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MANAGEMENT OF ROOT-KNOT NEMATODE MELOIDOGYNE HAPLA ON STRAWBERRY PLANT WITH SOME PLANT EXTRACTS

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ПРИМЕНЕНИЕ ЭКСТРАКТОВ РАСТЕНИЙ ДЛЯ КОНТРОЛЯ ЗА ГАЛЛОВОЙ НЕМАТОДОЙ *MELOIDOGYNE HAPLA*, ПАРАЗИТИРУЮЩЕЙ НА РАСТЕНИЯХ ЗЕМЛЯНИКИ

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Резюме

Было изучено действие спиртовых экстрактов из 6 видов растений на систему галловых нематод, паразитирующих на растениях земляники. Была сделана оценка нематостатического и нематоцидного действия этих экстрактов. По результатам исследований, проведенных в производственных условиях было установлено, что наибольшую активность показал 0.6% экстракт *Tanacetum vulgare*. Благодаря его применению, была достигнута минимальная плотность *M. Hapla.* При обработке растений *Tanacetum vulgare* и оксамилом не было установлено разницы в значениях индекса обилия и численности нематод в корневой системе и почве. Не выявлено никакого статистически достоверного различия между группой растений, обработанной *T. Vulgare* и незараженной контрольной группой.

Ключевые слова: нематостатическое и нематоцидное действие, Meloidogyne hapla, земляника, экстракты растений.

Summary

The activity of alcohol extracts from 6 plant species was investigated with respect to the root-knot nematode-strawberry system. An evaluation was made of the nematostatic and nematicidal activity of the extracts. In production conditions, the most active was the extract *Tanacetum vulgare* at concentration 0.6%. It had the lowest *M. hapla* population density, with gall index and root/soil population densities not different than the oxamil treated plants and there were no stastically proved difference between *T. vulgare* and non-infested control.

Key words: nematostatic and nematicidal activities, *Meloidogyne hapla,* strawberry, plant extracts.

Introduction

Root-knot nematodes, *Meloidogyne* spp., are known to infect many agricultural crops including strawberry (*Fragaria x ananassa Duch.*). *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*, are among the most economically important species of root-knot nematodes. In particular, *M. hapla*, the northern root-knot nematode, is a serious pest of strawberries in the countries with moderate continental climate [11, 23]. In Bulgaria, a recent survey of nematodes associated with strawberry conducted in major strawberry producing regions show that *M. hapla* was one of the frequently encountered nematode plant pests with frequency of occurrence 26 to 41% and range of population density from 35 to 560 nematodes/100 cm^3 soil [22]. Control of these nematodes was achieved predominantly by synthetic nematicides, some of which are environmentally undesirable [23]. Therefore, it is necessary to find new alternative control strategies for protecting plants against attacks by these pests. Addition of organic materials can change physical and biological properties of soils and improve the plant resistant to soil-borne diseases and nematodes affecting plants. Toxicity effects from plant against nematodes may also occur [3]. Plants that have nematicidal effects are an alternative management for nematode suppression. There are many researches related with control of plant parasitic nematodes [3, 6, 8, 16]. Some plant extract were evaluated with laboratory and pot experiments for their nematicidal potentials. They can reduce ability of egg hatching and cause juvenile immobility and mortality, and also can improve plant growth [1, 10, 14, 21].

The objectives of our study were to evaluate some plant extracts, from amongst Bulgarian flora, for nematicidal or nematostatic activity on *M. hapla* in vitro, to use a strawberry - *M. hapla* model to evaluate at planting prophylactic applications on nematode population development and plant growth, and to identify plant extracts as potential replacements for oxamyl in strawberry production

Materials and methods

Plant materials were collected from mature plants of *Allium ursinum, Artermisia absinthium, Juglans regia, Salvia officinalis, Tagetes patula, Tanacetum vulgare* (Table1).

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Table 1.

