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EVALUATION OF NEMATICIDAL EFFECTS OF MONOTERPENES AGAINST ROOT-KNOT NEMATODE, *Meloidogyne incognita*

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ABSTRACT

Nematicidal activity of 12 monoterpenes namely, camphene, (*R*)-camphor, (*R*)-carvone, 1-8-cineole, cuminaldehyde, (*S*)-fenchone, geraniol, (*S*)-limonene, (*R*)-linalool, (*1R,2S,5R*)-menthol, citral and thymol was evaluated against root-knot nematode, *Meloidogyne incognita* J2 in-vivo and in-vitro on eggplant cv. Black Beauty (*Solanum melongena* L.). In laboratory experiments, all of the tested compounds caused significant mortality of the second larval stage (J2). It was, also, noticed that the tested compounds reduced egg hatching. Carvone, cuminaldehyde, thymol, geraniol, and citral showed the highest nematicidal activity among the tested monoterpenes in vitro. The results of greenhouse experiment clarified that the tested monoterpenes; carvone, cuminaldehyde, thymol, geraniol, and citral, significantly, reduced numbers of root galls, egg masses and population of nematodes of eggplant at concentration 250 mg/kg soil compared to the inoculated control. Carvone was the most effective treatment in reducing root galls, egg masses and final population of the nematode except for oxamyl. Results revealed that all of the tested treatments increased various measures of plant growth characters with the reduction in the root-knot infestation. Among all of the tested monoterpenes, carvone followed by cumminaldehyde were the most effective treatments to increase both fresh shoot & root weights and lengths. None of the compounds was phytotoxic at the tested treatments. Results stated that monoterpenes exhibited significant nematicidal activity in-vitro and in-vivo experiments and could be considered as useful natural namaticidal agents.

Keywords: monoterpenes, nematicidal activity, *Meloidogyne incognita*

INTRODUCTION

Plant parasitic nematodes are mostly microscopic organisms. They cause significant damage for almost all crops. Global crop loss caused by plant parasitic nematodes is more than $100 billion annually (Khan *et al.,* 2008). Root-knot nematodes attack more than 2000 species of plants, including almost all cultivated plants and reduce world crop production by about 5 %. Losses in individual fields, however, may be much higher (Agrois, 1997). Root-knot nematodes, *Meloidogyne* spp., are the most pathogenic species of nematodes to the most crops and could cause up to 64% yield reduction (Khan *et al.,* 1996). *M. incognita* (Kofoid and White) Chitwood (Tylenchida: Heteroderidae) is a major plant-parasitic nematode species affecting the quantity and quality of the crop production in many annual and perennial crops. Root-knot nematodes spend part of their life in soil either as eggs or as second-stage larvae. The latter enters the roots and establishes feeding sites in susceptible hosts, inducing roots swelling with a characteristic “knotty” appearance. Root galling can drastically limits water and nutrient uptake leading to several symptoms, like malnutrition, chlorosis, and stunting, causing considerable quantitative and qualitative losses in several crop plants. The population of plant-parasitic nematodes in the field can be minimized through several approaches such as using natural enemies (Khan and Kim, 2007), enhancing cultural practices (Okada and Harada, 2007), cultivating resistant cultivars (Williamson and Kumar, 2006), and applying pesticides [Browning *et al*., 2006].

