SZENT ISTVÁN UNIVERSITY

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

PhD Dissertation

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I dedicate this PhD thesis to my beloved father Late. Shri Pravin Ramnikal Doshi.

You shall always be in our hearts Pappa.

Table of Contents

1. INTRODUCTION

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.) Berlese 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. LITERATURE REVIEW

The book 'Silent Spring' (Carson, 1963) highlighted and evoked consciousness about the hazards caused by pesticides on human health and environment. To reduce the hazardous effects of the pesticides, attempts were made to explore different options which can be equally effective or better than the chemical pesticides without hampering the environment. Biological control or biocontrol (henceforth used interchangeably in the text) which is primarily done by use of biological living organisms to control pest organisms, is looked upon as an alternative to chemical pesticide control; with reduced risk to human and environment (Thomas and Willis, 1998). 'Self- perpetuating', 'self-dispersing' and 'irreversible' are the benefits that were particularly attributed to biological control (Barratt et al. 2010). Biological control is mentioned as the third principle within the eight principles of Integrated Pest Management laid down by Barzman et al. (2015) under non-chemical methods.

2.1. Biological Control

As per Van Lenteren (1993, 2000), there are three main different techniques of biological control that exist, which are a. *classical* ('inoculative' biological control) which is usually used against invasive pests that established in different parts of the world, b. *augmentative* (with a distinction between 'inundation' and 'seasonal inoculation' is made) wherein natural enemies are introduced after their commercial production at appropriate periods, c. *conservation* control which is done with the help of indigenous predators and parasitoids to control pests.

Biological control is found to be a main component of 'systems approach' to integrated pest management (IPM), minimizing use of pesticides, to counteract insect-resistant pests (Bale et al. (2008). Biological control reduces the pests rather than eradicating them, thus keeping the pest population and natural enemy in low densities (Bale et al. 2008; Lamichanne et al. 2016). Biocontrol is the most appropriate strategy to control widely established pests which are resistant to different chemical pesticides or those who have the potential to infest on a wide scale (but already ahead of point of eradication) (Delfosse, 2005). There are several published articles on biological control discussing 'risks' without historical knowledge of the biological control process. But these discussions are often misinterpreted and are not based on a) several successful case studies and b) evolution of biological control agents (Delfosse, 2005). In the past, mankind has had a huge ecological and economic benefits from using the exotic biological control agents with several non-native species suppressing weeds and agricultural and forestry pests in different parts of the world (De Clercq et al. 2011).

Although biological control is becoming a huge success story with respect to controlling different pests, the effects of different biological control agents on the non-target organisms requires equal and/or more attention in practice. As stated by Topping et al. (2020) "*The overall picture is of a need to move to a more holistic (systems) view, which integrates far more than current risk assessment*". For that reason, one of the agenda in classical biological control is its effects on non-target organisms (Ehler, 2000). According to Hopper (2001), in order to consider the non-target impacts, it is long argued that impacts on all species should be evaluated. Some argue that only some species should be taken under special consideration while some others argue that only species under study should be considered and others should be ignored. So, the basic question is what makes a biological control agent successful? In order to search an answer to this question, Stiling and Cornelissen (2005) qualitatively reviewed 2 major biocontrol journals namely *Biological Control* and *Environmental Entomology* literature for the past 10 years. Meta-analyses of separate data revealed that negative impacts of biocontrol agents on non-target species were much smaller than those for target species, but such results should be interpreted with caution. In addition, they concluded that more generalist biocontrol agent gave better biocontrol efficacy than the specialists.

One such 'generalist' biocontrol agent used to study in this thesis was *Azadirachta indica* A. Juss commonly known as 'neem'. The neem tree was described and taxonomic position of *A. indica* was done by De Jussieu (1830) as follows:

Order	Rutales
Suborder	Rutinae
Family	Meliaceae (mahogany family)
Subfamily	Melioideae
Tribe	Melieae
Genus	*Azadirachta*
Species	*indica*

2.2. Description and Characteristics of *Azadirachta indica*

A. indica is a popular tree found in the tropical and sub-tropical countries with its origin thought to be in India or in dried inland forests of Burma (Myanmar) (Puri, 1999). It grows much in South East Asia and West Africa and cultivated in countries like Pakistan, Singapore, Philippines, Australia (Nishan and Subramanian, 2014). *A. indica* was introduced in the Caribbean nations by Indian immigrants. Neem is tolerant to high temperatures and thrives in nutrient poor soil but is susceptible to frost. However, neem cannot tolerate water-logged soils and does not grow in soils

which have high proportions of very fine sand and silt (Koul et al. 1990). Neem is hardy, fast-growing evergreen tree with straight trunk, long spreading branches and moderately thick fissure barks (Sharma, 1998; Ogbuewu et al. 2011) (Fig. 1).

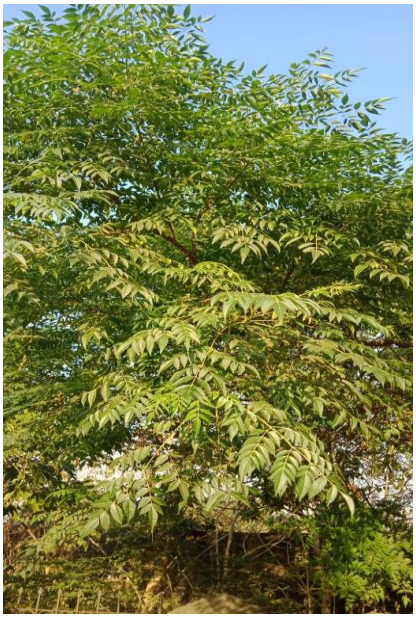

Figure 1: The neem tree (Source: Own photo)

A mature tree can reach up to 7-15 m in height (Sharma, 1998; Ogbuewu, 2008) and the tree starts producing yellow ellipsoidal dupes (fruits) (Fig. 2) in about 4 years and becomes fully mature in 10 years and may live for more than 200 years (Ogbuewu et al. 2011).

Figure 2: Mature neem fruits (Source: Own photo)

Leaves are compound, imparipinnate and comprises of 15 leaflets arranged alternatively with terminal leaflets (Ogbuewu, 2008) (Fig. 3). Maturation stage of neem flowers is from January to August and fruits mature from May to August (Koul et al. 1990) in India.

Figure 3: Fresh neem leaves (Source: Own photo)

Neem is called by various names such as “Heal all”, “Divine tree”, “Village pharmacy” and “Nature’s drugstore” (Puri, 1999). It was said in an old proverb about neem that,

“The land where the neem tree abound,

Can death there be found?”

The neem tree is considered in Sanskrit as *Sreva roga nivarni* which means ‘the panacea of all diseases’ (Puri, 1999; Hossain and Nagooru 2011). Neem regenerates naturally with mature seeds being dispersed by birds under natural conditions. Artificial propagation can be done by direct sowing or seedling transplantation or rootstock. It is a versatile tree which is highly useful to mankind, since every part of it is used in different forms (Sharma, 1998).

2.3. Plant parts of neem and their properties

Girish and Bhat (2008) listed the different uses of neem products as follows:

a. **Seeds**: Neem seeds are used to make oil and seed cake.
b. **Neem oil**: Neem oil is used for medicinal purpose as it is known to possess properties such as analgesic, antihelminthic, bactericidal, antipyretic, antiviral, fungicides, insecticides, insect repellents in the veterinary medicines. It is also used for technical purpose such as in cosmetics, hair oils, lubricants, propellants, shampoos, soaps, toothpaste.
c. **Neem leaves**: Neem leaves possess antidermatic, antifungal, anticlotting agent, antihelminthic, antituberculosis, antitumour, antiseptic, antiviral, contraceptive, cosmetics, fertilizers, insecticides, nematicides, insect repellents properties.
d. **Twigs (Fig. 4):** Neem twigs are traditionally and still used in India as oral deodorant, toothache reliever, tooth cleaners.

Figure 4: Neem twigs (Source: Google images)

e. Bark: Neem Bark is known to possess antiallergenic, antidermatic, antifungal, antiprotozoal, antitumor, deodorant properties.

2.4. Chemical compounds derived from neem

The biological activity of neem has been studied intensively over the past many years and in general extracts of different parts of neem tree is shown to possess different activities and properties such as insect repellent, antifeedant, nematicidal, fungicidal (Jacobson, 1989; Schmutterer et al. 1981; Schmutterer, 1990; Schmutterer 1995). These properties are the results of the different compounds found in different parts of the neem tree. The following types of major compounds are reported (Puri, 1999):

Terpenoid constituents*:* Protolimonoids, Limonoids, Tetratripernoids, Pentatriterpenoids, Hexatriterpenoids

Non terpenoid constituents: Hydrocarbons, Fatty acid, Steroids, Phenols. Flavonoids, Other compounds.

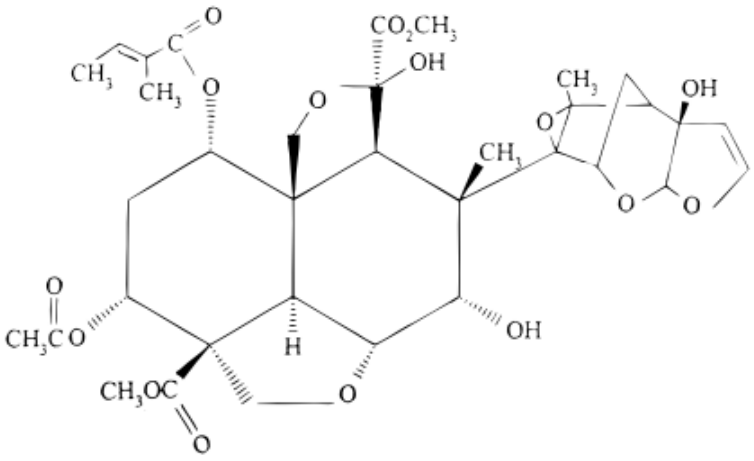

Figure 5: Chemical structure of azadirachtin A

Azadirachtin is one of the most intensively studied neem metabolites for its array of effects in plant protection (Rangiah et al. 2016). Apart from azadirachtin as shown in Fig. 5, several other bioactive compounds are found in neem namely nimbidin, nimbin, nimbolide, mahmoodin, gallic acid, margolone, nimbolide, salannin that have shown to possess a variety of effects (Biswas et al. 2002).

2.5. Applications of Neem

Due to its vast benefits and different uses in medicine, agriculture and industrial field, focus on the importance of neem products is increasing. It is the most researched tree and said to be the most promising tree in the 21st century (Ogbuewu et al. 2011). An overview of different applications is given by Mondal and Chakraborty (2016):

2.5.1. Neem and medicine

Neem has been used traditionally to treat a variety of diseases. Recent clinical and pharmacological studies of different parts of neem and their commercial products are done and the results in almost all cases are highly satisfactory (Bramhachari, 2004). In the Indian tradition, *A. indica* is used for treatment of diabetes. Blood sugar level is significantly decreased by aqueous extracts of neem leaves, thus preventing adrenaline- and glucose-induced hyperglycaemia (Murty et al. 1978). Neem leaf extract is known to possess antiulcer and antisecretory effects in rats (Jena et al. 2002). Nimbidin, a bitter principle of neem, has proved to heal ulcers in duodenum and relieving pain in epigastric region (Divakar et al. 2002). Neem is also used to treat malaria as it is found to possess antimalarial property (Iwalewa et al. 1994; Subapriya and Nagini, 2005). Several dermatological effects of neem are also seen against common skin diseases like acute and chronic eczema, ringworm and scabies (Bramhachari, 2004). All parts of neem are used effective against worms, wounds in mouth, *Escherichia coli (*Migula, 1895) Castellani and Chalmers 1919, *bacillosis*, intestinal wounds (Ketkar and Ketkar, 1995).

2.5.2. Neem and agriculture

Neem used as fertilizer and manure: Seed cakes made after squeezing out oil from the seeds is used as a bio-fertilizer which provides nutrients to the plants. It is widely used to increase the yield. Combining neem cake with urea fertilizer improves the fertilizer efficiency in crop production by gradual release of nitrogen to crops (Ketkar, 1983). Because neem is rich in sulphur, calcium, nitrogen, potassium etc, neem cake is used to make high quality manure to nourish the soil and to provide macro and micro-nutrients to plants. Plant debris is a good organic manure source (Bramhachari, 2004). Neem leaves are good source for preparation of vermicompost with fertilizer and pesticidal properties (Gajalakshmi and Abbasi, 2004a).

Neem as fumigant: Neem tree has been used as fumigant against household, crop and storage insect pest. The major advantage of this fumigant is that it is eco-friendly, kills the pests, do not develop any resistance to it and does not leave any residue on the plant (Grace, 1991; Lokanadhan et al. 2012).

Neem as animal feed for poultry, cattle and sheep: Asian countries, particularly India is exploiting neem's potential for commercial production of animal feed (Parmar and Ketkar, 1996; Dhaliwal et al. 2004). Processed neem seed meal rich in protein was palatable to buffalo at 25% concentration and was eaten at 10-20% level without any effect on milk constituents during lactation or on red and white blood cell counts (Koul et al. 1990). Calcium and phosphorus content in the blood was enhanced with a gradual increase in neem cake ration (Gangopadhyay et al. 1981) and de-oiled neem cake up to 25-50% could be added in maize diet for sheep without toxic effects (Gupta and Bhaid, 1981).

2.6. Role of Neem in Plant Protection

Neem is the most widely studied plant for its broad range of effects towards different plant pests and pathogens (Biswas et al. 2002). It tops the list of 2400 botanical pesticides in the world (Jagannathan et al. 2015). Neem-derived pesticides and products are effective against more than 350 arthropod species, 12 nematode species, 15 fungi species, 3 viruses, 2 snail species and 1 crustacean species (Ogbuewu et al. 2011), some of which are discussed below in separate sections.

2.6.1. Neem against different orders of insect pests

Neem has been used in Indian Sub-continent since the ancient times in plant protection. It is documented that desert locusts *Schistocerca gregaria* Forsskål, 1775 do not eat the leaves of *A. indica* (Morgan, 2009). A research was conducted by Keele University in 1966 to isolate substances deterrent to desert locusts. Accidently, they observed that desert locust did not eat the leaves treated with neem extracts and hence the pure compound with most potent anti-feedant property was named azadirachtin, from its botanical name (Ascher, 1993; Morgan, 2009). This compound was isolated from seeds of neem tree by Butterworth and Morgan in 1968 and its definite structure was explained previously as stated by Schmutterer (1988). Different neem plant parts possess a wide range of properties which are useful in plant/crop protection. Neem oil generally extracted from neem seeds has a strong antifeedant and oviposition deterrent activity (Benelli et al. 2015). Lokanadhan et al. (2012) observed the inability of female insects to lay eggs during storage. Babendreier et al (2020) tested two commercial neem-derived pesticides Ozoneem and Grow-Safe against fall armyworm and found that neem pesticides were as efficient as emamecitin, a chemical insecticide as control. Usharani et al. (2019) summarized the recent

research with neem oil to exploit its pesticidal property on different pests and pathogens in agriculture and a compilation of effects of neem-derived pesticides on different insect pests is presented in (Table 1).

Table 1: Recent research on neem essential oil for pesticidal potential in agriculture (Source: Usharani et al. 2019)

Crop/Plant	**Pest/Pathogen/Disease**	**Treatment**	**Reference**
Mango	Powdery Mildew and Mango Malformation	Neem oil (1%)	Ismail, 2016
Brinjal	Shoot and fruit borer, *Leucinodes orbonalis* Guenee	Neem oil	Singh and Sachan, 2015
Cowpea	*Maruka vitrata* (Fabricius, 1787)	Multinucleopolyhedrovirus + neem oil	Sokame et al. 2015
Kinnow mandarin	*Penicillium digitatum* (Pers.) Sacc. and *Penicillium italicum* Wehmer, (1894)	Neem essential oil	Jhalegar et al. 2015
Cotton	Cotton pests (*Helicoverpa armigera* Hübner, 1808, *Haritalodes derogate* Fabricius, 1775, *Aphis gossypii* Glover, 1877, *Polyphagotarsonemus latus* Banks, 1904, *Pectinophora gossypiella* Saunders, 1844, *Thaumatotibia leucotreta* Meyrick	*Beauveria bassiana* + neem oil	Togbe et al. 2015
Cabbage	Cabbage aphid (*Brevicoryne brassicae* Linnaeus, 1758	Neem oil (1%)	Pissinati and Ventura, 2015
Okra	Whitefly (*Bemisia tabaci* Gennadius)	Mineral oil+ Neem oil	Sridharan et al. 2015
Western white pine	*Zootermopsis augusticollis* Hagen, 1858 (Dampwood termite)	Neem oil	Fatima and Morrell, 2015
Cashew trees	*Toxoptera odinae* (van der Goot, 1917)	Neem oil	Ambethgar, 2015b

Crop/Plant	Pest/Pathogen/Disease	Treatment	Reference
Stone fruit	*Monilinia fructicola* (G.Winter) Honey	Neem oil	Lalancette and McFarland, 2015
Watermelon	*Aphis gossypii* Glover, 1877	Neem oil	Souza et al. 2015b
Coconut	*Aceria guerreronis* Keifer, 1965	Neem oil (3%)	Balaji and Hariprasad, 2015
Jasminum auriculatum	*Eriophid mite*	Neem oil 30 mL/L	Devi et al. 2015
Tomato	*White fly and Leaf minor*	Neem oil	Chavan et al. 2015
Cashew	*Ferrisia virgata* Cockerell, 1893	Neem oil	Ambethgar, 2015a
Okra	*Bemisia tabaci* Gennadius, 1889	Neem oil	Kumar et al. 2015
Tomato	*Tuta absoluta* Meyrick, 1917	Neem seed oil	Salem and Abdel-Moniem, 2015
Cultivated crops	*Helicoverpa armigera* Hübner, 1808	Neem oil	Ahmad et al. 2015
Cultivated crops	*Helicoverpa armigera*	Ponneem (neem+ pongania oil, 1:1 ratio)	Packiam et al. 2015
Chickpea	*Helicoverpa armigera*	Nimbicidine and Neemarine	Singh and Yadav, 2007
Phaseolus vulgaris	*Bemisia tabaci*	Neem oil	de Almeida Marques et al. 2015
Cotton	*Spodoptera exigua* Hübner, 1808	Agroneem, Ecozin, and Neemix, non-commercial neem leaf powder	Greenberg et al. 2005
Cowpea (Brazil)	*Spodoptera eridania* Stoll, 1781 (southern armyworm)	Neem oil (0.35% and 0.7%)	Rodrigues et al. 2015
Maize	*Spodoptera frugiperda* J.E. Smith, 1797	Neem oil (0.006, 0.05, 0.4%)	Roel et al. 2010
Ash trees	*Agrilus planipennis* Fairmaire, 1888	Neem leaf extract	Kreutzweiser et al. 2011
Potato	*Leptinotarsa decemlineata* Say, 1824	Azadirachtin extract and neem oil	Kaethner, 1992
Tomato	*Nezara viridula* Linnaeus, 1758	NeemAzal T/S and Neem oil	Durmusoglu et al, 2003

2.6.2. Neem against plant-parasitic nematodes

Neem is known to possess nematicidal (Schmutterer, 1985) and nematostatic properties (Nile et al. 2017). Akhtar (1998) tested different neem derived pesticide products such as neem leaf

powder, sawdust and oilseed cake and urea against 4 different plant-parasitic nematodes namely *Hoplolaimus indicus* Sher, 1963, *Helicotylenchus indicus* Siddiqi, 1963, *Rotylenchulus reniformis* Linford and Oliveira, 1940 and *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949 juveniles. He found that soil amendments with these products significantly suppressed these plant-parasitic nematodes. Abbasi et al. (2005) witnessed 67-90% reduction in *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941 and root-knot nematode *Meloidogyne hapla* Chitwood, 1949 in tomato grown in three different soils. Under field conditions, 1% neem cake reduced the number of lesion nematodes by 23% in corn roots and 70% around the roots in the soil. Musabyimana and Saxena (1999) successfully controlled *Pratylenchus goodeyi* Sher & Allen 1953 and *Meloidogyne* spp in banana and found that neem cake at 100g/ plant gave results which comparable to Furadan 5G (carbofuran). Khanna and Kumar (2006) tested five different neem formulations against *M. incognita in vitro* and found that neem seed kernel extract and Econeem, a commercial product consisting of azadirachtin A and B, gave the highest juvenile mortality (73-77%). Sahu et al. (2018) treated tomato with different concentrations of neem cake. They found that neem cake applied at rate of 100 g/m^2 increased the morphological characteristics of tomato and significantly reduced the number of root galls. Water extracts of neem leaves was found to be directly toxic to *Pratylenchus brachyurus* Godfrey, 1929 Filipjev & Schuurmans Stekhoven, 1941 in *in vitro* tests under laboratory conditions (Egunjobi and Afolami, 1976). They also found a significant reduction in *P. brachyurus* under semi-field conditions and concluded that neem contains nematicides which can be systemic.

