Efficacy of Neem Formulations for Controlling Aphids and Whiteflies on Cabbage and Tomatoes

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DEDICATION

This thesis is dedicated to

My parents Julius and Ruth Karanja,

Husband James and son Ryan Gacheru.

SUMMARY

Extracts from the neem tree *Azadirachta indica* are widely used as plant protection products. The active ingredient, Azadirachtin (AZA), has been shown to affect many important pests of different crops, and is classified as one of the most important bioactive compounds of neem, with systemic feeding deterrent, repellent and growth-regulating properties. Most commercial neem formulations are however, developed for foliar treatments thus their persistence is limited by the high sensitivity for photodegradation and risks for contamination of non-target organism foraging in the crop canopy cannot be avoided. Formulations applied to the growing medium making use of the systemic properties of AZA might be more effective, in particular in controlling of sucking pests such as aphids and whiteflies. Soil treatment formulations have been developed which are hydrophilic formulated; NeemAzal-T an aqueous solution without surfactants and NeemAzal granules formed from powdered NeemAzal with an inert carrier material. The potential of neem applications to the soil and the use of the systemic properties of these special formulations in controlling whiteflies and aphids were investigated in this study.

In the first part of the thesis, the efficacy and persistence (residual effect) of the NeemAzal formulations in the control of whiteflies was evaluated. The effects of the soil applied formulations, NeemAzal-T and NeemAzal granules was compared to a foliar spray, NeemAzal T/S® for the control of *Aleyrodes proletella* L. on cabbage and *Trialeurodes vaporariorum* West on tomato. The treatments were done 0, 3, 5, 7, and 14 days before the plants were exposed to adult whiteflies. All treatments caused high acute mortality of whitefly immature stages but efficacy of foliar formulation significantly decreased with time. On the other hand, soil applied formulations attained fast efficacy and long persistence indicating effective uptake and systemic translocation of AZA and improved stability of the

active ingredient in the greenhouse. In particular, the granular formulation provided reliable efficacy over a longer period and showed decrease in efficacy only after 14 days. NeemAzal T and NeemAzal T/S did not differ in their efficacy against *A. proletella* in a field experiment. Application 4 weeks after sowing caused clear effects with significantly lower number of whiteflies on treated plants.

In the second part of the study, the effect of organic matter on the efficacy and dose-response as well as persistence of substrate-applied azadirachtin was investigated. The two soil-applied products, NeemAzal-T and NeemAzal granules, were evaluated against two whitefly species, A*leyrodes proletella* and *Trialeurodes vaporariorum* on Brussels sprouts and tomatoes, respectively. The plants were grown in two substrates; a commercial substrate (CS) composed of 15% humus, 35% clay, and 50% peat and a substrate with lesser amount of organic matter, a mixture of commercial substrate and sand (CS+sand) in 1:1 ratio. For application, granules of NeemAzal were mixed with the culture substrates at 75mg/kg (=5.25 mg AZA/kg), 150mg/kg (=10.5 mg AZA/kg) and 300mg/kg (=21 mg AZA/kg) per kilogram of substrate. NeemAzal-T was drenched to the plant substrate as 1ml/kg (=10mg AZA/kg), 1.5ml/kg (=15mg AZA/kg) 2ml/kg (=20mg AZA/kg). To study the residual effect and persistence of azadirachtin, treatments were done 0, 5, and 10 days before the plants were exposed to adult whiteflies. The efficacy of azadirachtin was dose-dependent, with the highest doses of NeemAzal granules (21mg AZA/kg of substrate) and NeemAzal T (20mg AZA/kg of substrate) achieving up to 100% mortality of immature stages of whiteflies. NeemAzal formulations caused significantly higher mortality in immature stages of both whitefly species with CS+sand mixture than with pure CS. These results demonstrate that the amount of organic matter in a substrate has an influence on the efficacy of azadirachtin. Persistence of the NeemAzal formulations was not influenced by the substrate type but rather

by age of the residuals, with significant decrease in efficacy when whiteflies were exposed to 10 day-old residuals.

