Priming effects of Artemisia absinthium Linnaeus essential oil on tomato plants against the nematode Meloidogyne javanica and on pepper plants against the aphid Myzus persicae

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Résumé

French

Dans la recherche d'alternatives aux pesticides conventionnels, le « priming » représente une découverte intéressante en tant que traitement prophylactique, à moindre coût pour les plantes cultivées. Très peu de recherches ont été effectuées dans cette optique, sur les huiles essentielles, dont les multiples propriétés sont pourtant bien connues et rencontrent un succès grandissant dans le domaine de l'agriculture. L'effet de « priming » de l'huile essentielle d'*Artemisia absinthium* L. (HEA) (de la variété *candial*® qui ne possède pas de thujone), administrée par enrobage de la graine, a déjà été démontré sur *Solanum lycopersicum L. contre Fusarium oxysporum.* C'est pourquoi, dans le cadre de ce travail, son effet contre les ravageurs communs *Myzus persicae* et *Meloidogyne javanica*, sur respectivement, *Capsicum annuum* L. et *S. lycopersicum* a été investigué. Les essais *in vitro* ont confirmé que l'HEA n'était pas nématicide, possédait une faible activité contre les pucerons et n'était pas phytotoxique pour le poivron. Elle était néfaste pour la germination des graines de tomate à partir d'une concentration de 5 mg/mL mais les expériences *in vivo* ont mis en évidence que les plantes en résultant étaient, visuellement, saines. En ce qui concerne les essais *in vivo*, d'une part, les observations biologiques n'ont montré aucun effet contre *M. javanica* mais une augmentation de la reproduction de *M. persicae* sur les poivrons traités et des différences significatives entre les poids des racines et des parties aériennes des poivrons testés. D'autre part, les analyses GC- et HPLC-MS des métabolites secondaires synthétisés dans les parties aériennes des poivrons et des tomates, ont révélé que l'enrobage des graines avec l'HEA, a bel et bien stimulé une réponse chez les plantes. Pour pouvoir attribuer les différences observées, entre plantes traitées, non-traitées, infectées et non-infectées, au « priming », des données supplémentaires devraient être récoltées. La piste la plus prometteuse pour approfondir les recherches futures serait d'appliquer le traitement de l'HEA à un stade de développement de la plante, plus avancé. Plusieurs suggestions sont formulées dans les perspectives.

Mots clés : ***huile essentielle, défense des plantes, « priming », nématode, puceron, tomate, poivron***

Abstract

English

In search for alternatives to conventional pesticides, priming appeared to be an interesting finding as a prophylactic treatment of crops, at low fitness cost. In this respect, only few research has been conducted on the priming potential of essential oils, which are already known for their multiple properties and are raising interest in agriculture. The priming effect of seed coating with (the thujone-free) *Artemisia absinthium* L. var. *candial*® essential oil (AEO) on *Solanum lycopersicum* L. has been proven effective against *Fusarium oxysporum*. Therefore, in this study, its effect against the common pests *Myzus persicae* and *Meloidogyne javanica,* respectively on *Capsicum annuum* L. and *S. lycopersicum*, was investigated. *In vitro* assays confirmed that AEO was not nematicidal, had a low activity against aphids and that it was not phytotoxic to pepper. It was detrimental for tomato seeds' germination from a concentration of 5 mg/mL but the *in vivo* experiments highlighted that the resulting plants were, visually, healthy. On one hand, the biological observations made during the *in vivo* assays showed no effect against *M. javanica* but an increase in reproduction of *M. persicae* on treated peppers and significant differences in weight of roots and aerials. On the other hand, GC- and HPLC-MS analyses of the secondary metabolites synthesized in the aerial parts of both peppers and tomatoes, revealed that the seed coating by AEO, did trigger a response. To allocate the differences observed between treated, non-treated, infected and non-infected plants, to priming of defenses, additional data need to be collected. The most promising route for further investigation would be to apply the AEO-priming-treatment at a later growth stage. Several suggestions are provided in the perspectives.

Key words: ***essential oil, plant defense, priming, nematode, aphid, tomato, pepper***

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List of abbreviations

ABA	Abscisic acid
ACh(E)	Acetylcholine(esterase)
ACO	1-Aminocyclopropane-1-carboxylic acid oxidase
AEO	*Artemisia absinthium* essential oil
ALA	5-Aminolevulinic acid
AMF	Arbuscular mycorrhizal fungi
APX	Ascorbate peroxidase
C	Control
CAT	Chloramphenicol acetyl transferase
CSIC	« Consejo Superior de Investigactiones Cientificas »
DAMP	Damage-associated molecular pattern
DE	Dry extract
DEP	Differentially expressed proteins
DET	Differentially expressed transcripts
DW	Dry weight
EO	Essential oil
EOC	Essential oil component
ET	Ethylene
ETI	Effector-triggered immunity
GABA(rs)	γ-Aminobutyric acid (receptors)
HEA	Huile essentielle d'*Artemisia absinthium*
ICA	« Instituto de Ciencias Agrarias »
IPM	Integrated pest management
IR	Induced resistance
ISO	International Organization for Standardization
ISR	Induced systemic resistance
J2	Second-stage juvenile
JA	Jasmonic acid
LOX	Lipoxygenase
MAMP	Microbe-associated molecular pattern
NI	Nodulation index
OA	Octopamine
PAMP	Pathogen-associated molecular pattern
PGPF	Plant growth-promoting fungi
PGPR	Plant growth-promoting rhizo-bacteria
PR	Pathogenesis related
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
T	Test
TA	Tyramine
VOC	Volatile
WW	Wet weight

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1. State of the art

1.1 Context

The need for global food production is increasing with population, only in 2020, the world produced a total of 38,125,680 tonnes of fresh fruits and 296,169,431 tonnes of fresh vegetables (FAOSTAT 2020, accessed on 11 April 2022). Besides the potential resistance that can be developed by pathogens to conventional pesticides, the extensive use of chemical pesticides has raised concern about the safety of its users and the environment[1–4]. Indeed, several studies have demonstrated their detrimental effects: pesticides can be spread and accumulate in soil and the atmosphere, contaminate water streams, and eventually enter the food chain[1,2,5] through different channels, described in the review of Tudi et al. (2021)[6]. They can cause a loss of biodiversity as they might harm nontargeted organisms, including humans[1,2,5,6]. Furthermore, recent research has drawn attention to the transformation products of pesticides which might be even more harmful than their parent form[7].

In the European Union "Farm to Fork" strategy, solutions to the reliance on conventional phytosanitary products are addressed among other sustainability issues of the agri-food sector. The Commission states that it "*will take additional action to reduce the overall use and risk of chemical pesticides by 50% and the use of more hazardous pesticides by 50% by 2030*" by for example revising "[…] *the Sustainable Use of Pesticides Directive, enhance provisions on integrated pest management (IPM)*[i] *and promote greater use of safe alternative ways of protecting harvests from pests and diseases*."[5].

Nevertheless, as Lamichhane et al. point out in their paper from 2016, finding alternatives to the current pest management is challenging because of the heterogeneity of the European agroecosystems[1]. It is therefore useful to develop a diverse choice of biocontrol methods, such as biopesticides. Over the past 20 years, essential oils (EO) have drawn interest as a potential biopesticide[8].

1.2 Essential oils

Essential oils are naturally occurring plant secondary metabolites[4,9–13], usually involved in plant defense against biotic and abiotic stresses[4,10,12–15], including insects[12–16]. They are stored in different parts of aromatic plants, depending on the species and stage of development: in secretory cells, cavities, canals, glandular trichomes or epidermic cells[4,11,12,16–18] of leaves, flowers, buds, roots, rhizomes, fruits, stems, seeds or even wood and bark[4,10–12,16–20] and can constitute between 0.01 to 10 % of the plant (all parts of the plant included)[4]. They are usually liquid at ambient temperature[4,12,15,16,18] and their

[i] European Commission definition of IPM: "Integrated pest management means careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment. 'Integrated pest management' emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms." (https://ec.europa.eu/food/plants/pesticides/sustainable-use-pesticides/integrated-pest-management-ipm_en#definition).

constituents have a low molecular weight. As they are volatile[3,12–15,18,20] and lipid-soluble or soluble in organic solvents with a lower density than water[12,18], they are extracted by steam or hydrodistillation[4,11–13,15,19–22] (ISO 9235:2021). Extractions by maceration, lipophilic solvent, supercritical fluid[11–13,19] or microwave[12] have also been studied but this results in plant extracts that cannot be called EO (ISO 9235:2021). The only exception is for citrus EOs which are extracted by cold expression[13,19–22]. The method applied is chosen in function of the plant material and the field of application[12]. More details on the extraction methods can be found in numerous books and reviews such as "*Essential Oil Research: Trends in Biosynthesis, Analytics, Industrial Applications and Biotechnological Productio*n", Malik (2019) and "*Handbook of essential oils: Science, Technology, and Applications*", Başer and Buchbauer (2020).

EOs composition is very complex[3,12]. They comprise of a mixture of ten to over two hundred components[17,21]. Most of them contain phenylpropanoids[16,20,21,23] and terpenoids[9,11,12,14–16,18–23] among which a majority of, oxygenated or not, mono- (two isoprene units, C10)[4,11,14,17,18,21] and sesquiterpenes (three isoprene units, C15)[11,14,17,18,21]. A variety of aromatic and aliphatic compounds are also included in the composition of EOs[4,12–14,19,22,23]. Nevertheless, they are usually characterized by two or three major components[11,12] representing 20 to 70 % of the EO composition[12]. Apart from the organ from which the EO is extracted[11,12,16], the phenological stage[10–12,15,16,22], the species[10,14,15] and the extraction process[10,15,16], other factors might influence the yield and composition (components and proportions), such as abiotic factors (climate i.e., hygroscopy, temperature, light, etc.)[4,11,12,15,16,20,22] but also the soil characteristics (minerals, microorganisms, pH, etc.)[11,12] and chemotype[4,11,14,16,21]. It is the components of the EO that confer its bioactive properties, either one component alone or most likely the combination of several major and minor components (with synergistic, additive or antagonist interactions)[12–14,16,21,22].

A series of biological effects have been described for EOs and include: antibacterial, antioxidant, anti-inflammatory, insect repellent, insecticide, acaricidal, herbicidal, deterrent, antifungal, antiviral, medicinal (antimalarial, antidepressant, antimutagenic, anticancer, hepatoprotective, anticonvulsant, analgesic and antipyretic), allelopathic, …[10,11,11,12,16,17,19,20,23,24]. Some properties have been discovered centuries ago, so EOs were already used in traditional medicine[11,15,19] and as food additives or flavoring agents[19]. Nowadays, three hundred out of the approximate three thousand known EOs, are used at an industrial level, especially in the flavor (beverages, food additives, cigarettes, …) and fragrance industry[4,9,10,12,16,17,19,21–23,25] but thanks to their multiple properties, they are also employed in a wide variety of other fields, from pharmaceutical[9,12,14,22,23] and cosmetics[3,4,9,12,14,16,17,19,21] to sanitary products[9,12,19], to the feed[9,22] and food industry (flavoring or preservative additives)[3,4,9,14,15,21], materials (incorporated in packaging[3,23], plastics, resins)[4] and in agriculture[3,9,12,19,21,23].

1.3 Essential oils in agriculture

1.3.1 Opportunities

As mentioned earlier, there has been an increased interest in greener alternatives to conventional pesticides. EOs present multiple promising properties since they are highly volatile natural products and therefore their persistence in the environment and crops is limited[3,4,22,26]. Besides, their mammalian toxicity is low[3,14,22,26]. In their review of 2021, Maes et al. concluded that the risk for acute poisoning of humans by EOs was very low and that even if the chronical poisoning is more challenging to assess, it is sensible to assume that since the accumulation in the environment is minor compared to synthetic pesticides, the long-term effects on human health will probably be less significant as well[21]. Moreover, the complexity of EOs composition and the synergy that has been observed between their components, provide the opportunity of a lower level of administration[20] and could result in their involvement in plant defense through various mechanisms[20,26,27], implying that pests would be less prone to resistance[3,20,26]. In this light, EOs have been studied in the last two decades and are still under investigation for their use in the agronomic field.

1.3.2 Some essential oils properties and their mode of action

Numerous works have studied the insecticidal properties of EOs and their components (EOC)[4]. In their review, Koul et al. (2008) cite the following effects against a variety of insects: lethal toxicity, feeding deterrence and oviposition deterrence, by direct contact or fumigation[4]. Besides, EOCs can also play a role of attractant that could be used in traps[4]. Regarding their mode of action, EOs, or at least some of their components, seem to have the ability to affect insects' nervous system[25,28]. Indeed, behavioral/locomotor changes have been observed in insects when exposed to certain EOs and EOCs[25,28]. Jankowska et al. detail three of them in their review of 2017. One of the proposed mechanisms consists in the interference with the γ-aminobutyric acid-gated (GABA) chloride channels[25,29,30]. GABA is a neurotransmitter that binds to GABA receptors (GABArs)[25]. Some EOCs (monoterpenoids) seem to allosterically (stereoisomers have different activities) inhibit, or less often stimulate, GABArs through potentiation or generation of Cl^- current[25]. A second investigated mechanism is the inhibition of acetylcholinesterase (AChE)[25,29,30] involved in the neuro-neuronal and -muscular junctions, which can be competitive and/or uncompetitive to acetylcholine (ACh) but then allosterically modify the ACh binding affinity, depending on the EO component(s)[25]. A third and more certain pathway is the inhibition of the octopaminergic system, octopamine (OA) being a molecule able to act as a neurotransmitter, a neurohormone and a neuromodulator in invertebrates[25,29]. EOs would be able to act as agonist of OA but also of the receptors of its precursor tyramine (TA), causing an increase in cAMP and Ca^{2+} levels and consequently a cascade of phosphorylations, through protein kinase A and C activation[25]. Going further, Renoz et al. (2020), analyzed the physiological changes induced in a surviving coleoptera after exposition to *Mentha arvensis* EO, with a label-free quantitative proteomic tool. They observed the upregulation of differentially expressed proteins (DEP) involved in cellular respiration, transcription, translation and folding processes, ensuring the recovery of the insect after exposure[28]. Among the observed DEPs, some play key roles in the nervous system, which complements previously proposed neurotoxic mechanisms, but others were also involved in the muscular system and several other bioprocesses such as cuticular sclerotization and chromatin remodeling, for instance[28].

Not only can EOs be used against insects[3,4], which is the most reviewed agronomic application[11], but their potential as antibacterial agent and their efficiency against some pre- and post-harvest fungal infections[3,4,11,26] have been examined in several research. De Clerck et al. (2020) performed a large

screening of EOs on commonly encountered plant pathogens, showing, *in vivo*, the diversity of EOs' effects: generalist or specific, dose-dependent or not, antifungal and/or bactericidal[26]. Their mode of action is probably linked to the lipophilic nature of EOCs[12,15,16]. Indeed, this characteristic allows them to interact with the cell membrane of bacteria and fungi[16,31]. By destabilizing the cell membrane and the cell wall, they make the cells permeable, resulting in the leakage of cell components[15,16]. They can interfere with DNA, RNA and protein and peptidoglycan biosynthesis[15]. These effects lead to osmotic and metabolic disorders[15]. They can also affect the mitochondria, disturbing levels of reactive oxygen species (ROS) and adenosine triphosphate (ATP)[12,15]. The inhibition of efflux pumps of the fungal plasma membrane can cause acidification and cell death[15]. These mechanisms, among others, are explained in more details in Bakkali's et al. (2008) and Nazarro's et al. (2017) reviews. Besides antifungal activity, Rashad et al. (2022) observed that the application of lavender EO might elicitate plant defenses against fungal attacks[32], this process will be developed further.

