SZENT ISTVÁN UNIVERSITY

COMPARISON OF THE EFFECT OF TWO NEEM-DERIVED PESTICIDES ON SELECTED TARGET AND NON-TARGET ORGANISMS

Thesis of PhD Dissertation

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1. INTRODUCTION AND OBJECTIVES

Agriculture is a key sector in the world to feed the growing population. Different crops are used as food source for their nutritional values which fall prey to various natural threat such as pests, plant diseases and competing weeds. For the past many years, most of these have been controlled by chemical pesticides and continue to be controlled with next generations of synthetic chemical pesticides. But the process of making these chemical pesticides and their use comes with a cost of polluting the natural environment, the use of fossil reserves and their unwanted (and frequently still unknown) off-target effects. Rigorous efforts are made to reduce this pollution and find alternative solutions. Additionally, the problem with synthetic chemicals (PPPs) is not only the pollution and, but also (or predominantly) adverse effects on human health.

Biological pest control is drawing significant attention of world-wide researchers as an alternative. It involves biological control agent(s) (BCA) which are defined as those agent(s) that are living organism(s) itself such as insects, microbes or derived from living organism(s) such as plant extracts. The criteria for a biological control agent to be a plant protection product are sustainability, cost-effectiveness, availability and compatibility with the environment where they are used.

One such biological control agent of plant origin is neem (*Azadirachta indica* A. Juss, 1830). In the veterinary, neem is used as animal feed to provide immunity to different livestock animals against different diseases. Due to presence of long chain fatty acids, neem is used as poultry feed (Girish and Bhat, 2008). Neem is known to possess different medicinal properties since time immemorial and is also mentioned in Ayurvedic and Homeopathic medicine. Different neem plant parts have shown to be effective against different human health problems. For example, neem leaves are known to possess antimalarial, antifungal, antibacterial properties. Neem bark is known in the ayurvedic literature to be useful against cough, fever etc. Different infections such as smallpox, chicken pox and warts are also treated with neem leaf paste (Girish and Bhat, 2008). Apart from medicine, neem oil is also used in soap technology. In recent times, we could find commercial soaps containing neem extracts. Also, neem extract is found in commercially available hair creams/oils which prevent against headlice.

Neem has been used for centuries in tropical and sub-tropical countries for its wide range of plant protection properties. In the developing countries till today, unprocessed extracts of different parts of neem tree are used for plant protection. It has been studied extensively in different parts of the world and some of the compounds have been patented as well. There are a lot of commercially available neem-derived plant protection products in addition to different biological control agents available in the market.

Despite numerous documents pointing out the benefits of neem as a botanical pesticide, it is still under-exploited and its full potential is yet to be harnessed. A review of factors affecting the content and bio-efficacy of neem is discussed by Gahukar (2014). According to him, the major constraints affecting the content of neem phytochemicals are geographic area and climate, genetic variability, agronomic conditions, plant morphology and physiology, collection and storage of plant material. Additionally, other factors which affect bio-efficacy of neem stated by him are degradation/conversion of phytochemicals, formulations and application, pest resistance, modes of action/ insect life stages. In another review, Isman (1997) discussed the barriers for neem and other botanical insecticides to commercialization. He stated that resource availability, standardization and quality control, regulatory requirements are some of the major hindrances which is leading to under-exploitation of neem-derived pesticide products.

It is very important to study and understand neem's interaction and to investigate their efficacy and compatibility with other agents. This will enable researchers about the future possibilities of their use in combination or single to combat different present and emerging plant pest and pathogens. It could be also important to conduct studies with domestic neem extracts and commercial product as the former has wider range of metabolites and compounds than latter. One can also find a plethora of literature about the effects of purified neem products on several target- and non-target organisms but the information on the domestic neem extracts are rather scarce.

Therefore, the goal of this thesis is to compare the effects of neem leaf water extract (without any additional chemicals) and a commercial product NeemAzal T/S (1% azadirachtin only as effective ingredient, registered in the EU) on different:

a. target organisms
    - *Plasmopara halstedii* (Farl) Berl De Toni
    - Root-knot nematode (*Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949)
    - Colorado potato beetle (*Leptinotarsa decemlineata* Say 1824)
    - Western corn rootworm (*Diabrotica virgifera virgifera* LeConte 1868)
b. and to check their effects on non-target/beneficial organisms
    - Entomopathogenic nematodes and slug-parasitic nematode
    - Isopod species (*Porcellionides pruinosus* Brandt, 1833)
    - Entomopathogenic and antagonistic fungi

2. MATERIALS AND METHODS

2.1 Neem-derived pesticides tested

2.1.1 NeemAzal T/S (denoted as AZA)

NeemAzal® T/S containing 1% azadirachtin A (10g/litre) corresponding to maximum of 4% NeemAzal, a compound extracted from natural neem kernel and marketed by Trifolio-M GmbH, Germany, was used as the commercial product which is registered in the European Union.

For the different experiments, different stock solutions (v/v) was prepared using distilled water and the working concentrations were prepared from the stock accordingly depending upon the test target and non-target organisms. In addition, stock and working concentrations were selected based on the rationale that that were not unrealistic and which could have an effect on the target and non-target organisms.

2.1.2 Neem leaf extract (NLE)

Fresh neem leaves were collected from a local market located in the sub-urban region of Mumbai city, Maharashtra, India. They were spread on a flat surface and air-dried in Mumbai under room temperature, packaged and imported to Szent István University, Gödöllő, Hungary.