In the third part, efficacy and dose-response, and effect on fecundity of neem extracts on cabbage aphid *Brevicoryne brassicae* was determined. The effects of four dose levels of NeemAzal granules 75, 150, 225 and 300 mg/kg of substrate and, NeemAzal-T 1, 1.5, 2 and 2.5 ml/kg of substrate were evaluated. The efficacy of the neem formulations was dose-dependent, with the highest doses of NeemAzal granules and NeemAzal T, (300 mg and 2.5 ml/kg of substrate, respectively), having up to 0% survival of aphids by 14 days after treatment. Moreover, evaluation of persistence and residual effect of the azadirachtin on cabbage aphid over time was done using the manufacturer's recommended doses, NeemAzal granules at 150 mg and NeemAzal-T at 1 ml/kg of substrate. After treatment application, Brussels sprouts were infested with one day old aphid larvae on the same day (D0), four days (D4) and eight days (D8) after treatment. Persistence NeemAzal-T decreased sharply with time. There was no difference in survival between control plants and those treated with NeemAzal T 8 days prior to aphid infestation, however NeemAzal granules were still effective up to 8 days after treatment. Azadirachtin cause significant reduction in number of offspring per female per day also in a dose-dependent manner. Even if an aphid survived on NeemAzal T and NeemAzal granules treated plants, their reproduction was greatly reduced by Azadirachtin.

In conclusion, this study demonstrated the high efficacy of systemically administered azadirachtin against whiteflies and cabbage aphids. Furthermore formulation type was seen to play a role in determining both efficacy and persistence of neem products. NeemAzal granules proved to be the most efficient formulation which can be adopted by growers in bio-production, with little regard to the type of substrate they are using. The Azadirachtin is

slowly released from the granules, taken up by roots and translocated acropetally to the feeding site of insects, providing fast efficacy and long persistence, which could provide efficient plant protection. Use of soil-applied NeemAzal is therefore a promising IPM tool in the management of these pests.

Key words: Azadirachtin, neem, whiteflies, aphids, efficacy, persistence.

ZUSAMMENFASSUNG

Extrakte des Neembaumes *Azadirachta indica* sind weitverbreitete Pflanzenschutzmittel. Es hat sich gezeigt, dass der Wirkstoff Azadirachtin (AZA) bei vielen bedeutenden Schädlingen an verschiedensten Kulturpflanzen wirkt und daher wird dieser Wirkstoff als die wichtigste bioaktive Verbindung von Neem eingestuft, die systemisch wirkt und fraßhemmende, abschreckende und wachstumsregulierende Eigenschaften hat. Die meisten kommerziell erhältlichen Formulierungen sind jedoch für Blattapplikationen entwickelt worden und ihre Persistenz ist durch ein hohes Maß an Photodegradation limitiert. Zudem kann eine Vergiftung von Nichtzielorganismen die im Blattraum aktiv sind nicht ausgeschlossen werden. Formulierungen, die direkt auf das Substrat appliziert werden und die die systemischen Eigenschaften von AZA ausnutzen, könnten daher insbesondere bei der Kontrolle von saugenden Schädlingen wie Blattläusen und Weißen Fliegen effektiver sein. Es wurden Formulierungen für die Bodenbehandlung entwickelt, die hydrophil sind: NeemAzal-T, eine wässrige Lösung ohne Netzmittel und NeemAzal Granulat, das aus pulverförmigem NeemAzal und einem inerten Trägermaterial hergestellt wird. In dieser Arbeit wurden das Potenzial von Bodenapplikationen mit Neem und die Nutzung der systemischen Eigenschaften dieser speziellen Formulierungen bei der Kontrolle von Weißen Fliegen und Blattläusen untersucht.

