Comparative study of two different neem-derived pesticides on *Meloidogyne incognita* under *in vitro* and pot trials under glasshouse conditions

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Abstract: Two different neem-derived plant protection products i.e neem leaf extract and a commercial product containing 1% azadirachtin were used to study their effects on *M. incognita* under *in vitro* and pot trails under glasshouse conditions. In the *in vitro* studies, highest concentration (1%) of neem leaf extract resulted in more than 90% mortality in J2 whereas the commercial product did not differ significantly in mortality compared to control. In the pot trials under glasshouse conditions, fresh shoot weight and number of fruits of both the landraces did not differ significantly for different treatments. Zeck scale was found to be the best for evaluation of gall index compared to other scales i.e Garabedian and Van Gundy scale and Mukhtar et al. scale. Gall index decreased in all the treatments compared to positive control and 0.1% azadirachtin was significantly different from the control. This shows that neem-derived pesticides can reduce the galling of roots and can help control *M. incognita* infestation with proper planning and implementing the treatments.
Keywords: neem leaf extract, azadirachtin, root-knot nematodes, biological control, tomato
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Introduction

More than 90 root-knot nematode (*Meloidogyne*) species are known worldwide (Hunt & Handoo 2009), from which 23 species are present in Europe (Wesemael et al. 2011). Root-knot nematodes are responsible for decreasing yields (Bernard et al. 2017) of nearly every cultivated crop in the world (Sasser, 1980). They have a wide range of host plants: with different sensitivity, but they are able to infect the roots of vegetables (Anwar et al. 2007), medicinal and culinary plants (Walker 1995; El-Sherif et al. 2012), ornamental plants (Dabaj and Jenser, 1990; den Nijs et al. 2004) and weeds as well (Rich et al. 2008).

If once their appearance is noticed in a field, their total eradication is an almost impossible task (Briar et al. 2016). It is especially so recently due to the restricted use of soil disinfecting chemicals (Briar et al. 2016). Moreover, certain species, like *Meloidogyne incognita* has several biological races with different pathogenicity and host plant preferences (Khan and Khan 1991). Consequently, mixed natural populations of *Meloidogyne* species can break the resistance of Meloidogyne-resistant varieties of crops (Eddaoudi et al. 1997; Tzortzakakis et al. 2016).

Neem as a pesticide is used for centuries in Asia and has known to possess several beneficial plant protective properties such as antifeedant, repellent, antifungal (Schmutterer 1988) and nematicidal (Nile et al. 2017; Yadav et al. 2018). Javed et al. (2007) investigated the efficacy of different neem formulations on *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 on tomato. They found that crude extracts of neem cake and leaves reduced the severity of the nematode infestation both under *in vitro* circumstances and in plants under glasshouse conditions. However, in the case of pure azadirachtin which is a refined neem product, neither the

immobilisation of nematodes nor increased mortality was observed under in vitro conditions. Similar results were obtained by Khanna and Kumar (2006) when they tested five different neem formulations against *M. incognita in vitro*. Out of the five different neem formulations tested, neem seed kernel extract and Econeem, a commercial product consisting of Azadirachta A and B, gave the highest juvenile mortality (73-77%) whereas the other formulations i.e Nimbecidine, NeemAzal T/S and Neem Gold were comparatively less effective.

During a study conducted by Lynn et al. (2010) on the effects of azadirachtin and neem based formulations to control sweet potato whitefly and *M. incognita*, they observed a reduction in the development of both the whiteflies and root-knot nematodes and recommended that the soil-based application would be the best to control both leaf-sucking and soil pests. Sahu et al. (2018) compared the efficacy of different oil cakes in pot culture experiment with tomato. It was concluded that the neem cake applied at a rate of 100 g/m2 increased the morphological characteristics of tomato and significantly reduced the number of root galls, thus it is considered a most promising management option against *M. incognita* infecting tomato. Singh et al. (1980) also found significant reduction in the abundance of different plant parasitic nematodes and fungi by coating the tomato seeds with oil cakes of *Ricinus communis* L., *Brassica campestris* L, *Azadirachta indica*, *Madhuca indica* and *Arachis hypogaea* L. Similar results were obtained by Siddiqui and Alam (1987) with seed dressing method using neem and Persian lilac (*Melia azedarach* L.) extracts to control the plant-parasitic nematodes *M. incognita* and *Rotylenchulus reniformis* Linford et Oliveira.