The extensive use of pesticides in control of nematodes led to environmental and health problems as well as the development of nematode resistance. Therefore, it has become an important issue to find alternative control strategies for nematodes. One of possible alternatives is the utilization of plant extracts, plant secondary metabolites and plant essential oils for nematode control (Abid *et al*., 2005; Pavaraj *et al*., 2012). Many plant constituents and metabolites including essential oils have been investigated for the activity against plant-parasitic nematodes (Albuquerque *et al.,* 2007, Echeverrigaray *et al.,* 2010, Pérez *et al.,* 2003, Walker and Melin 1996). A wide variety of plant species, representing 57 families have been shown to nematicidal compounds (Sukul, 1992), which includes isothiocynates, thiophenics glycosides, alkaloids, phenolic and fatty acids (Gommers, 1973**).** Plant terpenoids are used extensively for their aromatic qualities. Terpenes form structurally and functionally different classes of compounds that are formed by coupling different numbers of isoprene units (5-carbon-base; C5), while terpenoids represent terpenes containing oxygen. The main structural classes of the terpenes are: monoterpenes (C10), sesquiterpenes (C15), hemiterpenes (C5), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) (Aharoni *et al.,* 2005). Monoterpenoids (C10), formed by two isoprene units, are the most representative molecules in more than 90% of the essential oils extracted from plants (Bakkali *et al.,* 2008). Nematicidal phytochemicals are generally safe for the environment and humans (Chitwood, 2002).

In this study, the nematicidal activity of 12 monoterpenes was investigated against the knot-root nematode, *M. incognita,* in laboratory and greenhouse, in order to find potential alternatives for the control of this important agronomical pest.

MATERIALS AND METHODS

Chemicals

Twelve monoterpenes, camphene (95%), (*R*)-camphor (98%), (*R*)-carvone (98%), 1-8-cineole (99%), cuminaldehyde (98%), (*S*)-fenchone (98%), geraniol (98%), (*S*)-limonene (96%), (*R*)-linalool(95%), (*1R,2S,5R*)-menthol (98%), citral (95%) and thymol (98%) were purchased from Sigma–Aldrich Chemical Co., Steinheim, Germany. Chemical structures of these monoterpenes are shown in Figure 1. Oxamyl (Vydate®24% L) (*N, N*-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio) acetamide) was supplied by Dupont company.

Nematode

M. incognita (Kofoid and White) Chitwood was isolated from inoculated plants of eggplant (*Solanum melongena* L.) in green house. Root-knot nematodes were identified using perineal patterns of adult females as well as the morphology of second stage juveniles (Hartman and Sasser, 1985; Jepson, 1987). Egg masses of root - knot nematode obtained from a pure culture maintained on eggplant roots were placed on sterilized distilled water of sodium hypochloride (NaOCl) solution (Hussey and Barker, 1973) and incubated for 48 h at room temperature at 25 ± 2C° for hatching. The hatched second stage juveniles (J2) were collected daily. Only freshly hatched J2 collected within 48 h were used for experiments. Second stage juveniles (J2) and eggs of *M. incognita* were used for toxicity evaluations.

Nematicidal activity

Nematicidal activity of monoterpenes was evaluated against second-stage juveniles (J2) of *M. incognita* under laboratory conditions. Four concentrations (125, 250, 500 and 1000 µg/ml) of each compound were prepared in distilled water containing 0.3% Tween 20. Four replicates of each concentration with about 100 specimens of *M. incognita* juveniles in each replicate were used. The control treatment contains distilled water with 0.3% Tween 20. Oxamyl was used as reference nematicide. The treatments were incubated at 25 ± 2C° and the mortality of nematodes was recorded after 48 h. The LC_{50} values were calculated according to Finney (1971).

Hatching inhibition

Approximately 100 eggs were transferred to the different concentrations of monoterpenes in glass vials containing distilled water. Monoterpenes were tested at concentrations of 125, 250, 500 and 1000 µg/ml. Each treatment was replicated four times. The glass vials were incubated at room temperature (25 ± 2C°) and the number of hatched juveniles was counted under a stereo microscope. Hatching inhibition percentages was observed after 7 days and IC_{50} values were calculated by probit analysis (Finney, 1971).