For the experiments, pre air-dried neem leaves obtained from Maharashta, India were grounded finely using an electric blender to make neem leaf powder. Depending upon the target and non-target organisms studied in different experiments, different stock solutions of neem leaf extract (w/v) was prepared by mixing neem leaf powder in distilled water and kept overnight in the a dark at room temperature. It was filtered the next day using muslin cloth and sieve to obtain the extract. It was further centrifuged at 5000 rpm for 5 mins with 6 acceleration to obtain a clear extract. The working concentrations (v/v) were prepared by diluting the stock solutions using distilled water respectively. In the case of in vitro experiments with fungi, the clear extract obtained after centrifugation was sterilized using SteriCup® and SteriTop® 0.22 µm millipore filter under aseptic conditions and working concentrations were prepared using sterile distilled water under aseptic conditions. Stock and working concentrations were selected based on the rationale that that were not unrealistic and which could have an effect on the target and non-target organisms.

2.1.3 Chemical analysis of the neem leaf extract

To determine the azadirachtin in the leaves of neem plant an HPLC analysis was conducted in the Food Analysis laboratory of the Szent István University Gödöllő, Hungary. Five grams of ground leaves were extracted by shaking for 15 min with

100 ml HPLC grade methanol or water followed by subjection to ultrasonication in a water bath ultrasonic device (Raypa Model UCD-150) at a maximum frequency of v=230 and W=450 for 5 min. The mixture was stored overnight in refrigerator. The supernatant was first filtered through filter paper and finally through a 0.22um, 25 mm hydrophilic PTFE syringe filter before injection onto HPLC instrument. For the standard, azadirachtin A (>95% pure, Sigma Aldrich) was used for comparison.

The HPLC runs and data processing were operated by EZChrome Elite software External standard solution of 250 µg/ml in methanol was used for the quantitative determination of azadirachtin and their possible derivatives. Peak identification was based on comparing retention time and spectral characteristics with those of standard material.

In order to find the factor which is needed to calculate the azadirachtin content, following formula was used:

250 (Std azadirachtin (µg/mL)/**5.5** (Area of std peak from chromatogram) × **100** (Total volume system mL)/**5** (weight of leaves in grams) = **909** (Factor)

The amount of azadirachtin A and the other peaks (which are suspected to be the derivatives or isomers of azadirachtin) in the leaf extracts were calculated by multiplying the area of the peaks with the factor. The final amount was calculated in mg/5 g neem leaves.

2.2. Effect of neem-derived pesticides on target organisms

2.2.1. *Plasmopara halstedii* (Farl.) Berl. De Toni

P. halstedii is an oomycete affecting and causing downy mildew in Sunflower. Infected sunflower cotyledons previously stored at -70ºC in Szent István University's Plant Protection Institute were used for the experiment.

a. Effect of neem-derived pesticides on *P. halstedii* sporangial germination under *in vitro* conditions

Infected sunflower leaves stored in deep freezer were soaked in 30 mL double distilled water to release the sporangia. One millilitre of sporangia suspension was diluted / mixed with 1 mL of each tested neem leaf extract or azadirachtin solutions and with 1 mL of mefenoxam for positive control in an Eppendorf tube. It was agitated gently to avoid bursting of sporangia and was incubated at 16 ± 1°C for 24h in the dark. After 24h incubation period, samples were checked with microscope with 200x magnification to check the effect of neem derived pesticides on the sporangia morphology and release of zoosporangia.

Microscopic examination was repeated 5 times for each tested treatment by counting first 50 sporangia/repetitions/treatment. Microscopic examination of sporangia in double distilled water served as a negative control. Based on the microscopic examination, we invented a germination scale (from 0-2) to identify the morphology of sporangia, wherein, 0 = Completely full sporangia, 1 = Partial empty sporangia, 2 = Completely empty sporangia. This scale is built and developed on the hypothesis that every single released zoospore capable infecting the host plant.

b. Pre- and post-treatment effect of neem-derived pesticides on *P. halstedii* in sunflower

Pre-treatment effect of neem-derived pesticides on *P. halstedii*

The Whole Seedling Immersion (WSI) method Cohen and Sackston (1973) was used for this experiment (Fig 11). Twelve treatments were used in the pre-treatment experiment as the following:

- Seedlings inoculated with *Plasmopara halstedii* sporangial suspension
- Seeds treated with bidistilled water (BW)
- Treated seeds (mefenoxam 3mg/Kg) inoculated with *Plasmopara halstedii* sporangial solution
- Treated seeds (mefenoxam 3mg/Kg) inoculated with bidistilled water (BW)
- Seeds pre-treated with AZA 0.1% inoculated with *Plasmopara halstedii* sporangial solution
- Seeds pre-treated with AZA 0.1% inoculated with bidistilled water (BW)
- Seeds pre-treated with AZA 0.01% inoculated with *Plasmopara halstedii* sporangial solution
- Seeds pre-treated with AZA 0.01% inoculated with bidistilled water (BW)
- Seeds pre-treated with NLE 10% inoculated with *Plasmopara halstedii* sporangial solution
- Seeds pre-treated with NLE 10% inoculated with bidistilled water (BW)
- Seeds pre-treated with NLE 20% inoculated with *Plasmopara halstedii* sporangial solution
- Seeds pre-treated with NLE 20% inoculated with double distilled water (BW)

Three-day old germinated sunflower seeds of susceptible sunflower variety 'Iregi szürke csíkos' (25 seedlings) were firstly immersed in NeemAzal T/S (0.1%, 0.01%) or neem leaf extract (10%, 20%) solutions, respectively, for 2 hours. These treated seedlings were further inoculated with *P. halstedii* by immersing them in the sporangial suspension which was adjusted to 50,000 sporangia/mL using haemocytometer and then incubated at 16°C for overnight in dark place. For negative control, germinated seeds were first immersed in different treatments for 2 hours followed by immersing in bidistilled water (BW) for 24 hours.

