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Investigating the Inhibitory Effect of Some Plant Crude Extracts against Root-Knot Nematode (*Meloidogyne javanica*) in Cucumber Plant

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ABSTRACT

Some plant extracts contain elicitors for inducing systemic resistances in treated plants like the lavender extract, whose protective effects against pathogen proved to be by activating the SAR systemic defense pathway. In this study, plant crude extracts including *Azadirachta indica* (neem), *Tagetes erecta* (marigold), *Thymus daenensis* (thymus), and *Carum carvi* (caraway) were used to control Root-Knot Nematode (RNK) *Meloidogyne javanica* in cucumber. First, the effect of these extracts on egg hatching and juvenile mortality was investigated, then, the effect of plant extracts on characters such as numbers of egg masses, nematode galls, and eggs per plant root was evaluated under greenhouse conditions. In the third part of this study, the effect of plant extracts on Polyphenol Oxidase (PPO), Peroxidase (POX), Catalase (CAT), Phenylalanine Ammonia-Lyase (PAL) and β(1,3) Glucanase (β-1, 3-Glu) enzyme activities was studied. Finally, the expression level of three stress enzymes genes including CAT, PPO, and β-1, 3-Glu β-1, 3-glu was evaluated by Real-time RT-PCR method. Results showed that, on the second day after treatment, 500 and 2,500 ppm concentrations of aqueous neem extract inhibited 64.79 and 73.48% of eggs hatching, respectively. In the greenhouse conditions, the four studied plant extracts (neem, marigold, Thymus, and caraway) at 1,500 ppm concentration significantly suppressed the development and reproduction of *M. javanica* terms of eggs/plant root, egg-masses, numbers of galls, nematode population in soil and, consequently, enhanced growth of the plants. In addition to the activity of the enzymes, the expression levels of these defense enzymes were also increased by the use of plant extracts.

Keywords: Egg hatching, Defense enzymes, Gene expression, Parasitic nematodes.

INTRODUCTION

Plant parasitic nematodes, especially Root-Knot Nematodes (RKN) of the genus *Meloidogyne*, are widely distributed and cause significant yield losses in a wide range of crops (Archidona-Yuste et al., 2018; Naserinasab *et al*., 2011).

Root-knot nematode, *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Heteroderidae) is a major plant-parasitic nematode species affecting the quantity and quality of the crop production in many annual and perennial crops (Siddiqui *et al*., 2001).

The defense responses reactions can include the damaging Reactive Oxygen Species generation (ROS). Plants-ROS such as Hydrogen peroxide (H_2O_2), superoxide (O^{-2}), hydroxyl radical, singlet oxygen, and alpha-oxygen produced mostly by products of various metabolic pathways that are done in different ranges of cellular section (Gimenez *et al*., 2010). In this case, under

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the stressful situations of cells, their formation could raise to excess the antioxidant scavenging capacity burden, therefore, creating oxidative stress pressure by reaction and damage to all the parts of the active cells (Gholamnejad, 2009). Many researchers believe ROS are the main agents that contribute to stress injuries and cause rapid cellular damage, especially when plants are subject to the stress factors such as drought stress, salt stress, biotic stress such as fungal pathogen, viruses and plant parasitic nematodes such as root-knot nematode, in comparison to animal parasitic nematodes (Aly and El-Beltagi, 2010; Afify and El-Beltagi, 2010). However, not much is known about the defense proteins employed by plant parasitic nematodes. Peroxidase, polyphenol oxidase and catalase have been discovered in some endoparasitic nematodes but not much is known about the roles of these proteins in the host-parasite interactions and none of these proteins are characterized in detail (Molinari and Miacola, 1997). Alterations of plant enzymes mainly peroxidase, polyphenol oxidase, catalase, superoxide dismutase, and protease in the tissues of susceptible and resistant host varieties infected with the nematodes were extensively studied (Molinari, 2009; Zacheo *et al*., 1983).

In cucumber and tomato, numerous reports in literature illustrated the influence of the root-knot nematode on roots phenol content, lipid peroxidation, antioxidants and peroxidase activities as defense mechanism against nematode infection (Aryal *et al*., 2011).

One of the possible alternatives is the utilization of nematicides from plant origin, known as natural and botanical pesticides (Anwar *et al*., 2009). Many phytochemical compounds with diverse structures have been isolated and detected from a large number of plant families (Portillo *et al*., 2013). A majority of these nematicidal phytochemicals have been isolated from the plant families including Acalyphoideae, Asteraceae, Meliaceae and Lamiaceae (Tsay *et al*., 2004).

The objective of current research was to determine the efficacy of plant crude extracts derived from *Azadirachta indica* (neem), *Tagetes erecta* (marigold), *Thymus daenensis* and *Carum carvi* as alternative to chemical nematicides and investigate the effects of four different plants extracts against *M. javanica* under *in vitro* and *vivo* conditions.

MATERIALS AND METHODS

Nematode

Eggs of Root-knot nematode, *M. javanica*, were extracted from infected cucumber (*Cucumis sativus*) roots in the greenhouse in the Mohammad-Abad village of the Yazd Province, Iran, using 10% NaOCl (Hussey and Barker, 1973). Second-stage juveniles emerging from eggs spread on a 30 µm-pore sieve were collected daily and stored at 10°C for no longer than 7 days.

Plant Extracts Preparation

Four dry plants used in this research were collected from rangelands of Yazd (Shirkuh

Table 1. The plant extracts applied for root-knot nematode management, scientific name, common name and part used in this study

Scientific name	Plant name	Part used
Azadirachta indica	Neem	Seeds
Tagetes erecta	*Tagetes*	Leaves, flowers
Thymus daenensis Celak	Thyme	Leaves
Carum carvi	Caraway	Seeds

rangelands) Province, Iran (Table 1). Plant extracts were prepared by soaking 20 g of powdered organs (Table 1) of the plant in 200 mL of distilled water in the dark bottle for a day. Plant material and solution were squeezed through cotton fabric and then the aqueous materials were filtered through Whatman filter paper No. 2. Then, the filtrate was allowed to centrifugation at 5000 rpm for 10 min to remove any debris. The supernatants thus collected were designated as 'stock solutions'. With these stock solutions, different dilutions of 2500, 1500, 1,000 and 500 ppm were prepared by adding the required amount of sterile distilled water (Khan *et al*., 2019).

