Article

Concentrations and application intervals of the essential oil of *Tagetes lucida* Cav. against *Nacobbus aberrans*

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Abstract

The scarce information on the effects of the essential oil of *T. lucida* against nematodes and the richness of native plant populations of this species in some localities at State of Mexico are favorable conditions to derive innocuous natural inputs that make it possible to face problems of galling by *N. aberrans* in the production of tomato. The objective of this study was to evaluate in greenhouse conditions the application of *T. lucida* oil from a natural population of Ixtapan of the Sal, State of Mexico in the formation of galls by *N. aberrans* in tomato seedlings. The essential oil was extracted by hydrodistillation at pilot level with dry weight yield of 0.4% (mL 100 g^{-1}) and analyzed by GC/EM identifying the following major compounds: geranyl acetate (40.8%), *β*-ocimene (15.1%), nerolidol (8.1%), β-cubebeno (5.1%) and caryophyllene (5.2%). Tomato seedlings in pot were inoculated with *N. aberrans* (10 mL kg^{-1} of substrate) and were dosed with oil concentrations from 0.01 to 10 mg mL^{-1}, as preventive and control treatments, in oil application intervals. 1, 2 and 3 weeks. Inhibition of root galling was consistent in the control (TC) treatment than in the preventive one. On TC, oil concentrations of 0.35 and 1 mg mL^{-1} produced 63 to 80% galling inhibition, and CL_{50} values of 0.06 mg mL^{-1} were obtained for intervals 1 and 2, and 0.13 mg mL^{-1} for the interval 3.

Keywords: *Tagetes lucida* Cav., *Nacobbus aberrans*, gill inhibition, oil concentration.

Reception date: January 2018
Acceptance date: March 2018

Introduction

Tomato is a vegetable that generates currency for Mexico (SIAP, 2014), it is also one of the hosts most affected by nematodes, among them *Nacobbus aberrans*, which cause yield losses of 36 to 55% in the greenhouse (Manzanilla *et al*., 2002) and from 50 to 100% in the field (Mendoza, 1999). Chemical control is the most used method against nematodes, but it induces resistance, in addition to eliminating natural enemies (Gutiérrez *et al*., 2013). On the other hand, nematicides are expensive and not easily accessible to small producers; consider that in the last decades the availability of efficient commercial products has been reduced (Sorribas and Ornat, 2011).

Given the economic importance of this vegetable and the consequences of using nematicides, alternatives are being explored such as biological control, genetic resistance of tomatoes and the use of vegetable substances. The use of aqueous extracts and essential oils, due to their cytotoxic properties, are potential inputs for the control of pests and diseases (Isman, 2000). In particular, essential oils have a promising use in the control of nematodes (Andrés *et al*., 2012).

Although there is great plant diversity in Mexico, the biological properties of several plants that could be useful in the control of nematodes are unknown (Silva *et al*., 2005). The most known botanical families for the presence of essential oils are: Asteraceae, Lamiaceae, Lauraceae, Labiateae, Myrtaceae, Poaceae, Rutaceae, Turaceae and Umbeliferae (Andrés *et al*., 2012), but Asteraceae is the most studied and recognized as a source of compounds with pesticide properties (Choi *et al*., 2003). Asteraceae represents 18.3% of the total species that make up the flora of Mexico (Villaseñor *et al*., 2005), in this family the *Tagetes* (*Tageteae*) stands out for its allelopathic potential against plant parasitic nematodes, documented for more than 75 years (Steiner, 1941).

In this regard, Tyler (1938) reports that 29 species of *Tagetes* are not attacked by *Meloidogyne* spp. Root-knot nematodes, because the excretions produced by the root, which reduce the incidence of nematodes in the soil, contain thiophenes, polyacetylene compounds that they are secondary metabolites responsible for the biological effect (Marotti *et al*., 2010). Due to these chemical properties of *Tagetes*, plants of this genus are used as an intercropped, imbricate, covert or rotating crop with other species of economic importance for nematode control (Serrato and Argomedo, 1993); On the other hand, the use of *Tagetes* essential oils as an applicable input against nematodes is being considered, since this natural resource is abundant in Mexico (Serrato, 2014). In this regard, there are several references to the effect of *Tagetes* essential oil on nematodes; For example, it is reported that *T. minuta* oil controls eggs and juveniles of *Meloidogyne incognita* (Adekunle *et al*., 2007), *T. erecta* has a toxic effect against populations of nematode eggs of *Haemonchus contortus* (Macedo *et al*., 2013) and the nematicidal effect of *T. zypaquirensis* oil was demonstrated (Álvarez *et al*., 2016).