2.6.3. Neem against different plant pathogens

It is documented that neem is known to possess anti-fungal properties and it has been used to control different plant pathogens. For instance, Moslem and El-Kholie (2009) tested ethanolic, hexane and methanolic extracts of neem seeds and leaves against *Alternaria solani* Sorauer, *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, *Rhizoctonia solani* J.G. Kühn and *Sclerotinia sclerotiorum* (Lib.) de Bary *in vitro* conditions. They found that both the extracts were effective against all the pathogens but *F. oxysporum* and *R. solani* were the most sensitive. Different concentrations (5%, 10%, 15% and 20 %) of aqueous, ethanol and ethyl acetate extracts of neem leaves were tested against early blight and wilt diseases of tomato (i.e *Alternaria solani* and *Fusarium oxysporum* respectively) under *in vitro* and *in vivo* conditions (Hassanein et al. 2008). It was found that all the used concentrations of neem extract suppressed the mycelial growth of both the fungi and the effect increased with increase in concentration. Through *in vitro* and *in vivo* tests, they concluded that neem extracts can be considered as a cheap and environmentally safe option to protect the tomato plants against early blight and wilt pathogens. Sitara et al. (2008)

reported that essential oils extracted from neem seeds showed fungicidal activity when tested against 8 different fungi namely *Aspergillus niger* van Tieghem, *Aspergillus flavus* Link, *Fusarium oxysporum*, *Fusarium moniliforme* J. Sheld, *Fusarium nivale* (Fr) Sorauer, *Fusarium semitectum* Berkely and Ravenel, *Drechslera hawiinesis* M.B Ellis and *Alternaria alternata* (Fr.) Keissl. Antifungal activity of neem leaf extract against *Aspergillus* and *Rhizopus* was also reported by Mondali et al. (2009). Hasan et al. (2005) investigated antifungal effects of neem along with nine other plant extracts against seed-borne fungi of wheat seeds. They found that alcoholic extracts of neem completely controlled the growth of *Bipolaris sorokiniana* (Sacc.), *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp. after the treatment on wheat seeds.

The anti-microbial properties of neem against different phytopathogenic bacteria are also documented. *Xanthomonas* is a very important genus of phytopathogenic bacteria that are known to cause diseases on the wide variety of crops (Mandavia et al. 1999; De Britto and Gracelin, 2011). The crude methanol extracts of neem showed growth inhibitory effects on *Xanthomonas campestris* (Pammel) Dowson 1939 (De Britto and Gracelin, 2011). Also, they observed significant inhibitory effects on *X. campestris* with methanolic extracts of leaves and fruits. In another study, Hulloli et al. (1998) tested the effects of different neem formulations i.e Plantolyte, Agricare, Neemark and RD-9 against *Xanthomonas axonopodis* pv. *malvacearum* (Smith 1901) Vauterin et al. that causes bacterial blight of cotton. They concluded that these neem-based formulations successfully control the bacteria and stated that neem can be used for eco-friendly management of bacterial blight of cotton. Abbasi et al. (2003) demonstrated that neem oil and fish emulsion reduced the symptoms of *Xanthomonas campestris* pv. *vesicatoria* (exDoidge) Vauterin et al. on tomato and bell pepper under greenhouse conditions with no phytotoxic effects of both the products. Verma and Agrawal (2015) tested 6 different medicinal plants including *A. indica* agains.t *Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye & Wilkie that causes bacterial blight in pea under *in vitro* conditions. They found that all the 6 medicinal plant extracts were significantly effective against the pathogen.

Neem has been used to treat several plant viruses in agriculture. Madhusudhan et al. (2005) demonstrated that tomato and bell pepper seedlings treated with 5% neem oil was effective in reducing the local legions caused by Tomato mosaic tobamovirus (ToMV) and Tobacco mosaic tobamovirus (TMV) compared to untreated ones. According to Saxena et al. (1985), application of neem oil and cake showed a reduction in rice seedling infection by rice tungro bacilliform and spherical virus particles.

2.6.4 Target organisms under study

***Plasmopara halstedii* (Farl.) Berl. & De Toni 1882**

Plasmopara halstedii (Farl) Berl. & De Toni 1882 is one of the most destructive pathogens infecting sunflower. It causes downy mildew disease in sunflower, causing huge economic losses. It is the 16th most important oomycete with respect to economics and science (Kamoun et al. 2015, Trojanová et al. 2018). The infection usually takes place under the soil by direct penetration in the roots (Virányi and Spring, 2011). Hypocotyls show the first symptoms of *P. halstedii* systemic infection in sunflowers than roots (Cohen and Sackston, 1973). The symptoms such as seed damping-off occurs by root infection. In addition, other symptoms such as stunting of plants (dwarfing), chlorosis on the leaves and white sporulation can be seen resulting in yield loss (Sackston, 1981; Gascuel et al. 2015). *P. halstedii* can rapidly develop races (pathotypes) that can overcome the resistance gene in sunflower (Sedlářová et al. 2016; Bán et al. 2018). Use of resistant cultivars, agrotechnical methods and chemical treatment are some of the measures to control downy mildew of sunflower (Albourie et al, 1998). Pathotypes have developed fungicide resistance and have overcome plant resistance genes (PI resistance genes) (Gascuel et al. 2015).

There have been studies to test effects of neem against different oomycetes. For instance, Rashid et al. (2004) tested neem leaf diffusate, neem leaf powder and neem seed cake against *Phytophthora infestans* (Mont.) De Bary and found that neem was effective in controlling the infection. Also, different neem products such as crude neem seed oil, crude neem seed oil terpenoid extract, nimbokil and neem leaf decoction has been tested successfully against *P. infestans* (Mirza et al. 2000). The only study to investigate effect of neem against *Plasmopara viticola* was conducted by Achimu and Schlösser (1992) where they successfully controlled the pathogen *in vitro* conditions.

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

Root-knot nematodes are known to infect and decrease the yield of almost every cultivable crop in the world (Bernard et al. 2017; Sasser, 1980). They have a wide range of host plants infecting mainly the roots of vegetables (Anwar et al. 2007), ornamental plants (Dabaj and Jenser, 1990; den Nijs et al. 2004), medicinal and culinary plants (Walker 1995; El-Sherif et al. 2012) and weeds as well (Rich et al. 2008).

Complete eradication of root-knot nematodes is impossible if they are noticed in the fields (Briar et al. 2016). The control is hampered 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). *Meloidogyne incognita* damages vegetables and is predominantly found infecting in warmer climates (Anwar and McKenry, 2010). *M. incognita* increases the severity of bacterial wilt disease caused by *Ralstonia solanacearum* Smith 1896 in solanaceous plants (Tsay et al. 2014). *M. incognita* infested farms frequently experience decline of subsequent crops (Tsay et al. 2014).

Colorado potato beetle (*Leptinotarsa decemlineata* Say 1824)

Colorado potato beetle (CPB) (*Leptinotarsa decemlineata* Say, 1824) is one of the main causes of significant economic losses worldwide. CPB destroys all the green vegetative parts of potato, sometimes resulting in 100% yield loss and is also a vector of bacterial potato ring rot disease *Clavibacter michiganensis* subsp. *Sepedonicus*, Smith 1910 Davis et al. 1984 (Alkan et al. 2015). CPB is a multivoltine insect and uncontrolled populations can destroy the whole yield during the growing season (Alkan et al. 2017). CPB feeds mostly on solanaceous crops as they contain high concentrations of toxic glycoalkoloids in their foliage which the beetle detoxifies and excrete them with the diet (Wimer et al. 2015). Management of CPB using chemical insecticides is a common control measure that is applied since many decades. (Alkan et al. 2017). As a result of regular chemical control, CPB is currently resistant to most classes of synthetic insecticides (Kutas and Nádasy 2005). This ability of detoxifying the active compounds can explain their ability to develop resistance to different insecticides (Wimer et al. 2015).

Combination of chemical insecticides is a simple approach to prevent the development of resistance (Trisyono and Whalon 1999), but the damage to the environment and the beneficial organisms dwelling in such environments are still inevitable. The growing challenges and concerns about the negative impacts on the environment and resistance to various insecticides lead researchers to look for alternative solutions to these. An alternative control method is biological control using entomopathogenic microbes such as *Bacillus thuringiensis* var. *tenebrionis* Berliner, 1915 (*Bt*). It is considered as a promising agent against CPB but frequent usage of *Bt* could result in resistance to it (Trisyono and Whalon, 1999). Apart from microbes, several plant extracts have been screened for their toxic and/or antifeedant effects on CPB. Plant derived pesticides and insect feeding inhibitors for crop protection are gaining attention (Kutas and Nádasy, 2005) but are still not exploited to their maximum potential. There could be several advantages of these plant derived pesticides like they are of natural origin, harmless to humans and non-target organisms and as such environmentally friendly. Combined application of *Bt* and plant derived insecticides can prevent the development of resistance to either of them. They represent a sustainable control method permitted in organic farming also (Skuhrovec et al. 2017).

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte 1868)

The western corn rootworm (WCR) *Diabrotica virgifera virgifera,* one of the most important insect pests of maize in USA (Berger, 2001), is now posed a grave threat to in Europe (Carrasco et al. 2010). It has been thought to originate from Mexico where several *Diabrotica* species occur (Szalai et al. 2011). *D. virgifera virgifera* LeConte or WCR is a univoltine insect that overwinters in egg stage (Berger, 2001; Carcasso et al. 2010) and the larvae hatch in spring (Toepfer and Kuhlmann, 2006). Feeding on maize roots by the larvae can lead to root injury, decreased plant growth and reduced yield (Meinke et al. 2009) while adults feed on maize leaves, silk and pollen (Toepfer et al. 2005). It has been known to cause great damage to maize crops especially in the high maize growing countries (Kehlenback and Kruigener, 2014). There has been vigorous research such as mark-release-recapture experiments have been undertaken to eradicate and to contain the pest (Carrasco et al. 2010).

Many approaches to control WCR have been undertaken. According to Gray et al. (2009), WCR was found to be adaptable to crop rotation where the maize and soybeans were rotated. The main reason for the failure to this management practices was attributed to a behavioural adaptation by a variant western corn rootworm that had lost fidelity to maize for egg laying. Wright et al. (2011) evaluated the soil insecticides in laboratory and field studies against larvae of an insecticide resistant population of WCR. They concluded that resistance previously documented in the adults, were found in the 3rd instars and resistance to methyl parathion in adults was evident in the larvae too. A similar experiment was carried out using two chemical insecticides namely - aldrin and methyl-parathion by Parimi et al. (2006), to study the resistance of WCR to these insecticides. They found that most of their laboratory reared and field collected populations of WCR were found to be resistant to both the insecticides. None of these experiments with chemical control discusses about the hazards of these chemicals on the environment which should be considered (Van Rozen and Ester 2010).

2.7. Mode of action of neem pesticides

2.7.1. Mode of action on insect pests

There are different modes of action of neem-derived pesticides against different plant insect pests. Some effects are seen on an animal level and some are seen at the cellular level where the basic lesion occurs (Mordue, 2004).

Asher (1993) and Mordue (2004), listed the non-conventional mode of action (or non-conventional effects) of neem-derived pesticides and different insect pests which are summarized as follows:

a. The most common effect of azadirachtin seen on different insect pests is the *antifeedant effect* for which numerous evidences and records exists. Insects belonging to different Orders exhibit different responses to the feeding deterrence level as suggested by Schmutterer (1985). He found that there are two ways in which anti-feedancy effect can be seen, 1. “Primary” (or gustatory) anti-feedancy, which is exhibited by inability to ingest resulting from perception of the antifeedant at a sensory level and 2. “Secondary” antifeedant effect, which causes an imbalance in hormonal and/or physiological systems which is due to ingestion, application or injection of the antifeedant.
b. The next important effect of neem pesticides is the delay in the development of immature stages after the treatment which is explained in detail by (Mordue and Blackwell, 1993) and consists of reduced growth, increased mortalities, abnormal moults.
c. Neem-derived pesticides also affect the metamorphosis of insects which was first reported and described in 1972 by Ruscoe (Asher, 1993).
d. Effects of azadirachtin on reproductive processes of male and female insects are also reported by (Koul, 1984; Schmutterer and Holst, 1987). Fecundity is reduced by neem treatment in larvae of *Spodoptera littoralis* Boisduval, 1833, nymphs of *Dysdercus fasciatus* Signoret and adults of *Epilachna varivestis* Mulsant, 1850 and *Leptinotarsa decemlineata* (Asher, 1993).
e. Studies have shown that azadirachtin disrupts normal synthesis, especially the transport system of the peptide hormones that control synthesis and release of ecdysteroid moulting hormones and juvenile hormone (Fig. 6), although thorough research is needed to understand the effects of neem pesticides on neuroendocrine system Mordue (2004).

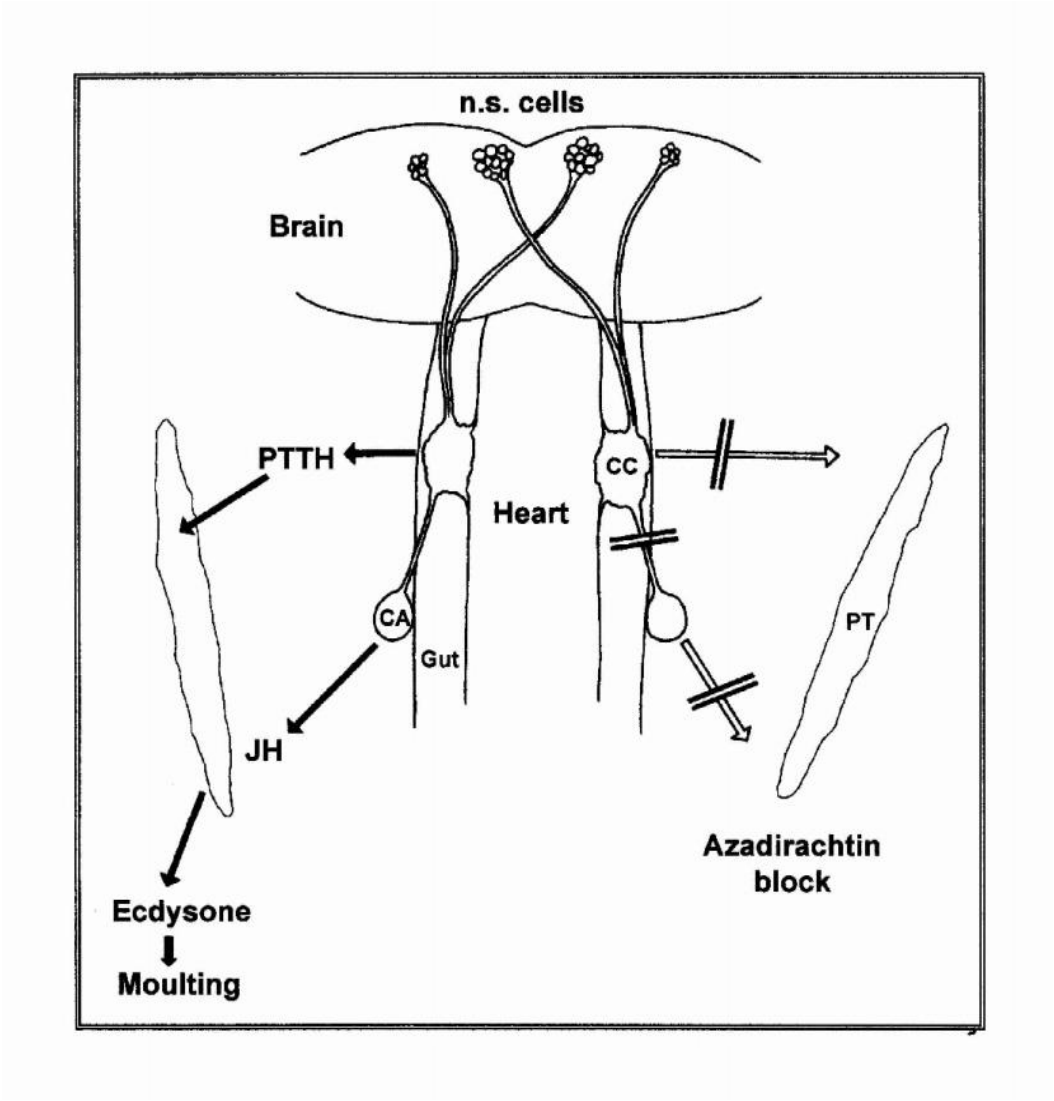

Figure 6: Diagrammatic representation of a dorsal view of the locust neurosecretory system showing azadirachtin treatment which is thought to block the transport and release of hormones affecting the synthesis and release of moulting hormone, and juvenile hormone; CC: corpus cardiacum; CA: corpus allatum; PT: prothoracic gland (Source: Mordue, 2004).

2.7.2. Mode of action on mycotoxin production

Apart from entomological findings, neem is known to possess anti-fungal and antimicrobial properties (Schmutterer, 1985). Neem leaf extract is found to inhibit aflatoxin producing strains of fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Asher, 1993). Bhatnagar and McCormick (1988) also found that neem leaf extracts inhibited the aflatoxin production in *A. parasiticus* up to more than 98%. Similar results were also obtained by (Zeringue and Bhatnagar, 1990), when they treated cotton bolls with neem leaf extract against aflatoxin producing *A. flavus*.

2.8. Neem effect on non-target organisms

A vast literature is available discussing about the effects of neem on target plant pests and pathogens. But it is necessary and of prime importance to study the effects of neem on beneficial organisms dwelling in soil or above soil surface.

Gajalakshmi and Abbasi (2004a) with an idea of producing vermicompost, fed the earthworms *Eudrilus eugeniae* Kinberg, 1867 with neem compost. They feared that due to nematicide property of neem, the neem compost will not be palatable to annelids but they found that earthworms fed voraciously on neem compost converting 7% vermicompost per day. Gajalakshmi and Abbasi (2004b) reported that the earthworms (*E. eugeniae*) also grew faster and reproduced rapidly in neem-fed vermireactors than the ones fed with mango leaf litter. Non-target effects of neem-based insecticides on aquatic macroinvertebrates was studied by Kreutzweiser (1997). He concluded that there was a little risk of direct toxic effect of neem-based insecticides contaminating the water bodies. Spyrou et al. (2009) conducted a study to see the effects of NeemAzal, a botanical pesticide containing azadirachtin as the active principle compound in the soil microbial community. They found that azadirachtin did not alter significantly in the soil microbial community under laboratory and field study. In a different study, Nawrocka (2008) tested the effects of neem on beneficial fauna that is naturally found on cabbage crops in field conditions. The results obtained did not show any influence on the larva or adult of Coccinellidae ssp., Syrphidae spp. and Chrysopidae ssp. and the percentage of cabbage aphid parasited by *Diaeretiella rapae* M'Intosh 1855 and the number of beneficial faunas in treated and control plots were the same.

Effects of neem-based pesticides have been tested on different spider species. Joseph et al. (2010) evaluated safety of neem products on tetragnathid spiders in rice ecosystem. They concluded that neem products can be used for management of rice pests without any effects on spiders. Mansour et al. (1986) assessed the toxicity of different alcoholic and water extract of neem seed kernel against the spider species i.e *Chiracanthium mildei* L. Koch. They found that at 2.5% all the extracts were non-toxic but at 4% the order of toxicity was pentane < acetone < ethanol << methanol = water (nontoxic). Neemgard, an acaricidal and fungicidal formulation obtained from neem seed kernel was tested by Mansour et al. (1997) on phytophagous mite *Tetranychus cinnabarinus* Boisduval, predacious mite *Phytoseiulus persimilis* Athias-Henriot, 1957, and the predatory spider *C. mildei* under laboratory conditions. They found that Neemgard was highly toxic to *T. cinnabarinus* and non-toxic to *P. persimilis* and *C. mildei*.

One of the most important non-target species are the foraging honey bees (*Apis* spp.) as they are natural pollinators. Various research has been done to test the effects of neem-derived pesticides

on honey bees. For instance, Naumann et al. (1994a) tested a standardised oil-free neem seed extract for its repellent effect against foraging honey bees and other pollinators on blooming canola. They found that foraging honey bees could discriminated between untainted sugar syrup and sugar syrup with neem but they found that there was no significant difference in the numbers of foraging honey bees collected in neem-treated, solvent-treated or untreated canola plots. Schmutterer and Holst (1987) found the enriched, formulated neem seed kernel extracts showed some degree of effect on relatively smaller bee colony consisting of queen and 200-300 worker bees but did not find any effects on a bee colony of ca. 3000 workers. Additionally, foraging bees on treated flowers did not show any damage or atypical behaviour. They concluded that under field conditions, serious effects of neem extracts to bees is unlikely but the neem extracts are not completely safe to bees and spraying higher doses of neem extracts on flowering plants should be avoided.

Schmutterer (1997) summarized the side-effects of neem products on different insect pathogens and natural enemy of spider mites and insects. He stated that neem products can be incorporated into Integrated Pest Management programs as it is improbable that neem-derived pesticides can damage the ecosystems. In conclusion, he also stated that neem-derived pesticides can contribute towards preservation of biodiversity in the ecosystem in spite of knowing that neem is not completely safe for some different stages of beneficial organisms.

2.8.1. Non-Target organisms under study

Entomopathogenic and slug-parasitic nematodes

Entomopathogenic (Heterorhabditidae, Steinernematidae) nematodes (EPNs) have lethal effects on several insect pests (Askary and Abd-Elgawad, 2017). Moreover, they can induce systemic resistance in plants against plant-parasitic nematodes by their presence (Jagdale et al. 2009). Among the most difficult insects to control are the white grubs and the Japanese beetle, *Popillia japonica* Newman, 1841 is found to be the most susceptible to EPNs (Klein et al. 2007). *Steinernema glaseri* Steiner, 1929 was the first EPN species used for control of scarabs by Gaugler et al. (1992). Different *Steinernema* and *Heterorhabditis* species were used to control Oriental beetle, *Anomala orientalis* Waterhouse, 1875 and *P.* japonica in different soil conditions and organic potting mix by Koppenhöfer and Fuzy (2006) and they found that *Heterorhabditis* spp were effective in organic potting mix while *Steinernema scarabaei* Stock & Koppenhöfer was most infective in loamy sand in the greenhouse. Williams and Walters (2000) conducted an experiment to test *Steinernema feltiae* Filipjev 1934 against three leaf miner species namely *Liriomyza huidobrensis* Blanchard, 1926, *Liriomyza bryoniae* Kaltenbach 1858 and *Chromatomyia syngenesiae* Hardy 1849 in vegetables and found that all the three species were

susceptible to *S. feltiae*. In the greenhouse conditions, *S. feltiae* controlled *L. huidobrensis* better than the chemical treatment by heptenophos.