The objective of this study was to test two different neem-derived products i.e. traditional aqueous neem leaf extract and a commercial product of azadirachtin, for their nematicide effect against *M. incognita in vitro* and in pot experiments. We wanted to compare the traditional water extract which can be easily prepared without any processing locally (being cost effective and easily available in nature) and the commercial product which is much more expensive to the farmers and growers. In addition, we also compared the different *M. incognita* infestation scales to get a better understanding about the severity of infestation.

Materials and Methods

Preparation of aqueous neem leaf extracts (NLE)

Pre-air dried neem leaves were obtained from the local market situated in Mumbai Sub-urban area, Konkan Division, Maharashtra, India. The method of Doshi et al. (2018) was followed with modified working concentrations. For *in vitro* studies, a stock concentration of 5% was prepared by suspending 5 g of air-dried neem leaf powder in 100 ml distilled water. It was filtered through muslin cloth and was centrifuged at 5000 rpm for 5 mins to remove the debris and leaf particles. Working concentrations of 0.01, 0.05, 0.1, 0.5 and 1% were prepared from the stock solution with distilled water.
For glasshouse trials, a stock solution of 20% was made by adding 200 g of neem leaf powder to 1000 mL of distilled water. It was followed by the same procedure as *in vitro* to get a clear solution. Working concentrations of 1, 10 and 20% were prepared from the stock solution with distilled water.

Preparation of azadirachtin (NAZ)

NeemAzal T/S (Trifolio-M GmBH) which contains 1% azadirachtin and is a registered product in the EU was used for preparation of azadirachtin. The methodology of Doshi et al. (2018) was followed with modified concentrations. For *in vitro* studies, the fol-

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lowing working concentrations were applied: 0.0001, 0.0003, 0.0005, 0.001 and 0.01% all in distilled water.
In pot trials, the working concentrations were increased to 0.001, 0.01 and 0.1% with a stock solution of 0.1% which is prepared by dissolving 100 mL of the product in 1000 mL distilled water.

M. incognita inoculum
Second stage juveniles (J_2) of *M. incognita* were obtained from egg masses previously collected from the infected Hungarian determinate tomato landrace cv. 'Dányi' grown in the greenhouse. In order to dissolve the gelatinous matrix and release the eggs, the egg masses were shaken by hand for 2 mins in 0.2% sodium hypochlorite (NaOCl) solution, then they were washed with tap water until the smell of NaOCl was removed. The eggs were suspended in tap water and kept at 24 ± 1°C in dark for hatching. After 14 days, the hatching of the eggs and viability of J_2 were checked under a dissecting microscope with transmitting illumination at a 40x magnification. Only moving and viable J_2 were picked up and were collected using a Pasteur pipette in a glass bottle with tap water and were stored in dark at 20°C ± 1°C for 24 hrs before using for the experiments.

Experiment 1: In vitro effect of neem-derived products on M. incognita (J_2)
A total of eight samples of each concentrations and control were applied. The entire experiment was performed in vitro in flat-bottom 96-well microplates (Kartell S.p.A., Italy) in three repetitions. Five J_2-s were put into each well with 60 μl of distilled water using a micropipette. Then 200 μl of different neem leaf extract or azadirachtin concentrations and 200 μl distilled water was added in the microplate wells as treatments and negative control respectively. Microplates were incubated at room temperature (25 °C) in dark for 24 hours. Nematode mortality was checked under dissecting microscope at 40× after 24 hours. In order to check the motility of nematodes as a sign of viability, pH was dropped by adding 10 μl of 5% lactic acid, a modification of the procedure described by Ciancio (1995). A maximum mortality of 20% in control was considered as a criterion for the validity of the tests (Kiss et al. 2018).

Experiment 2: Effects of neem-derived products on M. incognita infestation under glasshouse conditions

One Hungarian determinate tomato landrace. 'Dányi' (RCAT057829) and a Hungarian indeterminate tomato landrace 'Ceglédi' (RCAT030275) were chosen for this experiment. For potting material, horticultural soil and sand in the ratio of 1:1 (henceforth called as 'mixture') was used. After filling the pots with the mixture, approximately 20 g of *M. incognita* infested soil was added in the middle by making a ditch followed by planting of 1-month old tomato plants. The average temperature recorded during the experiment in the glasshouse was between 25 - 28°C and the relative humidity was between 55-60%. For positive infected control (henceforth called as positive control), only inoculation was done but no treatment was performed. Each treatment was replicated 5 times for both the landrace. The plants were watered daily. The first treatment was done by adding 50 ml of the different concentrations of neem derivatives by soil drenching method after 7 days from planting. In the case of negative control, plants were potted just with the mixture and watered with the rest of the plants. Plants were watered only after the treatment to help spread and mix everywhere in the pots. The treatments were repeated once per week on every 7th day after the previous treatment, for a period of 6 weeks altogether. Experiments were terminated 9 weeks after the setup. Gall index was measured using three different scales by Zeck (1971), Garabedian and Van Gundy

