls on the roots. The evaluation method measuring the number of galls per gram of root witness a trend of a higher number of galls on the roots. The results of those two methods put together thus might indicate that potential plant resistance led to a higher number of galls situated in a more concentrated location on the roots. However, the increase in the number of galls was not statistically verified thus raising doubts about those results. Furthermore, in the literature, nematode resistance comes with a decrease in gall number[19,37,90]. Nevertheless, in the future, a visual analysis on fresh material should be performed in detail before metabolomic analysis as roots' pictures are not clear enough to be analyzed.

Even if interpersonal errors were avoided for nematode infestation evaluation, standard deviations around SMix data for the number of galls per gram of root remain important. Indeed, Dr. Maria Fé Andres was the only person processing nematode results over the years but the variability in the development of tomato plants induced variability in roots weight and the variability in the capacity of nematodes to infest the roots led to variation in galls counts. All

in all, the results of nematode infestation evaluation for the SMix treatments have too much variability to even discussed the trends showed by the means. As for SMix Sm treated plants especially, even if NI showed a reduction in global infestation, this decrease is most probably due to variability errors as these values were calculated based on Figure 6's data.

In summary, SMix treated plants with the EOs used alone display a growth inhibition due to phytotoxicity of the EOs and no discussion can be made for a possible effect of the treatment on the ability of the plants to resist nematode as variability of the results is too high. The mix of the two EOs had a biomass production promoting effect probably due to a synergistic effect of the two oils. This promoting effect might have allowed plants to better resist the nematode infestation but, once again, no statistical result supports a potential nematode inhibition thanks to that treatment. As for the plants where EOs were applied by SC, Aa EO might have had a small biomass production promoting effect, but nothing can be discussed on nematode infestation as only NI results were available which showed no effect of the treatment. Finally, Sm EO applied by SC may have moderately increased plants resistance by significantly reducing the number of large galls but with a possible cost on AP and R FW.

The inhibition of nematode development is a viable hypothesis, but the effect of the treatment is not important enough to use this treatment as a viable way to fight root knot nematodes. Chemical nematicides, even if less environmentally friendly, remains way more efficient as they show high nematode population reduction[61]. Other studies on the seed priming of tomato plants showed interesting results of nematode resistance. Those studies used jasmonic acid[91], melatonin, spermidine[92] or sodium nitroprusside[93] as priming agents but the protocols were very different from the one used in this work. Indeed, nematode inoculation occurred after seed germination in the same Petri plates as the germination occurred and the infestation evaluation took place seven [91,92] or ten days later[93] raising concerns about the real priming effect of the treatments which might be a direct nematicidal effect. Neem leaves extract was also tested as a seed priming agent on bread wheat and reduced the number of galls of 24% of treated plants compared to the control[94]. To cite a hypothesis that was first stated by Juliette Cassart in her work and that led to the modification of the treatment application method (as explained in section 2.4), it is possible that the potential priming state set by the essential oils do not last long enough to allow tomato plants to defend themselves against nematodes. For example, the paper of Soudani et al. (2022) measured priming proves up to 12 days after EO treatment with no measurement at larger time scale while in this work, nematodes were inoculated

approximately one month after EOs treatments. As an example, Molinari et al. (2019) showed that primed state in tomato plants initiated by a mix of beneficial bio-control agents against *Meloidogyne incognita* did not last more than 7 days[95]. The nematode resistant effect that was measured on big galls per gram of root with Sm EO applied by SC may have been greater if the nematodes were applied sooner during the growth period. The studies mentioned sooner in this paragraph witness the lack of studies following the priming state in tomato plants on a longer time scale. Nonetheless, the study of Martínez-Medina et al. (2017) has demonstrated the priming role of *Trichoderma harzianum* on tomato plants against the nematode *M. incognita* with inoculation three weeks after treatment application and up to 42 days after nematode infestation[96].

Nevertheless, this work is the first work demonstrating a potential priming effect of the essential oil of *Satureja montana* L. on tomato plants *Solanum lycoprsicum* L. against root knot nematode *Meloidogyne javanica* (Treub) Chitwood.