List of Plants used against *Meloidogyne hapla* in the experiment

SN	Botanical name / Family	Plant part used
1	*Allium ursinum* L. / Amaryllidaceae	Leaves
2	*Artermisia absinthium* L. / Asteraceae	Foliage
3	*Juglans regia* L. / Juglandaceae	Small green fruits
4	*Salvia officinalis* L. / Limaceae	Leaves
5	*Tagetes patula* L. / Asteraceae	Flowers
6	*Tanacetum vulgare* L. / Asteraceae	Flowers

Preparation of Plant Extracts

Methanol extract. Air-dried powdered aerial plant parts of the 6 plant species (Table 1) were extracted by maceration with 80% methanol at room temperature for 24 h two times. After evaporation of the solvent the crude extract was subjected to subsequent analysis.

Concentrations of 0.15, 0.3 and 0.6%, used in our experiments were prepared with deionized water (DW) distilled water [18].

Culture of Meloidogyne hapla

The root-knot nematode *M. hapla,* originally isolated from *strawberry* cv. Marmolada (location *Berkovitca),* was cultured (from single egg mass) on tomato cv Tiny Tim in a glasshouse at 22-24°C. Egg masses of *M. hapla* were collected from galled tomato roots raised in the nursery. Eggs were extracted using the methods described by Hussey and Barker [9]. Eggs obtained were transferred into DW in a 50 ml beaker forming the egg suspension and their concentration determined by dilution counts. Second stage juveniles (J_{S2}) of *M. hapla* were extracted from galled tomato roots with egg masses using the methods described by Whitehead and Hemming [29]. Only freshly hatched J_{2S} were used for experiments.

In vitro toxicity experiment

At the experiment extracts from 6 plants (Table 1) in concentration 0.15, 0.3 and 0.6% and control - DW water or an aqueous solution of 0.006% (a.i.) oxamyl (Vydate) were used.

Effect of plant extract on egg hatching and juvenile immobility

Two mL of each extract concentrations and controls was pipetted into 60 mm/d watch glass (preliminary placed in Petri dish) 0.1 mL suspensions containing 100 J_{2S} or 100 eggs of *M. hapla* placed in each watch glass. Petri dishes with a watch glass were covered and incubated in the dark at 24°C. In each experiment, four replicates were evaluated for each treatment and the trial was conducted twice. For each evaluation, watch glasses were agitated to disperse the nematodes or eggs and placed on a gridded plastic counting sheet and immobility of J_{2S} or hatching nematodes from eggs / in each plate were observed at stereomicroscope. J_{2S} that were moving actively or touched gently with a nematode pick at each time interval were recorded as mobile. J_{2S} mobility was evaluated after 24, 48 and 72 h and hatching J_{2S} – after 5 days of exposure to the plant extracts and control (DW) or an aqueous solution of oxamyl), respectively. After 72 h or – 5 days contents with J_{2S} or eggs and hatching nematodes of each dish were poured into a submerged 20 µm /5 µm sieve and rinsed under a gentle stream of DW, respectively. The contents of the each sieve with 10 mL DW were rinsed into clean Petri dish. The mobility of J_{2S} was evaluated after 24 h and the hatching J_{2S} after 10 days.

In the studies, the percentage of immobile J_{2S} or hatching J_{2S} in each treatment was standardized by subtracting the percentage of immobile J_{2S} or hatching J_{2S} in the DW control treatments at each time interval.