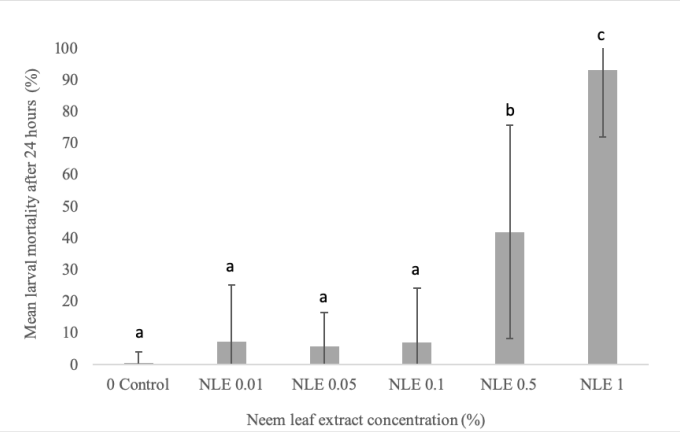

Figure 1. Mortality effect (%) of different concentrations of neem leaf extract (NLE) (%) on *Meloidogyne incognita* J_2 larvae under in vitro conditions after 24 hours. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data are the mean mortality values of 3 replications of the whole experiment, i.e. 24 replicates.

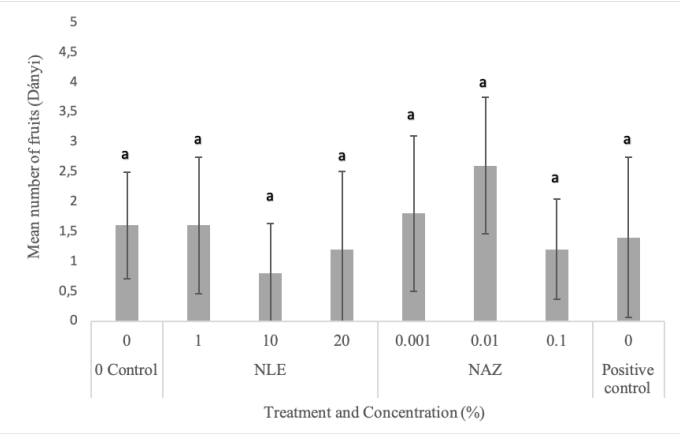

Figure 2. Mean number of fruits produced by *Meloidogyne incognita* infested 'Dányi' determinate tomato landrace after treatment with different neem leaf extract (NLE) and azadirachtin (NAZ) concentrations. Same letters indicate no significant difference at 95% confidence level ($p<0.05$). Data is average of five individual plants per treatment.

(1983) and Mukthar et al. (2013). Morphological characteristics such as fresh shoot weight and number of fruits were measured and recorded.

Data analyses

In the case of Experiment 1, post-hoc Tukey's test was performed after arcsine square root transformation of the data. In the case of Experiment 2, post-hoc Tukey's test was used in R software (R Core Team 2017) for all the three scales. With this approach, a more complete picture from root damage was given. Graphs and tables were made in excel sheet. In addition, we used post-hoc Welch test followed by Tukey's test to compare the two tomato landraces with respect to the root damage caused by *M. incognita* depending on three different scales and to select the best scales for evaluation.