The nematicidal activity of several EOs has also been demonstrated[14,33–35]. Common observations are the paralysis activity against second-stage juvenile (J2) nematodes and the inhibition of egg differentiation and hatching[14,34–37]. The extensive review of Andrés et al. (2012), gather different research conducted on the multiple EOs showing nematicidal activity and explain that it has been demonstrated that the combination of EOCs in a certain ratio and the position of the functional group and/or the double bond were determining for the bioactivity of EOs[14]. The EO effects on nematodes are dependent on: their concentration of course, their chemical composition (leading to synergy or antagonism and depending on species, ecotypes and chemotypes, as mentioned previously), the nematode species and their physiological stage, eggs being usually less sensitive[14,34,35]. The modes of action of EOs against nematodes might be similar to the insecticidal[37] and antibacterial and -fungal modes of action explained here above i.e., interference with the nervous system[4] and the cell membrane[14,37].

Some EOs also manifest phytotoxic effects[11,20,27,38]. They can therefore be used as herbicide, but this might in contrast impair their use as other pests control since they could affect non-targeted plants too[20]. Phytotoxicity manifests itself through inhibition of germination and growth, chlorosis, leaves burning and necrosis[11,38]. In their review, Amri et al. (2012) gather studies on the phytotoxic modes of action of EOs[38]. They discuss the following: interaction with lipid bilayer of cells causing electrolyte leakage, disordering and membrane thinning effect, increase of lipid peroxidation activity and induction of ROS-induced oxidative stress, inhibition of glucose-dependant respiration, impairment of mitochondrial metabolism, photosynthesis inhibition, microtubule disorganization and alteration of cell wall biosynthesis[38]. These modes of action are also well described and illustrated in the review of Werrie et al. (2020) (figure 1), who also explain that the effects of EOs on plants vary from EO characteristics (components, vapor pressure, molecular weight, hydrophobicity), from plant and from application method[20]. Lins et al. (2019) adopted another approach for the understanding of the underlying mechanisms of EOs by investigating *in silico* and *in vivo*, the site of action of some EOCs in plant and collected interesting results which might open a path to a better molecular understanding of EO interactions with plants and optimal exploitations of their potential[27].

In figure 1 from Werrie et al. (2020), the following cellular mechanisms are represented:

- interaction with the wax cuticle and the cytoskeleton leading to the stomates closure inhibition and so water loss,

- alteration of the lipids' structure, enzymes and ion channels of the plasma membrane, causing depolarization, disruption but also changes in gene expression, oxidative burst, and increase in unsaturated fatty acids and stigmasterol in the membrane,

- oxidative stress caused by lipid peroxidation and ROS generation linked to the alteration of LOX activity, chloroplast (metabolism alteration and photosystem degradation) and mitochondria (uncoupling of oxidative phosphorylation and electron transfer inhibition), which results in photosynthesis and energy production inhibition, ROS generation, membrane disruption, programmed cell death and consequently growth inhibition, leaf chlorosis and necrosis,

- mitotic disruption due to microtubule depolymerization, chromatin disorganization which affects chromosomes integrity or inhibition of DNA synthesis,

- various enzymatic hindrance[20].

Werrie et al. (2020) also mention that at non-phytotoxic concentrations, some EOs might act as biostimulant. This concept is developed in section 1.4 with a focus on priming[20].

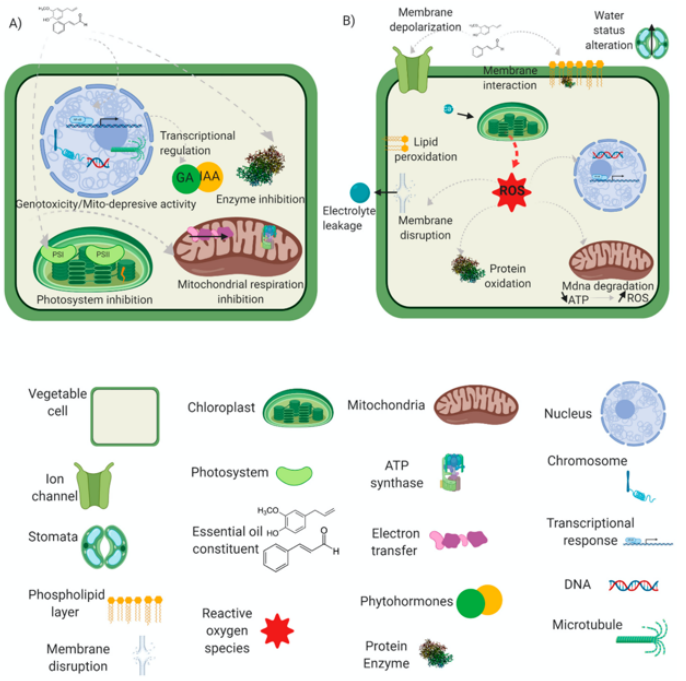


Figure 1. Mode of action of essential oil at the cellular level. **(A)** Photosynthesis and mitochondrial respiration inhibition, microtubule disruption and genotoxicity, enzymatic and phytohormone regulation. **(B)** Water status alteration, membrane properties and interactions, reactive oxygen species induction.

Figure 1. EO mechanisms from Werrie et al., 2020[20].

1.3.3 Essential oils on the market

Only a few EO-based pesticides are available on the market. In the United States, where their commercialisation has been facilitated compared to Europe, Mycotech Corporation and EcoSMART Technologies are offering several EO-based aphicides/miticides/fungicides/insecticides/herbicides[4,11]. Koul et al. (2008) also mention Apilife VAR™ which is produced in Italy and used against *Varroa* mites[4]. On the European market, pest control products based on clove, mint or sweet orange EOs have been registered, namely BIOXEDA, BIOX-M, LIMOCIDE-OROCIDE-PREV-AM and ESSEN'CIEL[11]. In 2021, the Integrated and Urban Phytopathology Laboratory of the Gembloux Agro-Bio Tech created the spin-off APEO (Agronomical Plant Extracts & Essential Oils) which will soon commercialize EO-based bioherbicides (in four to six years) (https://www.apeosolutions.com).

Besides complicated regulation processes and production costs[3,11], their loss of efficiency in the field might be partly responsible for the uncompetitiveness of EO-based pesticides[11]. As a matter of fact, their volatility and low molecular weight allow EOs not to persist in the environment, which is an advantage in an ecological point of view, but they are susceptible to degradation and actually, these features involve that larger amounts or repeated application might be required, that they might reach untargeted-crops and that a limited choice of application methods are available[3,11,14,20,21]. To cope with this drawback, scientists recently focused on new formulations and release control of EOs[3,14,20,39,40].

Oliveira-Pinto et al. (2021) demonstrated the efficacy of an EO against *Xanthomonas* spp. and studied the effect of its encapsulation in zein nanoparticles[39]. Nguyen et al. (2022) showed that the nematicidal effect of encapsulated EOs in lipid nanoemulsion formulated by homogenization combined with sonication was better than in their native form[40]. As for Mondéjar-López et al. (2022), they obtained promising results in the coating of seeds with chitosan-nanoparticle-encapsulated garlic essential oil for fungal control[31]. Nanotechnologies could be a solution to optimize EOs' efficiency, however Raveau et al. (2021) raise awareness on their possible adverse effects which should be investigated[11]. In that respect, the priming potential of EOs could represent another promising lead to tackle the formulation difficulty.

1.4 Priming

1.4.1 Theoretical remainder

Plants' immune system has been notably explained through Jones and Dangl's model called the "zig-zag model", which illustrates the co-evolutionary arms race between plants and pathogens[41,42]. Plants' first line of defense is called the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)[41–43]. It enables plants to sense the attack by a pathogen through the recognition of the PAMPs it emits (or thanks to damaged-associated molecular patterns resulting from an attack, DAMPs) and consequently trigger various defense mechanisms[41,42]. Some pathogens have developed the ability to escape/divert plants' PTI with effector molecules which forces the plant to deploy its second line of defense, the effector-triggered immunity (ETI)[41–43]. The activation of ETI involves the intracellular detection of these effectors by resistance proteins encoded by single resistance genes able to neutralize the pathogen by initiating rapid responses often leading to hypersensitive cell death[41–43]. PTI and ETI initiate relatively early local defenses such as the generation of ROS and the reinforcement of the cell wall, then later-acting defenses, monitored by the interaction of plant hormones (e.g., salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA))[41,43]. Plant immunity is complemented with another type of defense: induced resistance (IR). Yassin et al. (2021) qualifies it as a form of systematically expressed phenotypic plasticity that can directly upregulate plants immune mechanisms but also prime the defenses[41].

Priming is the physiological conditioning of a plant to a future stress enabling it to deploy faster, stronger and longer-lasting immune responses, liken to a kind of memory, at a relatively low fitness cost compared to a simple elicitation[41,43–46] (figure 2). It involves epigenetic changes in RNA and DNA and could sometimes be transgenerational[43,46]. Plants' primed state can be triggered by a variety of stimuli that activates various metabolic pathways which are dependant from the host and the attack (pathogen or abiotic stress, type of elicitor)[41,45,46]. These pathways are still under investigation, but some mechanisms are described in the reviews of Conrath et al. (2006), Martínez-Medína et al. (2016), Yassin et al. (2021), and Tan et al. (2022). They mention that priming can arise from:

- Systemic acquired resistance (SAR) stimulation, which is the ability of the plant to confer resistance in several organs after a local triggering of PTI or ETI. SAR priming is governed by SA which mediates the expression of pathogenesis-related (PR) genes[41–44].

- Induced systemic resistance (ISR) stimulation. This type of priming results from the interaction of the plant with non-pathogenic plant growth-promoting rhizo-bacteria (PGPR) or plant growth-promoting fungi (PGPF), which provoke an increased sensitivity of the plant to JA, among other hormones, and consequently the potentiated expression (expression upon attack) of PR genes[42–44].

- Chemical inducers. For example, β-aminobutyric acid (BABA)[46], a xenobiotic nonprotein amino acid, can prime plants for the formation of papillae against several biotic (microbes, nematodes, insects) and abiotic stresses through, inter alia, SA, ET and JA signalling pathways[43,44]. Other priming chemicals include also but are not limited to SA, JA[43,46], azelaic acid[43], hydrogen peroxide, GABA and 5-aminolevulinic acid (ALA)[45]. Priming by volatile organic compounds (VOC) is also possible as their emission is a plant natural defense mechanism[43,44,46]. Werrie et al. (2020) mention a priming mechanism by monoterpenoids too[20].

Pastor et al. (2013) provide a few examples of early-stage defenses resulting from priming such as stomatal closure, suppression of targeted genes, production of ROS or callose accumulation and later-acting defenses consisting in sensitization to hormones such as JA, SA and ABA[43] (figure 3).

The two following figures display an overview of the priming event in plants.

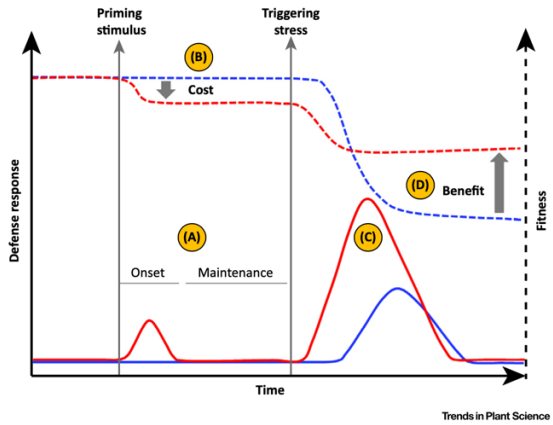


Figure 1. Scheme of the Relation between Defense Responses (Solid Lines) and Fitness (Dashed Lines) in Primed (Red) versus Unprimed (Blue) Plants. Analysis of defense priming requires a set of steps encompassing both the assessment of plant defenses and the associated cost–benefit balance. Here, we suggest some criteria that may help in deciding whether defense priming is present: (A) Memory: two sequential environmental events are required for asserting memory in the absence of molecular markers: the priming stimulus and the triggering stress. During priming and in the primed state (before the triggering stress), plant defenses are expected to be only transiently and generally faintly induced. (B) Low fitness costs: the maintenance of the primed state (before the triggering stress) has low fitness costs compared with the direct activation of defense. (C) A more robust defense response: in response to the triggering stress, primed plants mobilize cellular defenses in a faster, earlier, stronger, and/or more sustained manner than do unprimed plants. (D) Better performance: primed plants are expected to defend better against a given stressor than unprimed plants. Therefore, priming enhances plant fitness in hostile environments. Adapted from [2,6] and https://tonlab.wordpress.com/.

Figure 2. Evolution of fitness and defense responses with time, in primed and unprimed state, from Martinez-Medina et al. (2016)[46].

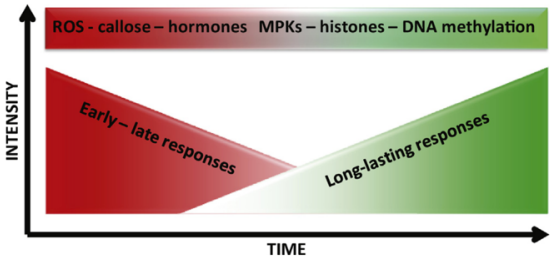


Fig. 2. Temporal events during priming. The main mechanisms of priming are temporally dissected. ROS and callose accumulation and hormonal responses happen relatively early after challenge, however, the accumulation of unphosphorylated MPKs, the modification of histones and DNA methylation are long lasting processes that can be transferred to the offspring. Additionally, the intensity of early responses can lose strength some time after challenge, while long term priming events gain relevance and are expressed stronger when the priming signals are maintained in time or repeated in descendants.

Figure 3. Plant response to priming, from Pastor et al. (2013)[43].

1.4.2 Some research on priming

Priming with EOs has been investigated against several pests but according to the review of Abd-Elgawad et al. (2021) on nematode control, never against nematodes[47]. An example is the effect of *Thymus vulgaris* EO against gray mold that has been demonstrated in the paper of Banani et al. from 2018[48]. The low expression of the pathogenesis-related gene PR-8 (encoding for a chitinase) at the moment of exposure to the EO and the resulting effectiveness of response of the apples, when confronted to the fungus, demonstrates the priming potential of this thyme EO[48].

More studies focussed on the effect of arbuscular mycorrhizal fungi (AMF) on plant defenses[49–52]. For instance, Medeirosa et al. (2015) observed the induction of resistance against the nematode *Meloidogyne javanica* thanks to the fungus *Pochonia chlamydosporia* which induced the reinforcement of roots by polyphenoloxidases and peroxidases, but further investigation needs to be conducted to confirm that it involved priming[52]. Hao et al. (2012) reported that, at first the root colonization of an AMF in grapevine did not/slightly induce(d) a response but that, after inoculation with an ectoparasitic nematode, it impaired its development via the activation of key genes such as a chitinase and the STS genes[49]. Molinari and Leonetti (2019), in turn, showed that a mixture of beneficial bio-control agents prevented the settling of a permanent feeding site by *M. javanica*[50]. The priming mechanism was deduced owing to, among others, the differential systemic over-expression of PR-1 and -5 (involved in SA-signaling) and ACO (involved in ET biosynthesis) genes, and the restriction of CAT and APX (involved in H_2O_2 activity), only after nematode inoculation[50]. These genes are usually respectively repressed and activated by nematodes[50]. Not only fungi have been reported to prime plant defenses, PGBR or their metabolites, such as surfactin, AHLs, NADB and pyoverdines have also been proven efficient[53].