5.3 Metabolomic analysis

Even if only the Sm treatment by SC moderately increase plant resistance to nematodes, interesting changes occurred in metabolites production. The study of those changes might uncover defense protection mechanism that were induced by the treatments that allowed SC-Sm treated plants to fight off the infection or that were maybe not strong enough to grant the plants to actively fight nematodes.

The goal of the calculation of corrected area counts was to highlight the changes in metabolite production between EO treated plants and controls. By doing this, it is possible to compare the metabolite production of plants treated with EOs and the ones treated both with EOs and infested with nematodes (+Me). A higher production of a metabolite for the infested treatment compared to the un-infested one could be due to a priming effect of the treatment as the only factor differentiating the plants is the seeds treatment. The treatment with EOs alone could lead to the triggering of the production of a certain metabolite in higher or lower amounts compared to the ethanol treated control. Plants treated with EOs and infested by nematodes could see their priming response triggered and so produce the same metabolite in higher amounts compared to the ethanol treated and infested control to better fight the pest. These types of modification of

production were observed on compounds 1, 3 and 9 and this is why they were selected for tandem mass spectrometry analysis.

As it was not possible to identify compounds 1 and 3, compound 9 will be the only one discussed. Indeed, this molecule revealed itself as being α-tomatine, a glycoalkaloid found in tomato unripe plants[97]. Among the many effects of α-tomatine, its ability to inhibit acetylcholinesterase (AChE)[97] is the most interesting one in this study. As stated in section 2.1.4, one of the possible nematicidal pathways of some molecules, even if the knowledge about those mechanisms is still scarce, could be through the hindrance of AChE activity. α-tomatine is known to be in high concentration during growth stage and the early reproductive growth and to allow the plant to resist diseases and pests[98].

Speaking of α-tomatine, its production seemed to be triggered by Sm EO when applied by SMix. This treatment may have shown little nematode resistance, but this hypothesis is not supported by any solid result. Moreover, the EO seems to have had a phytotoxic effect on the plants. The production of α-tomatine may have been triggered by the effect of both nematode infestation and phytotoxicity to help the plant overcoming the two stresses. For SMix Aa EO treated plants, no resistance to the invasion was noticed and Aa EO seemed to have an inhibition effect on biomass production. For this treatment only compound 1 was found to be produced in an interesting way but, as stated before, this molecule is believed to be in the injection peak. As for the mix of the EOs, resistance may have occurred thanks to stimulation of biomass production, but no evidence of priming has been noticed with the metabolomic analysis. For SC treatment application method, *in vivo* measurements noticed a nematode resistance for Sm EO treated plants. However, metabolomic analysis shows no interesting production of any metabolite except for a peak of 770 m/z. Nonetheless, no UPLC analysis was able to detect this mass. Finally, Aa EO treatment shows also an interesting production of α-tomatine. The treatment might have triggered this molecule to fight off the infestation but, once again, no solid result was able to confirm that the plants had increased their resistance to nematodes. Conversely to SMix treatment with Sm EO, SC Aa treatment did not have a negative effect on plants' weight. Continuing on SC Aa treatment, multiple interesting peaks were also analyzed that were different from the ones studied in other treatments, i.e. compounds number 2, 5, 6 and 8. This difference might be explained by the fact that metabolite extraction was performed on leaves and stems conversely to the extractions of the other treatments that were only realized on leaves. These molecules might thus be present in higher amount in the stems of tomato plants.

Gas chromatography mass spectrometry (GC-MS) was performed to assess an untargeted analysis of fatty acids, alkyl chains and related molecules. The results were displayed as corrected area percentages. The results are only preliminary results as only the interesting changes in the major components were studied. The choice of presenting those results this way was made as concerns were raised upon the quality of the identification of the molecules. Indeed, even if the peaks were identified by comparison of the masses and the retention times of the molecules with NIST and Willey databases, the identification were given by selecting the molecule suggested by the databases with the highest identification percentage. Thus, giving the same identification to some peaks with very different retention times.