Greenhouse experiment

The experiment was conducted in a greenhouse near Plovdiv, Central Bulgaria. Tissue culture strawberry plants, cultivar „Totem" were grown in 8 cm pots in soil-less media for 30 days prior to the trials. At the start of the experiment, plant roots were washed free of potting media and roots were trimmed ~ 11 cm. Plants were sorted by size and those of similar size were selected for each replicate. Three and half liters pots were filled with 3000 mL of steam-pasteurized loam soil mixed 2:1 (v/v) with washed sand, and strawberry plants were planted. Three days, after that a suspension of M. hapla was adjusted to ~ 450 eggs or 450 J_2s / mL and 10 mL of each suspension per pot were applied with an injection on 10 places, dispersing the solution on equal parts on the whole height of the soil layer. Population density of *M. hapla* was 9/mL soil (9000 eggs/J_2s per pot). Ten ml of each plant extracts (concentration 0.6%) and oxamyl 6 mg/kg soil were applied per plant/pot. Plant extracts was added to a 150-200 mL water and were applied as soil drenches. Soil was allowed to dry for 3 days before each subsequent application. Oxamyl was applied on humid soil with incorporation. Greenhouse experiments included: - Non-infested control; - Infested control; - oxamyl was applied 1 days before planting (once); - application of each plant extracts (at dose 10 ml per plant) on 1th, 15th, 30th, 50th, 70th and 90th day after planting (A) and application of each plant extracts (at dose 10 ml per plant) on 1th, 30th, 60th and 90th day after planting (B). The experiments were planted on 20 February 2016 and nematode and plant data were collected 120 days after nematode application.

Plants were arranged on greenhouse benches in a randomized block design with eight replicates. The greenhouse temperature during the cropping period was 14.5-32.6ºC (21.2ºC±4.4)] and humidity [24.5-78.2% (37.5%±8.9)]. Plants were irrigated every three days and fertilized bi-weekly. Runners and flowers were removed from plants throughout the study.

The following observations were made: 1. During the cropping season - numbers of females of *M. hapla* (50th day after planting the strawberry); 2. At the end of the experiments - weighed crowns and roots, root gall index, numbers of J_{2S} in the soil (mL), and numbers of eggs/J_{2S} in the roots (g). The soil samples on 50th day were taken by means of an auger (1.5 cm/d) at a distance 6 cm from the plant, upon which the roots were separated, washed, stained in acid fuchsine and the numbers of females were counted by direct examination of the roots using a stereomicroscope and determined per gram of fresh roots. At 120th day after planting, for each pot, the root system was removed and soil carefully shaken from the roots. The soil was

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screened through a 1 cm screen, mixed, and weighed. The nematodes were extracted from soil samples using a modified Baermann funnel technique [25]. Root gall index of was assessed according to a 0 to 10 scale [2]. Eggs were extracted from roots samples according to hypochlorite procedure [9]. Crowns and roots were ovendried for 24 h at 70°C and then weighed.

Statistical analysis

Analyses of variants were applied to the obtained data by using SPSS software. The found effects were compared to the controls and means of data groups were separated by Duncan multiple range test ($P_{0.05}$).

Results and discusion

In vitro toxity experiment

Juveniles immobility: There were no immobile J_{2S} in controls with DW after 24, 48, 72, 96 hours. Nematode movement in oxamyl solutions was consistent within all trials (Fig. 1). All nematodes exposed to oxamyl were quiescent, with straight bodies at each observation period. Some nematodes responded to touch with slow, often single, movement. After being touched, between 73 - 82% of nematodes were judged immobile after 24, 48, and 72 h in oxamyl solution. Following rinsing and incubation in water for 24 h, 74% of nematodes remained immobile (Fig. 1).