As mentioned in section 1.4.1, volatile organic compounds (VOCs) have also been investigated as priming agents. For example, Song and Ryu (2013) studied the effect of some VOCs on cucumber seedlings. Besides fruit yield promotion, they observed a species-dependent ability to induce resistance against a bacterium and simultaneously the ability to attract aphid predators through the regulation of oxylipin pathway genes[54]. Without identifying it as priming, Smart et al. (2013) observed a similar event when treating tomato seeds with JA since it led to the emission of an attractive volatile compound for a predator of the studied herbivorous mite[55]. Furthermore, they noticed that direct defense activation was different according to the plant cultivar[55].

In their review, Tan et al. (2022) described how ALA-priming can confer resistance to abiotic stresses under a certain threshold concentration and explain how this can be used on the field to promote growth, the effects being different in function of the mode of application (seeds coating, foliar spraying or rhizospheric application by soil watering)[45]. Luna et al. (2016) also noticed different responses to elicitors depending on the mode of application and the hydrophobicity of the elicitor[56]. Indeed, BABA was able to prime tomatoes against *Botrytis cinerea* both while coated or applied on the seedling but JA, on the contrary, only triggered immunity when applied on the seedling[56]. The application of these two molecules did not cause any detrimental effects (neither on germination and growth nor on AMF colonization) at low concentrations but when applied on the roots, the higher the concentration, the more growth inhibition[56]. To complete this study, Bruce et al. (2017) determined the duration of BABA effect: the inhibition of *B. cinerea* lasted six weeks[57]. Moreover, they also identified a cultivar dependency and the activation of defense against *M. incognita*[57].

With regards to the mode of application of the priming agent, even if the efficiency of a seed treatment seems variable depending on the priming agent and the plant species[45,56], its potential has been acknowledged since acting so early provides protection at the most vulnerable stage of growth and impacts the whole lifecycle (storage, germination, growth, resistance to stress) and it could reduce the environmental exposure to the treatment[58]. The work of Kulak et al. (2021) showed that SA seed-priming could confer increased shoot growth and water stress resistance[59]. Besides, seed-coating has been studied for protective microbial inoculum delivery[60] and recently, the emergence of nanoparticle technologies led to the development of nano-priming[58].

Finally, in 2018, Gully et al. introduced a new concept: de-priming. Indeed, after demonstrating the priming effect of an SA analogue (BTH) on *Arabidopsis* and apples (observation of long-term transcriptional memory of certain differently expressed transcripts (DET)), they showed that subsequent exposure to a MAMP (flg22) could reverse the process i.e., up-/downregulate the DETs tuned by priming, maybe to limit fitness cost[61].

Research on this relatively new and not yet unraveled concept need to be carried on, as priming has great potential in IPM thanks to its broad spectrum of targets, the multitude of genes involved in the mechanism and the lower fitness cost than direct elicitation[43].

1.5 Focus of this study

1.4.1 Objective

The objective of this work was to assess the priming potential of seed-coating with the essential oil of *Artemisia absinthium* Linnaeus (var. *®Candial*) on *Solanum lycopersicum* L. (var. *Marmande*) against the nematode *Meloidogyne javanica* and on *Capsicum annuum* L. (var. *Teson* F1) against the aphid *Myzus persicae*.

This study was meant to determine if essential oils can prime plants of agricultural interest against recurrent pests which, to the best of our knowledge, is a very innovative subject that has not been investigated widely. It could therefore be of great interest to open the way to further studies on EO priming and subsequently go deeper in the understanding of the molecular mechanisms behind it.

1.4.2 Host plants

The experiment was conducted on tomato plants as it is one of the most consumed horticultural products worldwide[62,63] with 186,821,216 tonnes produced globally in 2020 (FAOSTAT accessed on 10 March 2022)[39]. Pepper plants were used instead of tomato plants for the experiments conducted with *Myzus persicae* for technical reasons explained further. These two plant species are dicotyledons, belonging to the *Solanaceae* family.

1.4.3 Plant parasites

Myzus persicae Sulzer is an insect from the family Aphididae, able to perform both parthenogenesis and sexual reproduction[64,65]. It is a sucking plant parasite, able to colonize more than four hundred plant species from several families[64,65]. Besides damaging its host by feeding, it is a vector of plant viruses[64–66]. It is a widespread scourge as it has developed high resistance against a wide range of pesticides by means of several mechanisms explained in the review of Bass et al. (2014)[64,65].

Meloidogyne javanica is an obligate biotrophic nematode belonging to the root-knot nematodes[67,68]. Plant parasitic nematodes from this genus owe their name to the galls they cause on their hosts roots while feeding, proliferating[67], and sometimes while laying egg masses[68]. *M. javanica* reproduces by mitotic parthenogenesis[67]. The second-stage juveniles (J2), the infectious stage, penetrate their host using their stylet and secreting enzymes, then migrate to the vascular cortex to settle permanently[67,68]. Root-knot nematodes affect a broad spectrum of crops widely[24,47,67–69], tomato among others[63], and due to the complexity of their life cycle[24] and adaptability[67], they represent an important economic challenge[14,24,68,69].

1.4.4 Essential oil

Artemisia absinthium Linnaeus (vernacular name, wormwood) is a perennial[70,71] dicotyledon plant from the botanical family *Asteraceae*[71]. It originates from several regions of the world[71] including mountain areas of Spain, where it is a ruderal species[70,72]. It has been used for folk medicine as it possesses numerous pharmaceutical properties, still exploited nowadays[70,72] and listed in the review of Batiha et al. (2020)[71]. For instance, it has recently been studied for its wound-healing properties[73]. Besides, wormwood was also used in the food industry e.g., in alcoholic drinks but one of its main components, thujone, is neurotoxic[70,71]. Thujone is not present in all varieties and is involved in some

of wormwood's EO properties reported in literature: antimicrobial[71], anthelmintic, acaricidal[71,72], antifungal, insecticidal, insect repellent and antifeedant[71,72,74].

To allow a wider use of *A. absinthium* in the food industry, besides its valorization as biopesticide, a Spanish research project focused on the domestication of a marketable thujone-free variety which has been registered under the name ®*Candial*[70,72,75]. The EO extracted from this new variety is mainly composed of *cis*-epoxyocimene, chrysanthenol, chrysanthenyl acetate, linalool and *trans*-caryophyllene[72,74–76]. It has been shown inactive against several plant parasitic nematodes but active against an animal parasitic nematode[72,76], it presents antiprotozoal activity, a low to moderate antifeedant activity and no phytotoxicity against *Lolium perenne* L. and *Lactuca sativa* L.[75]. Nevertheless, depending on the population[72,74,76,77], the mode of extraction and extraction parameters, different fractions with quantitative and qualitative differences can be obtained and consequently display distinct bioactivities[72,76,77]. Indeed, a patent has been filed for the production and use of the fungicidal supercritical CO_2 extract of *A. absinthium* var. ®*Candial* and its hydrolate which holds nematicidal properties[78]. This essential oil from *A. absinthium* var. ®*Candial* primed tomato seedlings germinated from coated seeds against *Fusarium oxysporum*[79], however, no other studies of its priming effect have been found in the scientific literature.

2. Materials and Methods

2.1 Plant material

Dried *Solanum lycopersicum* seeds var*. Marmande* were obtained from Ramiro Arnedo s.a. (Calahorra - La Rioja, Spain, lot 0231604 F) and stored at 4°C.

Dried *Capsicum annuum* seeds var. *Teson* F1 were obtained from Ramiro Arnedo s.a. (Calahorra - La Rioja, Spain, lot 0233190) and stored at 4°C.

2.2 Essential oil

2.2.1 Plant material

Artemisia absinthium Linnaeus var. ®*Candial* originating from Teruel (Spain, 2019, PEO) were used for the experiment. As mentioned earlier, it is a domesticated thujone-free variety registered under Decision No EU 36714 of 27 January 2014 at the Community Plant Varieties Office[70].

2.2.2 Essential oil extraction

The leaves were dried in the shadow at ambient temperature and the essential oil extracted by vapor pressure in a stainless-steel semi-industrial plant with two distillation vessels of 3,000 L[75,79]. The essential oil was recovered by decantation.

2.2.3 Essential oil characterization

Artemisia absinthium Linnaeus var. ®*Candial* (AEO) was analyzed by gas chromatography (Shimadzu GC-2010) coupled with mass spectrometry (Shimadzu GCMS-QP2010 Ultra mass detector, electron ionization, 70 eV). The conditions of analysis are reported in the publication of Soudani et al. (2022)[79].

2.3 Aphids and nematodes

2.3.1 Myzus persicae

Myzus persicae aphids are reared on pepper plants, at the *Instituto de Ciencias Agrarias* (ICA, CSIC Madrid) on a regular basis, in the following conditions:

Temperature	23 ± 1°C (L) and 20 ± 1°C (D)
Relative humidity	> 70 %
Photoperiod	16:8 h (L:D) indirect light

* L = light; D = dark

New colonies are formed twice a week by reinfecting new pepper plants. The number of newly infected plants depends on the size of the aphid colony at that time.

2.3.2 Meloidogyne javanica

At the ICA, tomato seeds (var. *Marmande*) are planted periodically in *jiffy®* pots filled with vermiculite. Twenty-five days after planting, the seedlings are transplanted to bigger pots filled with quartz sand. Three days later, the tomato plants are inoculated with nematodes by inserting an infected root in the pots, close to the roots. After 2 months, the roots are washed, and the eggs collected manually under a microscope. The eggs masses are then incubated in a closed opaque box on filter paper, semi-immerged in distilled water, in a growth chamber. The eggs hatch subsequently during a month and the juveniles (J2) are collected every three days.

The conditions of the "clean" growth chamber (no insects) are the following:

Temperature	23.5°C ± 1°C (L) and 20 ± 1°C (D)
Relative humidity	> 70 %
Photoperiod	16:8 h (L:D) indirect light

* L = light; D = dark

2.4 In vitro phytotoxicity assay

The toxicity of the AEO on tomato seeds has been assessed by applying the protocol described in the paper of Martín et al. (2011)[77], routinely used at the ICA.

In a 12-wells plate, ten seeds per well (2 cm^2) were placed on a paper filter (Whatman® nº 1, 2.0 cm of diameter) impregnated with 20 µL of AEO solution (10 mg/mL) or with ethanol (control). The wells were then humidified with 500 µL distilled water. Each treatment was done in four replicates. After sealing the plate, it was placed in a growth chamber (conditions in section 2.3.1) for 6 days. The germination was monitored every day and the hypocotyl length measured in the end of the experiment, with the program ImageJ (https://imagej.nih.gov/ij/download.html).

The phytotoxicity on pepper seeds was assessed following the same protocol but the seeds were placed in water for one week before the experiment to boost their germination as it is slower than tomato seeds and mold might appear before the end of the experiment. The duration of the hydration period before the experiment has been determined based on observations of germination in distilled water, when different times in water are applied beforehand. The experiment was ended after 21 days in order to obtain a sufficient radicle length for measurement[80].

2.5 In vitro assays

2.5.1 Aphids settling

Following the protocol of Burgueño-Tapia et al. (2008)[81], two fragments of pepper leaves with the same size were disposed on the lid of a box, filled with agar as support and to prevent desiccation of the plant material. On one leaf, 10 µL of the treatment (10 mg/mL AEO) was spread and on the other, 10 µL of control (ethanol). At least ten aphids were placed in each box and stored in a growth chamber for 24 hours (conditions in section 2.3.1). The day after, the settled aphids were counted on each leaf fragment. A settling inhibition index was then defined as:

$$SI\% = \left(1 - \frac{T\%}{C\%}\right) * 100$$

in which, T% corresponds to the number of aphids settled on the leaf treated with AEO and C%, the number of aphids settled on the control.

2.5.2 Nematicidal effect

This experiment was based on the protocol of Navarro-Rocha et al. (2020)[82] adapted from Andrés et al. (2012)[14]. In a sterile 96-U-shaped-bottom well plate for cellular culture (Becton Dickinson Labware Falcon®, Franklin Lakes, NJ, USA), 95 µL of a solution of around 1 *M. javanica* (J2) per µL distilled water and 5 µL of treatment (AEO at 20 mg/mL in DMSO and 0.6 % Tween 20, or only DMSO + 0.6 % Tween 20 0.6 % for the control) were placed in a well[ii]. Each modality was repeated four times and the replicates were grouped in a square (cf. appendix 6). Around the samples, the empty wells were filled with distilled water to avoid desiccation and border effects. The plates were then sealed with parafilm, wrapped in aluminum foil to protect them from light and placed in the clean growth chamber (conditions in section 2.3.2) for 72 hours. After the incubation time, the corrected percentage of death was determined using the formula of Schneider-Orelli:

$$\%\text{death corrected} = \frac{\%T - \%C}{100 - \%C} * 100$$

in which, %T is the mean percentage of immobile nematodes in the treated sample and %C is the mean percentage of immobile nematodes in the control.

2.6 In vivo infection tests

2.6.1 Seed coating and plant growing

The tomato and pepper seeds were kept in a refrigerator at 4°C, at least 24 hours before use. For each assay the seeds were coated with distilled water as blank, with ethanol (EtOH) as negative control and the different concentrations of the studied AEO (0.5 mg/mL, 1 mg/mL, 5 mg/mL, and for pepper, 10 mg/mL as well) diluted in EtOH. The priming of the seeds was carried out as following: dipping of each seed in the treatment for about one second, drying on aluminum foil and planting in *jiffy®* pots filled with humidified vermiculite. The pots were then placed in the clean growth chamber (conditions in section 2.3.2).

The growth/phytotoxicity (height, lesions, burnings) was visually monitored while watering the plants.

2.6.2 Aphids (M. persicae) on pepper

2.6.2.a. Choice experiment

Twenty *M. persicae* were placed in a small container between two two-leaves stage pepper plants, planted at the same time: one treated with 5 or 10 mg/mL AEO by seed-coating and one control with EtOH, in a sealed box filled with humidified vermiculite (cf. set up in appendix 16-19). After 24 hours

[ii] DMSO (Dimethyl Sulfoxide, Fisher bioreagents, USA); Tween® 20 (Fisher bioreagents, USA).

in a growth chamber (conditions in section 2.3.1), the number of aphids on each plant was counted to determine the settling index (cf. section 2.5.1). The aphids were then removed, and an extraction of the metabolites synthesized in the aerial parts of the plants was performed for chromatographic analysis (detailed in sections 2.6.4, 2.6.5 and 2.6.6). This experiment was performed twice for each concentration, at one week interval and every time in six replicates.