Greenhouse experiment

The tested compounds, citral, geraniol, thymol, cuminaldehyde and carvone, were applied to evaluate their efficacy on (*M. incognita*) at a concentration of 250 mg/kg and oxamyl at recommended rate (4L per fedden). Seedlings of similar age and size of eggplant (*S. melongena* L. cv. Black Beauty), 21-day-old were singly transplanted on a plastic pot (20 cm diameter and 15 cm depth), filled with 3 Kg mixture of autoclaved sand: clay soil (3:1, v: v). Plants were allowed to recover from transplanting shocks for 10 days. Each pot was inoculated with an initial inoculum level of (5000 eggs/pot) of root-knot nematode in holes of 5-7 cm depth around the plant within the radius of two centimeters. There were four replicates for each treatment including the untreated un-inoculated and inoculated controls. Greenhouse temperature ranged between 25-30°C. After 60 days, plants root systems were gently cut from the stem. The parameters including number of galls and egg masses per root system, final J2 population were recorded. The reduction in the galls, egg masses and nematode population density expressed as a percentage was calculated at the end of the experiment according to Henderson and Tilton's (1955) equation. Roots were stained for 15 minutes in an aqueous solution of Phloxine B stain (0.15 g/l water) (Holbrook *et al.,* 1983), then gently washed in tap water. Plant growth parameters expressed by shoot and root lengths (in centimeter), and fresh weights (in grams), were recorded and calculated as a percentage of increase.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 11.00 software (SPSS Inc., Chicago, IL, USA). Duncan's Multiple Range Test (DMRT) was employed to test for significant differences between the treatments (Duncan, 1955), and the LC_{50}, IC_{50} values were calculated using probit analysis.

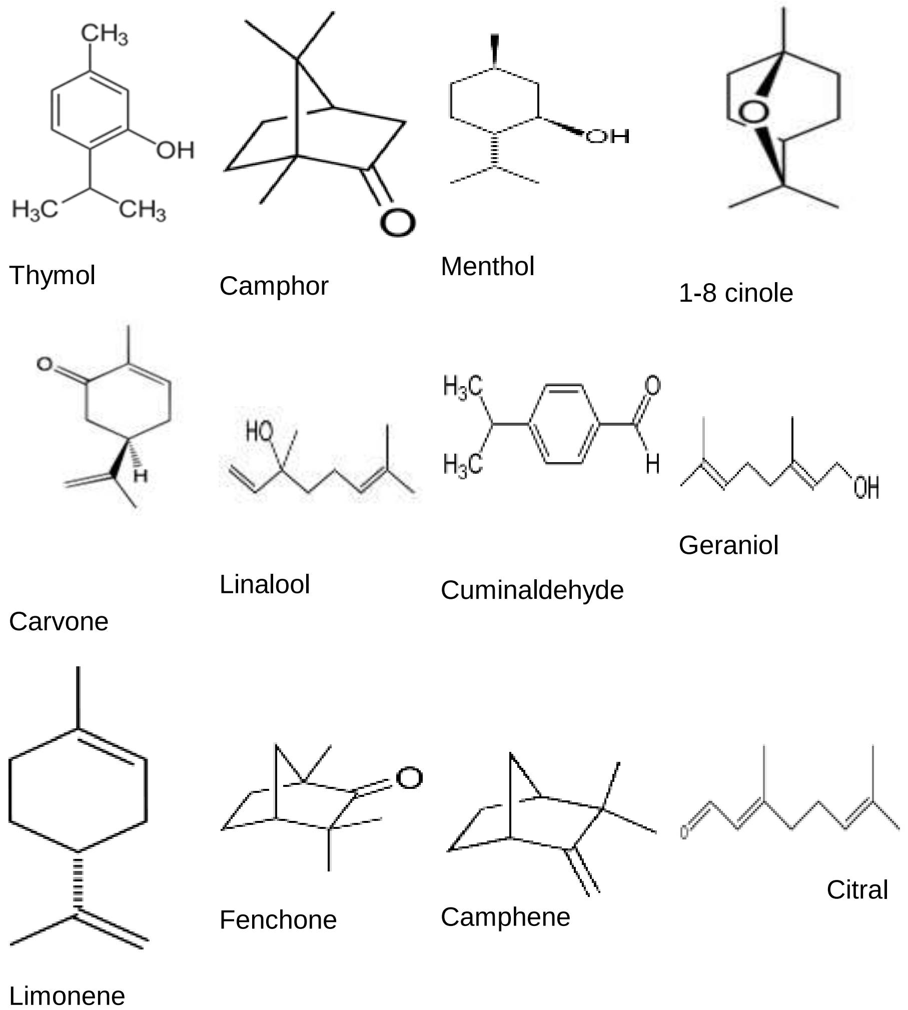

Figure 1. The chemical structure of the tested monoterpenes.