In another study conducted by Grewal and Richardson (1993), *S. feltiae* was found to successfully control mushroom fly *Lycoriella auripila* Winnertz, 1867. Other species of Steinernematidae were reported to be successful to control different pests. Journey and Ostlie (2000) concluded through their experiment that all strain of *Steinernema carpocapsae* Weiser, 1955 can control western corn rootworm larvae in dryland corn and their efficacy can be enhanced with proper timing of application. Edmondson et al. (2002) studied the biocontrol of back vine weevil *Otiorhynchus sulcatus* Fabricius, 1775 in outdoor strawberry plants using cold-active *Steinernema kraussei* Steiner, 1923 (isolate L_{137}). They found after 3 months of application that 81% weevil control was achieved at the highest rate at 60,000 nematodes/pot and they also suggested that *S. kraussei* (L_{137}) has the potential to control black weevil under low temperatures.

Slug-parasitic nematode (SPN) *Phasmarhabditis hermaphrodita* Schneider, 1859 can attack the members of Arionidae, Milacidae, Limacidae and Vagnulidae (Wilson et al. 1993; Rae et al. 2007; Askary et al. 2012). Wilson et al. (1993) tested *P. hermaphrodita* for its biocontrol effects against different slug species namely *Deroceras caruanae* Lessona & Pollonera, 1882, *Arion distinctus* Mabille, 1868, *Arion silvaticus* Lohmander, 1937, *Arion intermedius* Normand, 1852, *Arion ater* Linnaeus, 1758, *Tandonia sowerbyi* Férussac, 1823, *Tandonia budapestensis* Hazay, 1880, and *Deroceras reticulatum* O. F. Müller, 1774. They found that *P. hermaphrodita* infected and killed all the tested slug species. Entomopathogenic and slug-parasitic nematodes are eco-friendly; they are hard to over apply, are not harmful to humans or wildlife (Askary et al. 2012), and are compatible with numerous biological and chemical pesticides (Lacey and Georgis, 2012).

Isopod species (*Porcellionides pruinosus* Brandt, 1833)

Woodlice species (Isopoda, Oniscidae) are ubiquitous saprophagous members of the soil fauna (Paoletti and Hassall, 1999). They are present in various densities both in conventional and organic farming systems (Paoletti and Hassall, 1999), and as such exposed to any pesticide treatment.

Porcellionides puinosus is cosmopolitan and synanthropic isopod which helps in decomposition of agriculture and cattle waste material (Loureiro et al. 2006). *P. pruinosus* can also be used for biomonitoring, both in contaminated or remediated areas (Loureiro et al. 2006). Isopods inhabit littoral zone, beach, grassland, woodland, desert, and more special habitats (Warburg, 1987). Adaptations to these environments are thought to be largely behavioural but it now appears that there are also well-established physiological adaptations, based on anatomical structures. Certain terrestrial isopod genera are able to detect chemical cues using their second antenna pair (Harzsch

et al. 2011). This can explain the results of Santos et al. (2011) where the binary combinations of dimethoate, glyphosate and spirodiclofen, an insecticide and an herbicide and an acaricide respectively, resulted a dose related avoidance response of *P. pruinosus*.

Entomopathogenic and antagonistic fungi

Entomopathogenic fungi

Beauveria bassiana (Bals.-Criv.) Vuill. 1912 is distributed worldwide and is a well-known entomopathogenic fungus. *B. bassiana* was discovered by Agostinio Bassi in 1835 as the cause of the devastating muscardine disease of silkworms (Xiao et al. 2012). It belongs to ascomycetous family Clavicipitaceae (Sung et al. 2007; Ownley et al. 2008). *B. bassiana* has been documented to inhibit different fungal pathogens also such as *Fusarium oxysporum* (Reisenzein and Tiefenbrunner, 1997; Bark et al., 1996), *Botrytis cinerea* Pers. 1794 (Bark et al., 1996), and *Rhizoctonia solani* Kühn 1858 (Lee et al., 1999). *B. bassiana* can be used against *Rhizoctonia solani* (Ownley et al. 2000; Ownley et al. 2004) and *Pythium myriotylum* Drechsler 1930 (Clark, 2006; Clark et al. 2006) infecting tomato seedlings. Insects and other arthropods acting as disease vectors, other crop pests such as whiteflies, caterpillars, grasshoppers and borers and invading pests such as fire ants and termites are controlled by *B. bassiana* (Holder and Keyhani, 2005).

Another entomopathogenic fungi widely used in plant protection is *Metarhizium anisopliae* (Metchnikoff) Sorokin. There are several commercial products available in the market containing strains of *M. anisopliae*. It is widely used in the field of biological control against insect pests. It is found worldwide and is part of natural soil flora (Zimmermann, 1993; Schrank and Vainstein, 2010). It was first identified on cereal chafer *Anisoplia austriaca* Herbst, 1783 and named *Entomophthora anisopliae* in 1879 and later Sorokin assigned it to the genus *Metarhizium* and is now known as *M. anisopliae* (Metsch.) Sorokin (Zimmermann, 1993). Toxins produced by *M. anisopliae* mainly known as ‘destruxins’ are a class of insecticidal, anti-viral, and phytotoxic cyclic depsipeptides (Schrank and Vainstein, 2010; Roberts and St. Leger, 2004). These toxins affecting excretion and leading to feeding and mobility difficulties by weakening the host immune system and damaging the muscular system and the Malpighian tubules (Schrank and Vainstein, 2010).

Antagonistic fungi

Among the most frequent isolated fungi from the plant root ecosystem are the *Trichoderma* species (Harman et al. 2004). *Trichoderma* spp. are opportunistic, avirulent plant symbionts and function as parasites and antagonists of many phytopathognic fungi (Vinale et al. 2008). According to Harman et al. (2004) and Vinale et al. (2008), strains of *Trichoderma* have several advantages in

agriculture such as colonization of the rhizosphere by the biological control agent ("rhizosphere competence") allowing rapid establishment within the stable microbial communities in the rhizosphere; control of pathogenic and competitive/deleterious microflora by using a variety of mechanisms; improvement of the plant health and stimulation of root growth. Different properties such as high reproductive capacity, ability to survive under very unfavourable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms is what makes *Trichoderma* a successful ubiquitous genus present in many habitats and at high population densities (Benítez et al. 2004).

2.9. Traditional neem-derived pesticides v/s commercial neem products

It is widely known that the insecticide activity of neem is due to azadirachtin, which is the most active principle compound found in neem (Isman, 2006). A variety of commercial products of neem with different formulations are available in the market (Boursier et al. 2011). A list of well-known commercial products summarised by Campos et al. (2016) from different countries are shown in Table 2. Azadirachtin based insecticides are gaining popularity due to its demand in cotton industry (Gahukar, 2000). Meanwhile, in the developing countries commercial neem products cannot be afforded due to its high cost and hence the neem preparations (neem oil and water extracts) are used instead (Boursier et al. 2011). To further support his argument, in Mali, the growers produce and use azadirachtin-based insecticides based on soaking 100 g seed kernels in 1l water for three or seven days to obtain an aqueous extract (Boursier et al. 2011). In India, traditionally, farmers in rural areas harvest and crush the neem seeds and leaves and soak them in water for 7 days to make seed and leaf extracts respectively which they use to treat the plants against different insect pests (see Fig. 7).

Table 2: List of neem commercial products available worldwide (Source: Campos et al. 2016)

Product	Manufacturer	Country	Application
Ozoneem Cake®	Ozone Biotech	India	Fertilizer
Parker Neem Coat	Parker Neem	India	Fertilizer
Neem Urea Guard®	Neemex	India	Fertilizer
Ozoneem Coat®	Ozone Biotech	India	Fertilizer
Ozoneem Oil®	Ozone Biotech	India	Agrochemical
NeemAzal Techinical®	E.I.D Parry Ltd.	India	Agrochemical
NeemDrop®	Neem India Products Ltd	India	Agrochemical
DalNeem®	Dalquim Ltda.	Brazil	Agrochemical
Azact CE®	EPP Ltda.	Brazil	Agrochemical
Azamax®	UPL Ltda.	Brazil	Agrochemical
Fortuneem Cake®	Fortune Biotech	USA	Fertilizer
AZA-Direct®	Gowan Company	USA	Agrochemical
Neemix 4.5®	Certis	USA	Agrochemical
Azatin XL®	OHP Inc.	USA	Agrochemical
Neem Cake®	Unibell Corporation	Russia	Fertilizer
BioNeem®	Woodstream Corporation	USA	Agrochemical

Figure 7: Indian farmer preparing neem leaf extract (Source: Google images)

Boursier et al. (2011) conducted a bioassay to check the effects of neem seed extract and the commercial product NeemAzal T/S against leafhopper *Macrosteles quadripunctulatus* Kirschbaum, 1868 and the moth *S. littoralis*. They concluded that the neem seed extract was as effective as the commercial product and both the insecticide preparations performed higher than pure azadirachtin A.

3. MATERIALS AND METHODS

3.1 Neem-derived pesticides tested

3.1.1 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. Following is the characterization of NeemAzal T/S (Trifolio-M, 2012):

1. Formulation: EC emulsifiable concentrate
2. Active ingredient: azadirachtin (HPLC-UV) 1,02 ± 0,15 % (m/m)
3. Physical and chemical properties: conform with FAO regulation for „EC"
4. Appearance: honey-yellow to brown in colour, characteristic odour, viscous liquid
5. pH (1% solution): 7
6. Density (20 °C): 0.985 g/cm3
7. Dynamic Viscosity (20 °C): 276.8 mPa·s
8. Dynamic viscosity (40 °C): 95.5 mPa·s
9. Surface tension (20 °C): 32.2 mN/m
10. Registered to use in:

    Greenhouse tomato – *Trialeurodes vaporarium* Westwood, 1856 (greenhouse whitefly) – 2.5 l/ha, max. 3 times / vegetation; effective for the larval stages and pupal stages.

    Apple – leaf miner moth species – 3 l/ha – max. 2 times; effective only for the control of first two instar larval stages
11. Toxicity category: R 52/53 harmful for water organisms, it causes long-lasting damage in water environment. It cannot be applied within the 5 m range of surface water.
12. Not harmful for honey bees.
13. Pre-harvest interval = 0 days
14. Re-entry interval: Greenhouse tomatoes = 1 day, Apple = not required for normal use

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.

3.1.2 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 (Fig 8). 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.

Figure 8: Grinding neem leaves prior to making neem leaf extract

3.1.3 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:

$$\frac{\textbf{250}\ \text{(Std azadirachtin (µg / mL)}}{\textbf{5.5}\ \text{(Area of std peak from chromatogram)}} \times \frac{\textbf{100}\ \text{(Total volume system (mL))}}{\textbf{5}\ \text{(Weight of leaves in grams)}} = \textbf{909}\ \text{(Factor)}$$

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.

3.2 Effect of neem-derived pesticides on target organisms

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

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

Preparation of neem leaf extract and NeemAzal T/S

The extraction and preparation of different concentrations of neem leaf extract and NeemAzal T/S was done as per Doshi et al. (2018) with modifications. The stock concentration of neem leaf extract (20% w/v) and of NeemAzal (0.1% v/v) was prepared using distilled water and the working concentrations used for neem leaf extract were 10 and 20% (v/v) and for azadirachtin 0.01 and 0.1% (v/v) using distilled water.

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 (Fig 9, 10).

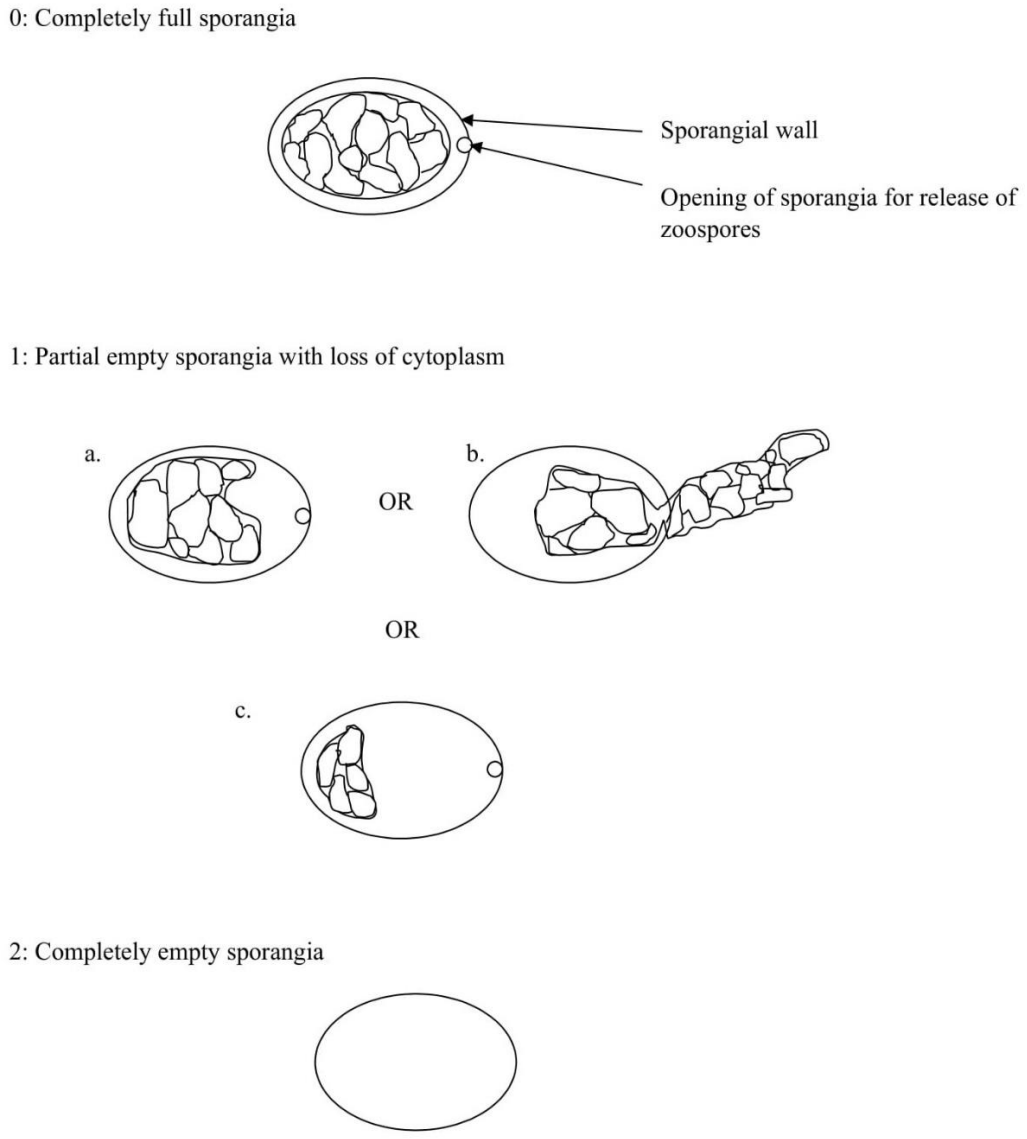

Figure 9: Diagrammatic representation of germination scale (from 0-2) to identify the morphology of sporangia, wherein, 0 = Completely full sporangia, 1 = Partial empty sporangia, 2 = Completely empty sporangia

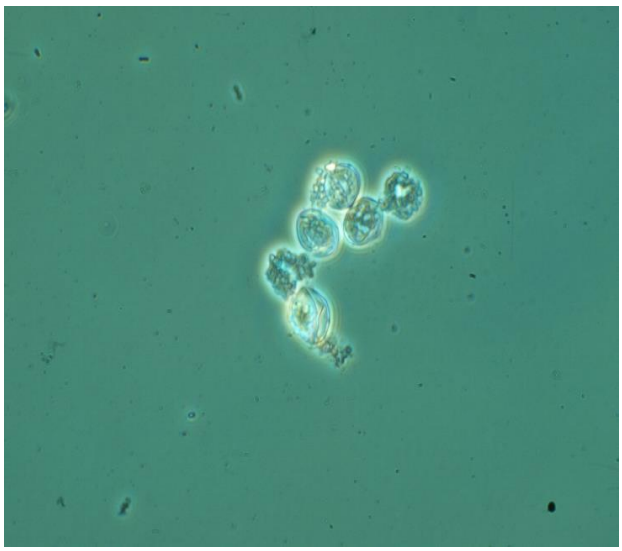

Figure 10: *Plasmopara halstedii* sporangia under transmission microscope

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.

Figure 11: *Plasmopara halstedii in vivo* experimental setup

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 (R Core Team, 2017) while graphs were made in Excel.

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

Preparation of neem leaf extract and NeemAzal T/S

The extraction and preparation of different concentrations of neem leaf extract and NeemAzal T/S was done as per Doshi et al. (2018) with modifications. The stock concentration of neem leaf extract (20% w/v) and of NeemAzal (0.1% v/v) was prepared using distilled water. For the *in vitro* experiment, the working concentration for azadirachtin was 0.0001, 0.001, 0.003, 0.005, 0.01% and for neem leaf extract was 0.01, 0.05, 0.1, 0.5, 1%. In the case of *in vivo* experiment, azadirachtin concentrations used were 0.001, 0.01 and 0.1% and for neem leaf extract were 1, 10 and 20%.

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 (Fig 12). 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 7^{th} 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.

Figure 12: *Meloidogyne incognita in vivo* experimental setup in the glasshouse

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.

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

Preparation of neem leaf extract and NeemAzal T/S

A 20% (w/v) stock concentration was prepared by mixing 20 g of dried neem leaf powder in 100 mL distilled water. The working concentrations of 1, 5, 10, 15 and 20% was prepared from the stock by diluting the stock solution with distilled water accordingly, a modified methodology of Doshi et al. (2018).

Stock solution with a concentration of 0.1% was prepared by dissolving 10mL NeemAzal T/S in 100 mL distilled water. The working concentrations (v/v) were 0.001, 0.003, 0.005, 0.01, 0.1%, a modified methodology of Petrikovszki et al. (2019) prepared by diluting stock solution to the respective working concentration.

Preparation of *Bacillus thuringiensis* var. *tenebrionis* (Btt)

Btt was prepared as a positive control. A 2% (v/v) solution of commercially available biopesticide Novodor (3.0% *Bacillus thuringiensis* var. tenebrionis) was prepared by mixing 2 mL of Novodor in 100 mL distilled water.

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 (Fig 13). Fresh undamaged potato leaves of the same potato variety were collected for different treatments and to serve as a food source.

Figure 13: Collection of *Leptinotarsa decemlineata* larvae from the infested field

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 (Fig 14) for a time period of 24, 48, 72, 96 hours.

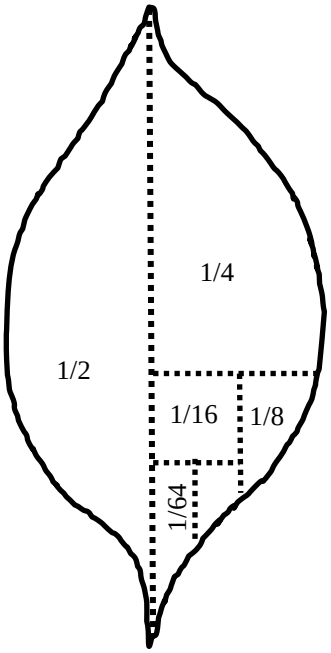

Figure 14: Diagrammatic representation of a potato leaf used to assess the feeding damage caused by different stages of Colorado potato beetle larvae.

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 (Fig 15). 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 (same as Fig 14) was observed and recorded for a time period of 24, 48, 72, 96 hours.

Figure 15: Choice test setup under *in vitro* condition for *Leptinotarsa decemlineata* larvae

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.

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

Preparation of neem leaf extract and NeemAzal T/S

A 20% (w/v) stock concentration was prepared by mixing 20 g of dried neem leaf powder in 100 mL distilled water. The working concentrations were prepared from the stock by diluting the stock solution with distilled water according to the experiment (written below), a modified methodology of Doshi et al. (2018).

Stock solution with a concentration of 0.1% was prepared by dissolving 10 mL NeemAzal T/S in 100mL distilled water. The working concentrations (v/v) were prepared using a modified method of Petrikovszki et al. (2019) by diluting stock solution to the respective working concentration using distilled water accordingly (mentioned for different experiments).

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 (Fig 16). 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.

Figure 16: *Diabrotica virgifera virgifera* egg experiment setup

(Photo credits: Dr. Stefan Toepfer, CABI)

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 (Fig 17).

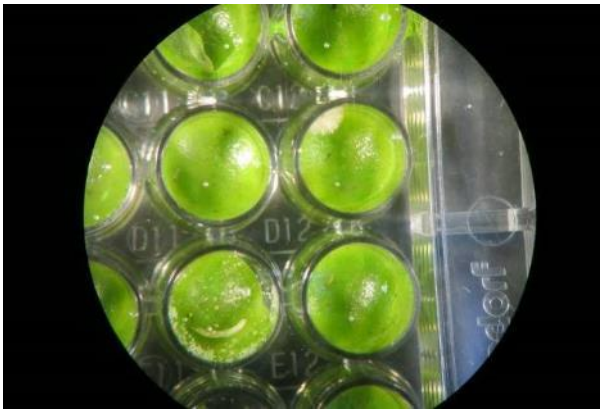

Figure 17: Observing the *Diabrotica virgifera virgifera* neonate larvae post-neem treatment under transmission miscroscope (Photo credits: Dr. Stefan Toepfer, CABI)

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 progam.

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

3.3.1. 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.

Preparation of neem leaf extract and NeemAzal T/S

The methodology followed a similar study (Doshi et al. 2018), with certain modifications. Pre-air-dried neem leaves obtained from India were ground to powder by a blender, and a stock concentration of 5% (w/v) solution using distilled water was prepared and kept in the dark at room temperature for 24 hours. The stock solution was filtered the next day using a muslin cloth to

obtain clear water extract. Further, it was centrifuged at 5000 rpm for 10 min to remove particulate matter. A maximum concentration of 1% of NLE was chosen, as a preliminary pilot study performed with 1% and 5% NLE resulted in 100% mortality of all tested nematode species. Therefore, range of concentration of NLE for the treatment was 0.1%, 0.3%, 0.6%, and 1%.