Germinated seeds were planted in pots placed in a tray containing the moistened perlite with 5 seeds/ pot and with the five repetitions and placed in the growth chamber with the controlled conditions (22°C, with the photoperiod of 12hr, RH=60%). Seedlings were watered regularly for 10 days. After 10 days of plantation, when the plants developed true leaves of about 1 mm, bidistilled water was sprayed using a hand sprayer on the plant leaves and enclosed in trays with lid and covered in the dark polyethylene bag (to saturate it with moisture) and kept overnight at 19°C under completely dark conditions to induce sporulation. The next day after sporulation, first evaluation was done based on the cotyledons bearing sporangia (white growth). Plant growth characteristics such as height was measured as well. Plants were kept back in the growth chamber at 22°C, with the photoperiod of 12hr, RH=60% and watered regularly. After 19 days of plantation, second evaluation i.e presence or absence of chlorosis, damping-off of seedlings was done and recorded.

Post-treatment effect of neem-derived pesticides against *P. halstedii* in sunflower

Whole Seedling Immersion method (Cohen and Sackston, 1973) was used for this experiment (Fig 11). Following 12 treatments were used in the post-treatment experiment:

- Seeds inoculated with *Plasmopara halstedii* sporangial suspension
- Seeds inoculated with bidistilled water (BW)
- Treated seeds (mefenoxam 3mg/Kg) inoculated with *Plasmopara halstedii* sporangial solution
- Treated seeds (mefenoxam 3mg/Kg) inoculated with bidistilled water (BW)
- Seeds inoculated with *Plasmopara halstedii* sporangial solution followed by AZA 0.1% solution
- Seeds inoculated with bidistilled water followed by AZA 0.1% solution (BW)
- Seeds inoculated with *Plasmopara halstedii* sporangial solution followed by AZA 0.01% solution
- Seeds inoculated with bidistilled water followed by AZA 0.01% solution (BW)
- Seeds inoculated with *Plasmopara halstedii* sporangial solution followed by NLE 10% solution
- Seeds inoculated with bidistilled water followed by NLE 10% solution (BW)
- Seeds inoculated with *Plasmopara halstedii* sporangial solution followed by NLE 20% solution
- Seeds inoculated with bidistilled water followed by NLE 20% solution (BW)

Three-day old germinated seeds of susceptible sunflower variety Iregi szürke csíkos were first inoculated with *P. halstedii* sporangial suspension for 24 hours which was adjusted to 50,000 sporangia/mL using haemocytometer followed by respective treatments for 2 hours. For negative control, germinated seeds were

first treated in bidistilled water (BW) for 24 hours followed by respective treatments for 2 hours. Germinated Seeds were planted in pots placed in a tray containing the moistened perlite with 5 seeds/ pot and with the five repetitions and placed in the growth chamber with the controlled conditions (22°C, with the photoperiod of 12hr, RH=60%). Plants were watered regularly. After 10 days of sowing, when the plants developed true leaves of about 1 mm, bidistilled water was sprayed onto the seedlings and enclosed in trays with lid and covered in the dark polyethylene bag (to saturate it with moisture) and kept overnight at 19°C under completely dark conditions to induce sporulation. The next day after sporulation, first evaluation was done based on the cotyledons bearing sporangia. Plant growth characteristics such as height was measured as well. Plants were kept back in the growth chamber at 22°C, with the photoperiod of 12hr RH=60% and watered regularly. After 19 days of sowing a second evaluation i.e presence or absence of chlorosis on true leaves, damping-off of seedlings was done and recorded.

Data analysis

For both, the *in vitro* and for the *in vivo* experiments, ANOVA followed by post-hoc Tukey test was performed to compare the different treatments in R software v 3.4.0 while graphs were made in Excel.

2.2.2. *Meloidogyne incognita* (Kofoid & White, 1919 Chitwood 1949)

Second stage juveniles (J_2) of *M. incognita* were obtained by dissolving gelatinous matrix of the egg masses previously collected from the infected Hungarian determinate tomato landrace cv. 'Dányi' by using 0.2% NaOCl followed by washing with tap water. For hatching, the eggs were suspended in tap water and kept in a thermostat at 24 ± 1°C in dark. After 14 days, viability was checked under transmission stereomicroscope. J_2 that were moving and viable were picked up and collected using a Pasteur pipette in a glass bottle filled with tap water. They were stored in a thermostat under dark conditions at 20°C ± 1°C for 24 hrs before using for the experiments.

a. Effect of neem-derived products on *M. incognita* (J_2) under in vitro conditions

A total of eight replicates of each concentrations and control were applied. The entire experiment was performed thrice in flat-bottom 96-well microplates (Kartell S.p.A., Italy) under laboratory conditions. Five J_2s were put into each well with 60µl of distilled water using a micropipette. Then 200µl of different treatments and 200 µl distilled water as negative control, was added. Microplates were incubated at room temperature in dark for 24 hours. Larval mortality was checked under dissecting microscope at 40x after 24 hours. In order to check the mobility 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).

b. Effects of neem-derived products on *M. incognita* infestation in tomato under glasshouse conditions

