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TITLE OF THE PHD THESIS

Exploration of New Oxidative Stress Nematicidal Compounds and Valorization of *Satureja montana* L. Essential Oils and Hydrolates as Plant Biopesticides

Scientific Disciplinary Sector

BIO15

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ABSTRACT

Plant diseases cause economic challenges because they are responsible for estimated pre- and postharvest losses of 16–28% of crops yearly. Control is aimed at the use of chemical protectants, which reduce or retard the growth of the pathogen population. Another option is the use of resistant varieties, which reduce the pest population or increase recovery from injury caused by the enemies. However, resistant varieties become susceptible after few years of cultivation due to pathogen adaptation and evolution to cultivated varieties.

Research of new environmentally benign products active against pests and diseases are required that control target organisms without harming the environment. In this thesis, I evaluated for the first time the nematicidal activity of new synthesized maleimide derivatives by structure-activity relationship (SAR), some selected haloacetophenones and transition metal ions. I also developed a new method to assess the metabolome alteration induced by these products on nematodes. Finally, I valorized a carvacrol chemotype domesticated plant of *S. montana* by evaluating its activity on insects, *Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*; and on the root-knot nematode *Meloidogyne javanica*.

The maleimide derivatives were easily synthesized in a one pot reaction; haloacetophenones were commercially available and metal ions present as sulphate or nitrate salts. The tested compounds showed strong nematicidal activity against *Meloidogyne incognita*, *X. index* and *G. pallida* with EC_{50} values lower than 5 mg/L. I also found a synergism action between maleimide and copper ion on one hand and between copper ions and tannins on the other hand. A GC-MS metabolomics analysis showed that these compounds might induce oxidative stress in nematodes by modifying the levels of fatty acids and acylglycerols. After a field experiment and phytotoxicity assays, these first reported nematicidal compounds could be used in crop protection against nematodes.

S. montana essential oil (EO) chemical composition was influenced by the fertilizers applied and the EO extraction method. Hence, the conventional agriculture plants showed a lower level of p-cymene with a higher level of carvacrol while more than 20 compounds showed significantly difference levels according to the method of extraction, laboratory or semi-industrial. The EOs and the organic phase of hydrolates with LC_{50} values of 20-65 µg/cm^2 modified the feeding behavior of *Spodoptera. littoralis*. *S. montana* EOs repel *Rhopalosiphon. padi* and *Myzus. persicae* with settlement inhibition LC_{50} values estimated at 25-60 µg/cm^2. The plant also inhibited the germination and growth of *Lolium perenne* at 10 mg/mL. Furthermore, ethanol extracts of *S. montana* did not show any activity on the pests. However, we successfully valorized the EO by product hydrolates on the insects and nematode.

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CHAPTER 1

INTRODUCTION

1.1. Plant parasitic nematodes: root-knot nematodes

Nematodes are members of the phylum Nematoda (round worms) and were found to have diverged from the lineage leading to arthropods and chordates at 1177 million years ago, making them one of the most ancient and diverse types of animals on earth.[1] They are thought to have evolved from simple animals some 400 million years before the "Cambrian explosion" of invertebrates able to be fossilized. The two nematode classes, the Chromadorea and Enoplea, have diverged so long ago, over 550 million years, that it is difficult to know accurately the age of the two lineages of the phylum.[2]

Nematodes are extremely abundant and diverse animals: exceeded only by insects. Most nematodes are free-living and feed on bacteria, fungi, protozoans and other nematode (40% of the described species); many are parasites of animals invertebrates and vertebrates (44% of the described species) and plants (15% of the described species). Nematodes were noted early in human history because some serious human diseases are caused by relatively large vertebrate-parasitic nematodes. Some of these nematodes were first described in the ancient Chinese scientific literature as early as 2700 B.C.[3] Another available record is that of Guinea worm (*Dracunculus medinensis*) in 'Ebers Papyrus,' written in Egypt 3,500 years ago. Hippocrates (460-375 B.C.), Aristotle (384-322 B.C.), Magnus (1193-1280 A.D.) also gave references of roundworms in their writings.

Borellus discovered the first free-living nematode *Turbatrix aceti* (vinegar eelworm) in 1656.

Since plant-parasitic nematodes (PPNs) often are small and subterranean, there are not many ancient references to phytoparasitic nematodes. The first report of a PPN being described dates back to 235 BC when an ancient Chinese symbol, resembling the shape of a typical adult-female cyst nematode, was made from an organism found on soybean roots. Needham (1743) was the first to observe a plant parasitic nematode, *Anguina tritici* in the cockles of wheat when

he examined a portion of the crushed cockle under his primitive microscope.[4] Berkeley (1855)[5] later identified root-knot nematodes on cucumber and Schacht (1859),[6] cyst nematodes causing "beet-tired" disease on sugar beets. To date, different PPNs are reported from different genera [7]. Our work tackled parasite nematodes from three different genera: *Meloidogyne* (root-knot nematode), *Xiphinema* (dagger nematode) and *Globodera* (cyst nematode).

Root-knot nematodes (RKNs) are members of the genus *Meloidogyne* (Göldi, 1892). *Meloidogyne* is of Greek origin and means 'apple-shaped female'. They are an economically important polyphagous group of highly adapted obligate plant parasites, distributed worldwide and parasitize nearly every species of higher plant. Typically they reproduce and feed on modified living plant cells within plant roots, where they induce small to large galls or root-knots, hence their vernacular name. The above-ground symptoms are not readily apparent and may be similar to those produced on any plants having a damaged and malfunctioning root system. Hosts may be heavily infected without showing external symptoms on the harvested products, e.g., symptomless potato tubers. The rapid rate of development and reproduction on good hosts results, in the majority of species, in several generations during one cropping season, leading to severe crop damage. Damage may consist of various degrees of stunting, lack of vigour, and wilting under moisture stress. Secondary infection by other pathogens often results in extensive decay of nematode-infected tissues. The common explanation for these above-ground symptoms is that *Meloidogyne* infection affects water and nutrient uptake and upward translocation by the root system. By disrupting the hostplant physiology, RKNs may not only reduce crop yield but also product quality (e.g. of potatoes and carrots) and therefore are of great economic and social importance.

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Root-knot nematode life cycle

The root-knot nematode life cycle is summarized in **Figure 1**. Females lay eggs into gelatinous masses composed of a glycoprotein matrix, which is produced by rectal glands in the female, keeps the eggs together and protects them against environmental extremes and predation. The egg masses are usually found on the surface of galled roots, although they may also be embedded within the gall tissue. The egg mass is initially soft, sticky and hyaline but becomes firmer and dark brown with age. Within the egg, embryogenesis proceeds to the first-stage juvenile, which moults to the infective second stage (J2). Hatch of the J2 is primarily dependent on temperature and sufficient moisture, although other factors, including root diffusate and generation, modify the hatching response so that the J2s hatch when conditions are favorable for movement and host location. J2s are attracted to roots, and there is evidence that when both resistant and susceptible plant roots are present, the susceptible ones are more attractive. The invasive J2 commences feeding after it has invaded the root, usually behind the root tip, and moved through the root to initiate and develop a permanent feeding site. The root cortex is penetrated near the zone of differentiation, the J2 migrating predominantly intercellularly toward the root tip and then making a 180° turn back towards the zone of differentiation. The feeding of the J2 on protoxylem and protophloem cells induces these cells to differentiate into specialized nurse cells, which are called giant cells. Once inside the vascular cylinder, the J2s initiate a series of three to eight giant cells, where they feed for 3–8 weeks and greatly increase in size by swelling into a sausage-shaped juvenile with a characteristically spicate tail region. The swollen J2s rapidly molt into short-lived third- and fourth-stage juveniles. Fourth-stage juveniles that will develop into males become vermiform after the third molt, but juveniles destined to become females remain swollen. Both types of fourth-stage juveniles molt once more to become either a mature male or a female. The morphology of the J2 and the adult male and female (see **Figure 2**) for comparison of the general appearance of these stages are

described according to body system. The combined time for the J3 and J4 stages is much shorter than for the J2 or the adult, typically 4–6 days. J3 and J4 lack a functional stylet and do not feed. Males, when present, are vermiform and there is no evidence that they feed. The specialized feeding sites are remarkable for their complexity. They are greatly enlarged from typical phloem and xylem parenchyma, or cortical cells, with final cell volumes nearly 100-fold greater than normal root cells. The giant cells are functionally similar to syncytia induced by other plant-parasitic nematodes that have sedentary adult females, but are distinct in their development. They are functional transfer cells, based on morphology.[9]

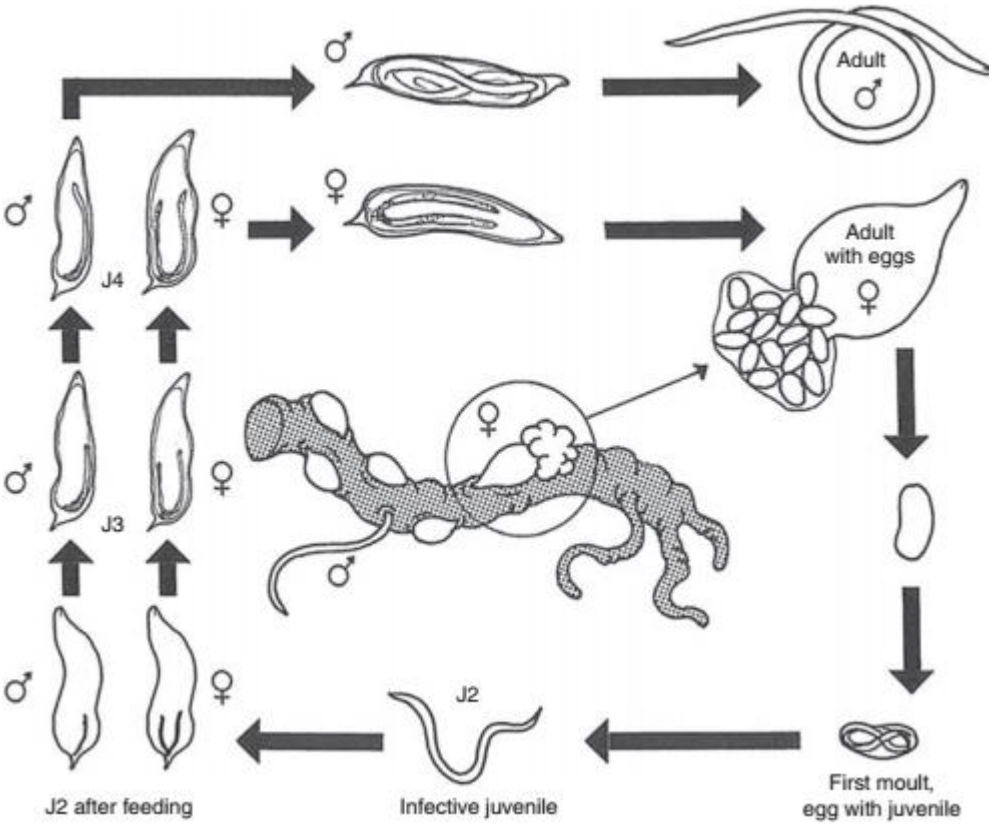


Figure 1: Diagram of the life cycle of the root-knot nematode, Meloidogyne. J2: second-stage juvenile; J3: third-stage juvenile; J4: fourth-stage juvenile. Figure reproduced from Moens et al. (2009). [8]

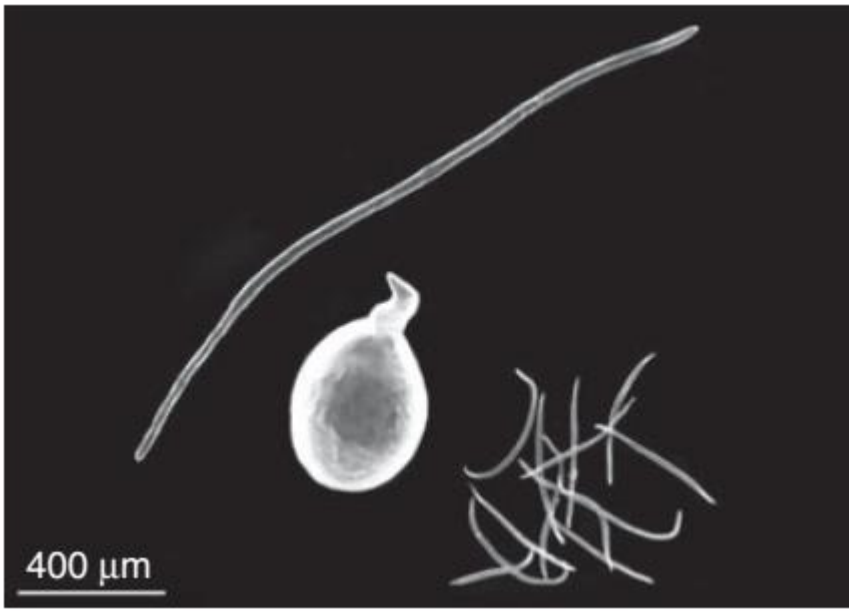


Figure 2: Scanning electron micrograph of root-knot nematode male (left), female (centre) and second-stage juveniles (right), showing general body shape and relative dimensions of the stages. Figure reproduced from Eisenback et al. (1991).[10]

Root-knot nematode general morphology

Near the end of the 19th century, recognition of the economic importance of root-knot nematodes stimulated several detailed morphological studies of these nematodes[11,12]. Although additional contributions on the morphology of all stages in the life cycle of RKN were made, the most significant study in this respect was by Chitwood (1949),[13] who revealed that the RKN comprised several different species, including *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949 and *M. hapla* Chitwood, 1949. Histological studies added important details to our understanding of the morphology of the root-knot nematodes, and, shortly thereafter, the use of the transmission electron microscope (TEM) greatly enhanced our knowledge of the fine structure of these nematodes, as well as the changes in morphology that occur during the onset of parasitism.[14] Additional observations on the external morphology of the various life stages were illuminated by the use of the scanning electron microscope (SEM). SEM of excised stylets

of the J2 revealed minute differences among the four best-known species (**Figure 3**).[15] The morphological details of RKN are important for the identification of species[13] and for identifying phylogenetic relationships. During the complex life cycle of the root-knot nematodes (**Figure 1**), the morphology changes from a one-celled zygote to vermiform males or swollen females.

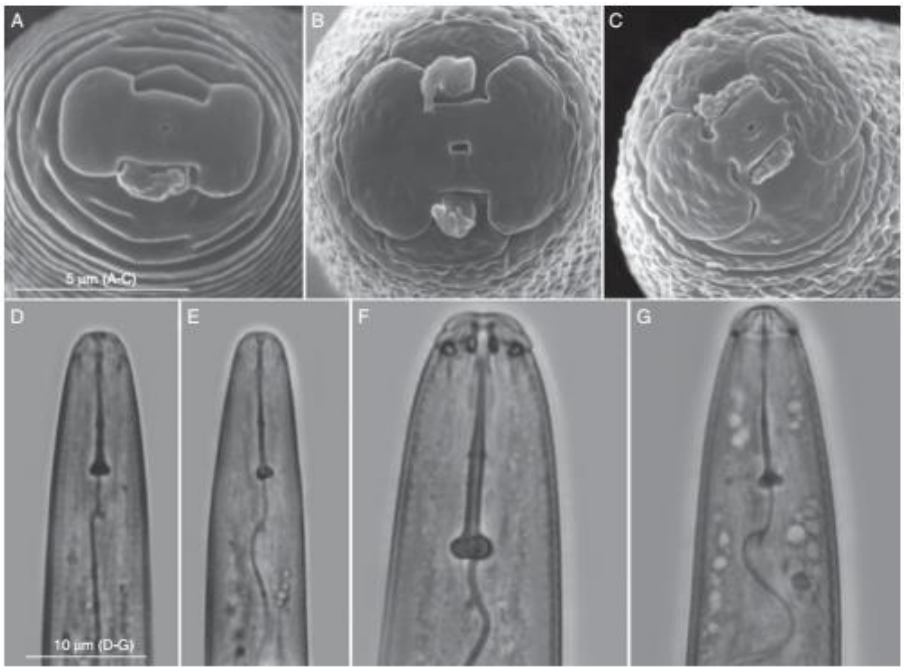


Figure 3: Micrographs of anterior end of second-stage juvenile root-knot nematode. Scanning electron micrographs. A: *M. incognita*; B: *M. brevicauda*; and C: *M. nataliae*. Light micrographs. D: *M. arenaria*; E: *M. hapla*; F: *M. brevicauda*; G: *M. nataliae*. Figure reproduced from Eisenback et al. (1988).[15]

Root-knot nematode damage

As they derive nutrients from tissues of plant organs, nematodes attacking plants are described as parasitic. The reaction of plants to a parasitic nematode differs greatly according to plant species and cultivar. Root-knot and many other parasitic nematodes are pathogens and cause disease that is expressed at histological, morphological, physiological and molecular levels, and which results in reduced growth, yield, lifespan and resistance to environmental stresses

of affected plants. Typical symptoms of nematode-incited disease include stunted growth, wilting, leaf discoloration (mostly yellowing) and deformation of plant organs. The degree of the pathogenicity of a nematode also depends upon its aggressiveness and the reaction of a plant species or cultivar. Crop damage from nematode-incited disease may consist of reduced quantity and/or quality of the yield (**Figure 4**). For instance, products from nematode-infested soil may have a larger unmarketable proportion because of small size or deformation. Also, sugar content of sugar beet tap roots from fields infested with *M. incognita* can be reduced, as is protein content of legumes in soil infested with *M. artiellia*.[16]

Plant species or cultivars that support nematode reproduction are referred to as susceptible. Among them, some may suffer greatly from nematode parasitism and are considered intolerant, while others will suffer much less and produce satisfactory yield, and are referred to as tolerant. Also, other plant species or cultivars may be infected by the nematode but then activate a defense mechanism that limits the nematode's development and reproduction; these are referred to as resistant. Different plant species or cultivars may express different degrees of resistance, susceptibility and tolerance.[16]

Information on the extent of damage a nematode may cause, especially the yield loss, is basic to implementation of the most appropriate control strategies. For example, to include a nematode in the list of quarantine organisms, the pathogenic potential of the nematode in a country must be demonstrated. Also, national policy makers will consider the impact a nematode may have on given crops and areas for their decisions. At farm level, information on the potential of the nematode populations to cause yield loss is a prerequisite to deciding if and when to apply a treatment and choosing the most appropriate control strategy.

Figure 4: Root-knot nematode disease damage on tomato plants. Figure reproduced from Reddy (2016).[17]

1.2. Control of nematodes

Nematode problems arise from contaminated soil or soil mixture used as a component of the growing medium, monocropping, and infested planting materials. Nematode management must be considered primarily as exclusion or avoidance. Once nematodes are introduced, it is difficult to manage them. Nematode management using physical approaches, host plant resistance, chemical nematicides, biological control, and integrated methods are discussed in this section.

1.2.1. Physical approaches

Because of the relatively low thermal sensitivities of nematodes and other soilborne pests (**Figure 5**), physical approaches which utilize heat have generally proved to be the most effective and widely used management strategies within greenhouses. On a relative temperature scale, nematodes and water molds would be considered relatively intolerant of high temperature, being effectively killed by 30-min exposure at temperatures as low as 120 °F.[18].

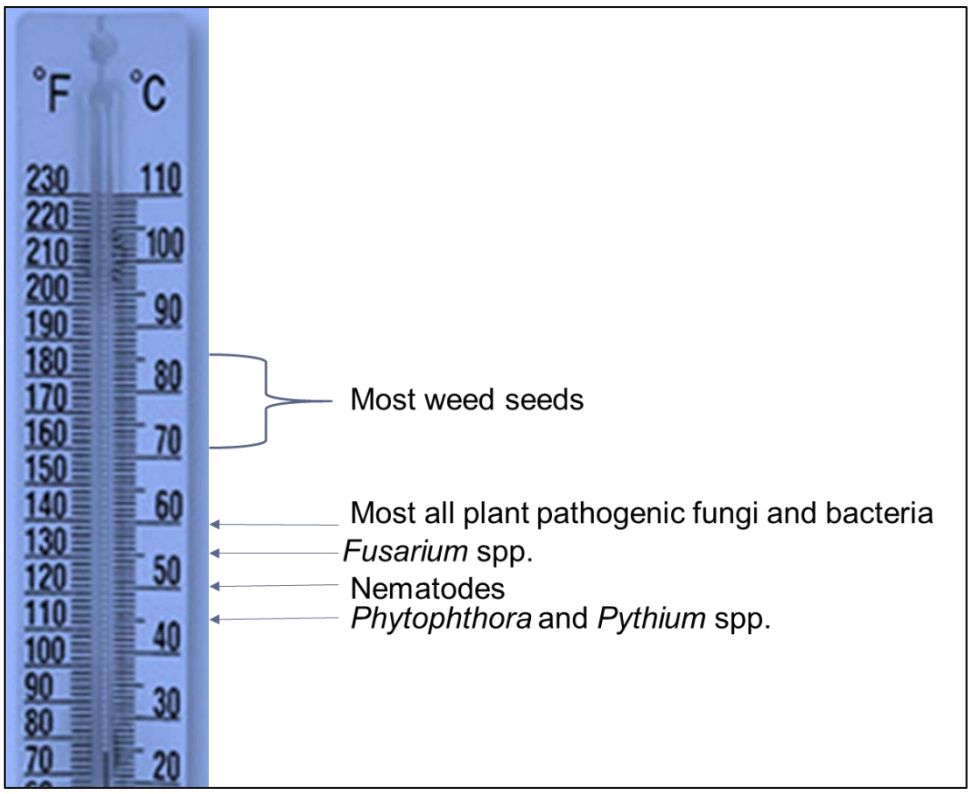


Figure 5: Generalized relationship of soil temperatures for 30-min exposures to kill various soilborne pests and pathogens.

Steam treatment

Steaming is the introduction of water vapor (>212 °F: 100 °C) into soil to elevate soil temperatures to lethal levels (160–180 °F) to soilborne pests including nematodes. Various methods have been developed to deliver and apply steam for soilborne pest and disease control. The most common methods include systems in which soil is brought to the boiler where steam was introduced into bins or chambers for bulk soil treatment. For other systems, a small portable steam boiler is brought to the treatment site where the steam is introduced directly, via a conduit, into buried perforated pipe, tile or winch-drawn plows or directly under an inverted pan, tray, or tarpaulin cover. Due to the high costs, heating inefficiencies, pest control inconsistencies, soil modification, and other treatment impracticalities, steam is not currently used for large-scale.

Soil solarization

Soil solarization is a nonchemical technique in which transparent polyethylene plastic mulch is laid over moist soil for a 4–6-week period to heat non-cropped, greenhouse soils to temperatures lethal to nematodes and other soilborne pathogens. Soil temperatures are magnified because of the trapping of incoming solar radiation under the clear polyethylene plastic. Solarization of bags of nursery potting mixes was also recently certified in California as an alternative to steam or fumigation with methyl bromide. But, it is known that soils with poor water holding capacity and rapid drainage can significantly inhibit heat transfer to deeper soil horizons. As a result, loss of pest control is often directly correlated to soil depth. The depth to which lethal temperature can be achieved (15–20 cm) is also dependent on the intensity and duration of sunlight and ambient temperature. At present, the only time to consider soil solarization for pest control is during hot summer months.

1.2.2. Host plant resistance

The complex morphological and physiological changes that occur during the establishment of feeding sites by nematodes are reflected by altered gene expression in the host (**Figure 6**).[19] Plants are defined as resistant to nematodes when they have reduced levels of reproduction. Nematode resistance genes are present in several crop species and are an important component in many breeding programs, including those for tomato, potato, soybean, and cereals. Use of nematode-resistant crop varieties is often viewed as the foundation of a successful integrated nematode management program. Commercially available nematode-resistant vegetable varieties are currently available for tomato, pepper, southern pea, and sweet potato. In many European and Mediterranean countries, grafting of susceptible plants onto nematode-resistant rootstocks is being used in protected culture systems for a number of annual greenhouse crops including tomato, eggplant, and various cucurbits. However, long-term resistance of a crop is

still a challenge because nematode populations often display a broad variation of virulent pathotypes.

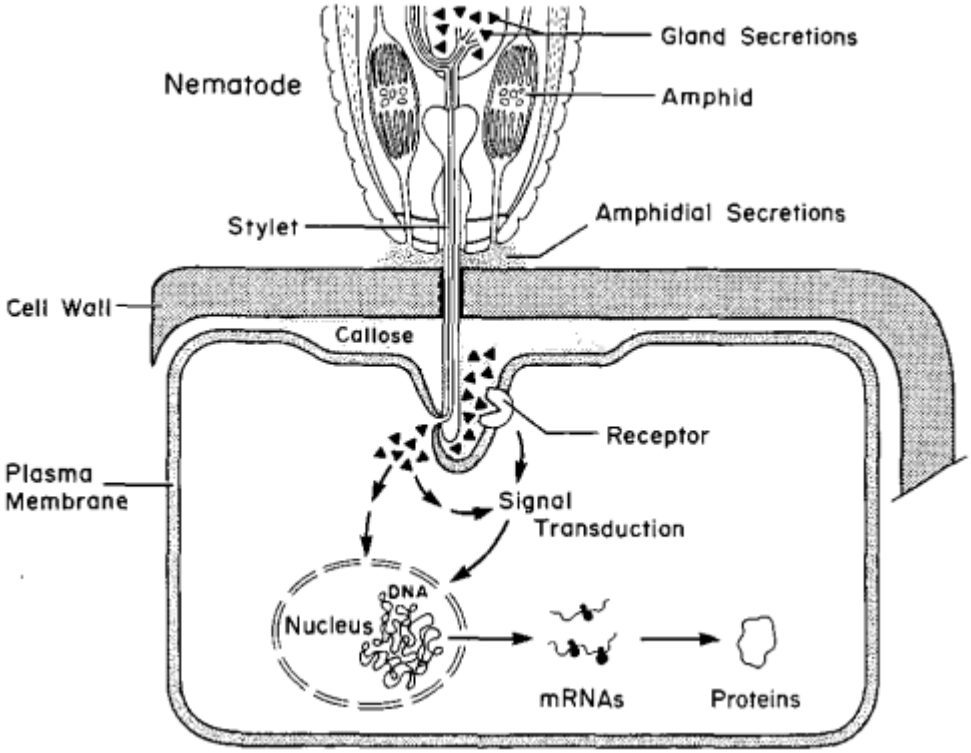


Figure 6: Schematic model of interactions of a nematode with its feeding cell. Figure reproduced from Williamson et al. (1996)..[19]

1.2.3. Biological control

Nematodes have a diverse range of natural enemies including other nematodes, Collembola and mites, but the literature on biological control has been dominated by research on microbial antagonists for a number of reasons. First, most microorganisms are readily cultured on defined media, and so they are amenable to study in the laboratory. Second, commercial mass production techniques are available for bacteria and fungi, and so they tend to be the first organisms selected for research programs on inoculative or inundative biological control. Third, organisms that exhibit a degree of specificity towards the target nematode have generally

been considered more effective biocontrol agents than generalists, and this has meant that research has focused on bacteria such as *Pasteuria*, and relatively specialized fungi capable of parasitizing the females and eggs of sedentary endoparasitic nematodes.