NeemAzal-T/S (Trifolio-M GmBH) is a commercial product registered in the European Union, containing 1% azadirachtin. According to the Hungarian approval document of NA, the maximum azadirachtin 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). In order to simulate overdosage, we examined concentrations of NA ranging between 0.001% and 0.01%, prepared by diluting the original product with distilled water.

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 (Kiss et al. 2018).

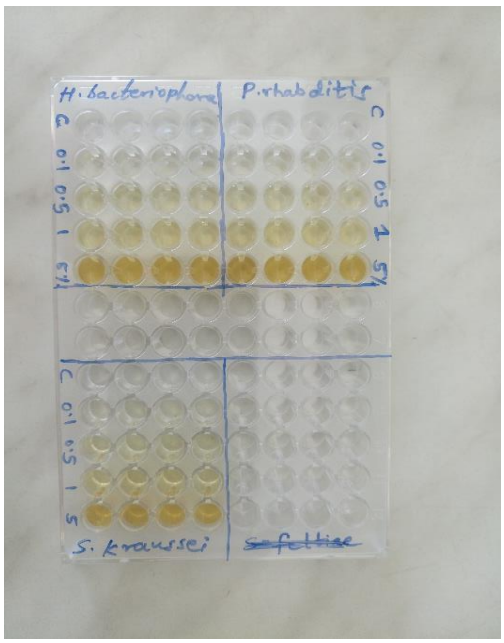

Figure 18: Experimental setup to study the neem effect on EPN and SPN under *in vitro* conditions

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.

3.3.2. 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).

Preparation of neem leaf extract and NeemAzal T/S

A stock concentration of 1% was prepared by soaking 1g of crushed dried neem leaves in 100 ml distilled water overnight and then filtered using a non-sterile filter paper. Different working

concentrations (0.05, 0.1, 0.25, 0.5, 0.75, and 1%) of neem leaf extract was prepared from 1% stock solution using distilled water in the laboratory and were used on the same day.

A stock concentration of 1% azadirachtin was prepared (from NeemAzal T/S which is 1% azadirachtin) by diluting 1 ml NeemAzal T/S in 100 ml of distilled water which equals to 0.01% azadirachtin. It was further diluted to get the 0.0005, 0.001, 0.0025, 0.005, 0.0075 and 0.01% azadirachtin concentrations respectively and was used on the same day. The registered dosage of azadirachtin ranges from 0.0025 to 0.005%, depending on the plant culture in the EU. A control with only distilled water was used for both experiments. The working concentrations and distilled water were sprayed using a hand sprayer under laboratory conditions.

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 (Fig 19).

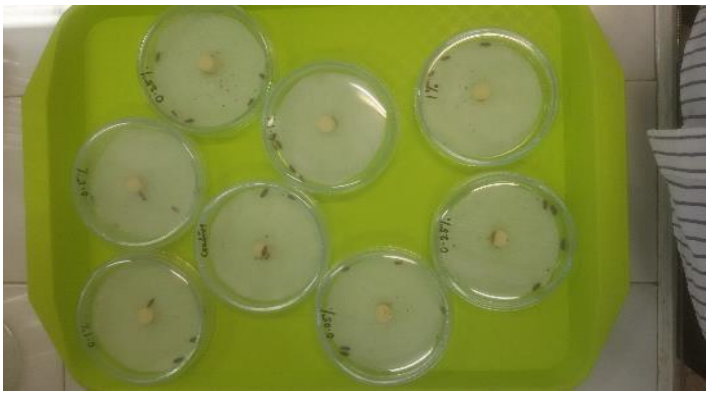

Figure 19: Experimental setup to investigate effect of neem on isopod *Porcellionides pruinosus*

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).

3.3.3. Entomopathogenic and antagonistic fungi

Preparation of neem leaf extracts and NeemAzal T/S

A 20% (w/v) stock solution was prepared by mixing 20 g of dried neem leaf powder in 100 mL distilled water. It was further filtered, centrifuged at 5000 rpm for 5 mins to obtain a clear solution by removing the coarse materials. This stock solution was sterilized using SteriTop® and SteriCup® filtration system under aseptic conditions to obtain sterile stock solution. Further working concentrations i.e 0.1, 0.5 and 1% was prepared from the stock solution using sterile distilled water under aseptic conditions.

A 0.1% stock solution of NeemAzal T/S was prepared by dissolving 10 mL in 100 mL distilled water. It was further sterilized in the same method mentioned above. Further working concentrations of 0.001%, 0.005% and 0.01% were prepared using sterile distilled water under aseptic conditions.

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.

4. RESULTS

4.1. HPLC analysis of neem leaf extract

The chromatogram of the analysis of the standard azadirachtin can be seen in Fig 20 while the chromatogram of neem leaf extract sample is shown in Fig 21. The peak 1 in both spiked and non-spiked graphs is the azadirachtin A concentration which can be verified when compared to the standard azadirachtin A and which had the same spectrum as azadirachtin A. The peaks 2-5 in both the spiked and non-spiked samples are thought to be the derivatives or isomers of azadirachtin. This was verified by comparing the spectrum of these peaks (2-5) with standard azadirachtin A peak which was 213-214 nm.

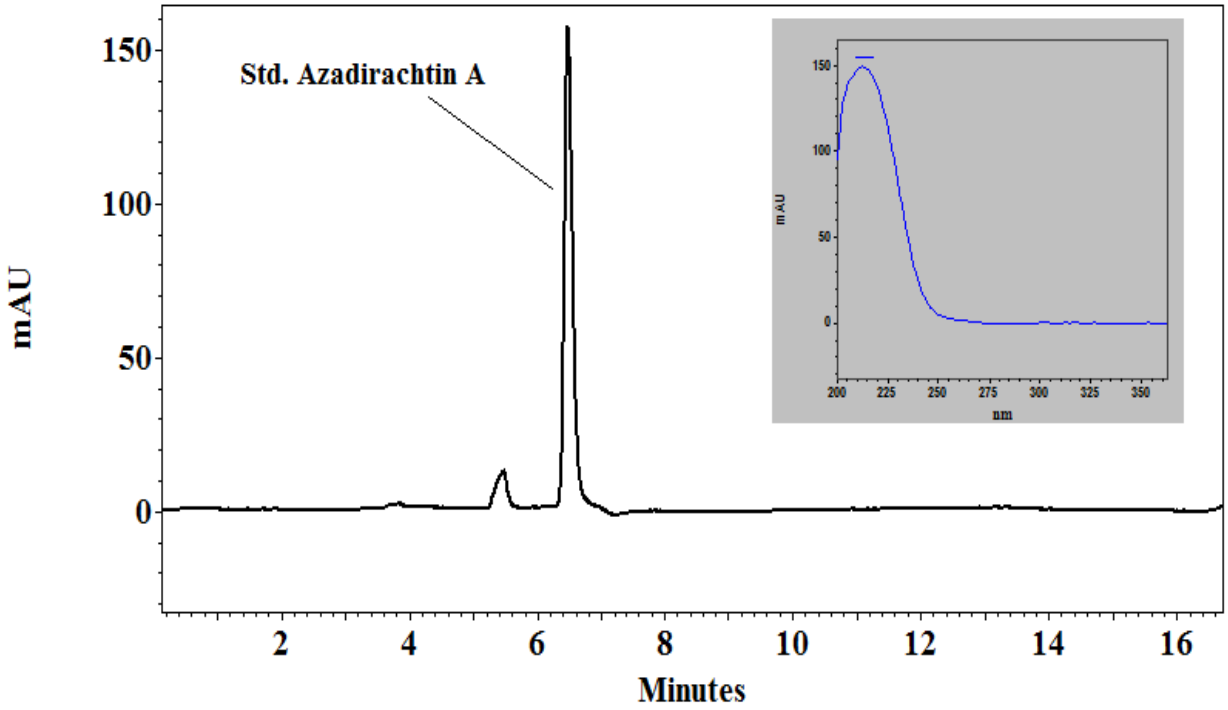

Figure 20: HPLC chromatogram of standard azadirachtin A

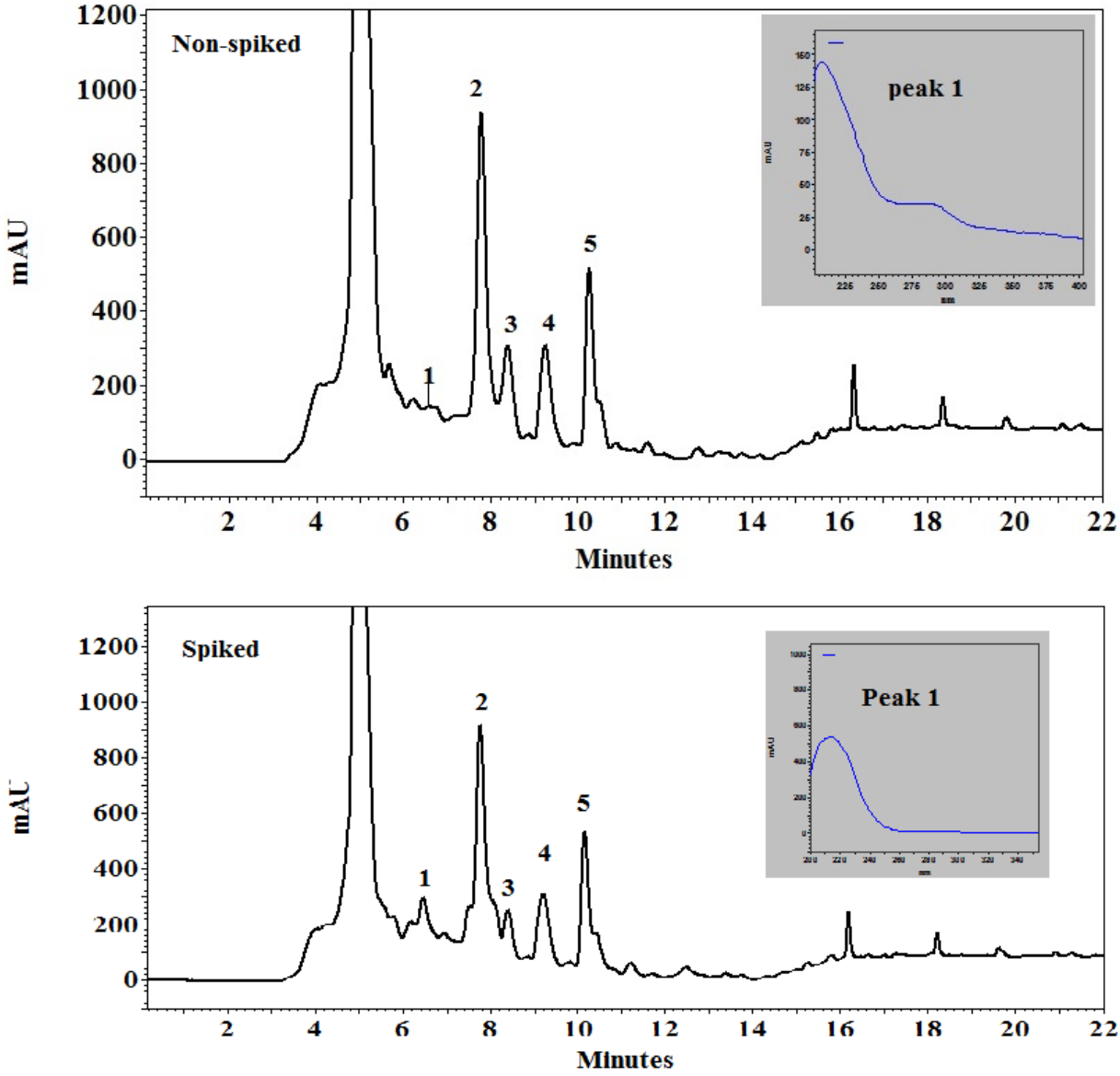

Figure 21: HPLC chromatogram of neem leaf extract. The top figure is "non-spiked" which means standard azadirachtin A solution was not added externally. The bottom figure is "spiked" which means standard azadirachtin A was added externally in the neem leaf extract sample before performing the test.

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 (Fig 21). 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 (Table 3).

Table 3: Table representing the area of the peaks as analysed from the chromatogram and calculating the amount of azadirachtin present in the given neem leaf extract samples. ** is the azadirachtin A concentration found in the neem leaf extracts.

Sample	Peak no	Area (from chromatogram)		Azadirachtin (µg/g)	Azadirachtin (mg/5g)
Non-Spiked	1	0.23	Area x 909 (factor)	209	1**
	2	47.2		42909	214.5
	3	15.5		14089	70
	4	18.06		16425	80
	5	18.6		16907	84.5
Spiked	1	5.5		4999	24.5**
	2	37.7		34269	171
	3	6.5		5908	29.5
	4	17.5		15907	79.5
	5	18.4		16725	83.5

4.2. Effects of neem-derived pesticides on target organisms

4.2.1. *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%.

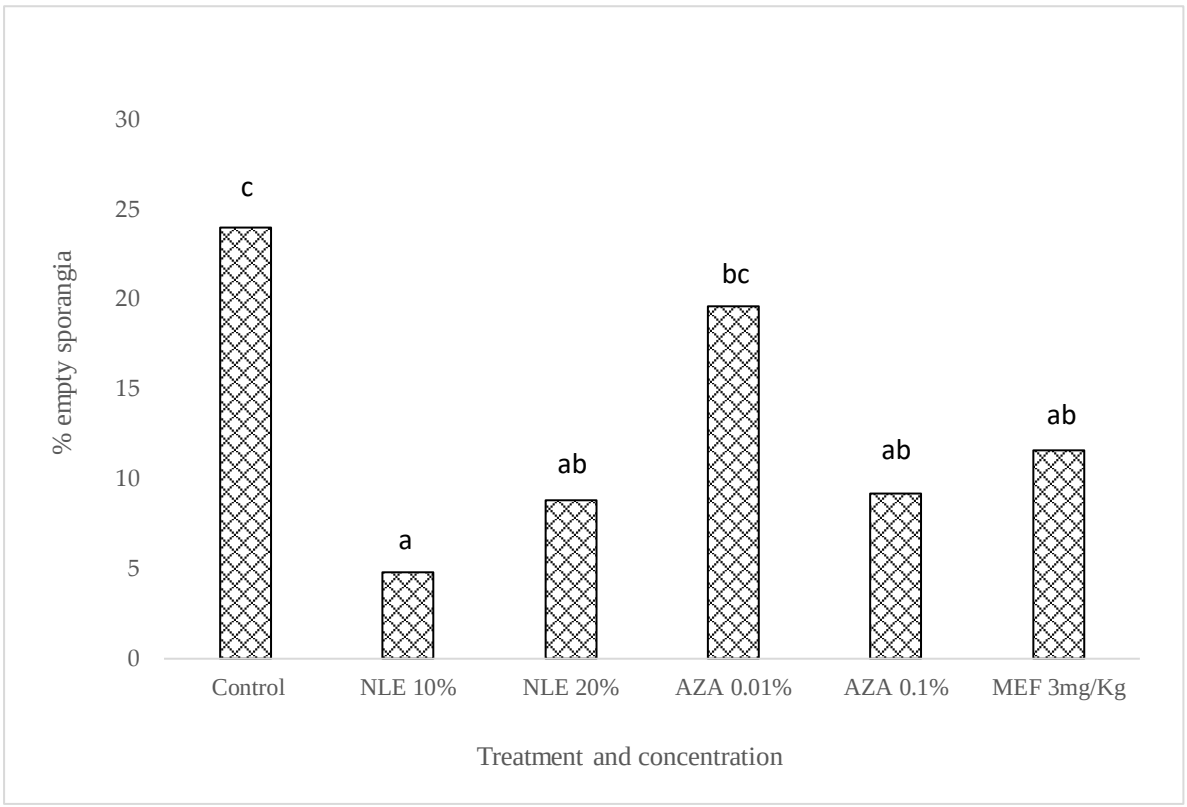

Figure 22: Effect of two different concentrations of neem leaf extract (NLE) and NeemAzal T/S (AZA), respectively, on the germination of *Plasmopara halstedii* sporangia (mefenoxam (MEF) was used as a positive control.

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

In the case of pre-treatment, it is evident that both concentrations of neem leaf extract and the highest concentration of NeemAzal T/S were found to significantly reduce the sporulation along with mefenoxam, while the lowest concentration of NeemAzal T/S (AZA 0.01%) was not significant in reducing the sporulation as compared to control (Fig 23).

In the case of post-treatment, the lowest concentration of both neem leaf extract and NeemAzal T/S did not reduce the sporulation and damping-off significantly as compared to control (Fig 23). However, the highest concentration of neem-derived pesticides significantly reduced the sporulation and the number of damped-off seedlings. The lowest sporulation and damping-off were observed in the mefenoxam treatment and was significantly different from all the other treatments.

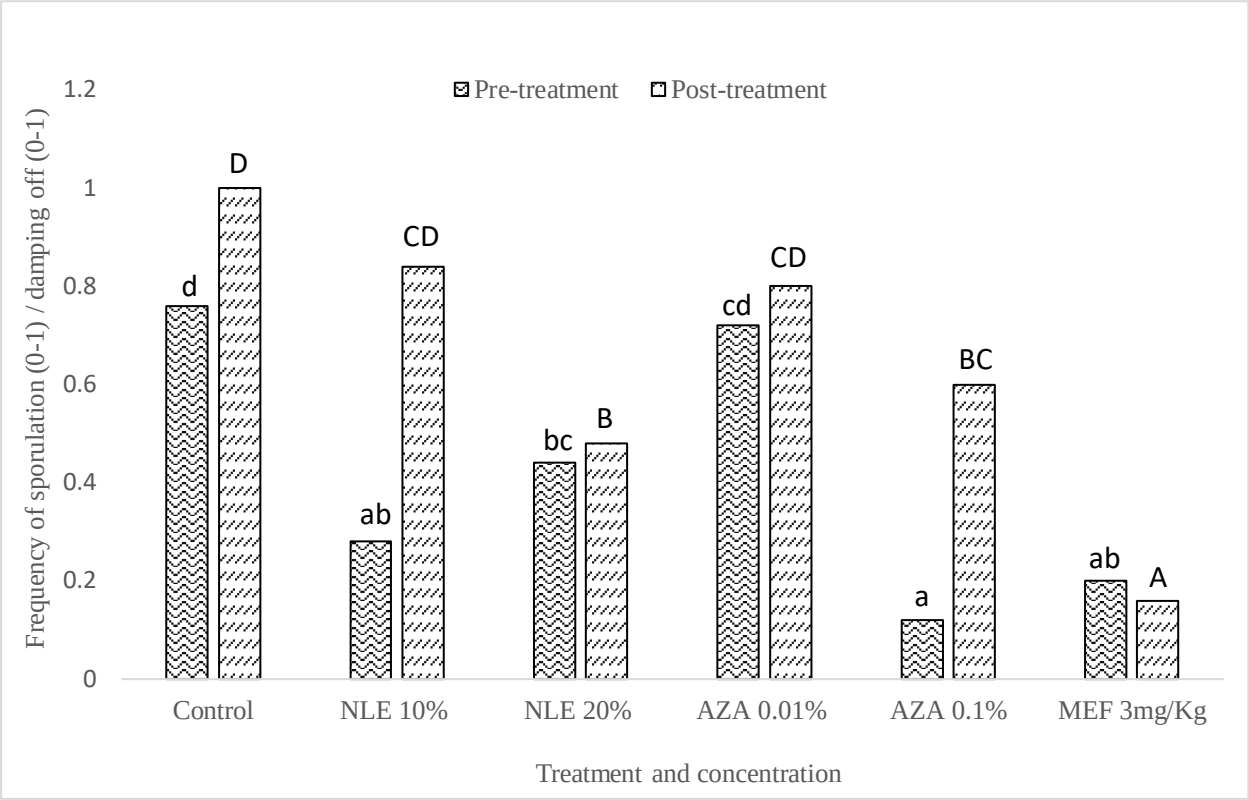

Figure 23: Pre- and Post-treatment effects of neem leaf extract (NLE) and NeemAzal T/S (AZA) on *P. halstedii* sporulation in susceptible sunflower seedlings. Different lowercase letters represent significant difference comparing the pre-treatment effect and the uppercase letters represent significant difference to compare post-treatment effect at 95% confidence level.

For initial plant height (10 days after sowing), in the case of pre-treatment, plant height measured for AZA 0.01% was significantly lower compared to other treatments of neem leaf extracts, AZA 0.1% and mefenoxam with inoculum but was not significant to the infected control (Seed + Inoculum). Plants pre-treated with different concentrations of neem leaf extracts, AZA 0.1% and mefenoxam and then inoculated with *P. halstedii* showed no significant difference to plants treated with bidistilled water except for AZA 0.01% + BW (Fig 24).