2.6.2.b. No choice experiment

Six two-leave stage pepper plants treated with 10 mg/mL AEO (highest concentration showing no growth inhibition or other symptoms) and six others with EtOH (control) were isolated in closed jars, infected with 20 synchronized *M. persicae* per plants (synchronization is explained in section 6.4.2 of the appendix) and placed in a growth chamber (conditions in section 2.3.1). Six plants of both treatments were kept uninfected as control and placed in the same conditions. Reproduction and mortality were assessed daily for 7 days, and the nymphs removed. On day 7, after the last record, all the aphids were removed. For the twenty-four plants, the aerials (leaves and green stems) and roots were separately weighed. The roots were placed in an oven at 40°C for 3 days for drying and then weighed again. The aerial parts were weighed and placed in methanol for maceration to extract the metabolites (cf. sections 2.6.4, 2.6.5 and 2.6.6).

2.6.3 Nematodes (M. javanica) on tomato

Fifteen seeds were planted per treatment and after 24 days, six plants per treatment were selected and transplanted in clay pots filled with 60 mL of quartz sand. After 3 days of adaptation in the new soil, the plants were inoculated with a 1000 juvenile *M. javanica* (J2) inoculum. Six plants treated with EtOH and six others with distilled water were kept non-inoculated, as blank. Thirty days after inoculation, the number of galls and infection sites were observed to determine a nodulation index in accordance with the publication of Hussey and Janssen (2002)[83]:

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

2.6.4 Extraction

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.

2.6.5 Sample preparation and HPLC-MS analysis[79]

The stored pepper and tomato extracts were injected at 0.5 mg/mL with a 5 µL injection volume by an automatic injector (SIL-20A XR). All extracts (0.5 µg/µL) were dissolved in 100 % MeOH for injection. The analyses were performed with a Shimadzu apparatus equipped with an LC- 20AD pump and a CTO-10AS VP column oven, coupled to a mass spectrometer with triple quadrupole as analyzer (LCMS-804) and an electrospray ionization source (ESI). 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 compounds were eluted with MeOH (LC-MS grade) with 0.1 % acetic acid (B) : MiliQ water with 0.1 % acetic acid. The solvent gradient started at 38 % B reaching 100 % in 45 min, followed by 100 % during 7 min and then 38 % B for 13 min before the next injection, at a flow rate of 0.5 mL/min. The nitrogen flow (drying gas for solvent evaporation) 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 = 100-850) at a potential of 1.66 kV, a capillary temperature of 250°C.

2.6.6 Sample preparation and GC-MS analysis[79]

Samples of the tomato and pepper 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. After dissolution to a concentration of 4 µg/µL in 100 % DCM, the analyses of these extracts were conducted by gas chromatography coupled with mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 µL) were performed using an AOC-20i, equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimetil- (5%) diphenylpolisiloxane. Working conditions were:

Split ratio	20:1
Temperature of the injector	300°C
Temperature of the transfer line connected to the MS	250°C
Initial temperature of the column	70°C
Heating at 6 °C/min intervals until	290°C
Temperature maintained for cleaning (15 min)	290°C

Electron ionization mass spectra and retention data were used to assess the identity of the compounds by comparing them with those found in the Wiley 229 and NIST Mass Spectral Database.

2.7 Statistical analysis

Microsoft Excel and Stat Graphics Centurion XVI version 16.1.18 and 18.1.16 (Stat Point Technologies, Inc., Warrenton, VA, USA) were used for the treatment of the results and statistical analysis.

The normality of the data sets was checked with the Shapiro-Wilk W test when the population size was smaller than 50 and with the Kolmogorov-Smirnov test when it counted fifty or more individuals.

Depending on the normality and the homogeneity of variances, a one-way analysis of variance (ANOVA) or a non-parametric analysis of variances (Kruskal-Wallis' test) was performed, with a 95.0 % confidential level. For non-independent observations i.e., data collected in pairs, a paired sample t-test was applied (*in vitro* settlement assay and *in vivo* choice experiment).

3. Results

3.1 *Artemisia absinthium* Linnaeus *var. Candial essential oil characterisation*

The characterization of the studied essential oil (same chemotype, same lot) has been performed by Soudani et al. and published in their article of 2022[79]. The main components they identified by GC-MS were *cis*-epoxyocimene (35%), *cis*-chrysanthenol (9.04 %), chrysanthenyl acetate (8.40 %), chamazulene (5.01 %) and *t*-caryophyllene (4.74 %). A complete table of AEO chemical composition can be found in their publication and matches previous analyses of AEO of this variety.

Table 1. Studied AEO chemical composition determined by GC-MS, from 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 isomer 1	15.520	3.37
Dihydrochamazulene isomer 2	17.672	1.03
Neointermedeol	18.526	1.20
Chamazulene	19.906	5.01
Geranyl-α-terpinene isomer 1	24.667	3.30
Geranyl-α-terpinene isomer 2	24.791	3.24

3.2 *In vitro phytotoxicity assay*

3.2.1 *AEO effect on Solanum lycopersicum* Linnaeus *var. Marmande germination*

It was decided that a growth inhibition lower than 50 % would be tolerated for further assays with AEO solutions. As shown in table 2, a concentration of 10 mg/mL AEO was toxic for the tomato seeds (60.14 ± 9.11 % inhibition) and a concentration of 5 mg/mL was close to the defined limit of tolerance of phytotoxicity i.e., 46.30 ± 8.97 % inhibition. A statistical non-parametric Kruskal-Wallis analysis (non-normality of the population and homogeneity of variances not always verified in the phytotoxicity data sets) was also performed on the raw data to assess the influence of the different AEO concentrations on the radicular growth (0, 0.5, 1, 5 and 10 mg/mL AEO). This test highlighted significant differences between radicle lengths for seeds treated with 10 and 5 mg/mL AEO compared to the control, with *p*-values of respectively 0.000 and 0.002, at a confidence level of 95.0 %. No significant differences with the control were observed for lower concentrations (*p*-value > 0.05). It was therefore decided to pursue the *in vivo* experiments with concentrations below 5 mg/mL, including the latter.

Table 2. *In vitro* study of the effect of different concentrations of AEO on *Solanum lycopersicum* L. (var. *Marmande*) radicular growth and germination.

AEO concentration	Growth - Radicular length at 144 hours		Germination at 144 hours	
(mg/mL)	$\%_{relative}$[a]	$\%_{absolute}$[b]	$\%_{relative}$[a]	$\%_{absolute}$[b]
0.5	- 14.61 ± 2.63	85.39 ± 15.34	- 5.13 ± 0.30	94.87 ± 5.48
1	0.97 ± 0.17	100.97 ± 18.13	- 2.56 ± 0.15	97.44 ± 5.70
5	- 46.30 ± 8.97*	53.69 ± 10.40*	- 2.56 ± 0.15	97.44 ± 5.70
10	- 60.14 ± 9.11*	39.86 ± 6.04*	- 7.69 ± 0.40	92.31 ± 4.81

Values of radicular length are means of 25 replicates.
Values of germination are means of 10x4 replicates.
[a] Relative radicular growth percentage: ((mean length treated - mean length control) / mean length control) x 100.
[b] Absolute radicular growth percentage: (mean length treated / mean length control) x 100.
*p-value < 0.05, Kruskal-Wallis' test, 95.0 % confidence level.

3.2.2 AEO effect on Capsicum annuum Linnaeus *var. Teson F1 germination*

The same approach as for the phytotoxicity against tomato was adopted to treat the phytotoxicity results on pepper. In this experiment, no phytotoxicity of AEO on pepper seeds radicular growth was observed. The *p*-value obtained for 5 and 10 mg/mL AEO compared to the control was higher than 0.05 (0.838).

Table 3. *In vitro* study of the effect of different concentrations of AEO on *Capsicum annuum* L. (var. T*eson* F1) radicular growth and germination.

AEO concentration	Growth - Radicular length at 21 days		Germination at 21 days	
(mg/mL)	$\%_{relative}$[a]	$\%_{absolute}$[b]	$\%_{relative}$[a]	$\%_{absolute}$[b]
0.5	28.18 ± 3.96	128.18 ± 18.01	2.70 ± 0.16	102.70 ± 6.16
1.0	7.27 ± 0.78	107.27 ± 11.54	11.43 ± 0.69	111.43 ± 6.73
5.0	3.31 ± 0.54	103.31 ± 16.75	2.70 ± 0.23	102.70 ± 8.89
10	- 8.23 ±1.35	91.77 ± 15.08	5.40 ± 0.46	105.40 ± 8.96

Values of radicular length are means of 25 replicates.
Values of germination are means of 10x4 replicates.
[a] Relative radicular growth percentage: ((mean length treated - mean length control) / mean length control) x 100.
[b] Absolute radicular growth percentage: (mean length treated / mean length control) x 100.
*p-value < 0.05, Kruskal-Wallis' test, 95.0 % confidence level.

3.3 In vitro aphid settlement

A threshold of 70 % is used in routine analysis of the ICA to qualify the tested product effective against *M. persicae* and further investigation. Considering the $SI\%_{corrected}$ obtained in this experiment (cf. table 4), AEO activity against *M. persicae* can be considered relatively low (i.e. 34.15 ± 8.82 % settling inhibition). In addition, a paired sample analysis was performed on the raw data set and revealed a *p*-value of 0.095, which confirms that there is no significant preference of the aphids for the treated leaf or the control.

Table 4. *In vitro* results of *M. persicae* settlement on AEO-treated pepper leaves.

AEO concentration (mg/mL)	$SI\%_{corrected}$[c]	T%[a]	C%[b]
10	34.15 ± 8.82	41.39 ± 5.61	58.61 ± 5.61

The value is the mean of 20 replicates.
[a] Percentage of aphids settled on the treated leaf after 24 hours.
[b] Percentage of aphids settled on the control after 24 hours.
[c] Corrected settlement inhibition: if SI% < 0, $SI\%_{corrected}$ = 0.

3.4 In vitro nematicidal effect

The corrected percentage of mortality of J2 *M. javanica* after 72 hours was very low (table 5), a usual threshold of 70 % being used to consider active the tested product. Hence AEO nematicidal activity was not tested at lower concentrations.

Table 5. *In vitro* nematicidal activity of AEO on *M. javanica* J2.

AEO concentration (mg/mL)	J2 mortality $\%_{corrected}$[a]
1	0.74 ± 0.81

The value is the mean of 4 replicates.
[a] Percentage of mortality of J2 *M. javanica* after 72 hours, corrected according to Schneider-Orelli's formula.

3.5 In vivo experiments with Myzus persicae

3.5.1 Choice experiment

Biological observations

As shown in table 6, the aphids did not show any preference for the treated peppers compared to the control, for neither of the tested concentrations of AEO. A paired samples statistical analysis was also run and showed no significant differences between treated and non-treated plants.

Table 6. *In vivo* settlement assessment of *M. persicae* on pepper plants.

AEO concentration (mg/mL)	Repetition	$SI\%_{corrected}$[c]	T%[a]	C%[b]	*p*-value[d]
10	(1)	26.39 ± 12.98	48.76 ± 8.42	51.24 ± 8.42	0.951
	(2)	16.67 ± 12.36	50.02 ± 6.72	49.98 ± 6.72	0.726
5	(1)	41.50 ± 13.55	38.08 ± 8.25	61.95 ± 8.25	0.187
	(2)	1.85 ± 1.85	61.87 ± 6.15	38.13 ± 6.15	0.082

The value is the mean of 6 replicates.
The repetitions of the experiments (2) were performed one week later and the control and treated peppers were not as similar in size as for (1).
[a] Percentage of aphids settled on the treated leaf after 24 hours.
[b] Percentage of aphids settled on the control after 24 hours.
[c] Corrected settling inhibition: if SI% < 0, $SI\%_{corrected}$ = 0.
[d] Paired sample analysis, *t*-test, 95.0 % confidence level.

Besides, the fresh weight of the aerial parts of the peppers used for this experiment were compared right before their maceration in methanol for metabolites' extraction. The only significant difference resulting from Kruskal-Wallis' analysis was observed for the second repetition of the choice experiment with pepper plants treated with 5 mg/mL AEO versus EtOH-treated peppers (control) (*p*-value = 0.010). However, it must be noted that at the beginning of the experiment, before infection, the difference in size of the treated plants versus the control was already noticeable.

Yields of the methanolic extracts prepared for chromatography coupled with mass spectrometry analyses were determined for each choice experiment but no general trend was observed.

3.5.2 No choice experiment

Biological observations

Birth and death of synchronized *Myzus persicae* placed on individualized pepper plants were monitored daily for a week.

The mean birth on treated plants relatively to the control was represented in a bar chart in figure 4. After 24 hours, the relative birth could not be represented since no nymphs were observed in the control plants (denominator equals zero). As the variability for the relative birth after 48 hours was very high, no reliable information could be extracted from this data point. Nevertheless, some information can be extracted from the observations made from 72 hours until the end of the experiment. A one-way analysis of variance was performed on the raw data (number of nymphs per plant, at each time, depending on the seed's treatment) for these times, and significant differences in the number of nymphs between the treated and control plants were observed. The *p*-values obtained with this test are reported in table 7.

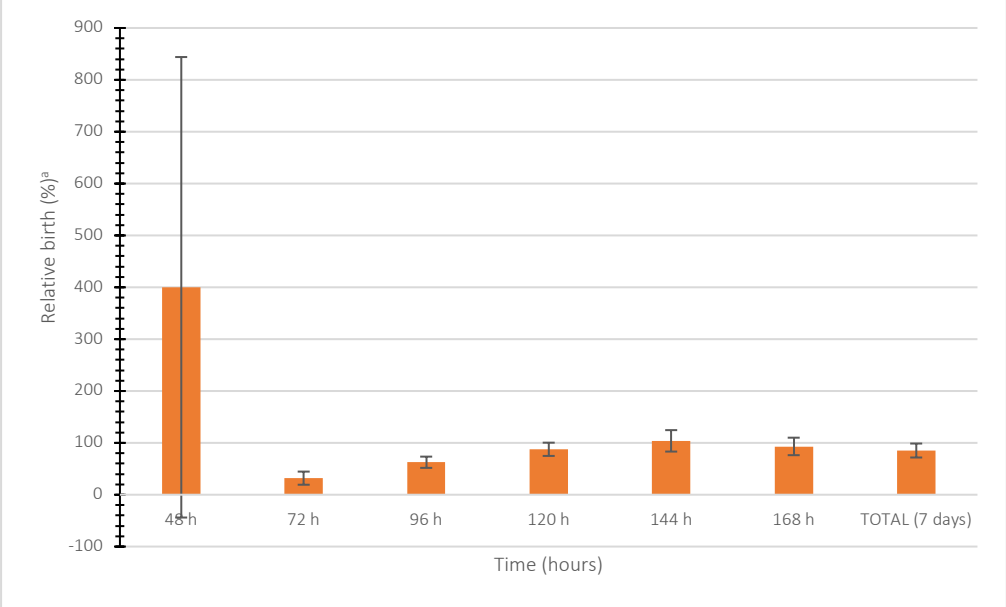


Figure 4. Mean relative percentage of birth of *M. persicae* on two-leaves stage pepper plants, over time. Values are means of six replicates.
[a] Mean percentage of birth on the treated peppers relative to the control: ((#T - #C) / #C) x 100, in which #T and #C is the mean number of births respectively in the treated peppers and in the control.

Table 7. Statistical analyses of the difference in birth of *M. persicae* on seed-treated and untreated two-leaves stage peppers, over time.