Im ersten Teil dieser Arbeit wurde die Wirksamkeit und Persistenz (Restwirkung) von NeemAzal Formulierungen zur Kontrolle von Weißen Fliegen evaluiert. Die Wirkung von Bodenapplikationen von NeemAzal-T und NeemAzal Granulat wurde mit Blattapplikationen von NeemAzal-T/S® zur Kontrolle von *Aleyrodes proletella* L. auf Kohl und *Trialeurodes vaporariorum* West auf Tomate verglichen. Die Behandlungen wurden 0, 3, 5, 7 und 14 Tage vor dem Einsetzen der adulten Weißen Fliegen durchgeführt. Alle Behandlungen

verursachten eine hohe akute Mortalität bei den Präimaginalstadien der Weißen Fliegen, aber die Wirksamkeit der Formulierungen für Blattapplikationen nahm signifikant mit der Zeit ab. Hingegen wirkten die Formulierungen für Bodenapplikationen schnell und waren lange wirksam, was auf eine erfolgreiche Aufnahme, systemische Translokation und eine verbesserte Stabilität des Wirkstoffs AZA unter Gewächshausbedingungen hinweist. Insbesondere die granuläre Formulierung wirkte über eine längere Zeit zuverlässig und zeigte erst nach 14 Tagen eine Abnahme in der Wirksamkeit. NeemAzal-T und NeemAzal-T/S unterschieden sich nicht in ihrer Wirksamkeit gegen *A. proletella* bei einem Feldversuch. Die Applikation vier Wochen nach dem Aussäen führte zu in deutlichen Effekten mit einer signifikant niedrigeren Anzahl von Weißen Fliegen auf behandelten Pflanzen.

Im zweiten Teil dieser Arbeit wurden die Auswirkungen von organischen Bodenbestandteilen auf die Wirksamkeit und Dosis-Wirkung sowie die Persistenz von Azadirachtin nach Substratapplikationen untersucht. Die beiden Produkte für Bodenapplikationen, NeemAzal-T und NeemAzal Granulat, wurden anhand der Wirksamkeit gegen *Aleyrodes proletella* auf Rosenkohl und *Trialeurodes vaporariorum* auf Tomate bewertet. Die Pflanzen wurden in zwei Substraten gezogen: ein kommerziell erhältliches Substrat (CS), das aus 15 % Humus, 35 % Ton und 50 % Torf bestand, und ein Substrat mit einem geringeren Anteil von organischer Substanz, nämlich einem Gemisch von kommerziell erhältlichem Substrat und Sand (CS+Sand) im Verhältnis 1:1. Für die Applikation wurden NeemAzal Granulat und die Substrate zu folgenden Konzentrationen gemischt: 75 mg/kg (= 5,25 mg AZA/kg), 150 mg/kg (= 10,5 mg AZA/kg) und 300 mg/kg (= 21,0 mg AZA/kg) pro Kilogramm Substrat. NeemAzal-T wurde mit folgenden Volumina angegossen: 1 mL/kg (= 10 mg AZA/kg), 1,5 mL/kg (= 15 mg AZA/kg), 2 mL/kg (= 20 mg AZA/kg). Um die Restwirkung und Persistenz von Azadirachtin zu untersuchen, wurden die

Behandlungen 0, 5 und 10 Tage vor der Zugabe von Weißen Fliegen durchgeführt. Die Wirkung von Azadirachtin war dabei abhängig von der Dosis, wobei die höchsten Dosen von NeemAzal Granulat (21 mg AZA/kg) und NeemAzal-T (20 mg AZA/kg) bis zu 100 % Mortalität bei Larvalstadien von Weißen Fliegen verursachten. Im Vergleich zu dem unvermischten Substrat führten NeemAzal-Formulierungen bei dem CS+Sand Gemisch zu signifikant höherer Mortalität bei Larvalstadien von beiden Weißen Fliegen-Arten. Diese Ergebnisse zeigen, dass der Anteil von organischer Bodensubstanz die Wirksamkeit von Azadirachtin beeinflusst. Die Persistenz der NeemAzal-Formulierungen wurde nicht durch den Substratart beeinflusst, sondern eher durch das Alter der Rückstände, denn die Wirksamkeit gegen Weißen Fliegen war nach 10 Tagen signifikant reduziert.