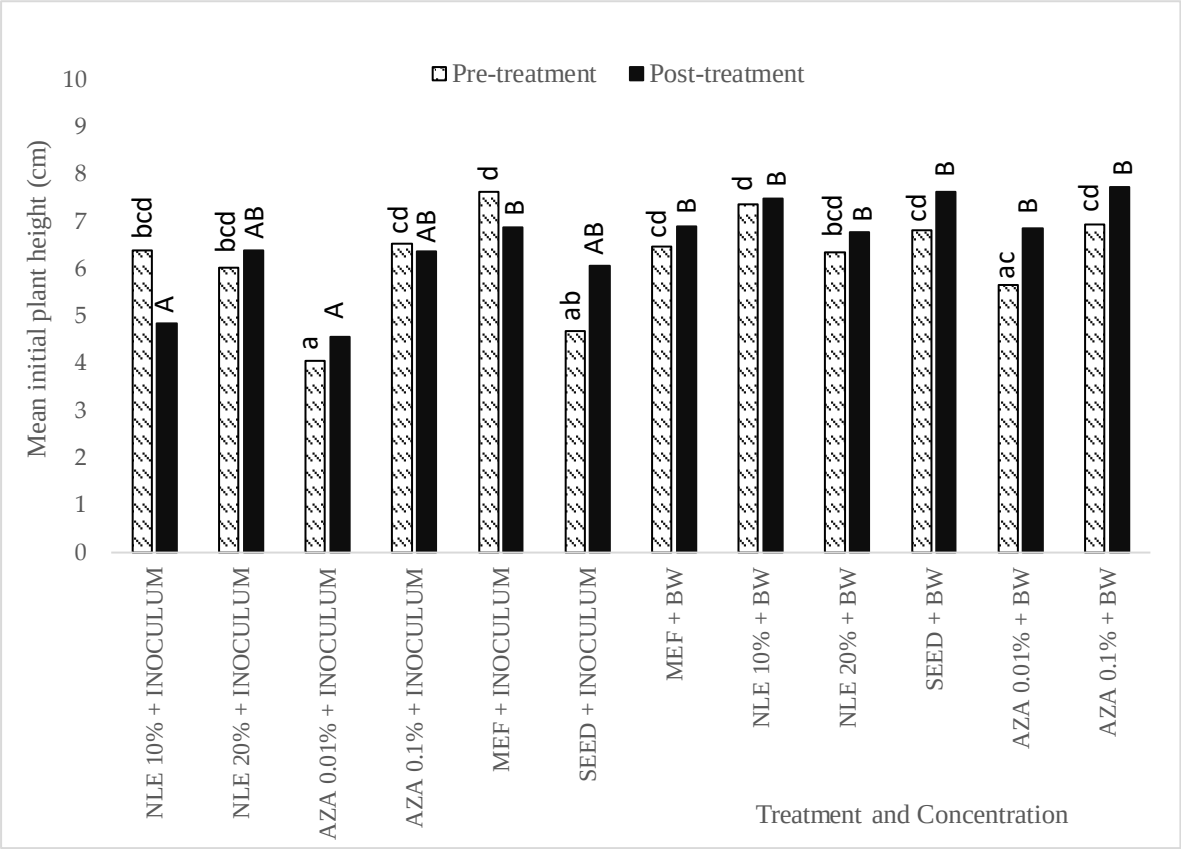

Figure 24: Measurement of initial plant height of seedlings pre- and post-treated with different concentrations of neem leaf extract (NLE) and NeemAzal T/S (AZA) as a part of first evaluation. Different lowercase letters represent significant difference comparing the pre-treatment effect and the uppercase letters represent significant difference to compare post-treatment effect at 95% confidence level

In the case for post-treatment (Fig 24), the lower concentration i.e NLE 10% and AZA 0.01% showed significant difference in the plant height as compared to mefenoxam 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) (Fig. 24). 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).

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

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

In case of NeemAzal T/S (AZA), 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 4).

Table 4: Different azadirachtin (AZA) 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 ± SE)
Control	0	0.69 ± 3.40 a
NeemAzal T/S (1% azadirachtin) (AZA)	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 case of neem leaf extract (Fig. 25), it is evident from that higher concentrations (i.e 0.5 and 1%) of NLE yielded in higher mortality. Mortality 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 6.

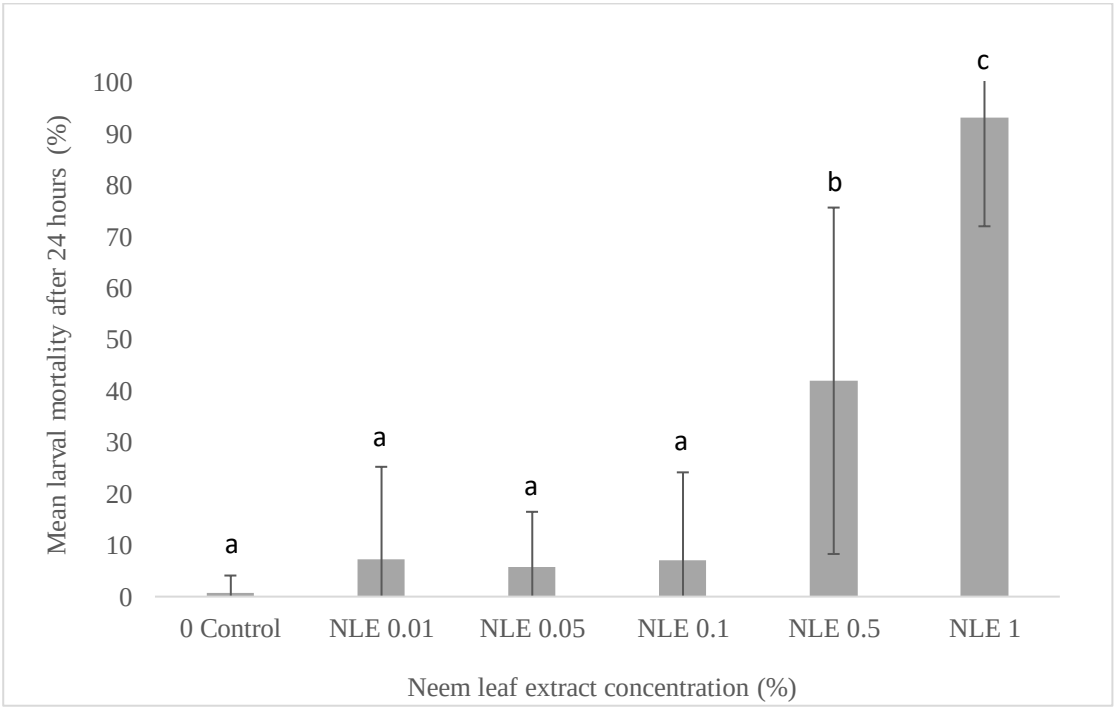

Figure 25: Mortality effect (%) of different concentrations of neem leaf extract (%) on *Meloidogyne incognita* J_2 larvae under *in vitro* conditions after 24 hours. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data are the mean mortality values of 3 replications of the whole experiment, i.e. 24 replicates

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 (Table 5).

Table 5: 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 Muhtar et al. (p-value: Welch test, CI 95%: 95% confidence level).

Tomato landraces	**Dányi**		**Ceglédi**	
M. incognita infection -/+	-	+	-	+
Replications	5	34	5	34
Zeck scale (0-10)				
mean ± CI 95%	0 ± 0	4.53 ± 0.60	0 ± 0	5.32 ± 0.40
p-value	$4.8*10^{-16}$		$1.69*10^{-23}$	
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	$5.12*10^{-16}$		$2.31*10^{-19}$	

In the case Dányi landrace, values of the root damage were inconsistent, since the values of Zeck and Mukhtar et al. scales of 0.1% azadirachtin 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 was similar to the negative control, but Zeck and Mukhtar et al. scales showed differences (Table 6). 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. On the other hand, average root damage with 0.1% AZA was significantly lower than only *M. incognita* infected treatment (Table 7).

Table 6: Average root damage caused by *Meloidogyne incognita* on Hungarian determinate tomato landrace 'Dányi', depending on three scales: Zeck, Garabedian and Van Gundy and Muhtar 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. Same letters indicate no 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 (1% azadirachtin) (AZA)	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

Table 7: Average root damage caused by *Meloidogyne incognita* on indeterminate Hungarian tomato landraces 'Ceglédi' depending on three scales: Zeck, Garabedian and Van Gundy and Muhtar et al. receiving the following treatments: 0.001, 0.01 and 0.1% of NeemAzal and 1, 10 and 20% of neem leaf extract. (ANOVA post-hoc Tukey's test. Same letters indicate no 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 (1% azadirachtin) (AZA)	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 ab	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

Neither in the case of Dányi (Fig 26) nor Ceglédi (Fig 27) landraces was there any significant difference in the average number of fruits with respect to different treatments and concentrations. In the case of Dányi landrace, the lowest average number of fruits was recorded in NLE 10%

whereas the highest was found in azadirachtin 0.01% (Fig 26). Further evaluation such as yield could had been possible as we did not wait for the fruits to ripen.

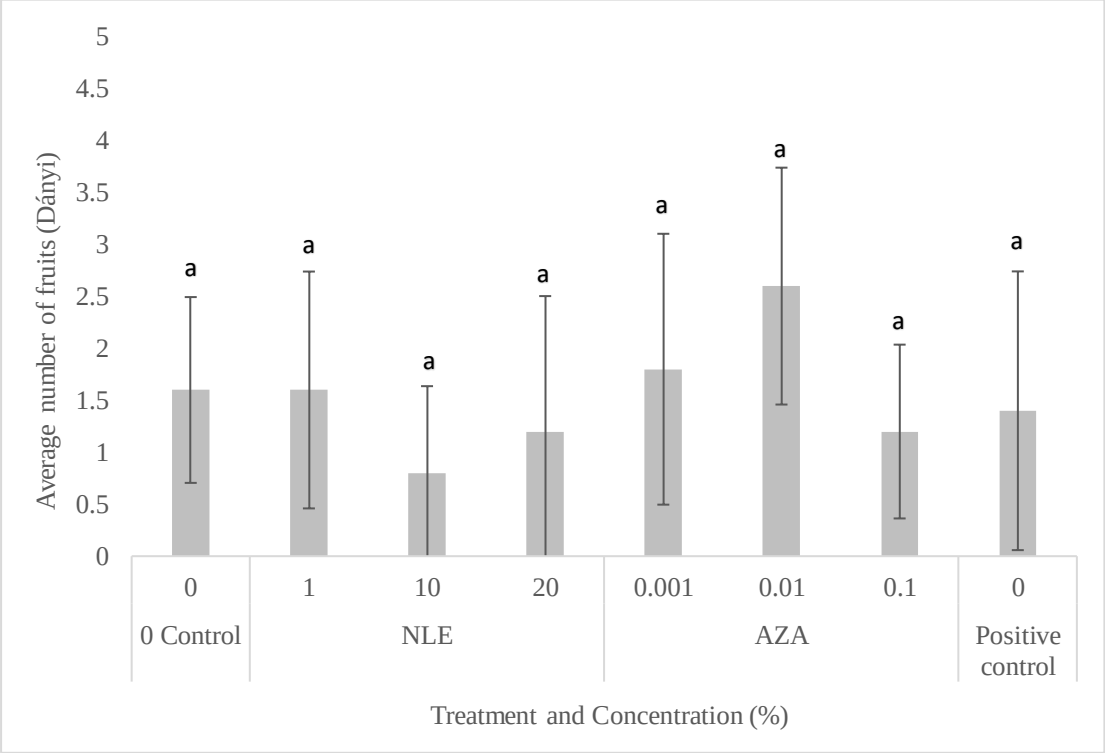

Figure 26: Average number of fruits produced by *Meloidogyne incognita* infested 'Dányi' determinate tomato landrace after treatment with different neem leaf extract and azadirachtin concentrations. Same letters indicate no significant difference at 95% confidence level ($p<0.05$). Data is average of five individual plants per treatment.

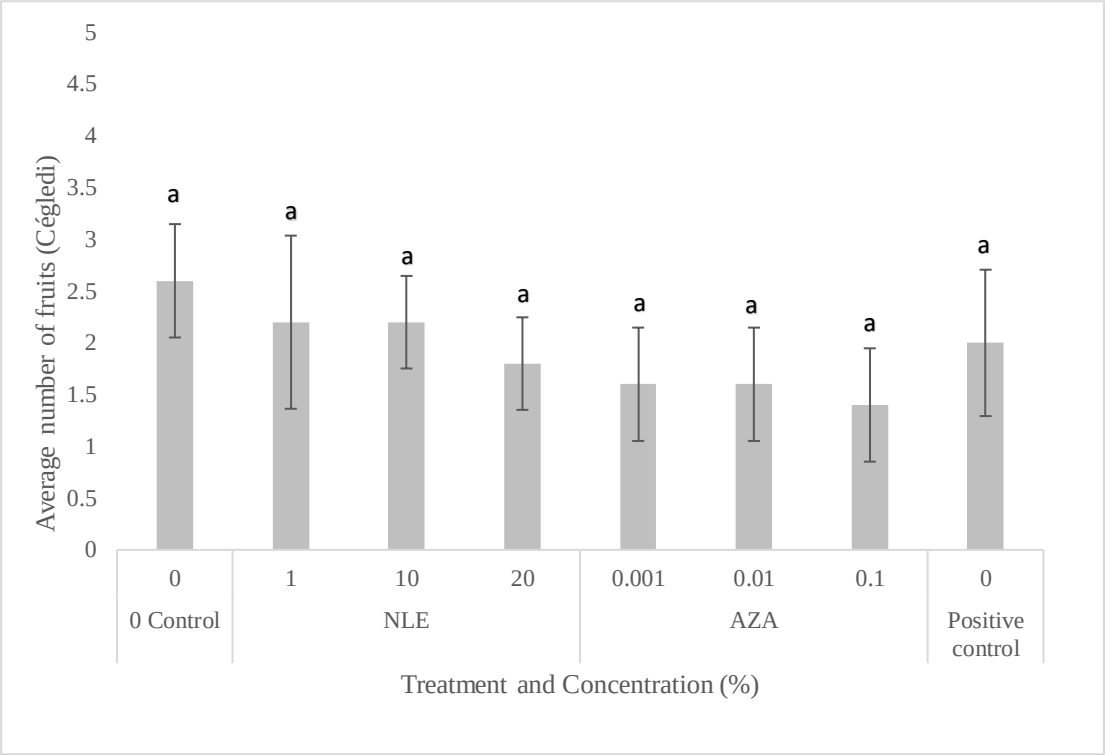

Figure 27: Average number of fruits produced by *Meloidogyne incognita* infested 'Ceglédi' indeterminate tomato landrace after treatment with different neem leaf extract and azadirachtin concentrations. Same letters indicate no significant difference at 95% confidence level ($p<0.05$). Data is average of five individual plants per treatment

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.

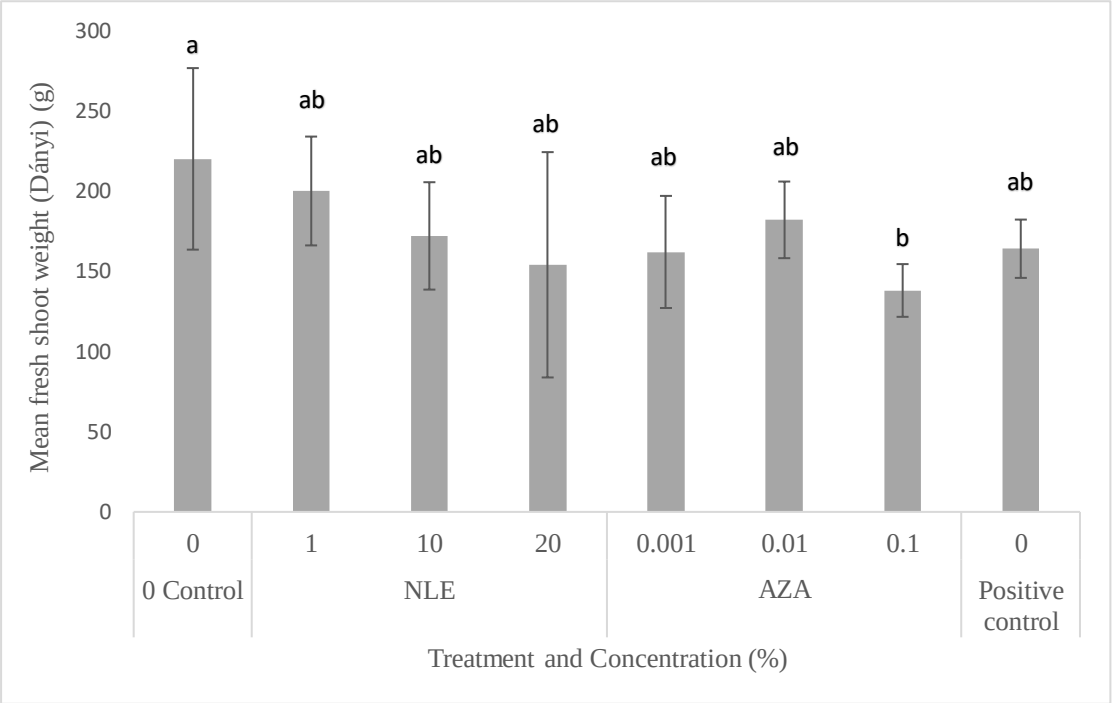

Figure 28: Mean shoot fresh weight in grams of *Meloidogyne incognita* infested 'Dányi' determinate tomato landrace after treatment with different neem leaf extract and azadirachtin concentrations. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data is replicate of five individual plants per treatment

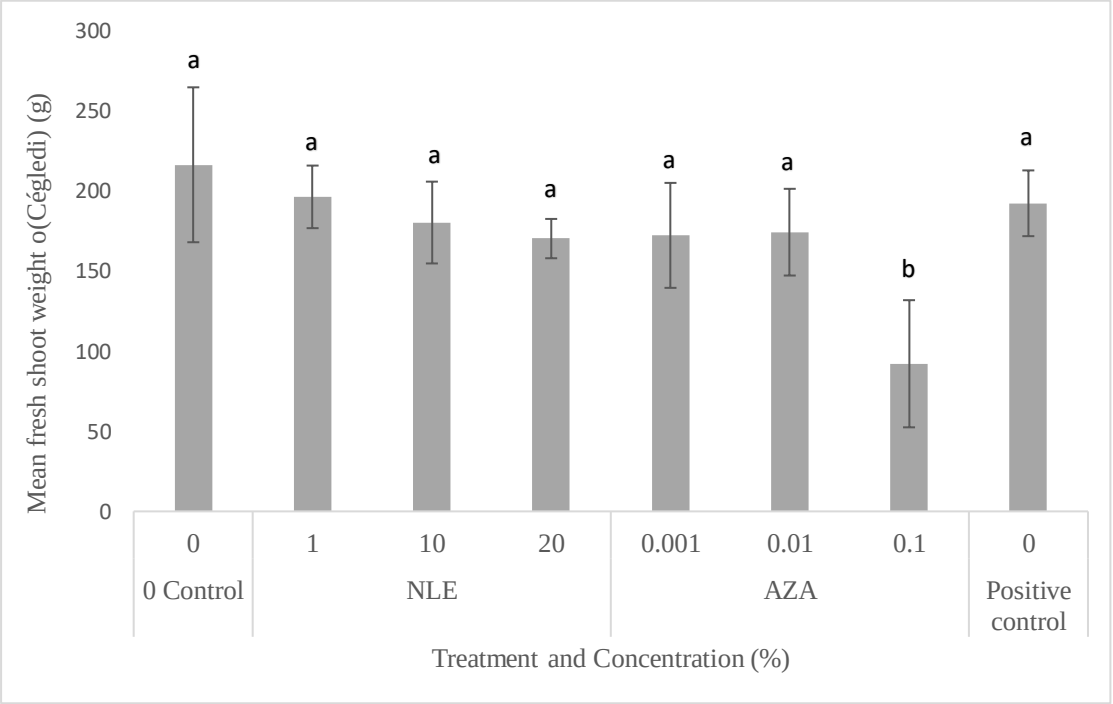

Figure 29: Mean shoot fresh weight in grams of *Meloidogyne incognita* infested 'Ceglédi' determinate tomato landrace after treatment with different neem leaf extract and azadirachtin concentrations. Different letters represent significant difference at 95% confidence level ($p \leq 0.05$). Data is average of five individual plants per treatment

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

a. No-choice test

In case of azadirachtin, there is no significant difference in the mortality after 96 hours post-treatment even at the highest concentration of 0.1%. The NLE was much more lethal as compared to AZA for CPB larvae. There was a significant difference ($p<0.05$) in mortality of CPB larvae with the increase in concentration as the time progressed. NLE 15% and 20% showed the highest mortality of 66 to 93% at 72h and 96h respectively and were significantly different from the rest of the treatments. Btt did not show any significant difference in the larvae mortality at the given working concentration (Table 8).

Table 8: Effect of different concentrations (%) of two different neem derived pesticides on mortality of *Leptinotarsa decemlineata* larvae (mean (%) ± SE) at different time interval under no-choice condition. Different letters represent significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates.

Treatment	Conc (%)	24h_mortality	48h_mortality	72h_mortality	96h_mortality
		(mean ± SE)	(mean ± SE)	(mean ± SE)	(mean ± SE)
Control 0	0	00 ± 00 a	00 ± 00 a	00 ± 00 a	00 ± 00 a
AZA	0.001	00 ± 00 a	00 ± 00 a	00 ± 00 a	00 ± 00 a
	0.003	00 ± 00 a	00 ± 00 a	6.66 ± 6.66 a	6.66 ± 6.66 a
	0.005	00 ± 00 a	7.00 ± 6.66 ab	6.66 ± 6.66 a	13.33 ± 13.33 a
	0.01	00 ± 00 a	7.00 ± 6.66 ab	13.33 ± 6.66 a	33.33 ± 6.66 a
	0.1	00 ± 00 a	00 ± 00 a	00 ± 00 a	00 ± 00 a
NLE	1	00 ± 00 a	00 ± 00 a	00 ± 00 a	00 ± 00 a
	5	00 ± 00 a	7.00 ± 6.66 ab	6.66 ± 6.66 a	6.66 ± 6.66 a
	10	00 ± 00 a	20.00 ± 20.00 ab	33.33 ± 13.33 ab	40.00 ± 11.54 ab
	15	00 ± 00 a	53.00 ± 24.03 b	66.66 ± 17.63 b	80.00 ±11.54 bc
	20	00 ± 00 a	13.00 ± 13.33 ab	66.66 ± 13.33 b	93.00 ± 6.66 c
Btt	2	00 ± 00 a	00 ± 00 a	6.66 ± 6.66 a	26.66 ± 13.33 a

After 24 hours post-treatment, there was no significant difference between the feeding damage caused by the CPB larvae throughout the different NLE concentrations. After 48 hours post-treatment, higher concentrations of NLE i.e 5 to 20% showed a significant difference in the feeding damage, whereas NLE 1% did not show any difference as compared to Control 0 (Fig 30). At 72h

and 96h post-treatment, NLE 1% showed the highest feeding damage as compared to other NLE concentrations or negative control. NLE 15 and 20% resulted the least feeding damage throughout which coincides with the high mortality as seen in Table 10 after 72 and 96h post-treatment respectively. Btt showed low feeding damage and was significantly different from NLE 1% at all the time intervals, from control at 48,72 and 96h and NLE 5% and 10% at 72h post treatment (Fig 30).