In particular, *T. lucida* or pericon is a species that is widely distributed in Mexico, mainly in agricultural lands such as arvense or ruderal at elevations of 800 to 2 700 meters above sea level (Villareal, 2003). The essential oil of the pericon can be used as an insecticide in larvae of *Aedes aegypti* L. (Vera *et al*., 2014), coleoptera such as *Sitophilus zemais* (Nerio *et al*., 2009) and adults of *Tribolium castaneum* (Olivero *et al*., 2013), as an extract obtained with solvents, has an effect against bacterial agents that cause respiratory infections (Caceres *et al*., 1991) and gastrointestinal infections (Céspedes *et al*., 2006) and with effect on nematodes (Siddiqui and Alam, 1988; Omer *et al*., 2015).

These last two references are the only ones on the effect of substances extracted from *T. lucida* against nematodes such as *Meloidogyne incognita*, *Rotylenchulus reniformis*, *Tylenchorhynchus brassicae*, *Hoploimus indicus*, *Helicotylenchus indicus* and *Tylenchus filiformis*, but *T. lucida* oil has not been evaluated against this type of organisms. The chemical composition of the essential oil of *T. lucida* is recorded since 1938, estragole is the compound first referred to in the oil of this species (Anonymous 1938, cited by Visbal *et al*., 2010).

This secondary metabolite, of the group of phenylpropanoids, has been identified as a high percentage compound (96.8% and 97.3%) for populations of Cuba (Regalado *et al*., 2011) and Costa Rica (Cicció, 2004), respectively; however, in addition to estragole, other major phenylpropanoids are reported in pericon samples from Guatemala, such as anethole and methyl eugenol (Bicchi *et al*., 1997). The chemical characterization of this natural resource of Mexico has not been sufficiently explored (Serrato, 2014).

In the State of Mexico *T. lucida* is present in 22 municipalities (Discover Life, 2014), it is a ceremonial and useful plant in traditional medicine (García *et al*., 2012). Considering that there is little background on the activity of the oil of this species against nematodes and taking into account the natural availability of the pericon in that entity, the objective was to evaluate the toxic activity of the essential oil of a natural population of pericon in the formation of galls by *N. aberrans* in the greenhouse, in order to assess whether this local resource, in specific doses, influences the process of galling in the roots of tomato plants, in addition to specifying the sequence in which this vegetable input could be applied , methodological aspect little referred in toxicological evaluations of this type.

Materials and methods

Tagetes lucida Cav. In the the Joya 3 of Mayo colony Linda Vista of the Ixtapan of the Sal municipality, State of Mexico, coordinates 18º 49' 31'' north latitude and 99º 41' 18'' E, altitude 1 853 m climate Cw_1(w)i'g, in October 2014, floral stems of ruderal plants were collected for the extraction of essential oil. Seeds of this plant population were entered into the National Plant Germplasm Bank (BANGEV) in the Department of Plant Technology of the Autonomous University of Chapingo (UACH) (JZE-*Tateges*-001) and specimens were also sent to the Herbarium-Hortorio "Jorge Espinosa Salas" of the Department of Agricultural Preparatory, UACH.

Tomato (*Solanum lycopersicum* L.). For the corresponding tests, the Rio Grande variety of the Edena®, brand was used, a variety susceptible to nematodes. In unicel trays of 200 (2.5 x 2.5 x 6.5 cm) and as peat moss substrate; one seed was planted per cell on May 7, 2015, the transplant was made on June 3 of the same year in plastic pots with a capacity of 1 L. Potted plants were established in the greenhouse area of the Institute of Horticulture, UACH.

