Università degli Studi di Cagliari

DOTTORATO DI RICERCA

In Tossicologia

Ciclo XXVIII

TITOLO TESI

Phytochemistry in the development of pesticides and biocides.

Settore scientifico disciplinari di afferenza

CHIM 10

Presentata da: Nikoletta Ntalli

Coordinatore Dottorato: Gaetano Di Chiara

Tutor: Pierluigi Caboni

Esame finale anno accademico 2014 – 2015

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my Professor and friend Pierluigi Caboni for the trust, guidance and collaboration both during the PhD but also earlier, since our first meet in 2009. Thank you for providing with facilities, support, creative discussions and friendly companionship.

I want to thank the Polish Professors Adamski Zbigniew and Grzegorz Rosinski as well as Dr Pawel Marciniak for giving me the possibility to work in their laboratory at the Institute of Animal Physiology and Development, of Adam Mickiewicz University, Poznań for the SEM and TEM experiments on RKN. My additional gratitude to Dr Slawek Cerbin of the Department of Hydrobiology, Collegium Biologicum of Adam Mickiewicz University, Poznań for the accommodation in his laboratory and help on the *Aphanizomenon gracile* bioassays.

I also thank the Greek Professor Menkissoglu-Spiroudi Urania of the School of Agriculture of the Aristotle University of Thessaloniki, and the Professor Tsiropoulos Nikolaos of the Department of Agriculture, Crop Production and Rural Environment of the University of Thessaly for our collaboration on the synergism of the nematicidal compounds against RKN as well as for the *Melia azedarach* field experiment and respective pot bioassays.

My special thanks to the researcher Karamaouna Filitsa, head of the laboratory of Pesticides Control & Phytopharmacy at the Benaki Institute of Phytopathology in Athens, for giving me the possibility to spend the last stage period, sympathy and help.

My deepest thanks to the Polish, Greek and Italian students without the help of whom many of the laboratory experiments presented herein wouldn't have been accomplished. In specific I want to thank the Greek MSc students Oplos Chrisostomos, Thanasseris Tasos, Kontea Dorothea and Michailidis Michalis; the Polish MSc students Agnieszka Kopiczko and Katarzyna Radtke and the Italian PhD students Murcia Antonio, Nadhem Aissani and Eloh Kodjo as well as the under graduate student Saba Marco.

I am grateful to the official reviewers of my PhD thesis, Professors Antonio Valverde of the University of Almeria, Spain and Milica Mojasevic of the University of Belgrade, Serbia for their valuable comments, suggestions and expert criticism on the manuscript.

My warmest gratitude to my family and friends for having always stood by me notwithstanding the distance.

TABLE OF CONTENTS

Chapter 2. Phytochemistry in the development of biocides.

I. Algicidal effect of essential oils on *Aphanizomenon gracile*

Chapter 1. Phytochemistry in the development of pesticides.

I. 1. Introduction - A field experiment with *Melia azedarach* as a nematicidal agent against root knot nematodes

Future agricultural and rural development is, to a large extent, influenced by the projected food needs of 2.5 billion people expected to swell the world population by 2020. Among agricultural pests suppressing crops, root-knot nematodes (RKN; *Meloidogyne* spp.) represent possibly the world's most damaging one with a host range, encompassing the majority of flowering plants, a short generation period, a high reproductive rate, and an ability to form disease complexes with other soil borne pathogens like fungi (Trudgill and Block, 2001; Back et al., 2002). The yield losses in tomato are associated with the completion of three nematode generations in spring plantings (Sorribas *et al.*, 2005), while losses are observed in autumn plantings when the nematode completes a single generation (Talavera *et al.*, 2009). Despite the usefulness of nematicidal compounds in agricultural practices, serious concerns have been raised in the recent years due to their excessive use and thus integrated pest management strategies are now favoured worldwide to maximize crop production while maintaining and contributing to agriculture sustainably (Ntalli and Caboni, 2012). In the recent years the EU has employed a fundamental reform of the Common Agricultural Policy (CAP) highlighting the respect to the environmental, food safety and animal welfare standards, imposing farmlands' cross-compliance with good agricultural and environmental conditions (Schillhorn van Veen, 1999). Additionally, due to environmental side effects and health concerns, many synthetic carbamate, organophosphate, and organophthalide nematicides have been banned (91/414/EEC) or are under evaluation (2009/128/EU). The most potent fumigant nematicide was methyl bromide, which has been banned according to the requirements of the Montreal Protocol due to its ozone depleting properties. On the other hand, industry does not easily sustain the economic cost of research and registration of new nematicides (Neale, 2000) even though in some cases like in the Netherlands, they represent more than 60% of the total pesticides used in agriculture (Chitwood, 2002). As a result there are only few nematicides still in use, and their limited number makes the same formulation applications' repetition, inevitable. This fact has favoured nematicides' biodegradation mechanisms in soil (Qui et al., 2004) as well as nematodes' resistance development (Meher et al., 2009), expressed in field as lack of

efficacy. All the above facts necessitate the urge for new and alternative nematode control methods (Chitwood, 2002).

Soil amending with botanical matrixes can act against RKN and the respective references published till now are reported hereafter. Intercropping with *Aeschynomene histrix*, *Crotolaria juncea*, and *Tagetes erecta* vanised *M.incognita* damage in intercropped tubers (Claudius-Cole et al., 2014). Of course the most characteristic plant material amended in soil for nematodes control are the Brassicaceae including many important economic plants rich in glucosinolates (GLSs) which contain sulfur. Glucosinolates coexist *in vivo* with glycosylated thioglucosidases, myrosinase(s), responsible of their hydrolysis with the production of nematicidal cognate isothiocyanates (ITC) (Avato et al., 2013). Lately, applications of fresh chopped whole plant of *Fumaria parviflora*, 15 days before transplanting, at the dose of 30g of material per kg of soil showed high nematicidal activity (Naz et al., 2013). The use of organic amendments against *Meloidogyne* spp. has also been identified in many animal wastes like poultry manure used in a carrot culture at 4 t/ha (Kankam et al., 2015). Previously, we have demonstrated the nematicidal activity of *M. azedarach* in pots as well as in *in vitro* bioassays (Ntalli et al., 2010a) and we have attributed it to the aldehydes as well as organic and carboxylic acids' contents (Ntalli et al., 2010b, Aoudia et al., 2012). Although various botanical products have been used to test for their activity against phytonematodes, experiments are usually performed in small scale *in vitro* bioassays rather than under field conditions thus not reflecting the real environmental conditions under which the nematodes need to be affronted. Possible reasons for the shortage of scientific articles on field experiments using botanical products might be the 1) shortage of botanical material needed to treat big soil surfaces, 2) demanding extraction procedures and equipment and 3) the cost effectiveness and handling of left over toxic organic solvents. The scope of this study was to evaluate 1) the *M. azedarach* nematicidal products, Melia Fruits Powder (MFP) and Melia Water Extract (MWE) under field conditions and to compare their activity with that of the commercial nematicides Furfural 99% Sigma and VYDATE® 10 SL, and 2) the impact on the micro organisms in soil, saprophytic nematodes included.

I. 2. Materials and Methods

I. 2. a. Field Experiment and Reagents. The study was performed in a commercial greenhouse on private land located in Volos, Thessaly, Greece in May 2013 and 2014. The field studies did not involve endangered or protected species, and no specific permissions were required for these activities. The soil texture was sandy loam with the following properties: pH (1:2 H_2O): 7.9, organic matter (%): 2.4, sand (%): 52.4, silt (%): 36.6, clay (%): 11. One experiment was established per year, designed in randomized block with four replicates (plots) per treatment as follows: (1) untreated control, 4 plots (x 4 plants); (2) Melia Fruit Powder (MFP), 4 plots (x 4 plants) (160 g/plant); (3) Melia Water Extract (MWE), 4 plots (x 4 plants) (440 ml/plant); (4) Furfural (F), 4 plots (3 ml/plant) and (5) VYDATE® 10 SL, 4 plots (1.5 ml/plant). Plots were 40 cm wide and 250cm long (4 plant per row). Nematodes free seedlings of tomato cv. Belladonna were transplanted when plants were 35 days old. Since transplant, prevention controls were performed on a daily bases for fungus infections and pests infestations together with watering with 2L/plant. Fertilization until the 20th of June was done, 1) every two days using $CO(NH_2)_2 \cdot H_3PO_4$. 15 kg/ha, KNO_3 10 kg/ha and $Mg(NO_3)_2$ 1kg/ha, and 2) on a weekly basis using $Ca(NO_3)_2$ 20 kg/ha (instead of using urea phosphate 15 kg/ha), $Mg(NO_3)_2$ 1kg/ha, KNO_3 and trace (B, Fe, Mn, Zn and citric acid) 1-2 kg/ha. *Bacillus thurigiensis* was used as pesticide to control insects.

VYDATE® 10 SL was purchased from DuPont Crop Protection Greece) and Furfural 99% from Sigma-Aldrich, Greece.

Treatments were performed using 500ml of carrier volume on a 20cm diameter cycle around the plant. Concerning the powder application, after PMF was distributed on that surface, 500ml of water were applied. The first application was performed 9 days before transplanting (DBT), while the others 12, 34 and 54 days after transplanting (DAT).

Nematode infestation assessments: Soil samples consisted of three soil cores at 0-15cm depth, 24mm width and were 50g each. Soil cores were pooled and passed through a 4mm *in situ* and this soil was used for nematodes count (*Meloidogyne* spp and saprophytic) after bringing to laboratory. Samples were collected on each application date before treatment, that is 9 DBT, 12 DAT, 34 DAT, 54 DAT and 71

DAT. Second stage juveniles of *Meloidogyne* were extracted from a subsample of soil (200 g) by the Baermann funnel method (Hussey and Barker, 1973) and were counted under a stereoscopic microscope. Saprophytic nematodes were separated from phytonematodes according to morphological characteristic.

Plant parameter assessments. Two samplings, 61 and 71 DAT, were performed on the harvested crop to assess for effects of treatments on plant parameters. In specific assessments were performed on plants height, shoot width, crop weight and number of fruits on the first and second cross. On the day of roots eradication root index was performed for nematodes infection assessment on host root.

I. 2. b. Nematode Population cultivation. A population of *M. incognita* originally obtained from tomato roots collected from a greenhouse in Vassilika, Thessaloniki, northern Greece, was reared on tomato (*Solanum lycopersicum* L.) cv. Belladonna, a cultivar that is very susceptible to root-knot nematodes. All plants were maintained in a growth chamber at 28-30 ^{o}C, 60% relative humidity, and 16 h photoperiod. Plants used for inoculations were 7 weeks old, at the five-leaf stage. After 40 days, the plants were uprooted, and the roots were washed free of soil and cut into 2 cm pieces. Eggs were extracted according to the sodium hypochlorite procedure, and second-stage juveniles (J2) were allowed to hatch in modified Baermann funnels at 28 ^{o}C (Hussey and Barker, 1973). All J2 hatching in the first 3 days were discarded, and thereafter J2 collected, after 24 h, were used in the experiments.

I. 2. c. Biological cycle arrest bioassay**.** The soil used for the pot experiment was a root knot nematodes free clay loam soil with 1.3% organic matter and pH 7.8 and was collected from a non cultivated field of the Aristotle University of Thessaloniki Farm. The soil was sieved through a 3-mm sieve and then was partially air dried overnight. Soil moisture and maximum water holding capacity (MWHC) were measured and then mixed with sand at a ratio of 2:1 to obtain the mixture hereafter referred to as soil. Then, the soil was separated into 6 plastic bags corresponding to treatments. MWE was mixed at 0.58 to 2.9% w/w for testing against *M. incognita* and *M. javanica*. A treatment with plain water served as control. Treatments were then incubated for 24 h. After a second sieving, the soil corresponding to each treatment was returned into the plastic bag and was inoculated with 2500 J2 of *M. incognita*, mixed thoroughly by shaking and left to equilibrate in the dark at room temperature

for another 24 h. Moisture content never exceeded the 24% of MWHC. After the incubation period, the soil was sieved again and then it was used for transplanting 7-week old, at the six-leaf stage tomato plants, cv. Belladonna into 200 g plastic pots. The experiment was maintained at 27 ^{o}C, 60% RH at 16 h photoperiod, and each pot received 20 mL of water every 3 days. Forty days later the roots were stained with acid fuchsin (Byrd et al., 1983) and the following variables were assessed: fresh root weight, fresh shoot weight and total number of female nematodes and galls per gram of root at 10 × magnification under uniform illumination by transparent light within tissue sample. The experiment was replicated once, and the treatments were always arranged in a completely randomised design with five replicates.

I. 3. Statistical analysis

The data from the pot experiments was expressed as a percentage decrease in the number of females or galls per gram of root corrected according to the control, using the Abbott's formula: corrected % = 100 × {1−[females number in treated plot/females number in control plot]}. It was fitted in the log-logistic model (Seefeldt et al., 1995) to estimate the concentration that caused a 50% decrease in females and galls per gram of root (EC_{50} value). In this regression equation, the *M. azedarach* products (% w/w) was the independent variable (x) and the female nematodes, or galls, (percentage decrease over water control) was the dependent variable (y). Because ANOVAs indicated no significant treatment by time interaction (between runs of experiments), means were averaged over experiments.

I. 4. Results and Discussion

According to the experimental results obtained from the field experiment all treatments decreased the number of *Meloidogyne* spp. J2 per gr of soil considering numbers assessed in the control treatment. Furfural was always significantly less infested and only in the second assessment the oxamyl treatment was found more effective than furfural. Treating tomatoes with MWE decreased nematode infestation considerably and interestingly in the first assessment even better than the commercial nematicide oxamyl. In the second and third assessment MWE was just as active as furfural, while PMF followed (**Table 1**).

Saprophytic nematode numbers in soil were considerably decreased after treating with oxamyl (first and second assessment) and furfural (last assessment). In soil treated with MWE and PMF the same effect was evident but only in the first assessment while in the successive ones they favoured saprophytic nematodes, and especially when treating in MWE (**Table 2**).

Treating RKN infested tomato plants with melia products had no significant effect on the plant parameters like plant height, shoot width, average fruit weight, green part and root weight. Only fruit weight and fruit number were significantly minor after treating with furfural which fact renders this treatment phytotoxic (**Table 3**). Similar results considering the phytotoxicity of furfural was evident in the second assessment date while all other treatments did not effect the plant growth and production (**Table 3**).

When MWE was tested in pot experiments against *M. incognita* and *M. javanica* a clear dose response relationship was established, *M. incognita* was less resistant and the efficacy higher (**Table 5**). In one case MWE applied at 2.9 % w/w favoured the growth of the aerial part but the effect was not constant.

According to the above furfural even though already commercialized seems to have phytotoxicity on the tomato plants and to decrease the number of saprophytic nematodes in the soil. Thus, although active on RKN, it seems less a coventient of a method while MWE is much more effective and without detrimental effects on crop and beneficials.

Table 1. Effect of field treatments with *Melia azedarach* products against *M. incognita* (J2/gr soil) on various assessment dates. Data are presented as average of four replicates with the respective standard deviations. Numbers followed by the same letters within column do not differ according to Tuckey's test for $P \leq 0.05$.

M. incognita (J2/gr soil)	18 June	14 July	24 July	11 Aug
Untreated	240.0 ± 18.7^{d}	1102.2 ± 45.5^{c}	3811.0 ± 128.3^{d}	3564.7 ± 478.6^{c}
Furfurale	11.5 ± 2.2^{a}	221.7 ± 31.3^{ab}	690.0 ± 31.9^{bc}	272.5 ± 63.0^{a}
MWE	70.2 ± 9.3^{b}	327.7 ± 88.3^{b}	882.7 ± 36.0^{c}	1056.5 ± 29.6^{ab}
PMF	101.0 ± 6.7^{bc}	920.0 ± 14.7^{c}	483.2 ± 6.2^{b}	1301.2 ± 79.1^{b}
OXA	145.0 ± 10.2^{c}	47.0 ± 10.6^{a}	202.5 ± 34.7^{a}	436.5 ± 45.1^{ab}

Table 2. Effect of field treatments with *Melia azedarach* products on saprophytic nematodes (J2/gr soil) on various assessment dates. Data are presented as average of four replicates with the respective standard deviations. Numbers followed by the same letters within column do not differ according to Tuckey's test for $P \leq 0.05$.

Saprophytic nematodes (J2/gr soil)	18 June	14 July	24 July	11 Aug
Untreated	8041.2 ± 692.5^{b}	2279.2 ± 264.4^{b}	3244.2 ± 171.9^{a}	1861.7 ± 49.7^{b}
Furfurale	6703.2 ± 135.9^{ab}	1635.2 ± 95.4^{b}	5966.2 ± 203.2^{b}	823.0 ± 30.9^{a}
MWE	5914.2 ± 104.9^{a}	3619.7 ± 146.7^{c}	11360.2 ± 470.3^{c}	3660.5 ± 163.8^{d}
PMF	5387.0 ± 164.6^{a}	3949.2 ± 249.2^{c}	4057.5 ± 77.2^{a}	2682.0 ± 179.8^{c}
OXA	6535.7 ± 148.0^{a}	359.5 ± 24.4^{a}	4272.7 ± 147.7^{a}	1439.0 ± 41.2^{b}

Table 3. Effect of field treatments with *Melia azedarach* products against *M. incognita* control, on crop parameters as assessed on 010814. Data are presented as average of four replicates with the respective standard deviations. Numbers followed by the same letters within column do not differ according to Tuckey's test for $P \leq 0.05$.

010814 (1st assessment)	Plant height	Shoot width	Fruit weight	Average fruit weight	Fruit number	Green part weight	Root weight
Untreated	146.0±4.0^{a}	15.0±0.3^{a}	911.2±40.4^{b}	164.5±5.2^{a}	5.6±0.2^{b}	1217.9±52.8^{ab}	50.3±5.5^{a}
Furfurale	109.6±20.4^{a}	11.3±2.4^{a}	441.8±122.0^{a}	108.5±24.7^{a}	3.6±0.5^{a}	817.6±119.9^{a}	2a6.5±6.4^{a}
MWE	133.3±10.7^{a}	13.2±1.4^{a}	688.6±59.5^{ab}	156.0±21.6^{a}	4.2±0.1^{a}	1039.7±62.9^{ab}	33.0±4.1^{a}
PMF	149.0±6.2^{a}	14.8±0.7^{a}	795.3±17.5^{b}	163.9±6.2^{a}	4.9±0.3^{ab}	1430.6±142.2^{b}	43.6±7.9^{a}
OXA	143.0±5.2^{a}	14.8±0.2^{a}	846.4±55.4^{b}	172.7±8.9^{a}	4.9±0.1^{ab}	1355.5±149.4^{b}	31.8±2.8^{a}

Table 4. Effect of field treatments with *Melia azedarach* products against *M. incognita* control on crop parameters as assessed on 110814. Data are presented as average of four replicates with the respective standard deviations. Numbers followed by the same letters within column do not differ according to Tuckey's test for $P \leq 0.05$.

110814 (2nd assessment)	Fruit weight	Average fruit weight	Fruit number
Untreated	1001.4±45.1^{b}	244.3±8.5^{b}	4.1±0.1^{a}
Furfurale	479.8±115.7^{a}	147.1±21.8^{a}	3.2±0.4^{a}
MWE	950.6±144.7^{ab}	224.1±24.4^{ab}	4.1±0.4^{a}
PMF	1018.7±127.3^{b}	226.8±24.8^{ab}	4.5±0.2^{a}
OXA	1047.9±107.3^{b}	233.0±10.9^{b}	4.5±0.3^{a}

Table 5. Tomato (*Solanum lycopersicum*) root infestation with *M. incognita* & *M. javanica*, and respective growth parameters, after treating with different rates of *Melia* Water Extract (MWE). Numbers followed by the same letters (column) are not statistically different for P≤0.05 according to Tuckey's test.

MWE (% w/w)	*M. incognita* (♀/gr root)	Aerial weight	Root weight	MWE (% w/w)	*M. javanica* (♀/gr root)	Aerial weight	Root weight
0.58	73.78 ± 4.0^{c}	8.07 ± 0.1^{b}	3.39 ± 0.2^{a}	0.58	82.36 ± 5.2^{d}	3.80 ± 0.1^{a}	0.90 ± 0.05^{a}
1.2	47.46 ± 3.1^{b}	7.97 ± 0.4^{b}	3.08 ± 0.1^{a}	1.2	51.02 ± 4.0^{c}	4.46 ± 0.2^{a}	0.87 ± 0.05^{a}
1.75	31.21 ± 1.2^{a}	$8.81 \pm 0.2b^{b}$	3.4 ± 0.2^{a}	1.75	20.24 ± 2.0^{b}	4.56 ± 0.3^{a}	0.88 ± 0.05^{a}
2.3	29.68 ± 2.1^{a}	9.63 ± 0.3^{ab}	3.71 ± 0.1^{a}	2.3	9.69 ± 1.2^{ab}	4.67 ± 0.02^{a}	0.96 ± 0.05^{a}
2.9	23.28 ± 2.2^{a}	10.90 ± 0.8^{a}	3.69 ± 0.2^{a}	2.9	5.39 ± 1.5^{a}	4.46 ± 0.3^{a}	0.99 ± 0.05^{a}

I. 5. References

Back MA, Haydock PPJ and Jenkinson P, Disease complexes involving plant parasitic nematodes and soilborne pathogens. *Plant Pathol* **2002**, *51*, 683-697.

Chitwood DJ, Phytochemical based strategies for nematode control. *Annu Rev Phytopathol* **2002,** *40*, 221-249.

Meher HC, Gajbhiye VT, Chawla G, Singh G. Virulence development and genetic polymorphism in *Meloidogyne incognita* (Kofoid & White). *Pest Manag Sci*. **2009,** *65*, 1201-7.

Neale M, The regulation of natural products as crop-protection agents *Pest Manag. Sci.* **2000**, *56*, 677-680.

Schillhorn van Veen TW, Agricultural policy and sustainable livestock development. *Int. J. Parasitol*. **1999**, *29*, 7-15.

Trudgill DL, Blok VC, Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol*. **2001,** *39*, 53-77.

Sorribas F.J., Ornat C., Verdejo-Lucas S., Galeano M., Valero J. Effectiveness and profitability of the *Mi*-resistant tomatoes to control root-knot nematodes. E*uropean Journal of Plant Pathology*, **2005**, *111*, 29–38.

Talavera M, Verdejo-Lucas S, Ornat C, Torres J, Vela MD, Macias FJ, Cortada L, Arias DJ, Valero J, Sorribas FJ, Crop rotations with *Mi* gene resistant and susceptible tomato cultivars for management of root-knot nematodes in plastic houses. *Crop Protection*, **2009**, *28*, 662–667.

Naz I, Palomares-Rius JE, Khan SM, Ali S, Ahmad M, Ali A, Khan A, Control of Southern root knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood on tomato using green manure of *Fumaria parviflora* Lam (Fumariaceae). *Crop Protection*, **2015**, *67*, 121-129.

Kankam F, Sowley ENK, Oppong NE, Effect of poultry manure on the growth, yield and root-knot nematode (*Meloidogyne* spp.) infestation of carrot (*Daucus carota* L.) *Archives of Phytopathology and Plant Protection* **2015**, ***48***, 452-458.

Claudius-Cole AO, Fawole B, Asiedu R, Coyne DL, Management of Meloidogyne incognita in yam-based cropping systems with cover crops . *Crop Protection* **2014**, *63*, 97-102.

Avato P, D'Addabbo T, Leonetti P, Argentieri MP., Nematicidal potential of Brassicaceae. *Phytochemistry Reviews* **2013**, *12*, 791-802.

Ntalli NG, Menkissoglu-Spiroudi U, Giannakou I, Nematicidal activity of powder and extracts of *Melia azedarach* fruits against *Meloidogyne incognita. Annals of Applied Biology* **2010,** *156*, 309-317.

Ntalli NG, Vargiu S, Menkissoglu-Spiroudi U, Caboni P, Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 11390-11394 .

Aoudia H, Ntalli N, Aissani N, Yahiaoui-Zaidi R, Caboni P, Nematotoxic phenolic compounds from melia azedarach against meloidogyne incognita. *Journal of Agricultural and Food Chemistry* 2012**,** *60*, 11675-11680.

Ntalli NG, Caboni P, Botanical nematicides: A review. *Journal of Agricultural and Food Chemistry* **2012,** *60*, 9929-9940.

Byrd KRB, Kirkpatrick T, An improved technique for clearing and staining plant tissues for detection of nematodes, *J. Nematol*. **1983**, *15*, 142-143.

Putener W, Manual for field trials, in: C. Geigy (Ed.), Plant Protection, 2nd ed., Basle, Switzerland, 1981, p. 205.

Seefeldt SS, Jensen JE, Feurst EP, Log-logistic analysis of herbicide dose-response relationships, *Weed Technol*. **1995**, *9*, 218-227.

II. 1. Introduction - Nematicidal activity of the volatilome of *Eruca sativa* on *Meloidogyne incognita*

Root-knot nematodes (*Meloidogyne* spp.) are among a vast array of pests causing annually significant crop losses in fruit and vegetable production (Reynolds et al., 2011). Among strategies to control these pests, natural nematicides isolated from plants or microorganisms tend to be successfully used as biocontrol agents to reduce non-target exposure to hazardous pesticides and to overcome resistance development (Isman, 2006, Tian et al., 2007). Plants can produce compounds that directly or indirectly affect their biological environment; these compounds, called allelochemicals, have a dramatic influence on the life cycle of the surrounding living organisms (Whittaker et al., 1971). Many scientific studies have reported data on the biological activity of plant secondary metabolites on root-knot nematodes (Ntalli and Caboni, 2012; Caboni et al., 2013). With this purpose, we recently discovered that methylisothiocyanate from *Capparis spinosa* and allylisothiocyanate from *Armoracia rusticana* were active on *Meloidogyne incognita* (Caboni et al., 2012; Aissani et al., 2013). Consistently, the tetrahydo-3,5-dimethyl-1,3,5-thiadiazine-2-thione, main component of the commercial fumigant nematicide Dazomet, is converted to methylisothiocyanate in the soil. *Eruca sativa* Mill. (salad rocket) with *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket), and *Bunias orientalis* (Turkish rocket) is a member of the Brassicaceae family (Bennett et al., 2006). These species, well represented in the Mediterranean basin, are widely used as an edible vegetable and spice. *Eruca sativa* Mill. is known to contain flavonoids, and D-thioglucosides, a class of glucosinolates (GLs) (Steinmetz and Potter, 1991). Importantly, when plants of rucola sustain tissue damage, vacuole glucosinolates are hydrolysed by myrosinase (EC 3.2.1.147), a thioglucosidase enzyme, to yield an aglycone which undergoes non-enzymatic rearrangements to produce volatile isothiocyanates, thiocyanates, indoles and nitriles (Fenwick et al., 1983). Bennet and coworkers found that leaves of *Eruca* and *Diplotaxis* contained high amounts of 4-mercaptobutylglucosinolate with lower levels of 4-methylthiobutlyglucosinolate and 4-methylsulfinylbutylglucosinolate (Bennett et al., 2006). Apart from having nematicidal activity, isothiocyanates and thiocyanates are also general biocides, whose activity is based on irreversible interactions acting as electrophiles that are subject to nucleophilic attack by cysteine residues in biologically critical proteins (Wu et al., 2014; Brown and Morra, 1997). Moreover, being glucosinolate volatile breakdown products, they could represent good candidates for the control of nematode as safe fumigants in integrated pest management procedures. Many of these volatiles have been shown to act as

attractants for certain insects seeking food or egg laying sites rather than to possess a direct insecticidal activity (Nair and McEwen, 1976; Feeny et al., 1970). In the past fumigants such us methyl bromide have been identified as responsible of producing volatile organic compounds (VOCs) contributing to the reduction of the ozone layer and overall leading to a poor air quality. These facts lead to new alternative nematode control practises such as anaerobic soil disinfestation and biofumigation. Both techniques involve incorporating in the soil of large quantities of fresh organic material. To qualify as a good cover crop for the management of plant-parasitic nematodes, the crop should be a poor host for the nematodes and lower the population after incorporation of the crop into the soil (Viaene and Abawi, et al., 1998). *Eruca sativa* cultivar Nemat, was found a poor host for *M. incognita, M. javanica* and *M. hapla* when tested as a cover crop in a greenhouse treatment (Edwards and Ploeg, 2014). Much of the scientific research on rucola extracts deals with their biological activities, but to the best of our knowledge, there are no reports on the nematicidal activity of *E. sativa* and its volatile phytochemicals on *M. incognita*. In the present investigation, we report on: (1) the chemical characterization of the volatilome of *E. sativa* by means of solid phase matrix extraction (SPME) followed by GC/MS analysis, (2) the nematicidal activity (EC_{50}) of the secondary metabolites of rucola, and (3) the disruption of the parasites' biological cycle in host roots treated with fresh rucola powder.

II. 2. Materials and Methods

II. 2. a. Chemicals. Carbon disulphide, erucin, iberin and sulforaphane were obtained from Santa Cruz Biotechnology (Dallas, Texas), butylisothiocyanate, isobutylisothiocyanate, 2-ethyl furan, 2-nonanol, benzaldehyde, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol, 2-furoic acid, ethyl-2-furoate,2-furanacetic acid, dimethyl sulphoxide and β-cyclocitral were obtained from Sigma-Aldrich (Milano-Italy). Methanol was of high-performance liquid chromatography grade.