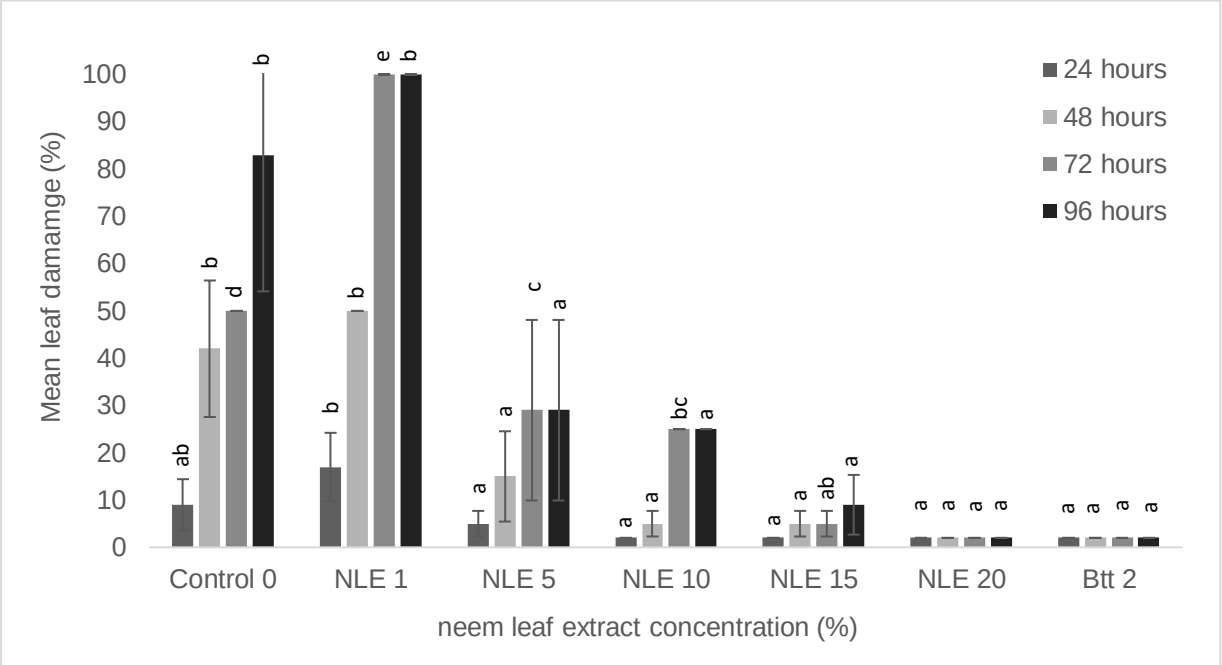

Figure 30: Effect of different neem leaf extract concentrations (%) on mean leaf damage (%) caused by *Leptinotarsa decemlineata* larvae at different time interval. Different letters indicate significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates

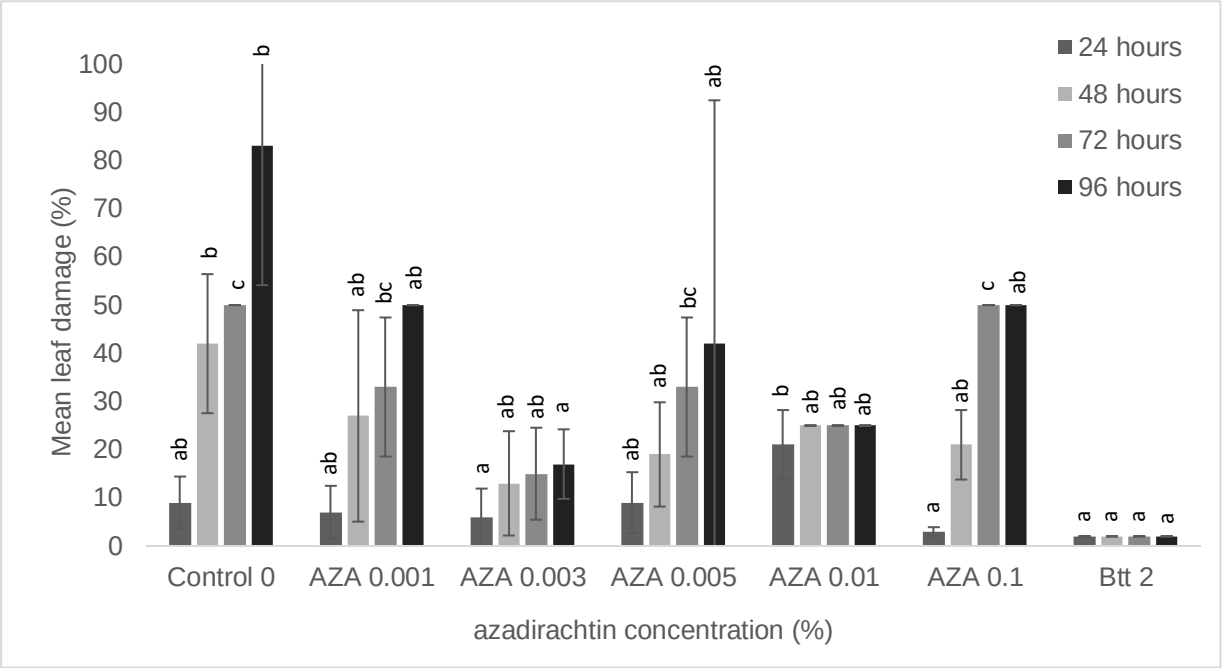

Figure 31: Effect of different azadirachtin concentrations (%) on the mean leaf damage (%) at different time interval caused by *Leptinotarsa decemlineata* larvae under no choice condition. Different letters indicate significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates

In the case of azadirachtin, the feeding damage was not significantly different from each other for all the time intervals which is also reflected in the mortality of CPB larvae in Table 10, except that mean leaf damage at AZA 0.003% was significantly different to the negative control at 72h and 96h post-treatment. Btt also significantly reduced the mean leaf damage from 48h until 96h (Fig 31).

b. Choice test

In this test, the effect of different neem derived pesticide products on the mortality of CPB larvae and the feeding damage can be investigated better (Table 9). There is no significant difference between different treatments for the entire time period throughout the experiment. NLE 5% showed no mortality even after 96 h post-treatment. The maximum mortality (%) was seen for AZA0.01 after 96 hr post-treatment followed by AZA 0.1% yet the difference was not significant.

Table 9: Effect of different concentrations (%) of two neem-derived pesticides on mortality of *Leptinotarsa decemlineata* larvae (mean (%) ± SE) at different time intervals under choice condition. Same letters represent no significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates

Treatment	Conc (in %)	24h_mortality (mean ± SE)	48h_mortality (mean ± SE)	72h_mortality (mean ± SE)	96h_mortality (mean ± SE)
Control 0	0	0.00 ± 0.00 a	13.33 ± 6.66 a	13.33 ± 6.66 a	13.33 ± 6.66 a
AZA	0.001	0.00 ± 0.00 a	0.00 ± 0.00 a	20.00 ± 11.547 a	20.00 ± 11.547 a
	0.003	0.00 ± 0.00 a	0.00 ± 0.00 a	13.33 ± 13.33 a	13.33 ± 13.33 a
	0.005	6.66 ± 6.66 a	6.66 ± 6.66 a	13.33 ± 13.33 a	26.66 ± 17.64 a
	0.01	13.33 ± 6.66 a	13.33 ± 6.66 a	40.00 ± 0.00 a	40.00 ± 0.00 a
	0.1	6.66 ± 6.66 a	20 ± 11.547 a	26.66 ± 6.66 a	33.33 ± 6.66 a
NLE	1	6.66 ± 6.66 a	13.33 ± 13.33 a	20 ± 11.547 a	26.66 ± 6.66 a
	5	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
	10	0.00 ± 0.00 a	6.66 ± 6.66 a	13.33 ± 6.66 a	13.33 ± 6.66 a
	15	0.00 ± 0.00 a	6.66 ± 6.66 a	13.33 ± 13.33 a	13.33 ± 13.33 a
	20	0.00 ± 0.00 a	6.66 ± 6.66 a	6.66 ± 6.66 a	13.33 ± 13.33 a
Btt	2	0.00 ± 0.00 a	6.66 ± 6.66 a	13.33 ± 6.66 a	26.66 ± 6.66 a

In the case of neem leaf extract, leaves treated with NLE 20% showed a significant difference in the leaf damage after 48h. In addition, it is also evident that all treatments had a significant reduction in the mean leaf damage at 96 h when compared to their respective untreated leaves (Fig. 32). Similarly, in the case of azadirachtin, all treatments had a significant reduction in the mean leaf damage at 96 h when compared to their respective untreated leaves (Fig 33)

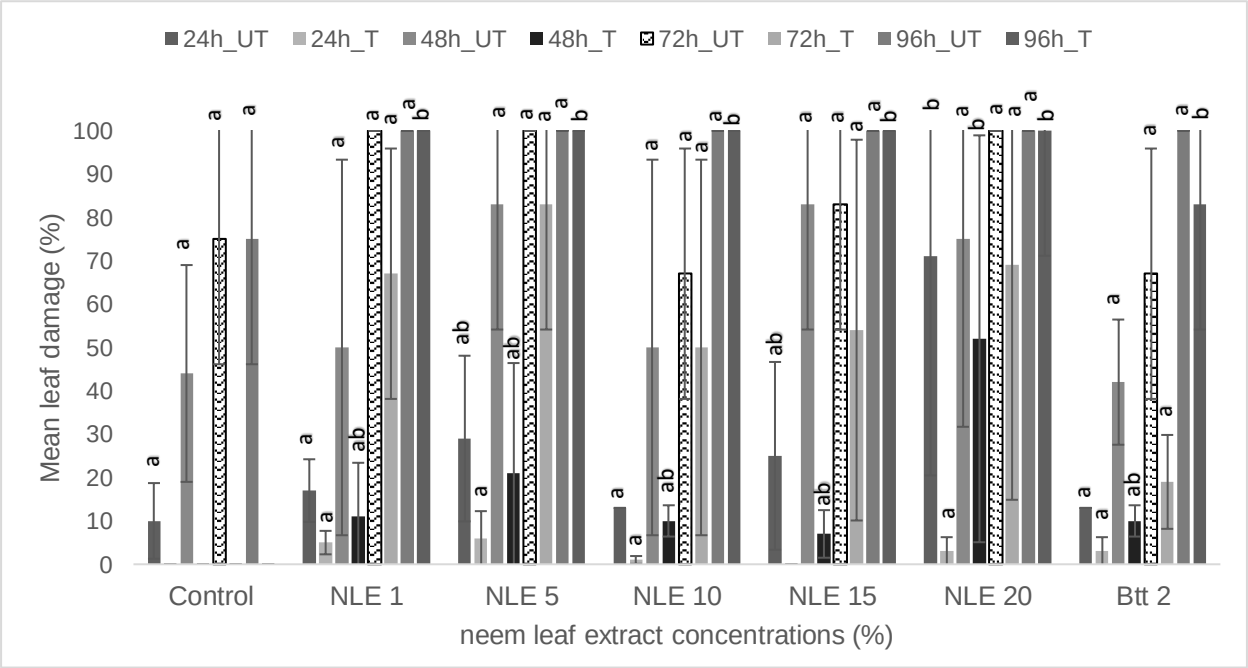

Figure 32: Effect of different neem leaf extract concentrations (%) on the mean leaf damage (%) at different time intervals caused by *Leptinotarsa decemlineata* larvae under choice condition. Different letters represent significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates

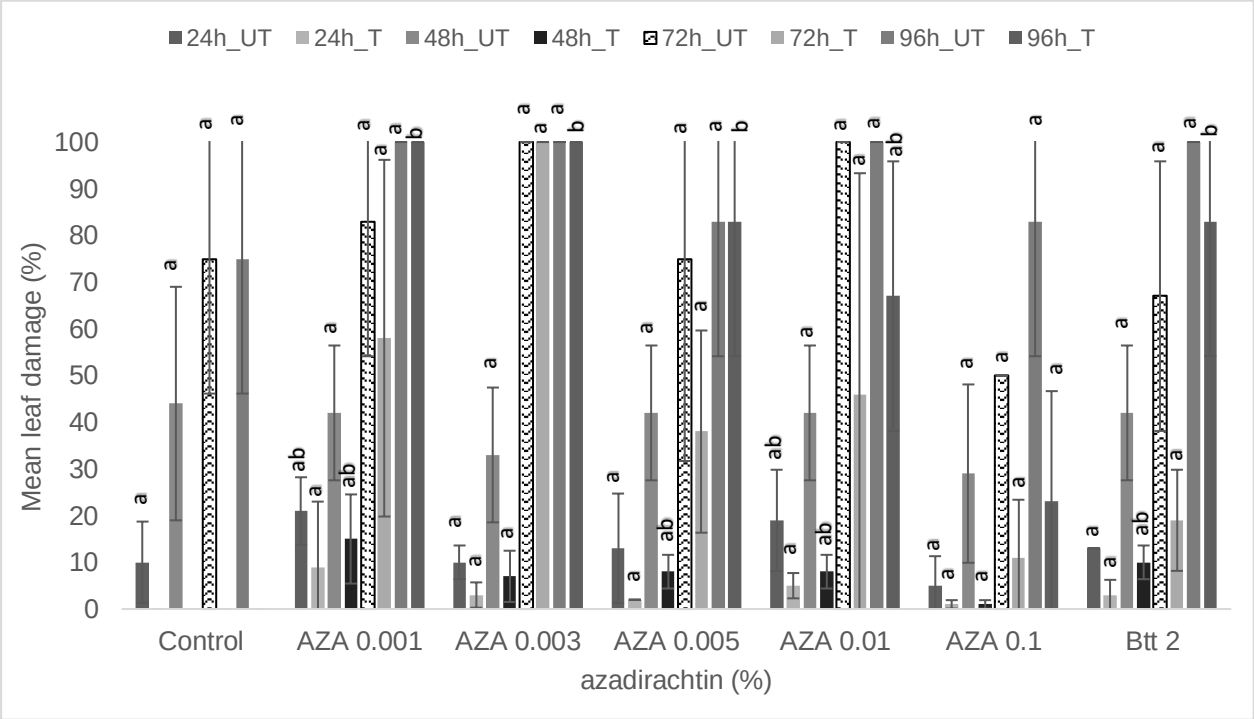

Figure 33: Effect of different azadirachtin (AZA) (%) on the mean leaf damage (%) at different time intervals caused by *Leptinotarsa decemlineata* larvae under choice condition. Different letters represent significant difference at 95% confidence level ($p<0.05$). Data are mean of 3 replicates

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

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

Different neem leaf extract and azadirachtin concentrations were tested against *Diabrotica virgifera virgifera* egg hatching *in vitro* condition. It was found that there was no significant difference in the egg mortality between each treatment measured at different time interval (Table 10).

Table 10: Table summarizing the effect of different concentrations of neem leaf extract and azadirachtin on *Diabrotica virgifera virgifera* egg mortality. The data is represented as mean±SD. Similar letters following SD represents no significant difference in the treatments at that time interval. D0-D7 represents the number of days post-treatment

Time interval	D 0	D 1	D 2	D 3	D 4	D 5	D 6	D 7
Treatments	Mean ± SD							
UC	12 ± 10.1 a	15 ± 12.6 a	16 ± 12.5 a	17 ± 13.6 a	18 ± 13.7 a	18 ± 13.7a	19 ± 14.6a	20 ± 15.6a
L 0.01%	10 ± 6.7 a	14 ± 10.5 a	18 ± 13.3 a	21 ± 15.1 a	21 ± 15.15	23 ± 15.6a	24 ± 16.8a	24 ± 16.8a
L 0.1%	10 ± 6.6 a	12 ± 7.6 a	16 ± 9.4 a	18 ± 9.7 a	19 ± 10.43 a	22 ± 10.5a	23 ± 11.1a	24 ± 12a
L 0.5%	10 ± 7.1 a	12 ± 8.2 a	14 ± 8.4 a	15 ± 9.1 a	16 ± 10.2a	17 ± 10.18a	19 ± 11.4a	20 ± 11.6
L 1%	14 ± 6.8 a	17 ± 10.2 a	20 ± 11.5 a	22 ± 12.6 a	23 ± 14.7a	23 ± 14.6a	24 ± 14.9a	26 ± 17.1
L 10%	11 ± 8.7 a	14 ± 10.7 a	17 ± 13.3 a	18 ± 13.5 a	19 ± 14.6a	20 ± 14.3a	20 ± 14.1a	21 ± 14.2
AZA 0.001%	17 ± 12.6 a	20 ± 16.2 a	22 ± 17.7 a	23 ± 17.6 a	24 ± 17.3a	26 ± 16.9a	27 ± 16.6a	29 ± 17.9
AZA 0.003%	12 ± 7.03 a	15 ± 10.2 a	17 ± 11.5a	18 ± 12.3 a	19 ± 13.12 a	21 ± 14.1a	22 ± 14.5a	23 ± 14.9
AZA 0.005%	10 ± 6.6 a	10 ± 6.4 a	12 ± 6.8a	14 ± 7.6 a	14 ± 8.8a	17 ± 11.8a	17 ± 11.8a	19 ± 12.8a
AZA 0.01%	11 ± 5.5 a	14 ± 8.08 a	15 ± 8.8a	18 ± 9.6 a	19 ± 10.8a	20 ± 11.1a	22 ± 13.7a	24 ± 15.1a
AZA 0.1%	15 ± 10.6 a	16 ± 11.6 a	18 ± 14.4 a	20 ± 16.1 a	21 ± 17.8	22 ± 17.8a	22 ± 17.5a	23 ± 17.9a
Imidacloprid 0.01%	13 ± 10.3 a	14 ± 10.7 a	16 ± 11.6 a	20 ± 12.7 a	23 ± 14.4a	25 ± 14.4a	27 ± 15.1a	27 ± 15.3a
Imidacloprid 0.1%	17 ± 7.8 a	20 ± 8.3 a	22± 9.29a	22 ± 9.8 a	24 ± 11.9a	25 ± 11.8a	26 ± 12.7a	27 ± 12.6a

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

The results revealed that azadirachtin caused high mortality in neonate larvae within 3 days. It can be concluded from the results that azadirachtin at 0.1% and 0.5% is as effective as Imidacloprid at 0.01µL concentration. In the case of neem leaf extract, no effect can be seen at all concentrations after day 3, whereas a slight effect was seen at day 5 only at the highest concentration i.e 20% (Fig 34).

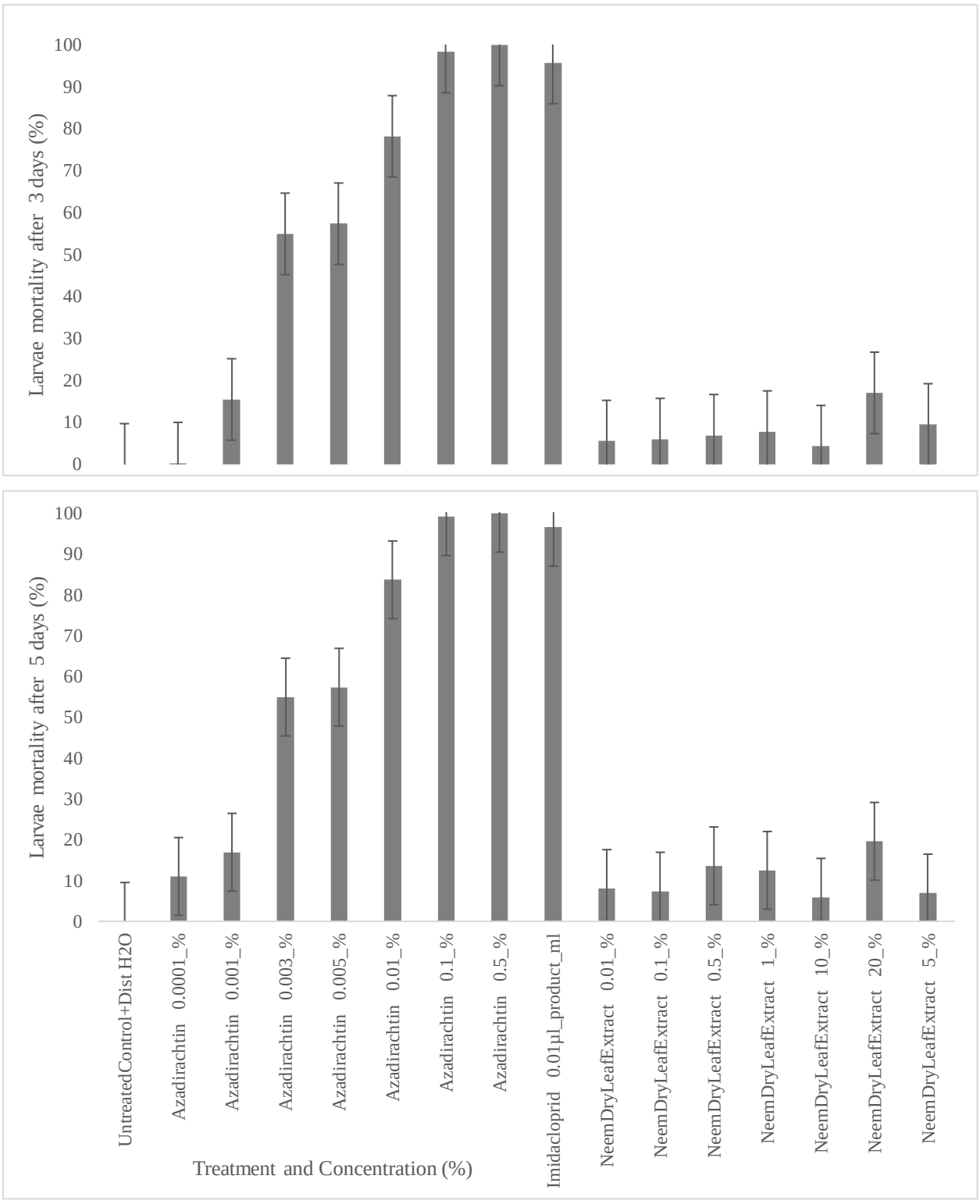

Figure 34: Mortality (%) of neonate *Diabrotica virigfera virgifera* larvae reported after 3- and 5-days post-treatment with neem leaf extract and azadirachtin.