Im dritten Teil wurde die Wirksamkeit, Dosis-Wirkung und Auswirkungen auf die Fekundität von Neemextrakten auf die Blattlausart *Brevicoryne brassicae* untersucht. Die Effekte von vier NeemAzal Granulat Dosisstufen (75, 150, 225 und 300 mg/kg Substrat) und NeemAzal-T (1, 1,5, 2 und 2,5 mL/kg Substrat) wurden untersucht. Die Wirksamkeit der Neem-Formulierungen war dosisabhängig, wobei die höchsten Dosen von NeemAzal Granulat und NeemAzal-T bei den Blattläusen 14 Tage nach der Behandlung zu einer Überlebensrate von bis zu 0 % führte. Darüber hinaus wurde die Persistenz und Restwirkung von Azadirachtin gegenüber der Mehligen Kohlblattlaus mit Dosen nach Herstellerempfehlung (150 mg NeemAzal Granulat bzw. 1 mL NeemAzal-T pro kg Substrat) ermittelt. Nach der Behandlung wurde Rosenkohl mit einen Tag alten Blattlauslarven am selben Tag (D0), nach vier Tagen (D4) und nach acht Tagen (D8) infestiert. Die Persistenz von NeemAzal-T war gering. Es gab keine Unterschiede bei der Überlebensrate von Aphiden zwischen Kontrollpflanzen und mit NeemAzal-T behandelten Pflanzen die acht Tage vor der Infestierung behandelt wurden, jedoch waren NeemAzal Granulat zu diesem Zeitpunkt noch

wirksam. Azadirachtin verursachte einen signifikanten Rückgang bei der Anzahl Nachkommen pro Weibchen und Tag ebenfalls abhängig von der Dosis. Selbst wenn die Blattläuse auf behandelten Pflanzen überlebten, wurde die Reproduktion durch Azadirachtin deutlich verringert.

Zusammengefasst zeigt diese Arbeit die hohe Wirksamkeit von systemisch appliziertem Azadirachtin gegen Weiße Fliegen und die Mehlige Kohlblattlaus. Zudem zeigte sich, dass die Formulierungsart sowohl bei der Wirksamkeit als auch bei der Persistenz eine entscheidende Rolle spielt. NeemAzal Granulat erwies sich als wirksamste Formulierung, die von biologisch wirtschaftenden Anbauern, relativ unabhängig davon welches Anbausubstrat sie nutzen, verwendet werden kann. Das Azadirachtin wird langsam aus dem Granulat freigesetzt, von den Wurzeln aufgenommen und akropetal zu den Saugorten der Insekten transloziert, was zu einer schnellen Wirksamkeit und langen Persistenz führt, so dass ein effizienter Pflanzenschutz möglich ist. Die Nutzung von NeemAzal durch Bodenapplikationen ist daher eine vielversprechendes IPM Maßnahme gegen diese Schädlinge.

Schlüsselwörter: Azadirachtin, Neem, Weiße Fliegen, Blattläuse, Wirksamkeit, Persistenz

ABBREVIATIONS

a.i.	Active ingredient
AZA	Azadirachtin
CS	Commercial substrate, Fruhstorfer Erde
CS-sand	Commercial substrate-sand mixture
GLM	Generalized linear models
L:D	Light: darkness photoperiod
OM	Organic matter
WF	Whiteflies
SE	Standard error

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CHAPTER 1

GENERAL INTRODUCTION

Neem and the approach of soil application

Neem products are derived from the neem tree, *Azadirachta indica*. The neem tree is native to southern Asia and can grow in most arid sub-tropical and tropical areas of the world (Copping and Menn, 2000). The tree belongs to the order Rutales and the family Meliaceae. It is a fast-growing tree, generally 15–20 m tall (sometimes up to 40 m tall), with a crown diameter up to 20 meters (Chamberlain et al., 2000). Several biologically active compounds have been isolated from different parts of neem trees. These include azadirachtin, salannin, nimbin, ammonia formaldehyde, phenols, fatty acids and tannins (Koul et al., 1990; Baidoo and Adam, 2012). Azadirachtin is thought to be the most bioactive ingredient with the highest concentration in the seeds (Thacker, 2002; Brunherotto et al., 2010; Egwurube et al., 2010; Metspalu et al., 2010).

