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Nematicidal activity of the essential oil of three varieties of *Tagetes minuta* from Argentina

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Essential oils composition of three *Tagetes minuta* varieties and a wild population (WP) from Argentina and their nematicidal activity against root-knot nematode, *Meloidogyne javanica*, *in vitro* and *in vivo* are described. All *T. minuta* EOs tested were very active against nematode juveniles (J2), by the strongest nematicidal effects were exhibited by the *Tm*V3 variety oil, characterized by a high content of (E)-ocimenone. High nematode egg hatching suppression (> 90%) was induced by *Tm*V3 EO after five days of incubation. *In vivo* tests on tomato seedlings showed a significant reduction of infection rate of *M. javanica* J2 treated with *Tm*V3 and WP oils at sub lethal dose. Therefore, EOs from stables new varieties of *T. minuta* could be environmentally friendly nematicidal agents.

Keywords: Essential Oils, *Tagetes minuta*, Nematicidal effects, (E)-ocimenone.

Root-knot nematodes (*Meloidogyne spp*.) are major threats to agriculture worldwide. In the last decades, environmental and human health concerns have steadily reduced the availability of efficient commercial nematicides. Essential oils (EOs) from medicinal and aromatic plants (MAPs) constitute an important source of bioactive compounds and have been proposed as an environmentally friendly alternative to synthetic pesticides [1-2].

The plant *Tagetes minuta* L. (Asteraceae), is native to South and North America but has a worldwide distribution. It yields a high-grade essential oil used in food, nutraceutical, perfumery, ornamental, and pharmaceutical industries [3]. In agriculture, tagetes oil is known for its arthropod repellency and its weedicidal, anthelmintal, insecticidal, fungicidal, antiviral, and many other antimicrobicidal properties, piperitone, piperitenone, limonene caryophyllene, β-ocimene, tagetone and (Z)- and (E)-ocimenones, are among the major constituents of this [3-4]. South Africa and France are the main tagetes oil-producing countries. Demand for tagetes oil is significantly increasing as an ingredient for the food industry [3]. The diverse bioactivities associated with tagetes oil may be due to its high degree of chemodiversity. EOs composition of Argentinean *T. minuta* accessions has been characterized [5] and recently, *T. minuta* varieties have been obtained based on chemical composition [6]. Additionally insecticidal, acaricidal and other biological effects have been reported for Argentinean *T. minuta* essential oil [7-10].

In this study, we report on the *in vitro* and *in vivo* nematicidal activity against root-knot nematode, *Meloidogyne javanica* of EOs from three cultivated varieties and a wild population of *T. minuta* from Argentina. Additionally, we correlated the nematicidal effects with the chemical composition of these EOs.

Table 1 show the chemical composition of the essential oils extracted from three *T. minuta* varieties (TmV1, TmV2 and TmV3) and a wild population (WP). The main constituents of WP essential oil were (E)-ocimenone (43.5%) and (Z)-β-ocimene (42.4%). TmV1 oil showed (E)-ocimenone (30.7%) and dihydrotagetone (30.3%) as major components, while (E)-ocimenone was the major one in TmV2 and TmV3 EOs. As in other compositions reported for *T. minuta*, (cis / trans)-ocimenone, (cis / trans)-ocimene, dihydrotagetone and (cis / trans)-tagetone dominated the chemical profile of the oils [7, 11].

Table 1: Proportion of the main components of the EOs of three *Tagetes minuta* varieties (*Tm*V1, *Tm*V2, and *Tm*V3) and a wild population (WP), from [6].

Main components	Essential oils (%)			
	WP	*Tm*V1	*Tm*V2	*Tm*V3
Limonene	1.95	1.71	4.57	2.82
(Z)-β-ocimene	42.38	18.08	14.72	11.87
dihydrotagetone	3.45	30.34	0.42	0.24
(E)-tagetone	3.75	3.88	4.13	0.61
(Z)-tagetone	1.14	10.82	3.07	1.02
(Z)-ocimenone	3.86	4.45	13.73	10.8
(E)-ocimenone	43.47	30.73	59.37	72.64

The EOs studied here showed strong nematicidal effects, causing maximum *M. javanica* J2 mortality at 1 mg/mL after a 12, 48 and 72 h exposure (Table 2). The TmV3 EO was the most effective one with the lowest LC_{50} (0.14 mg/mL) and LC_{90} values (0.24 mg/mL), followed by TmV2. On the contrary, the TmV1 oil exhibited values of LC_{50} and LC_{90} higher than the WP EO.