One Hungarian determinate tomato landrace. 'Dányi' (RCAT057829) and a Hungarian indeterminate tomato landrace 'Ceglédi' (RCAT030275) were selected for this experiment. Horticultural soil and sand in the ratio of 1:1 (henceforth called as 'mixture') was used for potting the plants. Approximately 20 g of *M. incognita* infested soil was added in the middle of the mixture by making a ditch using hands followed by planting of 1-month old tomato plants. For positive infected control (henceforth called as 'positive control'), only inoculation with *M. incognita* soil was done but no treatment was performed. Each treatment was replicated for 5 times on 5 different plants for both the landraces. 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 *sans M. incognita* soil 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 every 7th day after the previous treatment, for a period of 6 weeks altogether. Experiment was terminated 9 weeks after the setup. Gall index was measured using three different scales by Zeck (1971), Garabedian and Van Gundy (1983) and Mukthar et al. (2013). Morphological characteristics such as fresh shoot weight was measured and recorded.

Data analysis

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.

2.2.3. Colorado potato beetle (*Leptinotarsa decemlineata* Say 1824)

Collection of Colorado potato beetle larvae

Freshly hatched, first and second instar larvae from the untreated leaves of potato cv. 'Balatoni Rózsa' were collected from the experimental field of Szent István University, Gödöllő campus. Fresh undamaged potato leaves of the same potato variety were collected for different treatments and to serve as a food source.

a. No-choice test

One fresh undamaged potato leaf was dipped in the different treatment solutions for 10 seconds and kept outside for 1 min for drying at room temperature before placing it on moist filter paper in each of the 9 cm glass Petri dishes. A total of 5 individuals, which included freshly collected mixed population of newly hatched and 1st instar larvae were placed on the top of the leaves using a fine brush. A negative control was performed by dipping the leaves in distilled water and positive control was performed in the same way by using 2% of Novodor. Each treatment was performed 3 times. The plates were kept at 25±2°C, 60±5% RH, 16L:8D conditions. Larval mortality and feeding damage on the leaves was observed and recorded for a time period of 24, 48, 72, 96 hours.

b. Choice test

The setup for this test was the same as the no-choice test except that it was performed in 15 cm diameter glass Petri dish with 2 fresh undamaged potato leaves, one treated with different treatments of neem leaf concentration and NeemAzal T/S (as mentioned earlier) and the other with distilled water and placed on the opposite side of Petri dishes on moist filter paper. Five individuals consisting random mixture of first, second and third instar larvae were placed in the centre of the Petri dish and the dish was closed with a glass lid. A negative control was performed by dipping both the leaves in distilled water and a positive control was performed by dipping one leaf in 2% Novodor solution and the other in distilled water. The controlled environmental conditions were the same as that in no-choice test. Larval mortality and feeding damage on the leaves was observed and recorded for a time period of 24, 48, 72, 96 hours.

Data analysis

One-way ANOVA post-hoc Tukey's test was performed on the data using RStudio v 3.4.0 (2017) and graphs were made in the excel.

2.2.4. Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte 1868)

Source of WCR eggs

US non-diapause strain of WCR eggs were provided by CABI from Hódmezővásárhely, Hungary and were used for all exp. Eggs were not surface-sterilised, just washed in clean water with few drops of NaOCl the day before use.

a. Effect of neem against WCR eggs under *in vitro* conditions

Different neem-derived pesticides were tested against WCR eggs in a Petri dish *in vitro* conditions. WCR ready-to-hatch eggs (10 – 17 days old) were introduced in a drop of water ca. 0.5 mL on a filter paper and later 1 mL of treatment was

sprayed over the eggs and filter paper using a hand sprayer. It was ensured that no free water was available after spraying the treatments. Five concentrations of neem leaf extract viz. 0.01, 0.1, 0.5, 1 and 10% (v/v) were prepared from a stock concentration of 20% (w/v) prepared using distilled water, filtered through a milk-filter paper and the extract was obtained. Five concentrations of azadirachtin (v/v) viz 0.001, 0.003, 0.005, 0.01, 0.1% was prepared from 0.1% NeemAzal T/S by dissolving 10 mL in 100 mL distilled water. As positive control served the neonicotinoid pesticide imidacloprid concentrations 0.01 and 0.1% and as a negative control served the distilled water. The plates were closed with lid and were sealed with parafilm tape to avoid the hatched larvae to fall off the plates. The plates were incubated in a controlled condition (24°C, R.H=80%, dark conditions). Egg mortality, hatching of larvae and contamination in the plates were recorded for a period of 1 to 7 days post-treatment.

b. Effect of neem against neonate WCR larvae under *in vitro* conditions

Neem-derived pesticides were tested against neonate WCR larvae in an artificial-diet bioassay under standardised conditions. The experiment was conducted in CABI laboratory in Hódmezővásárhely, Hungary. The diet used for the experiment was by Frontier Ltd. F9800B commercial diet + lyophilized maize root powder (GLH5939 from USA, 1.5g/100ml diet) + KOH (10% w/v, 2.5ml/100ml diet, pH 6.3). Seven concentrations of neem leaf extract viz. 0.01, 0.1, 0.5, 1, 5, 10 and 20% (v/v) were prepared from a stock concentration of 20% (w/v) prepared using distilled water, filtered through a milk-filter paper and the extract was obtained. Seven concentrations of azadirachtin (v/v) viz 0.0001, 0.001, 0.003, 0.005, 0.01, 0.1 and 0.5% was prepared from 1% NeemAzal T/S by dissolving appropriate amounts in distilled water. As positive control served the neonicotinoid pesticide imidacloprid (2 µg a.i /ml) and as a negative control served sterile distilled water.