Of all the natural enemies of nematodes, the nematophagous fungi are the most diverse. They are found in many different taxonomic groups within the fungal kingdom and use a variety of mechanisms to capture and kill nematodes. Fortunately, there is evidence that progress is being made, as products based on *Purpureocillium lilacinum*, *Trichoderma* spp. *Bacillus firmus*, various other *Bacillus* spp. and in vitro-produced *Candidatus Pasteuria usgae* are now registered as biopesticides in some countries. However, one limitation of much of the work to date is that evidence of efficacy has generally been obtained under relatively controlled experimental conditions. Thus, there is an urgent need to confirm that the registered products are efficacious and cost-effective in the target markets.[20] Commercially available bionematicides and key applications are summarized in the **Table 1**.

Table 1: Key commercially available bionematicides, target nematodes, crops and applications rates.

Active ingredient	Product name	Specified Crops	Specified target nematodes
Bacillus firmus	VOTiVO™	Corn (maize)	*Heterodera* spp.
		Cotton	*Hoplolaimus* spp.
		Sorghun	*Criconema* spp.
		Soybean	*Pratylenchus* spp.
		Sugarbeet	*Meloidogyne* spp.
	Nortica	Turfgrass	*Meloidogyne* spp.
			Belonolaimus spp.

			Hoplolaimus spp.
Myrothecium verrucaria	DiTeRa DF®	Citrus fruit	*Meloidogyne* spp.
	DiTeRa WP®	Bedding plants	*Heterodera* spp.
		Ornamentals	*Globodera* spp.
		Leafy vegetables	*Pratylenchus* spp.
		Cole crops	*Tylenchulus semipenetrans*
		Pome fruits	
		Stone fruits	*Trichodorus* spp.
		Tree nuts	*Longidorus* spp.
		Grape	*Paratylenchus* spp.
		Pineaple	*Rotylenchulus* spp.
			Xiphinema
			Belonolaimus spp.
			Criconemoides spp.
			Criconemella spp.
			Tylenchorhynchus Spp.
			Hoplolaimus spp.
			Rotylenchus spp.
			Helicotylenchus Spp.
			Radopholus spp.
Pasteuria usgae	Econem™	Turfgrass	*Belonolaimus longicadatus*
Purpureocillium lilacinus	BioAct WP	Vegetables	*Meloidogyne* spp.

	BioAct WG	Strawberries	*Rapholus similis*
	MeloCon	Pineaples	*Heterodera* spp.
	NemOut	Ornementals	*Globodera* spp.
		Tobacco	*Rotylenchus reniformis*
		Citrus	*Nacobbus* spp.
		Tree nuts	*Helicotylenchus* Spp.
		Peaches	*Belonolaimus* spp.
		Grapevines	*Pratylenchus* spp.
		Bananas	

1.2.4 Chemical control

Chemical controls can be categorized according to their method of application as fumigant nematicides, non-fumigant nematicides and those derived from naturally occurring biotic sources.

Fumigants

The fumigant nematicides are generally liquids that volatilize to a gaseous phase upon entering the soil. The chemistry of fumigant nematicides currently being used includes either compounds containing halogenated hydrocarbons or those that discharge carbon disulfide or methyl isothiocyanate. Examples of halogenated hydrocarbons include methyl bromide, chloropicrin, methyl iodide and 1,3-dichloropropene. Metam sodium and dazomet decompose into the major active compound methyl-isothiocyanate when applied to moist soil. Tetrathiocarbonate is classified as an inorganic fumigant, which breaks down in the soil to form carbon disulfide; this compound is not as volatile as the halogenated hydrocarbons and is dependent on soil moisture to move through the soil profile to manage nematodes.[21] Until

recently, and for many years, methyl bromide was the most widely used multi-purpose fumigant nematicide because of its wide spectrum of activity against nematodes, other soil pathogens and weeds. Because of the partial ban on methyl bromide, a number of different alternatives are being evaluated in Europe and the USA against pathogens; these include the use of antagonistic crops. A partial list of chemical alternatives to methyl bromide includes methyl iodide, propargyl bromide, ozone, formaldehyde, sodium tetrathiocarbonate, carbon disulfide, anhydrous ammonia, inorganic azides, natural compounds, propylene oxide, sulfur dioxide, peroxyacetic acid and acrolein.

Non-fumigant

Non-fumigant nematicides do not suppress nematode populations as effectively as fumigant nematicides because they do not have broad-spectrum activity. These chemicals include aldicarb, oxamyl, ethoprophos, fenamiphos, carbofuran, fosthiazate and terbufos. These products have been available globally, but many of the registrations have since been revoked. Unlike fumigants, non-fumigant nematicides are not volatile and must disperse in the soil water phase (via irrigation and/or rainfall) to be active against nematodes. Non-fumigants are divided into two main chemical classes, which include organophosphates and carbamates. These nematicides are mainly considered to be 'nematostatics' because they paralyze nematodes, rather than killing them, or affect different aspects of their behavior, such as attraction to hosts, root penetration and feeding. Therefore, it is important that the nematicide remains in contact with the nematode for approximately 4–8 weeks to inhibit nematode infection sufficiently to enable plant growth with minimal impact due to nematode parasitism. Repeated applications are required and these are generally uneconomical and can lead to enhanced biodegradation of the chemical by soil microorganisms. Numerous other chemical compounds have been tested and found to exhibit nematicidal properties, but most have not been commercialized for a variety of reasons.[21]

Botanical nematicides

The development of plant secondary metabolites as tools in crop protection evolved through the observation of their activities when they were used in traditional practices and eventually by the identification of the active molecules as well as by the systematic screening of botanical families followed by biological tests to explore potential active molecules. Some botanicals used as pesticides pose less risk to humans and animals than their synthetic analogues, have a selective mode of action, are environmentally benign, and avoid the emergence of resistant races of pest species and therefore can be used in integrated pest management (IPM) programs.[22] Current studies investigate the encapsulation of essential oils as a potential controlled release vehicle with sites-specific delivery to maximize the properties of the oils. In recent years, the only commercial botanical registered for phytomenatodes control had been neem (*Azadirachta indica* A. Juss)-based formulations. Natural occurring compounds active on nematodes include aldehydes and ketones, alkaloids, glycosides, glucosinolates and isothiocyanates, limonoids, quassinoids, saponins, organic acids, phenolics, flavonoids, quinones, piperamides, polyacetylenes and polythienyls. For example, aldehydes and ketones such as *p*-anisaldehyde, benzaldehyde, trans-cinnamaldehyde, (R)-(+)-pulegone, and furfural exhibit high nematicidal activities against *M. javanica*. Specifically, the EC_{50} values of trans-cinnamaldehyde for juvenile immobilization and hatching inhibition in vitro were as low as 15 and 11.3 μL/L, respectively. In pot experiments, trans-cinnamaldehyde, furfural, and benzaldehyde at a concentration of 100 mg/kg greatly reduced the root galling of tomato. Under field conditions, soil treatment with trans-cinnamaldehyde (50 mL/m^2) reduced the galling index and increased the root weight of tomato plants.

1.3 *Satureja montana* and plant extracts in crop protection

The early discovery of synthetic pesticides and their utilization in crop protection was crucial in the “green revolution” characterized by a remarkable increase in crop yields. However, the

pesticides potential harmful effect on human health and the environment raised some concerns leading to modifications in registration procedures. These new rules reduced drastically the number of synthetic pesticides commercially available for farmers. Consequently, the systematic use of these chemicals in agriculture for pest control is considered. Integrated pest management, which is defined as the selection, integration and implementation of pest control based on predicted economic, ecological and sociological consequences, with maximum use of naturally occurring control agents including natural product-based pesticides, is considered to palliate the synthetic products restrictions.[23] Among natural products, plant essential oils are widely reported for pest and disease controls. Recent investigations in different parts of the world report not only insect repellent activity of essential oils, but also the contact and fumigant insecticidal actions against specific pests, and fungicidal actions against some important plant pathogens. [24-27]

Satureja montana L., commonly known as winter savory or mountain savory, belongs to the family Lamiaceae, subfamily Nepetoidae, and tribe Mentheae.[28] The genius *Satureja* L. contains about 200 species of aromatic herbs and shrubs that contain more than 0.5% essential oil (EO). *S. montana* is dwarf, hardy, perennial, glabrous or slightly pubescent under shrub, semi-evergreen herb (20–30 cm high) native to warm temperate regions of southern Europe, the Mediterranean, and Africa. It has been known in Great Britain since 1562. The plant grows better in a poor, stony soil than a rich one. In a rich soil, plants take in too much moisture to stand the severity of the winter. It is propagated either from seeds, sown and root divisions.[29] It is generally used as aromatic and spice and has evolved many morphological and physiological structures in response to dry climate conditions.[30]

In Italy, the aerial part of winter savory is traditionally used as a fumigant against the cold and the crushed aerial part as food for animals to reactivate rumination in livestock.[31] *S. montana* is also traditionally used to treat different disorders including male sexual dysfunction. In fact,

when the hydroalcoholic extract was orally administered acutely or repetitively for 8 consecutive days to rats at the doses of 25 and 50 mg/kg, their ejaculation latency increased significantly.[32] The EO of Spanish *S. montana* showed antimicrobial [33, 34] and antioxidant activities [35 36].

The chemical composition of the essential oil of *S. montana* was studied by different authors.[30, 37, 38]. The most important constituents varied from authors, geographic area, harvesting period and subspecies; carvacrol, thymol, p-cymene and linalool may frequently be reported as main components. Recently, Andrés et al. reported the in vitro nematicidal activity of the essential oil of *S: montana* against *Meloidogyne javanica* with an EC_{50} value of 41 mg/L.[25] To the best of our knowledge, none is known about antifeedant, phytotoxicity and in vivo nematicidal activity of the winter savor essential oils and hydrolates.

1.4. OBJECTIVES

1.1. To study the chemistry of RKN-plant interactions using mass spectrometry metabolomics

- Infest tomato seedlings with *Meloidogyne incognita*
- Extract polar metabolites of plants and identify them using GC/MS and ESI/Q-TOF
- Apply multivariate data analysis to identify possible up and down-regulating compounds

1.2. To develop new nematicidal compounds

- Synthesize new maleimide derivatives and determinate their nematicidal activity on *M. incognita.*
- Select possible nematicidal acetophenone derivatives and evaluate their nematicidal activity on *M. incognita* and *Globodera pallida*.
- Evaluate nematicidal activity of transition metal ions on *M. incognita* and *Xiphinema index*

1.3. Mass spectrometry metabolomics to identify possible metabolic pathway impacts induced in the different genera nematodes by nematicidal compounds

- Develop a protocol to extract and characterize nematode metabolites by GC/MS
- Apply multivariate data analysis to identify possible biochemical pathways altered by nematicidal compounds.

1.4. Test the biocidal activity of *Satureja montana* L.

- Determinate the insecticidal, antifeedant and nematicidal activity of different *S. montana* extracts from plants acclimatized in different geographic areas and treated with different fertilizers.

- Identify possible agricultural conditions to enhance biocidal activity of the plant.

CHAPTER 2

METHODOLOGY

2.1. Chemicals used and investigated

2.1.1. Maleimide derivatives

Background

Maleimide (**1**) is a five-membered ring compound belonging to the cyclic imides class. Its derivatives are emerging as potent pharmacophores showing different biological activities.[39,40] When investigated on *Escherichia coli* and *Staphylococcus aureus*, maleimides were approximately 30 times more active than succinimides. When tested on *Sclerotinia sclorotiorum*, different synthesized maleimide derivatives showed an interesting fungicidal activity.[41] Moreover, by acting like thiol reagents, maleimides like *N*-ethylmaleimide (**2**) and related *N*-arylcyclic imides showed herbicidal activitiy.[42] At low concentrations (1−2 μM), *N*-ethylmaleimide, a covalently binding cysteinyl reagent,[43] is considered an inhibitor of the eukaryotic vacuolar (V-type) ATPases, while at higher concentrations (0.1−1 mM), it inhibits the P-type ATPases, F-type ATPases that are resistant to inhibition by **2**.[44, 45] In the present study, starting with the hypothesis of the involvement of maleimide in the inhibition of the nematode V-ATPase activity, we prepared various maleimides and succinimides with different substituents and assessed their nematicidal activity in vitro.

General procedure for the synthesis of new nematicidal maleimide derivatives

All synthetic precursors and solvents were purchased from Sigma-Aldrich (Milan, Italy). Maleimide derivatives and succinimides **1**, **2**, **3**,[46] **4**,[41] **5**,[47] **8**,[47] **9**, **10**,[47] **11**,[48] **12**,[49] **13**,[49] **15**,[50] **16**,[49] **17**,[51] **18**,[41] **19**,[51] **21**,[48] **24**,[49] **26**,[49] **27**,[52] and **28**[47] were commercially available or obtained as previously reported.

A mixture of maleic anhydride (0.196 g, 2 mmol) and the appropriate amine (2 mmol) in dichloromethane (5 mL) was stirred at room temperature for 1 h. The solvent was removed in vacuo, and the residue, maleamic acid, was then dissolved in 3 mL of acetic anhydride. To this

solution we added sodium acetate (0.10 g, 1.22 mmol), and the mixture was then heated for 2 h at 100 °C and cooled with water afterward. The aqueous solution was extracted with Et_2O (3 × 10 mL), and the organic layers were collected, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give N-substituted maleimides **2**−**8** and **11**−**28**.

1-(Heptan-2-yl)-1H-pyrrole-2,5-dione **(*6*)**. Yield 82%, colorless oil. ^{1}H NMR (DMSO-d_6): δ 6.95 (s, 2H, CH), 4.00-4.03 (m, 1H, CH), 1.79-1.86 (m, 1H, CH_2), 1.52-1.59 (m, 1H, CH_2), 1.27 (d, J = 7.0 Hz, 3H, CH_3), 1.09-1.23 (m, 6H, CH_2), 0.81 (t, J = 7.0 Hz, 3H, CH_3). IR (neat) 1710, 1457, 1405, 1369 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{11}H_{17}NO_2$) 196.1332, found 196.1335. Calcd: C, 67.66; H, 8.78; N, 7.17. Found : C, 67.67; H, 8.76; N, 7.18.

1-(6-Methylheptan-2-yl)-1H-pyrrole-2,5-dione (*7*). Yield 79%, colorless oil. ^{1}H NMR (DMSO-d_6): δ 6.95 (s, 2H, CH), 4.00-4.03 (m, 1H, CH), 1.78-1.84 (m, 1H, CH_2), 1.51-1.55 (m, 1H,CH_2) 1.42-1.47 (m, 1H, CH_2), 1.27 (d, J = 6.5 Hz, 3H, CH_3), 1.06-1.15 (m, 4H, CH_2), 0.80 (t, J = 7.0 Hz, 6H, CH_3). IR (neat) 1704, 1460, 1405, 1367 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{12}H_{19}NO_2$) 210.1489, found 210.1487. Calcd: C, 68.87; H, 9.15; N, 6.69. Found C, 68.89; H, 9.13; N, 6.70.

1-(4-Fluoro-3-nitrophenyl)-1H-pyrrole-2,5-dione (**14**). Yield 82%, mp 115-118 °C. ^{1}H NMR (DMSO-d_6): δ 7.24 (s, 2H, CH), 7.72- 7.83 (m, 2H, Ar), 7.85 (s, 1H, Ar). IR (Nujol) 1714, 1594, 1538, 1503 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{10}H_5FN_2O_4$) 237.0306, found 237.0302. Calcd: C, 50.86; H, 2.13; F, 8.04; N, 11.86. Found C, 50.87; H, 2.15; F, 8.03; N, 11.88.

1-(4-(Methylthio)phenyl)-1H-pyrrole-2,5-dione (**20**). Yield 63%, mp 76-79 °C. ^{1}H NMR (DMSO-d_6): δ 7.31 (s, 2H, CH), 7.35 (d, J = 8.5 Hz, 2H, Ar), 7.26 (d, J = 8.5 Hz, 2H, Ar), 2.49 (s, 3H, CH_3). IR (Nujol) 1793, 1768, 1706, 1665 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd

for ($C_{11}H_9NO_2S$) 220.0427, found 220.0429. Calcd: C, 60.26; H, 4.14; N, 6.39; S, 14.62. Found C, 60.25; H, 4.12; N, 6.36; S, 14.63.

1-(4-Ethoxyphenyl)-1H-pyrrole-2,5-dione (**22**). Yield 75%, mp 78- 80 °C. ^{1}H NMR (DMSO-d_6): δ 7.38 (d, J = 8.5 Hz, 2H, Ar), 7.18 (s, 2H, CH), 6.98 (d, J = 8.5 Hz, 2H, Ar), 4.05 (q, J = 7.0 Hz, 2H, CH_2), 1.32 (t, J = 7.0, 3H, CH_3). IR (Nujol) 1793, 1763, 1713, 1666 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{12}H_{11}NO_3$) 218.0811, found 218.0810. Calcd: C, 66.85; H, 5.10; N, 6.45. Found: C, 66.83; H, 5.12; N, 6.46.

1-(3-Chloro-4-methoxyphenyl) 1H-pyrrole-2,5-dione (**23**). Yield 56%, mp 132-135 °C. ^{1}H NMR (DMSO-d_6): δ 7.49 (s, 1H, Ar) 7.21 (d, J = 8.5 Hz, 1H, Ar), 7.16 (s, 2H, CH), 7.10 (d, J = 8.5 Hz, 1H, Ar), 3.88 (s, 3H, CH_3). IR (Nujol) 1770, 1712, 1685, 1560 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{11}H_8ClNO_3$) 238.0265, found 238.0262. Calcd: C, 55.60; H, 3.39; Cl, 14.92; N, 5.82. Found: C, 55.62; H, 3.36; Cl, 14.90; N, 5.84.

1-(Naphthalen-2-yl)-1H-pyrrole-2,5-dione (**25**). Yield 72%, mp 105 °C. ^{1}H NMR (DMSO-d_6): δ 7.87−8.03 (m, 3H, Ar), 7.78 (s, 1H, Ar), 7.47−7.63 (m, 3H, Ar), 7.16 (s, 2H, CH). IR (Nujol) 1787, 1764, 1711, 1675 cm^{-1}. HRMS (ESI-TOF) (m/z) $[M + H]^+$ calcd for ($C_{14}H_9NO_2$) 224.0706, found 224.0705. Calcd: C, 75.33; H, 4.06; N, 6.27. Found: C, 75.31; H, 4.08; N, 6.30.

1-Benzylpyrrolidine-2,5-dione (**10**). A mixture of benzylamine (0.21 g, 2 mmol), succinic anhydride (0.2 g, 2 mmol), and anhydrous triethylamine (1 mL, 7.2 mmol) in 25 mL of toluene was refluxed with a Dean−Stark apparatus for 36 h. The reaction mixture was cooled then concentrated under reduced pressure. The residue was dissolved in ethyl acetate (25 mL) and washed with saturated aqueous $NaHCO_3$ solution (3×10 mL) and then with water (3 × 10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to give

the title compound in 80% yield. Analytical and spectroscopic data were in accordance with literature values.[47]

2.1.2. Nematicidal haloacetophenones

Background

Acetophenones are pervasive in the vegetal world. Frequently present as *O*-glucosides or prenylated derivatives[53], naturally occurring acetophenones are frequently used for the treatment of various diseases and for pest control.[54, 55] Moreover, the phenyl methyl ketone, the first representative of the series, is readily biodegradable in the environment, so has an insignificant potential for bioaccumulation and therefore is not classified as dangerous/hazardous for the environment (Reach consortium report). Remarkably, acetophenones are produced by plants as secondary metabolites to reinforce their allelochemical armory or they are sometimes generated in the soil as degradation products by microbial activities. Interesting examples are picein [3-(β-D-glucosyloxy)-hydroxy-acetophenone], pungenin [3-(β-D-glucosyloxy)-4-hydroxy-acetophenone, pungenol (3′,4′-dihydroxy-acetophenone) and piceol (4′- hydroxyacetophenone) that confer resistance to *Picea glauca* against spruce budworm.[56] In this work, based on a structure-activity relationship, we selected different haloacetophenones active against two plant parasitic nematodes.

Selected haloacetophenones

The **Figure 7** shows the selected haloacetophenones compounds. All were purchased from Sigma Aldrich (Milan, Italy) and Alfa Aesar, Thermo Fisher Scientific.

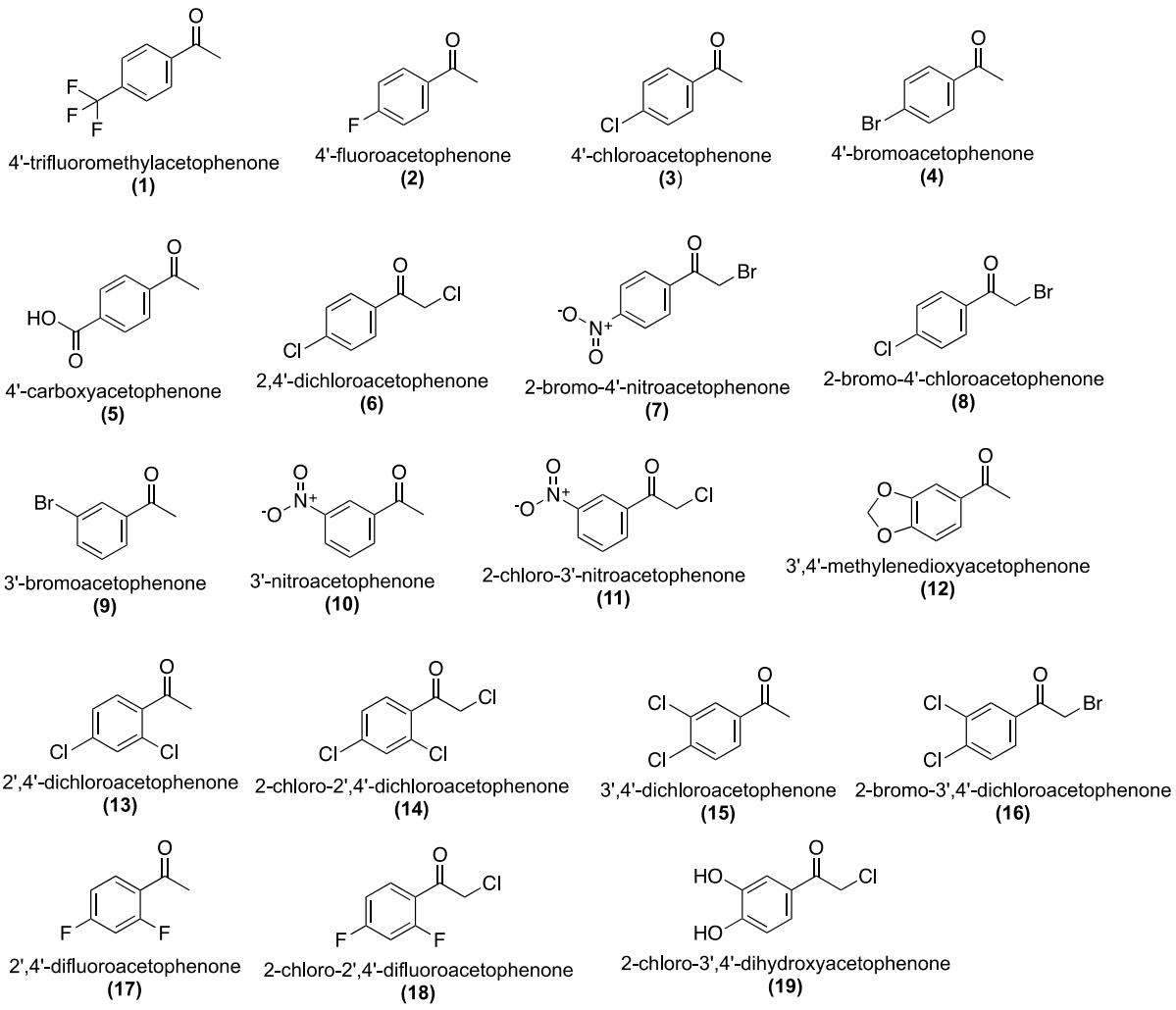


Figure 7: Selected haloacetophenones tested against *M. incognita*.

2.1.3. Oxidative stress induced by metal ions

Background

Recently, the key role of metal ions to the vital functions of living organisms, hence their well-being and health, has increasingly become evident. Consequently, the "bioinorganic chemistry" field, long-neglected is now developing rapidly. The metabolism and transport of metal ions and their complexes are being studied by research centers, and new models for complicated natural structures and processes are being designed and tested.[57] It is in this perspective of idea that we tested different transition metal salts solely and in combination with tannins on *M. incognita* and *X. index*.

Selected metal ions

Chemicals including copper (II) and zinc (II) oxides were obtained from Sigma-Aldrich (Milano, Italy) while solvents were of high performance liquid chromatography purity. A wettable powder of tannins (75% w/w) was kindly provided by Sadepan Chimica (Viadana, Italy).

2.2. Nematode populations

2.2.1. *Meloidogyne incognita*

All the nematode populations used in this work were kindly provided by Dr. Nicola Sasanelli from the CNR of Bari. *M. incognita* populations were maintained on susceptible *Solanum lycopersicum* L. plants (var. Marmande) in pot cultures at 25 ± 1 C, >70% relative humidity reared for two months in a greenhouse located in Cagliari, Italy. Infested plants were uprooted, and roots showing galls and egg masses were washed to remove soil. The roots were then cut into 2 cm pieces and the egg masses hand-picked. Batches of 20 egg masses of similar size (averaging 20000 eggs) were placed on 2 cm diameter sieves (215 μm), and each sieve was put in a 3.5 cm diameter plastic Petri dish; we then added 3 mL of distilled water (natural hatching agent for *Meloidogyne* spp.), sufficient to cover the egg masses, to allow the eggs to hatch. The dishes were incubated in a growth cabinet at 25 °C.[58] All second-stage juveniles (J2) which hatched in the first 3 d were eliminated, and only the J2s hatched after 24 h or more were collected and used to verify the in vitro nematicidal effect of the selected the nematicidal compounds and extracts.

2.2.2. *Meloidogyne javanica*

A field-selected *M. javanica* population from Barcelona, Spain, was maintained on *Solanum lycopersicum L.* plants (var. Marmande) in pot cultures at 25 ± 1 C, >70% relative humidity, at CSIC/ICA, Madrid. Egg masses of *M. javanica* were handpicked from infected tomato roots

two months after inoculation of the seedlings. Second-stage juveniles (J2) were obtained by incubating egg masses in a water suspension at 25 °C for 24 h.