Nematode inoculum

From tomato plants established in greenhouses of the Tlapeaxco Production Unit, Irrigation Department, UACH, those infested for extraction of galls by *N. aberrans* were selected, from which inoculum was obtained by means of the methodology described by Castaño (1998), with modifications of Carrillo (Carrillo, FC Com. Per.) May 2015, Department of Agricultural Parasitology, UACH). The 10 mL kg^{-1} of substrate was used for the inoculation of nematodes in

the tomato plants in a pot. The inoculum was applied 4 days after the transplant (preventive treatment) and it was also applied five days later (control treatment, 24 h after the application of *T. lucida* oil).

Extraction of essential oil

The transfer of the flower stems from the collection point to the work area (Salvador Sánchez Colin Foundation Winery, CICTAMEX, SC Coatepec of Harinas, State of Mexico) for the distillation was carried out on the same day. For the extraction of oil at the pilot level, 50 kg of plant was used, which was cut into pieces of approximately 2 cm using a mechanical mincer; the chopped tissue was taken to a stainless-steel distiller with a capacity of 60 kg, the distillation handling sequence is described by Serrato *et al*. (2014). From the first drops in the condenser outlet tube, 1 h of distillation was allowed to pass, obtaining an essential oil volume of 80 mL in 50 kg of vegetable mass.

Chromatographic analysis of essential oils

The identification of the compounds was done by gas chromatography with mass detector (Adams, 2000), by means of a gas chromatograph CG 7890A (Agilent Technologies, USA) coupled to a selective mass detector 5975C Inert MSD with a triple axis detector (Agilent Technologies, USA), with electric impact ionization (IE) of 70 eV; an HP-5ms® column (California, USA), packed with 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm Ø x 0.25 μm) was used. The injector and detector temperatures were maintained at 250 °C and 280 °C, respectively, and were reached at a speed of 10 °C min^{-1}.

The temperature of the oven started at 70 °C, was maintained for 1 min and was programmed to reach the temperatures and speed previously indicated. The flow velocity of the carrier gas (helium) was maintained at 1 mL min^{-1}. Diluted samples (1/100) were injected in acetone (v/v) of 1 μL, manually in automatic "split" mode (to dilute) by means of a 7683D injector (Agilent Technologies, USA). The data of relative abundance of each compound were obtained from the total percentage of the area of all the chromatographic peaks and then dividing the area of each peak among the total area, the result multiplied by 100.

As major compounds were considered those with more than 5% relative abundance (Mora *et al*., 2009) and trace elements those with less than 5% relative abundance. The mass range detected was from 35 to 500 m/z. Four samples were processed and the identification of the components was performed by comparing the relative retention indexes, plus the mass spectra compared in the NIST 05 database of the GC-MS system (National Institute of Standard and Technology) and with the Spectral data published by Carol Stream Corp., USA (Adams, 2000).

Preparation and application of essential oil concentrations

From the pure oil, concentrations of 0.01, 0.035, 0.1, 0.35, 1, 3.5 and 10 mg mL^{-1} (0.001 to 1%) were made by means of subsequent dilutions. To facilitate dilutions of the oil in water, Tween® 20 at 0.1% was added, stirring it manually, generating an emulsion. In the case of the control, which corresponded to distilled water, Tween® 20 was also added. The application of the different concentrations obtained from the oil was punctual; that is, at the foot of the seedling and applying

50 mL seedling^{-1}. The treatments were applied in two ways: preventive (treatment-inoculum) and control (inoculum-treatment). For the first, the concentrations were dispensed at 96 h before inoculation and for the second, 96 h after inoculation. In each of these experimental scenarios, three different application intervals were implemented, at one, two and three weeks. In each interval, the treatments of essential oil and the control were applied.

Experimental design

For the establishment of the treatments (range of application and concentrations) in each application form (preventive and control) the design of divided plots was followed where: large plot corresponded to the intervals and small plot to the concentrations. The control and the seven treatments were repeated five times, the experimental unit was a pot per repetition.

Statistical analysis

The number of plants with root galls was a necessary variable to obtain the percentage of galling inhibition, which was obtained by means of the equation Abbott (1925), we used the analysis of variance of the percentage of inhibition using the GLM procedure of SAS (1999) and also used the comparison of means by the Tukey test ($p\leq 0.05$). The data obtained in each of the application intervals were processed with the Probit analysis technique (SAS, 1999) to determine the dose-Probit log response lines, and thus the values of the Mean Lethal Concentration (CL_{50}).