In Vitro Experiments

The nematicidal activity of the four plant extracts was assayed at the four concentrations including 500, 1000, 1500, and 2,500 ppm in a water (distilled water) against RKN. For egg hatching assay, eggs suspension was adjusted to 150 eggs per mL of distilled water and distributed in 24-multiwell plates (250 µL per well). For the treatment, 250 µL of the aqueous extract prepared with phosphate buffered saline (PBS) at different concentrations was added and incubated at 23±1.5°C for 2, 4, 6 and 8 days. After incubation, hatched juveniles were counted under light microscope (Nicon, E200, Japan) and egg hatching and inhibition percentages was measured using the following fomula (Naserinasab *et al*., 2011:

Inhibition of egg hatching percentages (%) = (c-t)/c×100 [1]

Where, *c* and *t* are the numbers of hatched nematodes in control and treated plants, respectively.

1.5 mL of distilled water contained double concentrations of tested extracts on glass vial were added to 1 mL of a nematode suspension containing 80 J2 of RKN in a 5 mL plastic tube. The numbers of the live and dead nematodes were counted under a light microscope 15, 30, 45 and 60 hours after treatments at 23±1.5°C (Khan *et al*., 2016). After the various exposure periods, J2 were transferred to distilled water for 24 h to°heck revival in nematode, and mortalized nematodes were counted by microscope. The corrected percentages of nematode mortality were calculated according to Abbott's (1925) Formula (2) as cited by (Pavaraj *et al*., 2012):

Juvenile mortality (%)= (a-b)/(100-b) ×100 [2]

Where, *a* and *b* are percentages of mortalized juveniles in the treatment and control, respectively.

Greenhouse Assay

This experiment was performed to investigate the effect of plant extracts on *M. javanica* in greenhouse conditions. The plastic pots (25 cm diameter) were filled with 5 kg soil (mixture of autoclaved sand and clay with 2:1, v:v) and two seeds of cucumber (*Cucumis sativus* cv. Negin) were sown. After 5 weeks, plants were thinned to two plants and Nemacur and Rugby were applied at the rate of 2.5 g pot^{-1}. Ten days after plant thinning, four pots for each treatment were incubated (soil drenching) with 100 mL of four plant extracts (neem, marigold, thymus and caraway) for pot with 1,500 ppm concentration, and after 24 hours, each seedling was inoculated (in five holes of 4 cm depth made around the plant) with 3,000 newly hatched J2 per pot. The plastic pots were arranged in a completely randomized design in a glasshouse at 24±1.5°C under natural daylight length conditions. Plants were irrigated every 48 hours, and fertilized twice a month with commercial fertilizer with N (20%), P (20%), K (20%) (Green More Company, Iran) at the rate of 2.5 g L^{-1} of water until the end of the experiment (Naserinasab *et al*., 2019).

Treated plants were uprooted 70 days after nematode inoculation and roots were washed with a tap water. The plant growth indicators including the root and shoot lengths (cm)

Table 2. Cucumber defense-related genes examined in this study and the specific primers used in quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR).

Gene names	Sequences	Melting temperature	Length
β (1,3) Glucanase	5' AAATCCCAGCTCTCCAACAT 3'-F	58	149
	5' AACAGGGATGTGTAGAACCT 3' -R	58	
Catatlase	5' AGCGCTCACCTGGTTAAGAAT 3'-F	60	138
	5' ACCAGTGGGATCCTGTCCTAAC -R	62	
PPO	5' CGATAGCCAGATTCCAAAGAC 3'-F	62	256
	5' AGGTTGCCAGCGACCTACAACG -R	66	
Tef1α	5' AATTATGCTGGCATAGTGAAG 3'-F	64	270
	5' GATGACACCAACAGTTAAAG 3'-R	62	

and fresh dry weights (g) were recorded. The plant roots were stained in Phloxine B solution (0.015%) for 20 minutes (Holbrook *et al*., 1983) and numbers of egg masses, nematode galls, and eggs per root were counted and recorded (Taylor and Sasser, 1978). J_2s were extracted and counted from 300 cm^3 soil per pot using the bucket sieving technique (Cobb, 1918).

Reduction percentage of nematode parameters compared to the control plants was calculated with the following formula (Abdellatif, 2016):

Reduction %= [(Numbers in the control treatment−Numbers in the treated plants)/Number in control treatment]×100.

Reproduction Factor (RF) was also calculated as follows:

(RF)= Pf/Pi,

Where, Pf= Final nematode population= Number of eggs/Plant+Number of J2/pot at the harvest time and Pi= Initial nematode population= 5,000 eggs and J2.

Defense Related Enzymes Activity Assay

The activities of five defense enzymes including Polyphenol Oxidase (PPO) (Shi *et al*., 2001), Peroxidase (POX) (Bania and Mahanta, 2012), Catalase (CAT) (Rahnama and Ebrahimzadeh, 2006), Phenyl Alanina Amonialyase (PAL) (Wang *et al*., 2006) and β(1,3) Glucanase (β-1, 3-Glu) (Ashwell, 1957) were evaluated. Cucumber root were sampled on 0, 3, 6, 9, 15 and 20 days after nematode inoculation. Sodium phosphate buffer (0.1 mM, pH= 6) was used to assess the POX, CAT, and PPO activities and β-1, 3-Glu enzyme activity was evaluated with sodium acetate (0.05 mM, pH= 5). Root samples were homogenized in an ice cooled solution contain 50 mM Tris-HCl buffer (pH 8.8) and 15 mM β-mercaptoethanol for PAL enzyme assay. The mixture was centrifuged at 10,000 rpm for 15 minutes at 22±3°C and the supernatant was collected to measure enzymatic activity. Total protein concentration was measured according to the Bradford method (Bradford, 1976) using BSA (bovine serum albumin) as standard protein.