Three to six bioassay plates of 96 wells and single neonate larvae had been set-up for each of the experiments (total plate number 15). The procedure was as following: From the diet 190 µl was poured in the well and let it dried for 30 mins and was stored overnight at 4°C. Twenty microlitre treatment was added the next day and it was dried using a ventilator for 1 hour. One neonate larva was added per well and the plate was sealed using qPCR seals. The plates were incubated in the dark with controlled conditions (24°C, R.H= 75%). Mortality, stunting of larvae and contamination was assessed after 3 and 5 days.

Data analysis

In the case of *Diabrotica virigfera virgifera* eggs experiment, One-way ANOVA post-hoc Tukey's test was performed on the data using RStudio v 3.4.0 (2017) and graphs were made in the excel. In the case of neonate larvae, mean along with standard error was calculated and graphs were presented from excel program.

2.3. Effect of neem-derived pesticides on non-target organisms

2.3.1. Entomopathogenic (EPN) and slug-parasitic (SPN) nematodes

Commercial EPN species (products of Biobest, Belgium) used in the experiments were *Heterorhabditis bacteriophora* (B-Green), *Steinernema carpocapsae* (Carpocapsae-System), *Steinernema feltiae* (Steinernema-System), *Steinernema kraussei* (Kraussei-System), and commercial SPN species (product of Biobest, Westerlo, Belgium) was *Phasmarhabditis hermaphrodita* (Phasmarhabditis-System). All products were stored in the refrigerator at 5ºC until they were used for the experiment.

Experimental setup

The experiment was performed in flat-bottomed, 96-well microplates (Kartell S.p.A.,
Noviglio, Italy) under laboratory conditions. Using a micropipette, five juveniles in 60 µL distilled water were placed into each well of the microplate followed by the different treatments by adding 200 µL of each concentration and 200 µL of distilled water serving as control. Eight replicates of each treatment were applied (Fig 18). The microplates were closed with lids and sealed by parafilm tape to avoid evaporation of the extracts and incubated in a thermostat in dark conditions at 20ºC ± 1ºC. After an exposure period of 24 hours, the wells were observed under a transmission stereomicroscope. Ten µL of 5% lactic acid was added which is a modified method of Ciancio (1995) to assess the nematode viability and movement stimulant. Maximum mortality of 20% in the control treatment was considered as a validity criterion for the tests.

Data analysis

Data were processed and square root arcsine-transformed in an Excel spreadsheet before statistical analysis using PAST3 (Paleontological Statistics) statistical software (Hammer et al. 2001). One-way ANOVA, more specifically Tukey's test and Mann–Whitney U test, were performed on the data, depending on whether the normality (Shapiro–Wilk test) was fulfilled. Graphs were made using excel program. Low-lethal (LC10) and sublethal (LC50) values for different NLE concentrations were calculated by AAT Bioquest® calculator.

2.3.2. Isopod (*Porcellionides pruinosus* Brandt 1888)

The methodology followed was according to Akca et al. (2015) with modifications.

Collection of isopod species

Porcellionides pruinosus adults were collected from Regional Waste Management Center Pusztazámor, Hungary, by hand sorting. Isopods were bred

and maintained at the Institute of Plant Protection of Szent István University, Gödöllő, Hungary. Species level identification was based on the taxonomic key developed by Brandt (1833) (Farkas and Vilisics 2013).

Experimental design

Five adults of *P. pruinosus* were placed in glass Petri dishes (13 cm in diameter), with 1 g of commercial horticultural soil (pH = 7.0) and approximately 1 g of fresh potato as a food source. Each treatment was replicated 10 times. Two mL of different working concentrations of neem leaf extract and azadirachtin were sprayed using a hand sprayer. After spraying, the Petri dishes were kept in the dark, checked after time periods of 1, 24, 48, 72, 96, and 120 hours post-application of neem leaf extract and azadirachtin respectively, and mortality data was recorded.

Data analysis

The mortality data obtained after 120 hrs was subjected to statistical analysis using R software (R Core Team, 2017). Logistic regressions were fitted (as the response was binary, i.e., the isopods were either dead or alive) to check the effect of the two different products on isopod mortality. To test whether the concentrations have significant effect on mortality, chi-squared tests were performed on model deviances. Prior to running the tests model diagnostic plots were investigated to assess homoscedasticity and residual normality (Faraway, 2002).

2.3.3. Entomopathogenic and antagonistic fungi

Sub-culturing of entomopathogenic and antagonistic fungi

Species of different entomopathogenic fungi namely *Beauveria brassiana* and *Metarizhium anisopliae* and antagonistic fungi *Trichoderma harzianum* were obtained from the Institute of Plant Protection of Szent Istvan University, Gödöllő, Hungary. They were sub-cultured on Potato Dextrose Agar (PDA) plates and were kept at room temperature for 7 days before using for *in vitro* experiment.

In vitro tests

The working concentrations used for neem leaf extract were 0.1, 1 and 2% were prepared by adding 2.5, 25 and 50 mL of neem leaf extract to 500 mL sterile molten PDA respectively and for NeemAzal T/S the concentrations of 0.01, 0.05 and 0.1% were prepared by adding 0.25, 1.25 and 2.5 mL respectively, mixed well and poured into 9 cm sterile Petri dishes under aseptic conditions and allowed it to cool. Sterile PDA plates without neem served as control. A 9 mm mycelial disc of different fungi were placed in the centre of the Petri plates. Zone diameter was measured by averaging two diameters at right angle for each colony (Alkhail, 2005).