2.2.3. *Xiphinema index*

X. index infested soils of a greenhouse located at the CNR of Bari, Italy were used for nematode extraction. Five hundred milliliter of the infested soil was put in a bucket and 4-5 liters of water were added. The suspension was thoroughly mixed using a wood stick. After 1 min, the aqueous suspension was poured through a sieve of 2 mm and placed on a bucket for the recovery of the suspension free of coarse debris. After another 1 min, the aqueous suspension was slowly poured through a sieve of 20 µm to keep nematodes. The contents of the sieve, thus recovered, were poured in a bucket with the aid of running water. Then, water was added and mixed for cleaning. The last operation was repeated until the water passing through the 20 µm sieve was clear. The debris and nematodes in the 20 µm sieve were collected with the aid of water and placed in another 20 µm sieve with toilet paper. To obtain a clear suspension containing the nematodes, the sieve was placed in a petri dish and water was added to the bottom to the base of the sieve. The Petri dish was left for 1 day and nematodes used for experiments were collected from the suspension.

2.2.4. *Globodera pallida*

The population of *G. pallida* was obtained from an infested field at Conversano (province of Bari, Apulia region, Italy). Cysts were collected from dried soil by the Fenwick method (Fenwick 1940).[59] Juveniles were obtained treating cysts with a 0.6 mM sodium metavanadate aqueous solution used as hatching agent.

2.3. Nematode bioassays

2.3.1. Bioassays on *M. incognita, X. index* and *G. pallida*

Maleimide derivatives, acetophenone derivatives and metal ions were tested for the nematicidal activity on the second stage juveniles of *M. incognita*, *X. index* and *G. pallida* and the corresponding EC_{50} values were calculated. Stock solutions of every sample were prepared using water, methanol, or DMSO (0.3 % Tween) as solvent, whereas working solutions were made by dilution with tap water. The final concentrations of the organic solvent in each well never exceeded 1% because preliminary experiments showed that the motility of nematodes exposed to those concentrations was similar to the motility of the controls. Tap water was used with the controls. Treatments were performed in 96-well plates (BRANDplates, Germany) using 30 juveniles of *M. incognita* and *G. pallida* and 15 juveniles of *X. index* in each replication. To avoid solvent evaporation, plates were covered and kept in the dark at 28 °C. After treatment, juveniles were moved to plain water and grouped into two distinct categories, motile or immotile, with the use of an inverted microscope at 40× after pricking the juvenile body with a needle. Nematodes that never regained movement after being moved to tap water and pricked were considered to be dead. Six replications were made, and the experiment repeated at least twice.

Synergistic activity of maleimide with metal salts and tannins was also studied. By assuming the linearity of the nematicidal assay, we used the method reviewed by Berenbaum.[60] The combination of two agents A and B is termed (d_a and d_b), where d_a and d_b are the concentrations of A and B, respectively. The effect is treated as a mathematical function E; thus $E(d_a,d_b)$ is the combined effect and $E(d_a)$ and $E(d_b)$ are the effects of the individual agents A and B, respectively. Zero interactive combination is observed when the summation of the effects of the individual agents is not significantly different from the combination effect, i.e., $E(d_a,d_b) = E(d_a) + E(d_b)$. If the combination effect is smaller or greater than the summation of the

individual agent effects, the combination is antagonistic or synergistic, respectively. The comparison was made at two concentration levels, of maleimide at 1 and 2 mg/L, in combination with copper and iron sulfates at different concentration levels, 50 and 75; 125 and 200 mg/L, respectively. For tannins, the comparison was made at two concentration levels of copper sulfate i.e. 50 and 75 mg/L in combination with tannins at 5 mg/mL for two immersion periods (24 and 72 h). The test compounds were prepared at that concentration level and were then paired in a 1:1 (*v/v*) ratio. The sum of paralysis obtained from bioassays performed using solutions of each test compound or salt separately was compared with the observed paralysis caused by immersion of *M incognita* J2 in the respective combination. The experiment was repeated twice.

2.3.2. Bioassays on *M. javanica*

The *S. montana* hydrolates were tested in vitro on *M. javanica* juveniles with and without serial dilutions (50%, 33%, 25% and 10%) in water (*v/v*) to calculate the active minimum concentrations. A nematode inoculum (500 J2 in water) was filtered (25 µm) and the nematodes suspended in 500 µL of *S. montana* hydrolate solutions. Four aliquots (100 µL) of the nematode suspension (approximately 100 J2) and controls (water) were placed in 96-well plates (BD Falcon, San Jose, CA, USA). The organic fraction of the hydrolates and the essential oils were dissolved in DMSO-Tween solution (0.6% Tween 20 in DMSO) to obtain a stock solution at 20 mg/mL. For the nematicidal activity, 5 µL of this solution was added to 95 µL of water containing 90 to 150 nematodes to obtain a treatment final concentration of 1 mg/mL.[25] The stock solutions of active extracts were diluted serially for minimum active concentration calculation. Treatments were replicated four times. As a control, four wells were treated with the water/DMSO/Tween 20 in the same volume as the tests. The plates were covered to prevent evaporation and were maintained in the dark at 25 °C. After 72 h, the dead J2 were counted

under a binocular microscope. J2 mortality was ascertained by transferring some of treated, apparently dead juveniles to distilled water and examining them after 5-6 h for any revival.

2.4. Insect bioassays with *S. montana*

2.4.1. Insects presentation

Background

Three crop pest insect species were used for the bioassays: *Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*.

The Mediterranean climbing cutworm (**Figure 8a**), *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae), is a polyphagous insect that feeds on a wide range of unrelated plant species including many important crops. The abundance of this pest is correlated to its wide host range. *S. littoralis* is found in Mediterranean Europe and Africa, where it is detrimental to subtropical agricultural plants. In Central and Northern Europe, *S. littoralis* is recognized as an imported pest species that invades nurseries and greenhouse environments.[61] Its resistance to many pesticides means that alternatives are urgently required.

Myzus persicae (Sulzer) (**Figure 8b**), known as the green peach aphid or the peach-potato aphid, is a small green aphid. It is the most significant aphid pest of peach trees, causing decreased growth, shrivelling of the leaves and the death of various tissues. *M. persicae* is outstanding in distribution, in host plant range, and as a pest which causes not only direct damage but is able to transmit over 100 virus diseases of plants on about thirty different families including many major crops such as beans, sugar beet, sugar cane, brassicas, potatoes, tobacco and citrus.[62] The use of chemical methods to prevent spread of viruses by controlling their aphid vectors has been either unsuccessful or only partially successful. Thus, there is a special need for the integrated control approach using for instance botanical compounds.

The bird cherry-oat aphid, *Rhopalosiphum padi* L. (Homoptera: Aphididae) (**Figure 8c**) alternates between a winter host, bird cherry *Prunus padus* (Rosaceae), and summer hosts, a range of grasses (Gramineae) including cereals. The aphid is an important pest of cereals, causing damage both as a plant virus vector and by direct feeding.[63]

Figure 8: Photos of insects used in the bioassays: a) *S. littoralis*, b) *M. persicae* and c) *R. padi*.

2.4.2. Bioassays

Insect colonies of *S. littoralis*, *M. persicae* and *R. padi* were reared on an artificial diet, potato (*Solanum tuberosum*), bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants, respectively, and maintained at 22 ± 1 °C, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber. Bioassays were conducted with newly emerged *S. littoralis* sixth instar larvae (10 replicates with 2 insects each) and adults of the aphids *M. persicae* and *R. padi* (20 replicates with 10 insects each).

Figure 9: *M persicae* population maintained on pepper.

Feeding or settling inhibition (% FI or %SI) were calculated as FI = [1-(T/C)] x 100, where T and C are the consumption of treated and control leaf disks, respectively, or as %SI = [1-10 (%T/%C)] x 100 where %C and %T are percent aphids settled on control and treated leaf disks, respectively, as described by Santana et al.[64] The antifeedant effects (FI/SI) were analyzed for significance by the non-parametric Wilcoxon signed-rank test. EC_{50} values (effective dose to obtain 50% feeding inhibition) were determined for EOs and hydrolates with FI/SI values > 60% from linear regression analysis (**Figure 10**).

Figure 10: Insect bioassays a) *S. littoralis* selectively feeding on pepper, b) *R. padi* selectively settling on barley c) *M. persicae* settling selectively on pepper leaf.

2.5. Phytotoxic activity with *S. montana*

The experiments were conducted with *Lactuca sativa* L. cv. Teresa and *Lolium perenne* L. seeds (10 seeds/test) in 12-well microplates as described previously.[64] The EOs and hydrolates were tested at 10 mg/mL. Twenty microliters of the test solution were deposited on a 2 cm Whatman paper and 500 µL distilled water was added to each well containing 10 seeds. Germination was monitored for 7 days, and the root length measured at the end of the experiment for *L. sativa* and *L. perenne*; for the latter, the aerial part was also measured (25 plants were selected randomly for each experiment, digitalized, and measured with the application ImageJ, http// rsb.info.nih.gov./ij/). A nonparametric analysis of variance (ANOVA) was performed on root and aerial length data.

2.6. *S. montana* essential oil effect on infection and reproduction of *M. javanica* population in tomato plants

A concentration of 0.5 mg/mL of the *S. montana* essential oil, acclimatized in Zaragoza and extracted with the semi-industrial method (SAMOC PEO), was tested in pot experiments, according to the method described by Oka et al.[65] Treatment stock solutions were prepared in ethanol and diluted 100 times with distilled water to obtain working solutions. Sandy loam soil (1.2 kg) was mixed with 100 mL of the treatment solution and transferred into 1000 mL plastic pots. Thereafter, 2000 *M. javanica* juveniles suspended in 2 mL water were inoculated in each treatment and incubated for five days in a growth chamber (25 ± 2 °C, 60% RH and 16 h photoperiod). Five replications were made for the treatment and the control consisted of soil treated with water: ethanol mixture (99:1 *v/v*). One-month-old tomato (cv. Marmande) seedlings were planted in the pots after the incubation period and maintained under the same conditions and fertilized with 50 mL of a 0.3% solution of 20-20-20 (N-P-K) every 10 days. After 60 days, seedlings were uprooted and roots were processed as reported by Verdejo-Lucas et al.[66] Number of egg mass per plant (EMs) was counted and the nematode infectivity was

estimated. The nematode infection frequency (NF) was calculated using the following formula: NF = (EMS)/(number of juveniles inoculated per pot). By extracting eggs from the entire root system, egg production was also obtained.[67] The multiplication rate (MR) of the nematode was afterwards estimated by dividing the number of eggs per plant (Pf) by the juveniles inoculum (Pi). The experiment was performed twice. Data were transformed [$\log_{10}$ (x+1)] before analysis of variance, and means separated by LSD at $P < 0.05$ (**Table 2**).

2.7. Nematode metabolomics analysis

Nematodes water soluble polar metabolites extraction for metabolomics analysis was performed (**Figure 11**).[68] In vitro experiments consisted of approximately 300 J2 of *M. incognita* or *G. pallida* and 20 J2 of *X. index* treated in a Cellstar 96-well plates. Different concentrations of the most active compounds reported in this work (**Figure 11**, **Table 2**) in a total volume of 200 µL were assessed. Untreated worms consisting of nematodes immersed in tap water served as controls. After 24h, samples were transferred to 1.5 mL Eppendorf tube and ultrasonicated with a Vibracell cell disruptor (Labotal Scientific Equipment, Abu Ghosh, Israel) for the nematode cuticle lysis. Ultrasonication was made twice with three times pulse of 20 seconds each at 60% of amplitude (130 Watt, 20 KHz). Finally, 800 µL of tert-butylmethylether (MTBE) were added and vortexed for 1 min. Samples were centrifuged at 18000 rpm at 25 °C for 15 min and the liquid supernatant of MTBE was taken and dried overnight in vials under gentle nitrogen stream. Dried extracts were derivatized for GC-MS analysis with a solution of methoxamine chloride dissolved in pyridine at 10 mg/mL for oximation of carbohydrates. After 17 h, 80 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were added for metabolite sylilation. After 1 h, 50µL of hexane containing 20 mg/L of 2,2,3,3-d4-succinic acid or 2-dodecanone as internal standard were added. All experiments were replicated at least 8 times for *M. incognita* and *G. pallida*.

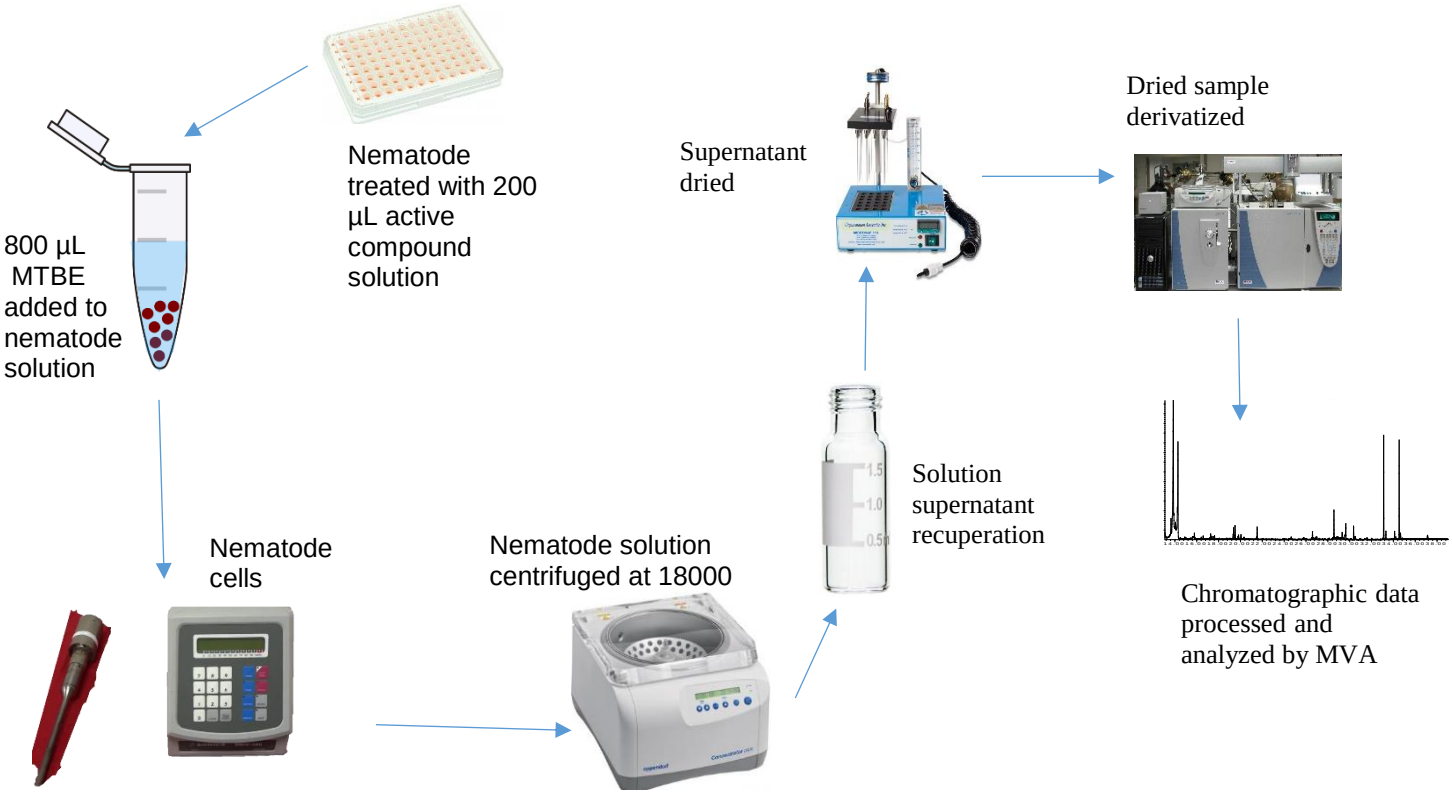


Figure 11: Metabolomics extraction scheme

Table 2: Metabolomics analysis with different chemical and nematode species.

Compound type	Selected compounds	Concentration (mg/L)	Nematode species
Maleimide derivatives	Maleimide	8	*M. incognita*
Acetophenone derivatives	2,4′-dichloro-acetophenone	100	*G. pallida*
Metal ions	copper sulfate	150	*M. incognita* and *X. index*
	silver nitrate	10	*M. incognita* and *X. index*

2.8. Metabolomics analysis of tomato plants after root-knot nematode infestation

Nematode Inoculum.

A race 3 *M. incognita* population was reared in sandy soil in 5 L pots for 2 months on tomato (*Solanum lycopersicum* L.) cv. Rutgers in a glasshouse at 25 ± 2 °C at the Institute for Sustainable Plant Protection National Research Council (CNR) located in Bari (Apulia region, Italy). Nematode inocula for the trial consisted of *M. incognita* eggs that were extracted from tomato roots with a 1% sodium hypochlorite aqueous solution (Hussey and Barker's method).[67] Eggs released from the roots were collected on a 25 μm pore size sieve and were counted under an optical microscope with nematode-counting slides. A total of 32 tomato seeds (cv. Rutgers) were sown, and after emergence, 45 day-old tomato seedlings were transplanted in clay pots filled with sandy soil (pH 7.2; sand, >99%; silt, <1%; clay, <1%, and organic matter, 0.75%) for the experiment. After 1 week, 16 tomato plants were inoculated with nematodes (10 000 eggs and juveniles/pot), and the plant material was harvested after 2 months. Plants not inoculated served as control (n = 16).

2.8.1. Sample Extraction

A total of 1 g of fresh plant material, leaves or stem, were chopped and extracted with 10 mL of methanol (HPLC grade) using falcon tubes. Samples were sonicated for 30 min and kept for 24 h for maceration. After that period, supernatants were filtered using cotton and centrifuged at 4000 rpm at 25 °C for 10 min. The liquid supernatant, consisting of a pool of low-molecular weight polar metabolites, was filtered using nylon syringe filters, 0.45 μm (Thermo Scientific, Rockwood, TN).

2.8.2. Sample derivatization for GC-MS analysis

A total of 200 μL of the methanol plant extracts was dried overnight in amber vials under a gentle nitrogen stream. Samples were then derivatized using a solution of methoxamine

chloride dissolved in pyridine at 10 mg/mL. After 17 h, 80 μL of *N*-methyl-*N*(trimethylsilyl)trifluoroacetamide (MSTFA) was added. After 1 h, 50 μL of hexane containing 5 mg/L 2,2,3,3-d4-succinic acid as the internal standard was added. Four replications were performed for every sample, and the experiment was repeated twice at different times.

2.8.3. Metabolic Pathway Analysis.

Metabolite log2 fold changes were calculated using Excel software. The significant differences were determined using the log2 fold change value (>0.4). Finally, to identify the metabolic pathways altered by the RKN infestation, pathway analysis of the identified potential biomarkers was performed using MetaboAnalyst 3.0[69] based on the pathway library of *Arabidopsis thaliana* (L.) Heynh.

2.9. *S. montana* plant material and extraction

2.9.1. Agronomic information

A domesticated and chemically stable population of *S. montana* (SAMO-0) was harvested in 2016 and 2017. The domesticated *S. montana* was acclimatized in 2 different geographic locations in Spain, in KIMITEC conditions at Almeria province (Southeast) and in Ejea de los Caballeros (Zaragoza, Northeast) and the wild population was harvested in Zaragoza in 2016 and 2017 (**Figure 12**).

Figure 12: Spain map showing the geographic areas of *S. montana* acclimatization: Almeria and Zaragoza

***S. montana* from Ejea de los Caballeros, cultivated in collaboration with the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Zaragoza.**

The plot was planted in December of 2014, in collaboration with CITA with details in the following scheme (**Figure 13**). CITA of Aragon is a public research organization belonging to the Department of Innovation, Research and University of the Government of Aragon, Spain, whose mission is to obtain benefits for the society for which it works, through technological development research, training and transfer.

In 2016, the plot received maintenance and control of weeds (manual clearing and pass of farmer between corridors). In addition, the park has been fertilized with organic BIOHUMIC fertilizer (1,200 kg / ha). Every year, the trials receive drip irrigation according to the needs of the plants or weather conditions (**Figures 14** and **15**).

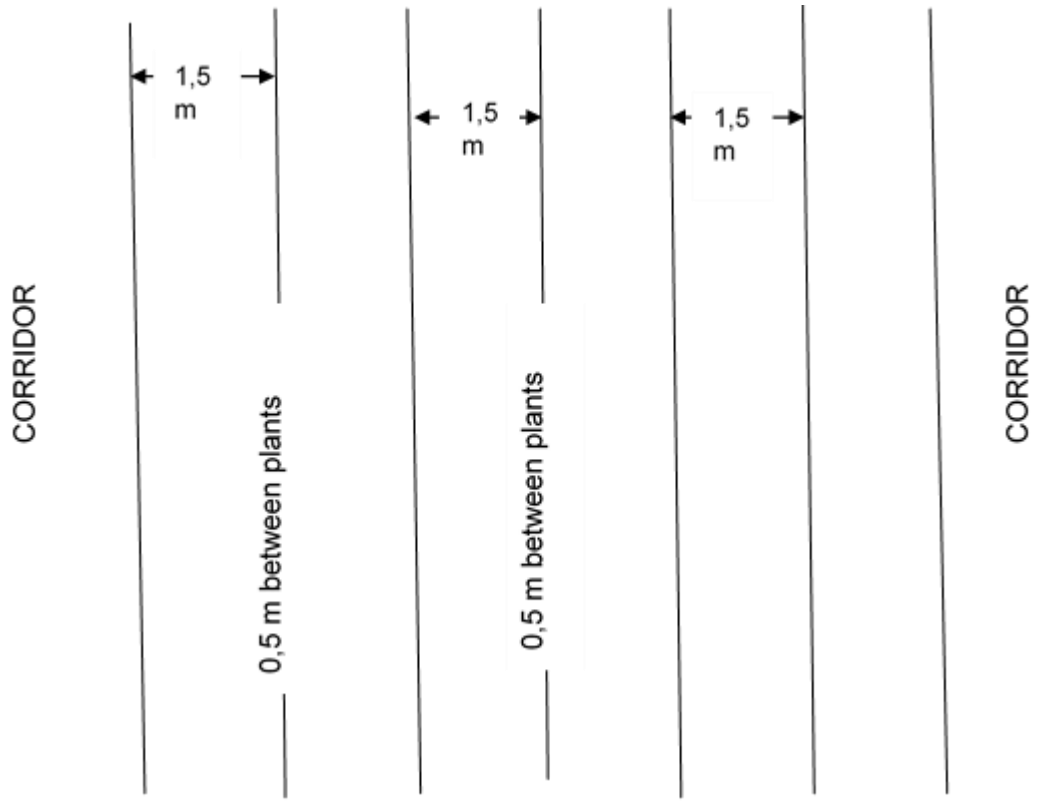


Figure 13: Sketch of the *S. montana* experimental plot in Ejea de los Caballeros. plantation frame: 1,5 x 0,5 = 0,75 m2/plant, Total: 140 plants x 7 lines (980 plants)

Figure 14: Vegetative development of a domesticated population of *S. montana* at the experimental field of CITA, Zaragoza, Spain.

Figure 15: Beginning of flowering of a domesticated population of *S. montana* at the experimental field of CITA, Zaragoza, Spain.

S. montana from Almeria, cultivated in collaboration with Kimitec

Experimental field was established in Almeria province (Southeast Spain) around 175 meters above the sea level, located at the following coordinates: 36º49´55.31´´N; 2º35´59.73´´W in 2015 in collaboration with Kimitec. Kimitec Group is a corporate biotechnology group specialized in the agricultural sector. The company is dedicated to researching, manufacturing and distributing organic plant nutrition, conventional plant nutrition and organic plant protection.

The area of *S. montana* covered 9.350 m^2 divided into four equal blocks with a sunny weather and good water availability (**Figure 16**). The block design was suitable to apply different treatments (irrigation, nutritional solutions, mycorrhizae, etc.) to accelerate crop adaptability and yield. By the end of November 2015, the land was developed according to a standardized process agreed between Kimitec and CITA/CSIC. The work was done on 30-50 cm tillage

depth to improve soil structure before planting. In order to assess soil fertility after tillage, soil samples at 30 cm depth were collected at random from each block of experimental field and further analysed. Full drip irrigation system was used.

Figure 16: Site preparation before planting in Almeria.

Around 11.000 *S. montana* seedlings provided by CITA, Zaragoza, were planted in March 2016 in furrows, at a depth of 15 cm, with 50 cm between plants and 150 cm amongst furrows like in Zaragoza (density= 0.75 m^2/plant). A randomized block design with four blocks was followed (**Figure 17**).

Figure 17: Planting pattern: 0.5 m between plants; 1.5 m between furrows in KIMITEC, Almeria.

Table 3: Descriptions of fertilizers used by KIMITEC from 2016 to 2017.

Treatment	Description
Co1: Conventional fertilization 1	- Ions: NO_3^-(10 mmol/l), $PO_4H_2^-$(1.5 mmol/l), K+ (6 mmol/l), Ca^{++} (2 mmol/l), Mg^{++} (1 mmol/l), Cl^- (0.9 mmol/l), H^+ (0.5 mmol/l) - KIMITEC product (RHYZO 1 kg/ha) (**Table 4**) - KIMITEC product (BOMBARDIER 10 l/ha)
Co2: Conventional fertilization 2	- Ions: NO_3^-(14 mmol/l), $PO_4H_2^-$(1.5 mmol/l), K+ (6 mmol/l), Ca^{++} (2 mmol/l), Mg^{++} (1 mmol/l), Cl^- (0.9 mmol/l), H^+ (0.5 mmol/l) - KIMITEC product (RHYZO 1 kg/ha) - KIMITEC product (BOMBARDIER 10 l/ha)
Co3: Conventional fertilization 3	- Ions: NO_3^-(10 mmol/l), $PO_4H_2^-$(1.5 mmol/l), K+ (8.5 mmol/l), Ca^{++} (2 mmol/l), Mg^{++} (1 mmol/l), Cl^- (0.9 mmol/l), H^+ (0.5 mmol/l) - KIMITEC product (RHYZO 1 kg/ha) - KIMITEC product (BOMBARDIER 10 l/ha)
M1: Microbiological 1	- KIMITEC products: A combination 1 including RHYZO, MYCOGEL, NITROCODE AZ+
M2: Microbiological 2	- KIMITEC products: A combination 2 including RHYZO, MYCOGEL, NITROCODE AZ+
O1: Organic 1	- KIMITEC products mixture 1: RHYZO, ESPARTAN, BOMBARDIER, KATON, TUNDAMIX, CAOS XT
O2: Organic 2	- KIMITEC products mixture 2: RHYZO, ESPARTAN, BOMBARDIER, KATON, TUNDAMIX

The plots harvested in 2016 were treated only with microbiological fertilizer or water as control while the plants harvested in 2017 were treated with whether water (control), microbiological, organic or conventional fertilizers. The different fertilizers used for 2017 samples are shown in the **Table 3**.

The commercial Kimitec products general composition is described in the **Table 4**.

Table 4: Kimitec fertilizer products general descriptions

Benefits	Bombardier	Caos	Espartan	Katon	Rhyzo	Tundamix
N-P-K	**		***	**	***	
Macro Nutrients		***				
Micro Nutrients						***
Amino Acids	***		*		***	
Rooting	*		*		***	
Vegetative	***	**	**		**	**

The full fertilization plan including both conventional and organic/microbiological fertilization was implemented in four blocks as follows (**Table 5**):

Table 5: Full fertilization plan

	Fertilizers	Blolck
O1	M2	Block **I**
Co3	Control plot (C)	Block **II**
Co2	M1	Block **III**
Co1	O2	Block **IV**

Figure 18 and **Figure 19** report the *S. montana* growth stages until their harvest in July 2017.