The area percentage of a molecule is based on the area count of its peak on the total area count of the chromatogram. As the total amount of detected molecules and the number of molecules detected per analysis is different, any sort of measurable metabolite production modification cannot be described between two treatments. Indeed, even if the amount of a metabolite production is the same between two plants treated differently, the final area percentage will differ. The corrected area percentage thus witness the variation of "importance" given by a plant to a specific compound compared to the others but does not exhibit a measurable value. In other words, variation in metabolite diversity and amount will differ in function of the treatment thus prohibiting the possibility to highlight real differences in compounds quantity when displayed as percentages.

Nevertheless, studying those differences is interesting as it may display the difference in stress management strategy between plants which depends on how they were influenced by the treatment and by the stress.

GC preliminary results show interesting changes of relative production of hexatriacontane, palmitic acid and tributyl acetyl citrate. Palmitic acid and hexatriacontane, a fatty acid and an alkyl chain respectively, are reportedly present in tomato plants[79,99] and have antioxidant properties[100]. Hexatriacontane was detected by Yang et al. (2016) while studying the variation of roots exudates before and after nematode infestation of three different cultivars of tomato plants with various nematode resistances. This study showed that hexatriacontane might play a role in tomato defense against nematode as its relative content in root exudates increases upon nematode infestation for highly resistant and moderately resistant varieties[101]. The relative quantity of hexatriacontane also increased in tomato plants when subjected to ultraviolet-B

radiations.[99] As for palmitic acid, it was discovered that it has nematicidal properties [102] and repellant effect [103] on *Meloidogyne incognita*. Also, palmitic acid is a C16 long fatty acid whose acylated derivative, along with C18 stearic acid acylated derivative, are major components of the surface layers of plants[104–107]. These layers are known to limit water loss and to be the first barrier against phytopathogens invasion[104,105,107]. Real increases in the amount of the detected molecule cannot be discussed but Rachidi et al. (2021) showed that the application of microalgal polysaccharides on tomato plants increased the production, among other molecules, of palmitic acid[107].

The response of the SMix-Sm treated plants to the infestation with the production of both hexatriacontane and palmitic acid might have allowed the plant to better face the infestation. However, this trend was not confirmed by statistical tests. For SMix-Aa treated plants, it seems that the higher relative percentage of hexatriacontane was insufficient to decrease nematode infestation.

Tributyl acetylcitrate may also show a nematicidal effect. Indeed, it was measured at up to 56% in a methylene chloride extract of *Colpomenia sinuosa*. This extract was tested against against *Meloidogyne incognita* and displayed a 87,5% mortality after 12 h and 100% mortality after 24 and 72 h of exposure[108]. Nevertheless, to the best of my knowledge, tributyl acetylcitrate is not produced by tomato plants which supports initial concerns about the identification quality of the various GC-MS peaks. Nevertheless, SC-Sm+Me treated plants, whose are the only ones showing a significative difference in nematode infestation reduction for big galls per gram of roots, show a very high relative composition of that molecule which has a retention time of 30,219 min.

The GC-MS results of the plants treated with Aa EO by SC did not show any interesting change in composition of their main components. As for GC-MS results, no interesting changes in the composition were detected because extractions were also performed on leaves and stem but also because the dichloromethane fraction was not directly extracted from fresh material but from the dried methanolic extract. This difference leads to variation in the polarity of the extracted molecules and thus on the results.

The metabolomic analysis carried on in this work might not have witnessed all the changes that might have occurred in the plants upon EO and nematode treatments. Indeed, the choice in the

extraction solvents impacts the polarity of the extracted metabolites. Furthermore, the extraction method also influences the volatility of the studied compounds. Lots of other molecules with different polarities and volatilities might thus have been missed.

6 Conclusion and perspectives

The present work describes the potential priming effect of *Satureja montana* L. and *Artemisia absinthium* L essential oils on tomato plant (*Solanum lycopersicum* L., var. Marmande.) when applied by seed coating or mixed with the substrate to face root knot nematode (*Meloidogyne javanica* (Treub) Chitwood) infestation.