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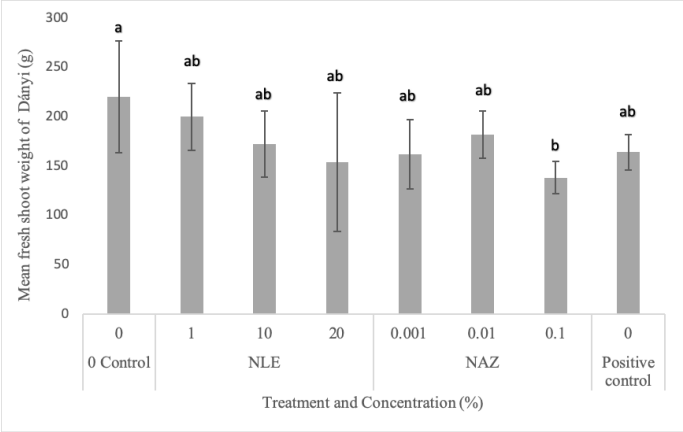

Figure 3. Mean shoot fresh weight in grams of *Meloidogyne incognita* infested 'Dányi' determinate tomato landrace after treatment with different neem leaf extract (NLE) and azadirachtin (NAZ) concentrations. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data is replicate of five individual plants per treatment.

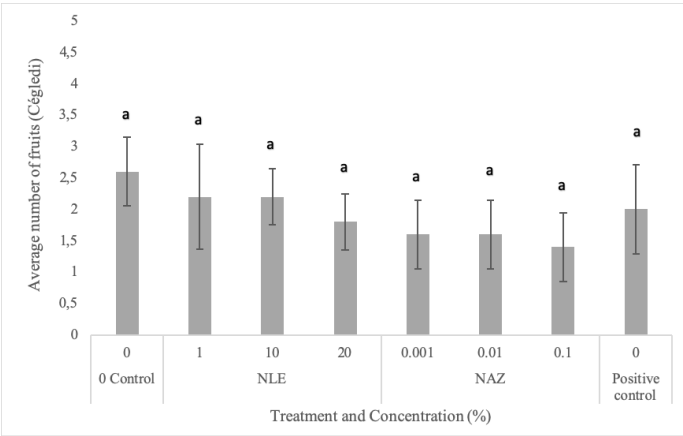

Figure 4. Mean number of fruits produced by *Meloidogyne incognita* infested 'Ceglédi' indeterminate tomato landrace after treatment with different neem leaf (NLE) extract and azadirachtin (NAZ) concentrations. Same letters indicate no significant difference at 95% confidence level ($p<0.05$). Data is average of five individual plants per treatment.

Results

Experiment 1: Effect of neem-derived products on *M. incognita* second stage juveniles (J_2)

The mortality effect of different concentrations of azadirachtin (NAZ) and neem leaf extract (NLE) on mortality of *M. incognita* J_2 larvae under in vitro conditions was demonstrated. In case of NAZ, the mortality of the larvae was inconsistent, wherein numerically the highest mortality was found at the lowest concentration i.e 0.0001% followed by 0.003% and not at the highest concentration of 0.01% as it would have been expected. However, all these mortality values were quite low with no significant differences (Table 1). In case of NLE, it is evident from Figure 1 that higher concentration of NLE yielded in higher mortality. Mortality

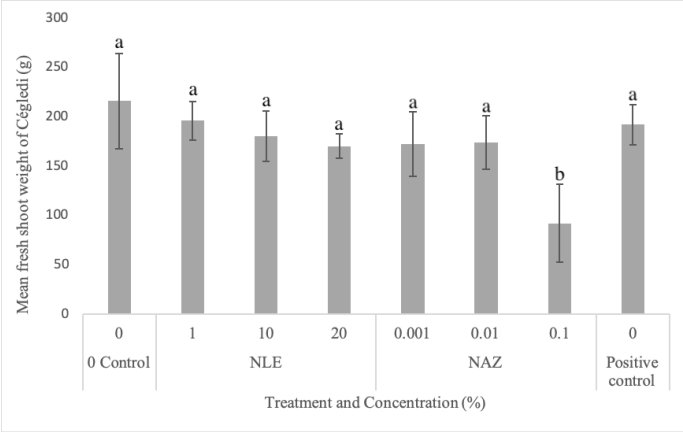

Figure 5. Mean shoot fresh weight in grams of *Meloidogyne incognita* infested 'Ceglédi' determinate tomato landrace after treatment with different neem leaf extract (NLE) and azadirachtin (NAZ) concentrations. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data is average of five individual plants per treatment.

Table 1. Different azadirachtin (NAZ) concentrations tested for mortality of *Meloidogyne incognita* J_2 larvae after 24 hours. Same letters indicate no significant difference at 95% confidence level ($p<0.05$). *Data are the mean mortality values of 3 replications of the whole experiment i.e. 24 replicates.