15 days after planting | 35 days after planting | 180 days after planting

Figure 18: *S. montana* plant vegetative development under KIMITEC conditions, Almeria, Spain.

9 months
after planting

before harvest (July
2017) (Start growing)

Figure 19: General view of different stages of *S. montana* plantation under KIMITEC facilities before harvest, Almeria, Spain.

The aerial parts of the plants were harvested (**Figure 20**) and dried for essential oil extraction

Figure 20: Optimal cutting height of *S. montana* plant material at the harvest at Kimitec experimental field, Almeria, spain.

Figure 21: Harvesting *S. montana* raw material in Kimitec experimental field, Almeria, Spain.

2.9.2. Extraction and fractionation

Dried plant materials were extracted by hydrodistillation in a laboratory scale (clevenger) to obtain essential oils from all the samples.

Large-scale (semi-industrial) was simulated by a pilot plant to extract the essential oil of the samples harvested in 2017 in Almeria, KIMITEC (**Figure 22**).

Figure 22: Extraction of essential oil from different *S. montana* plots at laboratory pilot plant-scale.

Semi-industrial extraction giving the used hydrolates

The semi-industrial (large scale) plant extractor equipped with two 3000 L vessels (**Figure 23**)[70] was used to extract EOs from all plant material harvested in CITA and KIMITEC conditions. A clear brown EO was obtained from our chemotype (**Figure 24**).

The decanted hydrolate (aqueous phase) obtained from the essential oil was filtered using a Whatman filter paper to eliminate the essential oil traces. Finally, four hydrolates from two *S. montana* populations of both Zaragoza crops harvested in 2016 and 2017 were obtained. Further, the hydrolates were subjected to liquid-liquid extraction using dichloromethane to obtain an aqueous phase (WP) and an organic phase (OF).

Figure 23: Extraction of essential oil from different *S. montana* plots at large-scale.

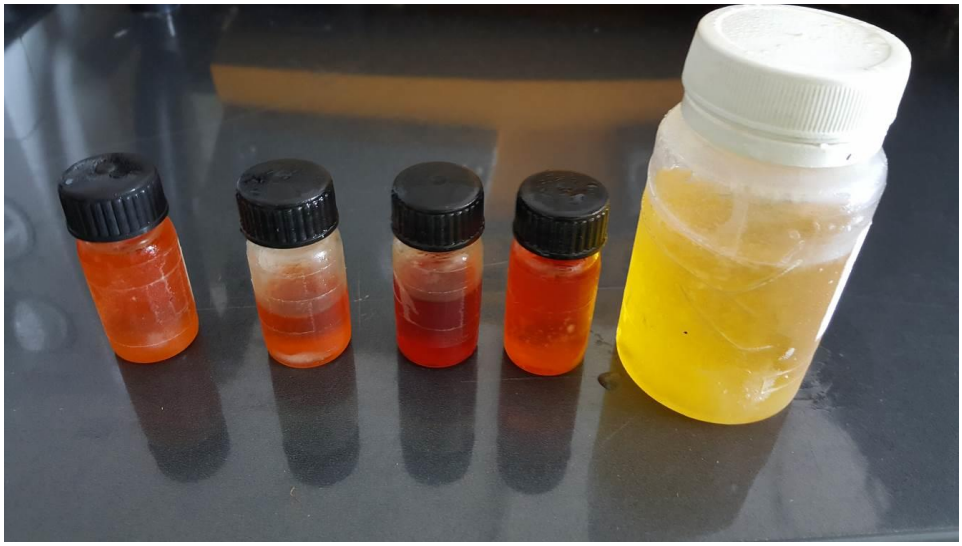


Figure 24: Characteristic color and viscosity of our *S. montana* chemotype compared with commercial sample.

Soxhlet extraction

Dried aerial parts of all harvested plant materials were extracted with ethanol using Soxhlet method. Twenty grams of every plant were introduced into a Whatman filter paper and extracted in a Soxhlet equipment with the corresponding ethanol volume during 20 h. After

cooling, the extract was concentrated to 1 ml using a rotary evaporator and further dried free of solvent using a gentle nitrogen stream overnight.

The overall plant extraction and fractionation of *S. montana* biomasses is summarized on the **Figure 25**.

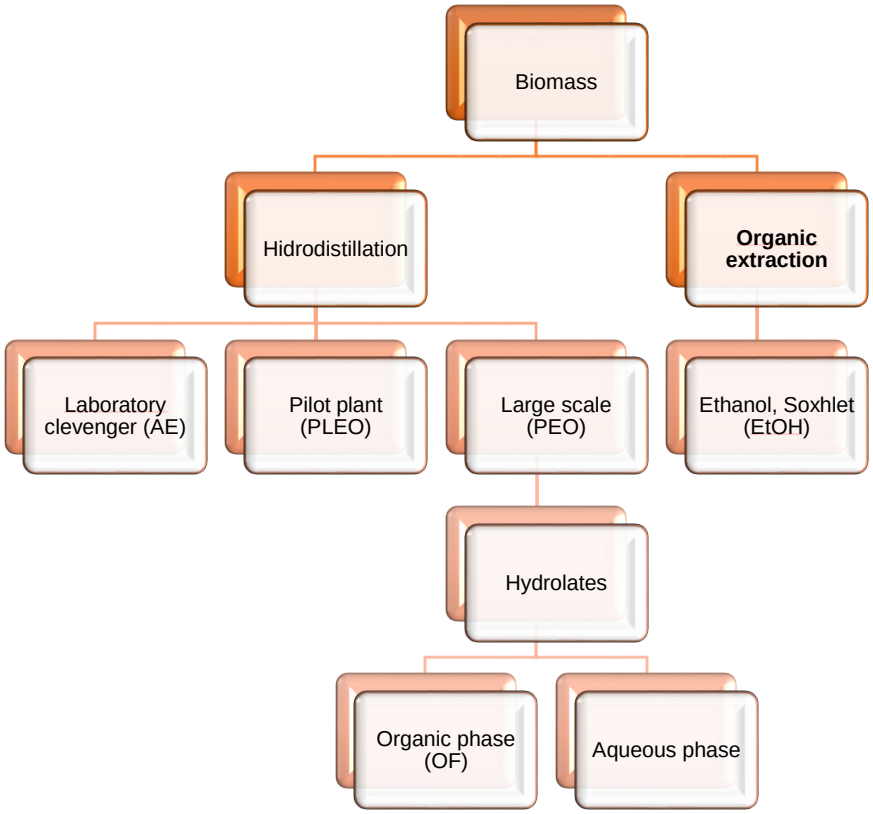


Figure 25: General process of obtaining different extracts of *S. montana*, CITA-KIMITEC.

2.10. GC-MS analyses

2.10.1. Metabolomics analyses

Derivatized samples from nematodes and tomato plant materials were analyzed by a Hewlett-Packard 6850 gas chromatograph, 5973 mass selective detector, and 7683B series injector (Agilent Technologies, Palo Alto, CA). Helium flow was the carrier gas at the flow of 1 mL/min. A total of 1 µL of samples was injected splitless and resolved on a 30 m × 0.25 mm × 0.25 µm DB5-MS column (Agilent Technologies, Palo Alto, CA). The temperatures for the

inlet, interface, and ion source were 250, 250, and 230 °C, respectively. The column temperature was held at 50 °C for 10 min, then increased by 10 °C/min to 300 °C, and held at that point for 4 min. Electron impact (70 eV) mass spectra were recorded from m/z 50 to 550. The resulting data were acquired after 10 min solvent delay and elaborated using MSD ChemStation. Raw data files were exported into the automated mass spectral deconvolution and identification system (AMDIS 2.1) for spectral deconvolution.[71] Database-search was then run against the National Institute of Standards and Technology (NIST) Mass Spectral Database (2.0a) and Golm metabolome database for metabolites identification.[72] Confirmation of sample components was performed by (a) comparison of their relative retention times and mass fragmentation to those of pure standards and (b) computer matching against NIST as well as retention indices as calculated according to Kovats for alkanes C9−C36 compared to those reported by Adams.[73]

2.10.2. Essential oils analyses

Essential oils (EOs) were analyzed by GC-MS using a PerkinElmer Clarus 600 gas chromatograph coupled to a Clarus 600 MS mass detector (electron ionization, 70 eV) and equipped with a 60 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) PerkinElmer DB-5MS dimethyl (95%)–diphenyl (5%). Working conditions were as follows: split ratio (1:150), injector temperature 250 ºC, temperature of the transfer line connected to the mass spectrometer 200 ºC, initial column temperature 60 ºC, then heated to 240 ºC at 5 ºC/min. Components were identified on the basis of comparison or their mass spectra with reference spectra in the NIST MS 2.0 and Adams[73] libraries.

2.11. HPLC-MS analyses:

2.11.1. LC-Q-TOF/MS analysis

Tomato plant extracts were analyzed by reverse-phase liquid chromatography on an Agilent 1200 series LC system using an Kinetex EVO C18, 100 Å, 5 µm, 150×2.1 mm (Phenomenex,

Castel Maggiore, Italy). The LC conditions were as follows: flow rate: 0.3 mL/min; solvent A: 0.1% formic acid in bi-distilled water; solvent B: methanol; and gradient was from 10% to 100% B over 10 min and kept 10 min. Eight microliter of filtered methanol samples were then analyzed by Electrospray ionization in positive and negative modes using an Agilent 6520 Time of Flight (TOF) MS. Mass spectral data were acquired in the m/z range of 100-1,500 with an acquisition rate of 1.35 spectra/s, averaging 10,000 transients. The source parameters were adjusted as follows: drying gas temperature 250°C, drying gas flow rate 5 L/min, nebulizer pressure 45 psi. Based on the original acquisition files, we performed a pre-processing step with MetAlign software used for automated baseline correction and alignment of all extracted mass peaks across all samples. Results were stored as CSV file. ESI/QTOF MS data were then analyzed using the molecular feature extraction algorithm of the MassHunter Workstation software (version B 03.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA). The molecular feature extraction algorithm took all ions into account exceeding 1000 counts with a charge state equal to one. Blank runs showed maximum 10 features with intensity threshold at 1000 counts. Isotope grouping was based on the common organic molecules model.

2.11.2. LC-MS analysis of hydrolates from *Satureja montana*

The hydrolates and the organic fractions of *S. montana* were analyzed by HPLC/MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and a Teknokroma, Mediterranea Sea 18 column (250×4.6 mm, 5 µm particle size) with an ACE 3 C18 analytical guard cartridge. The extracts were eluted with methanol (MeOH) (A): 0.1% acetic acid in milli-Q water (B). The gradient consisted of 38 to 100% of B in 45 min, kept isocratic for 10 min and decreased to 38% in 13 min at 0.5 mL/min and 15 L/min nitrogen (drying gas for solvent evaporation) flow rates. The electrospray capillary potential was set to +4.50 kV and ESI was conducted in the Full

Scan positive mode (m/z = 145-545) with a potential of 1.30 kV and a capillary temperature of 250 °C. Organic extracts stock solutions (250 mg/mL) were prepared in MeOH for sample injection (10 µL). All the solvents used were HPLC-MS grade.[74]

2.12 Statistical analysis

2.12.1. Nematicidal activity

The percentages of dead J2s were corrected by eliminating the natural death in the water or 5% DMSO control according to the Schneider Orellis formula:[75] The mortality in control never exceeded 5% of total number of J2.

$$\text{corrected \%} = \frac{\text{mortality \% in treatment} - \text{mortality \% in control}}{100 - \text{mortality \% in control}} \times 100 \quad (1)$$

and they were analyzed (ANOVA) after being combined over time. Since ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of death J2s treated with tested compounds were subjected to nonlinear regression analysis using the loglogistic equation proposed by Seefeldt *et al* in 1995:[76]

$$Y = C + \frac{D-C}{1+e^{b\log x - \log EC_{50}}} \quad (3)$$

where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the test compounds concentration required for 50% death/immotility of nematodes after elimination of the control (natural death/immotility). In the regression equation, the test compounds or extract concentration (% *w/v)* was the independent variable (x) and the immotile J2 (percentage increase over water and 5% DMSO control) was the dependent variable (y). The mean value of the six replicates per compound concentration and immersion period was used to estimate the LC_{50} and LC_{90} values.

2.12.2. Multivariate analysis

From the data obtained through GC-MS analyses, data matrixes consisting of chromatographic peak areas of each compound was obtained. Peak areas were normalized with respect to the internal standard (2-dodecanone). When a variable showed an abnormal distribution, it was discarded using a logarithmic transformation validated from the Skewness test; statistical test was provided by the SIMCA software (version 13.0, Umetrics, Umea, Sweden). Prior to analysis, data arrays were subjected to centering through the centering unit variance. The matrices obtained were subjected to multivariate analysis. Using software SIMCA-P, the following analyses were made: principal components analysis (PCA) and a regression method (PLS-DA) and its extension orthogonal (OPLS-DA). The quality of the statistical model has been validated based on the parameters R^2X (change in X explained by the model), R^2Y (the total of Y explained) and Q^2 sum parameter in cross-validation experiment.

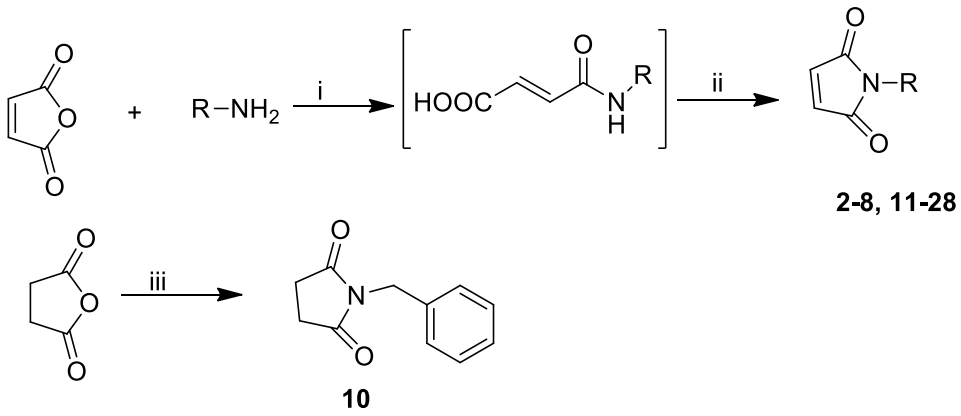
CHAPTER 3

RESULTS AND DISCUSSION

3.1. New chemical compounds for nematode control

3.1.1. Maleimide derivatives

We report in this part, for the first time, the nematicidal activity of maleimide derivatives. A series of maleimide and succinimide derivatives were synthesized through the one-pot two-step procedure described in **Figure 26**, and their nematicidal activity was tested in vitro on second stage juveniles of the root-knot nematode *M. incognita.*



compd	R	compd	R
2	Et	**17**	3,4-diCl-phenyl
3	i-Pr	**18**	3,5-diCl-phenyl
4	i-Bu	**19**	2,3-diCl-phenyl
5	n-Bu	**20**	4-SMe-phenyl
6	1-Me-hexyl	**21**	4-OMe-phenyl
7	1,5-diMe-hexyl	**22**	4-OEt-phenyl
8	benzyl	**23**	3-Cl, 4-OMe-phenyl
11	4-NO_2-phenyl	**24**	4-iPr-phenyl
12	4-Cl-phenyl	**25**	2-naphthyl
13	4-F-phenyl	**26**	4-OH-phenyl

14	4-F, 3-NO_2-phenyl	**27**	4-COOH-phenyl
15	4-CF_3-phenyl	**28**	4-COOEt-phenyl
16	2-Cl-phenyl		

Figure 26: Synthetic route to maleimides. Reagents and conditions: (i) CH_2Cl_2, 1 h, rt; (ii) $(CH_3CO)_2O$, $NaOCOCH_3$, 2 h, 100 °C; (iii) TEA, Toluene, 36 h, reflux.

Initially, we explored the nematicidal activity of the maleimide derivatives testing each compound at the concentration of 100 mg/L after 72 h treatments (**Table 6**). Compounds showing a paralyzing activity greater than 90% were then subjected to $EC_{50/72h}$ determination (**Table 7**). For comparison, abamectin and fosthiazate were used as chemical control with $EC_{50/72h}$ of 2.1 ± 1.2 and 0.6 ± 0.2 mg/L, respectively.

The maleimide, **1**, showed an $EC_{50/72h}$ value of 2.6 ± 1.3 mg/L while the succinimide, **9**, was not active. The *N*-ethylmaleimide, **2**, *N*-isopropylmaleimide, **3**, and *N*-isobutylmaleimide, **4**, showed 100% mortality in the primary screening experiment and $EC_{50/72h}$ values in the 2.6-19 mg/L range (5.1 ± 3.4, 5.1 ± 3.4 and 19.0 ± 9.0, respectively). On the contrary, reduced activity was produced by the *n*-butyl derivative, **5,** as well as by the introduction of a long chain as in compounds, **6**-**8**. Among *N*-arylmaleimides, the 4-nitrophenyl, **11**, the 4-chlorophenyl, **12**, and the 4-carboxyethylphenyl, **28**, were the most effective ($EC_{50/72h}$ = 23.8 ± 3.0, 53.4 ± 3.0 and 60.9 ± 20.6, respectively).

Table 6: Percentage nematicidal activity of tested maleimide and succinimide derivatives.

compd	mortality (%)± SD	compd	mortality (%)± SD
maleimide(1)	100 ± 0.0	**15**	6.9 ± 5.0
2	100 ± 0.0	**16**	12.0 ± 4.0
3	100 ± 0.0	**17**	7.7 ± 3.9
4	100 ± 0.0	**18**	11.0 ± 9.7
5	28.0 ± 8.2	**19**	8.9 ± 3.0
6	35.0 ± 6.6	**20**	7.5 ± 2.9
7	40.0 ± 7.1	**21**	18.8 ± 4.8
8	15.7 ± 9.7	**22**	20.6 ± 10.0
Succimide(9)	NA	**23**	11.0 ± 5.4
10	NA	**24**	22.8 ± 7.5
11	100 ± 0.0	**25**	11.3 ± 2.5
12	100 ± 0.0	**26**	40.5 ± 8.0
13	10.8 ± 2.3	**27**	NA
14	6.4 ± 0.8	**28**	92.3 ± 3.3

NA = not active, SD = standard deviation

The shift of chlorine to the 2-position, **16**, or the introduction of a further chlorine atom, as in maleimides, **17**-**19**, led to a reduction in activity. The nematicidal activity dropped to 6-20% of mortality after 72 h at 100 mg/L when a 4-nitro or a 4-chlorine group was replaced by a fluorine, **13**, trifluoromethyl, **15**, ether, **21**-**22**, or thioether, **20**, groups. Furthermore, the introduction of a nitro, **14**, or a chlorine, **23**, group on the aryl ring failed to enhance the nematicidal activity of maleimides, **13** and **21**, respectively. The replacement of the ester moiety of maleimide, **28**, with a carboxyl group produced the inactive derivative, **27**. The

introduction in the same position of a hydroxyl group produced maleimide, **26**, which also showed a reduced nematicidal activity.

Metabolomics is one of the latest -omics sciences that allows the unsupervised or supervised determination of low molecular mass and polar metabolites in biological samples. In "unsupervised" multivariate analysis, groups of objects that are of a-priori interest are not specified in advance when in "supervised" analysis, groups are specified and the analysis forced to focus on these.

Recently, it is reported the use of metabolomics in detecting upregulated or downregulated metabolites in *M. incognita* treated with different nematicidal arylhydrazones.[68] Using a GC-MS metabolomics approach we detected different endogenous metabolites such as carbohydrates, amino acids, fatty acids, and monoacylglycerols. Statistically significant polar metabolites, deemed important for the model by higher weight, were studied using the software SIMCA-P (Umetrics, Umea, Sweden, ver. 14.0.0.1359). The first step was to perform a PCA to examine the interrelations between groups, clustering and outlier diagnostics among the samples. In the second step, a PLS-DA was performed to maximize the difference of metabolic profiles between treated and control samples and allowing metabolite recognition. The final step of the statistical analysis was to perform a supervised OPLS-DA with the goal to separate samples in two clusters and identify potential biomarkers among nematodes treated with maleimide (**1**) at 8 mg/L for 24 h and the control group. Statistical parameters for the OPLS-DA model were: Q^2=0.63, R^2X=0.60 and R^2Y= 0.80 (**Figure 27**). The permutation test parameters relative to PLS-DA were: R^2=0.61 and Q^2=-0.47, respectively.

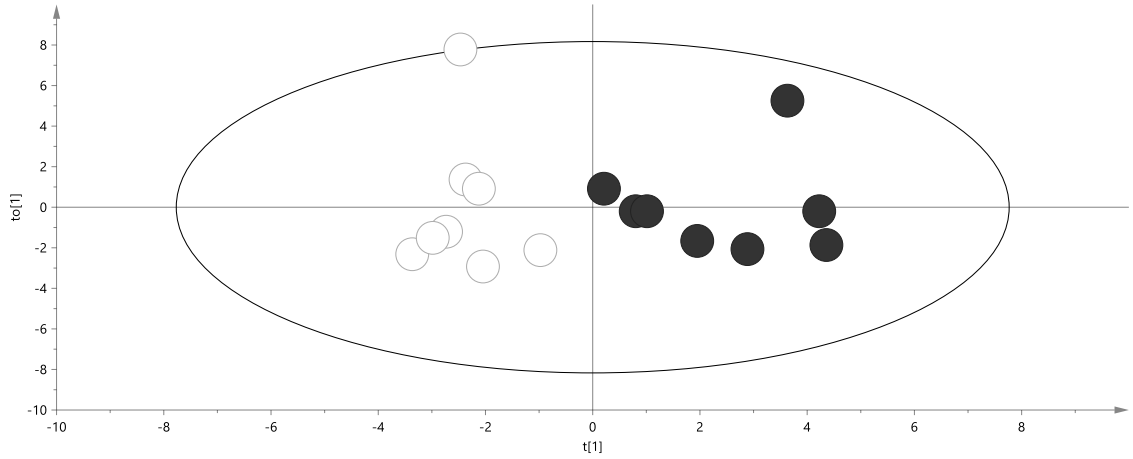


Figure 27: Score plot of OPLS-DA of nematodes treated with maleimide **1** (black circles) vs controls (white circles).

Upregulated metabolites for *M. incognita* nematodes treated with maleimide were in decreasing order oleic acid and palmitic acid, while the downregulated metabolite was 1-monopalmitin. Altered levels of fatty acids are probably related to the monounsaturated fatty acids that are essential components of membrane and storage lipids. Their biosynthesis depends on the conversion of saturated fatty acids to unsaturated fatty acids by Δ^9-desaturases.[77] Biosynthesis of oleic acid starting from palmitic acid in our inhibition experiment with maleimide may be a scavenger strategy of the nematodes to cope with reactive oxygen species.[78, 79] Considering the small pool of metabolites identified in this part of the work, further metabolomic studies were made to generate a different nematode metabolome from other nematode species and genera to contribute to a mechanistic assessment.

Moreover, considering that *vma* yeast mutants, lacking V-ATPase subunits, are hypersensitive to multiple forms of oxidative stress,[80] suggesting an antioxidant role of V-ATPase, we treated nematodes with copper and iron as sulfates, two redox active metals known to be able to produce superoxide radicals.[81] In our experimental conditions, as shown in more details later in this thesis, metal salts were highly toxic to *M. incognita* with EC_{50}s of 48.6 ± 29.8 and

126 ± 48 mg/L for copper and iron sulfate, respectively (**Table 7**). Furthermore, we measured the synergistic activity of maleimide, **1**, with copper and iron sulphate.

Table 7: $EC_{50/72h}$ of the most active maleimide derivatives and copper and iron sulphate solutions.

compd	$EC_{50/72h}$±SD (mg/L)
1	2.6 ± 1.3
2	5.15.1 ± 3.4
3	16.2 ± 5.4
4	19.0 ± 9.0
11	23.8 ± 3.0
12	53.4 ± 3.0
28	60.9 ± 20.6
copper sulfate	48.6 ± 29.8
iron sulfate	126 ± 48
fosthiazate	0.6 ± 0.2
abamectin	2.1 ± 1.2

After 72 h, we observed a synergistic nematicidal activity with maleimide **1** and copper ions with an effect increased by a factor of 10 (**Table 8**).

Table 8: Synergistic activity of maleimide **1** in combination with Cu^{2+} and Fe^{3+} after 24 and 72 h.

		mortality (%)±SD			
		24 h		72 h	
combination	concentration (mg/L)	expected	observed	expected	observed
1/Cu^{2+}	1/50	2.2 ± 2.6	84.6 ± 8.7	8.8 ± 2.5	100
	1/75	25.7 ± 5.5	61.7 ± 4.2	100	100
	2/50	11.4 ± 9.5	93.5 ± 5.8	100	100
	2/75	35.2 ± 9.0	100	100	100
1/Fe^{3+}	1/125	3.6 ± 2.7	35.1 ± 6.5	7.2 ± 2.1	78.2 ± 3.7
	1/200	34.6 ± 5.9	33.2 ± 7.0	89.3 ± 7.3	93.9 ± 5.2
	2/125	12.9 ± 6.2	95.6 ± 3.5	30.7 ± 3.4	100
	2/200	100	100	90.0 ± 5.6	100

Consistent with reported experiments with (*E*)-1-((3-methylthiophen-2-yl)methylene)-2-phenylhydrazine,[68] when nematodes where treated with the V-ATPase inhibitor **1** at 2 and 6 h, we measured a 3.5 fold increase in the excretion of ammonium (**Table 9**). On the other hand, levels of sodium, potassium, magnesium and calcium were not altered.

Table 9: Excretion levels of some cations when nematodes were treated with maleimide **1** at 8 mg/L After 2 and 6 h.

		concentration (mg/L) ± SD*	
cation	time (h)	**1**	control
Na^+	2	2.9 ± 0.3	2.6 ± 0.1
	6	2.5 ± 0.1	2.6 ± 0.2
NH_4^+	2	0.057 ± 0.007	0.016 ± 0.004
	6	0.076 ± 0.012	0.020 ± 0.003
K^+	2	1.1 ± 0.5	0.7 ± 0.1
	6	0.6 ± 0.0	0.6 ± 0.1
Mg^{2+}	2	1.9 ± 0.1	1,9 ± 0.1
	6	1.8 ± 0.2	1,7 ± 0.1
Ca^{2+}	2	6.9 ± 0.8	7.0 ± 0.5
	6	6.2 ± 0.5	6.7 ± 0.3

*SD = Standard deviation

Assuming that maleimide derivatives were able to inhibit the nematode V-ATPase at low EC_{50} values and considering that their strength is greatly increased when used in combination with redox metals such as copper and iron, these compounds may potentially be used to develop new active ingredients with nematicidal properties or be used in the control of root knot nematodes in the field conditions.