Results

Chemical composition of *T. lucida* essential oil

In the essential oil of the Ixtapan population of *T. lucida*, 19 compounds were identified, five of them major and the rest as traces. The major compounds were: geranyl acetate (40.83%), *β*-ocimeno (15.14%), nerolidol (8.19%), caryophyllene (5.29%) and *β*-cubebeno (5.17%) (Figure 1, Table 1).

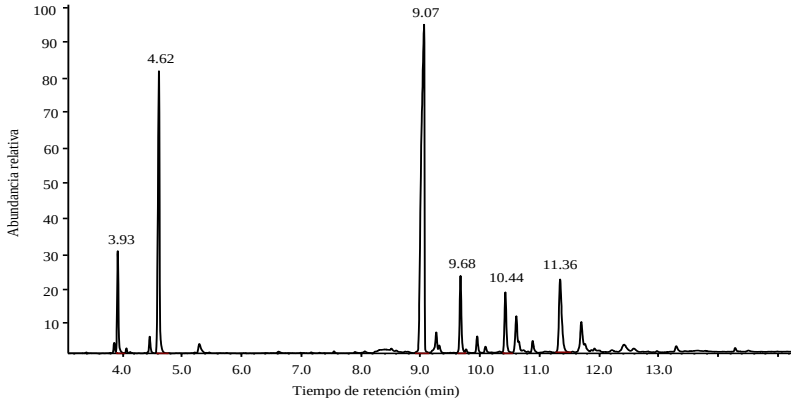

Figure 1. Chromatogram showing the peaks of: *β*-myrcene (retention time, Rt, 3.93) *β*-ocimene (Rt, 4.62), geranyl acetate (Rt, 9.07), carifilene (Rt, 9.68), *β*-cubebeno (Rt, 10.44) and nerolidol (Rt, 11.36).

Table 1. Chemical composition of the essential oil of floral stems of the Ixtapan population of *T. lucida*.

Peak	Compound	Tr	Area	(%)
1	*β*-Pineno	3.87	1 225 630	0.55
2	*β*-Mirceno	3.93	11 923 332	4.79
3	Acetato de alcohol de hojas	4.07	556 487	0.29
4	3,6,6-2-Norpineno	4.47	2 270 961	0.88
5	*β*-Ocimeno	4.62	41 693 860	15.14
6	Linalool	5.3	2 222 315	1.23
7	Geranyl acetate	9.07	92 446 673	40.83
8	Humuleno-(v1)	9.28	3 692 376	1.57
9	†Biciclo [4.3.0]nonan-2-eno, 8-isopropylideno-	9.33	1 165 226	0.55
10	Caryophyllene	9.68	11 650 343	5.29
11	Copaeno	9.78	473 212	0.21
12	Farneseno	9.97	2 449 168	1.27
13	*α*-Caryophyllene	10.1	1 011 519	0.45
14	*β*-Cubebeno	10.44	10 046 965	5.17
15	*γ*-Elemeno	10.62	6 598 214	3.97
16	*δ*-Cadinne	10.89	2 055 984	0.86
17	Nerolidol	11.36	18 323 420	8.19
18	Caryophyllene oxide	11.71	6 250 778	1.68
19	tau-Cadinol	12.43	2 935 784	1.19

†= no trivial name was found.

Inhibition of galling and lethal concentration (CL_{50})

In the control condition, the oil treatments inhibited root galling, such inhibition in the three intervals was increasing as the oil concentration increased (Table 2), the CL_{50} in the application intervals 1 and 2 it was the same (0.06 mg mL^{-1}) and almost double (0.13 mg mL^{-1}) in the third interval. In the condition of preventive management, it was not appreciated that the inhibition of the gills was proportionally associated with the concentration, for example, in interval 1 there was no difference between treatments and the control, and in intervals 2 and 3, only with the concentration of 10 mg mL^{-1} difference was observed in relation to the control; an irregular trend that, in the case of interval 3, made it difficult to calculate the CL_{50} (Table 2). In general, the most consistent results in the CL_{50} value were obtained with the control treatment, with a value of the slope of the regression line less than 0.7, highlighting the essential oil concentrations of 0.35 and 1 mg mL^{-1} for inhibition of galling by *N. aberrans*.