Treatment	Concentration (%)	*Per cent juvenile mortality after 24 hours (mean ± SD)
Control	0	0.69 ± 3.40 a
Azadirachtin (NAZ)	0.0001	10.97 ± 4.83 a
	0.001	4.58 ± 2.40 a
	0.003	9.26 ± 4.41 a
	0.005	6.37 ± 2.44 a
	0.01	6.98 ± 2.11 a

in the case of the two highest concentrations of NLE, i.e. 0.5% and 1% was significantly higher ($p <0.05$) as compared to azadirachtin in Table 1.

Experiment 2: Effects of neem-derived products on *M. incognita* infestation in pot experiment under glasshouse conditions

Neither in the case of Dányi (Fig 2) nor 'Ceglédi' (Fig 4) tomato landraces was there any significant difference in the number of fruits with respect to different treatments and concentrations. Further evaluation such as yield could had been possible as we did not wait for the fruits to ripen.

Having said that, azadirachtin (NAZ) 0.1% showed lower fresh shoot weight with a significant difference in both 'Dányi' (Fig 3) and 'Ceglédi' (Fig 5) varieties with respect to 0 control. Apart from this difference, there was no significant difference between the other treatments. All the three scales showed significant difference as compared to non-infected control. In the case of both 'Dányi' and 'Ceglédi', Zeck scale proved the strongest next to the scales of Mukhtar et al.

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Table 2. Average root damage caused by *Meloidogyne incognita* on two Hungarian landraces tomato, the determinate 'Dányi' and the indeterminate 'Ceglédi' depending on three scales: Zeck, Garabedian and Van Gundy and Mukhtar et al. (p-value: Welch test, confidence interval (CI) 95%: 95% confidence level).

Tomato landraces	Dányi		'Ceglédi'	
M. incognita infection -/+	-	+	-	+
Replications	5	34	5	34
Teck scale (0-10)				
mean ± CI 95%	0 ± 0	4.53 ± 0.60	0 ± 0	5.32 ± 0.40
p-value	$4.8*10^{-14}$		$1.69*10^{-23}$	
Garabedian and Van Gundy scale (0-5)				
mean ± CI 95%	0 ± 0	2.21 ± 0.34	0 ± 0	2.53 ± 0.37
p-value	$2.64*10^{-14}$		$7.95*10^{-15}$	
Mukhtar et al. scale (0-6)				
mean ± CI 95%	0 ± 0	4.06 ± 0.54	0 ± 0	4.62 ± 0.48
p-value	$5.12*10^{-16}$		$2.31*10^{-19}$	

Table 3. Average root damage caused by *Meloidogyne incognita* on Hungarian determinate tomato landrace ''Dányi", depending on three scales: Zeck, Garabedian and Van Gundy and Mukhtar et al. receiving the following treatments: 0.001, 0.01 and 0.1% of NeemAzal T/S and 1, 10 and 20% of neem leaf extract. ANOVA post-hoc Welch test followed by Tukey's test was performed. Different letters indicate significant difference at 95% confidence level ($p<0.05$).

Treatments	Concentration (%)	Zeck (0-10)	Garabedian and Van Gundy (0-5)	Mukhtar et al. (0-6)
Negative control	0	0 ± 0 a	0 ± 0 a	0 ± 0 a
Positive control	0	5.8 ± 0.96 c	2.8 ± 0.96 b	5.6 ± 0.78 c
NeemAzal T/S	0.001%	5.2 ± 0.73 c	2.4 ± 0.78 b	4.4 ± 1 bc
	0.01%	4.6 ± 1.47 bc	2.6 ± 1 b	4.4 ± 1 bc
	0.1%	2 ± 0.8 ab	1.25 ± 0.44 ab	2 ± 0.72 ab
Neem leaf extract	1%	5.4 ± 0.48 c	2.4 ± 0.48 b	4.2 ± 0.96 bc
	10%	5 ± 1.52 bc	2.4 ± 0.78 b	4.4 ± 1.33 bc
	20%	3.2 ± 2.09 bc	1.4 ± 1 ab	3 ± 1.96 c

and Garabedian and Van Gundy (Table 2).

In the case 'Dányi' landrace, values of the root damage were inconsistent, since the values of Zeck and Mukhtar et al. scales of NeemAzal T/S 0.1% concentration were significantly different from positive control, however, the scale of Garabedian and Van Gundy said the opposite. Moreover, according to the Garabedian and Van Gundy scale, the 20% concentration of neem leaf extract

Table 4. Average root damage caused by *Meloidogyne incognita* on indeterminate Hungarian tomato landraces 'Ceglédi', depending on three scales: Zeck, Garabedian and Van Gundy and Mukhtar et al. receiving the following treatments: 0.001, 0.01 and 0.1% of NeemAzal T/S and 1, 10 and 20% of neem leaf extract. ANOVA post-hoc Welch test was performed followed by Tukey's test. Different letters indicate significant difference at 95% confidence level ($p<0.05$).