As a plant protection product, Azadirachtin is known to be broad spectrum in its mode of action against many insects orders including Orthoptera, Lepidoptera, Coleoptera, Diptera and Hemiptera (Schmutterer, 1990; Isman, 2006; Siddiqui et al., 2009; Degri et al., 2013; Shannag, et al., 2014; Mondédji et al., 2014). It acts as a growth regulator by preventing insects from molting by inhibiting production of insect hormone ecdysone (Weinzierl and Henn, 1991). Moreover, it has been shown to have anti- feedant and oviposition deterrent properties (Abou-Fahkr Hammad et al., 2001; Hilje, et al., 2003; Kumar et al., 2005; Kumar and Poehling, 2007; Wen et al., 2009).

The magnitude of these effects would of course be determined by, among other factors, concentration of azadirachtin (Jaglan and Khokhar, 1997), formulation of the active principle

(Stark and Walter, 1995; Daly, 2004), application method (Kumar and Poehling, 2006; Kumar et al., 2008; Li et al., 2009) and the target species (Maredia, et al., 1992; Lowery and Isman, 1994).

Most of the commercial Neem products in the market are formulated for foliar spray application. Despite evidence of translaminar translocation and high efficacy when in direct contact with the target organism, their duration of activity, hence efficiency and persistence, is influenced by abiotic factors and environmental conditions (Kumar and Poehling, 2006).
In particular, Azadirachtin, the bioactive component against insects, is rapidly degraded under high temperatures and UV light. Frequent application would therefore be necessary especially under field conditions, to ensure sufficient pest control. Furthermore, the foliar treatments may cause residual and topical exposure of natural enemies in the crop canopy and cause toxic effects to sensitive species (Arnó and Gabarra, 2011; Biondi et al., 2012; Gontijo et al., 2014).

Application of neem products to the soil for root uptake and subsequent systemic distribution to the insect feeding sites would therefore be more desirable to eliminate such limitations.
However, most commercial neem products contain high amounts of lipophilic compounds such as vegetable oil to achieve a complete distribution on the hydrophilc leaf surfaces and facilitate uptake in the leaf via the cuticula. The sensitive and not cuticula protected roothair however could be agglutinated or even destroyed by such compounds as well as the importand microorganism community in the rhizosphere although azadirachtin by itself has been shown to have no or even synergistic effect on some soil microorganisms (Gopal et al., 2007; Spyrou et al., 2009). Formulations that provide high quantity and long-term supply for uptake of AZA into the root system without detrimental side effects are key demands and soil

application would minimize direct toxicity to natural enemies, thus allow their use in IPM strategies.

In the view of these challenges, special soil treatment formulations have been developed which are hydrophilic. A first test compound was NeemAzal®-U, (17% azadirachtin), which resulted in strong systemic effects against different life stages of *Liriomyza sativae* Blanchard on *L. esculentum* (Hossain et al., 2008) and *Bemisia tabaci* (Kumar and Poehling, 2006). The authors attributed the efficacy of these treatments to uptake of AZA by the intact root system. Follow-up compounds developed for use in hydroponic cultures or for soil treatments by Trifolio GmbH, Lahnau, Germany were NeemAzal T, which is a water - based liquid formulation, and NeemAzal granules, a solid formulation, consisting of AZA blended with a solid and inert carrier material which enhances continuous release and uptake through the root (Daly, 2004, Farah, 2009).