Four plants (replicates) per treatment were randomly collected at each specific sampling time, rinsed with demineralized water and stored at -80°C for further analysis. The same plants used for enzyme activity were also used to measure gene expression.

Gene Expression of the Defense Enzymes Using Real-time PCR

Total RNA was extracted from the treated plants at 0, 3, 6, 9, 15 and 20 days after nematode infection using RNXplus isolation kit (Cinnagen, Iran) according to the manufacturer's instructions. Extracted RNA was treated with DNase using a Qiagen RNase-Free DNase Set (Qiagen, Valencia, CA). Total RNA was visualized on a 1.6%

agarose gel and then quantified and assessed for purity using a UV–visible spectrophotometer (CECIL 9500 Model, UK). Then, first-strand cDNA was synthesized from total RNA with oligo dT primers, dNTPS and RevertAid Reverse Transcriptase (Thermo Scientific, Germany) according to the standard instructions. The expression of *β (1,3) glucanase, catatlase and polyphenol oxidase* genes were evaluated by Real-time PCR. The cucumber TeF1α gene was used as internal reference (housekeeping) gene (Paolacci *et al*., 2009; Gimenez *et al*., 2010). The following primers were used in the RT-PCR reaction, as shown in the Table 2.

The Real-time PCR Reaction (20 µL) contained 10.5 µL of 2×Yekta tajhiz Azma SYBR® Green RT Mix (Yekta tajhiz Azma, Iran), 1 µL of 10 nmol μL^{-1} of each primer, 1 µL of template cDNA (50 ng), and 7.5 µL of RNase free water. The time schedule of Real-time PCR reaction included an initial denaturation at 95°C for 10 minutes followed by 42 cycles of denaturation at 95°C for 12 seconds, annealing at 60, 62, 58 and 57°C for 30 seconds and extension at 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. The reaction was performed using a Rotor-Gene 6000 (Corbett RG-6000, Australia). Relative quantification of gene expression was performed by (ΔCT= CT–Reference gene, ΔΔCT= CT–Control, and ΔΔCT expression= $2^{(-\Delta\Delta CT)}$ (Livak and Schmittgen, 2001). The expression levels of the target genes were normalized relative to *TeF1α* gene and relative expression of untreated control plants at each time were set as ΔΔCT. The control plants were treated only with pathogen, and distilled water was used instead of plant extract. Gene expression experiments consisted of two technical replications and two biological replications.

Statistical Analysis

All experiments were carried out in a completely randomized design. Each treatment consisted of four replicates. When the analysis was statistically significant, Duncan's Multiple Range Test (SSR Test) was used to test mean separations among mean values of each treatment.

RESULTS AND DISCUSSION

The plant extracts affected the nematode hatching rate. In the control group, hatching rate was increased from 29 on the second day to 82 eggs on eighth day. The nematicidal effect of the used concentrations (500, 1,000, 1,500 and 2,500 ppm) of neem, thyme, marigold and caraway as well as the control nematicide Vapam (2×1000), on the egg hatching and J2 mortality of *M. javanica* was evaluated. Results showed that, on the second day of treatments 500 and 2,500 ppm, neem extract inhibited 64.79 and 73.48% of eggs hatching, respectively. All the studied plant extracts at the concentrations of 500, 1,000, 1,500 and 2,500 ppm suppressed ($P \leq 0.01$) the egg hatching at 2, 4, 6 and 8 days after nematode inoculation compared to the untreated control plants (Table 3). However, *T. erecta* extract showed the most effect in decreasing eggs hatchability, as egg hatching inhibition rate increased from 3–5 to 75.14–84.28% after the exposure for 2 days. Relatively acceptable reduction in egg hatching and inhibition of egg hatchability was observed with the increase in plant extract concentrations. Vapam showed severe suppression in nematode eggs hatchability (0.41-3.30%) with 97.86-93.94% inhibition rate upto 2 days after treatment (Table 3). Moreover, Vapam (2×1000) caused severe suppression of the hatchability of nematode eggs (0.62-4.96%) with 97.86-93.95%, after treatment with the Vapam and the largest inhibition in egg hatching was recorded for the *T. erecta*, which was 4.56-19.63 and 84.28-76.06%, respectively. These findings agreed with the results obtained by Krishna and Dabar (2004), who studied different concentrations of shade-dried leaves, bark and kernels of neem for their inhibitory roles

Table 3. Effect of plant extracts against egg hatchability and inhibition percentages (%) of *Meloidogyne javanica* under *in-vitro* conditions.[A]