***p*-values**							
Kruskal-Wallis' test		One-way ANOVA					
24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**	**Total (7 days)**
0.902	0.367	0.500	0.019*	0.004*	0.008*	0.005*	0.003*

Regarding death over time, it should be borne in mind that it was deduced from the number of living aphids that could be found on each plant, as the dead bodies of the aphids could not be found on the surface of the vermiculite. These results are therefore to be taken with caution. In figure 5, the mean percentage of death on treated peppers corrected with the mean death on the control (Schneider-Orelli's formula) is represented for each time point. The biggest difference with the control was observed at 72 hours, at which death was 10.48 ± 1.87 % lower than on the control. A Kruskal-Wallis test revealed no significant differences between the number of deaths, at each time, in treated and control plants except at 72 hours (table 8).

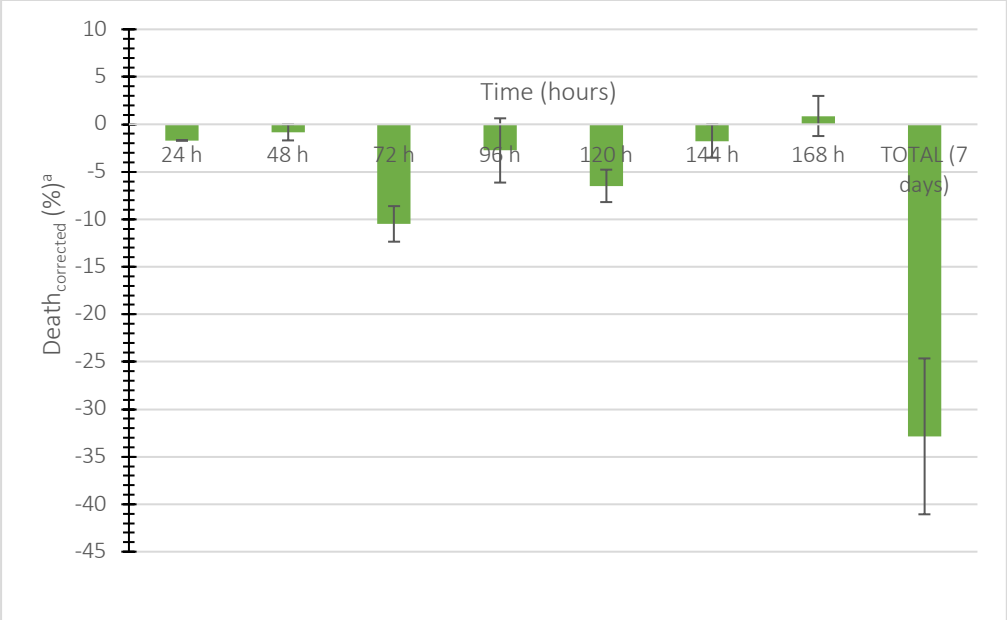


Figure 5. Mean percentage of death corrected of *M. persicae* on two-leaves stage pepper plants, over time.
Values are means of 6 replicates.
[a] Mean of the percentages of death corrected with the control according to the Schneider-Orelli's formula.

Table 8. Statistical analyses of the difference in death of *M. persicae* on treated and untreated two-leaves stage peppers, over time.

***p*-values** (Kruskal-Wallis' test)							
24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**	**Total (7 days)**
0.317	0.523	0.011*	0.412	0.208	0.730	0.652	0.024*

After the seven days of experiment, the roots and aerials weight were determined and then the yield of dried methanolic extract obtained from the aerials of pepper plants. The differences between weights of infected treated and infected non-treated peppers on one hand, and the differences between non-infected treated and non-infected non-treated peppers on the other hand, were assessed with a Kruskal-Wallis' test (95.0 % confidence level). Significant differences were observed between the roots weight of infected non-treated and treated plants but not among the roots of the

peppers that were not infected with aphids. Furthermore, significant differences were observed between the aerials weight of treated and control plants for both infected and non-infected plants. Nevertheless, it has once again to be highlighted that differences in the size of aerials were already observed visually between the treated and non-treated peppers before and during the experiment.

Finally, other differences can be observed in the yield of extractions: it was higher for the infected plants (0.4737 and 0.7789 gDE/gWW) and even higher for the infected treated plants (0.7789 gDE/gWW). Two elements have to be noted. Firstly, the replicates were combined for the methanolic extraction, which is why only single values for each modality were obtained. Secondly, the extracts had a viscous aspect, which was either the natural texture of the sample, or because some solvent was still present.

Table 9. Organs weight and methanolic extracts of treated and non-treated pepper plants of the "no choice" experiment.

Coating - treatment	Yield of extraction (gDE/gWW)[a]	Root mean weight (g)	Aerial parts mean fresh weight (g)*
EtOH	0.1899	0.0494 ± 0.0086 (WW) 0.0045 ± 0.0008 (DW)	0.0507 ± 0.0079 (WW)
EtOH + *Myzus persicae*	0.4737	0.0563 ± 0.0054 (WW)* 0.0044 ± 0.0004 (DW)*	0.0745 ± 0.0074 (WW)
EO 10 mg/mL	0.1815	0.0642 ± 0.0076 (WW) 0.0056 ± 0.0007 (DW)	0.0835 ± 0.0132 (WW)
EO 10 mg/mL + *Myzus persicae*	0.7789	0.0947 ± 0.0152 (WW)* 0.0070 ± 0.0010 (DW)*	0.1268 ± 0.0162 (WW)

Values are means of 6 replicates.
[a] yield of dried methanolic extract obtained per gram of the aerials of the pepper plants used in the experiment (DE stands for dry extract, WW for wet weight, DW for dry weight).
* Significant differences (p-value < 0.05), Kruskal-Wallis' test:
- aerials: p-value = 0.016 (difference between treated and untreated infected peppers); 0.037 (treated and untreated non-infected)
- roots: p-value = 0.037 (treated and untreated infected peppers fresh weights); 0.037 (treated and untreated infected peppers dry weights).

Metabolomic analysis

The methanolic extracts of the aerial parts of the peppers used in the "no choice" experiment were analyzed by GC-MS and HPLC-MS (replicates combined, so only one analysis per modality). Several differences in composition were observed between the samples. An attempt to find the function of some identified compounds and a possible explanation for the observed fluctuations can be found in section 4.

In figure 6 summarizing the GC-MS results, the most remarkable differences (> 10 %) can be observed for neophytadiene (2) and oleoamide (10). Minor changes (< 10 %) can be observed for methyl 4,8,12-trimethyltridecanoate (5), ambrettolide (6) and squalene (12).

The major pepper component detected by GC-MS (10) decreased with AEO treatment (2.64 times) and infection (2.04 times), followed by AEO+infection (1.45 times). As for compound 2, it increased with AEO treatment (5.36 times), infection (2.56 times) and AEO+infection (1.98 times).

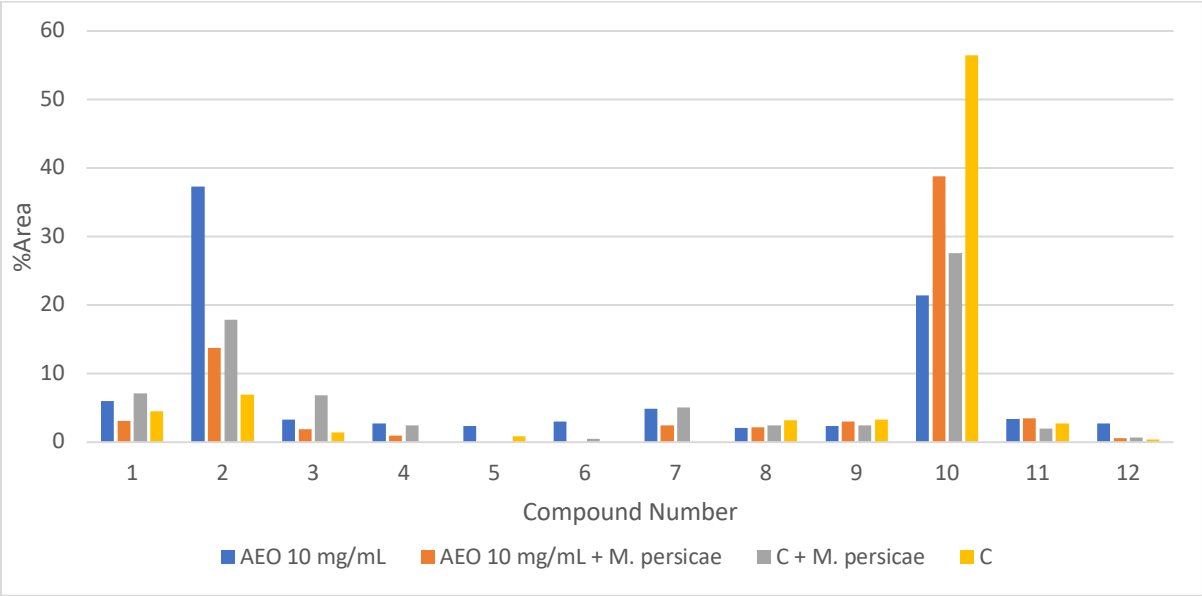


Figure 6. GC-MS identified compounds for the pepper methanolic extracts, reextracted with DCM, of the "no choice" experiment.

1: Acrylic acid tetradecanyl ester; **2**: Neophytadiene; **3**: Palmitic acid; **4**: Methyl linolenate; **5**: Methyl 4,8,12-trimethyltridecanoate; **6**: Ambrettolide; **7**: Methyl-11,14,17-Eicosatrienoate; **8**: Oleic acid; **9**: Hexadecanamide; **10**: Oleoamide; **11**: n-Hexatriacontane; **12**: Squalene.

AEO: pepper plant of which the seed has been coated with *Artemisia absinthium* L. EO.

C: control pepper plant of which the seed has been coated with ethanol.

Compounds 1, 2, 3, 4 (caffeic related) and 9 increased with AEO treatment (by 1.37 times, 1.21 times, 0.76 times, 1.20 times and 1.13 times, respectively). Infection or AEO+infection reduced compounds 1 (1.36 and 2.43 times), 2 (2.67 and 4.47 times), 3 (2.57 times and to 0 area unit), 4 (5.52 and 4.44 times) and 9 (isorhamnetin-3-(4-O-rhamnosyl)rutinoside) (from 125,571,794 area units for the control to 0 area units for both infection and AEO+infection). It is important to note that compound 8 (ferulic acid) appeared while 7 (luteolin C-pentosyl-C-hexoside) disappeared for AEO+infection.

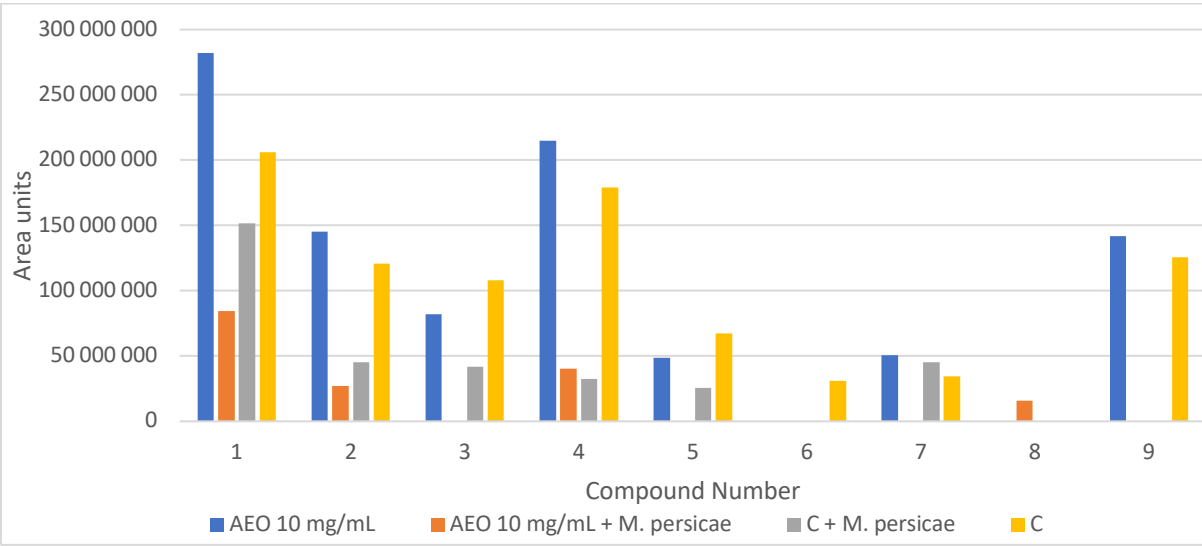


Figure 7. HPLC-MS identified compounds for the pepper methanolic extracts of the "no choice" experiment.

1, 2, 3, 4: caffeic acid derived; **5** and **6**: unidentified; **7**: Luteolin C-pentosyl-C-hexoside; **8**: Ferulic acid; **9**: Isorhamnetin-3-(4-O-rhamnosyl)rutinoside.

AEO: pepper plant of which the seed has been coated with *Artemisia absinthium* L. EO.

C: control pepper plant of which the seed has been coated with ethanol.

3.6 *In vivo experiment with Meloidogyne javanica*

Biological observations

As explained in "*Material and methods"* section 2.6.3, a nodulation index was determined for the one-month inoculated tomato roots, comparing the treated ones to the control. As represented in figure 8, no effect of the AEO seed-coating, no matter the concentration, was observed against the nematodes infection.

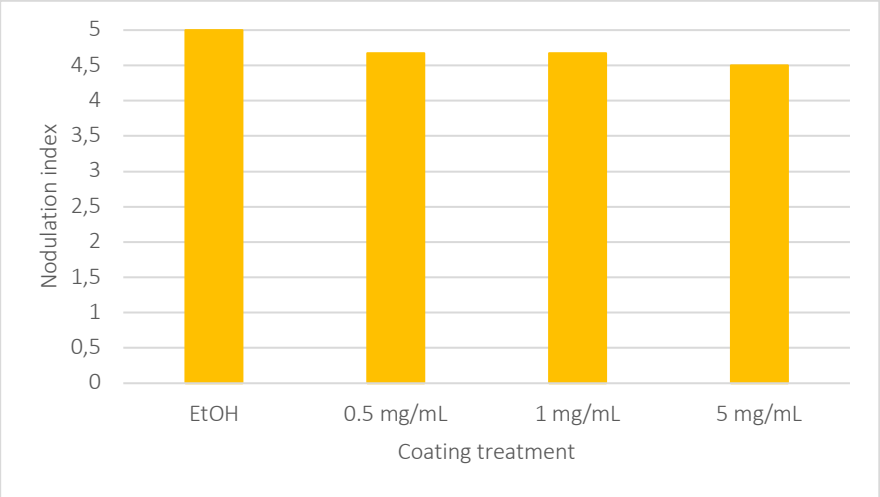


Figure 8. Nodulation index (0-5) of tomato roots after one month inoculation with *Meloidogyne javanica*.
Note that the commas stand for decimals in this bar chart.

A Kruskal-Wallis test was applied on the roots weight of the inoculated plants and no significant difference was observed. The difference in roots weight between non-inoculated tomato plants treated by seed-coating with 5 mg/mL AEO and EtOH- and water-treated ones, of the same age as the inoculated ones, was also analyzed by scientific curiosity but no significant difference was observed (Kruskal-Wallis, 95.0 % confidence level).