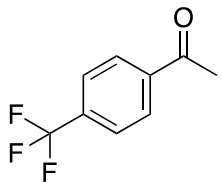
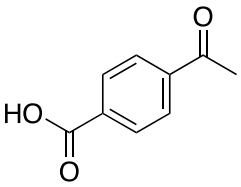
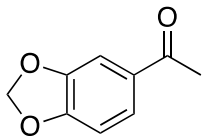
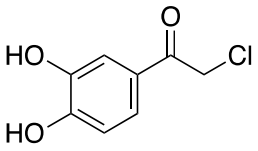
3.1.2 Nematicidal activity of haloacetophenones

Recently, a research reported on the nematicidal activity of α-methylene-γ-butyrolactone (tulipaline A) and some other structurally related compounds, hypothesizing a strict connection

between their activity and ability to hamper V-ATPase functionality.[82] It was postulated a chemical interaction between the nucleophilic thiol groups of the V-ATPase-cysteine residues at the catalytic site of subunit A and the carbonyl group of tulipaline A. In fact, carbonyl groups are known to form covalent adducts with endogenous proteins. This initial proof of concept prompted the investigation of a series of aromatic ketones, with the aim of identifying the structural features required for the explanation of their biological activity. For nematicidal paralysis assays, a series of differently substituted acetophenones bearing electron withdrawing or electron donor groups were selected (**Figure 7**).

Almost all the tested compounds were highly active after 24 h (EC_{50} between 4.42 and 54.8 mg/L) as well as after 72 h (EC_{50} between 3.24 and 65.62 mg/L). The 3',4'-methylenedioxyacetophenone (**12**), was slightly active while compounds 4-acetylbenzoic acid (**5**), and 2-chloro-3',4'-dihydroxyacetophenone (**19**) were completely inactive. Besides, 4'-trifluoromethylacetophenone (**1**) showed a reversible activity, i.e. nematode regaining motility after 72 hours (**Table 10**).

Table 10: Nematicidal activity of acetophenones **1**, **5**, **12** and **19** on M. incognita J2s, tested at 12.5 mg/L after 24 and 72 h.

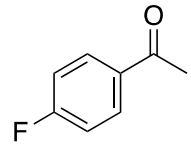
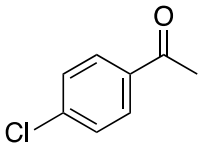
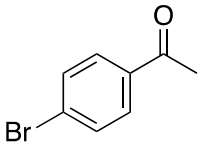
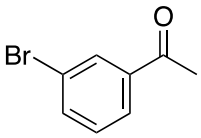
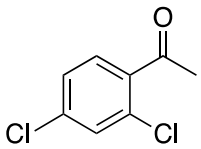
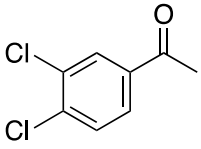
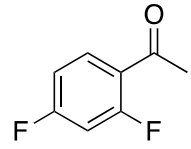
Compound	Structure	$EC_{50/24h}$ ± SD mg/L	$EC_{50/72h}$ ± SD mg/L
1		23.5±15.7	n.a.[a]
5		n.a.[a]	n.a.[a]
12		n.a.[a]	65.6±28.4
19		n.a.[a]	n.a.[a]

a) Not active

Interestingly, the presence of halogens positively affected the nematicidal activity. Since position 4' seemed to be the most promising,[83] the activity of 4'-fluoroacetophenone (**2**), 4'-chloroacetophenone (**3),** and 4'-bromoacetophenone (**4**) were evaluated. The latter, with an $EC_{50/72h}$ of 4.50 ± 3.60 mg/L was the best performing one. This experimental evidence led to the hypothesis that, the less electronegative the substituent the more active the derivative. This agrees with the fact that the thioacetalization reaction can be considered as a soft−soft

interaction, mainly orbital controlled.[84] Then, intrigued by the role of the substituent's position, the 3'-bromoacetophenone (**9**) was tested resulting in a significant decrease of the activity ($EC_{50/72h}$ of 20.66 ±18.70 mg/L).

Table 11: Nematicidal activity of acetophenones **2**, **3**, **4**, **9**, **13**, **15** and **17** against *M. incognita* J2s, tested at 12.5 mg/L after 24 and 72 h.

Compound	Structure	$EC_{50/24\ h}$ ± SD mg/L	$EC_{50/72\ h}$ ± SD mg/L
2		54.8±21.5	17.7±12.3
3		20.2±13.9	11.2±7.3
4		13.01±10.48	4.50±3.60
9		51.09±37.43	20.66±18.70
13		31.32±20.12	26.95±21.40
15		2.49±13.71	2.32±5.49
17		45.22±28.76	22.40±15.32

To determinate if the introduction of another halogen atom onto the aromatic ring might improve the efficacy of the acetophenones, we tested compounds 2',4'-dichloroacetophenone (**13**), 3',4'-dichloroacetophenone (**15**), and 2',4'-difluoroacetophenone (**17**). It was evident that the nematicidal activity was not influenced by the presence of a second halo-atom in position 2', whereas a ring walking of the chlorine from 2' to 3' position, drastically enhanced the efficacy of compound (**15**) (**Table 11**)

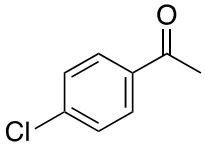
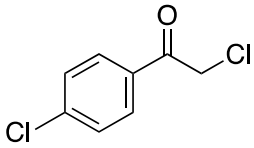
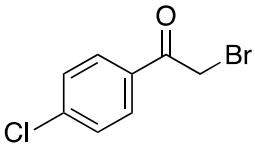
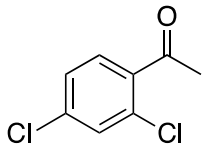
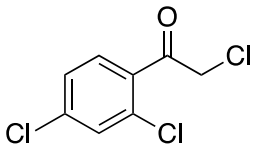
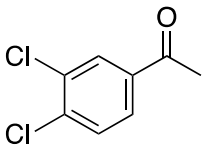
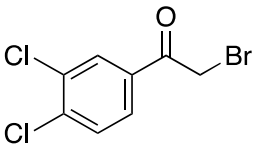
Recent research on novel covalent modulators of V-ATPase, studied the structure–activity relationship of some electrophilic chloroacetamido quinazolines. It was demonstrated that their role was as covalent probes able to inhibit the V-ATPase function through a nucleophilic attack of the cysteine thiol group C138, located at the beginning of the ATP6V1A NHR, at the chloromethylacetamido group.[85] Collectively this interesting research and previous studies on covalent interactions between small molecules and V-ATPase,[82] prompted us to determine a possible role of a halomethyl function directly linked to the acetophenones carbonyl group.

Thus, the nematicidal activity of compounds (**3**), 2,4'-dichloroacetophenone (**6**), and 2-bromo-4'-chloroacetophenone (**8**), was investigated first. The introduction of chlorine at the 2- position as in compound (**6**), significantly increased the activity ($EC_{50/72h}$ = 4.01 ± 2.67 mg/L), while the substitution of chlorine with a bromine in compound (**8**), ($EC_{50/72h}$ = 10.53 ± 9.60 mg/L), did not influence the activity in comparison with (**3**) (Error! Reference source not found.).

The implication of the chlorine in the biological activity of the acetophenones was validated also for the compound 2-chloro-2',4'-dichloroacetophenone (**14**) and particularly evident for 2-chloro-2',4'-difluoroacetophenone (**18)** and 2-chloro-3'-nitroacetophenone (**11**) (**Table 9** and **10**). On the other hand, the introduction of a bromine in compounds 2-bromo-3',4'-dichloroacetophenone (**16**) and 2-bromo-4'-nitroacetophenone (**7**) seemed to decrease the activity. Based on this experimental evidence, we suppose that, if a nucleophilic substitution

reaction is involved, the halogen role is related to its ability to enhance the electrophilicity of the carbon atom in 2-position rather than its leaving group attitude.

Table 12: Nematicidal activity of acetophenones **3**, **6**, **8**, **13**, **14**, **15**, **16**, **17** and **18** against *M. incognita* J2s after 24 and 72 h.

Compound	Structure	$EC_{50/24h}$ ± SD mg/L	$EC_{50/72h}$ ± SD mg/L
3		20.21±13.92	11.24±7.32
6		4.42±2.54	4.01±2.67
8		18.83±14.08	10.53±9.60
13		31.32±20.12	26.95±21.40
14		41.89±37.14	22.97±30.16
15		2.49±13.71	2.32±5.49
16		14.88±9.49	13.49±8.54

It the first part of this work, we reported on the use of metabolomics in detecting upregulated or downregulated metabolites in *M. incognita* model treated with different nematicidal compounds. Using a GC-MS metabolomics approach, we detected 42 different endogenous metabolites such as carbohydrates, amino acids, fatty acids, and monoacylglycerols in another model system consisting of the potato nematode *G. pallida* (**Figure 28**) after treatment with the most potent acetophenone derivative for 24h. This approach is unprecedented and may initiate other studies.

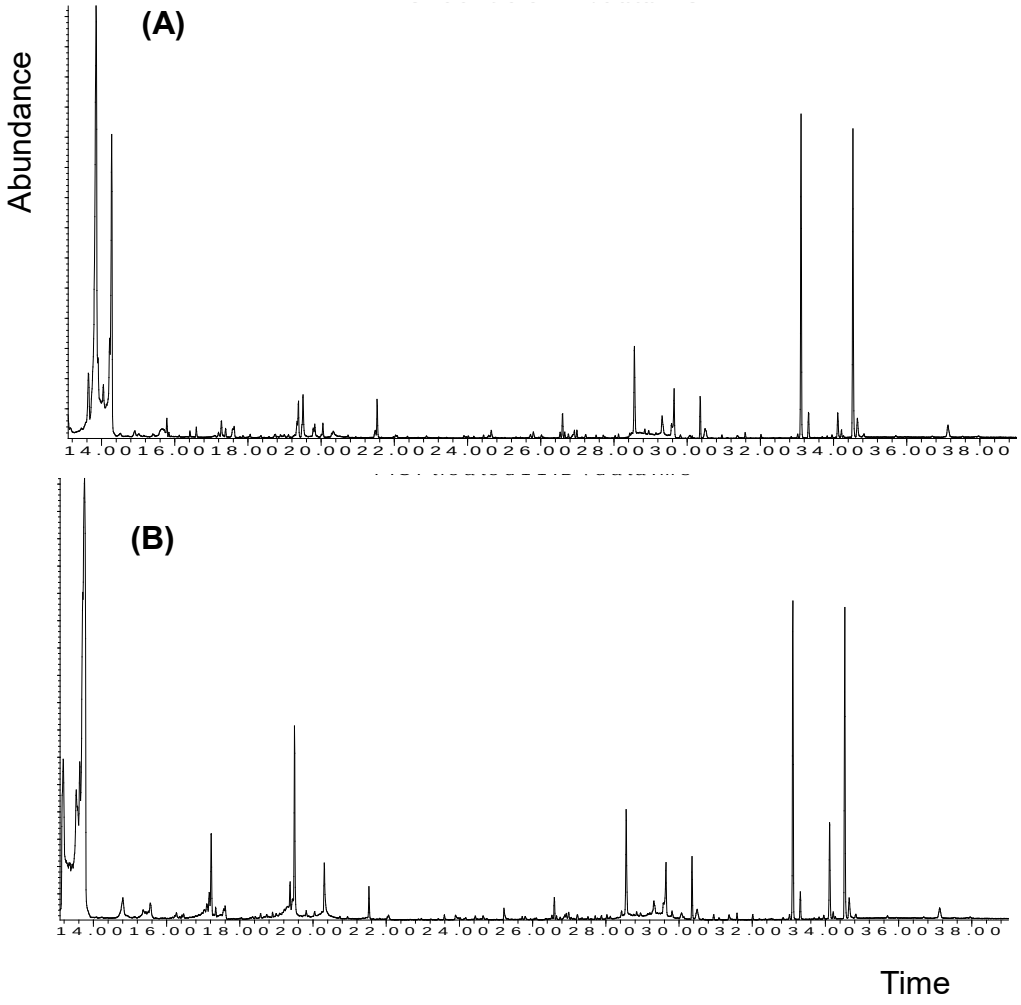


Figure 28: Representative total ion chromatograms of *G. pallida* nematodes extracts used as controls (**A**) compared with nematodes treated with 2,4'-dichloroacetophenone (**6**) (**B**).

Table 13 reports the identified compounds and the non-identified metabolites were referred to as unknown and numbered from U1 to U16.

Table 13: The GC-MS characteristics of nematode extracts.

Compounds	**LRI**	**Quantitative ion**	**EI-MS, *m/z* (amu) (% relative ion abundances)**
lactic acid 2TMS	973	147	147(999), 73 (925), 117 (673)
hexanoic acid TMS	979	117	75(999), 73(954), 117(497)
oxalic acid 2TMS	988	147	73(999), 147(837), 133(320)
U1	989	147	73(999), 147(833), 133(330
3 methyl pentanoic acid 2TMS	997	159	73(999), 159(833), 58(714)
urea 2 TMS	1223	147	147(999), 189(599), 73(545)
glycerol 3TMS	1236	147	73(999), 147 (641), 205 (465)
isonicotinic acid TMS	1241	180	180(999), 106(369), 78(365)
succinic acid 2TMS	1259	147	147(999), 73(547), 75(277)
nonanoic acid TMS	1283	215	75(999), 73(877), 215(681)
U2	1443	147	73(999), 147 (494), 58(291)
pyroglutamic acid 2TMS	1462	156	156(999), 73(717), 75(266)
U3	1483	243	75(999), 73(927), 243(769)
U4	1489	247	247(999), 249(668), 173(258)
laurate acid TMS	1625	257	73(999), 75(958), 117(709)
U5	1634	138	138(999), 58(630), 79(451)
U6	1685	61	61(999), 154(740), 58(533)
galactopyranose 5TMS	1819	204	204(999), 73(733), 191(433)

myoinositol 6TMS	1823	217	73(999), 217(447), 147(379)
fructose 5TMS	1830	217	73(999), 103(469), 217(374)
U7	1837	242	242(999), 73(651), 270(300)
galactose 6TMS	1838	319	73(999), 319(404), 147(362)
mannose TMS	1842	204	204(999), 73(977), 191(506)
U8	1854	147	
inositol 3TMS	1868	318	73(999), 318(593), 305(527)
U9	1877	299	299(999), 74(487), 73(302)
U10	1893	279	61(999), 58(744), 110(644)
U11	1898	200	61(999), 200(913), 58(792)
U12	2014	61	61(999), 58(502), 140(461)
palmitic acid TMS	2021	313	
U13	2042	140	61(999), 73(121), 140(500)
U14	2062	200	61(999), 140(437), 200(421)
stearyl-alcohol TMS	2076	327	327(999), 58(757), 75(531)
U14	2079	279	61(999), 279(260), 110(150)
stearic acid TMS	2220	341	73(999), 117(994), 341(927)
U15	2279	230	61(999), 230(572), 207(534)
1-monomyristin 2TMS	2292	343	343(999), 73(749), 147(410)
2-monopalmitin 2TMS	2471	149	149(999), 57(571), 129(523)
1-monopalmitin 2TMS	2488	371	371(999), 73(342), 372(300)
Sucrose 8TMS	2501	361	361(999), 73(877), 217(458)
U16	2558	204	204(999), 73(705), 191(605)
1-monostearin 2TMS	2578	399	399(999), 73(418), 400(337)

Statistical significant polar metabolites were studied using the software SIMCA-P. Principal component analysis (PCA) of all the samples (n = 20 samples; 10 controls, 10 treated and 42 metabolites) was performed. No separation between controls and treated samples was evident in this unsupervised PCA. However, orthogonal partial least squares discriminant analysis (OPLS-DA) models, validated by permutation analysis, showed good separation between the controls and treated nematodes (Q^2) (cumulative) = 0.623, total amount of variance explained in the x matrix (R^2X) (cumulative) = 0.443; total amount of variance explained in the y matrix (R^2Y) (cumulative) = 0.94; (**Figure 29**).

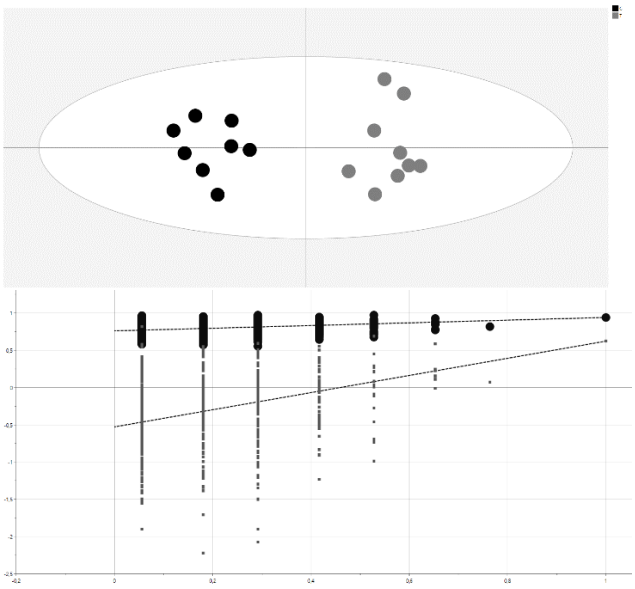


Figure 29: Orthogonal projections to latent structures-discriminant analysis of nematode samples: score plot (upper) of treated with 2,4'-dichloroacetophenone (**6**).(grey circles) and controls (black circles) samples. The lower panel shows the permutations test with validation parameters R^2=(0.0, 0,76), Q^2=(0.0, -0,528).

Upregulated metabolites for *G. pallida* nematodes treated with the test compound were, in order of importance: 1-dodecanol and talose, while the downregulated metabolites were pyroglutamic acid, 1-monopalmitin and 1-monostearin. Increased levels of 1-dodecanol could be explained by V-ATPase dysfunction as described by Yilan et al. that fatty acid starvation

induced fatty alcohol production in *Escherichia coli*.[86] On the other hand, when Zhang et al. treated *Phaeodactylum tricornutum* with bafilomycin A1, a V-ATPase specific inhibitor, fatty acid biosynthesis was downregulated.[87] Since it has been demonstrated that sugar induced hyperosmotic conditions prevent survival of *C. elegans*,[88] we suggested that *G. pallida* produced talose to cope with the osmotic stress, a result of defective vacuolar acidification induced by the nematicidal compound.[89] Furthermore, lipids such as 1-monopalmitin and 1-monostearin were downregulated because fatty acids in the form of neutral lipids provide reservoirs for membrane formation and maintenance in times of stress or nutrient deprivation.[90] We previously reported with maleimides the depletion of monoacylglycerols levels when nematodes were treated with V-ATPase inhibitors in the *M. incognita* model. Our data also showed that 5-oxoproline, a derivative of γ-glutamyl amino acid in cells, was a downregulated discriminant in treated samples. It is known that γ-glutamyl amino acid is formed from glutathione normally transported out of cells where transpeptidation occurs in the presence of amino acids.[91] From this fact, we speculated that pyroglutamic acid diminution might be promoted by glutathione depletion.

In summary, we demonstrated that haloaromatic ketones are highly potent in inducing paralysis and death of the root-knot nematode *M. incognita* and *G. pallida*. In particular, the α-chloro carbonyl moiety emerged as a key residue in improving acetophenones' activity, suggesting that both carbon atoms C1 and C2 might be possible sites for nucleophilic attack of V-ATPase cysteine thiol groups. Noteworthy, almost all the tested compounds exhibited their best nematicidal activity after 72 hours. This propensity to generate a delayed effect indicates that a further work is warranted to determine the mode of action for a possible application for a long-term crop treatment. Acetophenones studied herein for the first time against two nematode species from two different genus, *Meloidogyne* spp. and *Globodera* spp., provide new insight

in the search for novel environmentally sound alternatives to conventional nematicidal compounds.

3.1.3. Nematicidal activity of metal ions on *Meloidogyne incognita* and *Xiphinema index*

We evaluated the in vitro nematicidal activity of different metal ions on juveniles of the root-knot nematode *M. incognita* and the root tips feeding nematode *X. index.* With the aim of exploring the potency of different metals, the percent nematicidal activity of salts was in vitro determined at 500 mg/L after 72 h of treatment. For the root-knot nematode, the most active metals were: silver, copper and iron, and further tested for $EC_{50/72h}$ determination (**Table 14)**. For *M. incognita*, silver nitrate showed an EC_{50} value of 0.05 ± 0.01 mg/L while copper and iron, as sulfates, showed EC_{50} values of 48.6 ± 29.8 and 126 ± 48, respectively. For *X. index*, silver nitrate showed the highest nematicidal activity with 100% J2 mortality at the concentration of 0.13 mg/L while copper, aluminum, iron, cadmium and nickel showed $EC_{50/72h}$ values of 5.1 ± 0.7, 46.0 ± 12.1, 76.3 ± 36.4, 60.7 ± 7.4 and 61.0 ± 7.5 mg/L, respectively.

Table 14: EC_{50} values against *M. incognita* and *X. index*.

Chemical species	$EC_{50/72h}$ ± SD (mg/L)	
	M. incognita	*X. index*
Ag^{+}	0.05 ± 0.01	<0.13
Cu^{2+}	48.6 ± 29.8	5.1 ± 0.7
Fe^{3+}	126.2 ± 48.1	76.3 ± 36.4
Al^{3+}	NA	46.0 ± 12.1
Cd^{2+}	NA	60.7 ± 7.4
Ni^{2+}	NA	61.0 ± 7.5
Zn^{2+}	NA	96.9 ± 6.0
Pb^{2+}	NA	235.1 ± 46.1
NH_4^{+}	NA	NA
Ba^{2+}	NA	NA
Na^{+}	NA	NA
Ca^{2+}	NA	NA
Mg^{2+}	NA	NA
Mn^{2+}	NA	NA
oxamyl	10 ± 0.5	<10

*NA = not active at 500 mg/L. All metals were used in the form of their sulfate salts while silver was used as nitrate salt.

We also tested, in combination with copper ions at 50 and 75 mg/L, the synergistic activity of tannins, known for their nematicidal activity,[92] at the concentration of 3750 mg/L (**Table 15**).

Table 15: Synergistic nematicidal activity on *M. incognita* of copper sulfate in combination with tannins at 3750 mg/L.

Cu^{2+}(mg/L)		mortality (%)±SD		
		expected	observed	fold
50	24h	4 ± 4	15 ± 6	3.75
75		36 ± 8	88 ± 6	2.4
50	72h	79 ± 6	100	1.27
75		100	100	-

Silver nitrate and copper sulfate showed the strongest activity on the root-knot nematode *M. incognita*. These results are corroborated by previous works reporting different relative bioactivities of Ag and Cu nanoparticles.[93-95] The antimicrobial effects of silver have been recognized for thousands of years.[96] Its applications range from water purification, medical treatments to consumer products. Since that nematodes are considered as the most promising candidates as soil bioindicator and contamination with heavy metals,[97] our results showed that different species of nematodes might respond differentially to metal ions. Despite little is known about the mechanism of action of metals in nematodes, their bioactivity is related to donor atom selectivity, reduction potential and speciation. On microorganisms metals cause discrete and distinct types of perturbations as a result of oxidative stress, protein dysfunction or membrane damage.[98]

Copper and Zinc oxide nanoparticles were reported for their antimicrobial activity.[99] To determine their nematicidal activity, we tested in vitro, CuO and ZnO in micro- and nanoscale forms at 500 mg/L on *M. incognita*. After 72 h, these oxides did not inhibit root-knot nematode mobility. Copper oxide at the tested concentration may release less Cu^{2+} to overwhelm

nematode defense system and induce paralysis activity. Using higher concentrations of the readily available copper oxide nanomaterials might not be suggested in crop protection against parasitic worms because of the copper oxide nanoparticles toxicity.[100]

Moreover, we tested the synergistic activity of metal ions with tannins. Tannins were not active at 5000 mg/L when tested in vitro on *M. incognita* but were able, in vitro, to strengthen copper ions nematicidal activity. In the literature, tannins were reported active against cyst nematodes and *L. elongates,* a virus vector of plant soil borne disease.[101] A formulation containing tannins and copper may have a multi-protection effect for crops.

Metabolomics is one of the last omics science allowing the unsupervised or supervised determination of low molecular mass metabolites in biological samples. Specifically, to study biological pathway modifications in nematodes with microscopic size, mass spectrometry-based metabolomics is a good tool for this type of investigation. In the first parts, we reported on the use of metabolomics in detecting upregulated or downregulated metabolites in *M. incognita* and *G. pallida* treated with different nematicidal maleimide and haloacetophenones. In this part, using a GC-MS metabolomics approach, we detected different endogenous metabolites such as carbohydrates, amino acids, fatty acids, and monoacylglycerols in *M. incognita* and *X. index* models (**Figure 30**).

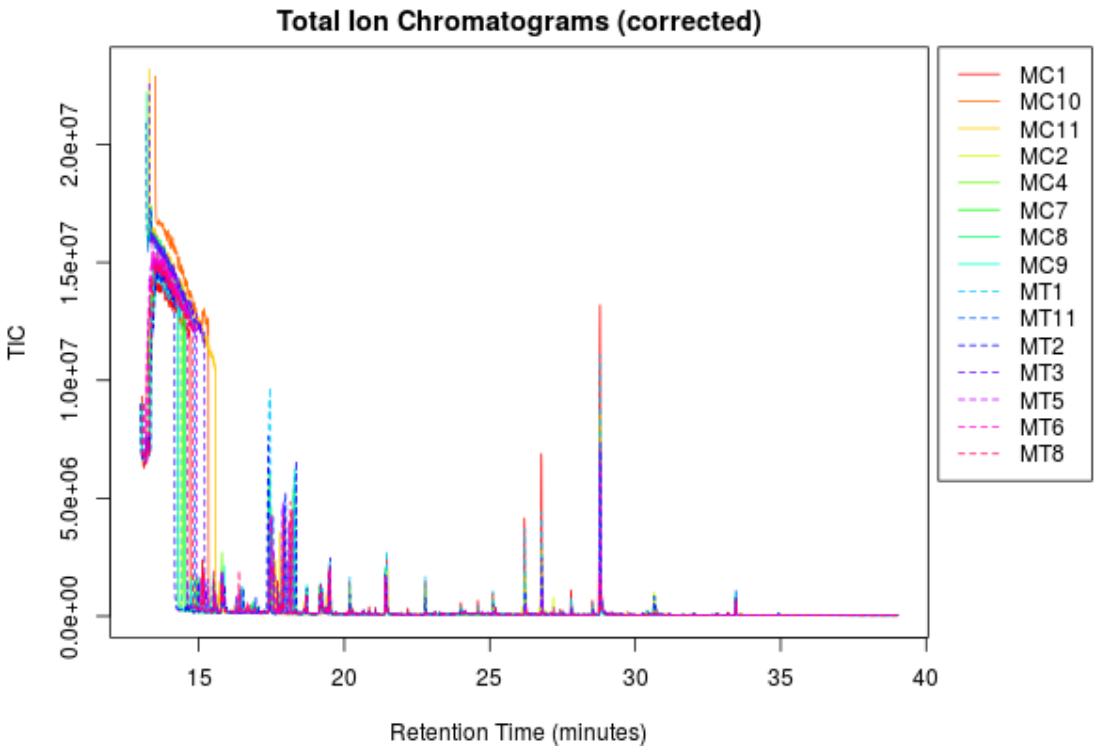


Figure 30: Retention time corrected total ion GC-MS chromatograms of overlaid chromatograms of polar metabolite extractions from *M. incognita* nematodes treated with silver nitrate (MT) salt and control (MC).