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

4.3.1. Entomopathogenic and Slug-parasitic nematodes

In the case of *H. bacteriophora*, a steep slope could be noticed; 0.1% NLE did not cause any lethal effect, while 0.3 – 1% NLE resulted in significantly higher 97.5% mortality (Figure 35A). NLE of 0.1% concentration did not cause any effect on the viability of *Ph. hermaphrodita* juveniles. Only at higher concentrations (i.e., 0.6 and 1%), could remarkable mortality be observed, respectively (Figure 36A). Only 13.75% of *S. carpocapsae* juveniles died by 0.3% NLE, while 0.6% and 1% NLE caused 80.36% and 79.64% mortality, respectively (Figure 37A).

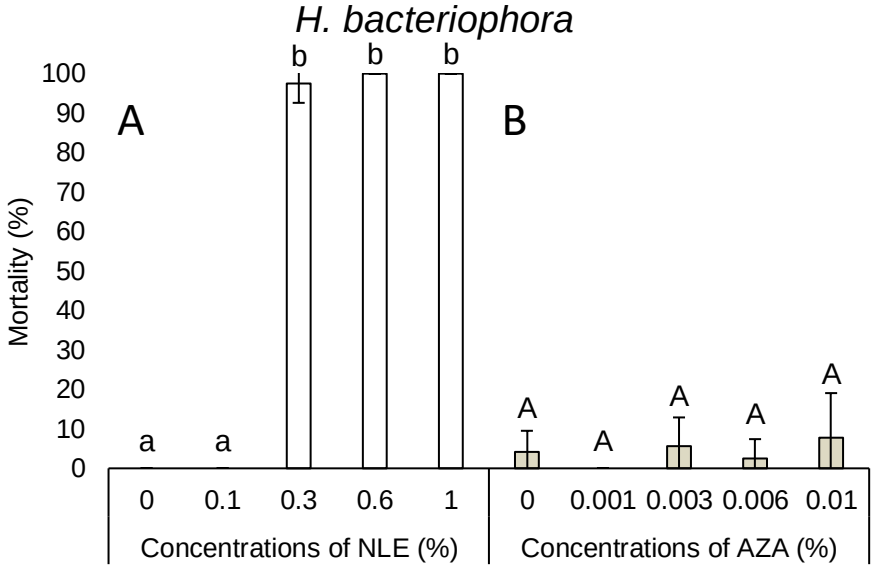

Figure 35: Mortality after 24-h exposure time of *Heterorhabditis bacteriophora* juveniles treated with neem leaf extract (NLE) (**A**) and NeemAzal-T/S (AZA) (**B**).

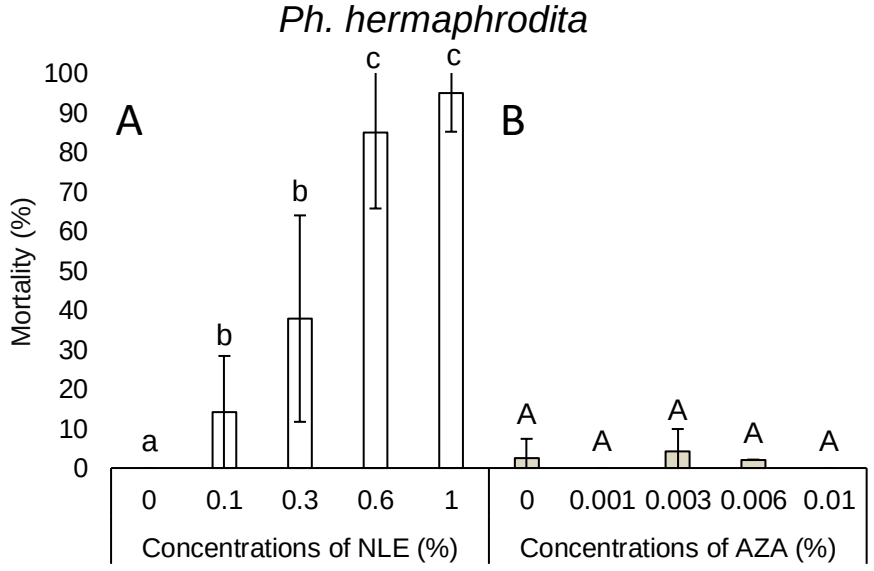

Figure 36: Mortality after 24-h exposure time of *Phasmarhabditis hermaphrodita* juveniles treated neem leaf extract (NLE) (**A**) and NeemAzal-T/S (AZA) (**B**).

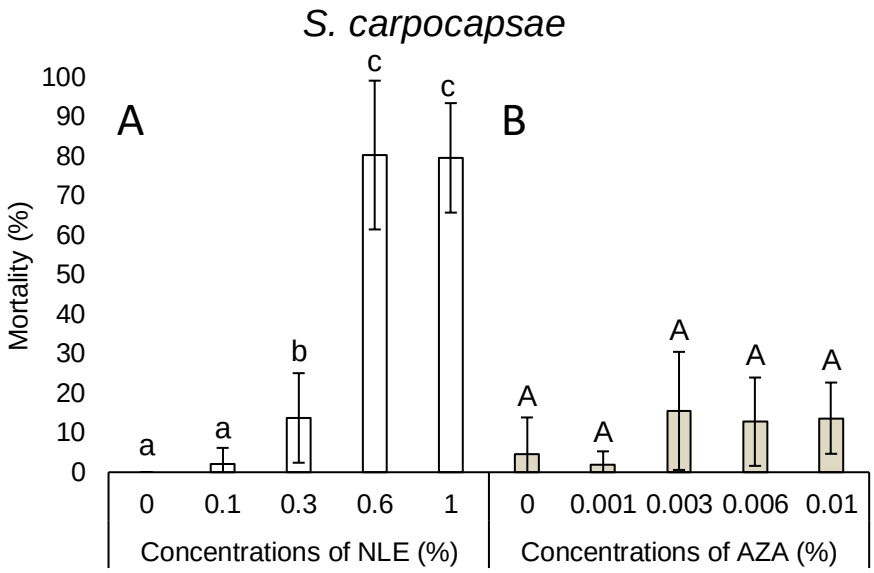

Figure 37: Mortality after 24-h exposure time of *Steinernema carpocapsae* juveniles treated neem leaf extract (NLE) (**A**) and NeemAzal-T/S (AZA) (**B**).

In the case of *S. feltiae*, there was a slight stepwise increase but no significant difference between mortality at control, 0.1%, and 0.3% NLE, with the highest average of mortality being 19.4%. Efficacy of 0.6 and 1% NLE concentrations showed between 70.5% and 90.8% mortality, respectively (Figure 38A). NLE of 0.1% did not have any effect on the survival of *S. kraussei* juveniles, while 0.3% NLE caused 46.5% mortality, whereas 95% and 100% mortality was observed in higher (0.6% and 1%) concentrations, respectively (Figure 39A).

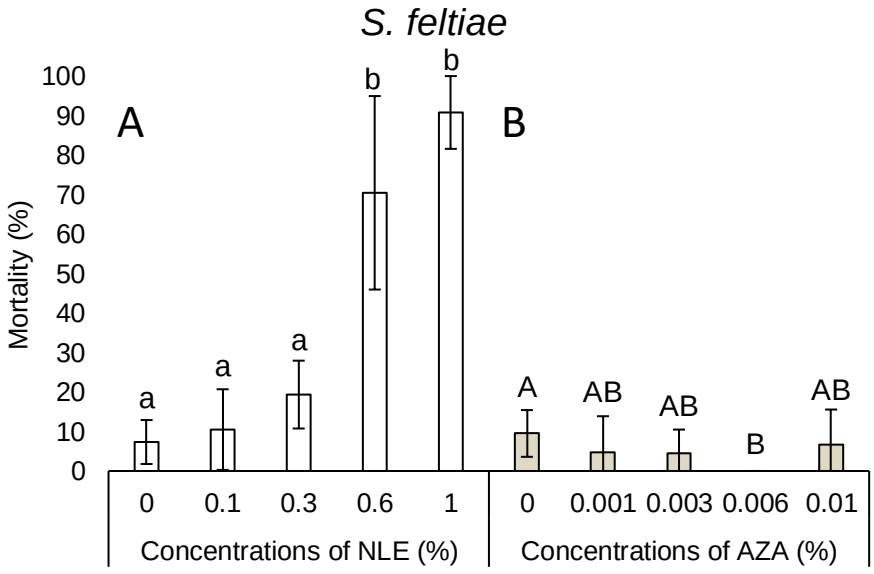

Figure 38: Mortality after 24-h exposure time of *Steinernema feltiae* juveniles treated neem leaf extract (NLE) (**A**) and NeemAzal-T/S (AZA) (**B**).

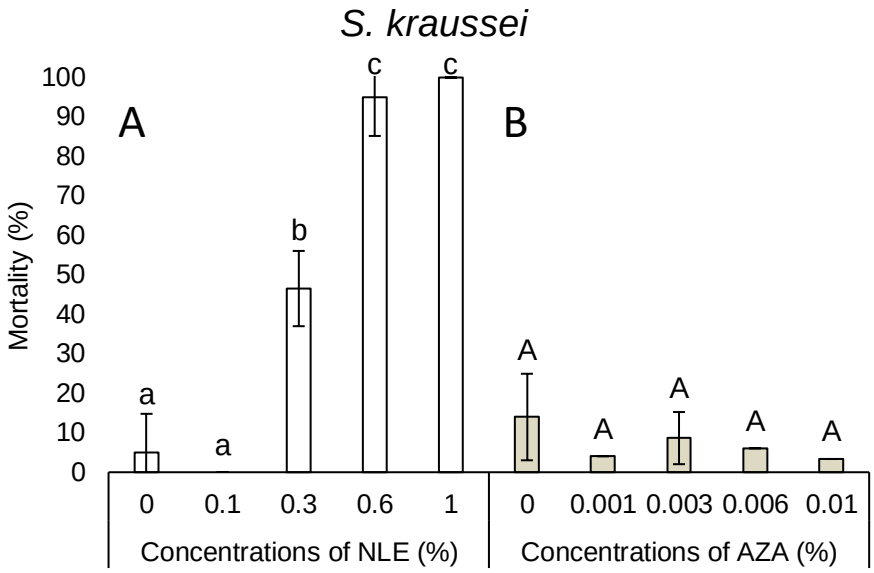

Figure 39: Mortality after 24-h exposure time of *Steinernema kraussei* juveniles treated neem leaf extract (NLE) (**A**) and NeemAzal-T/S (AZA) (**B**).

Since the mortality was inconsistent at every concentration of azadirachtin, with even the highest values being much lower than 50%, LC values were not calculated. On the other hand, values of neem leaf extract 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 other 3 nematodes, LC_{50} values were similar. 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* (0.132%) (Table 11).

Table 11: LC_{10} and LC_{50} values for neem leaf extract (%) in case of *Heterorhabditis bacteriophora Phasmarhabditis hermaphrodita, Steinernema carpocapsae, S. feltiae* and *S. kraussei*

Species	LC_{10}	LC_{50}
Heterorhabditis bacteriophora	0.179%	0.217%
Phasmarhabditis hermaphrodita	0.132%	0.366%
Steinernema carpocapsae	0.293%	0.330%
Steinernema feltiae	0.172%	0.480%
Steinernema. kraussei	0.185%	0.313%

4.3.2. Isopod (*Porcellionides pruinosus* Brandt 1888)

The mortality of *P. pruinosus* was generally low in all treatments. In the case of NeemAzal T/S (1%azadirachtin) even after 120 hours post-treatment, zero mortality was observed in seven replicates of 0.0005% concentration, eight replicates at 0.001%, nine replicates of 0.0025%, seven

replicates of 0.005 and 0.0075% each, and four replicates of 0.01% (Table 12). The same was observed in the case of neem leaf extract, after the time period of 120 hours: zero mortality in case of five replicates of 0.05% concentration, nine replicates of 0.1%, six replicates of 0.25%, seven of 0.5%, four replicates of 0.75%, and five replicates of 1% (Table 13).

Table 12: Effect of NeemAzal T/S on the mortality of *Porcellionides pruinosus* expressed as cumulative mean for different time intervals

Treatment	Conc (%)		Mean mortality rate after time interval					
			1 hr	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
NeemAzal T/S (1% azadirachtin)	0	mean	0.1	0.1	0.1	0.1	0.1	0.2
		SD	0.32	0.32	0.32	0.32	0.32	0.42
	0.0005	mean	0.2	0.3	0.3	0.3	0.3	0.5
		SD	0.42	0.67	0.67	0.67	0.67	0.85
	0.001	mean	0	0.3	0.3	0.3	0.4	0.4
		SD	0	0.95	0.95	0.95	0.97	0.97
	0.0025	mean	0	0	0	0	0	0.1
		SD	0	0	0	0	0	0.32
	0.005	mean	0.1	0.2	0.2	0.2	0.2	0.3
		SD	0.32	0.42	0.42	0.42	0.42	0.48
	0.0075	mean	0.2	0.2	0.2	0.2	0.2	0.3
		SD	0.42	0.42	0.42	0.42	0.42	0.48
	0.01	mean	0	0.1	0.5	0.5	0.5	0.7
		SD	0	0.42	0.71	0.71	0.71	0.67

Key: conc = concentration, SD= Standard deviation. Each value is an average of ten replicates

Table 13: Effect of neem leaf extract on the mortality of *Porcellionides pruinosus* expressed as cumulative mean for different time intervals

Treatment	Conc (%)		Mean mortality rate after time interval					
			1 hr	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
neem leaf extract	0	mean	0	0	0	0	0.1	0.33
		SD	0	0	0	0	0.33	0.5
	0.05	mean	0	0	0	0.1	0.2	0.527
		SD	0	0	0	0.32	0.42	0.52
	0.1	mean	0	0	0	0	0	0.1
		SD	0	0	0	0	0	0.32
	0.25	mean	0	0	0	0	0.2	0.4
		SD	0	0	0	0	0.42	0.52
	0.5	mean	0	0	0	0	0	0.4
		SD	0	0	0	0	0	0.7
	0.75	mean	0	0.1	0.2	0.2	0.2	0.7
		SD	0	0.32	0.42	0.42	0.42	0.67
	1	mean	0	0.1	0.2	0.3	0.4	0.7
		SD	0.32	0.42	0.67	0.7	0.95	0.32

Key: conc = concentration, SD= Standard deviation. Each value is an average of ten replicates

The mortality slightly increased with the increase in concentration but this observed increment was not statistically significant. Unusually high values (i.e., higher mortality) were occasionally observed both in NeemAzal T/S and neem-leaf extract treatments. These can be attributed to either the juvenile mortality of *P. pruinosus* (Dangerfield and Telford, 1995) or suboptimal conditions. NeemAzal T/S and neem leaf extracts were compared to check their respective effects on the mortality of the isopods. Neither azadirachtin nor neem leaf extract affected the observed isopod mortality (p-values are 0.43 and 0.39 and McFadden's pseudo R^2: 0.04 for azadirachtin, 0.05 for neem leaf extract respectively) (Figs 40 & 41).

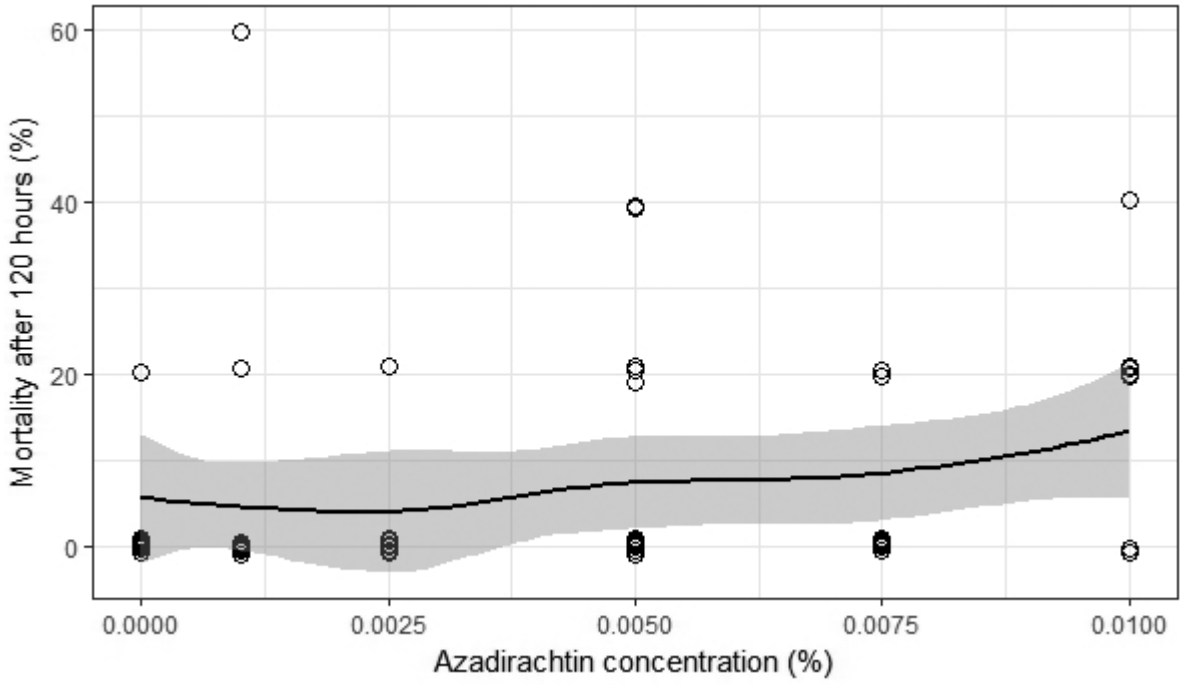

Figure 40: Mortality rate of the isopod *Porcellionides pruinosus* after 120 hours at different concentrations of NeemAzal T/S (1% azadirachtin). The vertically jittered circles (to avoid perfect overlapping) indicate the individual isopods whereas the line indicates the trend of the mortality with respect to increasing concentrations and the grey area represents the 95% confidence level

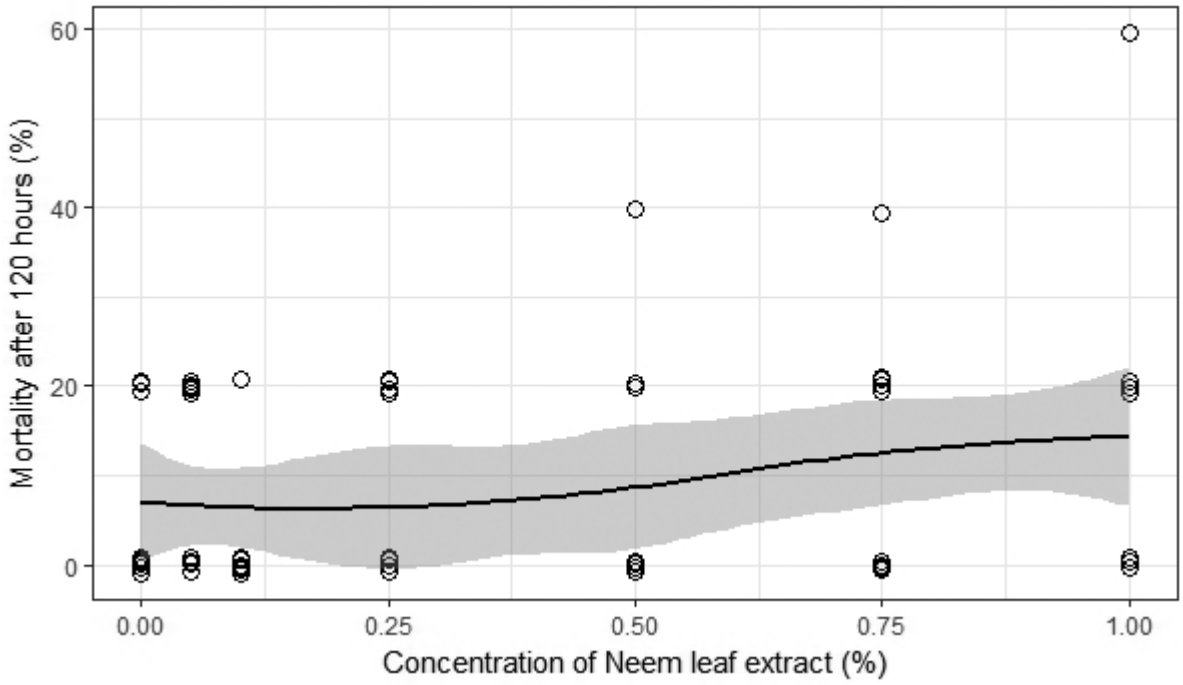

Figure 41: Mortality rate of the isopod *Porcellionides pruinosus* after 120 hours to at different concentrations of neem leaf extract. The vertically jittered circles (to avoid perfect overlapping) indicate the individual isopods whereas the line indicates the trend of the mortality with respect to increasing concentrations and the grey area being the 95% confidence level

4.3.3. Entomopathogenic and antagonistic fungi

It can be seen that 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 (Table 14).