Metabolomic analysis

The extracts of the tomatoes of the "nematodes experiment" were also analyzed by GC- and HPLC-MS. As no effect of the treatment had been observed while examining the nodulation on the roots, the extract of the tomatoes treated with the highest concentration of AEO and the controls were analyzed, assuming that the metabolic changes (if any) would be more pronounced.

In the GC-MS results, the major compounds detected were neophytadiene (2) and oleoamide (14). Acrylic acid tetradecanyl ester (1), was not present in the sample treated with AEO and inoculated. Compound 5 (palmitic acid) increased with inoculation (4.60 times for the control) and with AEO+inoculation (5.28 times). In turn, compound 7 (neophytadiene isomer 2) increased with AEO treatment (from 0.35 to 12.06 %area, or 34.46 times). With respect to 8 (9-eicosyne), it was only detected in the AEO+inoculation extract (5.43 %area). Ethyl linoleolate (9) increased for the three treatments (inoculation, AEO, AEO+inoculation), with both AEO-treated samples being the strongest inducers (8.21 times for the AEO treated and 10.05 times for AEO+inoculation). Compound 10 (phytol isomer) only appeared in the inoculated control (7.86 %area). Finally, 14 (oleoamide) decreased with all the treatments (1.49 times upon inoculation, 1.91 times when AEO-treated and 2.22 times for AEO+inoculation).

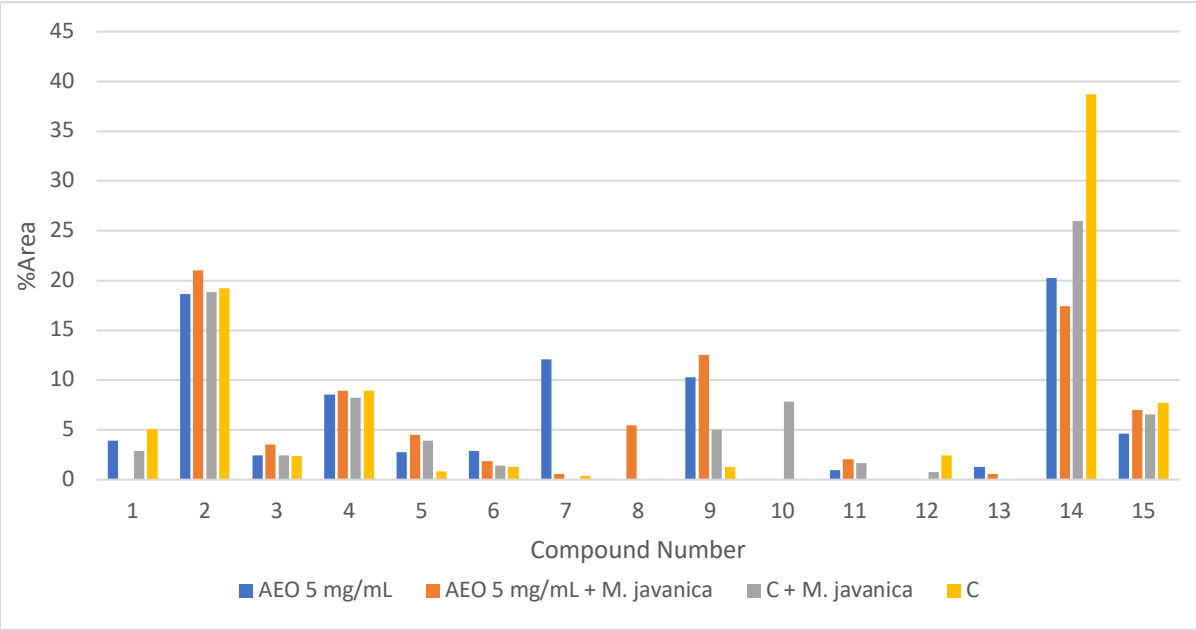


Figure 9. GC-MS identified compounds for the tomato methanolic extracts, reextracted with DCM, of the "nematode experiment".
1: Acrylic acid tetradecanyl ester; **2**: Neophytadiene; **3**: Dihydrophytol; **4**: Neophytadiene isomer 1; **5**: Palmitic acid; **6**: Ethyl linoleolate; **7**: Neophytadiene isomer 2; **8**: 9-Eicosyne; **9**: Ethyl linoleolate; **10**: Phytol isomer; **11**: Palmitic acid; **12**: Dodecanamide; **13**: Hexadecanamide; **14**: Oleoamide; **15**: n-Hexatriacontane.
AEO: tomato plant of which the seed has been coated with *Artemisia absinthium* L. EO.
C: control tomato plant of which the seed has been coated with ethanol.

Regarding the HPLC-MS results, AEO+inoculation increased 1, 3, 4, 5, (8,) 9 and 10, with 9 (lycopene-related) being the major component. The caffeic acid-related compound 1 increased for AEO+inoculation (1.19 times) and decreased upon infection (1.27 times) or when AEO-treated (1.86 times). Compound 9 was increased 1.34 times by AEO+inoculation and the inoculation and the AEO-treatment decreased its content (1.29 times and 2.33 times, respectively). Lastly, palmitic acid (5) increased a little more when AEO+inoculation (2.30 times) than inoculation only (1.56 times) and was not detected when AEO-treated.

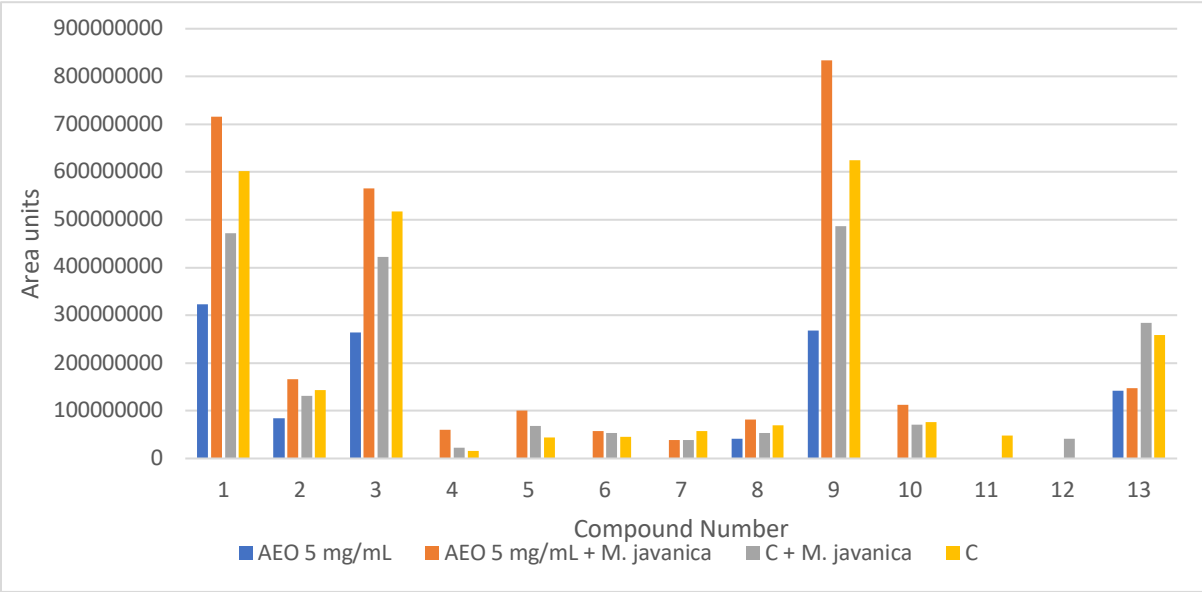


Figure 10. HPLC-MS identified compounds for the tomato methanolic extracts of the "nematode experiment".
1: Caffeic acid derived; **2**: unidentified; **3**: 4-hydroxybenzoic acid; **4**: unidentified; **5**: Chlorogenic Acid; **6**: unidentified; **7**: Rutin; **8**: unidentified; **9**: Lycopene-related; **10, 11, 12**: unidentified; **13**: Isorhamnetin-3-(4-O-rhamnosyl)rutinoside.
AEO: tomato plant of which the seed has been coated with *Artemisia absinthium* L. EO.
C: control tomato plant of which the seed has been coated with ethanol.

4. Discussion

4.1 In vitro assays

The results of the *in vitro* assays showed that the studied essential oil i.e., *Artemisia absinthium* var. *candial* EO (AEO), had no nematicidal effect on *Meloidogyne javanica*, had a low activity against *Myzus persicae*, was not phytotoxic to pepper and that the phytotoxicity to tomato started at a concentration of 5 mg/mL AEO. Nonetheless, the *in vivo* experiments conducted on tomato, showed that even treated at a concentration of 5 mg/mL with AEO, tomato seeds germinated at the same rate and resulted in plants as healthy (visual observation) as the ones treated with lower concentrations of AEO, water and ethanol. As already mentioned in the introduction, AEO had already been studied for these properties and comparable results were reported. Julio et al. (2015) obtained similar results to Bailen et al. (2013) and observed a moderate to low antifeedant effect of both AEO extracted by hydrodistillation and vapor pressure and no phytotoxicity to *Lactuca sativa* and *Lollium perenne*[72,74,75]. Though Julio et al. (2015) mentioned a moderate growth inhibition of *L. perenne*. Biological activities of the acetone extracts, hydrolate and supercritical extracts were reported but their composition was different from the EO of the plant[77,84]. The activity of the crude acetone extract against *M. persicae* was attributed to sequiterpene lactone hydroxypelenolide and casticin which were not reported in AEO GC-MS chemical profile[84]. The supercritical extract was an effective antifeedant and moderately phytotoxic to monocotyledons[77]. In 2017, the nematicidal activity of AEO against *M. javanica* was also ruled out[72] but the activity of its hydrolyte was demonstrated by Julio et al. and could be attributed to (5Z)-2,6-dimethylocta-5,7-diene-2,3-diol which is one of its major components (31.2 %)[78].

4.2 In vivo experiments with Myzus persicae

During the *in vivo* experiment assessing the priming effect of AEO on *Capsicum annuum* L. against *Myzus persicae*, no preference for, and no significant difference in death on treated and non-treated plants were observed. Yet, a surprising result was the significant difference in birth. Indeed, as represented in figure 4 and table 7, the number of newborn nymphs was more important in the AEO treated plants than in the control (85.23 ± 13.46 % more births than in the control, in average for the 7 days). Yet, when a product is tested for its potential efficiency against aphids, the usual results expected from this kind of experiments, when the product is active, is a repellent effect, an increased number of death and a reduction of reproduction[85]. In their article of 2018, Kang et al. concluded to the efficiency of β-ocimene against *M. persicae* after observing, among other, an inhibition of reproduction and a preference for non-treated plants[86]. Fingu-Mabola et al. (2021) also observed an increased death and a reduction of fecundity, when studying the impact of endophytism of entomopathogenic fungi[87]. Similar observations were made by Javed et al. (2021) and Dardouri et al. (2020) when studying respectively proteins elicitors[88] and plant-derived VOCs[89] against *M. persicae*. Hence, to explain the increase in reproduction observed during the present experiment, three hypotheses were formulated.

Firstly, it was considered that it could be a defense mechanism of the aphid to maintain the species, in stress conditions. However, a more common behaviour, which is observed in case of stress such as exposure to a predator[90] or bad nutritional quality of the host plant[91], is the generation of a greater proportion of winged offspring, along with a lower reproduction.

Secondly, in the event of hormesis, an increase in descendants' production has been observed. According to Rix and Cutler (2022), hormesis can result from a wide range of stress i.e., chemical, biological and abiotic stresses in mild amounts, and impact reproductive behavior, among others[92]. Nonetheless, records of hormesis of aphids found in literature involve sublethal exposure to pesticides[93,94]. Besides, in their paper, Sial et al. (2018) refer to "continuous asymmetric" exposures to hormetic concentrations[93]. Therefore, these results might not be comparable to the ones collected during the "no choice" experiment and more extensive data about the aphids' behaviour would be needed.

The last explored scenario is that the treatment applied to the plants changed their composition and/or had a phytostimulating effect that consequently made them a better food source for aphids. Some studies indicate that the fertilization of the soil with some nutrients or beneficial microorganisms can improve plants health but is not always accompanied with reinforced immune defenses, they might favor pests such as aphids[95,96]. Furthermore, plants with improved composition would supposedly be more attractive to insects but in the choice experiment, no preference was observed for the treated plants.

Despite the lack of evidence to prove any of these speculations, the comparison of the weights of the different parts of the pepper provides additional arguments in favor of the growth-promoting action of the AEO priming. Indeed, besides the visual observation that the treated peppers were bigger, the weights of the aerials of the treated peppers of the "no choice" experiment were significatively higher than of the non-treated controls. This would be an innovative subject of investigation as no research on the plant-growth-promoting effect of essential oils has been found in the scientific literature.

Nevertheless, among the identified compounds detected by GC- and HPLC-MS, none were reported as growth-promoting in literature. Soudani et al. (2022), who also studied the priming effect of AEO, detected coumarin and vanillic acid in the primed tomato plants which have not been detected in the peppers, neither in the tomatoes of the present research project. The only compound that could have been involved in such an effect, that was present in higher proportions in AEO-treated pepper solvent extracts, is squalene which modulates campesterol, among other sterols[97], a precursor of an important phytohormone for growth and development[98].

Regarding the differences in yield of extraction and the fact that the aerials weight of infected peppers was higher compared to uninfected ones remain unexplained. It suggests that the combination of AEO seed treatment and infection by *M. persicae* triggered a singular response in the peppers. Anyhow, these experiments should be repeated with more replicates to confirm the validity of this information.

Finally, among the compounds identified by chromatography coupled to mass spectrometry and exhibiting differences between treated/non-treated and infected/non-infected plants, the function of neophytadiene (2, GC-MS), oleoamide (10, GC-MS), squalene (12, GC-MS), caffeic acid derivatives (1-4, HPLC-MS) and luteolin C-pentosyl-C-hexoside (7, HPLC-MS) will be discussed below.

Neophytadiene (2, GC-MS) is a volatile terpenoid linked to the JA pathway[99,100]. It has been reported that its synthesis was triggered by salt stress[99] and viruliferous whitefly infestation[100] in tomato plants. Shi et al. (2019) observed that the content in neophytadiene was negatively correlated with the JA content and therefore, as the neophytadiene increased upon attack by viruliferous whiteflies, the plants became more attractive for other whiteflies which prefer lower JA levels[100]. Moreover, Rachidi et al. (2021) and El Arroussi et al. (2018) noted the induction of neophytadiene production in tomato plants treated with a microalgae polysaccharide[99,101]. This observation matches the presence of a higher content of neophytadiene in the AEO-treated peppers and the infected control but on the

contrary, is not in line with the lower neophytadiene level in the treated plants infected with *M. persicae*. Besides, the aphids did not exhibit any preference for the AEO-treated peppers in the choice experiment (composition assumed to be similar to the "no choice" experiment), which would suggest that either, unlike whiteflies, they are not sensitive to that compound, that its level was under their limit of detection or that it was synthesized inside the plant but not emitted (as a reminder, the analyzed samples come from methanolic extracts of plant aerials, re-extracted with DCM).