Treatments	Inhibition egg hatchability (%)					Exposure time (Days)			
	Concentration (ppm)	2	%	4	%	6	%	8	%
Conrol		29.00a	0.00	43.00a	0.00	66.00a	0.00	82.00a	0.00
Azadirachta indica	500	10.21jk	64.79	22.31hi	48.12	32.12hij	51.33	59.62gh	27.29
	1000	9.63kl	66.79	17.62kl	59.02	24.96lmn	62.18	37.12mn	54.73
	1500	8.36lmn	71.17	16.21lm	62.30	22.36mno	66.12	33.25no	59.45
	2500	7.69mn	73.48	12.96oq	69.86	19.63no	70.26	29.65pq	63.84
Thymus daenensis	500	22.12b	23.72	39.16b	8.93	53.20b	19.39	75.54b	7.87
	1000	20.32bc	29.93	37.12bc	13.67	49.36bc	25.21	72.12bc	12.05
	1500	18.21cd	37.21	32.21de	25.09	44.25cde	32.95	64.32ef	21.56
	2500	17.52de	39.59	30.62ef	28.79	43.21de	34.53	62.85fg	23.35
Tagetes erecta	500	7.21mno	75.14	15.25mn	64.53	23.54mn	64.33	32.58nop	60.27
	1000	6.96no	76.00	13.96no	67.53	19.85no	69.92	27.85qr	66.04
	1500	4.98qr	82.83	10.25qr	76.16	15.52q	76.48	24.20rs	70.49
	2500	4.56r	84.28	9.06rs	78.93	12.89r	80.47	19.63t	76.06
Carum carvi	500	16.98ef	41.45	31.25def	27.33	45.96cd	30.36	68.25de	16.77
	1000	14.85gh	48.79	28.42fg	33.91	39.85fg	39.62	58.96ghi	28.10
	1500	11.52ij	60.28	24.89	42.12	33.20hi	49.70	49.85jk	39.21
	2500	9.36kl	67.72	19.26ijk	55.21	27.44jk	58.42	41.85lm	48.96
Vapam	2.5×1000	0.62s	97.86	1.20s	97.21	3.78s	94.27	4.96u	93.95

[A] Each value is the mean of four replicates. Means within a column followed by a common letter are not different according to Duncan's multiple range test ($P \leq 0.01$).

in egg hatching of *M. incognita*. In general, with the increase in concentration and exposure period, there was a significant reduction in egg hatching. Neem kernels S concentration (Stock solutions), proved to be most effective and did not allow hatching even up to 11 days after exposure, and its lowest concentration (S18) allowed 69.22% hatching (91.66) compared to the control. Neem bark was the least effective among the other treatments as its S, S/4 concentrations allowed 69.8 and 88.1% eggs hatching, respectively. Our result showed that after *T. erecta* extract, *A. indica* extract had the highest effect for egg hatching with 2,500 ppm concentration and 73.48% inhibition of egg hatchability.

The second stage of juvenile's results revealed that all studied plant extracts significantly ($P \leq 0.01$) increased J_2 mortality after 15, 30, 45 and 60 hours compared to the untreated inoculated control plants. In addition, there was an increase in J_2 mortality percentages with increasing the concentrations and exposure time. Both marigold and neem extracts appeared to be the most effective plant extracts, as they caused 81.36 and 70.85% mortality of J_2 juvenile *in vitro* after 15 hours of exposure to the concentration of 2,500 ppm, respectively, the nematicidal activity of

marigold was significantly higher than vapam (69.25%).

In the greenhouse conditions and into the soil, the four studied plant extracts (neem, thyme, marigold and caraway) at 1,500 ppm concentration, and the comparable nematicide vapam at 5 µL mL^{-1}, significantly suppressed (at $P< 0.01$) the development and reproduction of RKN , in terms of eggs/plant root, egg-masses, numbers of galls, and nematode population in soil. Consequently, they enhanced the growth of plants ($P< 0.01$) with no toxic effects on the cucumber plants. All plant extracts significantly ($P\leq 0.01$) reduced the number of eggs/plant root, egg-masses, numbers of galls, number of J2/300 cm^3 of soil than the untreated inoculated plants (Table 5). As shown in Table 5, the nematode suppressive effect of *T. erecta* was superior to all other botanicals in terms of number of eggs/plant, i.e. the least egg masses number was found in the roots of plants grown in soil amended with *T. erecta* (21698), followed by *A. indica* (45879), and *C. carvi* (68542), while *T. daenensis* was found least effective against nematode with 80,254 eggs/plant as compared to untreated inoculated control (106985).

Generally, the nematicide vapam (5 mL L^{-1}) had the greatest effect on reducing the number of galls per root by 96.89%; while *T. erecta* was the most effective plant extract, resulting in 78.10% reduction, followed by *A. indica*, *C. carvi* and *T. daenensis*, with 74.05, 58.91, and 43.24% reduction, respectively (Table 5 and Figure 1). The same trend was observed in number of egg masses/plant root, number of eggs/plant root, and number of J2/300 cm^3 of soil. The reduction percentages recorded in egg masses per plant root due to the same mentioned treatments were 98.66, 77.58, 73.89, 63.30, and 43.34, respectively. These treatments reduced number of eggs/plant root by 99.36, 79.71, 57.11, 35.93, and 24.98%. Treatments also suppressed nematode J2 in the soil by 98.33, 77.38, 67.61, 55.00, and 27.38%, respectively; with significant difference between all of the cucumber plants treated with four plant extracts. The highest reduction percentages in Reproduction Factor (RF) were recorded by Oxamyl 24% SL (5 mL L^{-1}) (99.6%) followed by *U. fasciata* (75.7%), C. officinalis (62.4%), and *C. mediterranea*(40.3%) (Figure 1). The effect of plant extracts and the nematicide vapam (5 mL L^{-1}) on growth parameters of nematode infected cucumber plants under greenhouse conditions is shown in Table 6.

Evaluating the growth parameters of treated cucumber plants showed that the plant extracts and vapam (5 mL L^{-1}) significantly ($P\leq 0.01$) increased dry and fresh weights of the treated cucumbers as well as the number of the shoots and the length of the roots compared to un-infected control. However, shoots and roots fresh weights and lengths were similar between the nematicide, vapam (5 mL L^{-1}) and *T. erecta*. The cucumber plants treated with Vapam were not significantly different from the uninfected control (healthy). There was a significantly ($P\leq 0.01$) difference between the cucumber treated with vapam and *T. erecta* extract for shoots and roots dry weights.