Treatments	Concentration (%)	Zeck (0-10)	Garabedian and Van Gundy (0-5)	Mukhtar et al. (0-6)
Negative control	0	0 ± 0 a	0 ± 0 a	0 ± 0 a
Positive control	0	6.4 ± 0.48 c	3.4 ± 0.48 c	5.2 ± 0.73 c
NeemAzal T/S	0.001%	5.6 ± 0.48 bc	2.6 ± 0.48 bc	5.4 ± 0.78 bc
	0.01%	5.8 ± 0.73 bc	3.4 ± 1.47 c	5 ± 1.07 c
	0.1%	4 ± 1.24 b	1.4 ± 0.78	2.6 ± 0.78 ab
Neem leaf extract	1%5 ± 1.52 bc	2.4 ± 0.78 bc	4.8 ± 1.57 bc	
	10%	5.6 ± 0.48 bc	2.6 ± 0.48 bc	5.2 ± 0.96 bc
	20%	4.75 ± 0.84 bc	1.75 ± 0.84 ac	4 ± 1.01 ac

was similar to the negative control, but Zeck and Mukhtar et al. scales showed differences (Table 3).

In the case of 'Ceglédi' landrace, concentrations of neem leaf extract did not differ from positive control, according to all the three scales. On the other hand, 0.1% of NeemAzal T/S was significantly lower than only *M. incognita* infected treatment (Table 4).

Discussion

Although Khan et al. (1974) attributed to the toxicity of neem formulations to azadirachtin, it is evident from our *in vitro* experiment results that neem leaf extract showed better nematicidal property. Azadirachtin did not show any significant difference in the nematicidal activity which was reported by Javed et al. (2008) and Ntalli et al. (2009). Our results contradict the study of Grandison (1992), where he could not observe any effect of neem seed on J_2 larvae of *M. javanica*. But our results are in line with Abo-Elyousr et al. (2010) and Agbenin et al. (2005) as they both concluded that the neem leaf extracts were lethal to *Meloidogyne* larvae. In accordance with our results, previous investigations by several different researchers have shown 70% - 100% mortality using aqueous extracts of neem formulations as mentioned by Javed et al. (2008). This might be due to the array of different phytotoxins and chemical compounds which might work individually or synergistically, and which are water soluble (Nile et al. 2017). It could not be found which compound was responsible for the 90% and higher mortality in the case of neem leaf extracts in our study, but according to Qamar et al. (1989), kaemptro and myricetin could be the chemical compounds responsible for nematicidal activity in neem leaf extracts.

As seen in the results, in the case of 0.1% azadirachtin (NAZ), fresh shoot weight for both the landraces was lower and significantly different compared to 0 control. This is probably because the roots were ad-

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versely affected by the emulsifier used to dissolve the commercial product containing azadirachtin (i.e if the azadirachtin concentration is 0.1%, then the concentration of the emulsifier is 10%). According to the Hungarian approval document of azadirachtin, the maximum concentration of the applied spray mixture could be 0.003% against glasshouse whitefly (*Trialeurodes vaporariorum* Westwood 1856) in protected tomato (04.2/4878-1/2012. Nébih 2018), but there is no further information about the maximum concentration that can be used.

The results of the glasshouse experiment are in accordance with Agbenin et al. (2005) who used 20% fresh neem leaf extract weekly for 8 weeks on tomato plants (Roma VF) against *M. incognita*, and treatment did not differ from untreated control. According to Kankam and Sowley (2016), neem leaf powder applied to the root zone of chili pepper plants resulted the lowest root gall index next to neem seed powder and neem cake.

In the laboratory experiment, when *M. incognita* larvae came in contact continuously to the leaf extracts or product solutions, leaf extracts have stronger lethal effect. By contrast, under glasshouse conditions with weekly application, neem leaf extracts did not show the same lethal effects on the *M. incognita* larvae as compared to the laboratory conditions. As a conclusion, neem leaf extracts could be more effective against *M. incognita* with continuous and timely application either by drip irrigation or soil drenching.

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