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The efficiency of *Thymus vulgaris* against citrus nematode *Tylenchulus semipenetrans*

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ABSTRACT
Tylenchulus semipenetrans is considered one of the most destructive plant parasitic nematodes (PPNs) in citrus. Chemical nematicides are often used to reduce PPNs population, although the continuous use of these compounds causes negative environmental impacts, necessitating the adoption of eco-freindly strategies. The present study aimed at assessing the potential of the aqueous extracts of *Thymus vulgaris* in the biological control against the citrus nematode *T. semipenetrans* in terms of juvenile J2 mortality and egg hatch inhibition. Obtained results showed that *Thymus vulgaris* exhibited high toxicity against *T. semipenetrans*. Indeed, the juvenile mortality ranging between 11.22% and 49.28 % after 24h of incubation, and the egg hatch inhibition varying between 5.33% and 26.66% after 96h of incubation increased steeply with increasing extract concentration and incubation period reaching complete egg hatch inhibition after 144h of incubation at extract concentration of 10%. As a result of this study, *T. vulgaris* can be used as a healthy nematicidal product highly effective against *T.semipenetrans*.

1. Introduction:

Citrus spp. (Sapindales: *Rutaceae*) is one of the major horticultural crops cultivated around the world and the most widely traded horticultural commodity globally **[1]**. They represent an essential part of our daily diet, not only because of their pleasant flavor but also due to their high nutritional value and health benefits **[2]**. Among citrus species, oranges occupy the majority share of world citrus production (55%), followed by mandarins (25%), lemons (13%), and grapefruits (7%) **[3]**.

Citrus plants are hosts of numerous plant-parasitic nematodes (PPNs) **[4]**. Among these PPNs, *Tylenchulus semipenetrans* is one of the most important root nematodes of citrus trees, it has been found in most citrus-growing regions of the world **[5-6]**, including Morocco **[7-8]**. Plants infected with *T. semipenetrans* show signs of stunted growth, yellowed leaves, and reduced foliage and fruit size. The feeder roots infected by *T. semipenetrans* are thicker than healthy ones and appear muddy due to dirt clinging to the gel-like substance excreted by the female nematode on the root's surface **[4]**. This infection reduces the ability of citrus trees to absorb water and essential nutrients needed for their proper development **[9]**.

The density of this pest, in the soil has successfully reduced by a various approach, including synthetic pesticides which have been proven to be extremely effective in the field. However, their repeated use causes countless environmental problems and induces pest resistance to their active substances **[10]**. Thus, more sustainable and eco-friendly strategies for the control of nematodes have been developed, such as the use of plant-derived nematicidal compounds **[11]**. Various

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botanical families have been evaluated for their nematicidal activity, due to their secondary metabolites, such as alkaloids, phenols, diterpenes, glucosinolates, fatty acids, isothiocyanates, tannins, steroids, thienyls, and others **[12]**. These compounds exhibit repellent and attractant properties similar to those present in contact pesticides **[13]**. According to several studies, plant species and their phytochemicals were found to have nematicidal effects by inducing the defense reactions of the host plants **[14]**. The objective of the present study is to evaluate the nematicidal potential of *Thymus vulgaris* in the control against citrus nematode *Tylenchulus semipenetrans* under greenhouse and laboratory conditions.

2. Materials and methods:

2.1. Collection and extraction of inoculum:

The nematode inoculum (eggs and J2) obtained from infested citrus roots were washed gently in tap water, cut into 0,5 cm segments, and thoroughly mixed. Root samples were macerated for 30 s at 8000 rpm in an electric blender **[15]**. The resulting suspension was passed through 100, 60, 45, 30, and 20 µm sieves. The inoculum was gently rinsed from the filter disks with sterile distilled water, collected in beakers, and stored at 4°C.

2.2. Preparation of aqueous plant extract:

Ten grams of dried *Thymus vulgaris* plant material were added to 100 ml of distilled water, and then macerated in an electric blender. After 24h, the extract was filtered through cheesecloth and sterilized by passing it through a 0.45 µm membrane filter (Cellulose nitrate, Whatman). The original plant extract concentration was designated as 10% (S) and a second extract was prepared by diluting the S extract to 5% (S/2).