Several studies revealed the nematicidal activity of the plant extracts because it contains different compounds that have inhibitory effect against the plant pathogen nematodes (Abubakar *et al*., 2004; Asif *et al*., 2014; Tariq *et al*., 2018). However, treatment of cucumber plants with these plant extracts can enhance growth parameters, which may happen because of the presence of some growth-promoting compounds in the plant extract (Ganai, 2014). The use of organic amendments and plant extracts are gaining interest because of being a safe and environmentally friendly approach.

Defense Enzyme Activities

Application of plant extracts has increased in both infected and uninfected cucumber plants. Enzyme activity in cucumbers treated

Table 4. Effect of four plant extracts on egg J_2 mortality of *Meloidogyne javanica* (%) of the root-knot nematode under *in vitro* conditions.

Treatments		Exposure periods (h)			
		15	30	45	60
Conrol	Concentration	12.98p	17.69r	18.63	21.69jk
Azadirachta indica	500	54.48^{j}	47.60^{k}	46.23^{hio}	37.74gh
	1000	60.98^{hi}	51.58^{ij}	49.17^{fgh}	42.25ef
	1500	65.85^{fg}	54.85^{hi}	53.20^{ef}	45.32de
	2500	70.85^{cde}	61.25^{ef}	58.95^{cd}	50.98c
Thymus daenensis	500	27.74^{o}	20.08^{q}	18.89^{o}	14.45m
	1000	30.98^{no}	25.32^{p}	19.19^{no}	16.84lm
	1500	34.17^{mn}	33.85^{no}	22.50^{n}	21.52jk
	2500	39.45^{l}	34.58^{no}	29.85^{m}	22.85ij
Tagetes erecta	500	69.38^{cde}	58.19^{fg}	50.25^{fg}	42.11ef
	1000	74.36^{cd}	64.85^{de}	56.89^{de}	46.96de
	1500	79.69^{ab}	70.12^{bc}	60.65^{c}	50.52c
	2500	81.36^{a}	71.85^{ab}	65.85^{b}	54.85b
Carum carvi	500	49.12^{k}	42.17l^{m}	36.98^{kl}	30.87
	1000	54.12^{j}	45.87^{kl}	38.98^{jk}	33.96
	1500	60.25^{hi}	51.85^{ij}	42.02^{ij}	41.70fg
	2500	66.34^{ef}	52.25^{ij}	48.85^{gh}	42.20ef
Vapam	2.5×1000	69.25^{de}	74.89^{a}	82.65^{a}	92.24a

Table 5. Effect of assessed leaf extracts on galls, egg masses, eggs on roots, and second stage J2 population of *Meloidogyne javanica* in soil of cucumber under the greenhouse conditions. [a]

	Number of eggs/Plant	Number of egg masses/Plant	Number of galls/Plant	Number of J2/300 cm^3 of soil
Treatment				
Azadirachta indica	45879d	106de	96de	136d
Thymus daenensis	80254b	230b	210b	305b
Tagetes erecta	21698e	91e	81e	95e
Carum carvi	68542c	149c	152c	189c
Infected Plants	106985a	406a	370a	420a
Vapam	680f	5.4f	11.6f	7f

[a] Each value is the mean of four replicates. Means within a column followed by a common letter are not different according to Duncan's multiple range test ($P \leq 0.01$).

with nematodes, extracts and combinations of these two agents increases over time. The results showed the activities of Peroxidase (POX), Polyphenol Oxidase (PPO), Catalase (CAT), Phenylalanina Amonialyase (PAL) and β(1,3) glucanase enzymes in the healthy plants, as control, remained relatively stable, while inoculation with *M. javanica* significantly increased the enzyme activities during 0 to 20 days after nematode inoculation.

All treatments significantly increased the defense enzyme activity compared to healthy and infected untreated tomato plants. These activities were significantly enhanced in plants treated with the *T. erecta*, *A. indica*, *C. carvi* and *T. daenensis* extracts and the nematicide vapam (5 mL L^{-1}). POX, CAT, PPO, PAL and β-1, 3-Glu activities of all treatments began to increase significantly at 3-days after nematode inoculation, reached the highest values at 9 days, and then decreased gradually at 15 and 20 days