Results show significative reduction of the number of big galls per gram of root compared to the infested control for plants which seeds were coated with *S. montana* essential oil. This means that this work is the first work demonstrating a potential priming effect of this essential oil on tomato plants against root knot nematodes. Conversely, no effect of nematode resistance was observed on the other treated plants. However, interesting behavior of possible synergistic effect triggering the bio stimulation of the plants' biomass production treated with the mix of essential oils by soil mixing was witnessed. Another growth stimulation effect was also observed for plants treated with *A. absinthium* essential oil by seed coating. Metabolomic analyses by HPLC-MS, UPLC-MS/Qtof and GC-MS show interesting increased production of some metabolites which identification remains, for some of them, unclear except for α-tomatine that was confirmed by tandem mass spectroscopy.

All in all, essential oils are good candidates to prime tomato plants against root knot nematodes but as the pathways allowing the active molecules of essential oils to prime tomato plants' defenses are still unknown, the choice of the oil remains quite random. Increase research in this domain might improve the knowledge on active priming molecules and lead to tailored choice in essential oils use. Nevertheless, the application of essential oils coming from domesticated plants on seed is a low cost, sustainable and easy way to fight against such pests. As in vitro tests on the phytotoxicity of the essential oils used in this work were already carried out by Emma Zoccolan in her master thesis, only a few new results are still needed to achieve this research journey.

Indeed, i*n vitro* test of α-tomatine on nematodes should be performed to assess the nematicidal activity of the molecule. Also, further investigations on the identification of the peak identified as tributyl acetylcitrate should be carried out to try to better understand the pathways of plant resistance to nematodes. Derivatization and analysis of a n-alkane mix for RI calculation could

improve analytes identification. However, the variability between the samples remains high and an increase of the number of replicates could be one way of handling those errors. Nevertheless, working with biological material such as tomato plants and nematodes will still impact the variability. Furthermore, assessing several analyses during the growing stages of the plants would enable a better monitoring of the plants' behavior depending on its treatment and so ensuring the detection of potential priming-related changes. SPME-GC-MS analytical set-up or dynamic headspace could be implemented for VOCs analysis. Root exudates are also a good lead to unraveled new insights of plants resistance against nematodes. The study of specific hormones such as salicylic or jasmonic acid but also a focus on transcriptomic by RNA sequencing are leading path to better understand the effect of essential oils on tomato plants when used as primers. Phytotoxicity research on *S. montana* essential oil could also be interesting to evaluate its safety utilization in the agronomic sector.

Personal contribution

The multiple objectives of this work were assigned by Professor Marie-Laure Fauconnier and Doctor Azucena González-Coloma, my two co-promotors.

The first goal of this work was to investigate the last parameter that was not considered by the previous master theses meaning the study of the essential oil from *Satureja montana* L. applied by seed coating. In order to do that, I first had to carry out bibliographic research. Once I gathered enough knowledge about the project I had to organize, with the help of Maria Fé Andrés and Azucena González-Coloma, the planning of the experiments. I then carried out the *in vivo* experiments on tomato plants. Problems with the rearing of nematodes prevent me from performing the various measurements and metabolomic analyses myself. Those manipulation were realized by Ruben Muñoz, Felipe De la Peña and Maria Fe Andrés. Nevertheless, I still caried out weight measurements, metabolite extractions and sample preparation on tomato plants to acquire laboratory skills and to be aware of the manipulations carried out in this project.

The second goal of this master thesis was to gather all the results generated by the previous works to compare them between each other and to synthetize everything that has been realized this far. This part of the work asked a lot of time and dedication as those results were generated by various people, on different years and with different protocols. Moreover, all the samples from Emma Zoccolan's metabolomic analysis had to be re-analyzed. Statistical analysis had also to be performed to verify the potential differences between the treatments.

Finally, a new analytical method was added to verify the analysis of the metabolites analyzed in Madrid. This step asked to work with TERRA scientists that helped me with this step.

My work has generated new data on the priming of tomato plants with essential oils, both from a phenotypic and metabolomic point of view, including the identification of a new molecule but also the discovery of an induced nematode resistance in tomato plants when seed coated with *S. montana* EO.