RESULTS

Toxicity of monoterpenes to J2 of *M. incognita*

In the bioassay test, the effects of 12 monoterpenes on J2 of *M. incognita* at concentrations of 125, 250, 500 and 1000 µg/ml were evaluated. As shown in Table (1), it was noticed that the inhibitory effect of a monoterpenes on nematode activity or mortality was concentration dependent, *i.e.* toxicity of the nematode increased by increasing of monoterpene concentration. The results revealed that carvone, geraniol, cuminaldehyde, thymol and citral were highly toxic with LC_{50} values ranged from 149.5 to 259.6 µg/ml. Linalool and menthol were moderately toxic with LC_{50} of 394.8 and 667.3 µg/ml, respectively.

Table (1): *In vitro* toxicity of monoterpenes against J2 of *M. incognita.*

Treatment	LC_{50} [a] (µg/ml)	95% Confidence Limits (µg/ml)		Slope ± S.E.[b]
		Lower	Upper	
Citral	259.6	217.1	305.5	1.91 ± 0.19
1,8-Cineole	976.7	788.3	1390.8	1.98 ± 0.34
Camphor	721.3	583.1	961.4	1.62 ± 0.21
Geraniol	170.9	143.8	198.5	2.44 ± 0.29
Limonene	898.8	724.6	1218.1	1.82 ± 0.23
Menthol	667.3	530.5	912.6	1.43 ± 0.20
Linalool	394.9	330.2	477.3	1.71 ± 0.19
Cuminaldehyde	183.3	157.0	210.4	2.65 ± 0.29
Fenchone	1066.5	835.7	1537.0	1.77 ± 0.24
Carvone	149.5	127.8	170.6	2.94 ± 0.31
Camphene	929.5	746.9	1269.1	1.83 ± 0.23
Thymol	198.6	171.5	226.3	2.7 ± 0.24
Oxamyl	58.46	50.12	70.32	4.73 ± 0.42

[a] The concentration causing 50% larval mortality.
[b] Slope of the concentration- mortality regression line.

Effect of tested monoterpenes on hatching inhibition

The results shown in Table 2 indicated that, the tested monoterpenes, significantly, reduced hatching of eggs at concentrations of 125 to 1000 µg/ml, and all the compounds tested, drastically, reduced hatching at 500 and 1000 µg/ml.

Carvone cuminaldehyde, thymol, and geraniol were highly efficient in inhibition of hatching. At the highest concentration (1000 µg/ml), these compounds caused 90% reduction in hatching. The results revealed that carvone, cuminaldehyde, thymol, and geraniol were highly toxic with IC_{50} values ranged from 95.9 to 218.3 µg/ml. Meanwhile, Citral and menthol were moderately toxic with IC_{50} values of 358.6 and 330.2 µg/ml, respectively.

Table (2): *In vitro* effect of monoterpenes on egg hatching inhibition of *M. incognita*