Data analysis

Data was processed in an excel sheet and mean of the colony diameter of three replicates were calculated for each treatment at the given time interval.

3. RESULTS

3.1. HPLC analysis of neem leaf extract

The chromatogram of the analysis of standard azadirachtin showed a peak at a spectrum of 213-214nm while in the case of chromatogram of spiked and non-spiked neem leaf extract samples, five peaks appeared at different time interval but at the same spectrum. The peak 1 from the spiked and non-spiked neem leaf extract sample was confirmed to be azadirachtin A when compared to the standard azadirachtin A as they had the same spectrum i.e 213-214 nm and retention time. There were other peaks namely from 2 to 5 which appeared on the chromatogram for the neem leaf extract sample. They are suspected to be the derivatives of azadirachtin as they too appeared in the same spectrum as that of the standard. The concentrations of these peaks were calculated using the formula mentioned in the materials and methods for both spiked and non-spiked samples. In the non-spiked sample, azadirachtin A concentration was found to be 1mg/5g of leaves while in the case of spiked sample, it was found to be 24.5mg/5g leaves.

3.2. Effect of neem-derived pesticides on target organisms

3.2.1. *Plasmopara halstedii* ((Farl.) Berl. & De Toni, 1888)

a. Effect of neem-derived pesticides on *P. halstedii* sporangial germination under *in vitro* conditions

All the treatments except the AZA 0.01% were found to be significantly different as compared to the control where no treatment was done (Fig. 22); in reducing the total number of empty sporangia (which includes completely empty and partially empty sporangia as per the proposed scale). Both concentrations of neem leaf extract i.e 10 and 20% and the highest concentration of NeemAzal T/S i.e 0.1% decreased the number of empty sporangia; which are comparable to mefenoxam. The fewer empty sporangia were found in NLE 10%.

b. *In vivo* experiment: Pre- and post-treatment effect of neem-derived pesticides on *P. halstedii* in sunflower

In the case of pre-treatment, both concentrations of neem leaf extract and highest concentration of NeemAzal T/S (AZA 0.1%) significantly reduced sporulation, which was comparable to mefenoxam. The lowest concentration of NeemAzal T/S (AZA 0.01%) was not significant in reducing the sporulation as compared to control. Initial plant height in the case of AZA 0.01% was significantly lower to other treatments but not significant to the infected control (Seed + Inoculum). Pre-treated AZA 0.1% and mefenoxam plants showed no significant difference to plants treated with bidistilled water except for AZA 0.01% + bidistilled water

(BW). In the post-treatment, lowest concentrations of NLE and AZA did not significantly reduce sporulation while the highest concentrations of NLE and AZA did compare to control. For initial plant height, NLE 10% and AZA 0.01% showed significant difference compared to mefenoxam treated and plants treated with bidistilled water. All the treatments did not show any significant difference in the plant height compared to the infected control (i.e Seed + Inoculum). No chlorosis was observed in any of the plants inoculated with P. halstedii after a period of 19 days (i.e at the end of the experiment).

3.2.2. *Meloidogyne incognita* (Kofoid & White, 1919 Chitwood 1949)

a. Effect of neem-derived products on *M. incognita* (J_2) under *in vitro* conditions

No significant difference in mortality was observed for NeemAzal T/S, while 0.5 and 1% of NLE showed significant difference in mortality.

b. Effects of neem-derived products on *M. incognita* infestation in tomato under glasshouse conditions

All the three scales showed significant difference as compared to non-infected control. In the case of both Dányi and Ceglédi landraces, Zeck scale proved the strongest next to the scales of Mukhtar et al. and Garabedian and Van Gundy. In the case of Dányi landrace, values of the root damage were inconsistent throughout the different scales. In the case of Ceglédi landrace, concentrations of neem leaf extract did not differ from positive control with respect to average root damage, according to all the three scales but AZA 0.1% showed significantly lower root damage. No significant difference was seen in the case of average number of fruits in both the landraces. In the case of fresh shoot weight, AZA 0.1% showed lower fresh shoot weight with a significant difference in both Dányi (Fig 28) and Ceglédi (Fig 29) varieties with respect to 0 control. Apart from this, there was no significant difference between the other treatments.

3.3.3. Colorado Potato Beetle (*Leptinotarsa decemlineata* Say 1824)

a. No-choice test

No significant difference in the mortality across azadirachtin concentrations while NLE 15% and 20% showed significantly high mortality of 66% and 93% after 72h and 96 h respectively as compared to other treatments. In the case of feeding damage, after 48h post-treatment, NLE 5% to 20% showed significant difference in feeding damage. NLE 15 and 20% resulted the least feeding damage throughout which coincides with the high mortality after 72 and 96h post-treatment respectively. No significant difference in feeding damage was seen in azadirachtin at all time intervals.

b. Choice test

No significant difference in the mortality across different neem leaf extract and azadirachtin concentrations. NLE 20% showed a significant difference in the leaf damage after 48h. All the treatments showed significant reduction in leaf damage after 96h compared to their untreated leaves.