After a multivariate analysis of the GC-MS data, an OPLS-DA was able to discriminate between controls and treated nematode, i.e. *M. incognita* and *X. index*, treated with copper at 150 mg/L and the control after 24 h of treatment (**Figures 31** and **32**). Q^2, R^2X and R^2Y values found for the two experiments were 0.65, 0.84, 0.99 and 0.81, 087, and 0.99, respectively, showing a good statistical and predictive model.

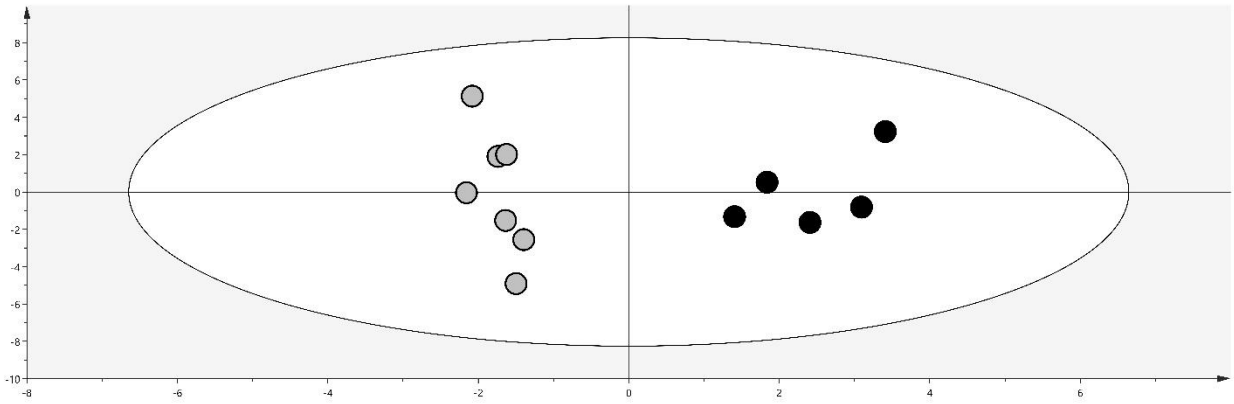


Figure 31: Score plot of OPLS-DA of M. incognita treated with Cu^{2+} at (150 mg/L) as sulfate salt– Each point represents a pool of 300 nematodes. Black circles represent treated nematodes with copper sulfate and gray circles represent control nematodes treated with tap water.

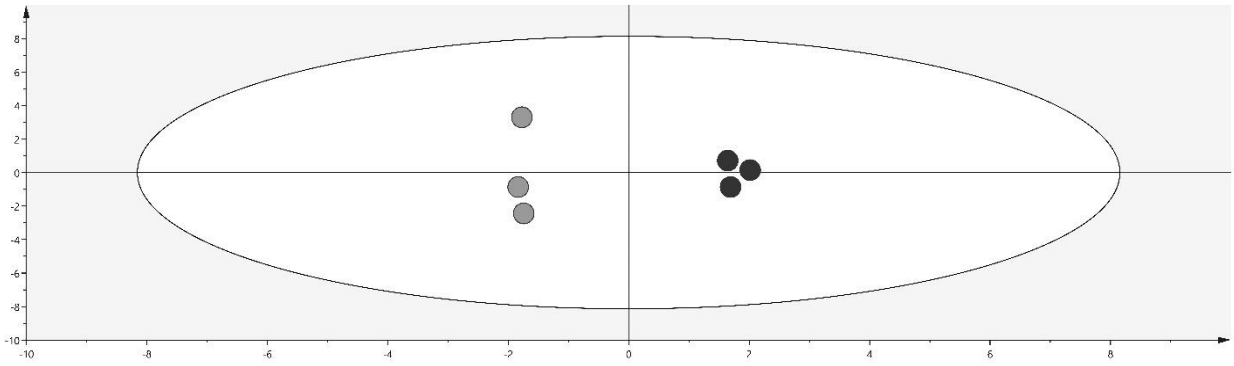


Figure 32: Score plot of OPLS-DA of X. index treated with Cu2+ at (150 mg/L) as sulfate salt– each point represents a pool of 20 nematodes. Black circles represent treated nematodes with copper sulfate and gray circles represent control nematodes treated with tap water.

Discriminant components between treated nematodes and controls were especially lipids. Upregulated metabolites for *M. incognita* nematodes treated with copper were in decreasing order 2-monostearin, palmitic acid and lactic acid while downregulated metabolites were methyl malic acid, 2-monomyristin and 2-arachidonoylglycerol (**Table 16**)

Table 16: Metabolomics of *M. incognita* treated with Cu^{2+} (150 mg/L) as copper sulfate. regulation of metabolites in treated samples.

Downregulated	**Upregulated**
methylmaleic acid	2-monostearin
1-monomyristin	palmitic acid
arachidonoylglycerol	lactic acid

On the other hand, discriminant compounds of the dagger nematodes treated with Cu^{2+} were similar to those of the root-knot nematodes, i.e. fatty acids and monoacylglycerols such as stearin, 2-arachidonoylglycerol and monomyristin levels were increased when 2-monostearin levels were decreased (**Table 17**).

Table 17: Metabolomics of *X. index* treated with Cu^{2+} (150 mg/L) as copper sulfate. regulation of metabolites in treated samples.

Downregulated	Upregulated
Stearin	2-monostearin
arachidonoylglycerol	
1-monomyristin	

Moreover, when we treated *M. incognita* with silver nitrate and data processed online by XCMS software package,[102] glycerol and glyoxylic acid were upregulated (**Table 18**).

Table 18: Regulation of metabolites respect to the controls when nematodes were treated with silver ions as copper sulfate at 10 mg/L for 24 h.

Compounds	Pvalue	Updown	Fold change
Glycerol	0.011	up	1.3
glycoxylic acid	0.028	up	1.3

Lipids levels might be perturbed because fatty acids play a key role in membrane formation and maintenance during oxidative stress triggered by metals.[103] O'Riordan et al. reported the importance of the glyoxylate pathway and lipid stores as an energy reserve for the non-feeding dauer larva of fat of *C. elegans*.[104] Our results are in agreement with those findings because the upregulated level of glyoxylic acid in treated nematodes with silver ions might be justified by a similar modification of glyoxylate cycle by *M. incognita* to adapt to a highly oxidation environment induced by metals. Subsequently, downregulation of monoacylglycerols levels matches with the previous study when *M. incognita* was challenged with nematicidal maleimide derivatives.[105] Furthermore, we observed an increased level of glycerol when we treated nematodes with silver ions. Worms under stress, may fail to control body liquids as described by an earlier study, reporting glycerol falling levels of *C. elegans* nematodes in air dry conditions.[106]

Finally, we measured up to eight folds change ammonia production by nematodes when they were treated with copper sulfate respect to controls (**Table 19**). Nematodes are likely to possess the ability to tolerate quite large variations in their internal osmotic pressure and capable of regulating the water or ionic content of their body fluids do to a wide environment fluctuation.[107] Furthermore, vacuolar H(+)-ATPase which plays an important role in oxidative stress is also involved in the acidification of eukaryotic intracellular compartments.[108] Thus,

ammonia production by the nematode might be an alternative way for the worms to cope with osmotic pressure variations in the case of copper salt stress.

Table 19: Ionic chromatography showing ammonia excretion by nematodes treated for 2 and 6 hours with copper ions as sulfate salt at 200 ppm.

ions	duration (h)	concentration (mg/L)	
		Cu^{2+}	control
Na^{+}	2	2.4 ± 0.1	2.6 ± 0.1
	6	2.3 ± 0.2	2.6 ± 0.2
NH_4^{+}	2	0.116 ± 0.001	0.016 ± 0.004
	6	0.100 ± 0.006	0.020 ± 0.003
K^{+}	2	0.6 ± 0.1	0.7 ± 0.1
	6	0.6 ± 0.1	0.6 ± 0.1
Mg^{2+}	2	1.9 ± 0.1	1.9 ± 0.1
	6	1.6 ± 0.2	1.7 ± 0.1
Ca^{2+}	2	6.6 ± 0.4	7.0 ± 0.5
	6	5.6 ± 0.6	6.7 ± 0.3

In conclusion, in the present work, we evaluated for the first time the toxicity of different metal salts on two nematode species: *M. incognita* and *X. index*. Silver and copper ions were the most active on both worms with relative low EC_{50} values. Finally, metabolomics experiments on J2 nematodes treated with Cu^{2+} and Ag^{+} allowed us to identify different metabolites linked to the oxidative stress. More studies may be needed to quantify those metabolites in a targeted experimentation and establish kinetics of metal toxicities to the worms. Moreover, field use of

formulations containing copper and silver may be suggested for the parasitic nematode controls.

3.2. Untargeted metabolomics of tomato plants after root-knot nematode infestation

Gas chromatography coupled to mass spectrometry unsupervised metabolite profiling was developed to try to understand the dynamics of the *S. lycopersicum* response to the infestation of the root knot nematode *M. incognita.* Two months after RKN infestation, different plant parts, i.e. stem and leaves, were extracted with methanol and after derivatization submitted to GC-MS fingerprinting metabolomics analysis comparing levels of each metabolite to the equivalent control. Representative GC-MS chromatograms of tomato leaves and stems are reported in **Figure 33**. A total of 50 low molecular weight polar metabolites were detected of which 8 were unknowns (**Table 20**).

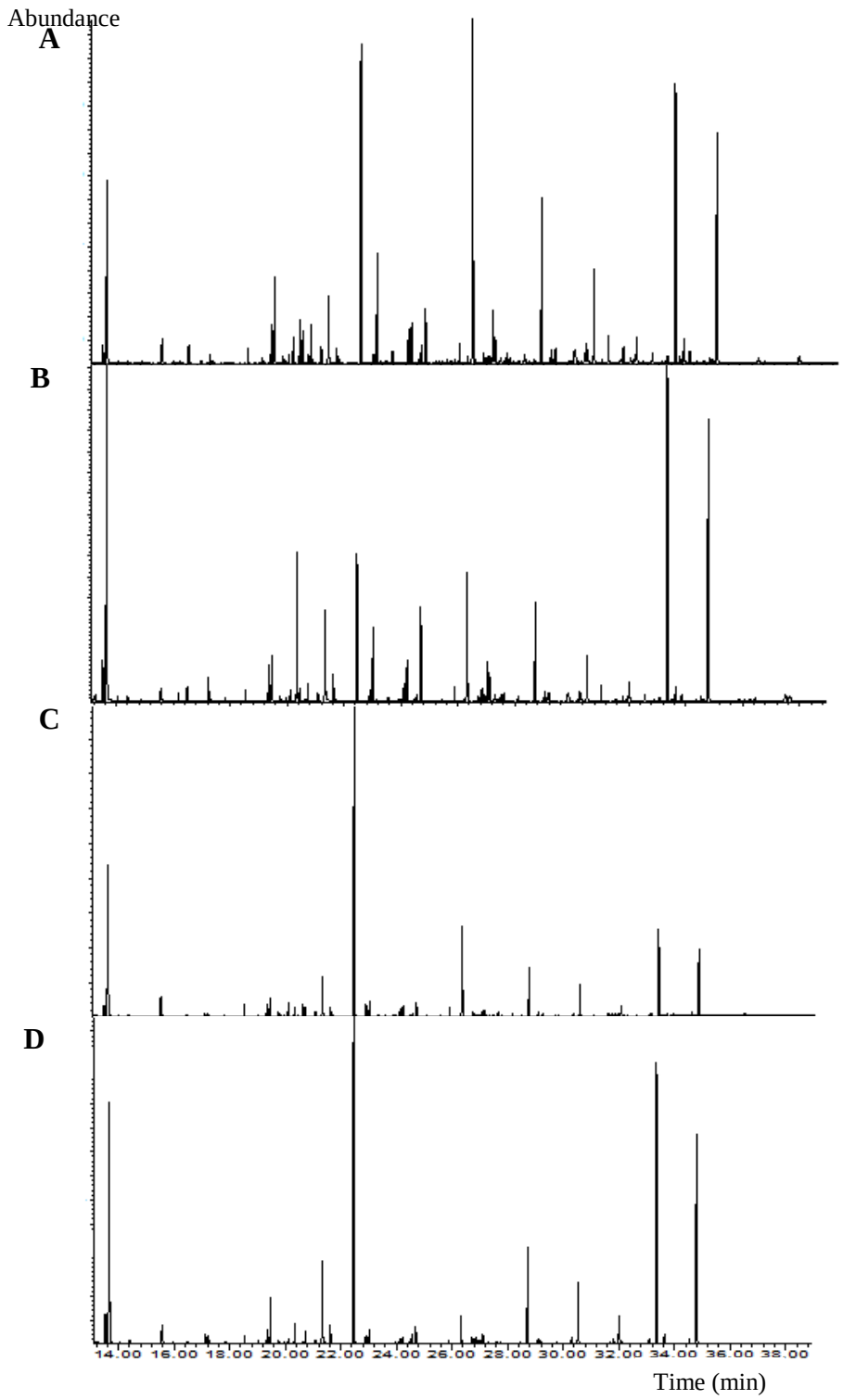


Figure 33: GC-MS TIC Chromatograms of *Solanum lycopersicum* cv. Rutgers plant extracts.**(A)** leaves of uninfested plants**, (B)** leaves of *M. incognita* infested plants, **(C)** stems of uninfested plants and **(D)** stems of *M. incognita* infested plants, analysis.

Table 20: GC-MS analysis of tomato plant after root-knot nematode infestation.

Cmpd[a]	***m/z***[b]	**LRI**[c]	**Cmpd**[a]	***m/z***[b]	**LRI**[c]
lactic acid $(TMS)_2$	117	973	unknown 3	103	1617
alanine $(TMS)_2$	116	1005	ribose	103	1629
2-hexanoic acid (TMS)	171	1024	glutamine $(TMS)_3$	156	1632
3-hydroxypropanoic acid $(TMS)_2$	177	1043	citric acid $(TMS)_4$	273	1691
3-hydroxy-2-methylpropanoic acid $(TMS)_2$	117	1056	myristic acid (TMS)	117	1813
valine $(TMS)_2$	144	1098	Fructose (MEOX) $(TMS)_5$	103	1832
phosphoric acid $(TMS)_3$	299	1148	galactose	331	1838
glycerol $(TMS)_3$	205	1149	glucose	117	1847
isoleucine $(TMS)_2$	158	1167	unknown 4	204	1851
proline $(TMS)_2$	142	1170	tyrosine $(TMS)_3$	218	1871
glycine $(TMS)_3$	174	1177	palmitic acid (TMS)	117	1876
succinic acid $(TMS)_2$	75	1186	inositol $(TMS)_6$	217	2033
glyceric acid $(TMS)_3$	189	1197	tryptophan $(TMS)_3$	202	2051
uracil $(TMS)_2$	241	1205	unknown 5	117	2217

fumaric acid $(TMS)_2$	245		stearic acid (TMS)	117	2220
serine $(TMS)_3$	204	1219	melibiose $(TMS)_8$	204	2234
threonine $(TMS)_3$	218	1225	unknown 6	204	2263
unknown 1	228	1247	glycerol myristate $(TMS)_2$	343	2393
β-alanine $(TMS)_3$	174	1413	1-monopalmitoyl glycerol $(TMS)_2$	371	2409
malic acid $(TMS)_3$	233	1415	sucrose $(TMS)_8$	361	2604
aspartic acid $(TMS)_3$	232	1446	unknown 7	217	2625
pyroglutamic acid $(TMS)_2$	156	1462	2-monostearin $(TMS)_2$	129	2640
γ-aminobutyric acid	174	1464	1-monostearin $(TMS)_2$	399	2689
glutamic acid $(TMS)_3$	246	1468	maltose $(TMS)_8$	85	2807
phenylalanine $(TMS)_2$	218	1612	unknown 8	217	3000

[a]MEOX, methoxime derivative; TMS, trimethylsilyl derivative. [b] Masses shown are those of the ion(s) selected for identification and quantitation of individual derivatized metabolites. [c] Values shown are linear retention indices based on linear interpolation of retention times between adjacent alkane retention standards.

Macroscopic foliar symptoms of nematode infestation of roots generally involve stunting and general unthriftiness, premature wilting and leaf chlorosis.[109] On the other hand, no macroscopic changes were observable on the plant material used for this study (**Figure 34**). From a metabolomics point of view RKN infestation appears to cause a metabolic response, mainly of primary metabolism and different from leaves and sink tissues.

Figure 34: Tomato plants: (A) infested and (B) not infested with *M. incognita*.

Statistical significant polar metabolites were studied using SIMCA-P. We first performed a PCA to examine interrelation between groups, clustering and outlier diagnostics among the samples. We discarded one outlier corresponding to a tomato leave from the control sample because outside of the Hotteling's T^2 area and after a DmodX test implemented by SIMCA-P. After this step, we performed a PLS-DA to maximize the difference of metabolic profiles between treated and control samples and allowing metabolite recognition. The following step of the statistical analysis was to perform a supervised OPLS-DA with the goal to separate samples in two clusters and identify biomarkers between the control and treated groups (**Figures 35** and **36**). Validation parameters for the two OPLS-DA were $R^2Y= 0.91$ and $Q^2Y= 0.84$ for stems while for leaves $R^2Y= 0.97$ and $Q^2Y= 0.89$.

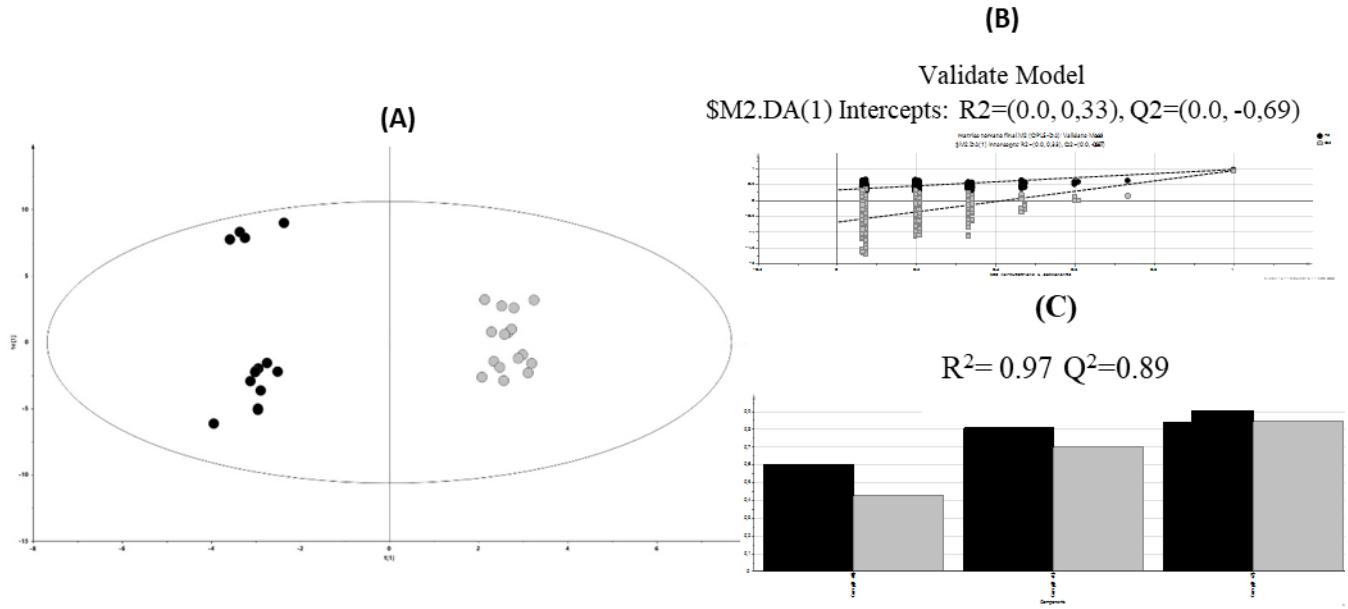


Figure 35: OPLS-DA of the not infested (black) vs infested leaves (white): (A) score plot (B) permutations analysis (C) multivariate statistic parameters.

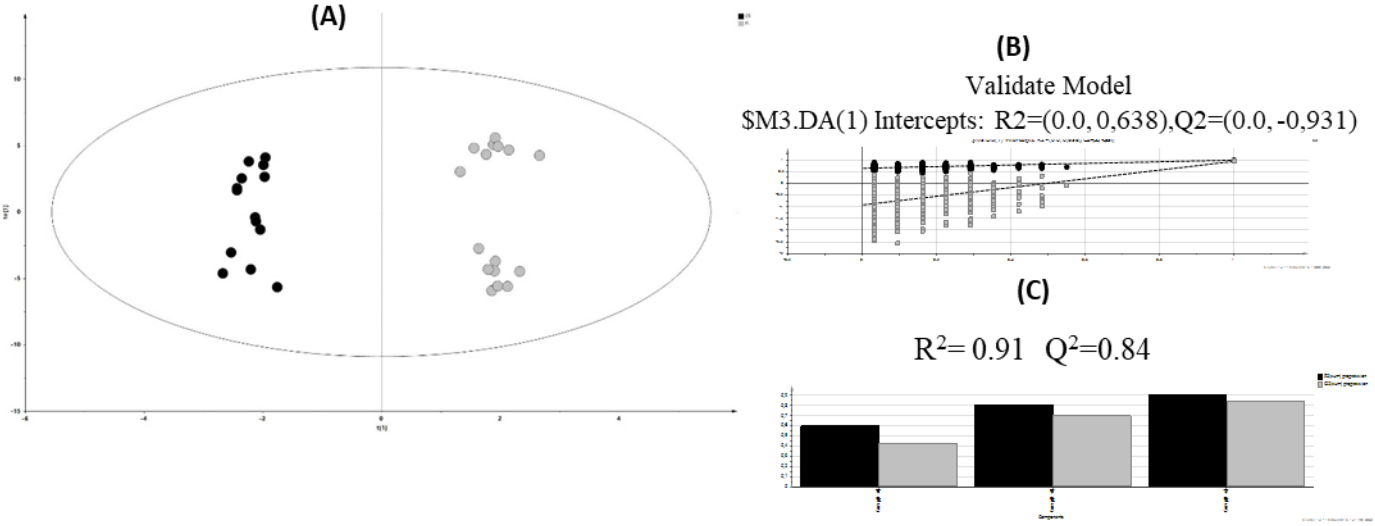


Figure 36: PLS-DA of the not infested (black) vs infested stems (white): (A) score plot (B) permutations analysis (C) multivariate statistic parameters.

Discriminating metabolites reported in **Table 21** were selected from the VIP-plot (selecting VIP>1). Metabolic pathway analysis was performed using the web platform MetaboAnalyst (ver. 3.0),[69] combining the topology with a powerful pathway enrichment analysis.

Table 21: Log2(fold-change) of discriminant plant metabolites after root-knot nematode infestation.

cmpd classes	**metabolites**	**leaves**	**Stem**
sugars	sucrose		1.41
	fructose		1.31
	ribose	-1.08	0.74
	melibiose	0.60	-0.50
	glycerol	-0.58	
	glucose		0.40
amino acids	β-alanine	1.89	
	glycine		-0.85
	phenylalanine	0.71	
fatty acids	myristic acid	-0.88	
	palmitic acid	-0.68	
dicarboxylic acids	fumaric acid		-1.47

The changes in the metabolite or potential biomarkers suggested that seven biochemical pathways were altered by nematode infestation. As shown in **Figure 37**, the most disturbed pathways were β-alanine metabolism, phenylalanine metabolism, pantothenate and CoA biosynthesis, glycine, serine and threonine metabolism, galactose and sucrose metabolism.

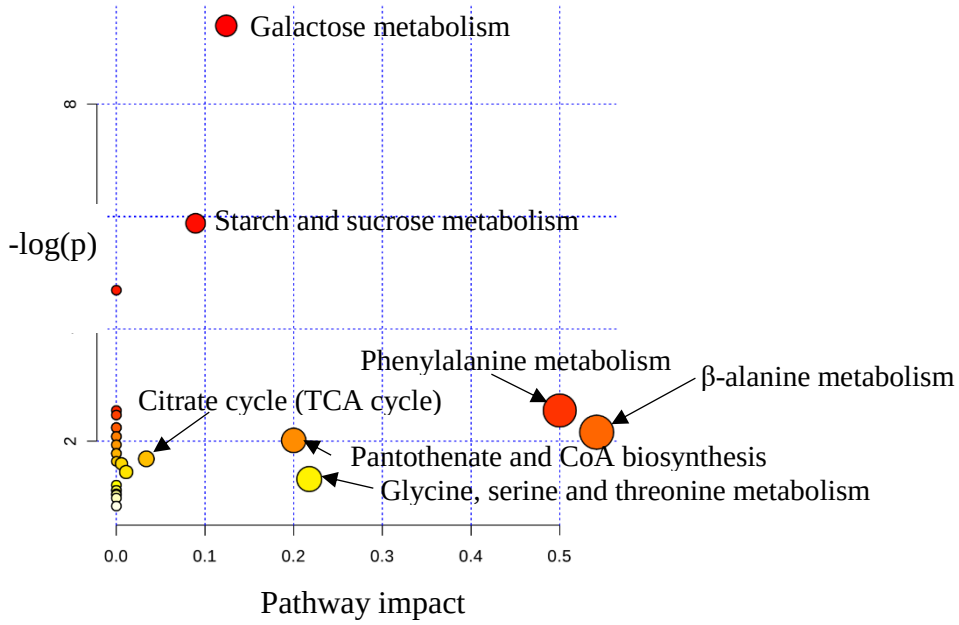


Figure 37: Pathway analysis showing changes in the metabolism of tomato plant after root-knot nematode infestation.