Treatment	IC_{50}[a] (μg/ml)	95% Confidence Limits(μg/ml)		Slope ± S.E [b]
		Lower	Upper	
Citral	358.6	308.9	416.7	2.15 ± 0.21
1,8-Cineole	675.6	555.8	870.5	1.73 ± 0.21
Camphor	1156.2	916.1	1645.1	2.02 ± 0.27
Geraniol	218.3	183.6	254.1	2.15 ± 0.21
Limonene	507.1	424.3	624.6	1.74 ± 0.20
Menthol	330.2	330.2	477.3	1.71 ± 0.19
Linalool	803.5	624.5	1160.5	1.41 ± 0.19
Cuminaldehyde	106.3	88.3	122.2	3.36 ± 0.41
Fenchone	1675.9	1160.0	3209.9	1.51 ± 0.24
Carvone	95.9	72.8	115.8	2.58 ± 0.35
Camphene	1333.3	997.5	2131.2	1.72 ± 0.25
Thymol	210.3	168.6	253.8	1.76 ± 0.22
Oxamyl	57.5	50.8	67.0	2.85 ± 0.42

[a]The concentration causing 50% hatching inhibition.
[b] Slope of the concentration-inhibition regression line.

In vivo nematicidal activity of monoterpenes to *M. incognita* on eggplant:

In pot experiments, the number of galls and egg masses per root system, and final population as affected by the tested monoterpenes are presented in Table 3. The nematicidal activity of the five selected monoterpenes, significantly, affected root galls, egg masses and final population of *M. incognita* infecting eggplant at the application rate of 250 mg/kg. After two months of a single application, *M. incognita* produced variable number of galls on roots of all tested compound. Untreated inoculated control (UI control) showed maximum number of galls and egg masses per root system, and final population. The number of galls produced by plants treated with citral (161) and thymol (125) were not significantly different .In addition, there were no significant differences among geraniol (119), thymol (125) and cumminaldehyde (95). On the other hand, plants treated with carvone produced the lowest number of galls (57) with 84.4 % reduction ,indicating that carvone was the highest effective treatment on root galls. All of the tested compounds were less active than oxamyl (92.6%) in reducing the number of galls per plant. Similarly, all of the tested compounds and oxamyl, significantly, reudced the number of egg masses . Carvone was the most effective treatment to suppress the number of egg masses with 84.5% reduction followed by cumminaldehyde and geraniol achieving 70.5 and 61.8% reduction, respectively. It was also, noticed that application of the tested compounds, significantly, decreased the population of J2 in soil with excepion of citral. Carvone was the superior treatment which suppressed final population of *M. incognita* with value of 49.4 reduction percent without any difference from cumminaldehyde in significance. Citral showed the less performance with value of 5.4% reduction .

Table (3): The efficacy of monoterpene against *M. incognita* galls, egg masses on roots and second stage J2 population in soil on eggplant.

Treatment	Number of Galls	Reduction %	Number of Egg Masses	Reduction %	Population J2 in soil	Reduction %
UU control	0[e]	-	0[e]	-	0[d]	-
UI control	367[a]	-	309[a]	-	9905[a]	-
Citral	161[b]	56.1	157[b]	49.1	9368[a]	5.4
Geraniol	119[c]	67.6	118[c]	61.8	6792[b]	31.4
Carvone	57[d]	84.4	48[d]	84.5	5010[c]	49.4
Cumminaldehyde	95[c]	74.1	91[c]	70.5	5870[bc]	40.7
Thymol	125[bc]	65.9	120[c]	61.2	7140[b]	27.9
Oxamyl	27[de]	92.6	25[de]	91.9	4624[c]	53.3

*** Data with the same letter(s) within a column are not significantly different according to Duncan's a new multiple range test. *UU control: untreated un-inoculated control, UI control: untreated inoculated controls**

In pot experiments as shown in Table 4 , *M. incognita* reduced all plant growth parameters in the untreated inoculated treatments as compared with the treated or uninoculated plants. Eggplant biomass was markedly increased by most of the used treatments. Oxamyl gave the greatest increases in fresh shoot & root weights and lengths achieving values 119 and 112.8%, respectively (Table 4). Beyond oxamyl, carvone followed by cumminaldehyde were the most effective treatments to increase both fresh shoot & root weights and lengths with 113.6 & 97.9% and 104.5 & 93.6% without any significant differences from each other, consecutively. Meanwhile, thymol recorded the intermediate value of fresh shoot and root weights and lengths (74.6 and 74.3 % increase). On the other hand, citral showed the least activity towards both fresh shoot & root weights and lengths with values of 61.6 and 62.4 % increase, respectively.