II. 2. b. General procedure for the synthesis of (E) or (Z) 3-hexenyl acetate.

(Z) 3-hexenyl acetate and (E) 3-hexenyl acetate were prepared as reported in literature, with slight modifications (Ward, et al., 1995). (Z) or (E) 3-hexen-1-ol (0.3 g, 2.99 mmols) and 4-(N,N- dimethylamino) pyridine (DMAP) (0.44 g, 3.59 mmols) were dissolved in 7.5 ml of diethyl ether. Acetic anhydride (0.37 g, 3.59 mmols) was then added drop by drop while stirring, rapidly producing a white precipitate. The reaction mixture was warmed up to 40°C and stirred for 4 hours. The crude mixture was then diluted with diethyl ether, washed with

water (10 mL), HCl 10 % (3 x 8 mL) and saturated $NaHCO_3$ (3 x 10 mL). The combined organic phase was dried over Na_2SO_4 and then concentrated under vacuum to provide the desired acetate as a pale yellow oil.^{1}H spectra were recorded on a Varian UnitInova 500 MHz spectrometer.

(Z) 3-hexenyl acetate

^{1}H NMR (500 Mhz, $CDCl_3$) δ: 5.51-5.47 (m, 1H), 5.33-5.29 (m, 1H), 4.05 (t, J= 7 Hz, 2H), 2.36 (q, J = 7 Hz, 2H), 2.07-2.05 (m, 3H), 2.03 (s, 3H), 0.96 (t, J = 7.5 Hz, 2H) ppm.

(E) 3-hexenyl acetate

^{1}H NMR (500 Mhz, $CDCl_3$) δ: 5.56-5.51 (m, 1H), 5.37-5.32 (m, 1H), 4.04 (t, J= 7 Hz, 2H), 2.28 (q, J = 7 Hz, 2H), 2.00 (s, 3H), 1.98-1.96 (m, 3H), 0.95 (t, J = 7.5 Hz, 2H) ppm.

II. 2. c. Plant Material. Commercial *E. sativa* was obtained from a local market in Cagliari (Sardinia-Italy) on March 2014. Samples were used fresh and a voucher specimen was deposited at the Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy, for species identification.

II. 2. d. GC-MS Analysis. SPME analysis was performed following the method proposed by Jirovetz et al (Jirovetz et al., 2002) with minor modifications. Prior to analysis the aerial parts of a sample of *E. sativa* were first ground to a paste in a mortar, 3g of it were then placed into a 20mL SPME vial, 75.5 X 22.5mm tightly closed with a septum and allowed to equilibrate for 5 minutes at 60°C and then the fibre was exposed to the headspace. The extraction was carried out on a 1cm DVB/CAR/PDMS 50/30 Stableflex (FFA57298-U, Supelco, Milan, Italy) SPME fibre. The fibre was pre-conditioned at 270°C for 1h in a Gerstel MPS bake-out station, according to the manufacturer's instructions. Prior and after each analysis, the fibre underwent to a further bake-out step for 5 minutes at 250°C. The extraction time was fixed to 30 minutes, based on a previous optimization. After the extraction, the fibre was desorbed for 2 minutes into a Gerstel CIS6 PTV injector operating at 250°C in a splitless mode. Compound separation and identification was performed on an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was performed on a VF-Wax 60m x 0.25mm i.d., 0.5µm film thickness column (Agilent), with the following temperature program: 40°C for 4min, then increased to 150°C at a rate of 5.0°C/min, held for 3min then increased to 240°C at a rate of 10°C/min, held for 12

minutes. Helium was used as the carrier gas at a constant flow of 1mL/min. The data was elaborated using a MassHunter Workstation B.06.00 SP1.

II. 2. e. *J2s* Paralysis Bioassays.

A population of *M. incognita* of Italian origin was reared on tomato (*Solanum lycopersicum* Mill.) cv. Roma VF for 2 months in a glasshouse at 25 ± 5°C. Batches of 30 egg masses (averaging 4500 eggs/batch) were collected from infected tomato roots and placed on 2cm sieves (215 μm diameter),positioned on a 3.5cm Petri dish. Distilled water was used as a natural hatching agent (Caboni et al., 2012). The eggs were then incubated in a growth chamber at 25 ± 2°C, in the dark. First emerging juveniles J2s (24 h) were discarded, and only J2s collected after 2 days were used for the experiments. Compounds identified in rucola, including carbon disulphide and erucin, were tested on J2s in a dose range of 1−1000 mg/L using fosthiazate as a chemical control. As part of this study, we also tested, on J2s, structurally related isothiocyanates. Stock solutions of pure compounds were prepared in methanol to overcome insolubility, using aqueous Tween 20 (0.3% v/v) for further dilutions. The final concentration of methanol in each well never exceeded 1% (*v/v*) since preliminary experiments showed that this concentration was not toxic to the nematodes (Caboni et al., 2013). Distilled water as well as a mixture of methanol and aqueous Tween 20 at 0.3 % (v/v) served as controls. In all cases, working solutions were prepared containing twice the test concentration and mixed in CellstarR 96-well cell culture plates (Greiner Bio-One) at a ratio of 1:1 (*v/v*) with a suspension of 25 J2s added to each well. Multiwell plates were covered to avoid evaporation and maintained in the dark at 20°C. Juveniles were observed with the aid of an inverted microscope (Zeiss 3951, Germany) at 20× after 1d and 3d for pure compounds and extracts of *E. sativa*, respectively. The nematodes were at this point moved to plain water after washing through a 20μm sieve to remove the excess of the test compounds. Numbers of motile and paralysed J2s were assessed by pricking the juvenile body with a needle, and they were counted. Paralysis experiments were performed three times, and every treatment was replicated six times.

II. 2. f. Experiment with containerized plants

For this experiment we used a clay loam soil, free of root-knot nematodes, with 1.3% organic matter and a pH of 7.8 that was collected from a non cultivated field at the Aristotle University of Thessaloniki Farm (Greece). The soil was sieved through a 3-mm sieve and partially air dried overnight. The soil was mixed with sand at a ratio of 2:1 to obtain the

mixture used for the bioassays. It was separated into 7 plastic bags, which received appropriate amounts of fresh rucola paste to achieve the concentrations from 4 to 100mg/g, while a treatment with plain water was used as control. Each soil sample was then inoculated with 2500 J2s of *M. incognita*, and was used for transplanting 7-week old, at the six-leaf stage, tomato plants cv. Belladonna into plastic pots containing 200g of soil, which were maintained at 27^{o}C, 60% RH at 16h photoperiod; each pot received 20mL of water every 3 days. Forty days later, root samples were stained with acid fuchsin (Byrd et al., 1983) and the following variables were assessed: fresh root and shoot weight and total number of females and galls of nematodes per gram of root at 10×, under uniform illumination by transparent light within tissue sample. The experiment was conducted twice, and the treatments were always arranged in a completely randomised design with five replicates.

II. 2. g. Field Proof Experiment

To verify the efficacy under field conditions *E. sativa* was planted in field and after having reached full growth it was incorporated in soil that successively harboured tomato plants. In specific the experiment consisted of two treatments, namely 1) rucola seeds planted in full field surface at the concentration of 5g/100m^2 and 2) an untreated control. Each treatment consisted of four replicates and each plot area was 10 m^2. One month later and when rucola had reached full growth it was mechanically incorporated in the soil. A week later 7-week old, at the six-leaf stage, tomato plants cv. Belladonna were transplanted. No other treatments were performed for nematode control during the cultural period and the efficacy was assessed 73 days post transplanting by uprooting the plants and categorising with root index for RKN infection.

II. 3. Statistical Analysis.

For containerised bioassays, data were expressed as a percentage decrease in the number of females and galls/g of root corrected to the control, according to the Abbott's formula: corrected % = 100 × {1−[females number in treated plot/females number in control plot]}. It was fitted in the log-logistic model (Seefeldt et al., 1995) to estimate the concentration causing a 50% decrease in females per gram of root (EC_{50} value). In this regression equation, the rucola paste concentration (mg/g) was the independent variable (x) and the number of females and galls of nematodes (percentage decrease over water control) was the dependent variable (y). Additionally, the Tuckey's test was used to separate treatment differences and

root or shoot fresh weight $P \leq 0.05$. Because ANOVA indicated no significant treatment by time interaction (between runs of experiments), means were averaged over experiments.

Since paralysis in solvent (methanol, Tween-20) did not significantly differ from that observed in distilled water, the percentages of paralyzed J2s were corrected by eliminating the natural paralysis in the water control (0−5% of total number of J2s) according to the Schneider Orelli's formula (Puntener, 1981): Corrected % =(mortality % in treatment) − (mortality % in control))/(100−mortality % in control))} × 100, and they were analysed (ANOVA) combined over time. Since ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2s treated with tested compounds were subjected to nonlinear regression analysis using the log−logistic equation proposed by Seefeldt et al. in 1995: $Y = C + (D - C) /\{1 + \exp[b(\log(x) - \log(EC_{50}))]\}$

Where C = the lower limit, D = the upper limit, b = the slope at EC_{50}, and EC_{50} = the test substances' concentration required for 50% death/paralysis of nematodes after eliminating the control (natural death/paralysis). In the regression equation, the test substances concentration was the independent variable (x), and the paralyzed J2s (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates per test substances' concentration and immersion period was used to calculate the EC_{50} value. Mean data values were reported with the respective standard deviations.

II. 4. Results and Discussion

SPME is a useful analytical technique for the semi-quantitation of volatile compounds in different matrixes, while GC/MS is used for compound identification. With these techniques, we identified 31 compounds in *E. sativa* paste; the most abundant compound was (*Z*)-3-hexen-1-ol, with a relative percent concentration of 12.64 ± 3.71, followed by an unknown compound eluting at 30.11 min tentatively identified as 4-methylpentyl isothiocyanate with a concentration of 12.06 ± 4.76 % **(Table 1)**. 3-hexen-1-ol stereoisomers (*E*, *Z*) are produced by plants when mechanical damage occurs and may have indirect plant defence signalling properties (Ruther and Kleier, 2005). The level of the isothiocyanate erucin in *E. sativa* volatilome was 8.40 ± 3.95 % while Jirovetz*et al*. reported levels of approximately 14.2% (Jirovetz et al., 2002).

According to Cataldi et al., 2007 the most abundant glucosinolates isolated from rucola leaves are glucoerucin, 4-methylpentyl-glucosinolate, n-hexyl-glucosinolate, glucoraphanin, 4-mercaptobutyl glucosinolate, progoitrin, sinigrin and glucobrassicin (Cataldi et al., 2007). In fact, three of the isothiocyanates deriving from the three first mentioned glucosinolates reported above were identified in *E. sativa* after GC/MS analysis, namely erucin, 4-methylpentylglucosinolate, and n-hexylglucosinolate**(Table 1)**.

When we tested the nematicidal activity of the volatilome of *E. sativa*, erucin, methylisothiocyanate,1-pentylisothiocyanate, 1-hexylisothiocyanate, (E)-2-hexenal, 2-ethyl furan and methylthiocyanate were the most active compounds($EC_{50/1d}$ =3.2 ± 1.7, 7.9 ± 3.1 11.1 ± 5.0, 11.3 ± 2.6, 15.0 ± 3.3, 16 ± 5 mg/L and 18.1 ± 0.6 respectively) followed by (*Z*)-3-hexen-1-ol ($EC_{50/1d}$ = 323 ± 91 mg/L) **(Table 2)**. On the other hand, furan derivatives such as 2-furoic acid, ethyl-2-furoate and 2-furanacetic acid were not active at 100mg/L (**Table 2**). Furthermore, β-cyclocitral found in trace amounts in leaves of *E. sativa* (Blaževic and Mastelic, 2008) was not active at 100 mg/L. Interestingly, (E, Z) methyl esters of 3-hexen-1-ol were not active at concentrations > 400mg/L. Among isothiocyanate derivatives, the most effective in paralysing *M. incognita* was benzylisothiocyanate, followed by allylisothiocyanate, and butylisothiocyanate, exhibiting $EC_{50/1d}$ values of 1.9 ± 0.3, 6.6 ± 2.5, and 12 ± 8mg/L, respectively. Noteworthy, isobutyl isothiocyanate and phenyl isothiocyanate were not active at concentrations >200 and > 1000 mg/L, respectively. Sulforaphane was fifty times less active if compared with its reduced isothiocyanate analogue erucin (EC_{50}=152 ± 35mg/L), while iberin showed an EC_{50} of 180 ± 21 mg/L. Finally, benzaldehyde, carbon disulphide and (*E*)-3-hexen-1-ol were not found active at concentrations >250, >500 and >700 mg/L respectively. Interestingly, in our *in vitro* conditions butylisothiocyanate showed a fumigant activity at 160 mg/L. Slight structural differences among isothiocyanates conferred significantly different nematicidal effects. This suggests that the biological activity is not only a concentration-dependent function but also highly related to the alkyl chain. Notably, Caboni and co-workers (Caboni et al., 2012) observed that J2s treated with different isothiocyanates, were paralyzed in a straight shape with evident internal vacuolization **(Figure 2)**. The same evidence was observed treating nematodes with some V-ATPase inhibitors such as α,β,γ,δ-unsaturated aldehydes and pyocyanin (Caboni et al., 2013; 2014). Conversely, nematodes treated with the organophosphorous fosthiazate were paralyzed in a coiling shape (Caboni et al., 2012).

When the methanolic extract of fresh rucola paste was tested against *M. incognita*, a clear dose−response relationship was established and significant paralysis/death of J2s was evident after 3 days of exposure with a calculated EC_{50} value of 93 ± 59mg/L (data not shown).

According to the results of our experiment with tomato plants infested with *M. incognita* grown in containerized plants, when *E. sativa* paste was incorporated to the growing medium it decreased root infestation and increased plant growth in a dose response manner. The EC_{50} values based on the numbers of females and galls were calculated at 20.02 and 20.75 mg/g, respectively **(Table 3)**. Most interestingly, the treatment of tomato plants with rucola paste induced fresh shoot weigh increase **(Table 4)** while no differences were observed on root weight. Additionally, rucola practiced as a soil amendant, fully controlled RKN under field conditions **(Figure 2).** In specific and based on galling index, all roots were severely knotted in control plants and the root system was absent (root index 10) due to secondary infections by soil pathogens while regarding rucola treated plants no knots were evident (root index 1).

Considering the bibliography reporting on the use of soil amendants in root-knot nematodes management, the family Brassicaceae is probably the most cited due to the isothiocyanates levels observed in its members (Matthiessen and Kirkegaard, 2006). This is the first report on the nematicidal properties of the volatilome of *E. sativa* on *M. incognita*, which shows the possibility of its potential use as an intercrop plant. Intercropping, being a cost effective, environmentally friendly pest management method can be easily used in developing countries. In our previous works, we largely stressed the importance of volatility in the nematicidal character of various botanical extracts (Ntalli and Caboni, 2012). This feature is essential for delivering the active substance to the inner parts of the soil infested by nematodes. Herein we report the high activity of rocket and attribute it to some of its isothiocyanates contents. Nevertheless, under field conditions the efficacy of the isothiocyanates decreases due to their short half-life, making mandatory the repetition of applications (Aissani et al., 2013), a fact that should not be underestimated. It is yet to be studied the fate in soil of pure isothiocyanates with regard to their half-life as ingredients in complex botanical soil amendments. This together with the cost effectiveness of respective treatments, could establish an nematode control method suitable for integrated crop management.

Abbreviation

SPME solid phase micro extraction, GC/MS gas chromatography mass spectrometry, GLS glucosinalates, ITC isothyocyantes

Table 1. Composition Percent (n=6) of Rucola Volatile Phytochemicals Determined by SPME Analysis, Listed in Order of Elution, followed by GC/MS analysis.

peak #	retention time (min)	linear retention index	compound	%
1	6.12	746	carbondisulfide	3.58±0.21
2	11.49	969	2-ethylfuran	1.84±0.87
3	12.3	995	3-pentanone	0.62±0.36
4	16.72	1128	tetrahydrothiophene	1.78±1.02
5	17.49	1152	2-pentenale*	0.30±0.18
6	18.78	1191	3-ethylthiophene	0.55±0.23
7	20.36	1242	2-(E)-hexenal	5.19±2.09
8	21.06	1265	methyl isothiocyanate	4.26±2.15
9	22.29	1305	methylthiocyanate	2.12±1.21
10	23.05	1331	3-(Z)-hexenylacetate	7.91±2.83
11	23.49	1347	2-(E)-hexenylacetate	0.33±0.22
12	24.02	1365	1-hexanol	0.46±0.33
13	24.4	1378	3-(E)-hexen-1-ol	0.72±0.61
14	25.07	1401	3-(Z)-hexen-1-ol	12.64±3.71
15	25.37	1412	nonanal	2.73±1.49
16	25.58	1419	2-(Z)-hexen-1-ol	1.54±0.23
17	26.83	1463	1-phenyl-1-butene	7.96±1.30
18	27.12	1473	3-(Z)-hexen-1-yl butyrate	1.18±0.25
19	27.47	1486	1-butene 4-isothiocyanatecoelutingwith z-3-hexenyl pentanoate	6.15±1.72
20	27.92	1501	3-(Z)-hexenylpentanoate	1.84±0.43
21	28.43	1518	n-pentyl isothiocyanate	1.92±0.80

22	30.00	1569	benzaldehyde	3.79±0.54
23	30.11	1573	4-methylpentyl isothiocyanate*	12.06±4.76
24	31.53	1625	1- hexylisothiocyanate	2.63±1.58
25	33.11	1691	(E)-hex-3-enyl (E)-2-methylbut-2-enoate	0.85±0.29
26	33.18	1694	3-methylhexyl isothiocyanate	0.67±0.30
27	34.47	1759	4-ethylbenzaldehyde	0.31±0.15
28	36.85	1898	cumaldehyde	1.72±0.19
29	37.25	1926	diethyldithiocarbamicacid	1.23±0.38
30	38.15	1990	3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)*	2.73±0.74
31	41.32	2216	erucin	8.40±3.95

Table 2. EC_{50} Values (±SD, n=4) of Tested Compounds against *M.incognita* Calculated after 1 day of Nematode Immersion in Test Solutions

compd	$EC_{50/1d}$ (mg/L± SD)
erucin	3.2 ± 1.7
methylisothiocyanate	7.9 ± 3.1
1-pentylisothiocyanate	11.1 ± 5.0
1-hexylisothiocyanate	11.3 ± 2.6
(E)-2-hexenal	15.0 ± 3.3
2-ethylfuran	16 ± 5.0
methylthiocyanate	18.1 ± 0.6
(*Z*)-3-hexen-1-ol	323 ± 91
3-pentanone	>100
tetrahydrothiophane	> 100
benzaldehyde	>250
3-(Z)-hexenylacetate	>400
3-(E)-hexenylacetate	> 400
carbon disulphide	>500
(*E*)-3-hexen-1-ol	>700
benzylisothiocyanate*	1.9 ± 0.3
allylisothiocyanate*	6.6 ± 2.5
butylisothiocyanate*	12 ± 8.0
sulforaphane	152 ± 35
iberin	180 ± 21
2-furoic acid	> 100
ethyl-2-furoate	> 100
2-furanacetic acid	> 100
β-cyclocitral	> 100
isobutyl isothiocyanate	>200
phenylisothiocyanate*	> 1000
fosthiazate	0.4 ± 0.3

* data published by Caboni et al., 2013

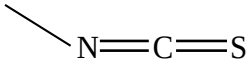
Table 3. EC_{50} Values (mg/L, n=2) of Rucola Paste Efficacy on *M. incognita* as Calculated in Containerised Experiments with Respective Standard Error and Confidence Interval Values

♀/g root			galls/g root		
EC_{50}	Stderror	95% ConfInt	EC_{50}	Stderror	95% ConfInt
20.02	1.65	16.64-23.41	20.75	1.84	16.99-24.53

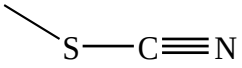
Table 4. Aerial Parts Weight Increase After Treatment With Rucola Paste in Nematodes Infested Tomato Plants

E. sativa paste (mg/g)	aerial part weight (g)
0	4.45 ± 0.24^{c}
4	4.99 ± 0.15^{cb}
8	5.61 ± 0.05^{cbd}
16	6.23 ± 0.36^{cbd}
32	7.35 ± 0.26^{d}
64	7.14 ± 0.16^{d}
128	6.95 ± 0.15^{bd}

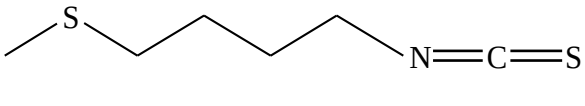
Data are presented as means of five replicates with standard deviations. Means followed by the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).



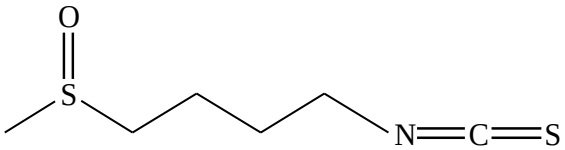
methylisothiocyanate



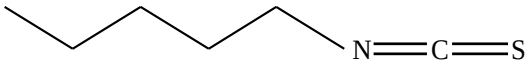
methylthiocyanate



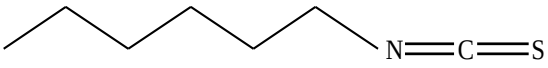
erucin



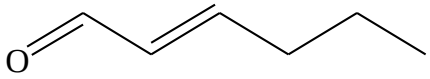
sulforaphane



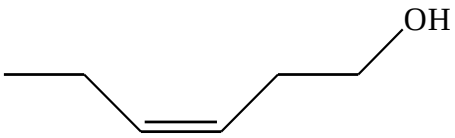
1-pentylisothiocyanate



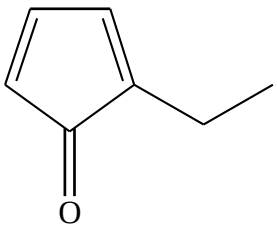
1-hexylisothiocyanate



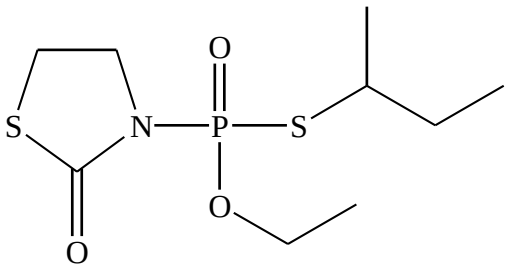
(E)-2-hexenal



(Z)-3-hexen-1-ol



ethyl furan



fosthiazate

Figure 1. Chemical structures of most active nematicidal compounds.

Figure 2.*M. incognita* J2s before (left) and after (right) 24 h immersion in erucin at 10 mg/L. After treatment, nematodes were paralyzed in a straight shape while internal vacuoles were evident.

Figure 3. A) Tomato root grown on rucola amended field and B) untreated control.

II. 5. References

Reynolds AM, Dutta, TK, Curtis RSC, Power SJ, Gaur HS, Kerry BR, Chemotaxis can take plant-parasitic nematodes to the source of a chemo-attractant via the shortest possible routes. *J. R. Soc. Interface.* **2011**, *8*, 568−577.

Isman MB, Botanical insecticides, deterrents, and repellents in modern agriculture and an creasingly regulated world. *Annu.Rev. Entomol*. **2006**, *51*, 45−66.

Tian B, Yang J, Zhang KQ, Bacteria used in the biological control of plant parasitic nematodes: Populations, mechanisms of action, and future prospects. *FEMS Microbiol. Ecol.***2007**, *61*, 197−213.

Whittaker RH, Feeny PP, Allelochemics: chemical interactions between species. *Science.***1971**, *171*, 757−770.

Ntalli NG, Caboni, P. Botanical nematicides: a review. *J. Agric. Food Chem.* **2012**, *60*, 9929−9940.

Caboni P, Saba M, Tocco G, Casu L, Murgia A, Maxia A, Menkissoglu-Spiroudi U, Ntalli N. Nematicidal activity of mint aqueous extracts against the root-knot nematode *Meloidogyne incognita. J. Agric. Food Chem.***2013**, *61*, 9784−9788.

Caboni P, Sarais G, Aissani N, Tocco G, Sasanelli N, Liori B, Carta A, Angioni A, Nematicidal activity of 2-thiophenecarboxaldehyde and methylisothiocyanate from Caper (*Capparis spinosa*) against *Melodogyne incognita*. *J. Agric. Food Chem.***2012**, *60*, 7345−7351.

Aissani N, Tedeschi P, Maietti A, Brandolini V, Vincenzo LG, Caboni P, Nematicidal Activity of Allylisothiocyanate from Horseradish (*Armoracia rusticana*) Roots against *Meloidogyne incognita. J. Agric. Food Chem.***2013**, *61*, 4723−4727.

Bennett RN, Rosa EA, Mellon FA, Kroon PA, Ontogenic profiling of glucosinolates, flavonoids, and other secondary metabolites in *Eruca sativa* (salad rocket), *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket), and *Bunias orientalis* (Turkish rocket) *J. Agric. Food Chem*. **2006**, *54*, 4005–4015.

Steinmetz KA, Potter JD, Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Cause. Control*. **1991**, *2*, 427-442.

Fenwick GR, Heaney RK, Mullin WJ, Glucosinolates and their breakdown products in food and food plants. CRC *Crit. Rev. Food Sci. Nutr.***1983**, *18*, 123-201

Wu H, Masler EP, Rogers ST, Chen C, Chitwood DJ, Benzylisothiocyanate affects development, hatching and reproduction of the soybean cyst nematode *Heteroderaglycines*. *Nematol.* **2014**, *4*, 495-504.

Brown PD, Morra MJ, Control of soil-borne plant pests using glucosinolate-containing plants. *Adv. Agron*. **1997**, *61*, 167−231.

Nair KSS, McEwen FL, Host selection by the adult cabbage maggot, *Hylemya brassicae* (Diptera: Anthomyiidae): effect of glucosinolates and common nutrients on oviposition. *Can. Entomol*. **1976**, *108*,1021-1030

Feeny P, Paauwe KL, Demong NJ, Flea beetles and mustard oils: host plant specificity of *Phyllotreta cruciferae* and *Phyllotreta striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Amer*. **1970**, *63*, 832-841

Vincent C, Steward RK, Effect of allyl isothiocyanate on field behavior of crucifer-feeding flea beetles (Coleoptera: Chrysomelidae) (*Phyllotreta cruciferae*, *Psylliodes punctulata*, *Phyllotreta bipustulata*, *Phyllotreta striolata*, rutabagas). *J. Chem. Ecol.***1984**, *10*, 33-39

Hovanitz W, Chang VCS, Honch G, The effectiveness of different isothiocyanates on attracting larvae of *Pieris rapae*. *J. Res. Lipid***1963**, *1*, 249-259

Huang X, Renwick JAA, Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracae*. *J. Chem. Ecol*. **1994**, *20*, 1025-1037

Viaene NM, Abawi GS, Management of *Meloidogyne hapla* on lettuce in organic soil. *Plant Disease***1998**, *82*, 945–952.

Edwards S, Ploeg A, Evaluation of 31 potential biofumigant brassicaceous plants as hosts for three meloiodogyne species. *J Nematol.***2014,***46*, 287-295.

Ward D, Villanueva LA, Allred GD, Payne SC, Semones MA, Liebeskind LS, Synthesis, Characterization, and Configurational Lability of (η^3-Allyl)dicarbonyl[hydrotris(1-pyrazolyl)borato] molybdenum Complexes Bearing Substituents at the Termini: Thermodynamic Preference for the Anti Stereoisomer *Organometallics*, **1995**, *14*, 4132–4156.

Jirovetz L, Smith D, Buchbauer G, Aroma compound analysis of *Eruca sativa* (Brassicaceae) SPME headspace leaf samples using GC, GC−MS, and olfactometry. *J. Agric. Food Chem*. **2002**, *50*, 4643−4646.

Caboni P, Ntalli GN, Aissani N, Cavoski I, Angioni A, Nematicidal activity of (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*. *J. Agric. Food Chem.***2012**, *60*, 1146−1151.

Byrd KRB, Kirkpatrick T, An improved technique for clearing and staining plant tissues for detection of nematodes. *J. Nematol*. **1983**, *15*, 142-143.

Seefeldt SS, Jensen JE, Fuerst EP, Log-logistic analysis of herbicide rate response relationships. *Weed. Technol*. **1995**, *9*, 218−227.

Puntener W, *Manual for Field Trials in Plant Protection*, 2nd ed.; Ciba Geigy, Basel, Switzerland,**1981**, p 205.

Ruther J, Kleier S, Plant-plant signalling: ethylene synergizers volatile emission in *Zea mays* induced by exposure to (Z)-3-hexen-1-ol. *J. Chem. Ecol.* **2005**, *31*,2217−2222.

Cataldi TR, Rubino A, Lelario F, Bufo SA. Naturally occurring glucosinolates in plant extracts of rocket salad (*Eruca sativa* L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry. *Rapid Commun Mass Spectrom.***2007**, *21*, 2374-2388.

Blaževic I, Mastelic J, Free and bound volatiles of rocket (*Eruca sativa* Mill.)

Flavour Fragr. J. **2008**,*23*, 278–285

Caboni P, Aissani N, Cabras T, Falqui A, Marotta R, Liori B, Ntalli N, Sarais G, Sasanelli N, Tocco G, Potent nematicidal activity of phthalaldehyde, salicylaldehyde, and cinnamic aldehyde against *Meloidogyne incognita*. *J. Agric. Food Chem.* **2013**, *61*, 4723−4727.