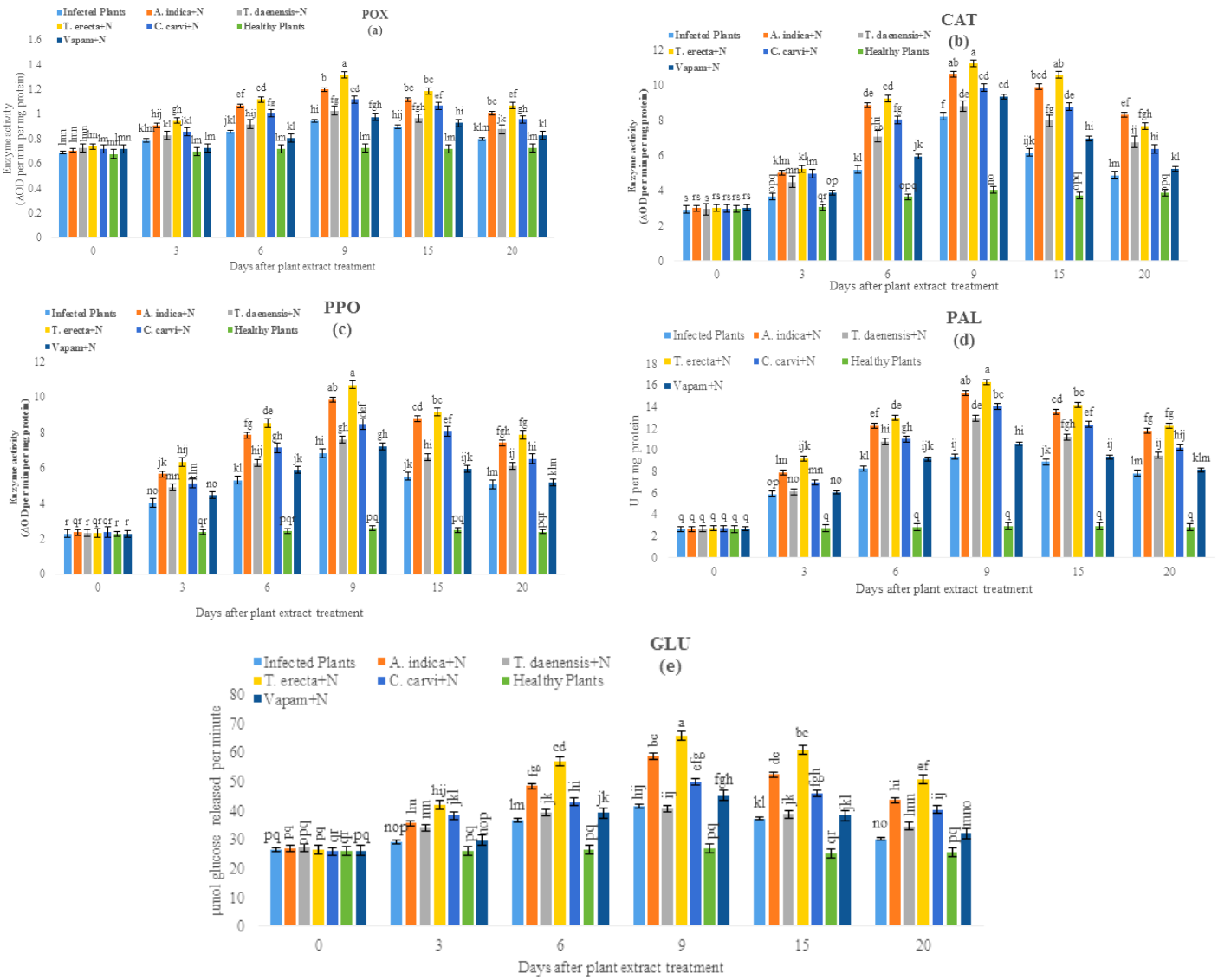

Figure 1. Activities of POX (Peroxidase) (a), Catalase (CAT) (b), Polyphenol Oxidase (PPO) (C), Phenylalanine Ammonia Lyase (PAL) (D) and β (1,3) Glucanase (Glu) enzymes in the roots of cucmber plants inoculated with *M. javanica* (N) and treated with *T. erecta*, *A. indica*, *C. carvi* and *T. daenensis* extracts and the Nematicide (N), vapam. Untreated un-inoculated plants served as control (Healthy control). Values are mean of three replicates, number in the same column followed by the same letter are not significantly different at P< 0.01 according to Duncan's Multiple Range Test.

after nematode inoculation, but still remained at a relatively higher level than day 0. Incubated cucumber plants treated with *T. erecta*+Nematode (Te+N) resulted in the highest activity level of all the studied enzymes. These activities reached a maximal level at 9 days after nematode inoculation and were approximately 1.38-,1.36-, 1.56-, 1.74-, and 1.58-fold higher than that in nematode-inoculated plants, and 1.80-, 2.76-, 4.13-, 5.60- and 2.42-fold that in un-inoculated control healthy plants for POX, CAT, PPO, PAL and GLU, respectively [Figure 1 (a-e)]. However, the activity of POD did not differ significantly be-tween *T. erecta* and *A. indica* at 0 and 9