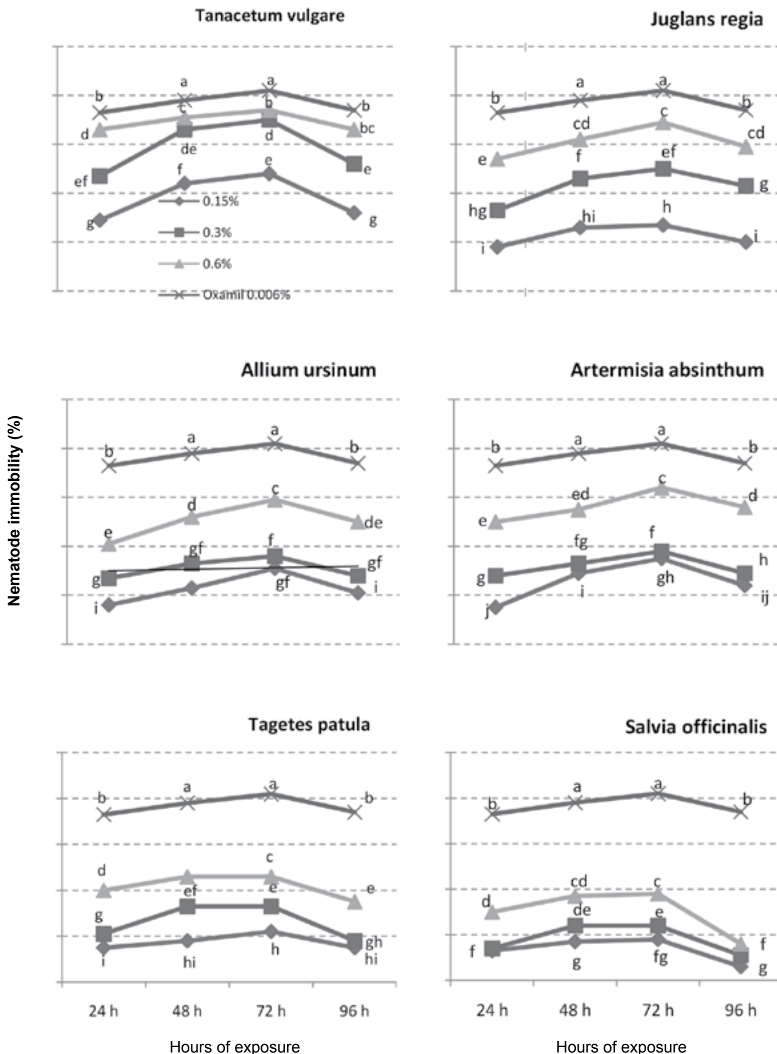

Fig. 1. Effect of plant extracts and oxamyl on the mobility of *M. hapla* in vitro. Movement of nematodes was observed after 24, 48, and 72 hours exposure in solutions of each extract/oxamyl, followed by a rinse in water. Responses of nematodes were observed after 24 hours incubation in water . Data were standardized by subtracting the percentage of immobile nematodes in the water control treatments at each time interval. Values followed by the same letter are not significantly different according by Dunnett's test ($P_{0.05}$).

All tested plant extracts were less effective as oxamyl in immobilizing nematodes. During 72 h exposure *in vitro* to oxamyl, 82% of the nematodes were immobile, which was similar to *in vitro* experiments with *M. hapla* population from Smolyan potato region after exposure 24 h [13] and show that the various populations of the parasites demonstrate different reaction to oxamyl and should be tested. The reaction of *M. hapla* individuals was not different during exposure to oxamyl and after rinsing with water. The quiescent nematodes that responded to touch did so with slow single movement.

Nematode immobility was similar among *T. vulgare* at concentration 0.6% and 0.0065% oxamyl during the 24 and 72 h of exposure (Fig. 1). As with oxamyl, greater part of the nematodes exposed to both higher concentrations of *T. vulgare* were quiescent and required touch to elicit movement. Thujone, monoterpenoid naturally occurring in aerial parts of *T. vulgare* [20], has previously possessed anthelmintic activity against *Ascaris lumbricoides* and *Fasciola hepatica in vitro* [12]. Therefore, this compound can be responsible for detected nematode immobility in our test. Nematode mobility was reduced, but to a lesser degree by exposure to concentrations 0.3% and 0.15% mg/mL *T. vulgare.* After rinsing and incubating nematodes in water for 24 hours, 66, 52 and 32% of nematodes exposed to 0.6, 0.3 and 0.15% were immobile, respectively. Similar revival of *Pratylenchus penetrans* individuals, following exposure to plant extracts of *T. vulgare*, was reported by Samaliev et al. [21].