Caboni P, Tronci L, Liori B, Tocco G, Sasanelli N, Diana A, Tulipaline A: Structure-Activity Aspects as a Nematicide and V-ATPase Inhibitor. *Pestic. Biochem. Phys*. **2014**, *112*, 33–39.

Matthiessen JN, Kirkegaard JA, Biofumigation and enhanced biodegradation: opportunity and challenge in soil borne pest and disease management. *Crit. Rev. Plant Sci*.**2006**, *25*, 235–265.

III. 1. Introduction - Nematicidal activity of furanocoumarins from parsley against *Meloidogyne* spp.

Meloidogyne is one of the most economically important plant-parasitic nematode genera (Jones et al., 2013). The feeding of these phytonematodes induces the formation of large galls or "knots" throughout the root system of infected plants. *Meloidogyne* spp. have a wide host range that includes most of the flowering plants and a short biological cycle; infection can be often followed by the attack of other soil pathogens (Trudgill DL and Blok, et al., 2001; Back et al., 2002). Crop quality and yield decrease severely with infection, resulting in significant economic losses. For decades, synthetic nematicides have been a major control method, although fumigants often readily disperse and non-fumigants often biodegrade in soils, sometimes making both ineffective. Eco-sustainable methods of nematode control involve resistant cultivars, crop rotation, cover crops, solarisation, fallow, or antagonistic fungi or bacteria; these methods tend to be adjuncts to traditional chemical control. The search for alternatives to chemical controls has been intensified with the screening of secondary plant metabolites (Chitwood, 2002; Isman, 2006; 2008). In some cases, cover crops and crop residue incorporation can take advantage of allelopathic and other endogenous plant compounds for economical pest management. Nonetheless, cover crops are usually added to soil as good sources of organic material rather than for their allelopathic potential. Cover crops are indeed beneficial to plant growth and nematode control because of improvements in the physical properties of soil and the enhancement of growth of nematode-antagonistic microbes. However, allelochemicals or secondary metabolites exuded in the plant rhizosphere have a great role in defence against abiotic and biotic stresses (Farooq et al., 2013). *Petroselinum crispum* or *P. crispum* (Apiaceae), commonly known as parsley, is native to the central Mediterranean region and is cultivated as a herb, spice or vegetable. Although it is infected by *M. arenaria* (Mennan et al., 2011) and *M. incognita* (Aguirre et al., 2003), it is resistant to *M.* javanica (Rosa et al., 2013) and contains substances of great biological interest with potential nematicidal activity. In specific, the aerial part of parsley yields a water extract rich in the flavonoids apigenin, apigenin-7-O-glucoside or cosmosiin, apigenin-7-O-apiosyl-(1→2)-O-glucoside (or apiin) and the coumarin 2″,3″-dihydroxyfuranocoumarin (or oxypeucedanin hydrate) (Chaves et al., 2011). Pinene is the main constituent of parsley seed essential oil (Kiralan et al., 2012), while α-pinene (25.5%), p-cymenene (17.7%), β-myrcene (16.9%), β-phellandrene (15.2%) and β-pinene (9.6%) are the major components of leaf oil (Gruszecki, 2009). Parsley has been reported to exhibit antimicrobial and antiradical activity (Kiralan et al., 2012; Gruszecki et al., 2009; Osman et al., 2009), but no reports exist on the activity against *Meloidogyne* spp. although phenols and terpenes are compounds possessing recognised nematicidal properties (Andrés et al., 2013). In addition, the aromatic

constituents of essential oils have received special attention as biopesticides, due to their simplicity and familiarity to the public as being less toxic than synthetic pesticides. In the United States, essential oil products are considered non-risk pesticides and do not undergo the same registration procedure of conventional pesticides because they meet the Criteria for FIFRA Exemption (http://www.epa.gov/oppbppd1/biopesticides/regtools/25b_list.htm). This is a first report on the nematicidal activity of parsley against *Meloidogyne* spp. In this study, we investigated the 1) chemical characterisation of the essential oil from the aerial parts of parsley, 2) furanocoumarin levels in the methanol extract of the aerial part by the use of mass spectrometer with a quadrupole and a time of flight analyser, coupled to a liquid chromatograph (LC-QTOF/MS), 3) *in vitro* paralysis caused by the essential oil and methanol extract, 4) paralytic activity of selected compounds, and 5) the effect of fresh parsley paste on gall formation and *M. incognita* reproduction in tomato roots.

III. 2. Materials and Methods

III. 2. a. Chemicals. Xanthotoxin, xanthotoxol, bergaptol, bergapten, psoralen, and apiole were purchased from Extrasynthese, France, while α-pinene, β-pinene, myrcene, α-phellandrene, β-phellandrene, and myristicin were purchased from Sigma-Aldrich, Italy. All solvents and reagents were of pesticide grade.

III. 2. b. Plant materials. *P. crispum* was bought from a local market (Cagliari, Italy). A voucher specimen was deposited at the Department of Life and Environmental Sciences, University of Cagliari (Erbario Farmaceutico dell'Erbario di Cagliari -CAG256/12).

III. 2. c. Nematode Populations. Three separate populations of *M. incognita, M. hapla* and *M. arenaria* originally obtained from tomato roots collected from a greenhouse in Vassilika (Greece) were reared on tomato (*Solanum lycopersicum* L. cv. Belladonna), and juveniles for bioassays were extracted in Baermann funnels at 28 ^{o}C (Hussey and Barker, 1973). All hatched J2 in the first 3 days were discarded; thereafter, J2 collected after 24 h were used in the experiments.

III. 2. d. Extract preparation. *Essential Oil Extraction.* Aerial fresh plant parts were subjected to water distillation using a Clevenger apparatus (Winzer) for 3 h according to the Fifth Edition of the European Pharmacopoeia (EU-Pharmacopoeia (Ph.Eur.), 2001), with 250 g of fresh plant material and 1.0 L of distilled water in a 2 L glass flask. The essential oil (EO) obtained was dried over anhydrous Na_2SO_4 and stored in dark glass vials with Teflon-sealed caps at -20 ^{o}C until use. The average yield of the EO obtained from the aerial parts for three replicates was 0.082 % w/w by wet weight. *Methanolic extract preparation*. Aerial parts of parsley (5 gr) were ground with a pestle in a

mortar with 50 ml of methanol, ultrasonicated for 15 min. and then filtered through Whatman 40 paper, and then dried over anhydrous Na_2SO_4 before use in bioassays.

III. 2. e. Chemical characterisation- GC-MS Analysis. The chromatographic separation for the identification of EO components was performed on an Agilent Technologies 6850 instrument coupled to a Mass Selective Detector 5973 and a 7683B Series injector autosampler. Sample injection was performed in splitless mode. Data were elaborated using MSD ChemStation D.03.00611. The 30 m x 0.25 mm column was coated with 5% phenylmethylpolysiloxane (DB-5); film thickness = 0.25 μm. Injector temperature was kept at 250 ^{o}C. The oven temperature was programmed from 50 to 230 ^{o}C (5 ^{o}C min^{-1}) in 38 min and kept at this temperature for 2 min. The carrier gas was helium with a flow of 1 mL/min; and 1 μL sample was injected after dilution (1:1000 v/v) of the EO with hexane. The MS conditions were as follows: ionization voltage, 70 eV; scan rate, 2.91 scan/s; mass range, 50-550; transfer line, 230 ^{o}C. The components of the EO were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against the NIST98 mass spectral library, as well as retention indices as calculated according to Kovats, for alkanes C_9-C_{24} compared with those reported by Adams (Adams, 2007).

III. 2. f. Chemical characterisation-LC-QTOF/MS analysis. An Agilent HPLC 1200 series instrument equipped with a 6520 QTOF detector was used. The chromatographic separation of the methanolic extract was performed on a Varian column XRS3 C8 (50 x 2 mm). The mobile phase consisted of (A) aqueous formic acid 0.1%, and (B) acetonitrile. The solvent gradient (v/v) was generated starting from 10 % B and reaching 40 % B in 10 min, then increasing to 80 % B at 20 min, then 90 % B at 25 min and maintaining 100% B for 10 min for a total run of 35 min. The methanolic extract was diluted 1:10 v/v with methanol and then 4 μL of this solution were injected into the electrospray interface (ESI) operating in the positive-ion detection mode. Mass spectral data were acquired in the range *m/z* 100-1500, with an acquisition rate of 1.35 spectra/s, averaging 10000 transients. The source parameters were as follows: drying gas temperature 250 °C, drying gas flow rate 5 L/min, nebulizer pressure 45 psi, and fragmentor voltage 150 V. Data acquisition and processing were done using Agilent Mass Hunter Workstation Acquisition v B.02.00 software.

III. 2. g. Paralysis bioassays. The nematicidal activity of the extracts and pure compounds, in terms of nematode juvenile motility suppression, was tested according to Ntalli et al. 2010. Based on the nematicidal activity of the extracts, the tested compounds were selected because they were major constituents to which hypothetically the activity could be attributed. The concentration range used to determine EC_{50} values for essential oil was from 4 to 100 mg/L; for pure substances, 10 to 1000

mg/L. Juveniles were separated into two distinct categories, motile or immotile, under an inverted microscope (Invertoskope, Zeiss, West Germany) at 40x after 24 h for pure compounds and 72 h for extracts and essential oil. Moreover, at that point, nematodes were moved to plain water after washing in tap water through a 20 μm pore screen to remove excess extract. Numbers of motile and paralyzed J2 were assessed by pricking the juvenile body with a needle, and they were counted.

III. 2. h. Effect of parsley paste on gall formation and *M. incognita* reproduction in tomato roots.

The soil used for the pot experiment was a root-knot nematode-free clay loam soil (33 % clay, 37 % loam, 30 % sand; pH 7.8) collected from an uncultivated field of the Aristotle University of Thessaloniki Farm. The soil was passed through a 3-mm-pore sieve and then was partially air dried overnight; soil moisture and maximum water holding capacity (MWHC) were measured. It was next mixed with sand at a ratio of 2:1 to obtain the mixture hereafter referred to as soil, which was then divided into 7 plastic bags corresponding to treatments. Appropriate amounts of fresh parsley paste, prepared in a mortar with a pestle, were mixed with the soil to achieve the concentrations of 4 to 100 mg g^{-1}, while a treatment with plain water served as control. Treatments were wetted with the same volume of water used in the control and incubated for 24 h. After a second sieving, the soil corresponding to each treatment was returned into the plastic bag and was inoculated with 2500 J2 of *M. incognita*, mixed thoroughly by shaking and left to equilibrate in the dark at room temperature for another 24 h. Moisture content never exceeded the 24% of MWHC. After the incubation period, the soil was sieved again and then it was used for transplanting 7-week-old, six-leaf-stage tomato plants (cv. Belladonna) into 200 g plastic pots. The experiment was maintained at 27 ^{o}C, 60% RH at 16 h photoperiod, and each pot received 20 mL of water every 3 days. After 40 days, roots were then stained with acid fuchsin (Byrd et al., 1983) and the following variables were assessed: fresh root weight, fresh shoot weight and total number of female nematodes and galls per gram of root at 10 × magnification under uniform illumination by transparent light within tissue sample. The experiment was replicated once, and the treatments were always arranged in a completely randomised design with five replicates.

III. 3. Statistical analysis.

All treatments in all bioassays were replicated five times and experiments were repeated at a different time. Data were subjected to statistical analysis according to Ntalli et al 2010. Briefly both paralysis and pot bioassay assessment data were corrected according to the bioassay data were fitted into a log-logistic model (Seefeldt et al., 1995) to estimate the concentration that caused a 50% decrease in females and galls per gram of root (EC_{50} value).

III. 4. Results and Discussion

According to the GC/MS analysis of the parsley essential oil the most abundant compounds were apiole, myristicin, α- and β-phellandrene, and myrcene (**Table 1**). In general, we detected 13 peaks (2 unknowns corresponding 0.41%) in accordance with Zhang et al., 2006 (Zhang et al., 2006). Compound identification and quantification were achieved by co-chromatography with authentic standards except for elemicin, which was identified with NIST08 mass spectral library at 92% probability. Additionally, we developed an HPLC-QTOF/MS method for the separation, identification and quantification of furanocoumarins in the methanolic extract of the aerial parts of parsley, using an external standard method. Levels of xanthotoxin, psoralen, bergapten and oxypeucedanin ranged from 1.77 to 46.04 mg kg^{-1} wet weight (**Table 2**). This is the first report on the nematicidal activity of furanocoumarins against *Meloidogyne* spp. According to our results, the methanolic extract of parsley was found highly nematicidal and the EC_{50} values after 72 h of nematode immersion in test solutions were 140 ± 15, 416 ± 25 and 611 ± 19 mg L^{-1} against *M. incognita*, *M. arenaria* and *M. hapla*, respectively (**Table 3**). The essential oil was found active only against *M. incognita* and the $EC_{50/72h}$ value was 795 ± 125 mg L^{-1}, while the water extract did not show any activity. Furanocoumarins are scarcely water soluble, and this fact may explain the activity of the methanolic extract (**Table 3**). The most active tested compounds against *Meloidogyne* spp. are shown in **Figure 1**. Xanthotoxol had an $EC_{50/24h}$= 68 ± 33 mg L^{-1} (**Table 4**) followed by psoralen and xanthotoxin ($EC_{50/24h}$= 147 ± 88 mg L^{-1} and 200 ± 21 mg L^{-1}, respectively). Apiole and myristicin showed lower $EC_{50/24h}$ values, 766 ± 67 and 812 ± 83 mg L^{-1}, respectively. In all cases to verify mortality, nematodes were transferred to water, after treatment, to assess for motility regain. In all cases paralysis was permanent even after pricking the juvenile body with a needle and thus herein we report on mortality. In the pot experiment, parsley paste exhibited activity against *M. incognita* at all doses tested. Numbers of galls and nematode females decreased with increasing treatment concentrations, and the EC_{50} values were calculated at 24.79 and 28.07 mg/g, respectively (**Figure 2**). Interestingly, treatment of tomato plants with parsley paste resulted in a significant increase in the fresh shoot and root weight (**Figure 3**). Some of the essential oil components reported herein namely, myristicin, α- and β-phellandrene, and apiole are known major components of *P. crispum* (Stan et al., 2013); while Osman *et al*. reported the myristicin and apiole contents in parsley as 24.10 and 16.65% w/w, respectively (Osman et al., 2009). Similarly, some furanocoumarins have already been identified in parsley by other groups (Chaves et al., 2011; Cherng et al., 2008; Manderfeld et al., 1997; Zaynoun et al., 1985). Furanocoumarins constitute a subfamily of coumarin compounds with important defensive properties against pathogens and insects, as well as allelopathic functions in plants (Chaves et al., 2011; Kiralan et al., 2012; Osman

et al., 2009; Andrés et al., 2013). In fact the importance of furanocoumarins is such that metabolic engineering is being employed to increase their secretion in plant tissues. In particular, expression of prenyltransferase (PcPT) and 4-coumaroyl CoA 2'-hydroxylase genes in *Nicotiana benthamiana*, which normally does not produce furanocoumarins, resulted in formation of demethylsuberosin, indicating that furanocoumarin production may be reconstructed by a metabolic engineering approach. In the same study, it was demonstrated that a single PcPT is involved in the biosynthesis of furanocoumarin in parsley (Karamat et al., 2014). In another study, a *Phytophthora sojae* 25-amino acid oligopeptide (Pep25) elicitor was found to affect the secondary metabolism of parsley cell cultures by inducing the accumulation of furanocoumarins (e.g., marmesin and bergapten) (Hagemeier et al., 1999). Coumarins and phenolics, i.e., apigenin, apiin and 6''-acetylapiin, as well as essential oil components such as myristicin and apiole, have been reported in the literature as active compounds in *P. crispum*. Particular biological activities of coumarins of agronomical interest are their antibacterial, antifungal and insecticidal effects (Farzaei et al., 2013). Psoralen and xanthotoxin have significant fungicidal activities (Boulogne et al., 2012); apiole showed acaricidal activity against *Dermatophagoides farinae*, *D. pteronyssinus*, and *Tyrophagus putrescentiae* (Song et al., 2011) and insecticidal activity against the storage pests *Sitophilus zeamais* and *Tribolium castaneum* (Chu et al., 2012) (Chu et al., 2012). Botanical nematicides have gained considerable interest in recent years (Ntalli and Caboni, 2012a; 2012b; Caboni et al., 2012; Ntalli et al., 2011). Single nematicidal compounds can be used directly after purification of botanical extracts or can serve as prototypes in the chemical synthesis of nematicidal formulates; while the nematicidal botanical extracts could be produced and applied directly by the farmer on the field as an ecofriendly, cost effect nematode control method. Additionally, synergism among extracts ingredients (Ntalli et al., 2011) could help overcome persistence problems. In the present study, parsley water extract was more effective than the essential oil, as previously reported by our group regarding the nematicidal activity of three mint species, namely *Mentha* × *piperita*, *M. spicata*, and *M. pulegium* against *Meloidogyne*. This fact strengthens the scenario of incorporating the use of amendments in integrated crop management programs and in particular by using them as green manures or in intercropping (Caboni et al., 2013). Research on essential oils remains valuable because their active ingredients are used by consumers as culinary herbs and flavourings (Isman, 2008). Recently, the use of essential oils in nematode control has been reviewed (Andrés et al., 2012). Last it is yet to be studied if parsley essential oil or it's specific compounds could be synergistic with the aqueous extract. Investigating the molecular structural basis for activity, we previously reported that salicylaldehyde bearing an hydroxyl group in the ortho position to the formyl group showed high nematicidal activity (Caboni et al., 2013). Similarly, in the present study

xanthotoxol was the most active furanocoumarin (**Table 4**) followed by psoralen, which lacks the hydroxyl group, and xanthotoxin, which has a methoxy group and was 3 times less active than xanthotoxol. In all cases nematodes died in a straight shape if compared with the organophosphorus nematicide fosthiazate. When effective, biodisinfestation with organic soil additives can be viewed as an environmentally friendly, low-cost and easy-to-apply nematode control method that additionally improves crop quality and yield. Recently, Ismail and co-workers reported on the use of cress (*Eruca sativa*), castor (*Ricinus communis*) and linseed (*Linum usitatissimum*) against *M. arenaria* in potato fields (Ismail et al., 2014). Organic manure (poultry, cow dung and domestic waste) used for the control of *M. incognita* on Ethiopian eggplant (*Solanum aethiopicum*) improved yield in greenhouse pot experiments (Abolusoro et al., 2014). Stirling suggested that conventional vegetable farming systems could be improved by annually incorporating commercially available organic amendments, or by mulching biomass from rotation crops onto the bed surface well before the vegetable crop is to be planted, or by leaving the bed undisturbed and covering those residues with plastic (Stirling, 2013). Organic amendments with *Brassica* species in combination with solarisation can sometimes be used as an alternative to conventional nematicides (Guerrero-Diaz et al., 2013). The commercial nematicide fosthiazate used on tomatoes at its commercial dose for *Meloidogyne* spp. control (2 mg g^{-1}) gave 100% efficacy (Ntalli et al., 2009). Interestingly, the most studied botanical species used as soil amendments for nematode control are the Brassicaceae that are usually used at between 0.5–9.3% w/w (Matthiessen and Kirkegaard, 2006) thus supporting the higher nematicidal potential of parsley as reported herein. Although parsley is susceptible to root-knot nematodes (Jerry, 2002; Dias-Arieira et al., 2012) it's EO has been already reported of nematicidal activity (Amin, 2005) in accordance with the results of our study. In conclusion, parsley has potential use as a soil amendment in *Meloidogyne* control, although field studies are necessary before any recommendations can be made. The current study demonstrated the successful use of parsley as a simulated intercrop in greenhouse-grown tomato for the control of *Meloidogyne* spp. Whole plant incorporation could ensure the availability of the high amounts of incorporated material needed for such a treatment.

ACKNOWLEDGEMENTS. We thank Dr. Nicola Sasanelli for providing *M. hapla* and *M. arenaria*. We also want to thank Vivaio Peterle (Italy) for providing tomato plants.

Table 1. Chemical composition of the essential oil from *Petroselinum crispum* according to GC/MS analysis. Values are the mean of three determinations.

compound	RT[a] (min)	KI[b]	%
α-pinene	6.36	0939	0.99 ± 0.15

β-pinene	7.25	0980	0.50 ± 0.18
myrcene	7.55	0991	5.85 ± 1.09
α-phellandrene	7.84	1005	2.70 ± 0.73
β-phellandrene	8.34	1031	19.99 ±4.32
p-mentha-1,3,8-triene[c]	9.96	1111	0.11 ± 0.05
myristicin	16.24	1520	23.66 ± 1.1
elemicin	16.75	1554	0.22 ± 0.09
apiole	19.19	1680	39.44 ±3.36
not identified	-	-	0.41 ± 0.30
Total	-	-	93

Compounds are listed in order of elution from a DB-5 (30 m, 0.25 mm, 0.25 μm) capillary column.
[a] Retention time.
[b] Kovats index.
[c] Identification by comparison of mass spectra with the respective data of NIST library in total ion current (TIC) and the literature, as well as retention indices.

Table 2. Furanocoumarins identified by LC-QTOF/MS in the methanol extract of the aerial parts of *Petroselinum crispum.*

[a]Retention time .

compound	**RT**[a]	**formula**	**log*P***[b]	**mg/kg**	***m/z***[c] **meas**	***m/z* calc**	**error**
xanthotoxin	13.14	$C_{12}H_8O_4$	1.9	1.77±0.12	217.0493	217.0494	-1.60
psoralen	13.63	$C_{11}H_6O_3$	2.3	3.55±0.75	187.0390	187.0389	-2.39
bergapten	15.61	$C_{12}H_8O_4$	2.3	10.21±1.29	217.0495	217.0494	-0.68
oxypeucedanin	16.59	$C_{16}H_{14}O_5$	2.6	46.04±5.50	287.0949	287.0950	-2.25

[b]Partition coefficient.
[c]*M*mass-to-charge ratio .

Table 3. EC_{50} values of different *Petroselinum crispum* extracts against *Meloidogyne incognita*, *M. arenaria* and *M. hapla* calculated after 72 h of immersion in test solutions, expressed as EC_{50} ± SD (mg/L).

extract			
stem methanol extract	140 ± 15	416 ± 25	611 ± 19
leaf methanol extract	505 ± 116	>500	>500
stem water extract	>500	>500	>500
leaf water extract	>500	>500	>500
essential oil aerial part	795 ± 125	NT	NT

NT means not tested.

Table 4. EC_{50} values of selected compounds tested against *Meloidogyne incognita*, the most sensitive of the three tested species to a methanolic extract of *Petroselinum crispum*, calculated after 24 h of immersion in test solutions.

compound	**EC_{50} ± SD (mg/L)**
xanthotoxol	68 ± 33
psoralen	147 ± 88
xanthotoxin	200 ± 21
apiole	766 ± 67
myristicin	812 ± 83
bergapten	>500
bergaptol	>500
β -pinene	>1000
myrcene	>1000
α -phellandrene	>1000
β -phellandrene	>1000
fosthiazate[a]	0.4 ± 0.3
salicylaldehyde[a]	11 ± 6

[a]Data reported in Caboni *et al*.

Figure 2. Regression curves of % decrease in females and galls per gram of root, following the exposure to parsley paste. Each point represents the average % decrease females or galls per gram of root, of five replications per treatment, with the respective standard deviations. Percentages are based on female and galls counts in the control. The five data point based lines represent the predicted function calculated by fitting a log-logistic regression model.

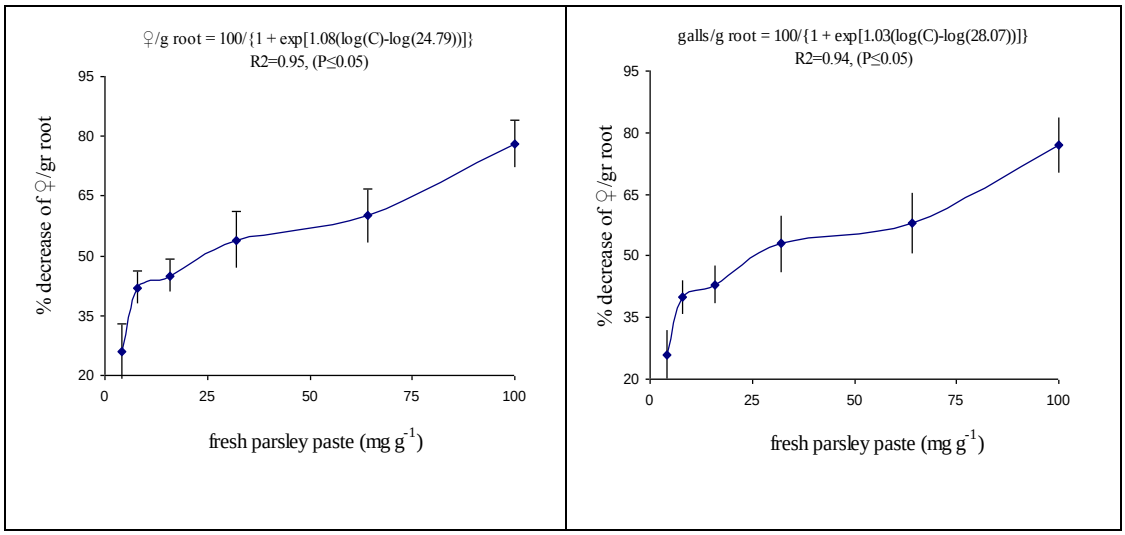
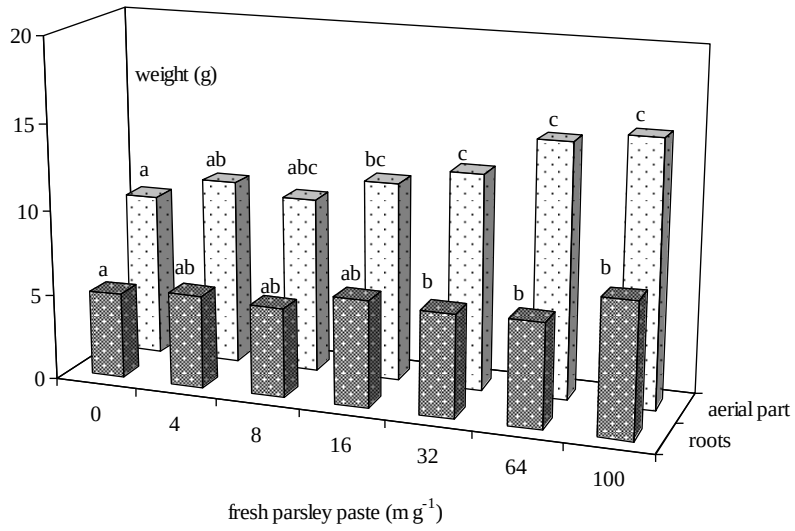


Figure 3. Tomato (*Solanum lycopersicum*) growth parameters in soil infested with root-knot nematodes and containing different rates of parsley (*Petroselinum crispum*) paste.



III. 5. References

Jones JT, Haegeman A, Danchin EGJ, Gaur HS, Helder J, Jones MGK, Kikuchi T, Manzanilla-López R, Palomares-Rius JE, Wesemael WML Perry RN, Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol Plant Pathol* **2013**, *14*, 946-961.

Trudgill DL, Blok VC, Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu Rev Phytopathol* **2001,** *39*, 53-77.

Back MA, Haydock PPJ, Jenkinson P, Disease complexes involving plant parasitic nematodes and soilborne pathogens. *Plant Pathol* **2002**, *51*, 683-697.

Chitwood DJ, Phytochemical based strategies for nematode control. *Annu Rev Phytopathol* **2002**, *40*, 221-249.

Isman M, Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu Rev Entomol* **2006**, *51***,** 45-66.

Isman M, Botanical insecticides: for richer, for poorer. *Pest Manag Sci* **2008**, *64***,** 8-11.

Ho JY, Weide R, Ma HM, van Wordragen MF, Lambert KN, Koornneef M, Zabel P, Williamson VM, The root-knot nematode resistance gene (Mi) in tomato: construction of a molecular linkage map and identification of dominant cDNA markers in resistant genotypes. *Plant J* **1992**, *2*, 971-982.

Barbary A, Palloix A, Fazari A, Marteu N, Castagnone-Sereno, Djian-Caporalino C. The plant genetic background affects the efficiency of the pepper major nematode resistance genes Me1 and Me3. *Theor Appl Genet* **2013,** *127***,** 499-507.

Nico AI, Jiménez-Díaz RM, Castillo P, Solarization of soil in piles for the control of *Meloidogyne incognita* in olive nurseries in southern Spain. *Plant Pathol* **2003**, *52*, 770–778.

Stapleton JJ, DeVay JE, Response of phytoparasitic and free-living nematodes to soil solarization and 1, 3-dichloropropene in California. *J Phytopathol* **1983,** *73***,** 1429-1436.

Chen P, Tsay TT, Effect of crop rotation on *Meloidogyne* spp. and *Pratylenchus* spp. populations in strawberry fields in Taiwan. *J Nematol* **2006**, *38*, 339–344.

Farooq M, Bajwa AA, Cheema SA, Cheema ZA. Application of allelopathy in crop production. *Int J Agr Biol* **2013,** *15***,** 1367-1378.

Mennan S, Aydinli G, Kati T, First report of root-knot nematode (*Meloidogyne arenaria*) infecting parsley in Turkey. *J Phytopathol* **2011**, *159*, 694-696.