Table (4): Effect of monoterpenes *against Meloidogyne incognita* on plant growth parameters of eggplant in the greenhouse.

Treatment	Length (cm)					Fresh weight (g)		
	Shoot	Root	Total	Increase %	Shoot	Root	Total	Increase %
UU control	44.50[a]	15.00[a]	59.50[a]	-	129.25[a]	30.88[a]	160.13[a]	-
UI control	17.25[c]	8.00[c]	27.25[e]	-	51.75[c]	16.00[c]	67.75[e]	-
Citral	35.50[b]	8.75[c]	44.25[d]	62.4	92.75[b]	16.75[c]	109.50[d]	61.6
Geraniol	38.75[ab]	10.25[bc]	49.00[bdc]	79.8	110.75[ab]	19.75[c]	130.50[bcd]	92.6
Carvone	42.00[a]	13.75[a]	55.75[ab]	104.5	117.00[ab]	27.75[ab]	144.75[abc]	113.6
Cumminaldehyde	40.25[ab]	12.50[ab]	52.75[abc]	93.6	108.75[ab]	25.38[ab]	134.125[abcd]	97.9
Thymol	38.00[ab]	9.50[c]	47.50[cd]	74.3	99.00[b]	19.31[c]	118.31[cd]	74.6
Oxamyl	43.50[a]	14.50[a]	58.00[a]	112.8	120.00[ab]	28.38[ab]	148.38[ab]	119.0

*** Data with the same letter(s) within a column are not significantly different according to Duncan's a new multiple range test.**
*** UU control: untreated un-inoculated control, UI control: untreated inoculated controls**
Therefore, these results indicated that none of the compounds was phytotoxic even at the tested concentration.

DISCUSSION

Monoterpenes are the main constituents of aromatic plant essential oils which responsible for most of the biological properties of these plants extracts (Bakkali *et al*., 2008, Sacchetti *et al*., 2005, Chedekal, 2013). Many essential oils and some of their main constituents possess nematicidal activity against *Meloidogyne* and other important phytonematodes (Kong *et al.,* 2007, Oka *et al.,* 2000). The results of this study indicated that twelve monoterpenes exhibited nematicidal activity against the root knot nematode *M. incognita.* The most efficient compounds were citral, geraniol, thymol, cuminaldehyde and carvone. The nematicidal activity of geraniol and citral were previously reported by Kong *et al.,* 2007 and Albuquerque *et al.,* 2007*.* Limonene showed antihatching activity differing from data obtained by Oka *et al.*, 2000, using the same concentrations. These results agree with findings of Ohri and Pannu 2009. Compounds with hydroxyl or carbonyl groups were stronger than the other monoterpenoids, indicating that functional group is very important for nematicidal activity. Among acyclic alcohols, geraniol with hydroxyl group at C_1 was more toxic than linalool, with hydroxyl group at C_3. The effect of double bond position on the nematicidal activity of terpenoids was previously reported by Park *et al*. 2007. Among the tested compounds, citral, geraniol, thymol, cuminaldehyde and carvone showed potential nematicidal activity *in vitro* experiments. These five compounds, significantly, reduced egg masses and galls produced by *M. incognita* nematodes at 250 mg/kg in greenhouse experiments. These results are similar to those reported by Echeverrigaray *et al*., 2010, and Oka *et al*. 2000, for the essential oils of five aromatic plants. Plant protection against phytonematodes has been obtained by the application of essential oils (Abo-Elyousr *et al.,* 2009, Onifade 2007 and Pérez *et al.,* 2003) or organic amendments from essential oil rich plants (Pérez *et al.,* 2003 and Silva *el al.,* 2006).