Table 14: Effect of neem leaf extract and azadirachtin on *Beauveria bassisana in vitro* on PDA plates

	Colony Diameter (cm)					
Treatment and concentration (%)	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7
Control 0	0	1.5	1.96	2.88	3.26	3.76
NLE 0.1%	0	1.53	2.1	2.6	4.2	5.2
NLE 0.5%	0	1.63	2.05	2.61	4.5	5.4
NLE 1%	0	1.66	3.2	3.11	4.53	6.03
AZA 0.001%	0	1.63	2.2	2.78	3.33	4
AZA 0.005%	0	1.7	2.16	2.55	3.23	4.03
AZA 0.01%	0	1.68	2.11	2.58	3.33	4.1

Neem-derived pesticides were also tested against another entomopathogenic fungus i.e *in vitro* (Table 15). The results showed that neither of the neem-derived pesticides had any negative effect on the growth of *Metarhizium anisopliae* (Table 15).

Table 15: Effect of neem leaf extract and azadirachtin on *Metarhizium anisopliae in vitro* on PDA plates

	Colony Diameter (cm)					
Treatment and concentration (%)	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7
Control 0	0	1.5	1.97	2.88	3.36	3.7
NLE 0.1%	0	1.53	2.08	2.6	4.2	5.2
NLE 0.5%	0	1.63	2.05	2.61	4.5	5.4
NLE 1%	0	1.67	3.2	3.11	4.53	6.03
AZA 0.001%	0	1.63	2.21	2.78	3.33	4
AZA 0.005%	0	1.7	2.16	2.55	3.23	4.03
AZA 0.01%	0	1.68	2.11	2.58	3.36	4.1

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* (Table 16).

Table 16: Effect of neem leaf extract and azadirachtin on *Trichoderma harzianum in vitro* on PDA plates

	Colony Diameter (cm)					
Treatment and concentration (%)	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7
Control 0	0	7.3	9	9	9	9
NLE 0.1%	0	6.68	9	9	9	9
NLE 0.5%	0	7.28	9	9	9	9
NLE 1%	0	8.1	9	9	9	9
AZA 0.001%	0	7.21	9	9	9	9
AZA 0.005%	0	6.51	9	9	9	9
AZA 0.01%	0	6.33	9	9	9	9

5. DISCUSSION

5.1. HPLC analysis of Neem leaf extract

It is evident from our result that the azadirachtin A content in the leaves is very low (0.2µg/g = 1mg/5g leaves). The same result was observed by Ghimeray et al. (2009) where they found trace amounts of azadirachtin content in the water extract in the neem leaves grown in the foothills of Nepal. However, it has been reported that azadirachtin is highly concentrated in mature seeds (Kumar et al. 1996). There were other peaks that were detected on the same spectrum as that of azadirachtin but the retention time was different as compared to azadirachtin A. It is suspected that these compounds are potential derivatives of azadirachtin A. Our potential azadirachtin derivative suspects can be confirmed by Kumar et al. (1996) as they stated that different analogues of azadirachtin A having similar biological activity has been identified to be azadirachtin B-L.

5.2. Effects of neem-derived pesticides on target organisms

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

All the treatments except the lower concentration of NeemAzal T/S showed a significant reduction in the sporangial germination *in vitro*. Our results are similar to those reported by Mirza et al. (2000) where they tested different neem products against different stages of an oomycete, *Phytophthora infestans* (Mont.) de Bary. They found that all the neem products namely crude neem seed oil, nimbokil (a commercial formulation of neem oil), crude neem seed oil terpenoid extract and neem leaf decoction were effective against mycelial growth, sporangial germination and sporangium production of *P. infestans*. Rashid et al. (2004) observed similar efficacy when they compared different neem products against two isolates of *P. infestans*. They found that all the neem products tested significantly inhibited the different developmental stages of this oomycete. Our results are also consistent with Mboussi et al. (2016) after they tested the effects of aqueous extract of neem on *P. infestans* and concluded neem extracts had the same effects as Ridomil Gold Plus, a chemical fungicide with effective ingredients mefenoxam and copper, against *Phytophthora megakarya* Brasier & M.J. Griffin. The results of this experiment are also in line with previous reports of Ngadze (2014) where *Azadirachta indica* (Neem) was found to be effective against *P. infestans* both under *in vitro* and *in vivo* conditions.

During *in vivo* tests, in the pre-treatment experiment, we found in our study that both the selected concentrations of neem leaf extract and higher concentration of NeemAzal T/S successfully reduced the infection in sunflower. Our results contradict the findings of Rovesti et al. (1992) where neem extract was found to be ineffective against *P. infestans* but they are in line with the results found by Ngadze (2014) where an *in vivo* experiment resulted that extracts of both onion

and neem were effective to control *P. infestans* in potato. Our results were also consistent with Achimu and Schlösser (1992), where neem seed extract and commercial neem products were highly effective against *Plasmopara viticola* in grapevine. Also, Krzyzaniak et al. (2018) found the same results as Achimu and Schlösser (1992) where they successfully controlled *P. viticola* using plant extract. It can be said that these results might be due to the presence of different biologically active compounds such as azadirachtin in neem leaves and other plant parts. Shakywar et al. (2012) also found similar results under *in vivo* conditions and they stated that the inhibitory action in the neem leaf extract may be due to azadirachtin present in all parts of the plant. The reduction in the infection in the pre-treatment might be the result of the sensitizing of sunflower defense response towards *P. halstedii* which was also reported by Fernandez et al. (2004), where they tested the essential oil obtained from *Bupleurum gibraltarium* against *P. halstedii*. They reported that the oil pre-treatment may activate the defense response of the seedlings against *P. halstedii.*

One of the possible reasons of controlling *P. halstedii* infection could be the systemic effect of neem. Systemic effect of neem is validated by various studies conducted by Naumann et al. (1994b) who found that the mountain pine beetle population in Lodgepole pine was reduced due to upward translocation of azadirachtin. Osman and Port (1990) and Marion et al. (1990) also observed translocation of azadirachtin in vegetable crops and in birch, *Betula* spp respectively. Goel et al. (2013) demonstrated the systemic acquired resistance in tomato induced by neem fruit extracts against bacterial speak cause by *Pseudomonas syringae* pv. Tomato (Okabe) Young, Dye & Wilkie, while Bhuvaneshwari et al. (2012) demonstrated the same in barley seedlings against *Drechslera graminea* (Rabenh.) S. Ito.

In the case of post-treatment, higher concentrations of both neem-derived pesticides inhibited the infection. This might be attributed to the curative effect of neem-derived pesticides observed by Achimu and Schlösser (1992) as he stated that inhibition of indirect germination of sporangia by preventing zoospore release and/or formation explains the efficacy of these products which can be validated through the *in vitro* results of this experiment. Perhaps azadirachtin in the neem leaf extract alone may or may not cause this inhibitory action. There might be more than one biologically active compounds working synergistically to control the infection that are different from azadirachtin and related substances (Lehmann, 1991; Biswas et al. 2002).

Plant extracts possessing different properties against pest and pathogens can prove to be beneficial where chemical pesticides fail, hence a thorough and extensive research in this field is needed. This is the first report of neem leaf extracts and commercially available azadirachtin exhibiting

strong antifungal activity against *P. halstedii*. It is a naturally available fungicide (the neem tree) and a promising alternative to chemical pesticides for controlling downy mildew in sunflower by seedling treatment.

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

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 *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949. 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, fresh shoot weight for both the landraces was lower and significantly different compared to 0 control. This is probably because the roots were adversely 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 NeemAzal T/S, the maximum azadirachtin 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.

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

It is evident that neem leaf extract is toxic to the newly hatched and first instar larvae. Intoxication of CPB larvae when treated with different but higher neem leaf extract concentrations showed delayed but high mortality as seen from the no choice test as compared to azadirachtin. Delayed larval mortality in the case of neem leaf extract might be due to the antifeedant activity of different compounds found in NLE and larvae as seen from the results. Another possible reason could be that the various compounds present in the NLE are slow in their action (Trisyono and Whalon 1999) or the accumulation of lower concentrations of neem compounds in the gut system and then acting on the hormonal system as suggested by Zehnder and Warthen (1988) and Trisyono and Whalon (1999).

On the contrary, weak mortality results were obtained in the case of azadirachtin in the no choice test for both the products in choice test. This might be because of the mixed population of the larvae and there is a possibility that the second and third instar larvae have more evolved gut system to digest neem and excrete out the toxic compounds Wimer et al. (2015), thereby sparing the untreated leaf for the first instar larvae with weaker gut system. Another possibility can be the uneven distribution of different compounds on the leaf extract. Perhaps there was not enough of concentration of different compounds found in neem leaves on the leaf surface which in turn was not enough for larval mortality. Another reason can be the slow toxic effect of the different neem compounds.

With respect to antifeedant properties, a strong antifeedant activity was observed in the case of neem leaf extract in the no choice experiment which might be due to different compounds present in the leaf extracts acting either alone or in combinations. Similar results were obtained by Alford et al. (1987) when they tested antifeedant activity of limonin against Colorado Potato Beetle larvae. Also, Zabel et al. (2002) found that neem extract had a strong antifeedant activity against Colorado Potato Beetle larvae under laboratory conditions which is like our results from the no choice test but contradicts the results from choice test.

In the case of azadirachtin, the antifeedant activity was weak in our experiment. Our results contradict the work done by Hiiesaar et al. (2000) where the azadirachtin from the same commercial product showed only 12% consumption. However, our findings are consonant with

the results reported by Klocke and Barnby (1989) and Hiiesaar et al. (2009), where they could not find any significant effect on feeding activity. Kutas and Nadasy (2005) experienced similar results of low antifeedant activity in the case of azadirachtin (NeemAzal T/S) and they argued that this can be possible due to the low concentration of azadirachtin used for the experiment while the recommended dose is 0.3-0.5%.

5.2.4. Western corn rootworm (*Diabrotica virgifera virgifera* LeConte 1868)

From the results, it is evident that the used concentrations of neem leaf extracts and azadirachtin were not effective and did not influence mortality of eggs. Our results contradict the study of Michaelides and Wright (1999), where they tested different chemical insecticides namely tefluthrin, carbofuran, terbufos and dieldrin against the *Diabrotica undecimpunctata howardi* Barber 1947, eggs in soil. Since it can be seen from our HPLC analysis results that the azadirachtin concentration in the neem leaf extract is very less, it can be a possibility that the concentrations sprayed on the eggs were not enough to permeate through the eggs and stop the embryonic development as seen by Michaelides and Wright (1999). Meanwhile, the results with azadirachtin were consistent with Boetel et al (1998) where they tested chemical insecticides namely chlorethoxyfos, tefluthrin, and terbufos on *Diabrotica barberi* Smith & Lawrence, and *Diabrotica virgifera virgifera*. They checked the egg viability in subsamples and found no difference across the treatment.

From the *in vitro* bioassays to control the neonate larvae, it was found that azadirachtin had a better role in controlling the neonate larvae than neem leaf extract. The mortality increased with an increase in azadirachtin concentrations. Similar results were obtained by Souza et al. (2015a) which are in line with our results who demonstrated that different formulations of *Melia azedarach* had same efficacy as the insecticide fipronil against *Diabrotica speciosa* Germar, 1824. Powder formulation of *M. azedarach* achieved above 80% larval mortality. Ventura and Ito (2000) tested different *M. azedarach* plant parts against *D. speciosa* and found a very strong antifeedant effect. Our results match the work of Xie et al. (1991) where they tested azadirachtin against *Diabrotica virgifera virgifera* larvae under *in vitro* conditions and pot trials and found a strong correlation between mortality of larvae and increasing azadirachtin concentration. Landis and Gould (1989) also reported that ethanolic extracts of plant seeds from the family Meliaceae were highly active feeding deterrents against *Diabrotica undecimpunctata*. The effect of two triterpenoids from neem was tested against *D. undecimpunctata* by Reed et al (1982) in laboratory and under greenhouse conditions. They found that azadirachtin, salannin and other bioactive compounds from neem seeds showed strong feeding deterrent activity against *D. undecimpunctata*. Our results are also similar to Gallo et al. (1996), where they tested *Mammea americana* extracts against *Diabrotica*

virgifera virgifera and *Trichoplusia ni* Hübner, 1800–1803 and found that both of them were susceptible to *M. americana*. Different concentrations of neem leaf extract did not show any significant effect in the results earlier but later only the highest concentration showed a slight effect. This might be due to the low concentration of azadirachtin present in the leaves which is indicated by our HPLC analysis results too.

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

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

There was species-specific variation in the response of the nematodes tested with the various concentrations of NLE, which was a similar finding when compared with the previous study in which EPN species could have different sensitivity against fungicides (Laznik et al. 2012). Considering LC_{50} values, *H. bacteriophora* seemed the most sensitive, which is in accordance with a previous study (Abdel-Razek and Gowen, 2002). Neem leaf extract had a stronger lethal effect than NeemAzal T/S on the examined nematode species. One of the possible reasons could be that leaf extract contains higher azadirachtin content than the commercial product. Another possible explanation could be that NLE does not consist only azadirachtin but other pesticidal active compounds known as triterpene', more specifically 'limnoids', e.g., nimbin, nimbidine, nimbinin, azadirachtol, salannin, and other such derivatives, which may exhibit a toxic effect (Mondal and Mondal, 2012). According to a previous study, aqueous extract of neem leaf had higher salannin content than azadirachtin content (Sadeghian and Mortazaienezhad, 2007). In addition, both nimbin and salannin have a nematicidal effect (Mojumder et al. 2002; NIIR Board of Consultants & Engineers, 2004). Moreover, compounds found in neem extracts from different parts of the neem tree may enhance the effect of each other by synergism (Mahmoud, 2007; Otieno et al. 2015; Meyer et al. 2012; Krishnayya and Grewal, 2002; Chen et al. 2012). Considering LC_{10} values, 0.1% of neem leaf extract might be used safely in combination with each examined EPN and SPN species. Since the mortality results of NeemAzal T/S were inconsistent and low, the low- and sub-lethal values could not be calculated. However, we demonstrated under *in vitro* conditions that concentrations of NeemAzal T/S three times higher than the recommended for field applications and neem leaf extract in low concentration did not harm either EPN or SPN species.

5.3.2. Isopod (*Porcellionides pruinosus* Brandt 1888)

While there is numerous literature available on the effect of neem and neem-derived products on target organisms, some of the studies reported data on nontarget organisms as well. For instance, Goktepe et al. (2004) carried out an ecological risk assessment of neem-based products on six aquatic animals through short-term acute toxicity tests and concluded that the risk values did not

exceed the criteria and were safe for use. In contrast, it has been noted that neem components do have adverse effects on non-target aquatic organisms such as *Daphnia* species Straus, 1820 as studied by Stark (1997) and fish (Tangtong and Wattanasirmkit, 1997). Scott and Kaushik (1998) assessed the effect of Margosan-O (a product of neem seeds) on non-target aquatic invertebrates. Their investigation revealed that there can be some effects of the product on non-target organisms at higher concentration but if applied in agricultural systems, Margosan-O may not reduce the survival or reproduction of the non-target aquatic organisms. Wagenhoff et al. (2013) studied the effects of NeemAzal T/S on the burying beetle *Nicrophorus vespilloides*, which co-occurs with the forest cockchafer *Melolontha hippocastani* Fabricius, 1801 and also feeds on the carcasses of *M. hippocastani*. In their study, they fed *N. vespilloides* with dead *M. hippocastani* which were previously fed with neem-treated leaves. They neither observed any impact on the mean larval weights nor on the morphology of *N. vespilloides*. Still, they authors did not dismiss the possibility of azadirachtin passing through the food chain and affecting other non-target organisms. Akca et al. (2015) investigated the effect of azadirachtin (NeemAzal T/S) on terrestrial isopod *Philoscia muscorum* Scopoli, 1763 and did not find any adverse effects on *P. muscorum*. The results of our experiments were found to be similar and this experiment for the first time investigated the effects of two different neem products on this non-target isopod species, i.e., *Porcellionides pruinosus*.

5.3.3. Entomopathogenic and antagonistic fungi

In the case of the entomophathogenic fungus *Beauveria bassiana,* it was observed that both the neem-derived pesticides at all concentrations enhanced the growth of the fungus. Results of this study were consistent with studies conducted by Bajan et al. (1998), where they tested the effect of botanical preparation BioNEEM ™ against *B. bassiana* and found that the fungus was active in the habitat where the treatment was present. While Mohan et al. (2007), reported that 23 out of 30 isolates of *B. bassiana* when tested with Margoside®, a commercial product containing neem oil, showed delayed conidial germination but not inhibition. But the combined treatment of *B. bassiana* with neem oil showed a synergistic effect against *Spodoptera litura* Fabricius, 1775. Findings of this study results matches with the study conducted by Depieri et al. (2005) also reported that neem seed and leaf extracts were compatible at all the concentrations with *B. bassiana*. The findings of this work are consistent with one part and contradict another part of the study carried out by Gupta et al. (1999) who tested 7 different commercial neem formulations viz. Margocide, Neem Gold, Neemark, Achook, Nimbicidine, Neemta and Field Marshal along with water and alcoholic extract of neem bark, dried leaves, neem cake against *B. bassiana*. They found that Margocide, Achook, Nimbicidine, Field Marshal, neem cake and neem leaf extract can be compatible with *B. bassiana*. The results of this work contradict the findings of Hirose et al.

(2001), where they reported that Neem oil exerted a negative effect on the colony diameter of two entomopathogenic fungi viz. *B. bassiana* and *Metarhizium anisopliae*. In the case of *Metarhizium anisopliae*, it was found that neem-derived pesticides enhanced the colony diameter of the fungus *in vitro* with the increase in time period. The findings in this work was in accordance to Schumacher and Poehling (2012) tested different pesticides including NeemAzal against *M anisopliae in vitro* as they found compatibility between the two. They are in consistent with work conducted by Gomes et al. (2015) as they too conclude that neem had no effect on conidial germination or fungal vegetative growth of *M. anisopliae in vitro*.

The neem-derived pesticides were found to be compatible with antagonistic fungus viz. *Trichoderma harzianum in vitro* at all concentrations. This result is similar to the findings of Bagwan, (2010), where he tested neem oil (5%), neem leaves extract (10%), wild sorghum leaves extract (10%), neem cake, castor cake against *Trichoderma harzianum* and found that they enhanced the growth of the fungus. The results in this study contradicts the findings of Sarkar et al. (2010) where they tested nimbecidine (0.03%), ponneem (0.05%), neem kernelaqueous extract (NKAE) against *T. harzianum in vitro* and found inhibition of growth in *T. harzianum* strain.

6. CONCLUSION 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.

7. 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*.

9. SUMMARY

Agriculture has been one of the most important occupation around the world. With ever-increasing population, it is difficult to keep the pace and the supply of food without hampering nourishment. Different pests and pathogens have either specific or wide-range of hosts which not only reduces yield but also makes huge economic losses to the countries producing it. Chemical control by using different insecticides, fungicides etc. being the traditional method to control these pests and pathogens, studies on their side-effects has become a necessity. Alternatives to the chemical control are researched and established to control the plant pests and pathogens. Biological control is gaining serious attention that uses natural predators and different naturally available plant extracts that are sustainable, environmentally friendly and economic in terms of their use.

In this thesis, a naturally available plant extract from *Azadirachta indica* commonly also known as 'neem' was used which has been known for its various effects since ancient times. The main objective of my thesis was to compare the effects of two neem-derived pesticides i.e neem leaf extract which was prepared in a traditional method by Indian farmers and a commercial product NeemAzal T/S (containing 1% azadirachtin) which is registered in the European Union, on target and non-target organisms. Neem-derived pesticides were tested on target organisms belonging to oomycetes, plant pathogenic fungi, plant-parasitic nematodes, insect pests which are known to cause a major damage to the yield and incur huge losses in the world.

The HPLC analysis of neem leaf extract showed that the azadirachtin A content was very low. However, there were other suspected derivatives of azadirachtin in the sample which could not be identified due to non-availability of the standard and appropriate testing.

The two neem-derived pesticides tested against *Plasmopara halstedii* showed significant effect in controlling the oomycete both in vitro and in vivo conditions. In the experiment with plant-parasitic nematode *Meloidogyne incognita*, *in vitro* results showed that higher concentrations of neem leaf extract increased the mortality of J_2 larvae. But in the *in vivo* experiment, although no significant decrease but a reduction in root infection was observed. Both the neem-derived pesticides had slight effect on plant shoot weight and height. In the experiment against *Leptinotarsa decemlineata*, neem leaf extract was as effective as Btt and superior to azadirachtin in controlling the larvae. Choice test revealed the antifeedant properties of neem leaf extract comparable to azadirachtin. *Diabrotica virgifera virgifera* eggs did not show any susceptibility in terms of morality towards both the neem-derived pesticides. *Diabrotica*

virgifera virgifera larval bioassays reported that azadirachtin was as effective as imidacloprid and superior to neem leaf extract in controlling larvae.

The effects of neem-derived pesticides were investigated against different non-target organisms. The tested entomopathogenic and slug-parasitic nematodes were more sensitive to neem leaf extracts as compared to azadirachtin *in vitro*. The isopod species i.e *Porcellionides pruinosus* was not found to be sensitive to all the concentrations of with neem leaf extract or azadirachtin.

Entomopathogenic and antagonistic fungi were not affected with all the neem-derived pesticides concentrations. In fact, their growth was enhanced in the presence of neem *in vitro* suggesting that they are compatible with neem-derived pesticides and can be used in integrated control of different pests and diseases.

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