Aguirre Y, Crozzoli R, Greco N, Effect of the root-knot nematode *Meloidogyne incognita* on parsley *Russian J Nem* 2003, ***11,*** 27-31.

Rosa JMO, Westerich JN, Wilcken SRS, *Meloidogyne javanica* reproduction on vegetable crops and plants used as green manure [Reprodução de *Meloidogyne javanica* em olerícolas e em plantas utilizadas na adubação verde] *Trop Plant Pathol* **2013,** *38*,133-141.

Chaves DSA, Frattani FS, Assafim M, de Almeida AP, Zingali RB, Costa SS, Phenolic chemical composition of *Petroselinum crispum* extract and its effect on haemostasis. *Nat Prod Comm* **2011,** *6***,** 961-964.

Kiralan M, Bayrak A, Abdulaziz OF, Özbucak T, Essential oil composition and antiradical activity of the oil of Iraq plants. *Nat Prod Res* **2012**, *26***,**132-139.

Gruszecki R, The effect of growth stage on essential oil composition of 'Hamburg' parsley. *Acta Hort* **2009**, *826***,** 391-396.

Osman YAH, Yaseen EM, Farag MM, Antimicrobial effect of some essential oils mixtures. *J Appl Sci Res* **2009**, *5***,** 1265-1276.

Andrés MF, González-Coloma A, Sanz J, Burillo J, Sainz P, Nematicidal activity of essential oils: A review. *Phytochem Rev* **2013**, *11***,** 371-390.

U.S. Environmental Protection Agency, Minimum risk pesticides: Criteria for FIFRA 25(b) Exemption. http://www.epa.gov/oppbppd1/biopesticides/regtools/25b_list.htm; last accessed 06/25/2014.

Hussey RS, Barker KR, A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. *Plant Dis Report* **1973,** *57***,** 1025−1028 ().

EU-Pharmacopoeia (Ph.Eur.) – Council of Europe, 67057 Strasbourg Cedex, France, 2001, 183-184.

Adams RP, Identification of essential oil components by gas chromatography/mass spectrometry, 4th ed.; Allured: Carol Stream, IL, 2007.

Ntalli NG, Vargiu S, Menkissoglu-Spiroudi U, Caboni P, Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. *J Agric Food Chem* **2010**, *58***,** 11390-11394.

Byrd DW, Kirkpatrick T, Barker KR, An improved technique for clearing and staining plant tissues for detection of nematodes. *J Nematol* **1983**, *15***,** 142-143.

Seefeldt SS, Jensen JE and Feurst EP, Log-logistic analysis of herbicide dose-response relationships. *Weed Technol* **1995**, *9***,** 218-227.

Zhang H, Chen F, Wang X, Yao H, Evaluation of antioxidant activity of parsley (*Petroselinum crispum*) essential oil and identification of its antioxidant constituents. *Food Res Int* **2006**, *39*, 833–839.

Stan M, Soran ML, Varodi C, Lung I, Copolovici L, Măruţoiu C. Extraction and GC determination of volatile aroma compounds from extracts of three plant species of the Apiaceae family. *AIP Conf Proceed* **2013**, *1565***,** 75-78.

Cherng JM, Chiang W, Chiang LC, Immunomodulatory activities of common vegetables and spices of Umbelliferae and its related coumarins and flavonoids. *Food Chem* **2008**, *106***,** 944-950.

Manderfeld MM, Schafer HW, Davidson PM, Zottola EA, Isolation and identification of antimicrobial furocoumarins from parsley. *J Food Prot* **1997**, *60***,** 72-77.

Zaynoun S, Abi Ali L, Tenekjian K, Kurban A, The bergapten content of garden parsley and its significance in causing cutaneous photosensitization. *Clin Expl Dermatol* **1985**, *10***,** 328-331.

Karamat F, Olry A, Munakata R, Koeduka T, Sugiyama A, Paris C, Hehn A, Bourgaud F, Yazaki K, A coumarin-specific prenyltransferase catalyzes the crucial biosynthetic reaction for furanocoumarin formation in parsley. *Plant J* **2014**, *77***,** 627-638.

Hagemeier J, Batz O, Schmidt J, Wray V, Hahlbrock K, Strack D, Accumulation of phthalides in elicitor-treated cell suspension cultures of *Petroselinum crispum. Phytochem* **1999**, *51***,** 629-635.

Farzaei MH, Abbasabadi Z, Ardekani MRS, Rahimi R, Farzaei F, Parsley: a review of ethnopharmacology, phytochemistry and biological activities. *J Tradit Chin Med* **2013**, *33*, 815-826.

Boulogne I, Petit P, Ozier-Lafontaine H, Desfontaines L, Loranger-Merciris G, Insecticidal and antifungal chemicals produced by plants: A review. *Environ Chem Lett* **2012**, *10***,** 325-347.

Song HY, Yang JY, Suh JW, Lee HS, Acaricidal activities of apiol and its derivatives from *Petroselinum sativum* seeds against *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Tyrophagus putrescentiae. J. Agric Food Chem* **2011**, *59***,** 7759-7764.

Chu SS, Cao J, Liu QZ, Du SS, Deng ZW, Liu ZL, Chemical composition and insecticidal activity of *Heracleum moellendorffii* Hance essential oil. *Chemija* **2012**, *23***,**108-112.

Ntalli NG, Caboni P, Botanical nematicides in the Mediterranean basin. *Phytochem Rev* **2012a,** *11***,** 351-359.

Ntalli NG, Caboni P, Botanical nematicides: A review. *J Agric Food Chem* **2012b**, *60***,** 9929-9940.

Caboni P, Ntalli NG, Aissani N, Cavoski I and Angioni A, Nematicidal activity of (*E,E*)-2,4-decadienal and (*E*)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*. J Agric Food Chem **2012,** *60***,** 1146-1151.

Ntalli NG, Manconi F, Leonti M, Maxia A, Caboni P, Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes. J Agric Food Chem **2011,** *59***,** 7098-103.

Ntalli NG, Ferrari F, Giannakou I, Menkissoglu-Spiroudi U, Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. *Pest Manag Sci* **2011**, *67*, 341-351.

Caboni P, Saba M, Tocco G, Casu L, Murgia A, Maxia A, Menkissoglu-Spiroudi U, Ntalli N, Nematicidal activity of mint aqueous extracts against the root-knot nematode *Meloidogyne incognita*. *J Agric Food Chem* **2013**, *61***,** 9784−9788.

Andrés MF, González-Coloma A, Sanz J, Burillo J, Sainz P, Nematicidal activity of essential oils: a review *Phytochem Rev* **2012,** *11***,** 371-390.

Caboni P, Aissani N, Cabras T, Falqui A, Marotta R, Liori B, Ntalli N, Sarais G, Sasanelli N, Tocco GJ, Potent nematicidal activity of phthalaldehyde, salicylaldehyde, and cinnamic aldehyde against *Meloidogyne incognita*. *J Agric Food Chem* **2013,** *61***,** 1794−1803.

Ismail AE, Mahfouz SA, Mohamed MM, Hassan RA, Abou-Setta LM, Environmentally safe substances for controlling the root-knot nematode, *Meloidogyne arenaria* infested potato and their influence on yield and alkaloidal content in Egypt. *Arch Phytopathol Plant Prot* **2014**, *47***,** 600-609.

Abolusoro SA, Abe MO, Abolusoro PF, Izuogu NB, Control of nematode disease of eggplant (*Solanum aethiopicum*) using manure. *Arch Phytopathol Plant Prot* **2014,** *78*, 327-330.

Stirling GR, Integration of organic amendments, crop rotation, residue retention and minimum tillage into a subtropical vegetable farming system enhances suppressiveness to root-knot nematode (*Meloidogyne incognita*). *Australas Plant Pathol* **2013,** *42***,** 625-637.

Guerrero-Diaz MM, Lacasa-Martinez CM, Hernandez-Piñera A, Martinez-Alarcon V, Lacasa Plasencia A, Evaluation of repeated biodisinfestation using *Brassica carinata* pellets to control *Meloidogyne incognita* in protected pepper crops. *Span J Agric Res* **2013**, *11***,** 485-493.

Ntalli NG, Menkissoglu-Spiroudi U, Giannakou IO, Prophetou-Athanasiadou DA, Efficacy evaluation of a neem (*Azadirachta indica* A. Juss) formulation against root-knot nematodes *Meloidogyne incognita. Crop Prot* **2009**, *28*, 489–494.

Matthiessen JN, Kirkegaard JA, Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. *Crit Rev Plant Sci* **2006, *25*,** 235–265.

Walker J T, Susceptibility of Eight Herbs to Common Root-Knot Nematodes *J Environ Hort* **2002,** *20*, 101–103.

Dias-Arieira CR, PL da Cunha T, Chiamolera FM, Puerari HH, Biela F, de M Santana S, Reaction of vegetables and aromatic plants to *Meloidogyne javanica* and *M. incognita*. *Hortic bras* **2012,** *30*, 322-326.

Amin AW, Nematicidal activity of some essential oils of plants of families Apiaceae and Lamiaceae on *Meloidogyne incognita* and *Rotylenchulus reniformis* infecting cowpea *Pak J Nematol* **2005,** *23*, 339-347.

IV. 1. Introduction - Nematicidal weeds, *Solanum nigrum* and *Datura stramonium*

In addition to competition for resources, some weeds interfere with the crop plants and the organisms living in the soil root sphere through production of chemical substances (allelochemicals) that inhibit their growth and development. The allelochemicals may include phenolic acids, terpenes, terpenoids, glycosides, alkaloids and flavonoids (Blum, 1996; Inderjit et al., 1999a,b; Whittaker and Feeny, 1971). To date, a top crop protection research issue is the study of herbal preparations, which do not produce any adverse effects in the non-target organisms and are easily biodegradable, as alternative nematode control measures. Most importantly, the use of nematicidal plant-based agronomical techniques, like green manures is suitable to extensive crop systems and is of particular interest if species to be incorporated are already present *in situ*, like weeds. *Solanum nigrum* Linn. and *Datura stramonium* Linn., commonly known as black night shade (Kakamachi) and Jimson weed, are two Solanaceae highly invasive and globally distributed weeds that exhibit biological properties and nematicidal activity (Abbasi et al., 2015, Sher et al., 2015, Shou et al., 2012). *Solanum nigrum* is affected by *Meloidogyne incognita* (Robab et al., 2012), nonetheless it's dried ground seeds powder lessens root galling index at the rate of 5 g/kg and increases shoot length (Radwan et al., 2012). The water extract of *S. nigrum* at a concentration of 10 mg/mL induces morphological changes in the body structure of the root-lesion nematode *Pratylenchus goodeyi* and greatly affects movement and causes mortality (Gouveia et al., 2014). Interestingly, roots extracts of *Solanum nigrum* are traditionally used in the treatment of worms and abdominal pain (Jagtap et al., 2013). *S. nigrum* is a major source for steroidal alkaloids compounds (Sammani et al., 2013; Jagtap et al., 2013), and glycoalkaloids like, solasonine, solamargine and solanigroside (Li et al., 2007; Ding et al., 2013). 4-quinolone waltherione and waltherione A, have been reported of larvicidal activity (EC_{50} values of 0.09 and 0.27 µg/mL at 48 h) and egg hatch inhibition activity (91.9 and 87.4% after 7 days of exposure to 1.25 µg/mL) against *M. incognita* (Jang et al., 2015). Additionally, an alkaloid found in the crude alkaloid extract named drupacine exghibited an EC_{50} va lue 76.3 µg mL^{-1} on M. incognita J2 and reduced eggg hatch by 36% after immersion in 1.0 mg mL^{-1} (Wen et al., 2013). Other S. nigrum components are saponins (Jagtap et al., 2013), like solanigroside A and solanigroside B (Zhou et al., 2007) as well as

oleanane-type triterpenoid saponins exhibiting LC_{50} values ranging from 70.1 to 94.7 μg/mL after 48 h against *M. incognita* (Li et al., 2013). Interestingly, in Europe there was available a saponin based commercial nematicide based on *Quillaja saponaria* of high activity on *M. javanica* under field conditions (Giannakou 2011). *S. nigrum* contains phenols like 4-methylphenol of significant *in vitro* activity against *M. javanica* (Yang et al., 2015), as well as fatty acids like linoleic acid (67.9% in *S. nigrum* seeds oil), palmitic, stearic, oleic acids (Dhellot et al., 2006) and linolenic acid, qualene, phytol (Mohy-ud-din et al., 2010). In our previous studies, we have demonstrated that acetic and hexanoic acid, as ingredients of high nematicidal extracts of *Melia azedarach*, are effective against RKN in terms of J2 paralysis activity (Ntalli et al 2010). Also Zhang and co-workers have demonstrated that fatty acids like caproic, caprylic, capric, lauric, myristic, and palmitic cause significantly high mortality of the second-stage juvenile of *M. incognita.* Moreover, phytochemical investigations of *S. nigrum* root shows also the presence of tannins (Jagtap et al., 2013) considered also of nematicidal activity against root knot nematodes (Hewlett et al., 1997). *D. stramonium* is also a host of root knot nematodes and under field conditions it even elevates the presence of *Meloidogyne* species, having thus a negative impact on crop production when not controlled timely and effectively (Ntidi et al., 2012). Nonetheless, seed extract of *D. stramonium* has a significant nematicidal activity. In specific, the hot water and ethanol extracts of *D. stramonium* tested at 25, 50 and 100 mg/ml caused 75-100% mortality on second-stage juveniles (J2) of *M. incognita* (Chaudhary et al., 2013). Leaf, seed and stem extracts of *D. stramonium* tested at 500 mg/L produced relatively high mortality rates of 68.8±4.5, 81.8±2.9 and 70.8±6.4 after 72 h of J2s exposure (Elbardi et al., 2008). Additionally, in a pot trial, dried ground weed leaves of *D. stramonium* mixed with soil at the rate of 1, 3, 5 and 10 g/kg soil significantly suppressed *M. incognita* populations and root galling as they decomposed, but the high rates showed phytotoxic effects (Radwan, et al., 2006). Moreover, pre-planting treatments with *Datura stramonium* leaf extracts at 0.5% to 1% significantly lessened the galls number at 2 to 5 per plant, considering 56 galls per plant assessed in the control plants (Mateeva and Ivanova, 2000). Last, aqueous leaf extracts of *D. stramonium* inhibited egg hatch and also killed the larvae significantly (Rao et al., 1986). Chemical composition studies on *D. stramonium* seeds revealed N-trans-feruloyl tryptamine, hyoscyamilactol, scopoletin, umckalin, daturaolone, daturadiol, N-trans-ferulicacyl-tyramine, cleomiscosin A, fraxetin, scopolamine, 1-

Acetyl-7-hydrox-beta-carbol-ine, 7-hydroxy-beta-carboline1- propionic acid (Li et al., 2012). Scopolamine is a muscarinic antagonist (Lee et al., 2000) and one of the most important alkaloids of *D. stramonuim* (Ma et al., 2015). The scope of this study was 1) to study the nematicidal activity of *S. nigrum* and *D. stramonium* in terms of a) J2 paralysis as well as b) egg hatch and c) biological cycle arrest in host roots

IV. 2. Materials and methods

IV. 2. a. Nematodes rearing and Chemicals. Populations of *M. incognita* and *M. javanica* both of Greek origin were reared on tomato (Solanum lycopersicum Mill.) cv. Belladonna. Freshly hatched (24 h) second-stage juveniles (J2) as well as eggs of different growth stages were extracted from egg masses according to Hussey and Barker (1973) from 60 days nematode infested roots, to be used for the bioassays. The egg masses were handpicked from the tomato roots under a stereoscope. Methanol was of high-performance liquid chromatography grade.

IV. 2. b. Plant material extraction procedure

Dry plant material, 5g of *S. nigrum* seeds and *D. stramonium* shoots, were extracted in 50 ml methanol for 30 min in a sonicator apparatus. After exhaustive evaporation of the solvent the yields in dry material were measured at 12,3 ± 0.07 and 12,6 ± 0.01 % (w/w) for *D. stramonium* and *S. nigrum*, respectively. The extracts were used directly for bioassays with nematodes and chemical composition analysis without evaporation.

IV. 2. c. Total phenolic compounds (TPC)

Phenols content in extracts was determined according to Folin-Ciocalteau assay (singleton, 1999). Briefly, 50mg of the extract were dissolved in 1,5ml methanol. From each sample 50µl were diluted in 450µl methanol and was mixed with 2.5 ml Folin-Ciocalteau reagent (diluted 1/10), followed by 2.0 ml of 7.5% Na2CO3. After 5 min incubation at 50OC, the absorbance was measured at 760 nm (SHIMADZU UV-1601, Kyoto, Japan). The phenol concentration was expressed as gallic acid equivalents (GAE/100g FW).

IV. 2. d. J2s Paralysis Bioassays.

J2s were extracted as described previously and were allowed to hatch in modified Baermann funnels. First two days hatched juveniles were discarded and then after,

every two days hatched J2, were used for the paralysis experiments. The *D. stramonium* and *S. nigrum* extracts were directly diluted in water and tested for paralysis activity in CellstarR 96-well cell culture plates (Greiner Bio-One) at a ratio of 1:1 (*v/v*). Distilled water served as control. Each well contained 15 J2s and the test concentrations were 10 to 1000 mg/mL. Border wells with J2s immersed in plain water served for fumigant activity control (Ntalli et al., 2012). Multiwell plates were covered to avoid evaporation and were maintained in the dark at 20°C. Juveniles were ranked into two distinct categories, moving and paralysed, with the aid of an inverted microscope (Zeiss 3951, Germany) at 20× after 1, 2d and 3d. Then, they were washed through a 20μm sieve, to remove the excess of the test compounds, and were immersed in water to study for regain of motility. Numbers of motile and paralysed J2s were assessed by pricking the juvenile body with a needle, and they were counted. Nematodes that did not move at this point were considered dead. J2 paralysis bioassays were performed three times, and every treatment was replicated six times.

IV. 2. e. Egg hatch arrest in free eggs treated with the test compounds

For the egg hatch arrest tests performed in microwell assays, nematodes were pipetted into 24-well cell culture plates (Greiner bio-one), with 0.5mL treatment at double the test concentration and 0.5mL nematode inoculum (20 eggs) in sterile distilled water per well. *D. stramonium* and *S. nigrum* water extracts were: 0.0 μg/mL test substance (water control), 0.0 μg/mL test substance (carrier control) and 1, 10, 100 and 1000 μg/mL, test substance in water. Five wells were used per treatment, and the plates were covered by plastic adhesive sheets to prevent volatiles escaping to adjacent wells. Hatch quantification was done by directly counting undifferentiated eggs and J2 in each well at day 0 using an inverted microscope at 40x. Thereafter, assessments were performed after 2, 6, 10 and 14 days. Cumulative percent J2 release was calculated using the formula: $((J2_{Dx}-J2_{D0})/total) \times 100$ where Dx = day after the start of the assay. Cumulative percent undifferentiated egg hatch was calculated using the formula: $((Eggs_{D0}-Eggs_{Dx})/total) \times 100$ where Dx = day after the start of the assay.

IV. 2. f. Biological cycle arrest.

Procedures were according to Ntalli et al., 2010. Briefly artificially inoculated with *M. incognita* tomato plants were treated with *D. stramonium* and *S. nigrum* water extracts in a dose response manner and after the completion of a biological cycle at 27 ^{o}C,

60% RH at 16 h photoperiod, roots were then stained with acid fuchsin (Byrd et al., 1983). The following variables were assessed: fresh root weight, fresh shoot weight and total number of female nematodes and galls per gram of root at 10 × magnification under uniform illumination by transparent light within tissue sample. The experiment was performed twice, and the treatments were always arranged in a completely randomised design with five replicates.

IV. 3. Statistical analysis.

Treatments of motility experiments were replicated six times, and each experiment was performed twice. The percentages of paralyzed J2 observed in the microwell assays after 1 h, were corrected by eliminating the natural death/paralysis in the water control according to the Schneider Orelli's formula: Corrected % = {(Mortality % in treatment-Mortality % in control)/(100-Mortality % in control)}x100 and they were analysed (ANOVA) after being combined over time. Since ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2 treated with the weed extracts were subjected to nonlinear regression analysis using the log-logistic equation proposed by Seefeldt *et al.* : $Y= C + (D - C)/\{1 + \exp[b (\log(x)-\log(EC_{50}))]\}$ where *C* = the lower limit, *D* = the upper limit, *b* = the slope at the EC_{50}, and EC_{50} = the test solution concentration required for 50% death/paralysis of nematodes after eliminating the control (natural death/paralysis). In the regression equation, the test concentration was the independent variable (*x*) and the paralyzed J2 (percentage increase over water control) was the dependent variable (*y*). The mean value of the six replicates per each test concentration and immersion period was used to calculate the EC_{50} value. The 95% confidence intervals ($CI_{95\%}$) were included for toxicity comparison. Egg hatch inhibition treatments were replicated 5 times, and each bioassay was performed twice. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. In egg hatch inhibition bioassays, both for free eggs and eggs in egg sacs, treatments means were compared using Tukey's test at $P \leq 0.05$. Statistical analysis was performed using SPSS 8.0. Pot bioassays were organised in a complete randomized design with five replications and were performed twice. Since ANOVAs indicated no significant treatment by time interaction (between runs of experiment), means were averaged over experiments. The data from the pot bioassays were expressed as a percentage decrease in the number of females or galls per gram of

root corrected according to the control, using the Abbott's formula: corrected % = 100 × {1−[females number in treated plot/females number in control plot]}. It was fitted in the log-logistic model (Seefeldt et al., 1995) to estimate the concentration that caused a 50% decrease in females and galls per gram of root (EC_{50} value). In this regression equation, the test compounds (% w/w) was the independent variable (x) and the female nematodes, or galls, (percentage decrease over water control) was the dependent variable (y). Because ANOVAs indicated no significant treatment by time interaction (between runs of experiments), means were averaged over experiments. Treatments means were compared using Tuckey's test at $P \leq 0.05$.

IV. 4. Results and Discussion

Also previously have the weeds been a subject of nematicidal activity studies in the frame of discovering alternatives to synthetic nematicides. Water and ethanol leaf extracts at 15 and 20% (w/v) concentrations of *Euphorbia hirta, Phyllanthus amarus* and *Cassia obtusifolia* and 20% (w/v) concentration of *Sida acuta* and *Andropogon gayanus*, provoked 100% mortality on *M. incognita* juveniles after 7 days of immersion (Olabiyi et al., 2008). These are rather high concentrations considering the EC_{50} values of *S. nigrum* and *D. stramonium* against *Meloidogyne* sp. as reported herein. In specific, paralysis activity was tested against *M. incognita* and *M. javanica* and at cases the most susceptible was *M. incognita* (**Table 1**). We have already demonstrated this difference in the susceptibility between the two species (Ntalli et al., 2012-Nasiou) but the biochemical mechanist lying underneath is yet to be delineated. Clear time and dose response relationships were established in all cases and the $EC_{50/96h}$ Values for *D. stramonium* and *S. nigrum* were 427 ± 75 and 418 ± 78 μg/mL for *M. incognita*; while 427 ± 23 and 954 ± 96 μg/mL for *M. javanica* respectively (**Table 1**). Also Elbardi and co-workers have reported on similar activity levels of *D. stramonium* seed extracts on *M. incognita* J2 (Elbardi et al., 2008); while Chaudhary and co-workers have reported on higher activity of seed *D. stramonium* extracts, namely test solutions of 25-100 mg/ml caused 75-100% mortality on second-stage juveniles (J2) of *M. incognita* (Chaudhary et al., 2013).

To the best of our knowledge this is the first report on the egg hatch inhibition activity of *S. nigrum* and *D. stramonium* against *Meloigyne* spp. The cumulative undifferentiated egg hatch decreased significantly by both *D. stramonium* and *S.*

nigrum extracts at 100 μg/mL since DAY 6, while in successive assessments the activity increased for *D. stramonium* and decreased for *S. nigrum* (**Table 2 and 3**). Concerning the percent of J2 released from eggs immersed in the two water extracts, again *D. stramonium* was more active since it differed from control at 10 μg/mL since DAY 2 while *S. nigrum* differed from control only at 100 μg/mL (DAY2). In the next assessment date (DAY 6) activity increased for *D. stramonium* and decreased for *S. nigrum* differing from control at 1 and 100 μg/mL, respectively. Since DAY 10 the percent J2 release in control decreased naturally and thus J2 release differences among treatments were not evident thereafter. Maybe this was because the hatch inhibition activity of the extracts was specified on early formed eggs rather than on J1 haching to J2. Similarly, water extracts of *Luffa cylindrica* and *Momordica charantia* significantly inhibited the hatching of *Meloidogyne* spp. eggs (Ononuju and Nzenwa, 2011).

Biological cycle arrest of *Meloidogyne incognita* in artificially inoculated tomato plants treated with the weeds extracts was evident and the EC_{50} values considering ♀/g root counts were calculated for *S. nigrum* and *D. stramonium* at 1.13 and 11.40 mg/g, respectively. Similar were the levels of the respective values considering galls/g root (**Table 6**), while no phytotoxicity was evident at the dose range of the treatments used for the bioassay. It has to be pinpointed that the EC_{50} value considering ♀/g root counted on *S. nigrum* treated tomato roots was the lowest, out of many extracts used under the same experimental conditions, ever reported by our group (Aissani et al., 2015; Caboni et al., 2015; Caboni et al., 2013; Caboni et al., 201; Ntalli et al., 2010). Also Radwan and co-workers have reported on *S. nigrum* powder activity on *M. incognita* but at higher concentration levels, namely 5 g/kg (Radwan et al., 2012).

Folin total phenolic compounds (TPC) were for *D. stramonioum* 34.42 μg/mg extract, or 4.48mg/g dry plant material and for *S. nigrum* 26.24 and 3.84, respectively (data not shown).

According to HPLC analysis the most abundant phenolic acids present in the extracts were chlorogenic acid in *D. stramonioum* and p-coumaric acid in *S. nigrum* followed by lower percentages of syringic and caffeic acid (data not shown). Previously, we have reported the activity of p-coumaric acid on *M. incognita* with the $EC_{50/48h}$ = 840

μg/mL while chlorogenic acid was not found active at concentrations less than 1000 μg/mL (Aoudia et al., 2012).

Interestingly, *S. nigrum* is more active than *D. stramonium* in the form of a paste thus revealing the complexity of interactions among ingredients.

Conclusively, farmers that already utilize waste resources, such as oil seed cake and gutter oil, rich in fatty acids for effective *M. incognita* management (Zhang et al., 2012), could also insert weed pastes to amend the soil such as *S. nigrum* and *D. stramonium*.

Table 1. EC_{50} ± SD (μg/mL) of *Datura stramonium* and *Solanum nigrum* water extracts against *M. incognita* calculated after 1, 2 and 3 days of nematode immersion in test solutions.

Immersion period	*Datura stramonium*		*Solanum nigrum*	
	M. incognita	*M. javanica*	*M. incognita*	*M. javanica*
1d	968 ± 98	>8000	409 ± 56	686 ± 98
2d	553 ± 85	581 ± 73	507 ± 72	792 ± 95
3d	427 ± 75	427 ± 23	418 ± 78	954 ± 96

Table 2. Effect of *Datura stramonium* water extract on cumulative percent hatch of *Meloidogyne incognita* undifferentiated egg calculated using the formula: (($eggs_{D0}$-$eggs_{Dx}$)/total)x100. Eggs (20-30 per well) were collected and distributed in 96-well plates and were incubated at 27°C for 14 days in test solutions or plane water. Eggs were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each day were compared using Tuckey's test and those followed by different letters are significantly different at (P≤0.05).

	Datura stramonium cumulative undifferentiated egg hatch			
(μg/mL)	DAY 2	DAY 6	DAY 10	DAY 14
1000	9 ± 1.0 [a]	9 ± 1.0 [a]	9 ± 1.5 [a]	9 ± 1.5 [a]
100	10 ± 5.0 [a]	10 ± 5.0 [a]	10 ± 5.0 [a]	10 ± 5.0 [a]
10	17 ± 6.5 [a]	23 ± 6.5 [ab]	23 ± 6.5 [a]	23 ± 6.5 [a]
1	14 ± 6.0 [a]	24 ± 4.5 [ab]	23 ± 6.5 [a]	23 ± 6.5 a
0	13 ± 8.0 [a]	35 ± 9.0 [b]	47 ± 9.0 [b]	47 ± 9.0 [b]

Table 3. Effect of *Solanum nigrum* water extract on cumulative percent hatch of *Meloidogyne incognita* undifferentiated egg calculated using the formula: $((eggs_{D0}-eggs_{Dx})/total)x100$. Eggs (20-30 per well) were collected and distributed in 96-well plates and were incubated at 27°C for 14 days in test solutions or plane water. Eggs were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each day were compared using Tuckey's test and those followed by different letters are significantly different at ($P \leq 0.05$).

	Solanum nigrum cumulative undifferentiated egg hatch			
(µg/mL)	DAY 2	DAY 6	DAY 10	DAY 14
1000	9 ± 0.5 [a]	8 ±0.0 [a]	9 ± 6.0 [a]	9 ± 6.0 [a]
100	15 ± 2.0 [a]	16 ± 4.0 [a]	16 ± 4.0 [a]	16 ± 4.0 [a]
10	15 ± 2.0 [a]	22 ± 1.0 [ab]	27 ± 1.5 [ab]	27 ± 1.0 [ab]
1	17 ± 2.0 [ab]	24 ± 3.5 [ab]	27 ± 5.0 [ab]	27 ± 5.0 [ab]
0	25 ± 4.0 [b]	35 ± 9.0 [b]	47 ± 9.0 [b]	47 ± 9.0 [b]

Table 4. Effect of *Datura stramonium* water extract on cumulative percent release of *Meloidogyne incognita* J2 calculated using the formula: $((J2_{Dx}-J2_{D0})/total)x100$. Eggs (20-30 per well) were collected and distributed in 96-well plates and were incubated at 27°C for 14 days in test solutions or plane water. Released J2 were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each day were compared using Tuckey's test and those followed by different letters are significantly different at ($P \leq 0.05$).