2.3. Juvenile mortality test:

A volume (1 ml) of water suspension containing 50 *Tylenchulus semipenetrans* J2 was dispensed using a pipette into individual Petri dishes (3 mm). Another 1 ml volume of 1%, 4%, 8%, and 10% was added to each Petri dish containing the juvenile suspensions. *Tylenchulus semipenetrans* J2 were exposed to aqueous plant extract concentration for 24, 48, 72, and 96 h. Distilled water was included as control. Five replicates were provided for each concentration at each exposure time and the experiment was a completely randomized design (CRD). The Petri dishes were maintained at 25 ± 2 °C **[16]**. The mortality of *Tylenchulus semipenetrans* juveniles was monitored at 24 h, 48 h, 72 h, and 96h under light microscope. During the experiment, dead nematodes were daily counted and removed from the treatments. The cumulative number of dead nematodes was used to calculate the percentage of mortality of the juveniles J2 of *T. semipenetrans*.

The corrected nematode mortality percentages were calculated according to **[17]**:

$$Mortality(\%) = \frac{m - n}{100 - n}$$

Where; m and n indicate the mortality in treatments and control, respectively.

2.4. Hatching eggs test:

A volume (1 ml) of water suspension containing 50 *Tylenchulus semipenetrans* Eggs was dispensed using a pipette into individual Petri dishes. Another 1 ml volume of *Thymus vulgaris* extract (1%, 4%, 8%, and 10%) was added to each Petri dish containing Eggs suspensions. Eggs were exposed to aqueous plant extract concentration for 48, 96, and 144 h. Distilled water was included as control. The experiment was a completely randomized design (CRD) and five replicates were provided for each concentration at each exposure time. The Petri dishes were maintained at 25 ± 2°C **[16]**. The number of eggs that hatched into juveniles was counted every 2 days using a stereo microscope.

2.5. Greenhouse experiments (In vivo tests):

The experiment was carried out in greenhouse (25–45 ◦C) at the faculty of sciences and techniques of Marrakech (Morocco) from August to December 2022 to evaluate the nematicidal properties of thymus against the citrus nematode *Tylenchulus semipenetrans*. One-year-old *Citrus volkameriana* cultivar seedlings were transplanted singly into plastic pots containing 5 kg of sterilized sandy clay soil and peat (2:1). Each pot was inoculated with 500 J2 populations of *T. semipenetrans* by pipetting them into four holes in the soil around the stem of the citrus seedling. Each plant was treated with 10% of leaf aqueous extract of *Thymus vulgaris*. After a period of 5 months, the tested plants were carefully taken out of the soil and their roots washed. The length, fresh and dry weights of shoots and roots were registered and the rate of nematode build up (Pf/Pi) was calculated according to the following equation:

$$Rate\ of\ nematode\ build\ up = \frac{Final\ nematode\ population\ Pf}{Initial\ nematode\ population\ Pi}$$

2.6. Statistical analysis:

Statistical analyses were performed using a statistical software package SPSS V21.0. A principal component analysis (PCA) was established to define the correlation between the different treatments. The data were subjected to ANOVA to test the significance of differences among treatments.

3. Results:

3.1. Nematicidal activity of the aqueous plant extract of Thymus vulgaris (Laboratory experiment):

Results data pertaining to the nematicidal activity of the aqueous extract of *Thymus vulgaris* against the citrus nematode *T. semipenetrans* are shown in table 1. Nematicidal activity varied significantly according to the extract concentration, the incubation period, and the development stage of *T. semipenetrans*. The aqueous extract of *Thymus vulgaris* exhibited high nematicidal activity against *T. semipenetrans* in terms of both J2 mortality and egg hatch inhibition. Indeed, during the first 24h of incubation, J2 mortality ranged between 11.22% at lower extract concentration (1%) and 49.28 % at high extract concentration (10%), compared to the control after 96 h of incubation. J2 mortality increased up to 31.2 % and 77.6 % respectively (table 1). In addition, T*hymus vulgaris* extract induced significant ($P<0.005$) decreases in egg hatch regardless of extract concentration and incubation period. Results revealed that Egg hatch (%) varied between 37.33% and 16.66% after 24h of incubation at extract concentrations of 1% and 10% respectively (table 2). Egg hatch decreased by increasing extract concentration and incubation time. Complete egg hatch inhibition was obtained after 144h of incubation at the extract concentration of 10%.

Table 1. Percentage of mortality of *Tylenchulus semipenetrans* second-stage juveniles (J2) after immersion in *Thymus vulgaris* aqueous extract at 1%, 4%, 8% and 10% during 24, 48, 72, and 96 hours.