After nematode infestation, *S. lycopersicum* leaves metabolites, β-alanine and Phe were upregulated with a Log2 fold change of 1.89 and 0.71, respectively. β-alanine and Phe are central components of the pantothenate biosynthesis and β-alanine metabolism, respectively (**Figure 37**). Ala can be derived from the degradation of polyamines such as spermidine and spermine.[110] Hewezi et al. reported that in *Arabidopsis*, spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*.[111] The subsequent degradation of spermidine through polyamine oxidase stimulates the induction of the plant antioxidant machinery, protecting the nematode feeding structure from reactive oxygen species that are produced as a common response of host plants to nematode infestation.[112] In leaves, melibiose was found upregulated while ribose was downregulated suggesting a possible inhibition of the plant glycosyl hydrolase.[110] In fact, protein extracts and exudates from a number of phytoparasitic and fungal-feeding nematode species contained cellulolytic, amylolytic, chitinolytic, and pectolytic enzyme activity, suggesting the potential for endogenous

production and secretion of cell wall-degrading enzymes from nematodes. Furthermore, glycerol biosynthesis was strongly downregulated in leaves. Levels of saturated fatty acids, such as palmitic acid and myristic acid, were reduced in leaves when compared to controls. This fact is corroborated by Agarrwal et al. who reported the downregulation of saturated fatty acids in rice as a response to the Asian rice gall midge infestation.[113]

On the contrary, in stems soluble sugars such as glucose, ribose, fructose, and sucrose were upregulated while melibiose was downregulated suggesting that melibiose is consumed by the gylcosyl hydrolase to yield glucose. Notably, modifications of sugar levels involved in the stress response pathways such as signaling, osmotic adjustment, and respiration for energy production were found by Merewitz et al. in *ipt* transgenic creeping bentgrass in a drought tolerance experiment.[114] Soluble sugars such as glucose, fructose, and sucrose are recognized as carbon and energy sources,[115] and as well as signaling molecules in plants.[116] After a comparative metabolomics analysis, Quian et al. reported that soluble sugars and glycerol were upregulated in *Arabidopsis* challenged with a bacterial pathogen.[117] Sugar signals may also contribute to immune responses against pathogens and probably function as priming molecules leading to pathogen-associated molecular patterns (PAMP)-triggered immunity and effector-triggered immunity in plants.[118]

Additionally, the metabolic data presented here showed that RKN infestation in tomato plants caused a marked reduction of glycine in stems. The latter is consumed by the glycine decarboxylase complex and functions in photorespiration producing ammonia. Moreover, fumaric acid was downregulated in stems and this fact is in accordance with Guo et al. who reported reduced levels of fumaric acid in response to salt and alkali stress in wheat seedlings.[119] The stem content of the tricarboxylic acid cycle intermediate fumaric acid was strongly reduced, reflecting a likely higher demand for reducing equivalents required for

defense responses reported in an *Arabidopsis* model challenged by soil-borne fungus *Verticillium dahliae* Kleb.[120]

We also performed a LC-QTOF metabolomics analysis of both plant tissues. After a supervised statistical analysis OPLS-DA we found that the most upregulated metabolite in tomato leaves was an *O*-acylsugar with a formula $C_{29}H_{48}O_{15}$ corresponding to *m/z* = 654.3335 $[M+NH_4]^+$ a non-volatile secondary metabolite reported for *S. lycopersicum* by Schilmiller et al.[121-123] O-acylsugars are specialized metabolites produced by glandular trichromes; epidermal secretory structures that play a crucial defensive role in many plant species.[123]

Concluding, a GC-MS metabolomics analysis provides a powerful and reliable approach to study the levels and changes of plant metabolites after RKN infestation. We have measured quantitative variation of different plant metabolites associated with nematode infestation. According to MetaboAnalyst, β-alanine metabolism, phenylalanine metabolism, pantothenate and CoA biosynthesis, glycine, serine and threonine metabolism and soluble sugars metabolism were found altered in our *M. incognita* infestation model. This is the first study to report a GC-MS metabolomics analysis of tomato plants infested with the root-knot nematode and our results indicated that metabolomics methods have adequate sensitivity and specificity to distinguish infested plant from controls. The combination of several -omic techniques (such as metabolomics, lipidomics, proteomics, genomics and transcriptomics) will be helpful in understanding the plant-nematodes interaction with the goal of improving plant health and growth while reducing nematode infestation. Moreover, assuming that we analyzed a partial set of *S. lycopersicum* plant metabolites, i.e., polar low-molecular-weight compounds, and at only one time of infestation, more studies are needed to have a complete view of the picture.

3.3. *Satureja montana*

Volatile oils were obtained from the classic hydrodistillation using the Clevenger apparatus in laboratory, a semi-industrial plant and a pilot plant. Average *S. montana* essential oil yields (% v/w, oil/plant biomass) for each extraction method, laboratory Clevenger, pilot and semi-industrial plants were 0.638%, 0.535 and 0.275%, respectively. Frequently reported *S. montana* essential oil extraction yield range in laboratory scale is between 0.4-0.7 %.[124, 125] The relative low yield obtained from the actual industrial plant may be due to reduced extraction time for energy management and losses due to essential oil recuperation.

The chemical composition of the different EOs of *S. montana* analyzed by GC-MS is presented in **Table 22**. Thirty-two compounds were identified in all the essential oils representing more than 98% of the total EOs with carvacrol (31-71%), *p*-cymene (4-23%), γ-terpinene (7-16%) and thymol (4.79-8.85%) the major components. The high level of these compounds is justified by the fact that the biosynthesis of all those compounds are related because γ-terpinene is converted to *p*-cymene, thymol and carvacrol. Carvacrol was reported as the major component of some subspecies of *S. montana* essential oils by other authors (30-85%)[34, 126, 127] while some authors usually report thymol, *p*-cymene or borneol as the major components.[37, 128, 129] However, the percentage of some individual constituents present in the EOs, extracted with different methods, differed significantly as reported in **Table 23** ($P<0.05$) and the Dendrogram of **Figure 38**. For instance, more than 5-fold differences were observed in the percentages of γ-terpinene, 1,8-cineol and thymol between laboratory and pilot scale extractions on one hand and the semi-industrial extraction on the other. The ANOVA result of the chemical composition of the EOs independently of the agronomic conditions indicated that the EO extraction methods play an important role in components recovery. The Dendrogram showed a better separation of Kimitec samples by EO extraction method than fertilizers. CITA samples have a similar chemical composition to Kimitec organic fertilized plants.

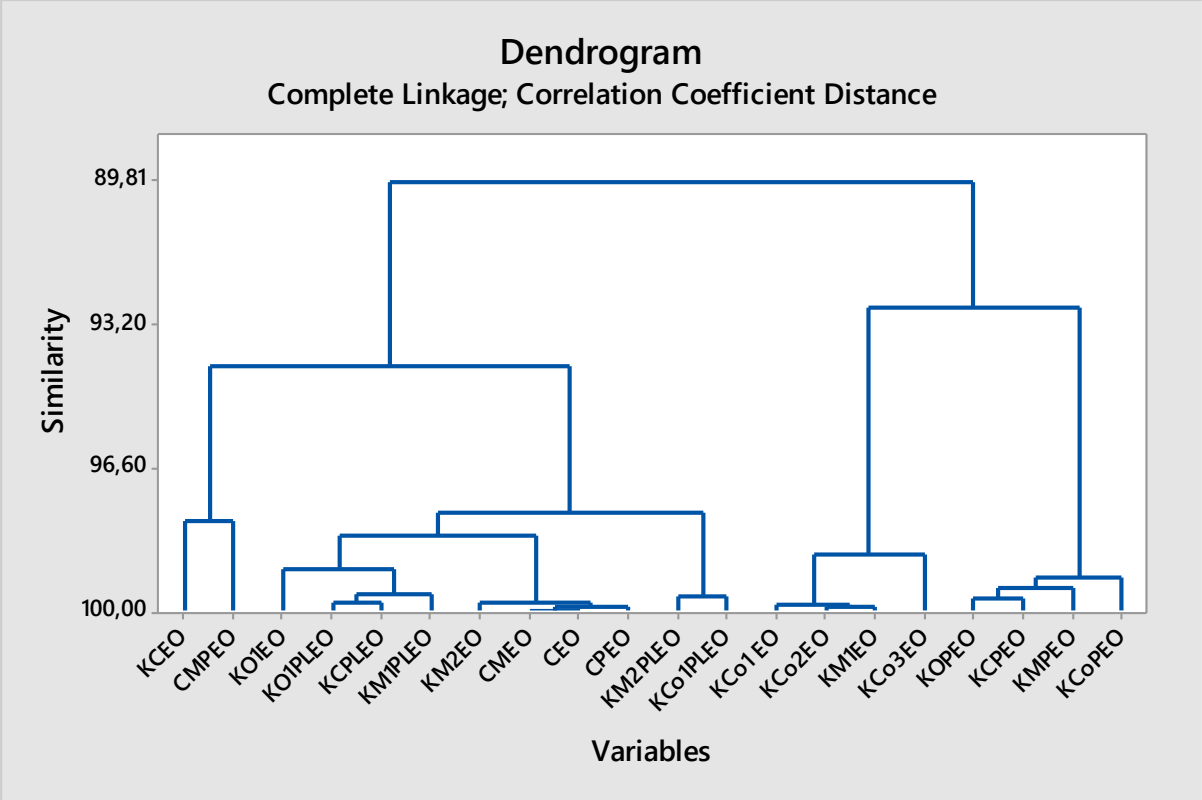


Figure 38: Dendrogram obtained from cluster analysis of chemical composition percentages of the essential oils obtained from the different populations of *S. montana*.

Table 22: Chemical composition (area %) of essential oils harvested in 2017.

			Clevenger										Semi-industriel plant					Pilot plant				
N°	**t_R**	**Compound**	KC	KO1	KCo1	KCo2	KCo3	KM1	KM2	CM	C	C	CM	KO	KM	KCo	KC	KO1	KM1	KM2	KCo1	KC
1	3.72	α-Thujene	1.22	1.39	1.21	1.4	0.29	1.48	1.18	1.65	1.75	1.6	1.76	0.35	0.29	1.2	0.78	1.64	1.82	1.94	2.31	2,26
2	3.83	(-)-α-Pinene	0.94	1.06	0.77	0.87	0.25	0.97	0.75	1.16	1.25	1.12	1.19	0.5	0.37	0.94	0.63	1.21	1.19	1.41	1.46	1,57
3	4.05	Camphene	0.22	0.49	0.28	0.73	-	-	0.47	0.4	0.43	0.39	0.54	0.42	0.2	0.35	0.3	0.52	0.55	0.61	0.62	-
4	4.37	1-octen-3-ol	1.67	1.5	0.77	1.66	-	0.73	1	1.03	1.07	1.01	0.97	0.39	0.58	1.11	0.9	1.45	1.34	1.62	1.54	1,93
5	4.45	β-Pinene	0.28	0.35	0.27	-	0.21	-	0.25	0.39	0.41	0.38	0.36	0.29	-	0.31	0.2	0.34	0.34	0.39	0.45	-
6	4.57	β-Myrcene	1.31	1.33	1.39		0.35	1.68	1.37	2.05	2.14	1.85	1.55	1.02	0.86	1.77	1.15	1.63	1.76	1.92	1.9	-
7	4.85	l-Phellandrene	0.25	0.23	0.22	-	-	-	0.21	0.34	0.36	0.33	0.28	0.17	-	0.31	0.22	0.29	0.31	0.35	0.37	-
8	5.05	α-Terpinene	2.08	2.01	1.74	2.25	0.71	2.28	2.11	2.92	3.06	2.79	2.35	1.36	1.37	2.47	1.84	2.43	2.85	2.9	3.22	3,27
9			21.0	18.9	10.7	10.7		12.4	19.0	17.7	17.4	16.4	27.0	15.6	14.8	17.5	16.5	21.2	18.3	21.5	23.4	20,4
	5.18	p-Cymene	7	5	3	5	4.5	7	4	6	6	2	7	7	4	8	2	9	1	8	4	2
10	5.26	Bornylene	-	1.32	0.96	-	-	-	0.79	-	-	-	-	0.98	0.94	1.21	0.99	1.53	1.3	1.88	1.29	-
11	5.29	Limonene	1.97	-	0.86	0.46	1.28		0.89	1.48	1.36	1.05	5.22	4.11	2.27	3.69	-	-	-	-	2,08	
12															10.0	13.4	11.1					
	5.31	1,8-Cineol	1.09	0.8	0.57	-	0.6	0.62	0.62	1.21	1.29	1.24	0.62	9.67	5	9	7	0.86	0.77	0.73	1.02	-

13			12.5	10.9	12.2	14.7		15.4	13.7	15.9	15.9	14.7					11.1	14.7	14.4	12.5	12,4
	5.78	ϒ-Terpinene	5	3	3	8	7.96	4	4	8	5	9	8.71	0.18		0.19		4	9	2	3	8
14		trans-Sabinene																			
	5.95	hydrate	0.91	0.4	0.48	-	0.63	-	0.52	0.85	0.9	0.9	0.77	0.17	3.22	1.96	2.34	0.82	0.41	1.08	0.34	1,25
15	6.53	Linalool	0.47	0.63	0.49	-	-	-	0.41	0.89	0.91	0.95	1.26	3.09	2.41	0.9	3.7	0.56	0.44	-	0.51	-
16	7.61	Camphor	0.13	1.88	-	-	-	-	-	0.14		0.16		1.92	2.27	1.72	2.14	-	-	-	-	-
17	7.99	Borneol	0.8	1.13	1.2	2.06	1.35	1.17	2.11	1.1	1.15	1.23	1.67	1.77	1.84	1.87	1.64	1.7	1.85	1.94	1.6	1,69
18	8.23	Terpinen-4-ol	0.74		0.68	0.95	0.52	0.75	0.91	0.66	0.71	0.75	0.96	0.93	0.78	0.48	0.64	0.73	0.91	0.72	1.2	1,06
19	8.52	α-Terpineol	0.25	0.24	0.15	-	-	-	0.18	0.14	0.16	0.18	-	-	-	0.14	-	0.2	0.21	0.18	0.25	-
20	9.80	Carvone	0.16	0.14	-	-	-	-	-	-	-	-	0.16	-	-	-	0.22	-	0.23	0.08	-	
21	10.7		16.2																			
	0	Thymol	9	0.14	4.79	6.2	8.05	4.53	8.85	8.37	8.47	9.21	7.85	5.52	8.98	5.23	4.17	1.76	0.20	0.20	2.19	2,80
22	10.9		31.1	49.5		54.8	70.9	54.3	41.3	38.6	37.8	40.2	32.5	32.1	35.1	34.4	36.8	39.2	40.5	36.4	32.5	39,2
	6	Carvacrol	6	9	56.3	5	3	8	6	5	2	7	5	8	5	5	4	2	2	9	8	1
23	12.7																					
	1	α-Copaene	-	-	-	-	-	-	-	-	-	-	-	0.13	0.15	0.15	-	0.17	0.17	0.14	0.16	-
24	13.6																					
	9	trans-Caryophyllene	0.90	1.54	2.08	1.43	1.61	1.15	1.36	1.07	1.13	1.18	3.44	0.21	0.22	0.15	0.17	0.22	0.24	0.19	0.17	-

25	14.8	β-cubebene																				
	9		-	-	-	-	-	-	-	-	-	-	-	-	4.47	4.11	3.41	3.58	3.98	3.77	4.27	3.66	4,89
26	14.8																					
	9	g-Muurolene?	0.13	0.18	0.19	-	-	-	-	0.16	0.18	0.18	0.35	0.17	-	0.13		0.46	0.19	0.63	0.17	-
27	15.0																					
	2	Germacrene-D	0.34	0.16	0.39	0.48	0.39	-	0.36		0.15		0.49	0.43	0.44	0.34	0.37	0.46	0.42	0.32	0.35	-
28	15.5																					
	3	β-bisabolene	1.06	0.98	0.29	0.73	0.29	1.07	0.95	0.94	1.0	1.07	2.26	0.87	0.58	0.4	0.5	0.54	1.03	0.63	0.73	-
29	15.7																					
	0	g-Cadinene	0.16	0.18	0.28	-	-	-	-	-	-	-	-	0.25	2.98	2.52	2.13	2.44	2.64	2.47	1.9	2.43	3,18
30	15.8																					
	7	d-Cadienne	0.16	0.29	-	-	0.28	-	0.22	0.24	0.26	0.28	0.54	0.4	0.38	0.28	0.27	0.37	0.35	0.21	0.29	-
31	17.0																					
	8	Spathulenol	0.42	0.17	-	-	-	-	-	-	-	-	-	0.75	0.79	0.62	0.65	0.78	0.76	0.5	0.59	-
32	17.1	Caryophyllene																				
	8	Oxide	1.12	0.83	-	-	-	-	-	0.48	0.51	0.36	1.16	1.48	1.14	0.48	0.66	0.86	0.34	0.55	0.5	-
		Total idendified	**99.8**	**98.8**	**100**	**99.4**	**100**	**99.8**	**99.5**	**99.0**	**100**	**100**	**93.9**	**98.6**	**98.0**	**98.5**	**100**	**99.6**	**99.9**	**98.0**	**98.1**	

Table 23: Comparison of chemical composition of essential oils according to extraction method.

RT	Compounds	Clenveger (AE)	PLEO	PEO
3,723	α-Thujene	1.29 ± 0.42[b]	1.99 ± 0.29 [a]	1.00 ± 0.63 [b]
3,836	(-)-α-Pinene	0.89 ± 0.29[b]	1.37 ± 0.16 [a]	0.79 ± 0.34 [b]
4,051	Camphene	0.36 ± 0.20[a]	0.48 ± 0.22 [a]	0.37 ± 0.12[b]
4,365	1-Octen-3-ol	1.07 ± 0.48 [ab]	1.58 ± 0.22 [a]	0.83 ± 0.28 [b]
4,448	β-Pinene	0.89 ± 0.29 [b]	1.37 ± 0.16 [a]	0.79 ± 0.34 [b]
4,569	β-Myrcene	1.31±0.67 [a]	1.48 ± 0.73 [a]	1.37 ± 0.41 [a]
4,847	l-Phellandrene	0.21 ± 0.11 [a]	0.28 ± 0.11 [a]	0.23 ± 0.09 [a]
5,045	α-Terpinene	2.13 ± 0.68 [a]	2.93 ± 0.34 [a]	2.03 ± 0.60 [a]
5,175	*p*-Cymene	14.75 ± 5.42 [a]	21.01 ± 1.87 [a]	18.02 ± 4.53 [a]
5,255	Bornylene	0.60 ± 0.34 [b]	1.28 ± 0.55 [a]	0.82 ± 0.34 [ab]
5,290	Limonene	0.85 ±0.62 [b]	0.6 ± 0.83 [b]	2.95 ± 1.65 [a]
5,312	1,8-Cineol	0.79 ± 0.34 [b]	0.73 ± 0.27 [b]	7.71 ± 5.42 [a]
5,782	γ-Terpinene	13.28 ± 2.67 [a]	13.07 ± 1.51 [a]	4.01 ± 6.3 [b]
5,951	*E*-Sabinene hydrate	0.54 ± 0.318 [b]	0.78 ± 0.40 [ab]	1.56 ± 1.14 [a]
6,533	Linalool	0.49 ± 0.28 [b]	0.38 ± 0.17 [b]	2.05 ± 1.19 [a]
7,607	Camphor	0.28 ± 0.6 [b]	0.07 ± 0.00 [b]	1.38 ± 0.99 [a]
7,985	Borneol	1.34 ± 0.45 [a]	1.76 ± 0.14 [a]	1.67 ± 0.23 [a]
8,231	Terpinen-4-ol	0.68 ± 0.21 [a]	0.92 ± 0.21 [a]	0.75 ± 0.18 [a]
8,518	α-Terpineol	0.15 ± 0.07 [a]	0.18 ± 0.07 [a]	0.10 ± 0.05 [a]
9,797	Carvone	0.06 ± 0.05 [a]	0.122 ± 0.10 [a]	0.06 ± 0.05 [a]
10,695	Thymol	7.30 ± 4.37 [a]	1.43 ± 1.18 [b]	6.83 ± 2.13 [a]

10,959	Carvacrol	48.34 ± 12.26 [a]	37.60 ± 3.17 [ab]	35.24 ± 3.0 [b]
12,710	α-Copaene	0.07 ± 0.00 [b]	0.14 ± 0.04 [a]	0.10 ± 0.04 [ab]
13,692	trans-Caryophyllene	1.36 ± 0.36 [a]	0.18 ± 0.06 [b]	0.90 ± 1.31 [ab]
14,889	β-cubebene	1.71 ± 0.00 [b]	4.11 ± 0.49 [a]	3.16 ± 1.19 [a]
14,892	g-Muurolene?	0.12 ± 0.057 [a]	0.30 ± 0.23 [a]	0.16 ± 0.11 [a]
15,017	Germacrene-D	0.269 ± 0.15 [a]	0.325 ± 0.15 [a]	0.36 ± 0.15 [a]
15,526	β-bisabolene	0.81 ± 0.31 [a]	0.62 ± 0.32 [a]	0.95 ± 0.69 [a]
15,700	g-Cadinene	0.12 ± 0.07 [b]	2.52 ± 0.46 [a]	1.73 ± 1.25 [a]
15,869	d-Cadienne	0.19 ± 0.09 [b]	0.26 ± 0.12 [ab]	0.36 ± 0.11 [a]
17,078	Spathulenol	0.13 ± 0.11 [b]	0.54 ± 0.28 [a]	0.50 ± 0.33 [a]
17,175	Caryophyllene Oxide	0.52 ± 0.39 [a]	0.48 ± 0.26 [a]	0.88 ± 0.44 [a]

Values are mean ± standard deviation of different essential oils from each method, analyzed individually in replicates of the different fertilizers. Means followed by different letters in the same row represent significant difference ($P < 0.05$).

In the **Tables 24**, **25** and **26** we report the insect antifeedant effects of *S. montana* harvested in 2 consecutive years (2016 and 2017) and 2 different sites on *S. littoralis, M. persicae* and *R. padi*. The essential oils and hydrolates showed strong antifeedant activity against all selected insects with LC_{50} values ranging from 20-50 μg/cm^2 for EOs. Organic phase (FO) extracted from the hydrolate solutions showed the strongest antifeedant activity with LC_{50} values of 7.93-30.99 μg/cm^2. Only the essential oil from conventional agriculture conditions plant extracted in laboratory (SAMOKCo1-17Ae) and ethanol extracts showed low activity against the selected pests. The antifeedant activity of *S. montana* extracts might be linked to their high concentration in carvacrol. In fact, when we tested carvacrol at 5 mg/mL against *S. littoralis, M. persicae* and *R. padi*, more than 90% activity was found. Bioactivity of carvacrol is widely

reported against pests[130 131] and its strong activity is due to the phenolic hydroxyl group.[132] The weak activity of SAMOKCo1-17Ae may be explained by its low percentage of *p*-cymene compare to others. Kisko et al.[133] demonstrated the synergic effects of carvacrol and its biological precursor *p*-cymene against *Escherichia coli* O157:H7. Agliassa et al.[130] reported a combined antifeedant activity of carvacrol and *p*-cymene from *Origanum vulgare* in the *S. littoralis* model conjugated with the insect increasing antioxidant enzymes activities and gene expression. This chapter of my thesis highlights the importance of fertilizers in the cultivation of bioactive plants. Concentration of active constituents might vary with the agronomic practices and influence particularly the insecticidal activity of plant extracts.

Table 24: Antifeedant effects of essential oils harvested in 2016 on *S. littoralis* larvae, *M. persicae* and *R. padi* apterous adults in choice tests.

Samples	*S. littoralis*	*M. persicae*	*R. padi*
	% FI[a] ± SE	% SI[a] ± SE	
	EC_{50} (µg/cm^2)[b]	EC_{50} (µg/cm^2)[b]	
Mycogel AE	83.63 ± 3.40	98.46 ± 1.08	97.64 ± 1.09
	5.66 (2.42-13.22)	26.06 (17.85-38.05)	4.19 (1.87-9.39)
Mycogel EtOH	42.83 ± 15.59	24.04 ± 7.97	43.29 ± 7.41
Control AE	76.93 ± 5.60	93.11 ± 3.73	99.31 ± 0.69
	24.99 (21.70-28.81)	4.23 (0.79-22.53)	10.78 (7.66-15.14)
Control EtOH	25.26 ± 8.10	57.12 ± 8.85	57.57 ± 8.36
CITA PEO	67.39 ± 11.42	70.47 ± 7.08	65.39 ± 7.62
CITA EtOH	16.96 ± 11.10	68.23 ± 5.74	65.39 ± 7.62
CM PEO	52.11 ± 13.09	64.02 ± 6.35	74.05 ± 5.15
CM EtOH	16.55 ± 7.75	58.84 ± 6.69	51.52 ± 8.63

Table 25: Antifeedant effects of essential oils harvested in 2017 in CITA on *S. littoralis* larvae, *M. persicae* and *R. padi* apterous adults in choice tests.

FINCA CITA 2017			*S. littoralis*	*M. persicae*	*R. padi*
			% FI[a] ± SE	% SI[a] ± SE	
			EC_{50} (µg/cm^2)[b]	EC_{50} (µg/cm^2)[b]	
SAMO CITA	Ae	SAMOC17Ae (5)	99.25 ± 0.48	86.42 ± 5.62	97.5 ± 1.17
			21.08 (7.52-59.04)	22.25 (8.38-57.73)	5.76 (1.54-21.51)
	PEO	SAMOC17PEO (15)	91.89 ± 5.13	78.24 ± 7.66	93.78 ±1.93
			38.22 (21.72-67.22)	27.96 (15.46-50.72)	14.67 (7.43-28.95)
	PH	SAMOC17PH (30)	93.80±2.78	92.50±5.47	98.89±0.76
			7.93 (0.70-89.59)	9.34 (3.41-25.55)	19.20 (12.29-29.96)
	EtOH	SAMOC17EtOH (21)	28.08±13.07	43.17±10.49	30.85±7.86
Cepas madre	Ae	SmCM-17Ae (6)	84.79 ± 4.18	88.97 ± 5.12	90.77 ± 3.03
			16.44 (5.24-51.58)	39.17 (31.27-48.64)	26.48 (18.14-38.66)
	PEO	SmCM-17PEO (16)	52.2 ± 11.44	89.30 ± 5.24	93.82 ± 1.70
				30.89 (25.08-38.32)	43.44 (34.17-55.19
	PH	SmCM17PH (31)	84.49±13.29	96.14±1.67	99.44±0.56
			30.99 (11.83-81.16)	0.31	21.26 (15.21-29.72)
	EtOH	SmCM17EtOH (22)	42.71±14.02	15.00±7.44	49.37±8.20

Table 26: Antifeedant effects of *S. montana* plant extracts harvested in 2017 in KIMITEC on *S. littoralis* larvae, *M. persicae* and *R. padi* apterous adults in choice tests.