Table 2. Average inhibition (%) of galling by *N. aberrans* at 52 days after the application of the essential oil of *T. lucida* in different concentrations to tomato seedlings and value of the CL_{50} according to treatment management and application intervals.

Application interval (weeks)	Preventive management		Control management	
	Concentration (mg mL^{-1})	Inhibition (%)	Concentration (mg mL^{-1})	Inhibition (%)
1	10	68.97 a	10	94.03 d
	3.5	53.45 a	3.5	77.61 cd
	1	59.77 a	1	64.93 bcd
	0.35	40.23 a	0.35	63.43 bcd
	0.1	45.4 a	0.1	53.73 cb
	0.035	26.44 a	0.035	46.27 cb
	0.01	42.53 a	0.01	38.06 b
	Control	0 a	Control	0 a
	CL_{50} 0.5 (mg mL^{-1}) (0.024 - 137.88)* b ±s= 0.27 ±0.09		CL_{50} 0.06 (mg mL^{-1}) (0.03 - 0.1)* b ±s= 0.49 ±0.05	
2	10	85.63 b	10	95.24 e
	3.5	59.88 ab	3.5	87.3 e
	1	44.31 ab	1	80.95 de
	0.35	19.76 ab	0.35	65.87 cd
	0.1	42.51 ab	0.1	61.11 c
	0.035	45.51 ab	0.035	41.27 b
	0.01	47.9 ab	0.01	31.75 b
	Control	0 a	Control	0 a
	CL_{50} = 0.37 (mg mL^{-1}) (-) b ±s= 0.26 ±0.18		CL_{50} = 0.06 (mg mL^{-1}) (0.04 - 0.08)* b ± s = (0.04 - 0.08) 0.68 ±0.06	
3	10	80.85 b	10	94.64 d
	3.5	-17.02 ab	3.5	76.79 cd
	1	-106.38 ab	1	63.39 bcd
	0.35	-151.06 a	0.35	47.32 bc
	0.1	-95.74 ab	0.1	39.29 b
	0.035	-104.26 ab	0.035	37.5 b
	0.01	-125.53 a	0.01	40.18 b
	Control	0 ab	Control	0 a
	CL_{50} (not obtained)		CL_{50} = 0.13 (mg mL^{-1}) (0.02 - 0.5)* b ±s= 0.53 ±0.12	

b= slope of the regression line; s= standard error; Control with Tween® 20, 50 mL $individual^{-1}$; *= 95% confidence limits; (-)= showed no confidence limits.

Discussion

The major compounds identified in the essential oil of the aerial part of flowering plants of *T. lucida* correspond to the chemical groups of monoterpenes (geranyl acetate and *β*-ocimeno) and sesquiterpenes (caryophyllene, nerolidol and *β*-cubebeno) (Lange and Ahkami, 2013). Monoterpenes and sesquiterpenes, in vegetable extracts of *Tagetes* obtained with solvents, may have biological activity against nematodes (Marotti *et al*., 2010) and it would be expected that their presence in the composition of the essential oil of *T. lucida* could have similar effect, as further on it is exposed (Table 2).

Geranyl acetate (40.8%) was the most abundant in the essential oil of the Ixtapan population of *T. lucida* (Table 1), this compound had not been reported in populations of Guatemala, Cuba, Costa Rica and Mexico (Bicchi *et al.*, 1997; Ciccio, 2004; Serrato *et al*., 2007; Regalado *et al*., 2011); this molecule and nerolidol are attributed toxic activity against *Aedes aegypti* (Muñoz *et al*., 2014), but there was no reference to their effect against nematodes. The compound *β*-ocimeno (15.14%) has antibiotic, anti-inflammatory and anti-oxidant properties, it also highlights its effect against pathogens and protects plants against insect pests (Adorjan and Buchbauer, 2010), but without a history against nematodes.