Differences in bioactivity among *T. minuta* EOs has been attributed to variations in their chemical composition [4]. The more active oils studied here (TmV3 and TmV2) are characterized by a high content in (E)-ocimenone, especially TmV3 oil (72.6%). The insecticidal and phytotoxic potential of this terpene has been previously demonstrated [9, 12]. On the other hand, dihydrotagetone and (Z)-β-ocimene showed nematicidal activity against eggs and juveniles of

M. incognita [13]. To our knowledge, this is the first report on the nematicidal effect of an essential oil rich in (E)-ocimenone.

Table 2: Effects of *Tagetes minuta* EOs (1 mg/mL) of a wild population (WP) and three varieties (*Tm*V1, *Tm*V2, and *Tm*V3) from Argentina on mortality of *Meloidogyne javanica* second stage juveniles (J2).

Essential oil	J2 mortality (%)[a]				
	24h	48h	72h	LC_{50} mg/mL[b] (95% CL[c])	LC_{90} mg/mL[b] (95% CL[c])
WP	100.0	100.0	100.0	0.18 (0.18-0.19)	0.30 (0.30-0.31)
***Tm*V1**	100.0	100.0	100.0	0.26 (0.26-0.27)	0.45 (0.43-0.46)
***Tm*V2**	100.0	100.0	100.0	0.16 (0.16-0.17)	0.27 (0.26-0.27)
***Tm*V3**	100.0	100.0	100.0	0.14 (0.14-0.15)	0.24 (0.23-0.24)

[a] Corrected according to Scheider–Orelli's formula. Values are means of four replicates. [b] Mortality was observed 72 h after treatment. Five concentrations were used to obtain LC_{50} and LC_{90}, and four replicates were used for each treatment. [c] CL denotes confidence limit. Values are means of four replicates.

We selected the more active oil, *Tm*V3, and the wild WP oil to further assess their nematicidal activity. The egg hatchability test (Table 3) indicated that both *Tm*V3 and WP oils significantly inhibited hatching, with a final suppression rate of 41.0% and 48.7%, respectively. The most active oil was *Tm*V3, which strongly suppressed *M. javanica* egg hatching (94.0%) after five days of incubation. This effect decreased with time after the egg masses were immersed in water. These results confirm that J2 are more sensitive to the effects of EOs than egg masses [14].

Table 3: Effects of *Tagetes minuta* EOs on *Meloidogyne javanica* egg hatching over time.

Essential oil	Relative egg hatch suppression rate (% ± DE)[a] with time [b]				
	0	7	14	21	28
WP	79.7 ± 6.4	63.6 ± 13.5	56.6 ± 10.5	46.5 ± 11.2	41.0 ± 10.7
***Tm*V3**	94.8 ± 3.0	81.6 ± 4.4	63.9 ± 12.3	51.0 ± 17.9	48.7 ± 18.7

[a] Each value represents the hatch inhibition rate in the respective treatment corrected according to the control (Schneider-Orelli's formula). Values are means of four replicates. [b] time 0: after 5 days of immersion in test solutions; time 7 and subsequent times: number of days of immersion in water after time 0.

The *in vivo* tests on tomato seedlings showed a strong suppression of J2 root penetration when treated with *Tm*V3 and WP oils at sub lethal concentration (LC_{50}: 0.1 and 0.2 mg/mL, respectively) (Fig.1).

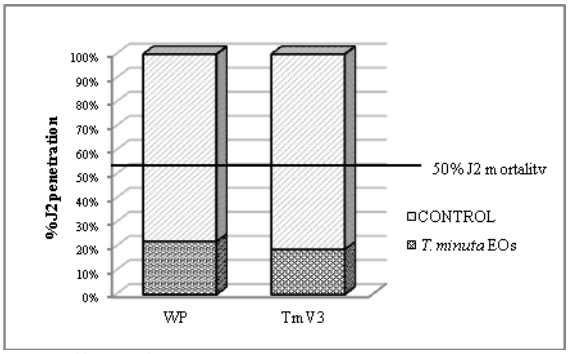

Figure 1: Effects of *Tagetes minuta* active EOs (at LC_{50} doses) on *Meloidogyne javanica* juvenile infection of *Solanum lycopersicum* root seedlings. Bars represent the penetration percentage of treated J2 with: WP at 0.2 mg/mL and *Tm*V3 at 0.1 mg/mL vs untreated J2: CONTROL.

Both treatments showed similar effects with a significant decrease of J2 root penetration (26.9 and 21.6% inhibition rate) respect to the control (inoculated with untreated J2). Therefore, *Tm*V3 and WP *T. minuta* oils at low concentration affected nematode behavior and strongly suppressed the infection capacity of *M. javanica* J2 on his host plant.