	Datura stramonium percent J2 release			
µg/mL	DAY 2	DAY 6	DAY 10	DAY 14
1000	3 ± 1.5 [a]	1 ± 1.0 [a]	1 ± 1.0 [a]	1 ± 1.0 [a]
100	6 ± 4.0 [a]	5 ± 3.5 [a]	4 ± 3.0 [a]	4 ± 3.0 [a]
10	20 ± 5.5 [ab]	5 ± 5.0 [a]	4 ±1.5 [a]	4 ± 1.5 [a]
1	31 ± 6.5 [bc]	5 ± 4.0 [a]	5 ± 3.5 [a]	5 ± 3.5 [a]
0	40 ± 5.5 [c]	20 ± 5.5 [b]	8 ± 4.5 [a]	5 ± 3.5 [a]

Table 5. Effect of *Solanum nigrum* water extract on cumulative percent release of *Meloidogyne incognita* J2 calculated using the formula: $((J2_{Dx}-J2_{D0})/total)x100$. Eggs (20-30 per well) were collected and distributed in 96-well plates and were incubated at 27°C for 14 days in test solutions or plane water. Released J2 were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each day were compared using Tuckey's test and those followed by different letters are significantly different at ($P \leq 0.05$).

	Solanum nigrum percent J2 release			
(µg/mL)	DAY 2	DAY 6	DAY 10	DAY 14
1000	2 ± 2.0 [a]	2 ± 2.0 [a]	2 ± 2.0 [a]	2 ± 2.0 [a]
100	11 ± 6.0 [ab]	5 ± 4.5 [ab]	2 ± 1.5 [a]	2 ± 1.5 [a]
10	23 ± 2.0 [bc]	6 ± 2.0 [ab]	2 ± 1.0 [a]	2 ± 1.0 [a]
1	33 ± 6.5 [c]	10 ± 5.0 [ab]	11 ± 6.5 [a]	6 ± 3.5 [a]
0	40 ± 5.5 [c]	20 ± 5.5 [b]	8 ± 4.5 [a]	4 ± 3.5 [a]

Table 6. EC_{50} values (mg/g soil) of weed paste (decomposing tissues) efficacy on *M. incognita* as calculated in pot experiments with respective standard error and confidence interval values

Datura stramonium					
♀/g root			galls/g root		
EC_{50}	Stderror	95% ConfInt	EC_{50}	Stderror	95% ConfInt
mg/g			mg/g		
11.40	0.92	9.48-13.32	12.85	1.19	10.39-15.33
Solanum nigrum					
♀/g root			galls/g root		
EC_{50}	Stderror	95% ConfInt	EC_{50}	Stderror	95% ConfInt
mg/g			mg/g		
1.13	0.17	0.78-1.48	1.15	0.17	0.79-1.51

IV. 5. References

Tellez MR, Dayan FE, Schrader KK, Wedge DE, Duke SO, Composition and Some Biological Activities of the Essential Oil of *Callicarpa americana* (L.) *J. Agric. Food Chem* **2000**, *48*, 3008–3012.

Robab MI, Shaikh H, Azam T, Antagonistic effect of Glomus mosseae on the pathogenicity of root-knot nematode infected Solanum nigrum. *Crop Prot.* **2012**, *42*, 351-355.

Radwan MA, Farrag SAA, Abu-Elamayem MM, Ahmed NS, Efficacy of dried seed powder of some plant species as soil amendment against *Meloidogyne incognita* (Tylenchida: Meloidogynidae) on tomato. *Arch. Phytopathol. Plant Prot*. **2012**, *45*, 1246-1251.

Gouveia M, Cordeiro N, Teixeira L, Abrantes IDO, Pestana M, Rodrigues M. In vitro evaluation of nematicidal properties of *Solanum sisymbriifolium* and *S. nigrum* extracts on *Pratylenchus goodeyi*. *Nematology* **2014**,*16*, 41-51.

Li M-H, Ding G, Meng Z-Q, Cai J, Liu J-J, Content determination of solasonine and solamargine in *Solanum nigrum. Chin J Nat Med*. **2007**, *5*, 360-362.

Zhou X-L, He X-J, Zhou G-X, Ye W-C, Yao X-S, Pregnane glycosides from *Solanum nigrum*. *J Asian Nat Prod Res.* **2007**, *9*, 517-523.

Dhellot JR, Matouba E, Maloumbi MG, Nzikou JM, Dzondo MG, Linder M, Parmentier M, Desobry S, Extraction and nutritional properties of *Solanum nigrum* L seed oil. *Afr. J. Biotechnol*. **2006**, *5*, 987-991.

Sammani A, Shammaa E, Chehna F, Qualitative and quantitative steroidal alkaloids of solanum species distributed widely in Syria by TLC and HPLC. *Int J Pharm Sci Rev Res.* **2013**, *23*, 23-27.

Jagtap CY, Prajapati PK, Rudrappa HC, Shukla VJ, Pharmacognostic and phytochemical investigation of root of *Solanum nigrum* Linn.: An ethnomedicinally important herb. *Int. J. Green pharm.* **2013**, *7*, 46-49.

Ding X, Zhu F, Yang Y, Li M, Purification, antitumor activity in vitro of steroidal glycoalkaloids from black nightshade (*Solanum nigrum* L.). *Food Chem* **2013**, *141*, 1181-1211.

Chaudhary KK, Haile A, Ayresea ZG, Semereab G, Weldegergish T, Nematicidal activity of Eritrean weed plants against the root-knot nematode *Meloidogyne incognita*. *Nematropica* **2013**, *43*, 207-215.

Ntidi KN, Fourie H, Mc Donald AH, De Waele D, Mienie C, M. S. Plant-parasitic nematodes associated with weeds in subsistence agriculture in South Africa. *Nematology* **2012**, *14*, 875-887.

Elbadri GA, Lee DW, Park JC, Yu HB, Choo HY. Evaluation of various plant extracts for their nematicidal efficacies against juveniles of *Meloidogyne incognita*. *J. Asia Pac. Entomol*. **2008**, *11*, 99-102.

Radwan MA, Abu-Elamayem MM, Kassem SM, El-Maadawy EK, Soil amendment with dried weed leaves as non-chemical approach for the management of *Meloidogyne incognita* infecting tomato. *Comm Agr Appl Biol Sci* **2006**, *71*, 25-32.

Mateeva A, Ivanova M, Alternative methods for control of root-knot nematodes, *Meloidogyne* spp. *Acta Hort* **2000**, *532*, 109-111.

Rao CV, Mani A, Rao PK, Effect of plant products on egg hatch and larval mortality of *Meloidogyne incognita*. *Animal Sci* **1986**, *95*, 397-401.

Li J, Lin B, Wang G, Gao H, Qin M, Chemical constituents of Datura stramonium seeds *Zhongguo Zhongyao Zazhi* **2012**, *37*, 319-322.

Ma L, Gu R, Tang L, Chen Z-E, Di R, Long C, Important poisonous plants in Tibetan ethnomedicine. *Toxins* **2015**, *7*, 138-155.

Mohy-ud-din A, Khan Z, Ahmad M, Kashmiri MA, Yasmin S, Asghar MN, Ahmad SR, Epicuticular waxes from solanum nigrum complex: Chemotaxonomic implications. *Asian J. Chem.* **2010**, *22*, 2919-2927

Aoudia H, Ntalli N, Aissani N, Yahiaoui-Zaidi R, Caboni P, Nematotoxic phenolic compounds from *Melia azedarach* against *Meloidogyne incognita*. *J Agric Food Chem* **2012**, *60*, 11675-11680

Zhou L, Wang J, Wang K, Xu J, Zhao J, Shan T, Luo C, Secondary metabolites with antinematodal activity from higher plants. *Stud Nat Prod Chem* **2012**, *37*, 67-114.

Ntalli NG, Nasiou E, Menkissoglu-Spiroudi U, Evaluation of Essential Oils from Rosemary, Orange, Lavandula and False Yellowhead on Hatching and Motility of Root-Knot Nematode *J Agric Sci Tech A*. **2013**, *3*, 603-616.

Hussey RS, Barker KR, A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. *Plant Dis Report* **1973,** *57***,** 1025−1028**.**

Olabiyi TI, Oyedunmade EEA, Ibikunle GJ, Ojo OA, Adesina GO, Adelasoye KA, Ogunniran TA. Chemical Composition and Bio-Nematicidal Potential of Some Weed Extracts on *Meloidogyne incognita* under Laboratory Conditions. *Plant Sci Res* **2008,** *1*, 30-35.

Ononuju CC, Nzenwa PO, Nematicidal effects of some plant extracts on egg hatchability and control of Meloidogyne spp. in cowpea (*Vigna unguiculata* (L.) Walp). *Afr J Plant Sci* **2011**, *5*, 176-182.

Zhang WP, Ruan WB, Deng YY, Gao YB, Potential antagonistic effects of nine natural fatty acids against *Meloidogyne incognita*. *J Agric Food Chem*. **2012**, *60*, 11631-7.

Ntalli NG, Vargiu S, Menkissoglu-Spiroudi U, Caboni P, Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. *J Agric Food Chem*. **2010**, *58*, 11390-4.

Wen Y, Meyer SL, Masler EP, Zhang F, Liao J, Wei X, Chitwood DJ, Nematotoxicity of drupacine and a Cephalotaxus alkaloid preparation against the plant-parasitic nematodes *Meloidogyne incognita* and *Bursaphelenchus xylophilus*. *Pest Manag Sci.* **2013**, *69*, 1026-33.

Yang X, Wang X, Wang K, Su L, Li H, Li R, Shen Q. The nematicidal effect of camellia seed cake on root-knot nematode *Meloidogyne javanica* of banana. *PLoS One.* **2015** 10(4):e0119700.

Li W, Sun YN, Yan XT, Yang SY, Lee SJ, Byun HJ, Moon CS, Han BS, Kim YH, Isolation of nematicidal triterpenoid saponins from *Pulsatilla koreana* root and their activities against *Meloidogyne incognita*. *Molecules*. **2013**, *18*, 5306-5316.

Giannakou IO, Efficacy of a formulated product containing Quillaja saponaria plant extracts for the control of root-knot nematodes. *Eur J Plant Patho*l **2011**, *130*, 587-596.

Hewlett TE, Hewlett EM, Dickson DW, Response of *Meloidogyne* spp., *Heterodera glycines,* and *Radopholus similis* to Tannic Acid. *J Nematol*. **1997**, *29*, 737-741.

Lee YS, Park YS, Nam S, Suh SJ, Lee J, Kaang BK, Cho NJ. Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. *J Neurochem.* **2000**, *75*, 1800-1809.

Aissani N, Urgeghe PP, Oplos C, Saba M, Tocco G, Petretto GL, Eloh K, Menkissoglu-Spiroudi U, Ntalli N, Caboni P, Nematicidal Activity of the Volatilome of *Eruca sativa* on *Meloidogyne incognita*. *J Agric Food Chem*. **2015**, *63*, 6120-5.

Caboni P, Saba M, Oplos C, Aissani N, Maxia A, Menkissoglu-Spiroudi U, Casu L, Ntalli N, Nematicidal activity of furanocoumarins from parsley against *Meloidogyne* spp. *Pest Manag Sci.* **2015**, *71*, 1099-105.

Caboni P, Saba M, Tocco G, Casu L, Murgia A, Maxia A, Menkissoglu-Spiroudi U, Ntalli N, Nematicidal activity of mint aqueous extracts against the root-knot nematode *Meloidogyne incognita*. *J Agric Food Chem*. 2013, 61, 9784-9788.

Caboni P, Ntalli NG, Aissani N, Cavoski I, Angioni A, Nematicidal activity of (E,E)-2,4-decadienal and (E)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica*. 2012, 60, 1146-51.

Ntalli NG, Vargiu S, Menkissoglu-Spiroudi U, Caboni P, Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. *J Agric Food Chem.* 2010, 58, 11390-4.

V. 1. Introduction - Strong synergistic activity and egg-hatch inhibition of (E, E)-2,4-decadienal and (E)-2-decenal on *Meloidogyne* spp.

Crop sustainability ensures food needs for today without endangering the production of tomorrow. Currently, there are serious constraints to the agricultural production and one of these is the high crop losses due to the plant parasitic nematode infestations. Plant parasitic nematodes are a major biotic stress affecting numerous crop productions. Root-knot nematodes (RKN: *Meloidogyne* spp.) heavily damage most solanaceous crops worldwide (Barbary et al., 2015). In the past, control of RKN involved the use of synthetic nematicides. However, high cost and harsh environmental impact resulted in a reduction of commercial nematicides. Recent research is focused in investigating the use of plant secondary metabolites, or compounds of bacterial origin, for the control of RKN. The major part of the scientific literature is focused on the paralysis effect on the second stage juveniles of RKN, representing the most notorious growth stage responsible for host infection (Ntalli and Caboni, 2012a; Ntalli and Caboni, 2012b). On the other hand, only a few are the studies that report on botanical's synergistic paralysis effects and egg-hatch inhibition. In that context the bibliography reports mainly some amino acids and only a few botanicals. Specifically, six amino acids namely DL-methionine, DL-valine, DL-serine, DL-phenylalanine, L-proline and L-histidine caused a reduction of hatch in egg masses of *M. javanica*. DL-phenylalanine was the most effective treatment followed by L-proline and L-histidine (Amdadul Hoque et al., 2014). Previously DL-*β*-aminobutyric acid was found to play a key role in plant defence responses to *M. javanica* as well as in decreasing egg-hatch (Ahmed et al., 2009, Fatemy et al., 2014). Hatch inhibition was reported for 3,4-dihydroxybenzoic acid from the bark of *Terminalia nigrovenulosa* on *M. incognita* (Nguyen et al., 2013).

Moreover, bacterial lytic enzymes are able to inhibit RKN egg hatch. In this case, a chitinase secreted by *Lysobacter capsici* YS1215 was able to inhibit egg hatch by chitinolysis and degradation of nematode eggs (Jung et al., 2014). 4-hydroxyphenylacetic acid, a lytic enzyme from *Lysobacter antibioticus* HS124, decreased the rate of *M. incognita* hatch (Lee et al., 2013). The polyphenol epigallocatechin gallate, an analogue of a compound found in nematode cysts, reduced hatch from *M. incognita* by determining J2 larvae retention in the egg (Masler et al., 2013). Substances extracted from *Chaetomium globosum* NK102

culture filtrate and chaetoglobosin A were found to inhibit hatch of *M. incognita* (Hu et al., 2013). Considering secondary metabolites acting as egg hatch inhibitors reports are as follows. Recently, isovitexin, a flavonoid glycoside isolated from butanol fraction of the leaf extract of *Kigelia pinnata*, was found to inhibit egg-hatch of *M. incognita* at the concentration of 0.16 mg mL^{-1} (Atolani et al., 2014). On the other hand, a crude alkaloid extract of *Cephalotaxus fortunei* (the plum yews) and its constituent drupacine reduced hatch of *M. incognita* (Wen et al., 2013). Root and stem extracts of *Fumaria parviflora*, containing alkaloids, flavonoids, glycosides, tannins, saponins, steroids and phenols, showed strong hatch inhibition activity against *M. incognita* (Naz et al., 2013).

In our previous works we demonstrated the activity of botanical aldehydes and ketones on RKN (Caboni et al., 2013, Caboni et al., 2012, Ntalli et al., 2011, Ntalli et al., 2010), at second juvenile stage with a potential inhibition of the vacuolar-type H+-ATPase (V-ATPase) (Caboni et al., 2014). In the present work, we focused on additional toxicity parameters on RKN. In specific, we tested: i) the nematicidal activity of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal on *M. incognita*, *M. javanica* and *M. arenaria* alone and in combination ii) the hatch inhibition on free eggs and those protected in egg masses, iii) the biological cycle arrest of *M. incognita* in tomato roots, testing aldehydes alone or as natural ingredients of *Altissima altissima* soil amendants and iv) the effects of aldehydes on J2 cuticle and eggs.

V. 2. Materials and methods

V. 2. a. Nematodes rearing and chemicals. *M. incognita, M. javanica* and *M. arenaria* were originally sampled from naturally infested tomato greenhouses in Heraklion, Crete. Populations were reared on tomato plants at the six-leaf stage tomato plants, cv. Belladonna and freshly hatched (24 h) second-stage juveniles (J2) and different growth stages eggs were extracted from egg masses according to Hussey and Barker (1973) from 60 days nematode infested roots. Egg masses were handpicked from roots using a stereoscope. (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal were purchased from Sigma Aldrich, while glutaraldehyde, sodium cacodylate and osmium tetroxide from Polysciences.

V. 2. b. Bioassays - *Paralysis activity of single test compounds*

The paralysis activity of the single compounds against J2 of *M. incognita* and *M. javanica* was tested in terms of nematode juveniles motility and EC_{50} values were calculated according to Ntalli et al., 2010.The assays were performed in Cellstar 96-well plates (Greiner bio-one), using 20-25 J2 per treatment well. Methanol was used to overcome solubility issues and it's concentration in test solutions never exceeded 1% (v/v) since preliminary tests showed that this concentration level of methanol had no effect on nematodes. The incubation was performed in the dark at 27 ^{o}C. Treatments were applied in separate plates to avoid cross contamination due to fumigant activity (Ntalli et al., 2010) and paralysis was recorded with the aid of an inverted microscope (Euromex, The Netherlands) at 40x after 1 to 4 days. The juveniles were separated to motile or immotile. Nematodes were transferred in plain water, and the body was prickled with a fine needle to observe vitality. Treatments of motility experiments (20-25 J2) were replicated six times, and each experiment was performed twice.

V. 2. c. Bioassays - *Paralysis activity of paired test compounds*

In order to study synergistic and antagonistic interactions furfural, 2-undecanone, (*E*)-2-decenal and (*E*,*E*)-2,4-decadienal were paired according to Ntalli et al., 2011. The expected and observed nematicidal activities were compared according to the effect addition model, that is: $E(d_1, d_2) = E(d_1) + E(d_2)$, where $E(d_1, d_2)$ is the effect at (d_1, d_2), and $E(d_i)$ is the effect of the compound alone at the dose d_i with i = 1, 2 (Berenbaum 1989). If the effect of the combination dose was greater, less or equal to the predicted by the additivity model (effect addition), the combination dose was characterized synergistic, antagonistic and additive, respectively (Lee et al., 2007). The comparison was made at one concentration level (providing a low nematicidal effect, less than 50%) for three immersion periods (1, 2 and 4 days). 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 separately was compared with the observed paralysis caused by immersion of J2 in the respective binary solutions. It was essential for paralysis not to exceed 50% in individual test compound tests because, clearly, the E (d1, d2) value could not be over 100%.

Stock solutions were prepared by dilution in methanol and further dilutions were made using distilled water containing Tween-20. Final concentrations of methanol and Tween-20 in treatment solutions never exceeded 1 and 0.3% v/v, respectively. Treatments were organized in a complete randomized design according to Ntalli et al., 2011 in 96-well cell culture plates (Greiner bio-one), using 20 - 25 J2 per treatment well. Juveniles motility was assessed as described above. As previously, motility regain was studied by transferring J2 in tap-water after the last assessment and observing again after 1 day. The results presented herein are those calculated before rinsing, since no differences were observed considering the paralyzed J2 immersed in test solutions for 4 day. Solutions preparation, experimental design and assessments were performed as mentioned in the previous paragraph.

V. 2. d. Bioassays - *Egg-hatch inhibition in free eggs treated with test compounds*

For the egg-hatch inhibition test, egg suspensions were chosen for experimentation instead of egg masses. The mixed-developmental stage eggs suspension (0.5mL) was pipetted into 24-well cell culture plates (Greiner bio-one) together with the treatment solution (0.5mL). Tested concentrations were: 0 μg mL^{-1} test substance (water control), 0 μg mL^{-1} test substance (carrier control) and 1, 10, 100 and 1000 μg mL^{-1}, test substance in carrier control. The plates were covered by plastic adhesive sheets to diminish volatilization and were maintained at 27±1 ^{o}C. Cumulative time – course of hatching data was done by directly counting undifferentiated eggs and J2 in each well, starting at day 0 using an inverted microscope at 40x. It must be noted that as undifferentiated eggs were considered those before cell division. Thereafter, assessments were performed after 2, 6, 10 and 14 days. Cumulative percent J2 release was calculated using the formula (1):

$$\text{Cumulative percent J2 release} = \frac{\text{J2Dx–J2D0}}{\text{total}} \text{ x } 100$$

where Dx = day after the start of the assay. Cumulative percent undifferentiated egg hatch was calculated using the formula (2):

$$\text{cumulative percent undifferentiated egg hatch} = \frac{\text{Eggs D0 – Eggs Dx}}{\text{total}} \text{ x } 100$$

where Dx = day after the start of the assay. Six wells were used per treatment per assay, and two separate assays were performed in time.

V. 2. e. Bioassays - Egg-hatch inhibition in egg masses treated with test compounds

Sixty days post tomatoes artificial inoculation with nematodes, mature egg masses were handpicked from roots and placed in small plastic extracting trays made by 6 cm Petri dishes. Solutions of the test compounds (1 - 1000 μg mL^{-1}), initially dissolved in methanol and brought to volume with water and Tween-20, were added to each extracting tray to cover three eggs masses. Attention was paid to use egg masses of the same age and size. The treatment lasted 5 days and then test solutions were removed, trays were washed and then filled with tap water. Extracting trays were covered to avoid evaporation and placed in an incubator at 27±1 °C. Assessments were performed every 7 days, when the water was replaced with fresh one. The experiment was terminated when egg hatch was over in the control treatment. The variable percentage of eggs remaining unhatched in the control was 10%. Successive assessment hatch values in the control were added, until hatch arrest, and their sum was considered 100%. This value was used to correct the hatch values in experimental treatments. The experiment was performed twice and every treatment was replicated 6 times.

V. 2. f. *SEM characterisation*

Freshly hatched J2 nematodes and eggs were treated for 1d by immersion in two 100 μL solutions containing respectively 100 mg L^{-1} of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal. Test concentration and immersion time were chosen after paralysis experiments. Thereafter, both treated and untreated nematodes, the latter used as negative controls, were immersed in a solution of 2% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.2) for 2 h at room temperature. Then, they were washed twice with 0.1M sodium cacodylate (pH 7.2), postfixed in a solution of osmium tetroxide (1%) in 0.1 M cacodylate buffer for 2 h and extensively washed with 0.1M sodium cacodylate (pH 7.2). The specimens were then dehydrated through a gradient ethanol series from 10 to 100% (at a 15min step, 4 ^{o}C) air dried, and mounted on stubs with double-sided sticky tabs. Lastly, they were coated with 10 nm gold by a sputter coater (SCD, Balzers Union, Liechtenstein), observed and

imaged using a Zeiss Evo 40 Scanning Electron Microscope (Carl Zeiss SMT Ltd., Cambridge, UK).

V. 2. g. Bioassays - Biological cycle arrest

Procedures were according to Ntalli et al., 2010. Briefly, artificially inoculated with *M. incognita* tomato plants were treated with *A. altissima* powder (tested at 1000 – 16000 mg kg^{-1}) and water extract (tested at 4000 – 64000 mg kg^{-1}) as well as with single aldehydes (tested at 30 – 625 mg kg^{-1}) in a dose response manner. Plants were kept at 27 ^{o}C, 60% RH and 16 h photoperiod, and after the completion of a biological cycle (45 days), roots were stained with acid fuchsine (Byrd et al., 1983) and the following variables were assessed: fresh root weight, fresh shoot weight and total number of female nematodes per gram of root at 10× magnification under uniform illumination by transparent light within tissue sample. The experiment was replicated once, and the treatments were always arranged in a completely randomised design with five replicates.

V. 3. Statistical analysis

The percentages of paralyzed J2 observed in the microwell assays, were corrected by eliminating the natural death/paralysis in the water control according to the Schneider Orelli's formula (Puntener et al., 1981):

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

and they were analysed (ANOVA) after being combined over time. Since ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2 treated with test solutions were subjected to nonlinear regression analysis using the log-logistic equation proposed by Seefeldt *et al.* (1995):

$$Y = C + \frac{D - C}{1 + \exp[b(\log(x) - \log(EC_{50})]}$$

where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the test compound(s) concentration required for 50% death/paralysis of nematodes after eliminating the control (natural death/paralysis). In the regression equation, the

test concentration was the independent variable (*x*) and the paralyzed J2 (percentage increase over water control) was the dependent variable (*y*). The mean value of the six replicates per test concentration and immersion period was used to calculate the EC_{50} value. The 95% confidence intervals ($CI_{95\%}$) were determined for toxicity comparison. For paired paralysis experiments, significance of difference was studied between observed and expected paralysis values using the least significant differences test at $P<0.05$.

Egg-hatch inhibition experiments were replicated 6 times, and each experiment was performed twice. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. For egg-hatch inhibition bioassays, both concerning free or contained in egg masses eggs, treatments means were compared using Tukey's test at $P \leq 0.05$. Data of J2 released from egg masses were expressed as a percentage decrease in the number of J2 released from control, according to the Abbott's formula:

$$\text{corrected } \% = 100 \text{ x } (1 - \frac{\text{J2 in treated plot}}{\text{J2 in control plot}})$$

It was fitted in the log-logistic model (Seefeldt et al., 1995) to estimate the concentration that caused a 50% decrease in J2 release (EC_{50}). In this regression equation, the test substance concentration (μg mL^{-1}) was the independent variable (x) and the J2 (percentage decrease over water control) was the dependent variable (y).

Biological cycle arrest in pot bioassays was studied in a complete randomized design with five replications and was performed twice. Since ANOVAs indicated no significant treatment by time interaction (between runs of experiment), means were averaged over experiments. The data from the pot experiments were expressed as a percentage decrease in the number of females or galls per gram of root corrected according to the control, using the Abbott's formula:

$$\text{corrected } \% = 100 \text{ x } (1 - \frac{\text{females number in treated plot}}{\text{female number in control plot}})$$

It was fitted in the log-logistic model (Seefeldt et al., 1995) to estimate the concentration that caused a 50% decrease in females and galls per gram of root (EC_{50} value). In this regression equation, the test compounds (% w/w) was the independent

variable (x) and the female nematodes, percentage decrease over water control, was the dependent variable (y).

V. 4. Results and Discussion

When compounds were tested individually on *M. incognita* and *M. javanica* J2, they achieved paralysis after one day of immersion (day 1) with the exception of (*E*,*E*)-2,4-decadienal that exhibited activity later (Table 1). (*E*,*E*)-2,4-decadienal, (E)-2-decenal and furfural were active on *M. arenaria* after day 1, while J2 immersed in 2-undecanone regained motility. At day 2 (*E*,*E*)-2,4-decadienal was the most active compound on *M. incognita* ($EC_{50/2d}$ = 15.30 μg mL^{-1}) and *M. arenaria* ($EC_{50/2d}$ = 13.24 μg mL^{-1}); and two days later (*E*,*E*)-2,4-decadienal was the most active of all compounds tested and against all nematode species, namely *M. incognita*, *M. javanica* and *M. arenaria* ($EC_{50/4d}$ = 8.06, 9.04 and 12.90 μg mL^{-1}, respectively). *M arenaria* was the most tolerant nematode species, since only for (*E*,*E*)-2,4-decadienal, (E)-2-decenal and furfural the $EC_{50/4d}$ were calculated at 12.90, 31.86 and 119.26 μg mL^{-1}, representing significantly different values to the control. While regarding the other two nematode species *M. incognita* was in most cases more sensitive than *M. javanica*, and the respective EC_{50} values were respectively lower (Table 1).

When we tested the activity of binary solutions we found that (*E*)-2-decenal and (*E*,*E*)-2,4-decadienal had a heavy synergistic effect in all assessments and nematode species (Table 2-4). Concerning *M. incognita* and *M. javanica* almost in all other than (*E*)-2-decenal and (*E*,*E*)-2,4-decadienal binary solutions, an antagonist effect was evident. We only perform a (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal binary solution, synergistic experiment for *M. arenaria* because other furfural and 2-undecanone exhibited nematostatic activity on *M. arenaria* and the paralysis was regained in time.

All compounds tested caused a considerable decrease of egg-hatch of *M. incognita* when tested at the concentration of 100 and 1000 μg mL-1, with the exception of furfural exhibiting activity only at 1000 on day 6 (Fig. 1).

At day 6 the most active compound was (*E*,*E*)-2,4-decadienal with a percent egg hatch of 22.6±2.5 on eggs treated with 10 μg mL^{-1}, if compared with the control (33.0±5.5 %). (*E*)-2-decenal and 2-undecanone were significantly active at 100 μg mL^{-1} showing a percent egg-hatch of 3.0±1.5 and 4.6±4.0, statistically different from the control

counting 14.0±3.5 and 32.0±5.5 (Fig. 1). On the other hand, furfural was effective at 100 μg mL^{-1} on day 10 with respective egg hatch percent of 3.9±1.5 while control counted 14.5±4.0. All treatments retained their efficacy on successive treatments and all tested compounds completely inhibited egg hatch at 1000 μg mL^{-1} since day 6.