The caryophyllene (9.6%) has an insecticidal effect against mosquito larvae (Jaenson *et al*., 2006) and *β*-cubebeno (5.17%), also has activity against *Escherichia coli* (Bezic *et al*., 2005); none of these compounds is related to effects against nematodes. Most of these compounds, in trace amounts, had already been described in the composition of *T. lucida* essential oil (Bicchi *et al.*, 1997; Ciccio, 2004; Serrato *et al*., 2007; Visbal *et al*., 2010; Regalado *et al*., 2011), the distinctiveness of the oil composition of the Ixtapan population of *T. lucida* was the high abundance of some of them and the presence of the compound geranyl acetate.

Considering that the root galling of tomato plants is a consequence of the presence of female nematodes and their penetration into the roots (Manzanilla *et al*., 2002), it was clear that the application of *T. lucida* oil reduced the process of galling by *N. aberrans* (Table 2), result that constitutes the first report for this nematode. Possibly the inhibition of galls was due to the fact that the essential oil affects the nervous system of the organism, consequently causes paralysis and finally death (Maffei, 2010), effect of immobility of nematodes previously reported with the application of root and part extracts of *T. lucida*, obtained with solvents (Siddiqui and Alam, 1988; Marotti *et al*., 2010; Omer *et al*., 2015); the effect of *T. lucida* essential oil against nematodes had not been evaluated before the present work.

The CL_{50} values were expected to be decreasing in proportion to the number of intervals, but the trend was not appreciated; when analyzing the number of plants with galls by factorial analysis, it was confirmed that there was no interval effect, so that applying once, or perhaps twice, is convenient. These results constitute a useful reference for the management of vegetable substances, since there is little information about them. From the practical point of view, the highest concentrations produced the best results, although the possible use of 0.35 to 1 mg mL^{-1} stands out; without phytotoxicity.

With the control treatment, the inhibition was proportional to the concentrations at which the oil was diluted and applied; this did not happen with the preventive application, therefore only the CL_{50} was obtained for the first two intervals. However, the low values of CL_{50} (0.06-0.13 mg mL^{-1}) in the control scheme, the slope was always lower than 0.7 in the three intervals, indicating that the population of individuals responded heterogeneously to the application of the oil , which could mean that oil molecules do not affect effectively the J2 females of *N. aberrans*, although it is also possible that a heterogeneous proportion of female individuals in the inoculum has dispersed to the root zone of the seedlings, even there have been differences in the parasitic ability of the populations of the nematode in the host, as well as the colonizing ability (Bourne and Kerry, 1999).

The essential oil of species such as *T. minuta* has been evaluated against *Anopheles gambiae* by recording CL_{50} of 1.49 mg mL^{-1} (Kyarimpa *et al*., 2014), of *T. patula* against *Aedes aegypti* with CL_{50} of 0.13 mg mL^{-1} (Dharmagadda *et al*., 2005) and of *T. lucida* against *Tetranychus urticae* with an CL_{50} of 0.016 mg mL^{-1} (Caramillo *et al*., 2008) such results and those achieved in this study in the case of control treatments with CL_{50} of 0.06-0.13 mg mL^{-1}, suggest strong biological action of *T. lucida* oil against *N. aberrans*.

To apply essential oil at a concentration of 0.06 mg mL^{-1} to one hectare would require 120 mL of oil in 199.8 L of water, while 260 mL would be required in 199.7 L of water for the 0.13 mg mL^{-1} concentration. In lands not cultivated in Ixtapan, it is estimated that in 1 m^2 of surface there are nine plants of *T. lucida* and in 1 ha, 90 000 of them with an oil yield of approximately 0.68 mL m^{-2} and production of 6.8 L ha^{-1}. The wide distribution of populations of *T. lucida* in the State of Mexico and its abundance in numerous localities such as Ixtapan, are favorable conditions for the use of this natural resource and low cost.

Conclusions

The essential oil obtained by hydrodistillation of flowering plants of the Ixtapan population of *T. lucida* presented the major compounds were geranyl acetate, β-ocimeno, nerolidol, β-cubebeno and caryophyllene, highlighting geranyl acetate that had not been reported as abundant. The essential oil of *T. lucida* influenced the process of galling of *N. aberrans* in tomato seedlings, the concentration effect being critical and the application interval little effective, in this way the best IC_{50} results of 0.06-0.13 mg mL^{-1} to inhibit galling were achieved by applying the oil as a control treatment.

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