KIMIFINCA 2017			***S. littoralis***	***M. persicae***	***R. padi***
			% FI[a] ± SE	% SI[a] ± SE	
			EC_{50} (µg/cm^2)[b]	EC_{50} (µg/cm^2)[b]	
SAMO KIMITEC	Ae	SAMOKCo1-17Ae (1)	22.92 ± 9.79	28.13 ± 7.63	36.06 ± 7.4
		SAMOKM2-17Ae (2)	82.69 ± 6.51 35.29 (17.48-71.22)	78.68 ± 5.94 30.75 (22.03-43.62)	97.22 ± 1.1 38.98 (31.97-47.53)
		SAMOKC-17Ae (3)	58.01 ± 14.76 42.83 (18.49-99.19)	86.94 ± 4.47 35.16 (26.79-46.15)	96.54 ± 1.43 47.05 (40.58-54.60)
		SAMOKO1-17Ae (4)	79.54±6.04 34.65 (26.37-45.53)	80.66 ± 6.19 11.85 (2.37-60.76)	97.22 ± 1.78 24.93 (17.18-36.17)
	PLEO	SAMOKCo1-17PLEO (7)	43.95±10.76	71.41 ± 6.8 25.67 (13.83-47.64)	97.78 ± 1.02 43.06 (36.51-50.79)
		SAMOKM2-17PLEO (8)	46.01±16.23	77.18 ± 5.94 22.18 (10.91-45.08)	83.50 ± 6.13 40.01 (29.58-54.13)
		SAMOKC-17PLEO (9)	60.05 ± 12.27 56.45 (36.83-86.52)	78.63 ± 6.72 26.71 (16.83-42.38)	98.89 ± 0.76
		SAMOKO1-17PLEO (10)	73.03 ± 10.12 35.11 (15.35-80.32)	60.32 ± 7.6	95.75 ±1.82 42.36 (37.82-47.45)

PEO	SAMOKCo-17PEO (11)	74.68 ± 8.56 47.38 (35.68-62.90)	69.38 ± 8.11 11.68 (5.62-13.12)	89.11 ± 3.98 34.54 (25.80-46.24)
	SAMOKM-17PEO (12)	98.15 ± 1.85 14.29 (3.23-63.21)	65.87± 7.54 25.33 (11.68-54.88)	85.03 ± 4.33 25.97 (17.47-38.60)
	SAMOKC-17PEO (13)	83.33 ± 16.67 36.43 (25.14-52.79)	70.12 ± 7.77 27.02 (12.67-57.59)	92.21 ± 3.18 33.89 (26.67-43.04)
	SAMOKO-17PEO (14)	88.5 ± 7.66 21.62 (6.79-68.78)	66.41 ± 7.48 8.98 (5.13-12.31)	90.77 ±3.03 44.09 (36.99-52.33)
EtOH	SAMOKCo-17EtOH (17)	50.91±16.80	29.93±8.64	34.63±8.34
	SAMOKO-17EtOH (18)	29.95±13.70	35.30±8.44	45.04±7.42
	SAMOKM-17EtOH (19)	65.80±12.29	33.57±9.27	18.97±7.28
	SAMOKC-17EtOH (20)	41.95±6.22	40.13±9.50	60.44±8.52

We further tested the nematicidal activity of the different essential oils and hydrolates on root-knot nematode *M. javanica* juveniles in vitro and in vivo. Results are reported in the **Tables 27** and **28**. All plant essences and hydrolates killed all the worms at 1 mg/mL after 72h. LC_{50} values were thus estimated and had a range of 0.13-0.32 mg/mL for essential oils and 0.07-0.10 mg/mL for hydrolate organic phases. Even though all essential oils from different

agronomic conditions showed strong nematicidal activity, SAMOKCo1-17Ae was the less effective with LC_{90} 95% confidence interval (0.415-0.451) different from others, confirming the possible interaction between *p*-cymene and carvacrol. Apart from SAMOKCo1-17Ae, the rest of the oils extracted in the laboratory showed better nematicidal activity with lower LC_{50} 95% confidence interval (0.120-0.146 mg/mL) than the pilot and semi-industrial methods (0.173-0.325 mg/mL). Our results still showing in general that nematicidal activity of *S. montana* is not influenced by the agronomic conditions. Nematicidal activity of *S. montana* essential oil from Portuguese flora was reported on the pinewood nematode, *Bursaphelenchus xylophilus* with LC_{100} value of 0.858 mg/mL.[134] Besides the exploratory activity on *S. montana* nematicidal activity,[25] and the recent reported nematicidal activity of carvacrol,[135] this work is the first complete evaluation of the winter savour extracts against root-knot nematodes. More, the data here reported suggests that floral water, the condensate water that remains after the extraction of *S. montana* essential oil by distillation might be valorised in agriculture against pests.

Table 27: Effects of *S. montana* essential oils, obtained from different sites in 2016 (KIMITEC, CITA) on mortality of second stage juveniles (J2) of *M. javanica* and their effective doses.

		J2 mortality (%)* at 1 µg/µl after 72h	LC_{50} mg/ml (95% CL)	LC_{90} mg/ml (95% CL)
KIMITEC	SAMO1K16CAe	100 ± 0	0.284 (0.272-0.296)	0.433 (0.413-0.457)
	SAMO2K16CAe	100 ± 0	0.130 (0.128-0.137)	0.142 (0.136-0.161)
	SAMO1K16EtOH	4.98 ± 1.28	-	-

	SAMO2K16EtOH	4.24 ± 1.03	-	-
	SAMOC16EtOH	8.66 ± 1.29	-	-
	SAMOC16PEtOH	2.31 ± 1.29	-	-
	SAMOC16PEO	100 ± 0	0.127 (0.122-0.133)	0.181 (0.171-0.191)
Cepas	SmCM16Ae (24)	100 ± 0	0.139 (0.134-0.151)	0.166 (0.153-0.200)
madre	SmCM16EtOH	9.01 ± 1.16	-	-
	SmCM16PEtOH	2.11 ± 1.21	-	-
	SmCM16PEO	100 ± 0	0.178 (0.174-0.183)	0.245 (0.238-0.255)

Table 28: Effects of *S. montana* essential oils, obtained from different sites in 2017 (KIMITEC, CITA) on mortality of second stage juveniles (J2) of M. javanica and their effective doses.

		J2 mortality (%)* at 1 µg/µl after 72h	LC_{50} mg/ml (95% CL)	LC_{90} mg/ml (95% CL)
K	SAMOKCo1-17Ae (1)	100 ± 0	0.323 (0.313-0.334)	0.432 (0.415-0.451)
I	SAMOKM2-17Ae (2)	100 ± 0	0.140 (0.135-0.146)	0.193 (0.184-0.204)
M	SAMOKC-17Ae (3)	100 ± 0	0.138 (0.133-0.144)	0.208 (0.199-0.219)
I	SAMOKO1-17Ae (4)	100 ± 0	0.125 (0.120-0.131)	0.189 (0.179-0.201)
T	SAMOKCo1-17PLEO (7)	100 ± 0	0.313 (0.302-0.325)	0.439 (0.423-0.459)
E	SAMOKM2-17PLEO (8)	100 ± 0	0.199 (0.190-0.209)	0.334 (0.317-0.357)
C	SAMOKC-17PLEO (9)	100 ± 0	0.204 (0.197-0.212)	0.301 (0.288-0.316)

	SAMOKO1-17PLEO (10)	100 ± 0	0.212 (0.205-0.221)	0.307 (0.294-0.323)
	SAMOKCo-17PEO (11)	100 ± 0	0.235 (0.225-0.245)	0.331 (0.315-0.352)
	SAMOKM-17PEO (12)	100 ± 0	0.190 (0.183-0.197)	0.283 (0.271-0.297)
	SAMOKC-17PEO (13)	100 ± 0	0.250 (0.240-0.262)	0.378 (0.359-0.399)
	SAMOKO-17PEO (14)	100 ± 0	0.232 (0.225-0.242)	0.329 (0.315-0.347)
	SAMOKCo-17EtOH	NA	-	-
	SAMOKO-17EtOH	NA	-	-
	SAMOKM-17EtOH	NA	-	-
	SAMOKC-17EtOH	NA	-	-
C	SAMOC-17Ae (5)	100 ± 0	0.179 (0.173-0.186)	0.265 (0.254-0.279)
I	SAMOC-17PEO (15)	100 ± 0	0.240 (0.230-0.251)	0.370 (0.352-0.392)
T	SAMOC17PHd (30)	100 ± 0	0.100 (0.097-0.103)	0.140 (0.136-0.146)
A	SAMOC17PH filtrated	100 ± 0	0.335 (0.323-0.347)	0.470 (0.458-0.484)
	SAMOC17EtOH	NA	-	-
C	SmCM-17Ae (6)	100 ± 0	0.180 (0.174-0.188)	0.271 (0.260-0.286)
.	SmCM-17PEO (16)	100 ± 0	0.206 (0.197-0.214)	0.316 (0.302-0.334)
M	SmCM17PHd (31)	100 ± 0	0.077 (0.075-0.079)	0.101 (0.0978-0.106)
A	SmCM17PH filtrated	100 ± 0	0.364 (0.347-0.381)	0.608 (0.583-0.639)
D	SmCM17EtOH	NA	-	-
R				
E				

Root-knot nematodes could adapt to extreme conditions in soil and plant environments and may respond differently in in vivo conditions. After the encouraging results obtained in vitro from the *S. montana* EOs, we decided to evaluate the effectiveness of the extracts in tomato plant infestation prevention in growth room. When we treated tomato plants with SAMOC-17PEO, we found a drastic reduction of egg masses and reproduction index after 60 days of infection respect to untreated plants (**Figure 40**). Complete protection of *S. lycopersicum* can be achieved by a concentration of 0.5 mg/mL or sand treatment of 50 mg/kg of *S. montana* essential oil. More, the essential oil treatment did not show phytotoxicity or growth inhibition effect to tomato plants. Despite the volatility of essential oil components, *S. montana* may be effective against nematodes in opened conditions for the crop duration. Our results are interesting when compare to results of Oka et al[136] who reported the in vivo nematicidal activity of *Origanum vulgare*, *O. syriacum*, *Coridothymus capitatus*, *Carum carvi*, *Foeniculum vulgar*e, *Mentha rotundifolia* and *Mentha spicata* and found a reduction of root galling of cucumber seedlings in pot experiments when the oils were mixed in sandy soil at concentrations of 100 and 200 mg/kg.

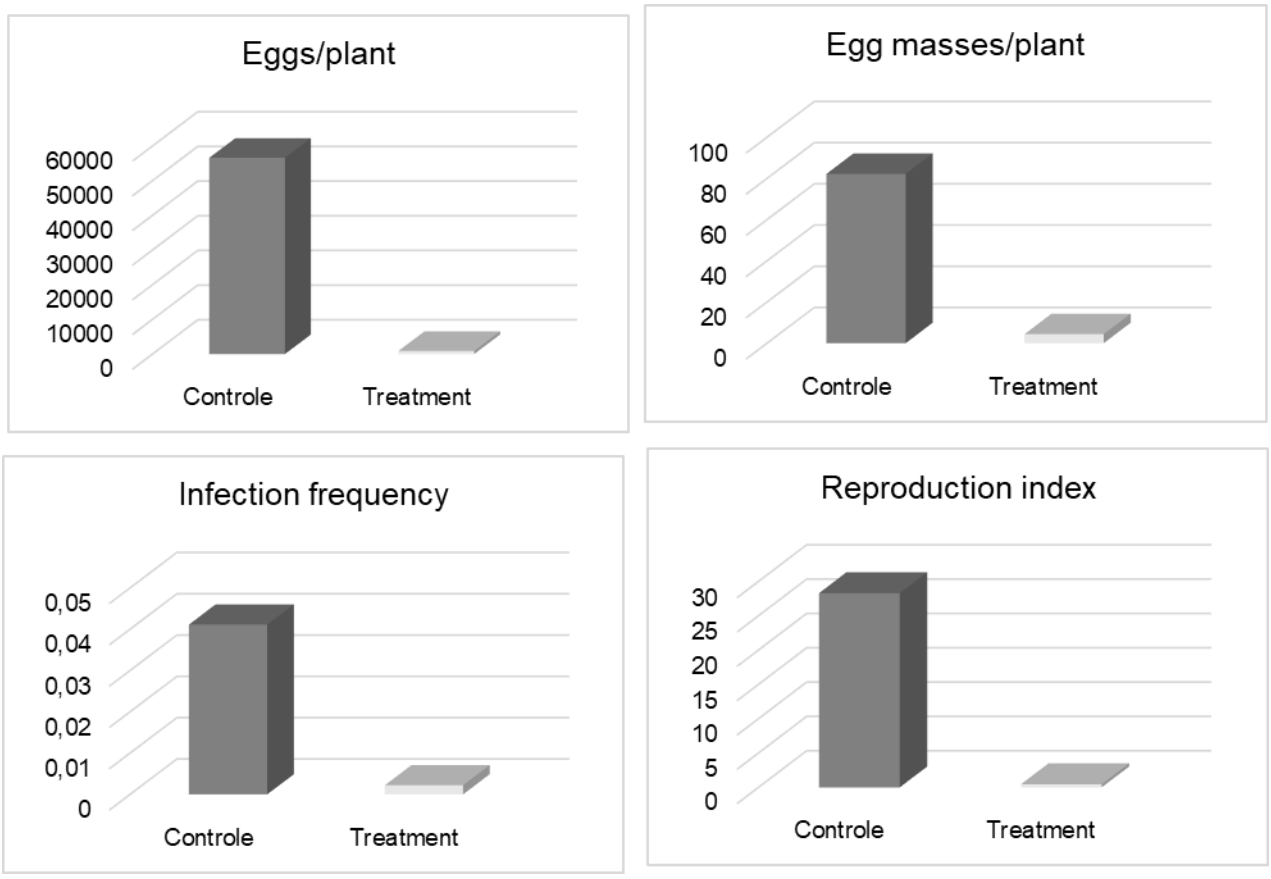


Figure 39: In vivo inhibition effect of S. montana essential oil SAMOC-17PEO on reproductive traits of M. incognita in tomato plants 60 days after inoculation with 2000 juveniles per plant maintained in a growth chamber.

To understand the contribution of the essential oil components to the biocidal activity, we ran a correlation test ($p<0.05$) with component chromatographic areas variations in all studied EOs.

The correlation values showed that the settling inhibition of *M. persicae* was positively influenced by bornylene (0.58), 1,8-cineol (0.50), Camphor (0.60) and α-copaene (0.50) and negatively influenced by γ-terpinene (-0.50) and thymol (-0.54). The activity on *R. padi* was positively correlated with the major component carvacrol (0.52) and negatively with more components, *p*-cymene (-0.53), carvone (-0.50), germacrene D (-0.72) and spathulenol (-0.50).

Finally, the antifeedant activity on *S. littoralis* was increasing with the percentage of β-bisabolene (0.50), σ-cadinene (0.66) and caryophyllene oxide (0.50) and decreasing with the concentrations of α-thujene (-0.50), (-)-α-pinene (-0.50), 1-octen-3-ol (0.72) and *p*-cymene (-0.60). On the other hand, no component area variation strongly affected the nematicidal activity of the essential oils on *M. javanica*.

Widespread use of synthetic herbicides to control weed could affect negatively human health and the environment. Natural compounds bioherbicides are plausible alternative because they are potentially safe and environmental friendly. *S. montana* was assessed for the first time in vitro for its phytotoxicity activity. The essential oil of *S. montana* was evaluated on the germination and root and aerial growth of *L. sativa* (lettuce) and *L. perenne* (ryegrass) and in acting at 10 mg/mL against *L. perenne* and *L. sativa*. Results are reported on the **Figures 40** and **41**. The germination and radicle growth of tested seeds of *L. perenne* were highly affected by all the essential oils and hydrolate organic phases except SAMOKCo1-17Ae and SAMOKCo1-17PLEO which showed less toxicity. The highest germination inhibition was found in general after 72h on the monocotyledon weed (100% inhibition). *L. sativa* was sensitive to the *S. montana* but was inhibited by hydrolates organic phases mostly after 24h (> 60%), while concurrently essential oils are showing stimulatory effects. When Angelini et al. [137] evaluated the germination inhibition potential of 3 essential oils from Mediterranean Lamiaceae and 4 monoterpenes on three different annual weeds and three crops, *S. montana* and carvacrol where found the most active. The study suggests that the essential oil from *S. montana* due to its phytotoxicity could be used in weed management programs.

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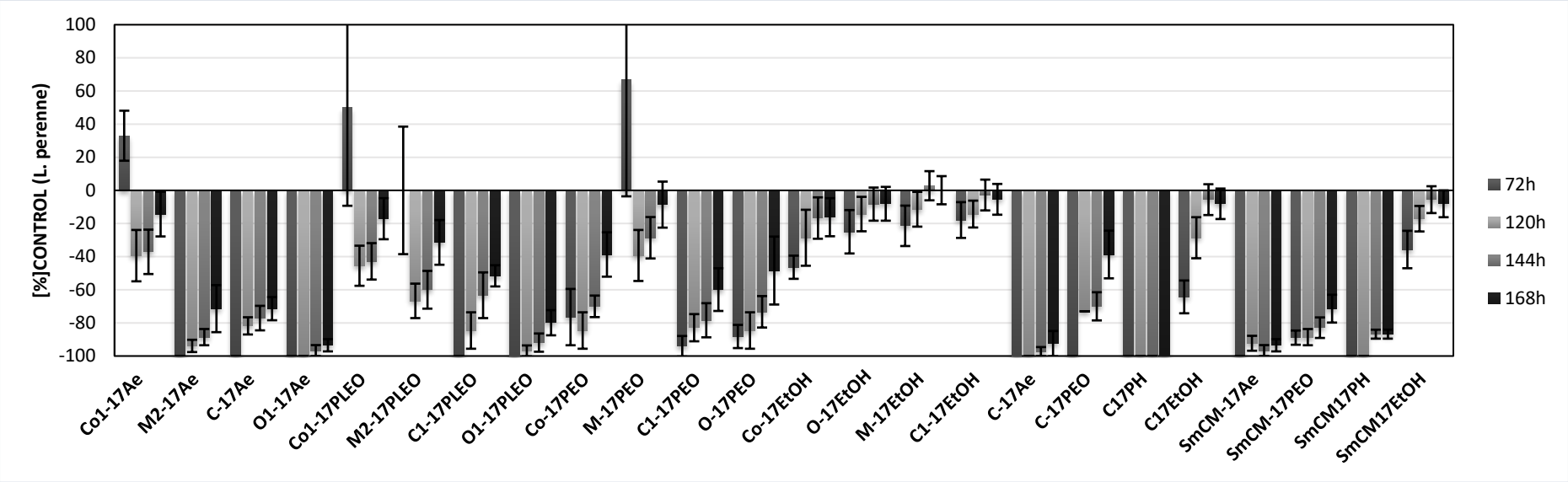

Figure 40: Phytotoxic activity of *S. montana* essential oils obtained with three different extraction methods in 2017 against *Lolium perenne*.

Germination and root length are expressed as % of growth inhibition respect to the control.

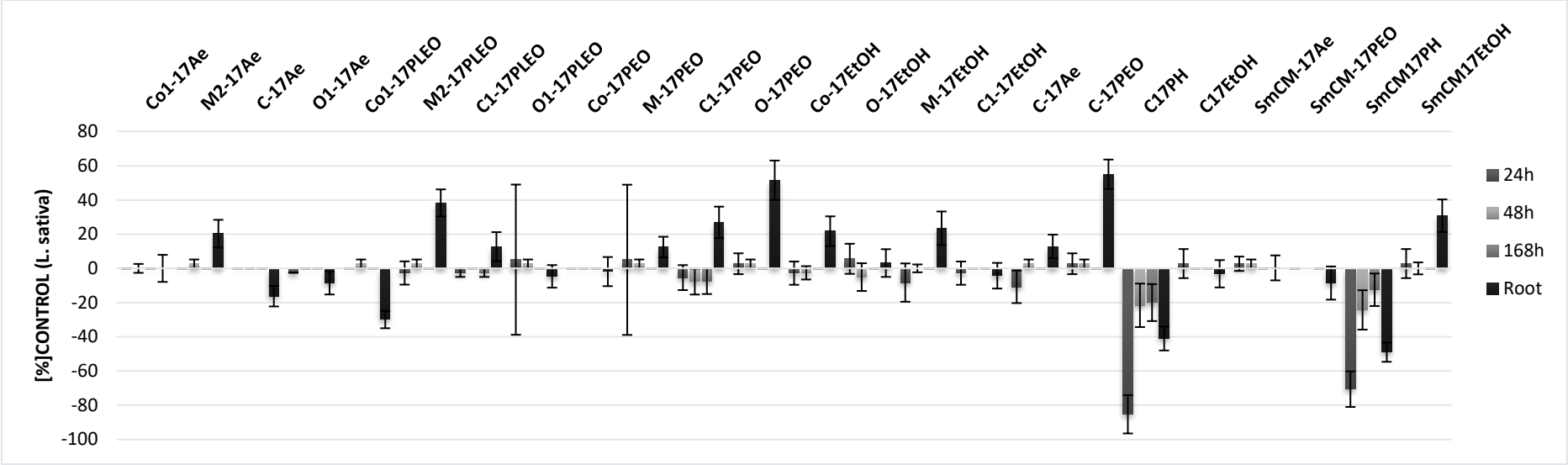

Figure 41: Phytotoxic activity of *S. montana* essential oils obtained with three different extraction methods in 2016 against *Lolium perenne*. Germination and root length are expressed as % of growth inhibition respect to the control.

CHAPTER 4

FINAL DISCUSSION

Plant-parasitic nematodes are easy to control during their active phase in soil when searching for host plants. Once endoparasitic species have reached a root, control with nematicides is more difficult as nematicide products have to be non-phytotoxic and preferably systemic. There are several reported compounds that can efficiently control nematode pests after infestation but they are only effective for management of nematodes when application is repeated. Only in certain cases will such treatments be environmentally benign and justified economically. Recently, dangers associated with the manufacture of nematicdes and their use become apparent due to environmental problems, human and animal health concerns. For example, effective nematicides such as EDB (ethylenedibromide) and DBCP (dibromochloropropane) have been withdrawn from the market due to their negative effects on humans and the environment. The most effective product and widely used fumigant methyl bromide, for nematodes control, has been banned. The highly toxic aldicarb used to control both insects and nematodes has been reported to contaminate ground water.

The development of new classes of nematicides with novel mechanism of action, that are effective when used against nematodes and are environmentally benign and specific to target pests is desirable. Such compounds require more research effort. They might have actions againt many plant-parasitic nematodes to be considered an economic proposition for the traditional agrochemical company. We propose herein the synthesis and nematicidal activity of new maleimide derivatives, selected haloacetophenones and some transition metal ions and their nematicidal activities were evaluated against three nematodes species: *M. incognita*, *X. index* and *G. pallida*. The nematicidal maleimide derivatives were synthesized in a one pot reaction and their chemical structures were guided by a structure-activity relationship study. The most potent compound was maleimide (**1**) with an $EC_{50/72h}$ of 2.6 ± 1.3 mg/L. By comparison other maleimide derivatives showed $EC_{50/72h}$ values lower than 100 mg/L. These results, first report of nematicidal activity of maleimide derivatives, suggest that these products

could be incorporated into an integrated pest management system against root-knot nematodes after a study to assess their safety to humans and developments of commercial synthesis and formulations to optimise their efficacy and stability to reduce cost.

Naturally occuring acetophenones are usually reported for their antimicrobial activities. Other studies reported their bioreduction by fungi and bacteria making them probable non persistent biopesticide candidates. Selected haloacetophenone derivatives herein showed strong activity against nematodes after 48 and 72 h. The most active acetophenone derivative, **15**, showed an $EC_{50/72h}$ value lower than 5 mg/L with almost all the active compounds showing an $EC_{50/72h}$ lower than 100 mg/L. Nematicidal activity of the haloacetophenones was enhanced when the halogenes were *para*- to the the ketone group. However, some *para*-haloacetophenone did not show high activity on nematodes. Further studies may be necessary to clarify the activity of haloacetophenones, their phytotoxity and the conditions of their eventual use in the protection of crops. The identification of natural compounds as haloacetophenones useful in nematode control and not harmful to beneficial soil microbiota can lead to new strategies for the management of soilborne plant pathogens. The in vivo experimental bioassays performed in this thesis must be considered a rapid first step evaluation of nematicidal activity of haloacetophenones in controlled conditions, and the information obtained from this screening of several substances must be confirmed by pot experiments and field trials.

Metals play essential roles in many biological processes but are toxic when present in excess. When we evaluated the nematicidal activity of some transition metal ions, silver and copper ions showed strong activities against *X. index* and *M. incognita* with $EC_{50/72h}$ as low as 0.05 mg/L. The association with maleimide, tannins and copper ions showed a strong synergism against *M. incognita*. If the ability of transition metal ions to generate ROS and induce apoptotic cell death is known, we demonstrated here for the first time their enhance activity by

maleimide and tannins. This study suggested that these compounds could be associated in a unique formulation to control nematodes after pots and field assays.

New strategies are needed to circumvent increasing outbreaks of resistant strains of plant pathogens including nematodes and to expand the dwindling supply of effective pesticides. A common impediment to nematicidal compounds development is the lack of an easy approach to explain the in vivo mechanism of action and efficacy of novel chemicals leads. Toward this end, we describe an unbiased approach to predict in vivo mechanisms of action of selected compounds from GC-MS metabolomics data. After treatment with selected nematicidal compounds, nematodes were extracted with MTBE and after GC-MS analysis, we successfully identified up and downregulated metabolites. These results were obtained using multivariate analysis. Fatty acids and mono acyl glycerols were involved in the nematicidal activity of the studied compounds, suggesting an oxidative stress activity. This method could be used to understand the mode of action of new nematicidal compounds.

Prone to attacks by pathogens and pests, plants exert intricate metabolic adaptations chemical defense mechanisms. Furthermore, many plant attackers manipulate the host metabolism to counteract defense responses and to create favorable nutritional conditions. Advances in mass spectrometry detectors have allowed the generation of extensive metabolic profiles in study of plant-pathogen and pest interactions. We reported nematode-plant chemical interactions using *M. incognita* and *S. Lycopersicum* by GC-MS and LC/ESI/QTOF platforms. Plant materials were harvested and extracted for chemical analysis after two months nematode infestation. By MVA, we were able to separate infested and non-infested plants and identify discriminant compounds of both groups. Amino acids and sugar discriminated and this may be due to the reprogramming of plants to cope with energy lost caused by nematode attack. Moreover, with LC-MS, we identified a potential biomarker in infested plants, an *O*-acylsugar with a formula $C_{29}H_{48}O_{15}$ corresponding to m/z = 654.3335 $[M+NH_4]^+$, a non-volatile secondary metabolite

reported for *S. lycopersicum*, a compound produced by tomato plants against insects. This study is the first report of tomato plant stress by *M. incognita*. Our method may require improvement to enlarge the metabolite pool. The clusters of identified compounds might serve as base in the quest of novel defense compounds and as markers for the characterization of the plants infestation state. Knowing plant infestation state is useful in agronomic applications where valuable markers are crucial for crop protection.

Pesticides with botanical origin represent an underused alternative to synthetic pesticides with almost limitless potential of new discovery. Plants have produced compounds, known as secondary plant products, to fight phytophagous insects because both coevolved. Herein, we studied the biocidal activity of domesticated and chemically stable chemotype of *S. montana* essential oil, ethanol extracts and hydrolate organic phase. The ethanol extract did not show activity on pests, suggesting the low bioactivity of the plant polar metabolites. On the other hand, EOs and the hydrolates showed strong activity against *S. littoralis*, *M. persicae*, *M. incognita* and *R. padi*. These extracts showed phytoxicity on *L. perenne* but not on *L. sativa*. We also compared the difference in chemical compositions of essential oils by fertilizer or extraction method. We found that the plant with conventional fertilizer showed low concentration in p-cymene and higher concentration in carvacrol. Relatively low difference was found due to the extraction method. The ability of these natural plant extracts to kill nematodes and control insects at relatively low concentrations might represent an alternative to the use of synthetic pesticides. These findings suggest that the carvacrol rich chemotype of *S. montana* could be used for various crop protection applications.

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