The decreasing effect of compounds on the cumulative percent J2 release of *M. incognita* was evident only in day 2 and day 6, while later J2 release ended in control treatments. Thereafter it was impossible to find differences between treatments. Concerning this assessment parameter again all compounds caused a considerable decrease when tested at 100 and 1000 μg mL^{-1} as evident in day 2 and day 6 with the exception of furfural (Fig. 2). The most active compound, were (*E*,*E*)-2,4-decadienal and 2-undecanone counting at day 2 and 10 μg mL^{-1} a cumulative percent J2 hatch of 15.8±3.5 and 8.1±1.5, statistically different from the control counting 51.0±2.0 and 60.4±2.5, respectively.

In all cases the trend of decrease of cumulative egg hatch and J2 release, remained stable in successive assessments.

For *M. incognita* the hatch inhibition in egg masses immersed in test solutions was evident since day 14 (Fig. 3). In previous assessments, the percent total hatch in control at day 7 was 2.8%) and thus differences regarding efficacy of treatments were not evident. Additionally, in all cases egg hatch inhibition in egg masses was time and dose dependant (Fig. 3). For all assessments, hatchability of *M. incognita* from egg masses was the greatest decreased by (*E*)-2-decenal and EC_{50} values were lower than 1 μg mL^{-1}. 2-undecanone, (*E*,*E*)-2,4-decadienal and furfural followed with EC_{50} values of 4.09 μg mL^{-1}, 4.87 μg $mL^{-1}$and 5.23 μg mL^{-1} day 14. At the maximum of hatching in control (day 21) the lowest efficacy was evident in (*E*,*E*)-2,4-decadienal (EC_{50} value = 3.62 μg mL^{-1}) followed by furfural, 2-undecanone and (*E*)-2-decenal.

Longwise the larval body retained in the eggs suspended in (*E*)-2-decenal and 2-undecanone there were evident ring malformations (tightening) (Fig. 4 a, c); while the lateral lines of J2 exhibited a "sunken" appearance with altered cuticle annular pattern showing conspicuous damages at the cuticle layers (Fig. 4 b, d). No such effects were evident on other treatments (data not shown) resembling to the water control (Fig. 4 e, f).

When pure substances were used to treat soil artificially inoculated with nematodes (*E*)-2-decenal exhibited the highest activity and the calculated EC_{50} value was 77.46 mg/kg, followed by (*E*,*E*)- 2,4-decadienal, 2-undecanone and furfural (EC_{50} was 114.47, 231.91 and 520.33 mg kg^{-1}, respectively) (Table 5). Most interestingly (*E*,*E*)-2,4-decadienal promoted plant growth (Table 6), while all other treatments had no significant effect on the aerial and root weight. On the other hand when the *A. altissima* botanical extract (AWP and AWE) were used as soil amendments, on tomato plants artificially infested with *M. incognita*, a clear dose response relationship was established and AWP was more active (Table 7). Both AWP and AWE inhibited plant growth at high concentration levels.

Botanical substances have since long been a subject of research for their activity on *Meloidogyne* spp. (Sousa et al., 2015; Douda et al., 2010; Ntalli and Caboni, 2012a; 2012b). Motility is a key physical behavior for nematode moving towards host and suitable root cell detection to establish feeding site (Bellafiore et al., 2008; Curtis, 2008). The ability to penetrate the host root is important for plant tissue invasion and successively infestation. Achieving paralysis at the parasite growth stage, which induces host penetration, limits host infection. Artificial blends of compounds such as (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal, can induce inhibition of nematode motility, and can thus constitute promising agents for pest management. The synergistic effect of two compounds can actually provide with greater activity dynamic products, of multiple modes of action based on the chemical structure. On the contrary, antagonism is a diminution in the biological activity of a mixture (Yamashita and Viglierchio, 1987) and interestingly can be evidenced even between compounds highly active when used individually. Care should thus be taken for such blends not to be desirable for pest management products. We previously reported on the synergistic effect of binary mixtures of terpenes to be used as botanical nematicidals. In particular, *trans*-anethole showed synergistic interactions with other terpenes, while L-carvone showed antagonistic interactions, with the exception of its mixture with *trans*-anethole. The synergistic activity of *trans*-anethole/geraniol is a most potent combination, followed by *trans*-anethole/eugenol, carvacrol/eugenol and geraniol/carvacrol (Ntalli et al, 2011).

On the other hand, egg hatch is a very critical stage for RKN reproduction in host tissue. As shown in this study, (*E*,*E*)-2,4-decadienal has an inhibition effect on eggs

hatch and in paralysing J2. Specifically (*E*,*E*)-2,4-decadienal is the best inhibitor of both undifferentiated egg hatch and J2 release. Between the four tested compounds, (*E*,*E*)-2,4-decadienal is the best inhibiting the first and last egg hatch evolution steps, namely cell division and J2 release. (*E*)-2-decenal is the second best active concerning inhibition of cell division in eggs; and 2-undecanone on the arrest of J2 release. Nonetheless, when the eggs are protected in the egg mass (*E*)-2-decenal becomes significantly more effective probably due to different lipophilicity of the compound and eventual different egg mass penetration ability. The egg mass is made of a gelatinous matrix protecting the eggs from the loss of water and thus permeability of water soluble substances is reduced. Interestingly, SEM experiments revealed tightening on the larvae body inside the egg treated with (*E*)-2-decenal but not on those treated with (*E*,*E*)-2,4-decadienal implicating differences in the biochemical mode of action of these two substances. Furthermore, (*E*)-2-decenal and 2-undecanone exhibit effects on *M. incognita* J2 (Fig. 4) similar to those reported for salycilaldehyde (Caboni et al., 2013).

(*E*)-2-decenal activity increases when applied on eggs contained in egg masses and the respective EC_{50} value was below 1 μg mL^{-1}. We previously studied the egg hatch inhibition activity of terpenes applied to egg masses, but they exhibit activity at higher concentration levels (500-1000 μg mL^{-1}) (Ntalli et al., 2013).

Furthermore, (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal arrest the biological cycle of *M. incognita* in tomato roots when used individually. Moreover, extracts of *A. altissima* used as amendments are nematicidal on *M. incognita*. Both soil amendands were phytotoxic at high concentrations but this is not restrictive for their use because they are active at much lower levels. The nematicidal activity of *A. altissima* extracts can be explained by the presence of (*E*,*E*)-2-decadienal and (*E*)-2-decenal (Caboni et al., 2012) and by-products produced in soil, as well as to other components playing a distinct role on the overall efficacy by acting together synergically or antagonistically. Plant promotion parameters, like aerial weight increase, exhibited by (*E*,*E*)-2-decadienal are to be considered together with the efficacy parameters in case it would be addressed as a pest management tool. Finally, cost effectiveness and large-scale production, plant biomass availability and fate of nematicidal compounds in soil contribute to the best choice of botanical product used as a nematicidal.

Conclusively, the differences in the efficacy of a compound on various nematode species should be taken into consideration when addressing the control of nematodes. The paralysis activity on J2 together with the hatch inhibition activity of free eggs and eggs contained in egg masses contribute to the overall nematicidal efficacy of a compound. On these bases, studying the chemical composition of the egg mass and how it can affect permeability of nematicidal compounds is mandatory. Other issues to be addressed concerning blends to be formulated are the synergistic and antagonistic interactions among compounds mixed together, as well as any attractant and repelled properties on nematodes in soil.

Acknowledgements: This work was partially financed by Research Committee of University of Thessaly in the frame of Targeted research Action Programs 2012-13 and also by a scholarship for PhD studies of the University of Cagliari. Special thanks are due to T. Koufakis and AGRIS SA for providing seeds and seedlings and to Tzortzakakis Emmanuel for the nematode species speciments.

Figure Captions

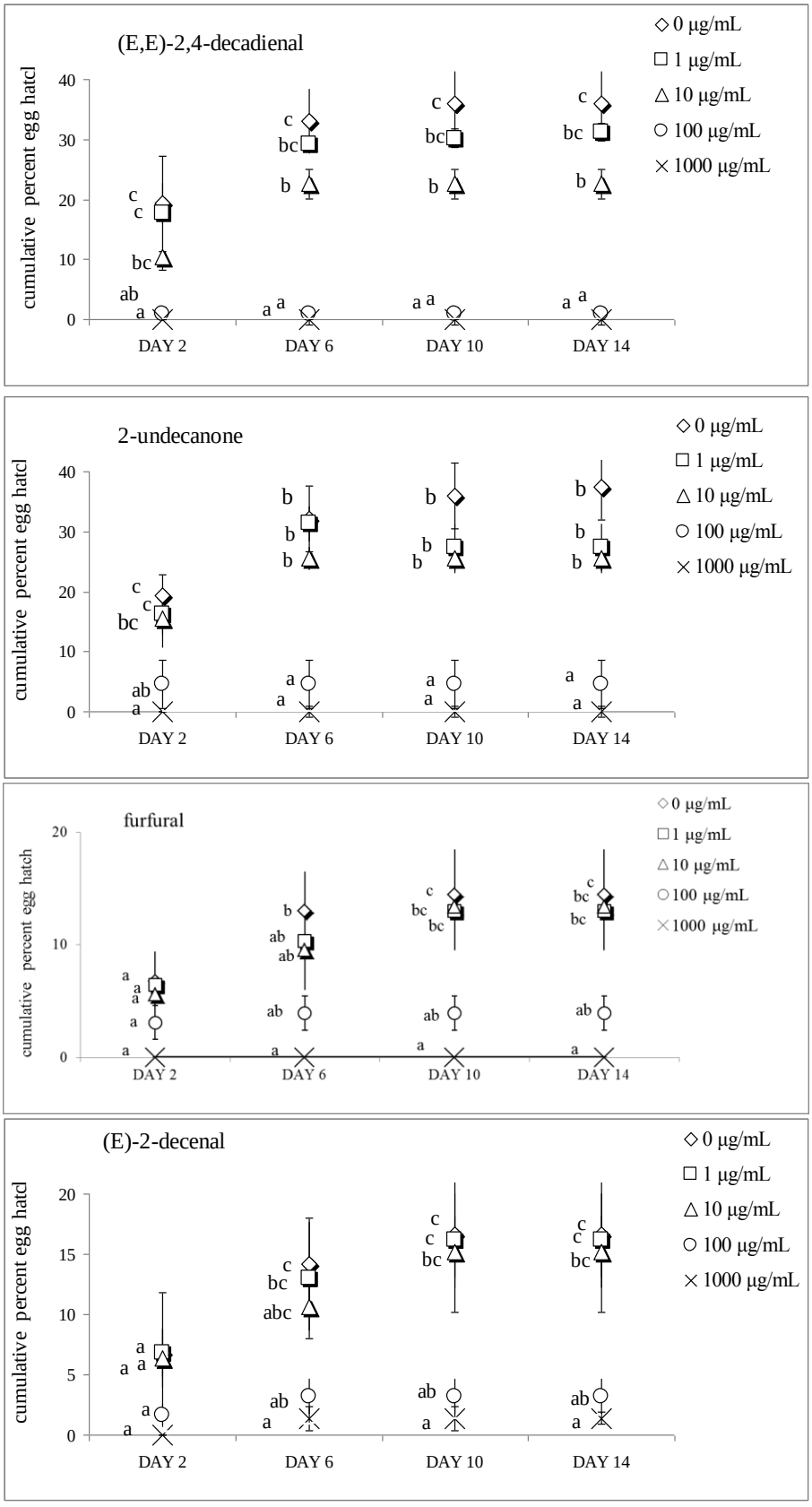
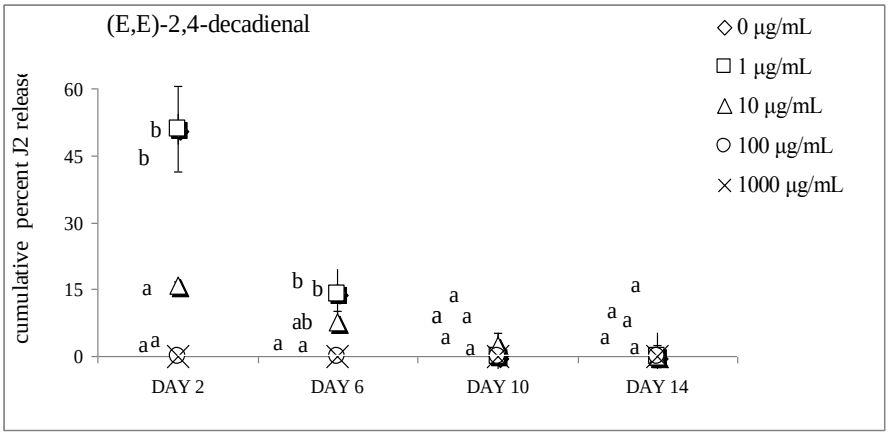
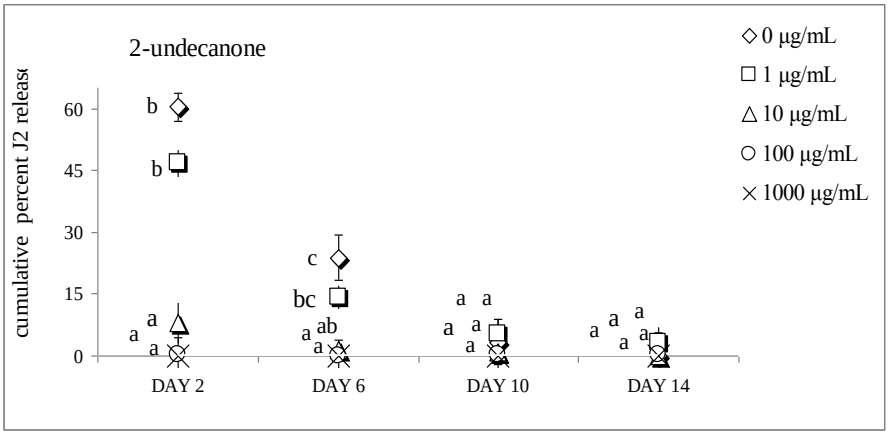
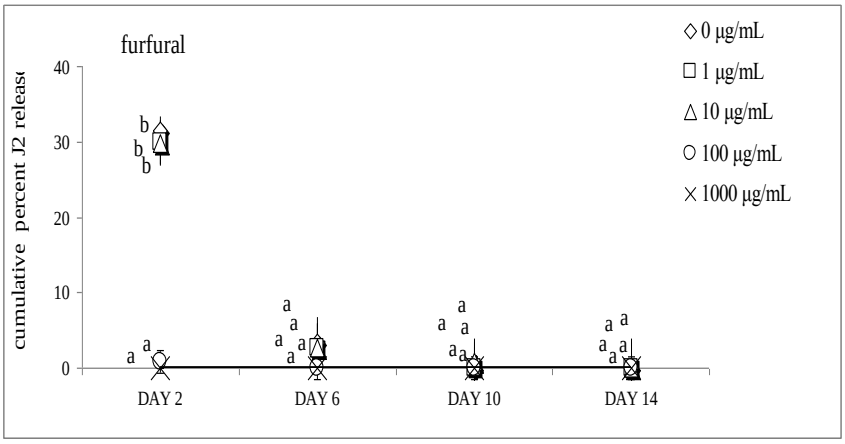


Fig. 1 Effect of test compounds on cumulative percent hatch of *Meloidogyne incognita* undifferentiated eggs calculated using the formula (1): Eggs (20-30 per well) were collected and distributed in 24-well plates and were incubated at 27^{o}C for 14 days in test solutions or water. Eggs were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SDEV from two experiments performed in time, with 6 replicates per treatment each. Values within each day were compared using Tukey's test and those followed by different letters are significantly different at ($P \leq 0.05$).


(E,E)-2,4-decadienal
◇ 0 µg/mL
□ 1 µg/mL
△ 10 µg/mL
○ 100 µg/mL
× 1000 µg/mL
cumulative percent J2 releas
60
45
30
15
0
DAY 2
DAY 6
DAY 10
DAY 14


2-undecanone
◇ 0 µg/mL
□ 1 µg/mL
△ 10 µg/mL
○ 100 µg/mL
× 1000 µg/mL
cumulative percent J2 releas
60
45
30
15
0
DAY 2
DAY 6
DAY 10
DAY 14


furfural
◇ 0 µg/mL
□ 1 µg/mL
△ 10 µg/mL
○ 100 µg/mL
× 1000 µg/mL
cumulative percent J2 releas
40
30
20
10
0
DAY 2
DAY 6
DAY 10
DAY 14

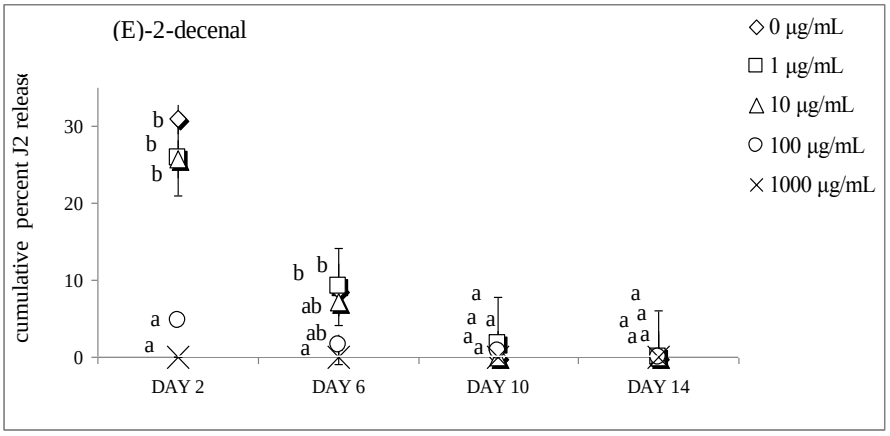
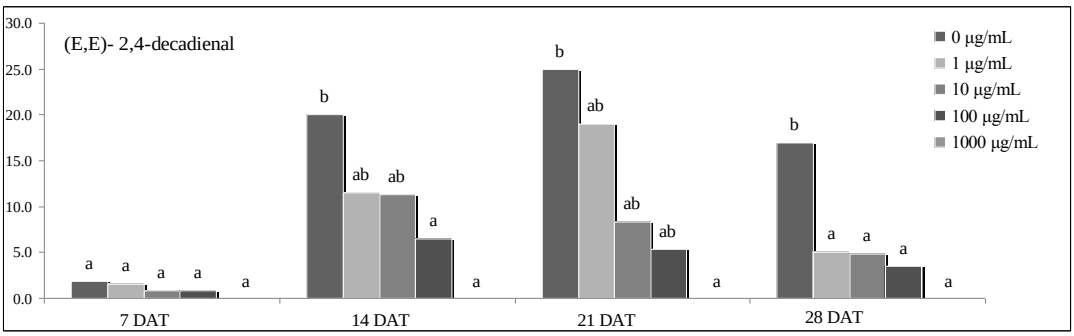
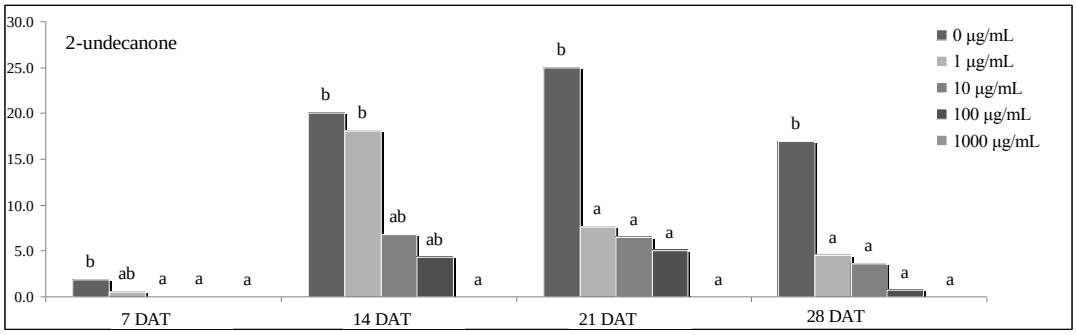

(E)-2-decenal
◇ 0 µg/mL
□ 1 µg/mL
△ 10 µg/mL
○ 100 µg/mL
× 1000 µg/mL
cumulative percent J2 releas
30
20
10
0
DAY 2
DAY 6
DAY 10
DAY 14

Fig. 2 Effect of compounds on cumulative percent release of *Meloidogyne incognita* J2 calculated using the formula (2):

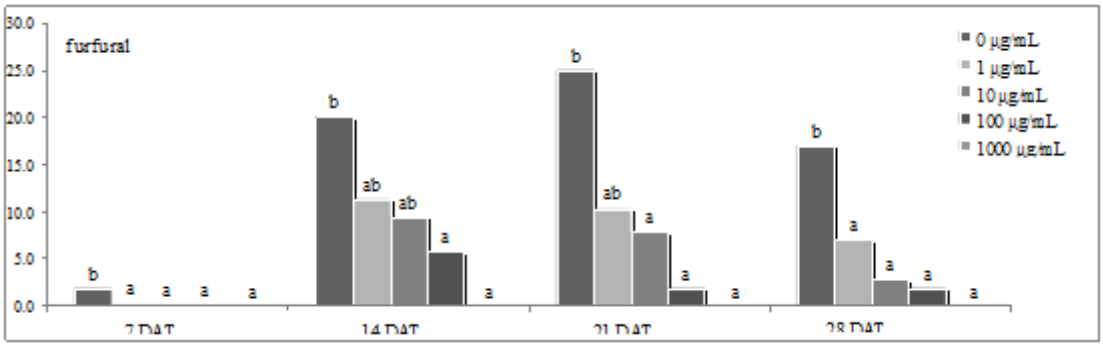
Eggs (20-30 per well) were collected and distributed in 24-well plates and were incubated at 27^{o}C for 14 days in test solutions or water. Released J2 were counted at 2, 6, 10 and 14 days post experiment establishment. Each percent data represent the mean ± SDEV from two experiments performed in time, with 6 replicates per treatment each. Values within each day were compared using Tukey's test and those followed by different letters are significantly different at ($P \leq 0.05$).



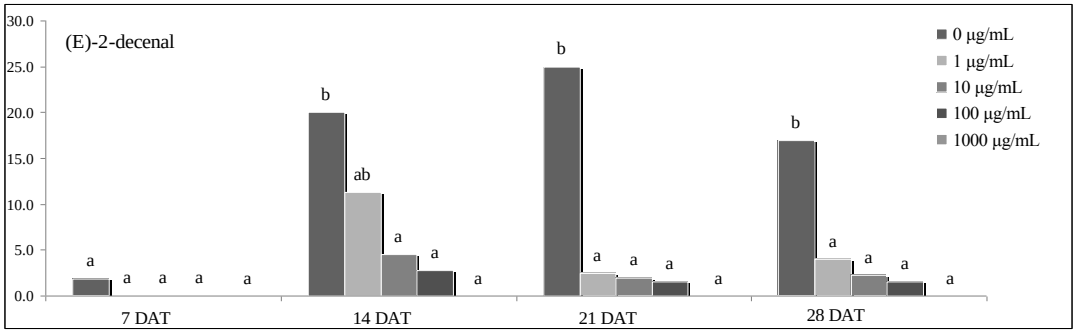
EC_{50}≤1 µg mL^{-1} b: na **EC_{50} = 4.87 µg mL^{-1} b: 1.023** **EC_{50} = 3.62 µg mL^{-1} b: 1.668** **EC_{50}≤1 µg mL^{-1} b: na**



EC_{50}≤1 µg mL^{-1} b: na **EC_{50} = 4.09 µg mL-1 b: 1.429** **EC_{50}≤1 µg mL-1 b: na** **EC_{50}≤1 µg mL^{-1} b: na**



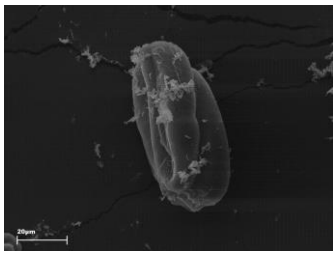
EC_{50}≤1 µg mL^{-1} b: na **EC_{50} = 5.226 µg mL^{-1} b: 1.134** **EC_{50}≤1 µg mL^{-1} b: na** **EC_{50}≤1 µg mL^{-1} b: na**



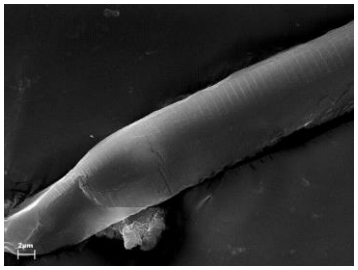
EC_{50}≤1 µg mL^{-1} b: na **EC_{50}≤1 µg mL^{-1} b: na** **EC_{50}≤1 µg mL^{-1} b: na** **EC_{50}≤1 µg mL^{-1} b: na**

Fig. 3 Effect of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal on *Meloidogyne incognita* hatch, after immersion of egg masses at the dose rates of 1, 10, 100 and 1000 µg mL^{-1} for 5 days. At the time of the assessment 7, 14, 21 and 28 Days After

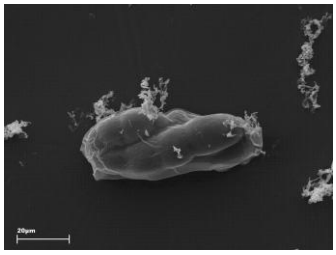
Treatment (DAT), the egg hatch at the control treatment was at 2.8, 31.5, 38.1 and 27.7 % over total corresponding to the maximum recorded on week intervals. Within each assessment date, bars followed by the same letter were not significantly different (P≤ 0.05). na: not available



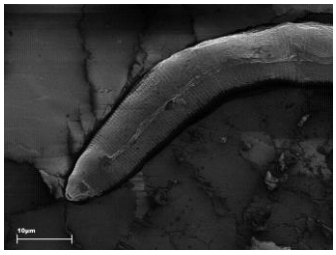
a. (E)-2-decenal



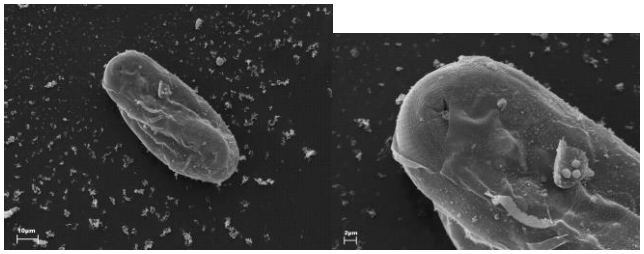
b. (E)-2-decenal



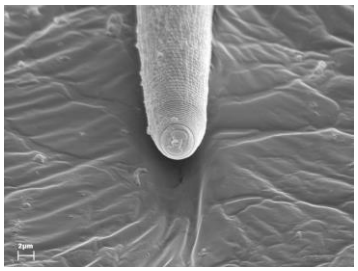
c. 2-undecanone



d. 2-undecanone



e. water



f. water

Fig. 4 ESEM topographical images of *Meloidogyne incognita* eggs (left) and second stage juveniles (right) immersed for 1 d in 100 mg L^{-1} of (**a** and **b**) (*E*)-2-decenal or (**c** and **d**) 2-undecanone, and (**e** and **f**) water control (untreated (control) specimens). **a** and **c** note the tightenings along the juveniles body inside the egg; **b** and **d** note the damaged (smooth) adjacent annuli of the cortical layer; (**e**) low magnification showing a macroscopic fracture of an untreated nematode body contained in a mechanically ruptured egg cell and (**f**) juvenile body surface (note the well formed annulated cuticle cortical layer).

Table 1 EC_{50} values of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal paralysis activity against *Meloidogyne incognita*, *M. javanica* and *M. arenaria* calculated for 1 to 4 days immersion periods in test solutions. The respective R^2, b_{EC50} and $CI_{95\%}$ values are given as well for toxicity comparison.