Oleoamide (10, GC-MS) is an oleic acid amide[102,103]. It has demonstrated inhibitory activity against some bacteria such as *Microcystis aeruginosa*[103]. Its concentration was higher in tomatoes (fruit) of improved quality parameters via exposition to LED lighting according to Gil et al. (2020)[102]. Furthermore, Celeste Dias et al. (2018) suggested that oleamide might have a defense role against UV-B_2[104]. Similarly, Soudani et al. (2022) observed in their study of the priming effect of AEO seed treatment against *Fusarium oxysporum* (*Fol*) that the oleoamide content was increased by the combination of AEO-treatment and *Fol* infection[79]. This corroborates the observation made by comparing the oleoamide content of the AEO-treated and AEO-treated and infected peppers.

Though the triterpene squalene (12, GC-MS) represented a smaller proportion of the analyzed pepper extracts, it is interesting to mention its higher content in AEO-treated plants because of its role in plant adaptation against biotic stresses[105]. Increased levels of this compound have been detected in SA-treated rose plants, which might be responsible for a better resistance to the aphid *Macrosiphum rosae*[106]. Salinas-Sánchez et al. (2021), in turn, suspected squalene to be partly responsible for *Ricinus communis* seeds hexanoic extracts' activity against the aphid *Melanaphis sacchari*, as it is one of its major components[107]. Besides, squalene is involved in sterols synthesis which are required by insects for their growth, the synthesis of signaling molecules or hormones such as ecdysone (molting) and as membrane inserts, as they are unable to synthesize them themselves[108–110]. Some derivatives from squalene, on the contrary might negatively impact insects' growth, molting and reproduction, depending on the species and therefore the dietary sterol profile of the plants is important[110]. For example, Toyofuku et al. (2021) mentioned the antagonist activities of a steroid (cucurbitacin B) on the molting hormone, ecdysone[111]. Chen et al. (2022) highlighted the importance of the ingested sterols mixture for growth, survival, and reproduction of diamondback moth caterpillars[112] and in their review, Behmer (2017) cited some studies which observed the influence of the ingested sterols on aphids reproduction[109].

Compounds 1, 2, 3 and 4 detected by HPLC-MS were identified as caffeic acid derivatives. Caffeic acid is a phenolic compound with antioxidant properties[113] and its derivatives present a variety of bioactivities[114]. It is also a precursor of lignin which reinforces plants cell-wall[114]. These compounds might therefore contribute to plants' resistance to biotic and abiotic stresses[114]. Its increase upon aphid infection in pepper plants has been observed by Florencio-Ortiz et al. (2021)[113] which is in contradiction with the observations made in the present work. Furthermore, Zhou et al. (2021) also observed an increase in caffeic acid in nanoselenium-induced resistant wheat plants upon aphid infection[115] and Zhang et al. (2017) in whitefly-infected tobacco plants showing resistance to *M. persicae*[116]. Nevertheless, Florencio-Ortiz et al. (2021) also noticed the reduction in some phenolic acids in time (from two days post infection onwards), which, according to them, might be the result of their consumption for the synthesis of other defense compounds or of a counter-defense strategy of the aphid[113]. This last observation could explain the decrease in caffeic acid derivates in the AEO-treated and infected plants as the extraction was performed seven days after infection.

Luteolin derivatives involvement in resistance against fungal infection has been highlighted by several studies cited in the paper of Mikulic-Petkovsek et al. (2013) who noticed an increase in this component in peppers upon infection by *Colletotrichum coccodes*[117]. This could be correlated with the slight

increase in luteolin C-pentosyl-C-hexoside (7, HPLC-MS) in the infected control compared to the non-infected control but cannot explain why none was detected in the AEO-treated infected pepper in this experiment. Besides, luteolin has shown detrimental effect on the aphid *Acyrthosiphon pisum* which suggests that if the AEO-treatment had induced a response against *M. persicae*, this compound would have increased[118].

Regarding ambrettolide (6, GC-MS), methyl 4,8,12-trimethyltridecanoate (5, GC-MS), methyl-11,14,17-eicosatrienoate (7, GC-MS) and isorhamnetin-3-(4-O-rhamnosyl)rutinoside (9, HPLC-MS), no relevant information could be found in literature for the study of priming.

A last observation, worth mentioning considering the results of this experiment, was made by Florencio-Ortiz et al. (2021). They observed that the plant response to aphids' infestation was dependent on the density of aphids: it was less significant in their experiment with 20 aphids per plant than with 200 aphids per plant[113].

4.3 In vivo experiment with Meloidogyne javanica

No effect of the seed-priming has been observed while inspecting the tomato roots. This would indicate that the treatment did not work as usually the resistance mechanism developed by the plant is supposed to prevent the permanent settling of the nematodes[67], recognizable by the nodules formed on the roots during the establishment of their feeding site.

One reason that could explain why this experiment did not show conclusive results, in terms of biological observation, is that the treatment occurred at a very early stage (seed) compared to the moment of inoculation (28 days after planting). Indeed, while pursuing the same experiment to prove the priming effect of AEO seed-coating on tomato against *Fusarium oxyporum*, Soudani et al. (2022) observed that the callose deposition and the ROS production triggered by the treatment, lasted for at least 12 days but they did not perform any analysis at later stages[79]. Molinari et Leonetti (2019) observed the loss of the priming state of tomato induced by BCA against *Meloidogyne incognita* after 7 days[50]. In other study, testing the nematicidal effect of different kinds of treatment, the time laps between treatment and inoculation was usually shorter. For example, Liu et al. (2021) irrigated rice plants with their treatment (K_2SO_4) one day before inoculation[119]. Several studies focused on the resistance induced by AMF and PGPRs but in these experiments, the exposure of the plant to the priming agent lasts longer and even though, the inoculation is not performed as late as in the present experiment (5 to 10 days)[50,52,63]. Therefore, as it is not known how long the primed state can last, a possible explanation to the lack of effect against the nematodes is that the time laps between induced resistance and infection was too long.

Nonetheless, the chromatographic analysis revealed some differences in the metabolites present in the aerial parts between treated/non-treated and infected/non-infected tomato plants. This suggests that the treatment did trigger a response in the plants but maybe not strong enough to lead to an efficient resistance against the nematode attack.

A first observation was the induction of palmitic acid (5 and 11, GC-MS) synthesis in the AEO-treated plants and even higher in both inoculated treated and control plants. Noticing a stimulation of *de novo* lipid synthesis after biostimulation of tomato plants with microalgae or cyanobacteria crude bio-extracts, Mutale-Joan et al. (2020) and Farid et al. (2019) explained that palmitic acid was one of the first fatty acid synthesized for the reinforcement of the lipid bilayer of the cell membrane and the cuticular wax[101,120] in order to protect them from abiotic stress (such as drought stress for the tomato fruits[121]) but also pathogen invasion[120]. On another note, Dong et al. (2018) reported the repellant

effect of palmitic acid secreted in root exudates[122] and Zhang et al. (2012) its toxicity against the nematode *M. incognita*[123].

As explained in previous section, the neophytadiene (7, GC-MS) increase after biostimulation has already been observed[99,101] which is in line with the higher content observed in the AEO-treated extract. However, it was not possible to explain why it was present is such low proportions in the other extracts based on literature.

Few relevant information has been found about 9-eicosyne (8, GC-MS), which was only present in the AEO-treated and inoculated plants. Zhang et al. (2022) found non-significant differences in that compound, among others, between plants exposed to the mirid *Macrolophus pygmaeus* and control plants[124] and Kumar et al. (2018) mentioned its antimicrobial and cytotoxic properties[125]. Its absence in the control extracts could be, for example, explained by its degradation during the extraction process. Besides as its function in plant physiology is uncertain, 9-eicosyne cannot be used to demonstrate AEO treatment effect on tomatoes' immunity.

The AEO treatment seem to influence ethyl linoleate (9, GC-MS) production, which was present in higher proportions in these samples. Bolivar et al. (2020) indicated that this compound might be implicated in the activity of *Papaver rhoeas* against nematodes[126]. It has been detected in tomato fruits but its effect on animal and not on plant immunity were studied[127].

A phytol isomer (10, GC-MS) was only detected in the inoculated control but not in the inoculated AEO-treated tomato extracts. This could indicate that AEO-treatment conferred protection to the tomatoes: Zarandi et al. (2022) associated a high phytol content to chlorophyll degradation[106]. Nonetheless, they studied the influence of the aphid *Macrosiphum rosae* on rose plants, which might not be comparable to the reaction of tomato plants to a nematodes' infection. In their investigation of the biostimulating effect of microalgal and cyanobacterial crude extracts, Mutale-Joan et al. (2020), in turn associated an increased phytol content to the chlorophyll degradation caused by the metabolite extraction[120].

Oleoamide (14, GC-MS) has already been discussed in the previous section but in the tomato extracts, the trend in oleoamide proportion seem to be the opposite of what was observed for peppers and by Soudani et al. (2022): it was smaller in controls than in AEO-treated plants and decreased slightly upon infection.

Even if chlorogenic acid (5, HPLC-MS) was not detected in high proportions in the extracts, it will be discussed as it is a major soluble phenolic compound in *Solanaceae*[128]. It is an antioxidant that has shown antibacterial, fungicidal[128] and insecticidal properties by altering cell membrane permeability or being oxidized into toxic quinones[128,129]. Chlorogenic acid was absent from the AEO-treated but non-inoculated sample but was detected in higher amount in the AEO-treated and inoculated sample compared to the three others. Several studies support the induction of chlorogenic acid synthesis in resistant and inoculated plants but do not explain the fact that this compound was absent from the AEO-treated non-inoculated extract. An increase upon infection by a root-knot nematode was observed in the experiment of Patel et al. (2018), in a susceptible tomato cultivar but not in the resistant one[130]. Atkinson et al. (2011) made a similar observation but measured the chlorogenic content of the fruits of tomato plants infected by *M. incognita*[131]. *Pseudomonas putida*-induced resistant tomato plants against *Clavibacter michiganensis* also showed a higher content of chlorogenic acid in the leaves upon infection[131]. El-Sappah et al. (2022) detected higher levels of chlorogenic acid, among other resistance related compounds, in tomato genotypes resistant to *M. incognita*[132] and more chlorogenic acid was measured in the roots of an inoculated resistant pepper genotype than in the non-inoculated and inoculated susceptible genotype by Pegard et al. (2005)[133]. In 1971, Hung and

Rohde suggested that the increase of chlorogenic acid upon nematodes infection was a defense mechanism of the plant as its oxidation products, generated by an enzyme secreted by the nematodes or the polyphenol oxidase of the plant, inhibited nematodes' activity and altered their penetration and settling in the roots[134].

Rutin (7, HPLC-MS) did not represent a major part of the extracts, but it is noticeable that it was absent from the AEO-treated and non-inoculated tomato extract. This compound might only have been produced upon attack as it is alleged to be a signal molecule in plant defense[135] and Atkinson et al. (2011) reported significantly higher rutin content in late-harvested tomatoes (fruit) exposed to *M. incognita*[131] but it is then surprising that it is present in the non-inoculated control.

Besides the functions described in the previous section, caffeic acid has been reported to have adverse effect against nematodes by Afifah et al. (2019)[136]. The increase in the detected derivative of this compound (1, HPLC-MS) in AEO-treated plants upon infection by *M. javanica* is therefore consistent and it coincides with Florencio-Ortiz et al. (2021), Zhou et al. (2021) and Zhang et al. (2017) results, who observed a similar effect, albeit upon insects' infection[113,115,116]. Yet, according to the paper of Afifah et al. (2019) who studied nematode-resistant tomato genotypes[136], the caffeic acid content should have been higher in AEO-treated plants than in controls if AEO had induced resistance of the tomatoes. Although, it can be noted that Afifah et al. (2019) analyzed the roots metabolites and not the aerials of the plant and besides, that innate resistance might not engage the same metabolites as primed defense.

One of the most abundant compounds detected in all samples was identified as a lycopene derivative (9, HPLC-MS). It is not surprising to find this compound in tomato plants as this carotenoid is a pigment conferring their red color to tomatoes[137]. It is an antioxidant[137] and its antifungal activity against *Candida albicans* has been displayed by Choi and Lee (2015)[138]. It was present in higher amount in the AEO-treated and inoculated sample and on the contrary, it was lower in the inoculated control compared to the non-inoculated control. The induction of the same lycopene-related compound has been observed by Soudani et al. (2022) in AEO-treated tomatoes infected by *Fol*[79]. This observation along with the results obtained in the current study confirm the potential effect of AEO-treatment on the lycopene-related compound content of tomatoes' aerials. Increase in lycopene in tomato fruits has been observed when treated with $CuSO_4$ that could be attributed to oxidative stress[139] and when inoculated with the PGPR *Bacillus subtilis* CBR05[140] as well. Choudhary et al. (2013) observed an increase in lycopene in tomato genotypes resistant to *M. incognita* when inoculated and the reverse evolution for susceptible genotypes[141]. Besides, in the experiment of Singh et al. (2013), the lowest lycopene levels in ripe tomatoes were observed in the controls inoculated with *M. incognita*, higher contents were measured in the non-inoculated control and plants inoculated and treated with a nematicide, and lycopene was even more abundant in plants colonized by a nematode-trapping endophytic fungi (*Arthrobotrys oligospora*) and inoculated with *M. incognita*[142].

Finally, regarding isorhamnetin-3-(4-O-rhamnosyl)rutinoside (13, HPLC-MS), for which the differences between the control and the AEO-treated tomatoes were interesting, no relevant information has been found in literature and the trend was different from what was observed in the pepper extracts.

5. Conclusion and Perspectives

This work aimed at determining the seed-priming potential of the essential oil of *Artemisia absinthium* Linnaeus (var. *Candial*) (AEO) against the aphid *Myzus persicae*, on *Capsicum annuum* L. (var. *Teson* F1) and against the nematode *Meloidogyne javanica*, on *Solanum lycopersicum* L. (var. *Marmande*).

No effect against *M. javanica* was visually observed but surprising results were obtained regarding the reproduction of *M. persicae* and plant growth. These last biological observations cannot be allocated to priming as they could be related to a growth-stimulation effect, for instance. Besides, the metabolomic analyses (HPLC-MS and GC-MS) revealed, for both experiments, that the AEO seed-coating did trigger a response of the plants. Yet, it is not possible at this stage, without complementary analyses, to state that they reflect a primed state of the plants. Indeed, the variations observed in the metabolites' content are difficult to explain and link with plant immune responses.

Overall, this first investigation provided encouraging results to pursue further pioneering research on this novel subject of priming by an EO, and especially with the essential oil of *Artemisia absinthium* L. of the registered trademark *Candial* variety, which, interestingly compared to most studied EOs for agronomic purposes, has no direct biological activity, neither as herbicide, biopesticide nor repellent.

In the future, it would be interesting to repeat this experiment and gather additional data on aphids' behavior after exposure to the treated plants and on the influence of their density, on the transcriptomic changes by RNA-sequencing to prove the theory of a priming effect and, on the metabolomics of the treated plants to confirm the present observations and detect suspected growth-promoting components. To find out if some active VOCs are resulting from AEO-primed defense, SPME analyses or dynamic headspace trapping of compounds emitted outside the plants could be intended. Performing analyses at earlier growth-stages of the plants and during the infection at several times, would be useful as well, in order to determine the duration of the potential primed-state and optimize the time span between priming of defence and infection.