The mode of action of essential oils and monoterpenes on nematodes is unclear. However, some essential oils have been reported to have genotoxic activity in *Drosophila melanogaster* to activate octopaminergic receptors (Enan, 2001, Kostyukovsky *et al.,* 2002), and to interfere with GABA receptors of insects (Priestley *et al.,* 2003). As typical lipophiles, essential oils and terpenoids interact with the cytoplasmic membrane of yeasts disrupting the structure of polysaccharides, fatty acids, and phospholipids, and provoking depolarization of the mitochondrial membranes resulting in leakage of radicals, cytochrome C, calcium ions, and proteins (Bakkali, *et al.,* 2008). In general, the *in vitro* cytotoxic activity of essentials oils has been attributed to the presence of phenols, aldehydes, and alcohols (Bruni *et al*., 2004 and Oka *et al.,* 2000).

In conclusion, the results obtained in this study indicate the remarkable nematicidal activity of monoterpenes such as citral, geraniol, thymol, cuminaldehyde and carvone against the root knot nematode, *M. incognita*. The need for new natural nematicides with different mode of action and the strong nematicidal activity of these compounds demonstrated in this study both in in-vitro and in-vivo may encourage further studies on their using as biodegradable and mammalian and environmentally safe nematode control agents.

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اجريت هذه الدراسة لتقييم النشاط النيماتودى لـ 12 مركب من المونوتربينات وهي كامفين، (R) كافور، (R)-كارفون، 1-8 سينول ، كيمون الدهيد، (S) فينشون ، جيرانيول، (S)-الليمونين، (R)- لينالول، المنثول، السترال والثيمول على نيماتودا تعقد الجذور فى الباذنجان(صنف بلاك بيوتى) وتم ذلك معمليا وداخل الصوبة. اوضحت التجارب المعملية، ان كل المركبات المختبرة تسببت فى موت الطور اليرقى الثانى لنيماتودا تعقد الجذور (J2). كما اظهرت النتائج انخفاض معنوى واضح فى نسب فقس البيض. و لقد أوضحت نتائج التجارب المعملية ان كل من مركبات الكارفون، كيمون الدهيد ، الثيمول، جيرانيول، والسترال قد اظهرت تاثير نيماتودى قوى عن بقية المركبات المونوتربين. ولقد تم اختبار مركبات المونوتربينات الاكثر كفاءة نيماتودية وهى الكارفون ، كيمون الدهيد ، الثيمول، جيرانيول، والسترال داخل الصوبة وعكست نتائج التجربة حدوث انخفاض معنوى كبير في أعداد العقد الجذرية ، كتل البيض وتعداد النيماتودا النهائى فى التربة لنبات الباذنجان باستخدام تركيز 250 ملجم / كجم تربة مقارنة بالكنترول المعامل. ولوحظ ان المعاملة بمركب الكارفون قد تسببت في أكبر انخفاض معنوى في أعداد العقد الجذرية و كتل البيض وتعداد النيماتودا النهائى فى التربة مقارنة بأي مركب آخرباستثناء المبيد الكاربامـاتى الاوكساميل . وتشير النتائج أن كل من المعاملات التي تم اختبارها قد احدثت زيادة معنوية بدرجات مختلفة فى قياسات نمو النبات مع تقليل الإصابة بينماتودا تعقد الجذور. وقد اظهرت النتائج انه من بين كل مركبات المونوتربين المختبرة كان الكارفون ويلية الكيمونالدهيد اكثر المعاملات فعالية للزيادة فى اوزان واطوال المجموع الجذرى والخضرى. هذا و لم تظهر أي من المركبات التى تم اختبارها اى سمية نباتية. بناءا على النتائج المتحصل عليها وجد أن مركبات المونوتربينات ذات نشاط نيماتودى قوى في كلا من التجارب المعملية وتجارب الصوبة ويمكن الاستفادة منها كمركبات نيماتودية طبيعية.
كلمات البحث: المونوتربينات , النشاط النيماتودى , نيماتودا تعقد الجذور

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