3.2.4. Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte 1868)

a. Effect of neem against *Diabrotica virgifera virgifera* eggs under *in vitro* conditions

No significant difference was observed in egg mortality for all the treatment across different time interval.

b. Effect of neem against *Diabrotica virgifera virgifera* larvae under *in vitro* conditions

Azadirachtin 0.1% and 0.5% was as effective as Imidacloprid at 0.01µL concentration in inducing mortality in neonate larvae at day 3 and day 5. A slight effect of neem leaf extract at 20% concentration was seen at day 5.

3.3. Effects of neem-derived pesticides on non-target organisms

3.3.1. Entomopathogenic and Slug-parasitic nematodes

In the case of *Heterorhabditis bacteriophora* and *Steinernema kraussei*, NLE 0.3% to 1% concentrations caused significantly high mortality. For *Phasmarhabditis hermaphrodita*, *Steinernema carpocapsae* and *Steinernema feltiae* NLE 0.6 and 1% showed significantly high mortality compared to control and other treatments. On the other hand, azadirachtin at all the tested concentrations did not have significant effect on any of the nematode species. LC_{50} were determined for the different nematode species. *H. bacteriophora* had the lowest value, while *S. feltiae* had the highest. In the case of LC_{10} concentrations, a different tendency developed. *S. carpocapsae* had the highest value, followed by *S. kraussei, H. bacteriophora, S. feltiae* and *Ph. hermaphrodita*.

3.3.2. Isopod (*Porcellionides pruinosus* Brandt 1888)

The mortality of *P. pruinosus* was generally low in all treatments. No significant mortality was observed in the case of different concentrations of neem leaf extract and azadirachtin.

3.3.3. Entomopathogenic and antagonistic fungi

All concentrations of neem leaf extract and azadirachtin enhanced the growth of *Beauveria bassiana* over the period of time. No effect of any of the neem pesticides was observed. Also, the results showed that neither of the neem-derived pesticides had any negative effect on the growth of *Metarhizium anisopliae*. The results were consistent with the antagonistic fungus *Trichoderma harzianum* and it was found that neem-derived pesticides did not exert a negative effect on *Trichoderma harzianum*.

4. NEW SCIENTIFIC RESULTS

1. Neem leaf extract prepared in the traditional way contained a low concentration of azadirachtin A, the most abundant, extensively studied and extremely effective ingredient of commercial neem-based plant protection products. However, suspected derivatives of azadirachtin were detected at much higher concentrations.
2. Commercial azadirachtin and neem leaf extract both inhibited the germination of *Plasmopara halstedii* sporangia *in vitro*. Efficacy of neem leaf extract was comparable to that of mefenoxam. Pre- and post-treatment of sunflower seedlings gave consistent results with neem leaf extract showing better results compared to azadirachtin concerning both the sporulation of the pathogen and the initial plant height.
3. Higher concentrations of neem leaf extract significantly increased the mortality of *Meloidogyne incognita* larvae *in vitro*. However, either neem leaf extract or azadirachtin have only slight *in vivo* effect on root infection, plant shoot weight and yield, respectively.
4. Neem leaf extract was as effective as Btt in controlling Colorado potato beetle. Choice test proved that neem leaf extract also has antifeedant effect on Colorado potato beetle, comparable to that of azadirachtin.
5. *Diabrotica virgifera virgifera* eggs treated with neem-derived pesticides under study did not show any significant signs of mortality. Meanwhile, the larvae bio-assay data revealed that azadirachtin induced high mortality which was comparable to insecticide imidacloprid. Neem leaf extract showed slight high mortality but not as effective as azadirachtin.
6. Entomo- and slug-parasitic nematodes (*Heterorhabditis bacteriophora*, *Phasmarhabditis hermaphrodita, Steinernema carpocapsae*, *S. feltiae*, *S. kraussei*) were significantly more sensitive to higher concentrations of neem leaf extract than to azadirachtin *in vitro*.
7. The isopod species under the study (*Porcellionides pruinosus*) wasn't sensitive to neem leaf extract and azadirachtin *in vitro*.
8. No adverse effect was observed in the case of both entomopathogenic and antagonistic fungi when treated with neem-derived pesticides. In fact, the neem enhanced their growth *in vitro*.

5. CONCLUSIONS AND RECOMMENDATIONS

The main objective of my thesis was to compare the effects of two neem-derived pesticides on target and non-target organisms. The target organisms selected for study were *Plasmopara halstedii, Meloidogyne incognita, Leptinotarsa decemlineata* and *Diabrotica virgifera virgifera* while the non-target organisms selected were entomopathogenic- and slug-parasitic nematodes, isopod (*Porcellionides pruinosus*) and entomopathogenic and antagonistic fungi.

The HPLC results of this study showed that the azadirachtin A component in the neem leaf extract was found to be low and also presence of possible suspected derivatives of azadirachtin. Although it must not be forgotten that there are other bioactive compounds in neem plant parts which do possess different properties as per the literature. There can be a synergistic or antagonistic effect of these compounds with azadirachtin. Their presence in the neem and their compatibility testing may provide us better answers as to why neem is such a widely studied as a plant protection agent. Therefore, a detailed analysis of different compounds present in the neem leaf extract should be done to estimate their concentration as different batch of neem leaves used for analysis may vary in the concentration of azadirachtin and other biologically active compounds.