To induce a stronger and effective immune response of the plants, priming at a later growth-stage should be considered as the infection by aphids and nematodes occurs later than with *Fusarium oxysporum*. Some clues to put this into practice would be treating the soil (the vermiculite) with the AEO, irrigating the soil or spraying the aerial parts with AEO at a later growth-stage or even tuning the newly emerged slow-release coating technologies to control the time of release of the AEO. This last option would be very promising as the initial idea of selling already treated seeds, which is very attractive on the market, would still be feasible. Finally, the combination of this priming agent with another biocidal essential oil could also be explored, converging with the research on synergy between EOs.

Personal contribution

After my promotor defined the main guidelines of my master's thesis with my supervisor at the ICA, Dr. GONZALEZ-COLOMA, I conducted bibliographic research to deepen my knowledge on the subject, I carried out the experiments agreed upon with my supervisor and described in this work, treated the results and discussed them, based on the scientific literature, always following my promotor's and supervisor's instructions and advice. My good integration at the ICA (receiving a student from GxABT for the first time), my collaboration with its team during my practical work and my commitment to the task that was assigned to me, allowed to support a good relationship with the ICA, for the future.

6. APPENDIX – Detailed Protocols

6.1 Phytotoxicity Assay

Treatments:

- Positive control (100% Distilled Ethanol, as used in the samples to be evaluated)
- Treatments to evaluate: 10.0, 5.0, 1.0, 0.5 mg/mL EO solution (dilution in EtOH)

Repetitions: 4 per treatment.

Conditioning of the wells plate: wrap the box with plastic film and place them in the plant growth chamber.

Preparation of the seeds:

Weigh 10 seeds and multiply that amount by the number of total replicates.

Soak the seeds (10 per repetition) in distilled water 24 hours before the test (less time for tomato seeds was enough, about 6 hours but more time for the pepper seeds was necessary to boost the germination, about 1 week).

Timing:

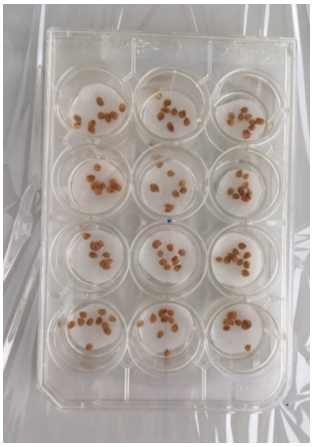
Solanum lycopersicum (dicotyledoneous): start test on Fridays since the first 2-3 days there is no germination.

Capsicum annuum: according to observations of the germination in distilled water, the germination started about 1 week in the plates and 21 days were necessary to obtain sufficient germination and radicle length.

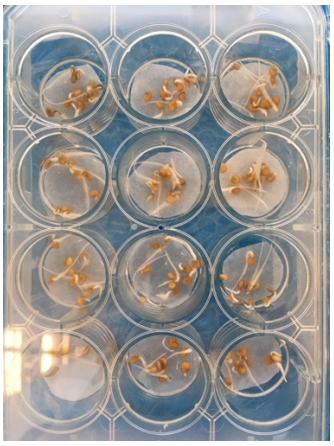
Process:

1. The treatments are applied to 2 cm diameter filter paper disks placed on aluminum foil. Apply 20 µL of solvent (control) or test solution, taking care that the entire surface of the disc is moistened. Allow the solvent to evaporate.
2. Prepare the 12-wells-plate (2 cm diameter), identify the treatments with a label, place the treated disks and the seeds accordingly.
3. Add 500 µL of distilled water to each well, seal the plate and place it in the growth chamber.
4. Control germination daily (number of germinated seeds).
5. On day 6 for tomato and 21 for pepper (counting starts the first day of germination), take 25 seedlings randomly selected from each treatment and measure the radicle length with ImageJ.

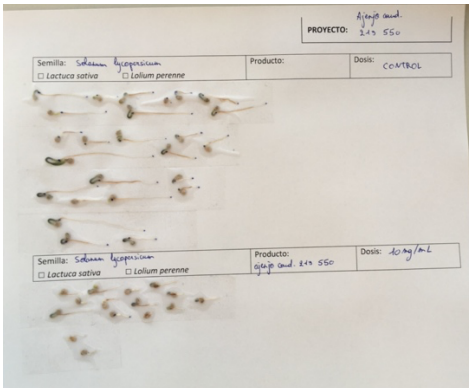
In ImageJ put the distance of pixels: 118 is equal to 1 cm (https://imagej.nih.gov/ij/download.html).



Appendix 1. Phytotoxicity test (tomato) on day 1.



Appendix 2. Phytotoxicity test (tomato) on day 6.

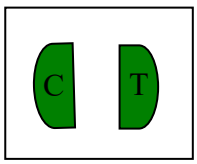


Appendix 3. Phytotoxicity test (tomato) on day 6 - radicle length measurement.

6.2 In vitro antifeedant assay

1. Prepare 20 boxes per treatment (each box: 3 x 3 x 1.5 cm, closed and aerated through a net).
2. Prepare the agar at a 10 g/L concentration. Heat up the mix until it boils consistently and pour it into de lids of the boxes, taking care to fill them completely, to prevent the aphids from escaping through the corners.
3. Before the agar cools down, place the plant material (pepper leaves for *Myzus persicae,* cut in disks of 1.0 cm^2 and then in half) in the box, avoiding contact between the control and the test leaves.
4. In each of the boxes, spread 10 μL control on one leaf (solvent of preference when preparing the samples) and 10 μL treatment on the other leaf (the initial concentration for the treatment is 10 μg/μL for plant extracts and will be reduced if the sample is active).
5. Once applied, add 10 aphids per box.
6. Incubate for 24 h (overnight).

   Count how many aphids have settled on the control and on the treated leaf, ignoring the ones on which the total amount of settled aphids is below five.



Appendix 4. Scheme of the box.

Appendix 5. Lid of the box with sample and aphids, the day after preparation.

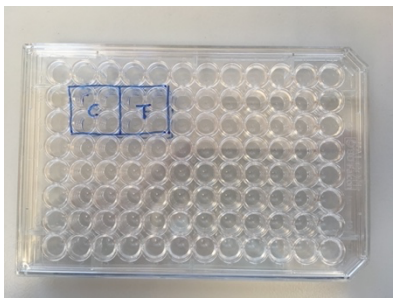
6.3 *In vitro nematicidal assay*

1. Prepare a stock solution of your sample at a concentration of 5 mg/mL in DMSO + 0.6 % Tween 20.
2. Place J2 *Meloidogyne javanica* in a berlin with a magnetic stirrer to homogenize the inoculum, adding distilled water to achieve a concentration of about 100 nematodes/100 µL aqueous solution.
3. Use sterile plates for cellular cultures with 96 wells and a U-shaped bottom and make 4 repetitions per treatment.
4. Distributing the treatments in groups of 4 (cf. appendix 6), in each well put:

- 95 µL solution with nematodes,
- 5 µL essential oil stock solution or DMSO + 0.6 % Tween 20 for the control.

=> final concentration of 1µg/µL per well.

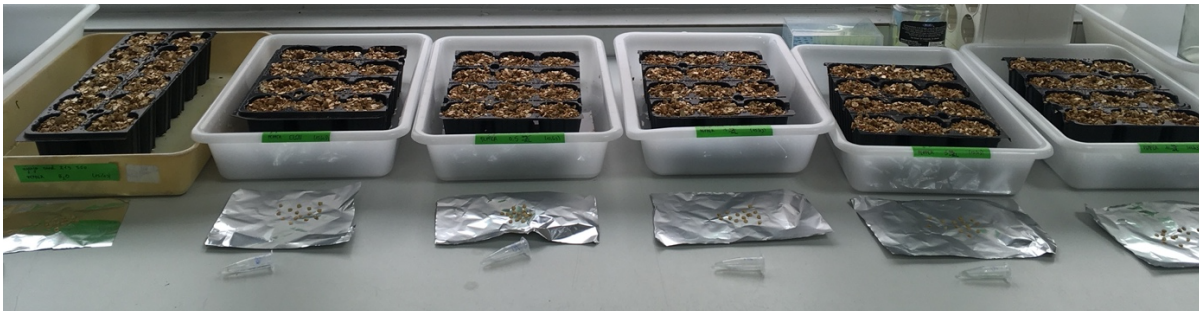
5. In the external columns, around the treatments, fill the wells with distilled water to avoid desiccation and border effect.
6. Sealing of the plate with parafilm.
7. Wrap the plate in aluminum foil to protect from light.
8. Place it in growth chamber (25 ± 1 °C, HR >70%, L:D 16:8 h) for 72 hours (also 24 and 48 h if the sample is active).
9. After the incubation time, count the number of dead and living nematodes under an electronic microscope.
10. Report the results in percentage of death J2, corrected with the Schneider-Orelli formula.
11. If the sample is active, start over lowering the concentration.
12. The LC_{50} and LC_{90} (efficient lethal dosis) are calculated by a Probit analysis on Statgraphics Centurion.



Appendix 6. 96-wells plate used for the *in vitro* nematicidal activity assay.

6.4 Infection tests

6.4.1 Seed-coating and plant growing



Appendix 7. Seed-coating and planting illustration.

Appendix 8. 18-days-old tomato plants in jiffy® pots filled with vermiculite.

Appendix 9. Tomato plants transplanted in quartz sand pots, 24 days after planting.

Appendix 10. 37-days-old pepper plants in jiffy® pots filled with vermiculite – seed-coated with 10 mg/mL AEO.

Appendix 11. 37-days-old pepper plants in jiffy® pots filled with vermiculite – seed coated with EtOH.

6.4.2 Aphids synchronization

Preliminary test to evaluate the time and number of adults required for the synchronization preceding the aphid infection test:

- In a 14 cm diameter petri dish, place a humidified filter paper (Whatman n°1).
- Place a fleshly cut tomato branch with a few leaves in an Eppendorf filled with water and put them on the petri dish.
- Introduce a noun number of adult aphids and place the petri dish for 24-48 h in the growth chamber (monitor every day).
- Remove the adults and leave the nymphs to grow for a few days before the experiment.

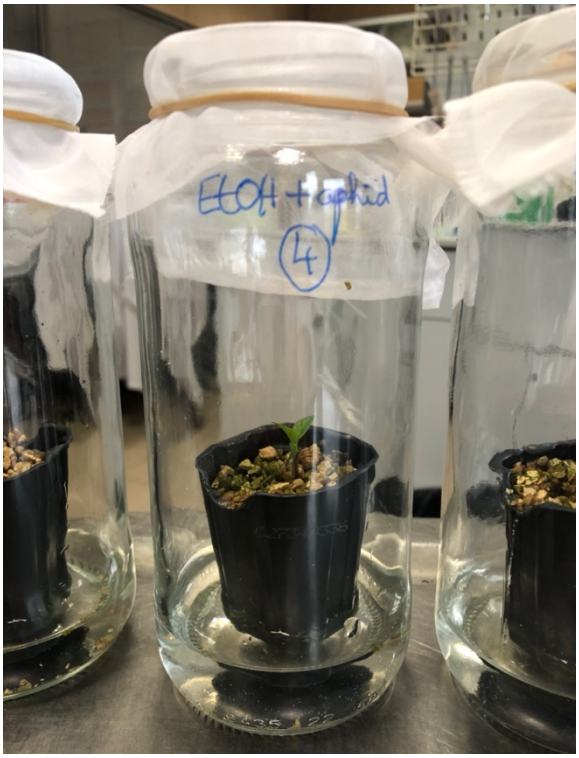
Results (the most limiting): 100 aphids gave birth to minimum 25 nymphs, in 24 hours and the newborn nymphs started reproducing after maximum 8 days.

The actual synchronisation followed the same steps as here above but the aphids were placed on a whole plant, instead of a leaf in a petri dish, as 240 synchronized aphids were required for the “no choice” experiment and 1.5 times more adults were used initially to make sure to obtain enough synchronized nymphs. The nymphs were 5-6 days old on the first day of the “no choice” experiment and were monitored every day until then to make sure they did not start reproducing (which they did not).

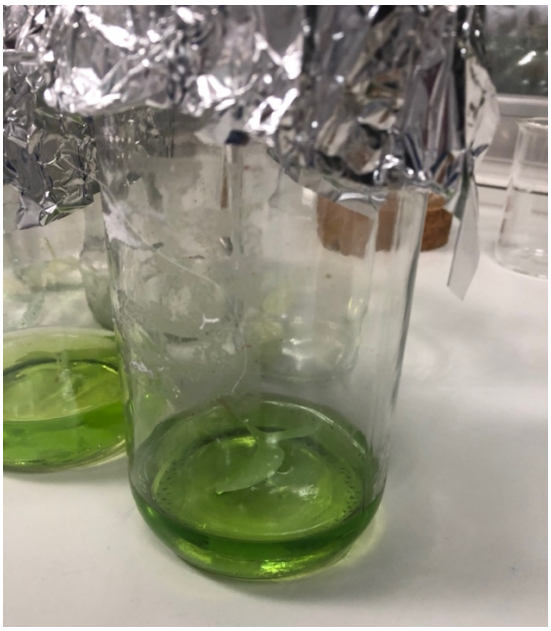
Appendix 12. Mock synchronisation test of *M. persicae*.

Appendix 13. Synchronisation of *M. persicae* for the “no choice” experiment – time 0.

6.4.3 Aphids "no choice" experiment – set-up



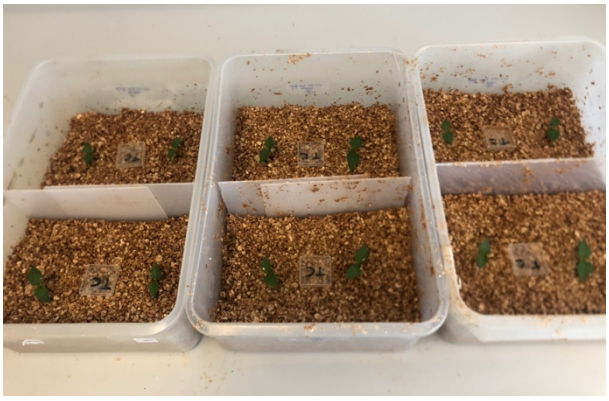
Appendix 14. Isolated two-leaves stage pepper plant + 20 synchronized *M. persicae*.



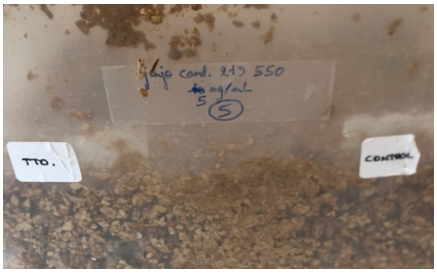
Appendix 15. Aerial part of the pepper plants macerating in methanol, after the experiment, for the metabolites' extraction.

6.4.4 Aphids choice experiment – set-up

Appendix 16. Treated and control two-leaves stage pepper plants + 20 *M. persicae* in between.



Appendix 17. The six repetitions of a choice experiment.



Appendix 18. Example of labelling of the boxes used for the choice experiment.

Appendix 19. Sealed box ready to be placed in the growth chamber overnight.

6.4.5 Nematodes experiment – roots NI

Appendix 20. Root of one of the six replicates of the non-inoculated tomato plants (negative control).

Appendix 21. Root of one of the six positive control, after 1 month inoculation with *M. javanica*.

Appendix 22. Tomato roots of which the seeds were coated with 5 mg/mL AEO, after 1 month inoculation with *M. javanica*.

Appendix 23. One of the six replicates of the tomatoes of which the seeds were coated with 5 mg/mL AEO, 1 month after *M. javanica* inoculation.

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