	M. incognita				*M. javanica*				*M. arenaria*			
compound name	$EC_{50/1DAY}$ (µg mL^{-1})	R^2	$b_{EC50/1DAY}$	$CI_{95\%}$	$EC_{50/1DAY}$ (µg mL^{-1})	R^2	$b_{EC50/1DAY}$	$CI_{95\%}$	$EC_{50/1DAY}$ (µg mL^{-1})	R^2	$b_{EC50/1DAY}$	$CI_{95\%}$
(*E*,*E*)-2,4-decadienal	> 20	-	-	-	> 50	-	-	-	13.38	0.98	2.87	13.09-13.68
2-undecanone	32.40	0.88	2.01	27.93-37.47	50.44	0.85	1.70	40.45-60.42	221	0.98	1.52	210,39-235.39
furfural	33.59	0.84	2.87	28.24-38.95	49.15	0.79	2.07	37.97-60.34	63.63	0.98	2.43	58.32-68.55
(*E*)-2-decenal	39.05	0.75	0.82	36.32-41.77	36.45	0.77	0.98	35.33-37.57	32.92	0.95	1.54	32.41-33.45

	M. incognita				*M. javanica*				*M. arenaria*			
compound name	$EC_{50/2DAY}$ (µg mL^{-1})	R^2	$b_{EC50/2DAY}$	$CI_{95\%}$	$EC_{50/2DAY}$ (µg mL^{-1})	R^2	$b_{EC50/2DAY}$	$CI_{95\%}$	$EC_{50/2DAY}$ (µg mL^{-1})	R^2	$b_{EC50/2DAY}$	$CI_{95\%}$
(E,E)-2,4-decadienal	15.30	0.86	2.01	12.04-18.56	> 50	-	-	-	13.24	0.83	1.23	12.95-13.54
2-undecanone	28.10	0.84	1.98	23.34-32.86	44.55	0.84	1.74	36.01-53.08	>300	-	-	-
furfural	28.78	0.81	2.78	23.55-34.00	44.27	0.73	1.88	32.29-56.24	79.74	098	1.72	66.42-93.41
(*E*)-2-decenal	35.15	0.95	0.88	32.98-37.32	33.78	0.30	0.88	31.95-35.62	32.19	0.90	1.23	31.68-32.72

compound name	*M. incognita*	*M. javanica*	*M. arenaria*

	$EC_{50/4DAY}$ (μg mL^{-1})	R^2	$b_{EC50/4DAY}$	$CI_{95\%}$	$EC_{50/4DAY}$ (μg mL^{-1})	R^2	$b_{EC50/4DAY}$	$CI_{95\%}$	$EC_{50/4DAY}$ (μg mL^{-1})	R^2	$b_{EC50/4DAY}$	$CI_{95\%}$
(*E*,*E*)-2,4-decadienal	8.06	0.91	2.73	6.89-9.23	9.04	0.92	2.66	7.81-10.27	12.90	0.92	1.95	11.69-14.21
2-undecanone	23.34	0.78	1.79	17.25-29.42	41.58	0.89	1.42	32.43-50.73	>300	-	-	-
furfural	29.39	0.86	2.46	24.01-34.76	46.7	0.82	1.78	35.95-57.64	119.26	0.98	1.89	107.96-134.84
(*E*)-2-decenal	30.04	0.81	0.90	27.77-32.31	< 10	-	-	-	31.86	0.94	1.85	30.43-33.35

Table 2 Synergistic and antagonistic interactions observed between (E,E)-2,4-decadienal, 2-undecanone, furfural and (E)-2-decenal against *M. incognita*

combination	µg mL^{-1}	*M. incognita* J2 paralysis over control, % (1DAY)		sign.	*M. incognita* J2 paralysis over control, % (2DAY)		sign.	*M. incognita* J2 paralysis over control, % (4DAY)		sign.
		observed[a]	expected[b]		observed[a]	expected[b]		observed[a]	expected[b]	
(*E*)-2-decenal /furfural	25/20	79.87±6.75	100.00±0.00	antagonism	76.79±6.80	92.35±6.22	additive effect	76.22±6.83	79.27±1.82	additive effect
(*E*)-2-decenal /(*E*,*E*)-2,4-	25/20	98.95±0.00	84.55±7.05	additive effect	100.00±0.00	73.73±5.45	synergism	100.00±0.00	64.14±2.50	synergism
(*E*)-2-decenal/2-undecanone	25/20	16.00±1.89	33.80±0.97	antagonism	16.60±1.89	33.8±0.97	antagonism	16.60±1.89	34.00±1.99	antagonism
2-undecanone /furfural	20/20	80.45±3.12	94.07±3.15	antagonism	91.48±1.78	100.59±3.48	antagonism	95.07±1.77	100.00±0.00	antagonism
(*E*,*E*)-2,4-decadienal	10/20	82.25±0.89	87.91±4.65	additive effect	91.73±1.86	96.05±2.23	additive effect	91.82±1.82	100.00±0.00	antagonism
(*E*,*E*)-2,4-decadienal /2-	10/20	80.68±0.78	88.44±3.44	antagonism	92.09±1.61	93.88±3.68	additive effect	92.88±1.79	100.00±0.00	antagonism

[a] Observed % paralysis, corrected according to the control, after immersion of J2 in solutions of paired test compounds

[b] Expected % paralysis, corrected according to the control, calculated as the sum of paralysis observed after immersion of J2 in solutions of single test compounds

[c] Significance of difference between observed and expected paralysis as presented by each row in table (P<0.05).

Table 3 Synergistic and antagonistic interactions observed between (E,E)-2,4-decadienal, 2-undecanone, furfural and (E)-2-decenal against *M. javanica*

combination	µg mL^{-1}	*M. javanica* J2 paralysis over control, % (1DAY)		sign.	*M. javanica* J2 paralysis over control, % (2DAY)		sign.	*M. javanica* J2 paralysis over control, % (4DAY)		sign.
		observed[a]	expected[b]		observed[a]	expected[b]		observed[a]	expected[b]	
(*E*)-2-decenal /furfural	25/20	25.63±6.00	27.73±1.83	additive effect	30.49±7.43	39.32±1.45	additive effect	35.79±2.50	71.51±1.77	antagonism
(*E*)-2-decenal /(*E*,*E*)-2,4-	25/10	100.00±0.00	49.05±1.75	synergism	100.00±0.00	62.66±2.11	synergism	100.00±0.00	76.31±1.45	synergism
(*E*)-2-decenal /2-undecanone	25/20	10.73±1.04	35.15±1.77	antagonism	24.15±6.50	38.86±2.32	antagonism	32.19±8.78	48.17±1.43	antagonism
2-undecanone /furfural	20/20	69.06±2.78	82.05±2.44	antagonism	78.08±2.73	89.04±1.84	antagonism	89.03±0.62	98.46±2.77	antagonism
(E,E)-2,4-decadienal	20/40	71.50±3.12	84.3±2.79	antagonism	76.19±2.1	89.87±2.66	antagonism	84.16±1.91	100.00±0.00	antagonism
(*E*,*E*)-2,4-decadienal /2-	20/40	70.44±2.03	86.15±1.52	antagonism	82.82±2.41	93.41±3.67	antagonism	92.88±1.41	100.00±0.00	antagonism

[a] Observed % paralysis, corrected according to the control, after immersion of J2 in solutions of paired test compounds

[b] Expected % paralysis, corrected according to the control, calculated as the sum of paralysis observed after immersion of J2 in solutions of single test compounds

[c] Significance of difference between observed and expected paralysis as presented by each row in table (P<0.05).

Table 4 Synergistic and antagonistic interactions observed between (E,E)-2,4-decadienal, 2-undecanone, furfural and (E)-2-decenal against *M. arenaria*

combination	µg mL^{-1}	*M. arenaria* J2 paralysis over control, % (1DAY)		sign.	*M. arenaria* J2 paralysis over control, % (2DAY)		sign.	*M. arenaria* J2 paralysis over control, % (4DAY)		sign.
		observed[a]	expected[b]		observed[a]	expected[b]		observed[a]	expected[b]	
(*E*)-2-decenal /furfural	na	-	-	-	-	-	-	-	-	-
(*E*)-2-decenal /(*E*,*E*)-2,4-	30/5	94.80±1.31	16.10±1.75	synergism	95.00±1.12	22.10±2.07	synergism	95.20±1.23	22.30±1.41	synergism
(*E*)-2-decenal /2-undecanone	na	-	-	-	-	-	-	-	-	-
2-undecanone /furfural	na	-	-	-	-	-	-	-	-	-
(E,E)-2,4-decadienal	na	-	-	-	-	-	-	-	-	-
(*E*,*E*)-2,4-decadienal /2-	na	-	-	-	-	-	-	-	-	-

[a] Observed % paralysis, corrected according to the control, after immersion of J2 in solutions of paired test compounds

[b] Expected % paralysis, corrected according to the control, calculated as the sum of paralysis observed after immersion of J2 in solutions of single test compounds

[c] Significance of difference between observed and expected paralysis as presented by each row in table (P<0.05).

na not available

Table 5 EC_{50} values of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal efficacy against *M. incognita* as calculated in pot experiments with respective standard error and confidence interval values.

(*E*,*E*)-2,4-decadienal (♀/g) EC_{50} (mg kg^{-1})	Std error	95% Conf Int	**furfural** (♀/g) EC_{50} (mg kg^{-1})	Std error	95% Conf Int
114.47	4.92	104.28-124.66	520.33	75.08	365.01-675.65
2-undecanone (♀/g) EC_{50} (mg kg^{-1})	Std error	95% Conf Int	**(*E*)-2-decenal** (♀/g) EC_{50} (mg kg^{-1})	Std error	95% Conf Int
231.91	9.78	211.67-252.16	77.46	10.02	56.72-98.21

Table 6 Tomato (*Solanum lycopersicum*) growth parameters, grown in soil infested with *M. incognita*, treated with different rates of (*E*,*E*)-2,4-decadienal, 2-undecanone, furfural and (*E*)-2-decenal. Numbers followed by the same letters (column) are not statistically different for $P \leq 0.05$ according to Tukey's test.

(*E*,*E*)-2,4-decadienal	Aerial weight	Root weight	2-undecanone (mg kg^{-1})	Aerial weight	Root weight	furfural	Aerial weight	Root weight	(*E*)-2-decenal (mg kg^{-1})	Aerial weight	Root weight
0	5.36^{a}	1.88^{a}	0	8.74^{a}	2.44^{ab}	0	5.36^{a}	1.88^{a}	0	9.94^{c}	2.42^{ab}
75	8.64^{b}	2.82^{b}	30	7.20^{a}	1.92^{a}	125	7.78^{ab}	2.04^{a}	30	10.00^{c}	2.66^{b}
112.5	10.12^{ab}	2.94^{b}	75	8.54^{a}	2.80^{b}	250	9.82^{b}	2.70^{a}	75	8.9b^{c}	2.62^{b}
150	11.52^{cd}	1.34^{a}	150	9.12^{a}	2.28^{ab}	375	8.50^{ab}	2.30^{a}	150	6.24^{ab}	2.34^{ab}
225	12.28^{cd}	1.42^{a}	225	7.98^{a}	2.24^{ab}	500	8.12^{ab}	2.70^{a}	225	5.58^{a}	1.68^{a}
300	10.68^{cd}	1.30^{a}	300	7.34^{a}	2.06^{ab}	625	9.88^{ab}	2.74^{a}	300	10.00^{c}	1.94^{ab}

Table 7 Tomato (*Solanum lycopersicum*) root infestation with *M. incognita* and respective growth parameters, after treating with different rates of *Ailanthus* Wood Powder (AWP) and *Ailanthus* Wood Water Extract (AWE). Numbers followed by the same letters (column) are not statistically different for $P \leq 0.05$ according to Tukey's test. Concerning aerial and root weight data the treatment of 1000 mg/kg did not differ from control (0 mg/kg) and thus in the table we present dose range from 1000 to 16000 mg/kg.

AWP	*M. incognita*	Aerial weight	Root weight	AWE	*M. incognita*	Aerial weight	Root weight
1000	73.89 ± 4.5^{c}	3.18 ± 0.1^{a}	0.49 ± 0.02^{a}	4000	81.22 ± 6.1^{c}	3.06 ± 0.2^{a}	2.04 ± 0.20^{a}
2000	37.65 ± 3.0^{b}	3.36 ± 0.1^{a}	0.43 ± 0.03^{a}	8000	62.38 ± 5.3^{b}	3.82 ± 0.3^{a}	1.86 ± 0.10^{a}
4000	28.84 ± 2.0^{b}	3.48 ± 0.2^{a}	0.44 ± 0.02^{a}	16000	57.81 ± 6.1^{b}	3.62 ± 0.2^{a}	1.72 ± 0.2^{a}
8000	13.57 ± 4.4^{a}	2.20 ± 0.1^{b}	0.37 ± 0.03^{a}	32000	49.59 ± 6.1^{b}	3.90 ± 0.4^{a}	1.78 ± 0.1^{a}
16000	5.54 ± 3.0^{a}	1.39 ± 0.1^{c}	0.41 ± 0.13^{a}	64000	0.00 ± 0.00^{a}	0.15 ± 0.0^{b}	0.0 ± 0.0^{b}

V. 5. References

Ahmed N, Abbasi MW, Shaukat SS, Zaki MJ Induced systemic resistance in mung bean plant against root-knot nematode *Meloidogyne javanica* by dl-β-amino butyric acid. *Nematol Medit* **2009,** *37*, 67-72.

Amdadul Hoque AKM, Rejwan Bhuiyan M, Ashik Iqbal Khan M, Mahmud A, Uddin Ahmad M Effect of amino acids on root-knot nematode (*Meloidogyne javanica*) infecting tomato plant. *Arch Phytopathol Plant Prot* **2014,** *47*, 1921-1928.

Atolani O, Fabiyi OA, Olatunji GA. Isovitexin from *Kigelia pinnata*, a potential eco-friendly nematicidal agent. *Trop Agric* **2014**, 91, 67-74.

Barbary A, Djian-Caporalino C, Palloix A, Castagnone-Sereno P Host genetic resistance to root-knot nematodes, Meloidogyne spp., in Solanaceae: from genes to the field. *Pest Manag Sci*. **2015,** *12*, 1591-1588.

Bellafiore S, Shen Z, Rosso MN, Abad P, Shih P, Briggs SP Direct identification of the *Meloidogyne incognita* secretome reveals proteins with host cell reprogramming potential. *PLoS Pathog*. **2008,** *4*, e1000192

Berenbaum MC What is synergy. *Pharmacol Rev* **1989,** *41*, 93–141.

Byrd DW, Krikpatrick T, Barker, KR. An improved technique for cleaning and staining plant tissue for detection of nematodes. J. Nematol **1983,** *15*, 142–143

Caboni P, Aissani N, Cabras T, Falqui A, Marotta R, Liori B, Ntalli N, Sarais G, Sasanelli N, Tocco G () Potent nematicidal activity of phthalaldehyde, salicylaldehyde, and cinnamic aldehyde against *Meloidogyne incognita.* J Agr Food Chem **2013**, *61*, 1794-1803.

Caboni P, Ntalli NG, Aissani N, Cavoski I, Angioni A Nematicidal activity of (E, E)-2,4-decadienal and (E)-2-decenal from *Ailanthus altissima* against *Meloidogyne javanica.* J Agr Food Chem **2012,** *60*, 1146-1151

Caboni P, Tronci L, Liori B, Tocco G, Sasanelli N, Diana A Tulipaline A: Structure-activity aspects as a nematicide and V-ATPase inhibitor. Pestic Biochem Physiol **2014**, *112*, 33-39

Curtis RH Plant-nematode interactions: environmental signals detected by the nematode's chemosensory organs control changes in the surface cuticle and behaviour. *Parasite* **2008,** *15*, 310–316

Douda O, Zouhar M, Mazáková J, Nováková E, Pavela R. Using plant essences as alternative mean for northern root-knot nematode (*Meloidogyne hapla*) management. J Pest Sci, **2010,** *83*, 217-221

Fatemy S, Moslemi F, Bernard F Seed treatment and soil drench with dl-*β*-amino butyric acid for the suppression of *Meloidogyne javanica* on tomato. Acta Physiol Plant **2014**, *36*, 2311-2317

Hu Y, Zhang W, Zhang P, Ruan W, Zhu X Nematicidal activity of chaetoglobosin a poduced by *Chaetomium globosum* NK102 against *Meloidogyne incognita.* J Agr Food Chem **2013**, *61*, 41-46

Hussey RS, Barker KR A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Dis Rep **1973**, *57*, 1025–1028.

Jung WJ, Kim KY, Park YS, Kim, SB, Lee YS, Anees M Purification and properties of a *Meloidogyne*-antagonistic chitinase from *Lysobacter capsici* YS1215. Nematol **2014**, *16*, 63-72

Lee JJ, Kong M, Ayers GD, Lotan R Determination interaction in combination therapy. J Biopharm Stat **2007**, *17*, 461–480

Lee YS, Anees M, Hyun HN, Kim KY Biocontrol potential of *Lysobacter antibioticus* HS124 against the root-knot nematode, *Meloidogyne incognita*, causing disease in tomato. Nematol **2013**, *15*, 545-555

Masler EP, Rogers ST, Chitwood DJ Effects of catechins and low temperature on embryonic development and hatching in *Heterodera glycines* and *Meloidogyne incognita*. Nematol **2013,** *15*, 653-663

Naz I, Palomares-Rius JE, Saifullah Blok V, Khan MR, Ali S, Ali S In vitro and in planta nematicidal activity of fumaria parviflora (fumariaceae) against the southern root-knot nematode *Meloidogyne incognita* Plant Pathol. **2013**, *62*, 943-952

Nguyen DM, Seo DJ, Kim KY, Park RD, Kim DH, Han YS, Kim TH, Jung WJ Nematicidal activity of 3,4-dihydroxybenzoic acid purified from *Terminalia nigrovenulosa* bark against *Meloidogyne incognita*. Microb Pathog **2013**, *60*, 52-59

Ntalli NG, Nasiou E, Menkissoglu-Spiroudi U Evaluation of essential oils from rosemary, orange, lavandula and false yellowhead on hatching and motility of Root-Knot Nematode. J Agr Sci Tech A **2013**, *3*, 603-616

Ntalli NG, Caboni P Botanical nematicides: A review. J Agric Food Chem 2012a, 60, 9929-9940

Ntalli NG, Caboni P Botanical nematicides in the mediterranean basin. Phytochem Rev 2012b, 11, 351-359.

Ntalli NG, Manconi F, Leonti M, Maxia A, Caboni P Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes J Agr Food Chem **2011**, *59*, 7098-7710

Ntalli NG, Vargiu S, Menkissoglu-Spiroudi U, Caboni P Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. J Agric Food Chem **2010**, *58*, 11390-11394

Ntalli NG, Ferrari F, Giannakou I, Menkissoglu-Spiroudi U Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. Pest Manag Sci **2011**, *67*, 341-351

Puntener W (1981) Manual for Field Trials in Plant Protection, 2nd edition. Ciba-Geigy Limited, Basle, Switzerland, 205 pp.

Seefeldt SS, Jensen JE, Fuerst EP Log-logistic analysis of herbicide dose response relationships. Weed Technol **1995**, *9*, 218–227

Sousa RMOF, Rosa JS, Silva CA, Almeida MTM, Novo MT, Cunha AC, Fernandes-Ferreira M. Larvicidal, molluscicidal and nematicidal activities of essential oils and compounds from *Foeniculum vulgare* J Pest Sci. **2015**, *88*, 413-426

Wen Y, Meyer SLF, Masler EP, Zhang F, Liao J, Wei X, Chitwood DJ Nematotoxicity of drupacine and a Cephalotaxus alkaloid preparation against the

plant-parasitic nematodes *Meloidogyne incognita* and *Bursaphelenchus xylophilus* Pest Manag Sci **2013**, *69*, 1026-1033

Yamashita TT, Viglierchio DR In vitro testing for non fumigant nematicide resistance in *Xiphinerna index*. Rev Nem **1987**, *10*, 75–79

Chapter 2– Developing Botanical Biocides

I. 1. Algicidal effect of essential oils on Aphanizomenon gracile

Introduction

Blooms of cyanobacteria in water bodies contribute to musty odor and the potential presence of harmful toxins (Rajasekhar et al., 2012). In the recent years scientific consensus has emerged concerning the increasing frequency and severity of cyanobacterial blooms in freshwater environments. Algae bloom, that is they grow rapidly, in the presence of nutrients in their environment and usually when phosphates are not a limiting factor. Blooms intensify as fertilizers wash out of agricultural soils, into the rivers and lakes (Newman 2013). The toxic metabolites are released into surface waters during cyanobacterial blooms, and *in vivo* assays have shown that they exhibit estrogenicity, neurotoxicity and teratogenic potency as reported on zebrafish embryos (Jonas et al., 2015). In fact, animal and human health problems associated with the ingestion of or contact with cyanobacterial scums have long been recognized. In 1996 an incident occurred in Brazil that led to the death of 50 people due to contamination with microcystins in the water used for hemodialysis (Jochimsen et al., 1998; Pourie et al., 1998). Some cyanobacteria, *Oscillatoria perornata* and *O. agarhii* (blue-green algae), are common in catfish production ponds, a ray-finned fish farmed for food in Africa, Asia, Europe, and North America (Tellez et al., 2000). These blue-green algae produce 2-methylisoborneol that is absorbed into the catfish flesh imparting a "musty" taste, thus rendering it unpalatable and unmarketable (Purcaro et al., 2009). Cyanobacterial blooms of *Aphanizomenon* spp. occur worldwide, with deleterious effects on aquatic communities (De Figueiredo et al., 2015). The main toxins of the cyanobacterium *Aphanizomenon gracile* are the saxitoxins (Ledreux et al., 2010) but also the cytotoxin cylindrospermopsin has become increasingly common in fresh waters (Kokociński et al., 2013) that is related to the presence of unknown tumor promoting metabolites (Novakova et al,. 2012). Moreover, there are some cyanobacteria infesting crop cultures like *Microcystis aeruginosa,* a blue-green alga growing in rice cultivating water, thus reducing the water temperature and inhibiting photosynthesis by blocking the light (Ateeque et al., 2013).

It is thus mandatory to find safe compounds that selectively control *A. gracile* so as to make safe the recreational and drinking water bodies, benefit the channel of the catfish industry and to prevent animal and human poisoning (Newman 2013).

The treatment of cyanobacterial blooms has traditionally been carried out using copper algicides, the herbicides diuron and copper sulfate. Unfortunately, these synthetic algicides also affect non-target species and can result in metal residue in the reservoir sediments. The toxicity considerations of the synthetic pesticides have created an impetus for the discovery and development of more environmentally benign and less hazardous pesticides, although the assumption "natural is safe-synthetic is hazardous" is invalid (Isman and Grieneisen, 2014). Alternative algicidal strategies involve sonication with impacts on both structure and function of the blue algae (Leclercq et al., 2014; Rajasekhar et al., 2012). In the frame of introducing alternative strategies to control the algae bloom, the use of natural substances of botanical origin might be a promising tool. The few references, available to date, reporting on the algicidal activities of botanicals are as follows.

Anthraquinones are aromatic organic compounds (Dave and Ledwani, 2012) and potential selective algicides, for which structural modifications are employed to increase their solubility in water and thus their activity (Nanayakkara and Schrader KK, 2008). Moreover, Ateeque et al. (2013) have reported on the allelopathetic activities of momilactones, identified in rice straw, against *M. aeruginosa* at 10ppm. Similarly, oleioyl-*β*-d-arabinoside, a compound isolated from a methanol extract of *Oryza sativa* straw was found to exhibit strong growth inhibition (92.6 ± 0.3%) against *Microcystis aeruginosa* UTEX 2388 at 100 ppm (mg/L) (Ahmad et al., 2013). *Ceratophyllum demersum* and *Vallisneria spiralis* are two aquatic macrophytes and their essential oils, rich in phthalates, lipids and terpenoids were proven of growth inhibitory activity to the cyanobacterium *Microcystin aeruginosa* (Xian et al., 2006). Furthermore, *β*-cyclocitral is a terpenoid exhibiting a characteristic lytic activity on cyanobacteria *Microcystis*. This volatile compound was found to be derived from the cyanobacteria when in co-culturing with the Gram-positive bacteria *Brevibacillus* sp. (Ozaki et al., 2008). In addition, urocanic acid a bacteria-derived algicidal compound, eliminated the cyanobacteria *Skeletonema costatum*, *Prorocentrum donghaiense* and *Heterosigma akashiwo* (Zhao, et al., 2014). Tingenone is a pentacyclic triterpene isolated from the botanical species *Maytenus gonoclada* exhibiting activity on

Microcystis novacekii and the median effective concentration was calculated at 12.2 μg L^{-1}, thus indicating that tingenone can be potentially applied in water management for public supply, replacing synthetic algicides (Silva et al., 2013). Structure activity relationship studies revealed that fatty-acids are a chemical group of substances exhibiting different algicidal activity based on their molecular surface area and shape (Huang et al., 2014). On the other hand, compounds carrying at least one carboxyl group exhibit algicidal activity as proven for some pinewood-derived biochar water-extracted substances that were found toxic to blue-green algae (cyanobacteria *Synechococcus*) (Smith et al., 2013). Anthraquinones are substances of algicidal activity against the cyanobacterium *Oscillatoria perornata* (Skuja), one of the major causes of musty off-flavor in farm-raised catfish in Mississippi (Schrader et al., 2003).

The essential oils (EOs) have a long history of human use, are easy to prepare (Isman and Grieneisen, 2014) and are included in a list of 31 compounds categorized as minimum risk, thus exempted from federal registration requirements in the US (Gillilan 2012). The EOs, distilled from Mediterranean aromatic plants, have a vast range of biological activities (Bakkali et al., 2008) but to the best of our knowledge this is the first report on their algicidal activity. However, their labile and volatile nature reduces the EOs activity under open air or long term storage conditions. Microencapsulation is a process of entrapping EOs within a shell like silica capsules or coating for controlling the release of the active ingredients (Sousa et al., 2014). Controlled release may be defined as a technique or method by which active chemicals are made available to a specified target at a rate and duration designed to accomplish an intended effect (Kenawy, et al.,1992). Microencapsulation is widely practiced industrially and has found use in many applications like drug delivery systems as well as controlled release of pesticides, flavors and other bioactives (Lachman, et al, 1976).

The scope of this study was the identification of new natural substances of botanical origin with algicidal growth inhibition properties against *A. gracile*. In specific we performed 1) the *in vitro* study of the algicidal activity of 11 essential oils on *A. gracile* by spectrophotometric estimation of chlorophyll content and transformation to carbon biomass after one day of algae immersion in test solutions, 2) the phytochemical composition analyses of the bioactive essential oils and 3) the *in vitro* study of the algicidal activity of the secondary metabolites (*E*)-anethole, 1,8-cineol,

linalool and carvacrol as major components of the most active EOs and comparison with other phytochemicals' activity, 4) the half-life calculation of the best active EO component, namely (E)-anethole and 4) the use of (E)-anethole to produce a formulated a slow release product by entrapping the active ingredient in polyurea microcapsules and test against *A. gracile*. This is the first report on the use of EOs and constituent terpens as alternative algicides.

I. 2. Materials and Methods

I. 2. a. Plant materials

The aromatic species used in this study were collected at the flowering stage from various locations in Greece. They were dried in the absence of light, at room temperature, and they were stored in sealed paper bags. Voucher specimens were deposited in the Department of Ecology, School of Biology Aristotle University of Thessaloniki, Greece.

Filamentous cyanobacterium *A. gracile* Lemmermann 1907, strain SAG 31.79 was obtained from the culture collection of algae (Sammlung von Algenkulturen (SAG)) at the University of Göttingen, Germany. This strain is not known for toxin production, and no cylindrospermopsin was detected when concentrations were measured using an ELISA test (96 well-plate, ABRAXIS, Warminster, PA, USA). The mean length of *A. gracile* filaments used in tests was 254.1 ± 146.1 μm (mean ± SD). The strain was cultivated in a walk-in phytotron (Conviron; Winniped, Canada, temperature: 20°C, light: PAR 44 μmol quanta m^{-2} s^{-1}, 16:8 h light-dark cycle) in a 2-L chemostats on WC medium (Guillard and Lorenzen 1972) pumped using the peristaltic pump (BVP Standard, Ismatec) at speed allowing maintain of exponential growth phase of the cyanobacteria.

I. 2. b. Bioassays of test compounds.

The possible toxic effects of the EOs and their terpene components on *A. gracile* were determined by immersing *A. gracile* in test solutions for one day and then quantifying the impact on photosynthesis by making a spectrometric estimation of chlorophyll a concentration at 750nm. Stock solutions were made in dimethyl sulfoxide and then brought to volume with deionized water. Working solutions were prepared at the concentrations of 5 to 500 μg mL^{-1} and 2 to 200 μg mL^{-1}, for essential oils and pure

compounds respectively. Working solutions were then micro-pipetted into wells (100μL solution per well) in 96-well polystyrene microplates (Greiner bio-one) containing 100μL of cyanobacterial culture material from continuous cultures. Deionized water was added to control wells. Final test concentrations in test solutions were 25 to 250 and 1 to 100 μg mL^{-1}, for essential oils and pure compounds respectively. Six replications were used for each treatment, while bioassays were performed in time twice. The plates were covered to prevent evaporation and were maintained in continuous light at 25^{o}C. Before measurement of absorbance the plates were agitated for 1 min. Optical densities of each well were measured at 750 nm. Absorbance values were transformed to carbon content (SLAWEK). Mean values and standard deviations of carbon content were calculated and used to compute the IC_{50} values, that is the test concentration degreasing to half the carbon content of algae immersed in the water control.

I. 2. c. Preparation of microcapsules

The microencapsulated geraniol was prepared according to methods described before (Michaelakis, et al., 2005.; Michaelakis, et al., 2006). For the preparation of polyurea microcapsules containing the non polar organic compound (geraniol) in a two phase suspension system formed by a nonpolar organic solvent and water. As complementary monomers are used a polyfunctional amine (e.g. DETA) and a di- or tri-isocyanate (e.g. TDI). The choice of the amine versus alcohol as the water soluble monomer is based on the rapid reaction (without the need of use of any toxic catalyst e.g. organic metallic salts) of the amino- groups with isocyanates leading to the formation of polyurea polymers. Non polar organic solvents with b.p.>100 ^{o}C (e.g. octane) may be used as the organic media in order to avoid evaporation of the solvent with subsequent distraction of the polymeric membrane. The microcapsules prepared had a rough rather than spherical surface as it was observed by Scan Electron Microscopy. Their stability in open-air or high vacuum conditions (10^{-6} mmHg) was also confirmed.