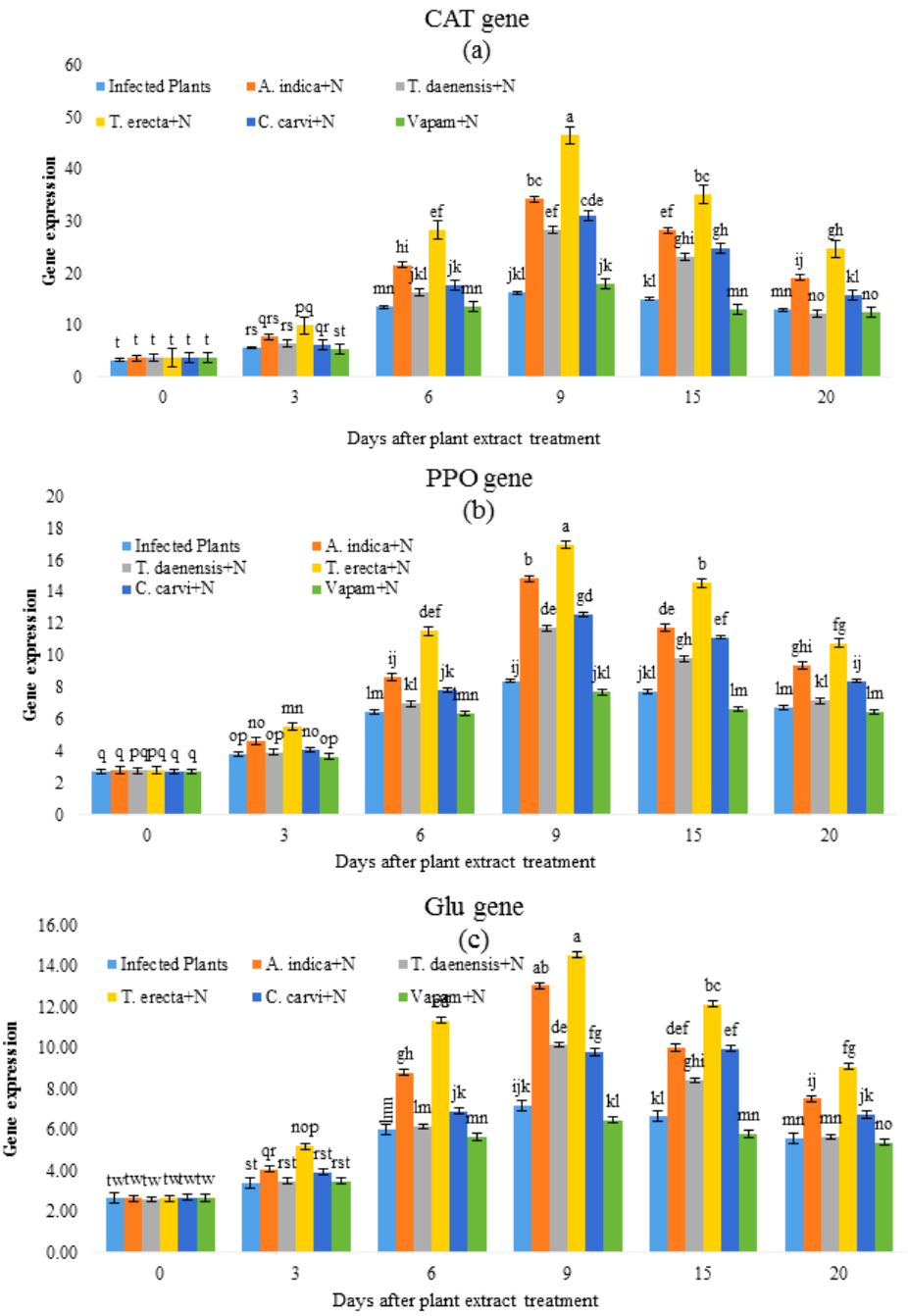

Figure 2. CAT (a), PPO (b) and GLU (c) relative gene expression in roots of cucumber plants inoculated with *M. javanica* and treated with *T. erecta*, *A. indica*, *C. carvi* and *T. daenensis* extracts and the Nematicide (N), vapam. Untreated un-inoculated plants served as Control (C). The expression level of the target genes were normalized relative to Tef1α gene and relative expression of untreated control plants at each time. Values are the mean of three replicates. The means with different letters have a significant difference at P≤0.01 according to Duncan's Multiple Range Test.

Table 6. Effect of four plant extracts on the cucumber plant growth indices infected by *Meloidogyne javanica* in relation to plant growth of cucumber in pots in the greenhouse.[A]

	Fresh weight (g)				Length (cm)				Dry weight (g)			
Treatment	Shoot	%	Root	%	Shoot	%	Root	%	Shoot	%	Root	%
Azadirachta indica	68.96d	49.13	33.92c	63.94	39.52cd	32.35	31.69cd	22.58	6.92d	66.35	4.01de	56.64
Thymus daenensis	51.78f	11.98	24.42de	18.03	32.69f	9.48	26.91ef	4.10	5.19f	24.76	3.27f	27.73
Tagetes erecta	74.20bc	60.47	38.96ab	88.30	43.85b	46.85	34.49bc	33.41	7.46c	79.33	4.36cd	70.31
Carum carvi	60.17e	30.13	27.12d	31.08	37.96de	27.13	29.25de	13.15	6.12e	47.12	3.89ef	51.95
Infected Plants	46.24g	-	20.69ef	-	29.86fg	-	25.85f	-	4.16g	-	2.56g	-
Healthy Plants	82.20a	77.77	41.35a	99.86	50.25a	68.29	39.75a	53.75	8.14a	95.67	5.89a	130.08
Vapam	79.65ab	72.25	40.25a	94.54	48.36ab	61.96	36.98ab	43.04	8.03ab	93.03	5.66ab	121.09

[A] Each value is the mean of five replicates. Means within a column followed by a common letter are not different according to Duncan's multiple range test ($P \leq 0.01$).

days after nematode inoculation (Figure 1-a). Moreover, a significant difference was observed in CAT activity between *T. erecta* and *A. indica* at 3 to 6 sampling days (Figure 2-b). PPO activity had the same rate with the activity of CAT and POX. Enzyme PPO activity showed a significant difference between treatments *T. erecta* and *A. indica* from the third to the ninth sampling days, but these two treatments on days 0, 15 and 20 did not show a significant difference in terms of enzyme activity. The activity of enzymes PAL and GLU increased until the ninth day and then decreased from the ninth to the twentieth day. All enzymes showed the highest activity in cucumber plants treated with *T. erecta* extract on all sampling days.nce

These findings confirm that the use of plant extracts induced the host plant defense system against the pathogen. However, plant extracts might help in stimulating the plants' enzymatic and non-enzymatic antioxidative systems, which play an important role in plant defense against the pathogens (Hammerschmidt, 2012). Also, the high production of the antioxidant compounds such as phenols, ascorbic acid, reduced glutathione, flavonoids, and carotenoids plays an important role in plant defense against the plant pathogens (Molinari, 2009; Gill and Tuteja 2010; Gholamnezhad *et al*., 2013).

Catalase, Polyphenol Oxidase, and Glucanases Gene Expression

Quantitative Real-time RT polymerase chain reaction was applied to determine the transcript levels of three defense related genes, which are commonly considered as responsive to RKN attack. Three expression levels of five defense related genes including CAT, PPO, and GLU were relatively measured by quantitative real-time PCR in roots of cucumber plants inoculated with *M. javanica* and treated with different plant extracts. The expression profile of these genes was analyzed at 0, 3, 6, 9, 15, and 20 days after nematode inoculation (Figure 2). All of the applied treatments induced the three studied genes including CAT, PPO, and GLU compared to the basal level in healthy plants as negative control. The highest gene expression level was observed on the ninth day after pathogen inoculation in all of the treated plants for five assessed genes compared to the control and, then, it decreased at 15 and 20 days after pathogen inoculation. However, the studied genes were induced early (3 days