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8 Appendix - supplementary materials

Some information of this part originated from Juliette Cassart's and Emma Zoccolan's master thesis. It is thus possible that some parts are identical as the work they originate from.

8.1 Rearing of nematodes - protocol

Meloidogyne javanica were reared on *Solanum lycopersicum* L. (var. Marmande) in a 1 L pot in growth chamber at a temperature of 23,5 ± 1°C during the day and 20 ± 1°C during the night. Relative humidity was above 70% and the photoperiod was 16h light and 8h dark. 21-days old 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 a filter immersed in distilled water for hatching in a closed opaque box kept in growth chamber. The second-stage juveniles were collected after a minimum of 3 days in distilled water.

8.2 Phytotoxicity of essential oils

In this work, two concentrations of *Satureja montana* L. (Sm) essential oil (EO) were tested as treatments. This choice was based on the results from Emma Zoccolan's master thesis as it showed a 47,97% reduction of the hypocotyl growth rate compared to the control at 1,25 mg/mL and nearly no inhibition at 0,625 mg/mL. The protocol and the results coming from Emma Zoccolan's master thesis are exhibited below.

If no germination problems were to be seen, only the plants whose seeds were treated with the 1,25 mg/mL Sm EO concentration will be kept. Indeed, it is believed that a higher concentration of EO in the seed coating solution might improve the priming effect of the EO.

8.2.1 Protocol

Originated from Emma Zoccolan's master thesis:

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 the one of the two EOs alone. The assay was conducted at 10, 5, 2,5, 1,25 and 0,625 mg/mL

for the mixture and both EOs. 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 for 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 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.

8.2.2 Results

Results from in vitro tests from Emma Zoccolan's master thesis show a 47,97% reduction of hypocotyl growth rate compared to the control at 1,25 mg/mL and nearly 100% germination rate at 0,625 mg/mL.

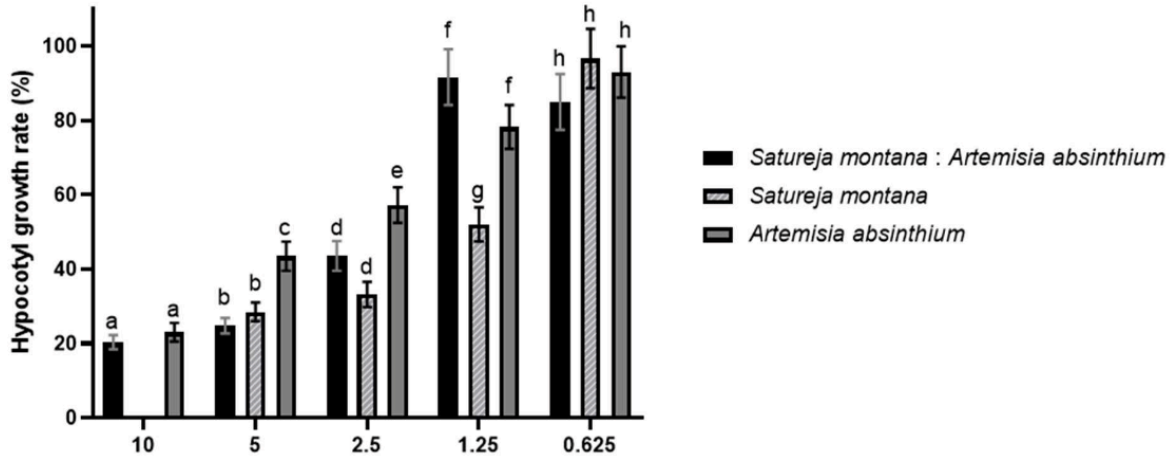

Figure S 1: Percentage growth rate of hypocotyl from Solanum lycopersicum L. seeds treated with S.montana EO, A.absinthium EO and the mixture of the two EOs (1:1 w/w) at 0,625; 1,25; 2,5; 5; and 10 mg/mL.
The hypocotyl lengths were measured after 7 days of experimentation and the growth rate is the relative 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).