Concentration (%)		**Incubation period (h)**			
		24	**48**	**72**	**96**
Thymus vulgaris	**1%**	11.22± 0.47^{k}	15.31 ±0.47^{j}	26.56 ±0.58^{h}	31.29 ±0.57^{g}
	4%	20.75 ±0.65^{hi}	24.49 ± 1^{h}	47.28 ± 0.88^{ef}	57.82± 0.33^{c}
	8%	28.57 ±0.28^{gh}	31.29± 0.88^{g}	53.06± 0.58^{cd}	67.35 ±0.58^{b}
	10%	49.28 ±0.47^{e}	33.33 ±0.88^{g}	57.82 ±0.33^{c}	77.55± 0.57^{a}
Control		1^{l}	1.2^{l}	11^{k}	13.6^{j}

Numbers in the same column followed by different letters are significantly different based on Tukey's test (*P* < 0.05).

Table 2. Percentage of egg hatch of *Tylenchulus semipenetrans* after incubation in the aqueous extract of *Thymus vulgaris* at 1%, 4%, 8% and 10% during 48, 96, and 144 hours.

Concentration (%)		**Incubation period (h)**		
		48	**96**	**144**
Thymus vulgaris	**1%**	37.33±0.09^{de}	26.66±0.73^{e}	11.33±0.45^{ghi}
	4%	31.33±0.65^{e}	18.66±0.57^{ef}	8.66±0.27^{hi}
	8%	18^{ef}	11.33±0.57^{ghi}	6.66±0.54^{hi}
	10%	16.66±0.43^{efg}	5.33±0.57^{hi}	0^{j}
Control		73^{c}	83^{b}	95^{a}

Numbers in the same column followed by different letters are significantly different based on Tukey's test (P < 0.05).

3.2. LC50 and LC90 analysis:

Obtained results showed that LC50 values ranged from 7.1 % (4.8%-13.1%) to 0. 88% (0.71%-1.04%), and the LC90 values varied between 9.68% (6. 64%- 21.41%) to 2.07 % (1.76% -2.56%), indicating an intense toxic effect of *Thymus vulgaris* extract on the population of *T. semipenetrans* (J2).

Table 3. LC50* and LC90 ** values (95 % confidence level) of *Thymus vulgaris* on the second-stage juveniles of *Tylenchulus semipenetrans* estimated from four dosages (five replicates/dosage) by PROBIT analysis.

	LC50% (Lower bound-upper bound)			
Thymus vulgaris	**24**	**48**	**72**	**96**
	7.1 (4.8-13.1)	6.5(5.6-7.58)	2.5 (2.1-3.3)	0.88(0.70-1.03)
	LC90%% (Lower bound-upper bound)			
	24	**48**	**72**	**96**
	9.6 (6. 6- 21.41)	18.17(15.39-22.9)	6.0(4.2-10.4)	2.0(1.76-2.56)

3.3. Greenhouse experiments:

This study evaluated the effect of the aqueous extract of *Thymus vulgaris* on the population of *T. semipenetrans* infesting *Citrus volcameriana* seedling under greenhouse conditions. Generally, results of the current work showed that compared

to the control, the extract of *Thymus vulgaris* reduced considerably the density of the J2 population of *T. semipenetrans* both in the rhizophere and the roots of *Citrus volcameriana*, (Table 4). Indeed, the nematode population was twice lower (453.33 J2 / 100 g of soil) in the soil treated with *T. vulgaris* extract compared to the control (997.33J2/100 g of soil). Moreover, extract of *Thymus vulgaris* resulted in a considerable reduction in the total number of *T. semipenetrans* nematodes in the roots of *Citrus volkameriana* seedlings, with a nematode build-up rate 1.8 times lower than in the control.

Table 4. Effect of Leaf extract of *Thymus vulgaris* on the J2 population of *T. semipenetrans* in the rhizosphere and the roots of *Citrus volkameriana* seedlings grown under Greenhouse conditions.

Treatment	Initial population number J2 per 100 g of soil (Pi)	Final population number of J2 per 100 g of soil (Pf)	Rate of build up (Soil)	Final population number of J2 per roots	Rate of build up (Roots)
Control	500	997.33	1.99	185	0.37
Thymus vulgaris	500	453.33	0.91	105	0.21

On the other hand, the application of the extract of *T. vulgaris* significantly improved the growth of *Citrus volkameriana* seedlings compared to the control (Table 5). Results showed that in the presence of *T. vulgaris* extract the shoot and roots length as well as the shoot and root dry weight were significantly similar to the control NIN (non-inoculated with nematode) but significantly higher than in the control IN (inoculated with nematode). About 24.06 % with aqueous plant extract compared to 18.66 g in control IN.