after nematode inoculation) in treated plants. The highest peak of CAT, PPO, and GLU accumulation (46.54-, 16.94-, and 14.56-folds) appeared at 9 days after nematode inoculation in nematode infected cucumber plants treated with *T. erecta* and nematode, respectively, higher than the control. When the cucumber roots were infected with nematode alone, the significant induction of five genes was observed but it was less compared to the plants inoculated with pathogen and plant extracts. Despite that, the gene expression was always higher than the plants treated with Vapam. Our results confirmed the research of Medeiros *et al*. (2009), who showed that leaf coffee extract formulation (NEFID) could control the bacterial spot in tomato caused by *Xanthomonas vesicatoria*. The transcriptional changes in tomato leaves elicited with NEFID was evaluated by using a tomato gene chip. It was observed that 268 genes were differentially regulated with a majority up-regulated and encoding defense responses, signal transduction, and transcription factors. Chitinases, polyphenol oxidase, glucanases and peroxidases, with reported activity against pathogens, were transcriptionally up-regulated and corresponding enzyme activities were over-expressed as early as 24 h post elicitation and remained elevated for up to five days after NEFID exposure.

The efficacy of savory and thyme essential oils was investigated against fungal pathogen *Botrytis cinerea* on apple fruit. Apple fruits treated with savory and thyme essential oils showed significantly lower gray mold incidence and severity. After 6 h of pathogen inoculation, thyme essential oil induced a 2.5-fold increase of PR-8 gene expression level compared to inoculated fruits. These results showed that thyme oil induces resistance against *B. cinerea* through the priming of defense responses in apple fruit (Banani *et al*., 2018; Gholamnezhad *et al*., 2016).

Increased activity of these enzymes is very essential for acquired resistance for plants defense against the infection nematodes, as confirmed by Arioli *et al*. (2015). However, it was well shown that phenol, flavonoid, and polyphenol compounds were synthesized at high amounts in resistant plants infected with nematodes because of the high level of these gene expression (Cabrera *et al*., 2014; Gholamnezhad, 2017; Gholamnezhad *et al*., 2019 and 2016).

Flavonoids can inhibit egg hatching and increase the larval motility (Wuyts *et al*. 2006), and rutin killed pre-parasitic stages of cyst nematodes (Faizi *et al*. 2011). Phenol and flavonoids levels increased in tomato roots infected with *M. Incognita*, and the increase was greatest in the resistant cultivar (Farahat *et al*., 2012).

CONCLUSIONS

In this study, extracts of plants including neem, tagetes, thyme and caraway were used against root knot nematode in cucumber plant. The studied plant extracts improved some plant features such as fresh and dry weights. The plant extracts also had an inhibitory effect on egg hatching, as well as decreasing the numbers of the eggs, egg masses, and gall per plant. When the activity of antioxidant enzymes in the treated plants were measured, all plant extracts, especially the targets, were able to increase the activity of the assessed enzymes compared to the control plants. Plant extracts are able to activate the plant's defense system against the nematodes and they can directly reduce their pathogenic parameters. Results of this research revealed that increased expression level of the studied defense related genes corresponded to the simultaneous increase in their coded enzymes activity.

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بررسی اثر بازدارندگی بعضی از عصاره‌های گیاهی علیه نماتد گره ریشه (*Meloidogyne javanica*) در گیاه خیار

ج. غلام‌نژاد، ف. ناصری‌نسب

چکیده

بعضی از عصاره های گیاهی، مانند گیاه اسطوخودوس، حاوی ترکیبات محرک، برای القا مقاومت های سیستمیک در گیاهان هستند، این عصاره ها باعث می شوند که مسیرهای دفاعی گیاه مانند SAR در گیاهان علیه بیمارگرها فعال شوند، و گیاه را در برابر بیمارگرها محافظت کند. در این مطالعه، از عصاره های گیاهی شامل چریش (*Azadirachta indica*)، گل جعفری (*Tagetes erecta*)، آویشن دنایی (*Thymus daenensis*) و زیره سیاه (*Carum carvi*) برای کنترل نماتد مولد غده (*Meloidogyne javanica*) در گیاه خیار مورد استفاده قرار گرفتند. در ابتدا، اثر عصاره ها بر روی تفریخ تخم و همچنین مرگ و میر لاروها بررسی، و سپس تأثیر آن‌ها بر روی صفاتی مانند تعداد توده تخم، تعداد گال نماتد و همچنین تعداد تخم در ریشه هر گیاه در شرایط گلخانه، مورد ارزیابی قرار گرفت. در قسمت سوم این مطالعه، تأثیر عصاره‌های مذکور بر فعالیت آنزیم‌های پلی فنل اکسیداز (PPO)، پراکسیداز (POX) ، کاتالاز (CAT) ، فنیل آلانین آمونیا لیاز (PAL) و همچنین β (1و3) گلوکاناز مورد مطالعه قرار گرفت و در نهایت میزان بیان ژنهای آنزیم‌های دفاعی با روش Real-time PCR ارزیابی شد. نتایج نشان داد که در روز دوم پس از اعمال تیمار، غلظت‌های 500 و 2500 ppm از عصارهٔ آبی چریش به ترتیب 64/79 و 73/48 درصد، به‌ترتیب، از تفریخ تخم نماتد جلوگیری کرد. در شرایط گلخانه، چهار عصارهٔ گیاهی مورد مطالعه (شامل چریش، گل جعفری، آویشن دنایی و زیرهٔ سیاه) با غلظت 1500 ppm، به طور قابل توجهی رشد و توسعهٔ نماتد *M. javanica* را از نظر شاخص تعداد هر تخم به ازای هر گیاه، تودهٔ تخم، تعئداد گال‌های نماتدی، جمعیت نماتدها در خاک و همچنین شاخص های رشد گیاه، کاهش دادند. علاوه بر افزایش فعالیت آنزیم‌ها، سطح بیان ژن این آنزیم های دفاعی نیز، با استفاده از عصاره های گیاهی افزایش یافت.