Mobility of nematodes exposed to concentration 0.6% of *J. regia* at 72 h was not different ($P_{0.05}$) from those exposed to oxamyl at 24 h. Effect declined to 59% at 72 h exposure (Fig. 1). Nearly 52% of nematodes exposed to concentration 0.3% were immobile after 48 h exposure, but this effect declined to 50% at 72 h exposure. *J. regia* at concentration 0.15% was not effective, with less than 31% of nematodes immobilized. The effect of *J. regia* at all concentrations was decreased to 20 – 59% after rinsing the nematodes in water. Fetterer and Fleming [7] had previously described antihelmintic activity of juglone, a quinone compound of *J. regia* pericarp, against Ascaris suum *in vitro*. Therefore, it is highly probable that juglone is accountable for nematicidal activity of *J. regia* extract to *P. penetrans* in our experiment.

Artermisia absinthium in concentration 0.6% immobilized greater than 52% of the nematodes after 48 and 72 h exposure and it was with only 9% less than oxamyl at 24 h. (Fig. 1).

Anthelmintic activity of artemisinin (a compound present in *A. absinthium*) against some species from class Trematoda (*Clonorchis sinensis, F. hepatica and Schistosoma japonicum*) was previously described by Fathy [5]. The 2.5 mg/mL extract of *A. absinthium* induced 71% mortality of second-stage juvenile (J_2) of *M. incognita* 24-hour exposure [4]. Olah et al. [17] reported that *Artermisia spp*. produces a very complex mixture of compounds, including ketone and t-anethole. Our results confirm the investigation of the cited authors and show that the extract of *A. absinthium* posseses nematicidal activity and against *P. penetrans in vitro*. However, the effect of the *A. absinthium* decreased to 24 – 56% after rinsing and incubation in water, with most nematodes regaining mobility.

Allium ursinum in concentration 0.6% also immobilized over 59% of the nematodes after 48 and 72 h exposure (Fig. 1). *A. ursinum* has an efficient action against many fungal species, such as *Aspergillus flavus*, *A. niger*, *Candida albicans*, *Fusarium laceratum*, *F. oxysporum, Microsporum canis*, *Mucor racemosus*, *Penicillium* spp., *Rhizopus nigricans*, *Saccharomyces* spp., *Trichophyton granulosum* [19]. According to data of the same authors the antifungal activity of the flower extract was stronger than that of the leaf extract, and this was correlated with a higher content of allicin. Therefore, the observed antinematode activity of the extract of *A. ursinum* in our experiment we base on the allicin content. The effect of the *A. ursinum* like as *A. absinthium* decreased to 21 – 49.8% after rinsing and incubation in water, with most nematodes regaining mobility.

Tagetes patula has minimal effect on nematodes at all concentrations (0.6, 0.3 and 0.15%), with 46%, 33% and 22% immobile nematodes during exposure, respectively (Fig. 1). Plants from the genus *Tagetes (T. erecta, T. minuta* and *T. patula)* are regularly used for nematode control. This is done especially in the form of mixed cultures of *Tagetes* spp. as interculture vegetables (cover crop grown before planting cash crop) and in commercial formulations, and their nematicidal effect has been known for a long time [27]. However, our results are similar to findings by Sasanelli and D’Addabbo [24] conduced with other species of nematodes. These authors did not observe nematicidal activity of *T. erecta* against the root-knot nematode *M. incognita* in an *in vitro* experiment. It is possible that the main plant compound responsible for the nematicidal effect of *Tagetes* species, a-therthienyl, was destroyed during the distillation process [28]. *Salvia officinalis* also has minimal effect on nematodes at all concentrations, with 38%, 37% and 30% immobile nematodes during exposure (Fig. 1). This extract was effective in the control of the second stage juveniles and egg hatch of *M. javanica* [15]. The effect of both extract (*T. patula* and *S. officinalis*) at all concentrations was decreased to less than 35 and 16% after rinsing, respectively.