From the results of testing the effects of neem on *P. halstedii*, it can be concluded that azadirachtin and NLE both inhibited the germination of *P. halstedii* sporangia *in vitro*. Efficacy of the applied concentration of NLE was comparable to that of mefenoxam and slightly superior to azadirachtin. Similarly, in the in vivo conditions, neem leaf extract at 10 and 20% concentration gave results which are comparable to mefenoxam while the highest concentration of azadirachtin gave better results. Further research is needed to test the effect of neem extracts and commercial products on different pathotypes of *P. halstedii* under *in vitro*, glasshouse and field conditions with different mode of application. In addition, an investigation on the systemic and/or curative effect of neem-derived pesticides against *P. halstedii* in sunflower by measuring different enzymatic activities in the plants, needs to be done. It is also recommended to test the freshly harvested seeds from the field that were previously treated with neem-derived pesticides to check the presence of different biologically active compounds of neem in them.

In case of *M. incognita*, it can be concluded that under *in vitro* conditions, neem leaf extract was found to be consistent with the previous literature undertaking this experiment. However, neem leaf extract and azadirachtin did lower the root infection, increased the plant shoot weight and height in number but the effect was not statistically significant. Neem leaf extract could be more effective against *M. incognita* with continuous and timely application either by drip irrigation or soil drenching

In the experiments with *Leptinotarsa decemlineata*, mixed results were found according to the antifeedant and lethal effects of commercial azadirachtin and

neem leaf extract, respectively. It was found that in these aspects traditional neem leaf extract was superior to the commercial product. The reason for it could be that it contains not only azadirachtin but many other biologically active different compounds which exhibit different plant protection properties. Field trials on a wider scale are necessary to validate our hypothesis.

Diabrotica virgifera virgifera eggs did not show symptoms of any effect due to application of neem leaf extract or azadirachtin. Meanwhile in the case of larvae, higher concentrations of azadirachtin was consistent and more promising and the results were comparable to the insecticide imidacloprid in controlling the larvae. Neem leaf extract showed a delayed effect in the mortality but not as high as azadirachtin.

The non-target organisms selected for this study were entomopathogenic and slug-parasitic nematodes, isopod (*Porcellionides pruinosus*) and entomopathogenic and antagonistic fungi. While checking the effects of neem leaf extract and azadirachtin, it was found that entomopathogenic and slug-parasitic nematodes were significantly more sensitive to higher concentrations of neem leaf extracts than to azadirachtin *in vitro*. As a conclusion, the commercial neem product may be applied with EPNs and/or *Ph. hermaphrodita* simultaneously as plant protection agents, although further research regarding its field application needs to be investigated. Furthermore, compatibility of neem leaf extract and beneficial nematode species also requires further evaluation.

From our results with *P. pruinosus* and neem-derived pesticides interactions, it can be concluded that neither NeemAzal T/S nor neem leaf extracts pose any risk to the terrestrial isopod species studied in the tested concentrations. However, further research is needed to test the possible effect of various neem products on the members of the isopods found in soil. Also, it can be concluded that NeemAzal T/S and domestic neem leaf extract do not differ in respect to their mortality effects on *P. pruinosus*.

The results from interaction of neem-derived pesticides with entomopathogenic and antagonistic fungi point us to the fact that they are compatible with each other *in vitro*. So far, plenty of research has been documented proving the compatibility of neem and entomopathogenic and antagonistic fungi both *in vitro* and *in vivo*. It may not be sufficient enough to provide their compatibility, hence greenhouse and field trials on a large scale with wider range of host plants against different soil-borne pathogens and pests is recommended.

RELATED PUBLICATIONS

Publications in the peer-review scientific journals

- **Doshi, P**., Tóth, F., Turóczi, G (2020). Effect of traditionally prepared and commercial neem-derived pesticides on Colorado potato beetle (L*eptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae) under laboratory conditions, Columella (in press).
- **Doshi, P**., Nisha, N., Yousif, A.I.A.Y., Körösi, K., Bán, R., Turóczi, G. (2020). Preliminary investigation of effect of neem-derived pesticides on *Plasmopara halstedii* pathotype 704 in sunflower under *in vitro* and *in vivo* conditions, *Plants*, 9, 535. DOI:10.3390/plants9040535 (IF = 2.632).
- **Doshi, P**., Tóth, F., Nagy, P., Turóczi, G., Petrikovszki, R. (2020). Comparative study of two different neem-derived pesticides on *Meloidogyne incognita* under *in vitro* and pot trials under glasshouse conditions, Columella, 7(1), 11-21. DOI: 10.18380/SZIE.COLUM.2020.7.1.11
- Petrikovszki, R., **Doshi, P**., Turóczi, G., Tóth, F., Nagy, P. (2019). Investigating the Side-Effects of Neem-Derived Pesticides on Commercial Entomopathogenic and Slug-Parasitic Nematode Products Under Laboratory Conditions, *Plants*, 8(8), 281. DOI: 10.3390/plants8080281 (IF = 2.632).
- **Doshi, P**., Póss, A.M., Tóth. F., Szalai. M., Turóczi, G. (2018). Effect of neem-derived plant protection products on the isopod species *Porcellionides pruinosus* (Brandt, 1833). In: Hornung E, Taiti S, Szlavecz K (Eds) Isopods in a Changing World. ZooKeys, 801, 415–425. DOI: https://doi.org/10.3897/zookeys.801.25510 (IF = 1.143).

Poster

- Presented a poster on "Effect of NeemAzal-T/S, a biological plant protection product on the isopod species *Porcellionides pruinosus"* in the 10th International Symposium on the Biology of Terrestrial Isopods, Budapest, Hungary

Conference abstract

- Effect of NeemAzal-T/S, a biological plant protection product on the isopod species *Porcellionides pruinosus"* in the 10th International Symposium on the Biology of Terrestrial Isopods, Budapest, Hungary

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