I. 2. d. Gas chromatography-Mass Spectrometry (GC/MS)

The chromatographic separation of EOs for component identification purposes was performed on an Agilent Technologies 6850 gas chromatograph coupled with a mass detector 5973 and a 7683B Series Injector autosampler, and the injection was

performed in splitless mode. The resulting data was elaborated using MSD ChemStation. The column was a Thermo Scientific TRACE TR-5 5% phenylmethylpolysyloxane (30 m x 0.25 mm; film thickness 0.25 μm). Injector temperature was kept at 250 °C. The oven temperature was programmed as follows: from 50 to 230 °C (5 °C/min in 36 min) and kept at this temperature for 2 min. The carrier gas was helium with a flow of 1 mL/ min; and 1 μL of the sample was injected. The sample was prepared by diluting 1 μL of EO with 1 mL of hexane. The mass detector settings were as follow: ionization voltage, 70eV; scan rate, 2.91 scan/s; mass range, 50-550; transfer line, 230 °C. The components of the EOs were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against NIST98, as well as retention indices as calculated according to Kovats, for alkanes C9-C24 compared with those reported by Adams (2007). Quantitative analysis of each component was carried out with an external standard method when available.

The residual life of (*E*)-anethole in time was studied after 1, 2, 4, 6, 12 and 24 hour exposure of test solution to open air conditions. Analysis was performed as discussed before.

I. 3. Statistical Analyisis

Treatments of *A. gracile* with EOs and single test compounds were replicated six times, and each experiment was performed twice. The carbon content of the treated *A. gracile* in the microwell assays was expressed as % content of the natural carbon content in *A. gracile* immersed in the water-control. Data were analyzed (ANOVA) combined over time. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of *A. gracile* carbon content treated with EO and pure compounds were subjected to nonlinear regression analysis using the log-logistic equation proposed by Seefeldt et al. (1995): $Y = C + (D - C)/\{1 + \exp[b (\log(x) - \log(IC_{50}))]\}$, where C = the lower limit, D = the upper limit, b = the slope at the test compound (s), and IC_{50} = the test compound (s) concentration required for 50% decrease in carbon content of *A. gracile* after elimination of the carbon content in the control (water). In the regression equation, the EO or pure compound concentration (μg/mL) was the independent variable (x) and the treatment carbon content (percentage decrease over water control)

was the dependent variable (y). The mean value of the six replicates per essential oil or pure compound concentration and immersion period was used to calculate the IC_{50} value.

I. 4. Results and Discussion

The aromatic plants yielded essential oils as reported in **Table 1.** The chemical composition analysis is reported in **Table 2** where the components are listed in order of elution from the Thermo Scientific TRACE TR-5. The botanical species, compound retention index and percent composition of the oils are presented. Ninety-four constituents were identified in total, accounting for 84.14 to 99.47 % of the total area in EOs. All EOs yielded mainly monoterpenoids and the highest percentages (> 94.1 %) were recorded for *C. sinensis* (10 compounds yielding 97.28%), *S. officinalis* (14 compounds yielding 98.46%), *O. vulgare* (13 compounds yielding 95.32%), *O. dictamnus* (15 compounds yielding 96.32%) and *L.angustifolia* (15 compounds yielding 94.1%). On the other hand, sesquiterpenoids yielded less than 3.13 % of the EOs total synthesis, with the exceptions of *O. basilicum* (6 compounds yielding 13.15%), *E. meliodora* (9 compounds yielding 24.14%) and *M. officinalis* (13 compounds yielding 30.00%). Last *P. anisum, O. basilicum* and *M. officinalis* yielded also phenylpropanes at the percentages of 98.97, 12.93 and 13.51 %, respectively. The major compounds in the oils, supposed those of more than 50 % of total synthesis, were limonene (91.8% in *C. sinensis*), 1,8-cineole (56.14% in *L. nobilis* & 58.16 % in *S. officinalis*), linalool (51.69% in *O. basilicum*), carvacrol (88.37 and 56.22% in *O. vulgare* and *O. dictamnus* respectively) and (E)-anethole (97.36% in *P. anisum*). The EOs and the single major compounds of the most active EOs were screened against *A. gracile* in 96 well microtiter assays, and the chlorophyll was estimated spectrometric ally. Chlorophyll absorbance values were transformed to carbon content and were used for IC_{50} values calculation. Our results indicate that most of the essential oils used for this study as well as some of their terpene components have high potential for use as alternative algicides against *A. gracile*. In specific, the essential oil of *O. vulgare* appeared as the most promising (IC_{50} = 168.43 μg mL^{-1}) followed by *O. dictamnus*, *O. basilicum*, *E. meliodora, M. officinalis* and *P. anisum* in a discending order (**Table 3**). When we tested the major components of the algicidal EOs individually, that is 1,8-cineole, linalool, carvacrol and (*E*)-anethole, we found that (*E*)-anethole was the most active terpenoid, exhibiting an IC_{50} value of 71.35 μg mL^{-1}.

When (*E*)-anethole was enclosed in the microcapsules the product had a concentration of 0.02% (w/w) and if applied at 500 μg/mL it exhibited 97% of activity after one day of *A. gracile* immersion in test solutions. This concentration of the formulated product in water corresponds to 13 μg/mL of active – ingredient-(*E*)-anethole in test solution which is a rather lower than the $IC_{50/1d}$ value calculated for the (*E*)-anethole used without microencapsulation. When the fate in time of (*E*)-anethole at ??? μg/mL was studied we found that after 2 hours there was 100% decomposition. This fact coheres to the fact that the activity of (*E*)-anethole on *A. gracile* is even faster than one day, used as assessment timing in our case. Thus microencapsulation procedure enhances the residual life under open air conditions and explains the enhanced activity after 1 day of *A. gracile* immersion in test solutions.

Moreover other phytochemicals were used for comparison and according to our results the descending order of activity was: hexadecanoic acid, acetic acid, decadienal and geraniol with respective IC_{50} values of 17.70, 29.91, 45.32 and 160.10 μg mL^{-1}. Interestingly 1,8-cineole, linalool and carvacrol did not achive 50% activity when tested up to 100 μg/mL, regardless of the fact that the EOs in which they are contained at high concentrations were active against *A. gracile* (**Tables 3 and 4**). Kobaisy and co-workers found that constituents of the EO of *Hibiscus cannabinus*, were of no activity on another cyanobacteria species, *Oscillatoria perornata*. In specific n-nonanal, benzene acetaldehyde, (*E*)-2-hexenal and 5-methyl furfural were not found active at concentrations lower than 250 μg mL^{-1}, which fact agrees to our results (Kobaisy et al., 2001). *Callicarpa americana* EO demonstrated selective activity against *Oscillatoria agardhii* at 285 μg mL^{-1} but it was not found active on the green algae *Selenastrum carpicornutum* (Tellez et al., 2000). Purcado and co-workers have reported on natural-product-based coumarins and alkaloids found in the ethyl acetate extract of the roots of *Swinglea glutinosa* (Purcado et al., 2009). According to our results, hexadecanoic and acetic acid were the most active compounds against *A. gracile*, and their EC_{50} values were calculated at 17.67 and 29.91 μg mL^{-1}.

According to the above, the activity of the EOs of *Origanum vulgare, Origanum dictamnus, Ocimum basilicum, Eucalyptus meliodora, Melissa officinalis* and *Pimpinella anisum* and (*E*)-anethole used in the present study is high and they can be of use under open air conditions if their residual life under open conditions permits

efficacy. On this bases, there could be produced microencapsulated formulates like the one we produced for (*E*)-anethole. Ongoing experiments are performed to study the residual life of (*E*)-anethole in the formulate, it's effect on acquatic organisms and the enclosure of EOs in similar matrixes rather than pure compounds.

Acknowledgements

The development of slow release formulations of essential oils employing polyurea microcapsules is a patent (No GR1008453, 2015)

Table 1. Yield of Essential Oil from the Eleven Aromatic Species

Plant species	Classification	Plant part used for water distillation	Yield (mL 100 g^{-1} dw)[a]
Ocimum basilicum	Lamiaceae	aerial part	0.82 ± 0.01
Origanum vulgare	Lamiaceae	aerial part	2.98 ± 0.12
Origanum dictamnus	Lamiaceae	aerial part	1.11 ± 0.08
Melissa officinalis	Lamiaceae	aerial part	0.20 ± 0.04
Eucalyptus meliodora	Myrtaceae	leaves	0.81 ± 0.03
Pimpinella anisum	Apiaceae	seeds	0.50 ± 0.04
Lavandula angustifolia	Lamiaceae	flowers	0.98 ± 0.12
Laurus nobilis	Lauraceae	leaves	2.10 ± 0.02
Rosmarinus officinalis	Lamiaceae	aerial part	0.21 ± 0.02
Citrus sinensis	Rutaceae	peel	0.45 ± 0.02
Salvia officinalis	Lamiaceae	aerial part	3.11 ± 0.10

[a] EO yields (%), based on dry materials, expressed as average values of three replications with relative standard deviations.

Table 3. In vitro IC_{50} Values (μg/ml) of Essential Oil on *Aphanizomenon gracile* with Respective Standard Error and Confidence Interval Values

	IC_{50} (μg/ml)	Std error	95% Conf Int
Origanum vulgare	168.43	20.03	126.35 - 210.51
Origanum dictamnus	174.48	17.20	138.33 - 210.62
Ocimum basilicum	182.03	16.90	146.53 - 217.53
Eucalyptus meliodora	208.39	18.71	169.06 - 247.71
Melissa officinalis	210.37	16.06	176.64 - 244.11
Pimpinella anisum	241.97	3.60	234.41 - 249.52
Lavandula angustifolia	> 250.00	-	-
Laurus nobilis	> 250.00	-	-
Rosmarinus officinalis	> 250.00	-	-
Citrus cinensis	> 250.00	-	-
Salvia officinalis	> 250.00	-	-

Table 4. In vitro EC_{50} Values (μg/ml) of Pure Substances on *Aphanizomenon gracile* with Respective Standard Error and Confidence Interval Values

	IC_{50} (μg/ml)	Std error	95% Conf Int
hexadecanoic acid	17.70	0.57	16.49- 18.91
acetic acid	29.91	5.22	18.94 - 40.89
decadienal	45.32	6.33	32.02- 58.63
anethole	71.35	5.05	60.70- 81.97
decenal	>100.00	-	-
furfural	>100.00	-	-
undecanone	>100.00	-	-
1,8-cineole	>100.00	-	-
linalool	>100.00	-	-
carvacrol	>100.00	-	-

Table 2. Chemical Composition and Percent Content of the Essential Oil Components of the anti-algae Essential Oils.

no.	Compound name in order of elution[b]	RI[c]	*P. anisum*	*C. cinensis*	*L. nobilis*	*S. officinalis*	*O. basilicum*	*R. officinalis*	*O. vulgare*	*O. dictamnus*	*L. angustifolia*	*E. meliodora*	*M. officinalis*
Monoterpenoids													
1	α-thujene	930	-	-	-	-	-	-	-	0.39	-	-	-
2	α-pinene	938	-	0.2	3.56	7.3	-	16.74	-	0.57	0.25	-	-
3	camphene	955	-	-	-	4.86	-	1.72	-	-	0.4	-	-
4	sabinene	977	-	0.26	4.96	-	-	-	-	-	-	-	0.54
5	*β*-pinene	982	-	-	5.59	2.51	0.41	0.23	-	-	-	-	1.39
6	*β*-myrcene	991	-	0.26	-	4.17	-	0.88	-	0.75	-	-	-
7	*δ*-3-carene	1011	-	0.89	-	-	-	1.23	-	-	-	-	-
8	α-terpinene	1020	-	-	0.19	0.22	-	-	-	1.77	-	-	-
9	*δ*-4-carene	1020	-	-	-	-	-	0.4	-	-	-	-	-
10	*p*-cymene	1030	-	-	0.32	1.02	0.83	1.35	0.98	17.91	0.32	14.55	1.8
11	limonene	1033	0.37	91.8	-	-	-	-	0.94	-	-	-	2.55
12	1,8-cineole	1040	-	-	56.14	58.16	10.71	21.89	2.3	-	23.38	6.84	-
13	moslene	1063	-	-	0.52	-	-	-	-	-	-	-	-
14	*γ*-terpinene	1063	-	-	-	-	-	0.25	0.55	13.72	-	0.25	0.38
15	*cis*-linalool oxide	1075	-	-	-	-	-	-	-	-	10.00	0.36	-
16	*cis*-sabinene hydrate	1076	-	-	0.41	-	-	-	0.25	0.41	-	-	0.61
17	terpinolene	1090	-	-	0.26	-	-	0.58	-	-	-	-	-
18	*trans*-linalool oxide	1092	-	-	-	-	-	-	-	-	-	0.36	-

Table 2. *(Continued)*

19	linalool	1100	0.18	1.6	5.92	0.39	51.69	3.91	0.52	2.5	27.83	2.28	0.49
20	(Z)-thujone	1112	-	-	-	3.64	-	-	-	-	-	-	-
21	*trans*-2-menthenol	1134	-	-	-	-	-	-	-	-	-	1.5	1.77
22	*trans*-pinocarveol	1152	-	-	-	-	-	-	-	-	-	-	1.86
23	neo-isopulegol	1153	-	-	-	-	-	-	-	-	-	1.34	-
24	citronellal	1157	-	0.26	-	-	-	-	-	-	-	-	-
25	(-)-camphor	1159	-	-	-	12.43	0.32	19.64	-	-	15.37	-	-
26	lavandulol	1168	-	-	-	-	-	-	-	-	0.58	-	-
27	pinocarvone	1173	-	-	-	-	-	-	-	-	-	-	1.38
28	menthone	1177	-	-	-	-	-	-	0.13	-	-	-	-
29	borneol	1183	-	-	-	1.19	0.42	8.09	0.21	0.24	11.01	-	0.66
30	(-)-4-terpineol	1190	-	0.25	2.29	0.47	-	-	0.44	0.89	1.74	4.97	6.13
31	cryptone	1203	-	-	-	-	-	-	-	-	-	14.66	-
32	α-terpineol	1204	-	0.52	2.7	1.77	1.37	2.57	-	-	-	3.06	-
33	myrtenol	1206	-	-	-	-	-	-	-	-	-	-	3.34
34	verbenone	1219	-	-	-	-	-	8.62	-	-	-	-	0.59
35	*trans*-piperitol	1220	-	-	-	-	-	-	-	-	-	0.64	-
36	bornyl formate	1241	-	-	-	-	-	-	-	-	0.41	-	-
37	thymol methyl ether	1244	-	-	-	-	-	-	0.16	0.21	-	-	-
38	neral	1244	-	-	-	-	-	-	-	-	-	-	1.35
39	linalool formate	1250	-	-	-	-	-	-	-	-	1.64	-	-
40	*p*-cumin aldehyde	1256	-	-	-	-	-	-	-	-	0.61	7.32	3.13
41	pulegone	1263	-	-	-	-	-	-	0.36	0.18	-	-	0.84

Table 2. *(Continued)*

42	thymoquinone	1260	-	-	-	-	-	-	-	0.28	-	-	-
43	piperitone	1268	-	-	-	-	-	-	-	-	-	0.61	-
44	(*E*)-neral	1273	-	1.24	-	-	-	-	-	0.28	-	-	2.05
45	*trans*-sabinil acetate	1274	-	-	0.18	-	-	-	-	-	-	-	-
46	bornyl acetate	1293	-	-	0.36	-	-	0.97	-	-	0.19	-	-
47	isobornyl acetate	1293	-	-	-	0.33	0.77	-	-	-	-	-	-
48	phellandral	1294	-	-	-	-	-	-	-	-	-	2.47	-
49	carvacrol	1306	-	-	-	-	-	-	88.37	56.22	-	5.39	6.88
50	methyl geranate	1323	-	-	-	-	-	-	-	-	-	-	0.41
51	carvacrol acetate	1370	-	-	-	-	-	-	0.11	-	-	-	-
52	geranyl acetate	1377	-	-	-	-	-	-	-	-	0.37	-	0.68
53	geranyl acetone	1452	-	-	-	-	-	-	-	-	-	-	0.47
	Total		**0.55**	**97.28**	**83.4**	**98.46**	**66.52**	**89.07**	**95.32**	**96.32**	**94.1**	**66.6**	**39.30**
Sesquiterpenoids													
54	*δ*-elemene	1320		-	0.48	-	-	-	-	-	-	-	-
55	α-cubebene	1358		-	-	-	-	-	-	0.24	-	-	-
56	copaene	1390		-	-	-	-	0.28	-	0.74	-	-	-
57	*β*-bourbonene	1400		-	-	-	-	-	-	-	-	-	0.6
58	*β*-elemene	1400		-	-	-	1.87	-	-	-	-	-	-
59	caryophyllene	1440		-	-	0.65	-	0.68	0.91	1.03	-	0.49	3.34
60	α-bergamotene	1444		-	-	-	5.79	-			-	-	-
61	α-guaiene	1450		-	-	-	0.73	-	-	-	-	-	-
62	(*Z*)-*β*-farnesene	1458		-	-	-	-	-	-	-	-	1.67	-

Table 2. *(Continued)*

63	humulene	1477		-	-	0.15	0.42	0.13	0.14	-	-	-	0.62
64	allo aromadendrene	1485		-	-	-	-	-	-	-	-	1.25	0.67
65	germacrene D	1491		-	-	-	-	-	0.08	-	-	-	0.54
66	α-curcumene	1492	0.12	-	-	-	-	-	-	-	-	-	-
67	(+)-epi-bicyclo sesqui-phellandrene	1501		-	-	-	2.53	-	-	-	-	-	-
68	*β*-bisabolene	1518		-	-	-	-	-	1.18	0.23	-	-	-
69	bicyclo germacrene	1519		-	-	-	-	-	-	-	-	1.29	-
70	*δ*-cadinene	1533		-	-	-	1.81	0.23	0.34	0.17	-	-	1.04
71	*trans*-nerolidol	1567		-	-	-	-	-	-	-	-		0.52
72	elemol	1568		-	-	-	-	-	-	-	-	0.37	-
73	spathulenol	1608		-	-	-	-	-	-	-	-	15.85	1.17
74	carophyllene oxide	1610		-	0.26	-	-	-	0.48	0.48	0.7	2.55	17.27
75	iso aromadendrene epoxide	1651		-	-	-	-	-	-	-	-	-	0.36
76	isospathulenol	1658		-	-	-	-	-	-	-	-	0.47	-
77	α-caryophyll -adienol	1664		-	-	-	-	-	-	-	-	-	3.07
78	ledene oxide-(II)	1697		-	-	-	-	-	-	-	-	0.2	-
79	α-bisabolol	1703		-	-	-	-	-	-	-	0.25	-	-
80	eudesma-4,11-dien-2-ol	1717		-	-	-	-	-	-	-	-	-	0.33
81	farnesyl acetate	1822	0.33	-	-	-	-	-	-	-	-	-	-

Table 2. *(Continued)*

82	perhydrofarnesyl acetone	1843		-	-	-	-	-	-	-	-	-	0.47
	Total		**0.45**	**0.0**	**0.74**	**0.8**	**13.15**	**1.32**	**3.13**	**2.89**	**0.95**	**24.14**	**30.0**
Phenylpropanoids													
83	estragole	1205	0.91	-	-	-	-	-	-	-	-	-	-
84	(*E*)-anethole	1304	97.36	-	-	-	-	-	-	-	-	-	13.51
85	eugenol	1361		-	-	-	12.93	-	-	-	-	-	-
	total		**98.27**	**0**	**0**	**0**	**12.93**	**0**	**0**	**0**	**0**	**0**	**13.51**
Others													
86	octanal	1005		0.42	-	-	-	-	-	-	-	-	-
87	decanal	1209		0.34	-	-	-	-	-	-	-	-	-
88	*p*-cumenol	1234		-	-	-	-	-	-	-	-	0.83	-
89	*p*-anisaldehyde	1272	0.2	-	-	-	-	-	-	-	-	-	-
90	*β*-(*E*)-damascenone	1389		-	-	-	-	-	-	-	-	-	0.86
91	*β*-(*E*)-ionone	1491		-	-	-	-	-	-	-	-	-	0.71
92	pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	1593		-	-	-	-	-	-	-	-	-	0.92
93	diisobutyl phthalate	1868		-	-	-	-	-	-	-	-	-	1.01
94	epimanoyl oxide	2035		-	-	-	-	-	-	-	-	-	0.3
	total		**0.2**	**0.76**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.83**	**3.80**
	final total		**99.47**	**98.04**	**84.14**	**99.26**	**92.60**	**90.39**	**98.45**	**99.21**	**95.05**	**91.57**	**86.61**

[a] Mean value of three determinations (three replicates) calculated from GC-MS areas; t (trace), relative content <0.1%; (-), not detected. [b] Compounds are listed in order of elution from a Thermo Scientific TRACE TR-5 capillary column. Identification by comparison of mass spectra with the respective data of NIST and Willey (30:70) libraries in total ion current (TIC) and the literature, as well as retention indices as calculated according to Kovats (1978) for alkanes C9-C24 compared with the ones reported by Adams (21). [c] Retention indices on a Thermo Scientific TRACE TR-5 capillary column.

I. 5. References

Jonas A, Scholz S, Fetter E, Sychrova E, Novakova K, Ortmann J, Benisek M, Adamovsky O, Giesy JP, Hilscherova K, Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular in vitro and zebrafish embryos assays. *Chemosphere* **2015**, *120*, 321–327.

Kokociński M, Mankiewicz-Boczek J, Jurczak T, Spoof L, Meriluoto J, Rejmonczyk E, Hautala H, Vehniäinen M, Pawełczyk J, Soininen J, *Aphanizomenon gracile* (Nostocales), a cylindrospermopsin-producing cyanobacterium in Polish lakes. *Environ Sci Pollut* R **2013**, *20*, 5243-5264.

Nováková K, Bláha L, Babica P, Tumor promoting effects of cyanobacterial extracts are potentiated by anthropogenic contaminants - Evidence from in vitro study. *Chemosphere* **2012**, *89*, 30-37.

Ledreux A, Thomazeau S, Catherine A, Duval C, Yéprémian C, Marie A, Bernard C, Evidence for saxitoxins production by the cyanobacterium *Aphanizomenon gracile* in a French recreational water body. *Harmful Algae* **2010**, *10*, 88-97

Li L, Guo H, Shao C, Yu S, Yin D, Gao N, Lu N, Effect of algal organic matter (AOM) extracted from *Microcystis aeruginosa* on photo-degradation of Diuron. *Chem. Eng. J.* **2015**, *281*, 265-271.

Newman J, All choked up. Planet Earth. Issue SUMMER, June 2013, Pages 30-31.

Ahmad A, Kim SH, Ali M, Park I, Kim JS, Kim EH, Lim JJ, Kim SK, Chung IM, New chemical constituents from oryza sativa straw and their algicidal activities against blue-green algae. *J Agric Food Chem* **2013**, *61*, 8039-8048.

Smith CR, Buzan EM, Lee JW, Potential impact of biochar water-extractable substances on environmental sustainability. *ACS Sustain Chem Eng*. **2013**, *1*, 118-126.

Rajasekhar P, Fan L, Nguyen T, Roddick F, A. A review of the use of sonication to control cyanobacterial blooms. *Water Res.* **2012**, *46*, 4319-4329.

Osman MEH, El-Sheekh MM, El-Naggar AH, Gheda SF, Effect of two species of cyanobacteria as biofertilizers on some metabolic activities, growth, and yield of pea plant. *Biol. Fert. Soils*. **2010**, *46*, 861-875.

Xian Q, Chen H, Zou H, Yin D, Allelopathic activity of volatile substance from submerged macrophytes on *Microcystin aeruginosa*. *Acta Ecol. Sin*. **2006**, *26*, 3549-3554.

Ozaki K, Ohta A, Iwata C, Horikawa A, Tsuji K, Ito E, Ikai Y, Harada KI, Lysis of cyanobacteria with volatile organic compounds. *Chemosphere*. **2008**, *71*, 1531-1538.

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CE, Antunes MB, de Melo Filho DA, Lyra TM, Barreto VST, Azevedo SMFO, Jarvis W , Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *New Engl. J. Med.* **1998**, *338*, 873–878.

Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreo VT, Ward CJ, Preiser W, Poon GK, Neild GH, Codd GA. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* **1998**, *352*, 21–36.

Zhao L, Chen L, Yin P, Algicidal metabolites produced by Bacillus sp. strain B1 against *Phaeocystis globose*. *J Ind Microbiol Biotechnol* **2014**, *41*, 593-599.

Leclercq DJJ, Howard CQ, Hobson P, Dickson S, Zander AC, Burch M. Controlling cyanobacteria with ultrasound. 43rd International Congress on Noise Control Engineering: Improving the World Through Noise Control, INTERNOISE 2014; Melbourne; Australia; 16 November 2014 through 19 November 2014; Code 110401. 2014. INTERNOISE 2014 - 43rd International Congress on Noise Control Engineering: Improving the World Through Noise Control

Huang H, Xiao X, Shi J, Chen Y, Structure-activity analysis of harmful algae inhibition by congeneric compounds: Case studies of fatty acids and thiazolidinediones. *Environ. Sci. Pollut. R.* **2014**, *21*, 7154-7164.

Silva FC, Guedes FAF, Franco MW, Barbosa FAR, Marra CA, Duarte LP, Silva GDF, Vieira-Filho SA, Algistatic effect of a quinonamethide triterpene on *Microcystis novacekii*. *J Appl. Phycol.* **2013**, *25*, 1723-1728.

Jonas A, Scholz S, Fetter E, Sychrova E, Novakova K, Ortmann J, Benisek M, Adamovsky O, Giesy JP, Hilscherova, K. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular in vitro and zebrafish embryos assays. *Chemosphere*. **2015**, *120*, 321-327.

De Figueiredo DR, Gonçalves AMM, Castro BB, Gonçalves F, Pereira MJ, Correia A. Differential inter-and intra-specific responses of *Aphanizomenon* strains to nutrient limitation and algal growth inhibition. *J Plankton Res.* **2011**, *33*, 1606-1616.

Adams RP, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed.; Allured: Carol Stream, IL,2007.

Puntener W, Manual for field trials. In Plant Protection, 2nd ed.; Ciba-Geigy: Basle, Switzerland, 1981; pp 205.

Seefeldt SS, Jensen, J. E.; Fuerst, E. P. Log-logistic analysis of herbicide rate response relationships. *Weed Technol.* **1995**, *9*, 218–227.

Schrader KK, Nanayakkara NP, Tucker CS, Rimando AM, Ganzera M, Schaneberg BT, Novel derivatives of 9,10-anthraquinone are selective algicides against the musty-odor cyanobacterium *Oscillatoria perornata*. *Appl Environ Microbiol*. **2003**, *69*, 5319-27.

Nanayakkara NP, Schrader KK, Synthesis of water-soluble 9,10-anthraquinone analogues with potent cyanobactericidal activity toward the musty-odor cyanobacterium *Oscillatoria perornata*. *J Agric Food Chem*. **2008**, *56*, 1002-1007.

Kobaisy M, Tellez MR, Webber CL, Dayan FE, Schrader KK, Wedge DE, Phytotoxic and fungitoxic activities of the essential oil of kenaf (*Hibiscus cannabinus* L.) leaves and its composition. *J Agric Food Chem*. **2001**, *49*, 3768-3771.

Ateeque A, Seung-Hyun K, Mohd Inmyoung P, Jin-Seog K, Eun-Hye K, Ju-Jin L, Seul-Ki K, Ill-Min C. New Chemical Constituents from *Oryza sativa* Straw and Their Algicidal Activities against Blue-Green Algae *J. Agric. Food Chem.*, **2013**, *61*, 8039–8048

Tellez MR, Dayan FE, Schrader KK, Wedge DE, Duke SO, Composition and Some Biological Activities of the Essential Oil of *Callicarpa americana* (L.) *Agric. Food Chem* **2000**, *48*, 3008–3012

Purcaro R, Schrader KK, Burandt C, DellaGreca M, Meepagala KM, Algicide constituents from *Swinglea glutinosa*. *J Agric Food Chem*. **2009**, *57*, 10632-10635.

Lachman L, Lieberman H, Kanig J, The Theory and Practice of Industrial Pharmacy; Lea and Febiger: London, 1976

Kenawy ER, Sherrington DC, Akelah A, Controlled release agrochemicals formulations: A review *Eur. Polym. J.* **1992**, *28*, 841-862.

Michaelakis A, Mihou AP, Couladouros EA, Zounos AK, Koliopoulos G, Oviposition Responces of *Culex pipiens* to a Synthetic Racemic *Culex quinquefasciatus* Oviposition Aggregation Pheromone. *J. Agric. Food Chem.* **2005**. 53, 5225-5229.

Michaelakis A, Mihou AP, Koliopoulos G, Couladouros EA. Attract-and-kill strategy. Laboratory studies on hatched larvae of *Culex pipiens*. *Pest Manag. Sci.* **2006**, 63, 954-959

Dave, H.; Ledwani, L. A review on anthraquinones isolated from Cassia species and their applications. *Indian J Nat Prod Resour*. **2012**, *3*, 291-319.

Isman MB, Michael L. Botanical insecticide research: many publications, limited useful data. Grieneisen, **2014**, *19*, 140–145.

Improvement of U.S. EPA minimum risk essential oils' pesticide activity through surfactant enhancement and synergy Gillilan, JoAnna, Ph.D., THE OHIO STATE UNIVERSITY, 2012, 178 pages; 3530136

Sousa FL, Santos M, Rocha SM, Trindade T, Encapsulation of essential oils in SiO2 microcapsules and release behavior of volatile compounds. *J Microencapsul.* **2014**, 31, 627-635.

Bakkalia F, Averbecka S, Averbecka D, Idaomarb M. Biological effects of essential oils – A review. *Food Chem Toxicol* **2008**, *46*, 446–475