Root exudates of *T. minuta* have nematicidal effects [15-16] and the plants have been used in intercroping schemes [17], but this is the first report on the nematicidal activity of *T. minuta* essential oil against root-knot nematodes. EOs from the three varieties and the wild population of *T. minuta* were very active against *M. javanica*, but the strongest nematicidal effects were exhibited by the TmV3 oil, characterized by a high content of (E)-ocimenone. Domestication and cultivation of MAPs has become necessary in order to avoid the natural morphological and chemical variability of wild populations which decreases the predictability of their bioactivity [18-19]. The nematicidal activity of EOs from chemically stable new varieties of *T. minuta* could significantly increase its commercial value, determined by sustainable supply of considerably sufficient volumes and a high quality of the oil [4].

Experimental

Plant material and essential oil: Plants used for this study were three of *T. minuta* varieties: Aromisky (TmV1), Serrano (TmV2) and Don Monje (TmV3) and a wild population (WP) from Argentina [6]. The wild population and the variety plants were harvested and cultivated respectively in Córdoba, Argentina [6, 20]. The varieties were obtained by a classical plant breeding process for three generation evaluated in different places [6] and are registered in INASE (National Institute of Seeds from Argentina). The EO was obtained from samples of dried aerial parts (flowers and leaves). Each sample was hydrodistilled during one hour using a modify Clevenger-type apparatus (with extraction chamber separated). The EO was dried over anhydrous sodium sulfate and stored at 4-6 °C until its chemical analysis.

GC-MS analysis: As has previously described [6], EOs were analyzed by GC-MS using a PerkinElmer Clarus 600 gas chromatograph coupled to a Clarus 600 MS mass detector (electron ionization, 70 eV) and equipped with a 60 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Perkin Elmer DB5 MS dimethyl (95%)–diphenyl (5%). Working conditions were as follows: split ratio (1:150), injector temperature 250ºC, temperature of the transfer line connected to the mass spectrometer 200ºC, initial column temperature 60ºC, then heated to 240ºC at 5ºC min [6]. Components were identified on the basis of comparison or their mass spectra with reference spectra in the NIST MS 2.0 and Adams (2007) libraries. The concentrations of the seven main compounds of the EOs were calculated by integrating the chromatogram area.

Nematodes: *M. javanica* population was maintained on *Solanum lycopersicum* plants (var. Marmande) in pot cultures at 25 ºC, and 70% RH. Egg masses were handpicked from infected tomato roots and J2 were obtained from hatched eggs by incubating handpicked egg masses in a water suspension at 25 ºC for 24 h.

Bioassays

In vitro* effect *on juveniles: Test solution of each treatment was prepared with EOs diluted in a DMSO-Tween solution (0.5% Tween20 in DMSO). Test solution (5 µL) was added to each well of 96-well plate containing 100 nematodes in 95 µL of water. The initial concentration tested was of 1 mg/mL. Four control wells with water/DMSO-Tween were included in each experiment. All treatments were replicated four times. The plates were covered to prevent evaporation and maintained in darkness at 25 ºC. After 72 h, the dead J2 were counted. In addition, the most nematicidal EOs, with mortality rates > 90%, were further tested to assess J2 mortality after 24 and 48 h. The nematicidal activity is presented as percent dead J2 corrected according to Schneider-Orelli's formula. Dose-response experiments were carried out to calculate lethal concentration LC_{50} and LC_{90} with Probit analysis.

In vitro effect on egg mass hatching: Three egg masses, of uniform size, were washed with sterilized distilled water and transferred to a 96-well plate containing the test solutions. Egg masses placed in sterilized distilled water with 5% DMSO-Tween solution were used as controls. Each experiment consisted of four replicates of treatment and control. The plates were covered to prevent evaporation and incubated in darkness at 25 ºC. After five days the hatched J2 were counted and the test solutions were removed and wells with egg mass were washed and filled with sterilized distilled water. The eggs were monitored during four weeks, until hatching was complete in the control. Relative hatching percentages (compared with the control) were calculated.

Effect on juvenile infection capacity: Tomato seeds (susceptible variety, Marmande) were germinated, incubated in a growth chamber (25 ºC, 60% RH and 16 h photoperiod) for three weeks and transplanted into 5-cm diameter clay pots filled with 10 mL of quartz sand. The seedlings were individually inoculated with 180-200 J2 untreated (control) or treated with EOs of three cultivars and a wild population of *T. minuta* at a sublethal dose (LC_{50}) and incubated for one week at the same environmental conditions. The seedlings were then removed from the pots and the roots stained with acid fuchsine [21]. Juveniles within the roots of each individual seedling were counted by examining the entire root system under a stereomicroscope. The experiment consisted of six replicas and was repeated three times. Relative percentages of J2 penetration (treated J2 vs untreated J2) were calculated to obtain inhibition rates of J2 infectivity [14].

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