8.3 Previously tested treatments

The first study focused on the use of *Artemisia absinthium* L. essential oil by seed coating. The seeds treatment consisted of the same treatment application method as described in section 3.3.1 with the same blank and controls. The concentration of the essential oil was 5 mg/mL.

For the two other studies, 100 mg 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. The EOs were also tested on their own in the same amounts. A volume of 80 mL of ethanol was also mixed with 80 g of vermiculite as control and a blank was also created accordingly but with distilled water. For all batches of vermiculite, the solvent was air-dried until complete evaporation (one day).

8.4 Plant growth conditions

The plant growth period before inoculation with second-stage juvenile nematodes were of 36 days with 7 days of acclimatization after transplantation for Emma Zoccolan and Sabrina Kesraoui experiments and 24 days with 3 days adaptation after transplantation for Juliette Cassart experiment. After inoculation with nematodes Emma Zoccolan and Sabrina Kesraoui experiments needed 20 days while Juliette Cassart experiment needed 30 days. The various

growth periods depend on plants' behavior. Each experimental period is thus different which might impact the reproducibility of the experiments.

8.5 Extraction method for plants treated with *Artemisia absinthium* essential oil by seed coating

Originated from Juliette Cassart's master thesis:

The extraction of the metabolites produced in the aerial parts (leaves and green stems) of the plants used in the infection experiments consisted in a maceration in methanol (MeOH), for at least one week. The replicates were first combined to obtain a higher mass for analyses (note: the subsequent chromatography analyses are consequently less robust as only one analysis per modality was performed). The liquid fraction was filtered on cotton and the solid fraction (i.e., the leaves and stem) was grinded with a mortar and pestle, also filtered on cotton and the filtrate added to the liquid fraction. Subsequently, the majority of the solvent was evaporated with a rotative evaporator, and the remaining extract was dried under an air flow after being transferred in a pre-weighed vial, to determine an extraction yield. The dry extracts were finally stored in the fridge until preparation for chromatography. Samples of the tomato dried methanolic extracts were re-dissolved in dichloromethane (DCM), filtered (reg. cellulose 0.2 µm, 17 mm, pk 100, Symta, Spain), dried and stored in the fridge until analysis.

8.6 Artemisia absinthium essential oil chemical characterization by GC-MS (Soudani et al., 2022)

Table S 1 :Chemical characterization of Artemisia absinthium essential oil by Soudani et al. (2022)

TABLE 1 | Chemical composition of the *Artemisia absinthium* var. candial essential oil tested.

Compound	Retention time (min)	Area (≥1%)
Linalool	6.451	2.03
(−)-(*Z*)-Epoxyocimene	7.088	34.85
(*E*)-Epoxyocimene	7.303	2.37
Camphor	7.447	1.97
(−)-*cis*-Chrysanthenol	7.765	9.04
Chrysanthenyl Acetate	9.930	8.40
trans-Caryophyllene	13.546	4.74
Germacrene-D	14.868	2.41
β-Selinene	14.990	1.45
Dihydrochamazulene	15.520	3.37
Dihydrochamazulene isomer	17.672	1.03
Neointermedeol	18.526	1.20
Chamazulene	19.906	5.01
Geranyl-α-terpinene	24.667	3.30
Geranyl-α-terpinene isomer	24.791	3.24

8.7 Illustrations of the studied tomato plants

Figure S 2 : Coated seeds ready to be sown into vermiculite

Figure S 3 : 31 days old tomato plants treated with Sm EO by SC before transplantation

Figure S 4 : 31 days old tomato plants treated with Sm EO by SC after transplantation and inoculation with nematodes

Figure S 5: Tomato roots only treated with water 32 days after transplantation

Figure S 6: Infested tomato roots treated with ethanol 32 days after transplantation and inoculation (positive control)

Figure S 7: Infested tomato roots treated with Sm EO 32 days after transplantation and inoculation

Figure S 8 : Closer look of one of the infested tomato roots treated with ethanol (left) or with Sm EO (right) 32 days after transplantation and inoculation