Eggs hatching: At the end of experiment there was 98% hatching of J_{2S} in the control with DW. When *M. hapla* eggs were exposed to oxamyl for 5 days following rinsing and incubation in DW for 10 days, there was 27.4% hatching of J_{2S} (Figure 2).

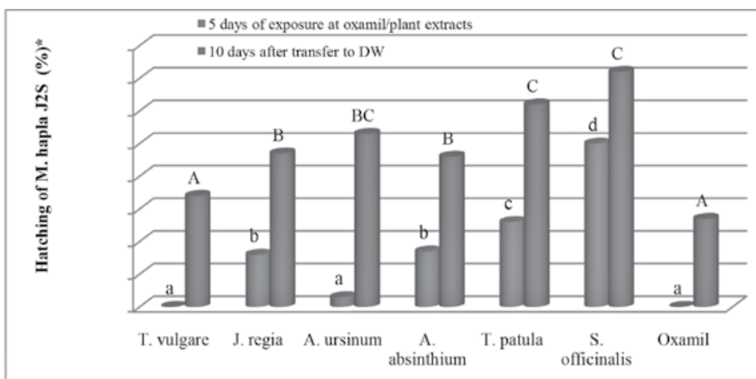

Fig. 2. Effect of plant extracts and oxamyl on the hatching of *M. hapla* J_{2S} in vitro. hatching of nematodes was observed 10 days after transfer to deionized water (DW), following 5 days of exposure in solutions of each extract (0.6%) or oxamyl (0.006%). Data were standardized by subtracting the percentage of unhatched nematodes in the water control treatments at each time interval. Values followed by the same letter are not significantly different according by Dunnett’s test ($P_{0.05}$).

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In the test with plant extracts after *M. hapla* eggs were exposed for 5 days at the concentration 0.6%, upon rinsing and incubation in DW for 10 days, there was eggs hatching in all variants with plant extracts. As with oxamyl, in greater part of the eggs exposed to the 0.6% concentrations of *T. vulgare,* eggs hatching was lesser (34.6), following *A. absinthium, J. regia* and *A. ursinum* (46.4, 47.6 and 53.0%, respectively). With *T. patula* and *S. officinalis*egss hatching was highest (62.6 and 72.0%, respectively) (Figure 2, $P_{0.05}$).

Hatching tests are useful in screening extracts for nematicidal activity, because counting hatched juveniles is more accurate than counting juveniles in a particular J_{2S} population [15]. In this tests all the plant extracts/oxamyl tested had a nematicidal effect and affected the hatching of *M. hapla* J_{2S}. When the eggs were transferred to DW alone, hatching resumed, but the degree of recovery was affected by the plant extracts/oxamyl to which the eggs had previously been exposed.

According to these results of laboratory-Petri dish studies, we decided to use only *T. vulgare*, *J. regia* and *A. absinthium* at 0.6% concentrations in the greenhouse trials.

Greenhouse Study

At the 50th day after *M. hapla* inoculation, in plants treated with oxamyl and the plant extracts (variants A and B) had significantly lower number of females present in the roots than the plants in the infected control. The lowest number of *M. hapla* females was observed in the treatment oxamyl followed by *T. vulgare* (6 and 15 (A) and 40 (B), respectively). *Juglans regia* and *A. absinthium* also reduced the number of females (48 and 55 in A and 69 and 71 in B, respectively) compared with the infected control (136) (Table 3, $P_{0.05}$).

Table 2.