Table 5. Effect of the leaf extract of *Thymus vulgaris* on *Citrus volkameriana* growth parameters under greenhouse conditions.

	Roots			Shoot			
Treatment	Fresh weight (g)	dry weight (g)	length (cm)	Fresh weight (g)	Dry weight (g)	length (cm)	Breast height (cm)
Control IN	18.60±0.70^{ab}	7.10±0.39^{b}	15.40±0.10^{d}	56.00±1.15^{c}	16.20±1.74^{c}	64.00±0.49^{b}	3.70±0.19^{a}
Control NIN	23.30±0.59^{ab}	11.60±0.75^{ab}	22.90±0.04^{ab}	96.80±1.63^{ab}	26.50±0.25^{ab}	76.80±1.34^{a}	3.60±0.13^{a}
T.vulgaris	24.00±0.92^{ab}	11.00±0.11^{ab}	22.20±0.27^{abc}	85.90±1.72^{ab}	21.60±0.77^{bc}	75.60±0.95^{a}	4.10±0.25^{a}

Numbers in the same column followed by different letters are significantly different based on Tukey's test ($P < 0.05$).

4. Discussion:

Developing new alternative environmentally-friendly practices to reduce and/or eliminate the use of synthetic chemical nematicides is now a major priority for global agriculture. Various investigations highlighted the benefits potential of plant ingredients to manage plant diseases **[14]**, **[18]**, **[19]**, but very few studies have dealt with the effect of natural products as nematicides against the citrus nematode *Tylenchulus semipenetrans* **[20]**. The present study showed acceptable levels of effectiveness of leaf extract of *Thymus vulgaris* against the citrus nematode *Tylenchulus semipenetrans* under laboratory and greenhouse conditions. This finding is consistent with several previous studies in which plant-parasitic nematodes were effectively suppressed by various plant extracts **[21-23]**. In this study, *Thymus vulgaris* showed a strong in vitro nematicidal activity against *T. semipenetrans* in terms of both J2 mortality and egg-hatching suppression. It can cause more than 77% mortality of juvenile and complete egg hatch inhibition of citrus nematode *Tylenchulus semipenetrans.* The efficiency of *Thymus vulgaris* varied significantly according to the extract concentration and time of incubation. Indeed, low extract concentration (1%) induced J2 mortality rate ranging between 11.22% and 31.29%, and egg hatch reduction of 56.33% after 24h and 48h of incubation respectively. This results agree with the finding of other studies **[24-26]** confirming the toxicity of thyme on plant-parasitic nematodes. According to LC50 and LC90, thyme achieved 7.12% and 9.68%, respectively after 24 and 48 hours. Massoud *et al.* (2022) **[27]** reported LC50 and LC90 values of 44.75% and 13.57% for thyme against *Meloidogyne incognita* after 24 and 48 hours respectively. In greenhouse experiment, thyme extract significantly reduced the nematode parameters. Results showed that the extract of *T. vulgaris* significantly increased the fresh weight, dry weight of roots and shoots compared to control IN (with nematode inoculation). Sasanelli *et al.* (2008) **[28]** reported that the application of aromatic and medicinal plants, as soil amendments, significantly suppressed several species of PPNs. Massoud *et al.* (2016) **[29]** indicated that the application of plant extracts increased the vegetative growth parameters such as shoot length, and shoot fresh and dry weights in citrus. The ability of plant extract to inhibit and control plant diseases is related to some of their natural compounds such as sterols, saponins, tannis, alkaloids and flavonoids **[30]**. Indeed, these compounds released into the soil penetrate root tissues thereby reducing the nematode population in terms of inhibiting egg hatching and inducing the motility of juvenile nematodes **[31-32]**.

4. Conclusion:

The aqueous extract of *Thymus vulgaris* exhibited high nematicidal activity against *T. semipenetrans* in terms of both J2 mortality and eggs hatch inhibition. The toxicity of thyme extract increased with increasing extract concentration and time of incubation, reaching 77% of J2 mortality after 96h incubation, and 0% egg hatch after 144h of incubation at concentration of 10 %. This suggests that *Thymus vulgaris* extract can act as a bio-control agent, reducing nematode population densities below the threshold level. Furthermore, it is a safer and more economical method of nematode management. However, further study is needed to verify the efficacy of this plant extract in field environment.

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