Effect of selected plant extracts (concentration 0.6%) and oxamyl (concentration 0.0065%) on numbers of *M. hapla* females, root-gall index, numbers of J2S in soil and root, and on the growth of "Totem" strawberry plants in greenhouse experiment

Treatments	At 50th day after transplanting	At the 120 after *M. hapla* application				
	Fameles g^{-1} root	Gall index	Eggs/J_{2S} per g^{-1} root	J_{2S} per g^{-1} soil	Root weight (g)	Crown weight (g)
Non-infested control	0.0 a*	0.0 a*	0 a	0 a	8.7 b	21.5 a
Infested control	136 d	5.2 e	5324 d	176 e	6.6 e	17.4 d
Oxamyl	6.0 a	1.7 b	1231 b	58 b	10.7 a	22.1 a
Plant extracts were applied 6 time (variant A)						
Tanacetum vulgare	15 a	2.3 b	1917 b	78 b	8.9 b	21.8 a
Juglans regia	48 b	3.5 c	2870 c	111 c	8.1 c	20.2 b
Artermisia absinthium	55bc	3.7 c	3315 c	118 c	8.2 c	20.1 b
Plant extracts were applied 4 time (variant B)						
Tanacetum vulgare	40 b	3.8 c	3527	118 c	7.9 cd	20.2 b
Juglans regia	69 c	4.3 d	4270	134 d	7.5 d	19.3 c
Artermisia absinthium	71 c	4.4 de	4315	145 d	7.6 d	19.1 c

*Values followed by the same letter are not significantly different according by Dunnett's test ($P_{0.05}$).

A hundred and twenty days after *M. hapla* application (at the end of experiment), the gall index varied from 5.2 for the plants in the control plots to 1.8 and 2.3 for the plants treated with oxamyl and *T. vulgare* (6 times application - A), respectively. In the variant A of *J. regia* and *A. absinthium* also reduced ($P_{0.05}$) *M. hapla* infection compared with the infested control (3.5 and 3.7 root gall index, 111 and 118 eggs/J_{2S} per g^{-1} soil and 2860 and 3315 eggs/J_{2S} per g^{-1} root, respectively) (Table 3, $P_{0.05}$)

At the variant B when the plant extracts were applied 4 time, the effect was to a lesser degree ($P_{0.05}$) in all three parameters of assessment (gall index, J_{2S} in the soil and eggs and J_{2S} in the plant roots), compared with variants with 6 time application of plant extracts (Table 3).

M. hapla population densities were lower when tested plant extracts applications started at planting 6 time at intervals 15 or 20 days than application 4 time with longer intervals – 30 days. Probably the nematostatic action of tested plant extracts continues to 15-20 days after application. Root-knot nematodes are more difficult to control once they enter the root. These data suggest that plant extracts the acted as prophylactics, in contrast to oxamyl, and had little therapeutic activity on nematodes once inside the roots. Plant extracts that inhibit nematode movement, orientation, and feeding behavior could reduce penetration of nematodes into the roots and delay population increase. *T. vulgare* at concentration 0.6% had the lowest *M. hapla* population density, with gall index and root/soil population densities not different than the oxamyl treated plants and there were no stastically proved difference between *T. vulgare* and non-infested control.

Application of plant extracts will not substitute directly for carbamate nematicides. They will require different strategies, such as multiple applications at critical times during flushes of root growth [23] or synchronized with egg hatch and when nematode population densities are high in the soil. We have demonstrated that nematodes once established in new strawberry roots were not affected by drenching the soil with plant extracts at 4 time in 30 days intervals. *M. hapla* can infest the strawberry roots throughout the vegetation year [26]. More frequent applications and higher concentrations may be required to manage nematode populations of *M. hapla*. In agricultural soils, plant extracts may degrade rapidly. In addition, plant extracts evaluated in this study were effective at the higher concentrations tested, which may not be economical in agricultural soils. Application of *T. vulgare* at 5 mg/mL demonstrated *in vitro* efficacy against *P. penetrans* [21] and *in vivo* in a raspberry

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(Samaliev, unpublished data). Research needs to be directed to adjusting application timing and concentrations for specific application methods and nematode pathosystems.

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