Granular solid formulations, in particular, could ensure longer periods of crop protection through a slow-release process of "piece by piece" solubilized Azadirachtin, when the carrier is progressively penetrated by the surrounding water. Typically, granules are formulated with with 70 - 98% carrier material, 2-30 % pesticide, 0-10% solvent or binder and 0-7% deactivator (Kalley et al., 1992; Goss et al., 1994). This study therefore, aimed at evaluating efficacy of different neem formulations, focusing on the soil treatments, in the control of the common greenhouse whitefly, *Trialeurodes vaporariorum* (West) on tomato and the Cabbage whiteflies, *Aleurodes proletella* (L) and the cabbage aphid, *Brevicoryne brassicae* (L) on Brussels sprouts. Whiteflies and aphids seem to be quite convenient pests in this regards since they intensively suck up assimilate and could easily ingest high amounts of systemically translocated AZA.

The selected pest – plants systems

Whiteflies, *T. vaporariorum* are important polyphagous pest of various crops (Byrne and Bellows, 1991; Johnson et al., 1992). Adults and larvae cause damage to plants by sucking the phloem sap, which encourages the growth of sooty molds on leaves, and transmitting some plant viruses (Coffin and Coutts 1995; Guzman et al., 1997; Mellor and Anderson, 1995; Jones, 2003; Laznik et al., 2011). Over reliance on persistent synthetic pesticides for control of these pests creates the risk of selecting resistant populations (Cahill et al., 2009; Springate and Colvin, 2012; Liang et al., 2012) and increased risk of higher residue levels. Moreover synthetic pesticides are completely banned for organic farming approaches that are of increasing socio-economic importance. Other important interventions in whiteflies control include use of natural enemies. Though a certain degree of success has been reported (Berndt and Meyhöfer, 2007; Messelink et al., 2008), use of predators and parasitoids may not be sufficient since their efficacy is not constant and reliable, if a broader range of crops is considered. Alternatively, use of biopesticides such as neem, which have very low human toxicity and persistence and satisfies the increasing consumer demand for insecticide residue-free produce (Byrne et al., 1992; Harris and Burress, 2000) could be promising. Neem products have been successfully used for integrated control of pests (Schmutterer 1990), and has been shown to be effective in controlling whiteflies (Coudriet et al., 1985; El Shaffe and Basedow, 2003). With development of the new neem formulations, a detailed study of persistence and efficacy under different application methods would help in choosing the optimal method and dose level of these products to higher level of reliability of neem biopesticides in management of whiteflies and aphids.

Cabbage whitefly, *A. proletella* is present on host plants in the infested areas throughout the year, has a wide range of host among the *Brassiceae* and is one of the most important

Brassica pests in Europe and particularly in Germany (Saucke et al., 2011; Springate and Colvin, 2012). It causes both direct damage by sucking phloem sap, which affects growth and yield of the cabbage crops and indirect damage by honeydew excretion. Wax and exuvia from whiteflies, provides substrate for the growth of sooty mould fungi and a sticky layer on the plant surface (Ramsey and Ellis, 1996). Such impurities can drastically reduce crop quality and cause additional costs for cleaning. Biocontol of this pest using parasitoid *Encarsia tricolor (*Foerster), is in general still not sufficient to keep the pest population below economical threshold levels (Zhang and Hassan, 2003; Saucke et al., 2011). It was therefore the aim of this study to evaluate Azadirachtin, in the afore mentioned soil -applied formulations in order to acquire a more intricate analysis of the potential use of neem soil application for the control of cabbage whiteflies in the greenhouse as well as in an open field.

Likewise, the cabbage aphid, *B. brassicae*, is also a phloem feeder and is one of the major pests of cabbage worldwide. Significant losses are associated with aphid infestation not just from direct feeding but also indirectly through accumulations of the exuviae, honeydew and the sooty mould that grows on honeydew (Griffin and Williamson, 2012; Opfer and McGrath, 2013). Damage and yield loss can reach up to 70 – 80% (Costello and Altieri, 1995; Wrzodak, 2009). It may attack the crop at any stage (Elwakil and Mossler, 2013). *B. brassicae* is native to Europe but has a worldwide distribution. Moreover they also vector virus of Cruciferae (Alford, 2005). *B. brassicae* can be controlled by natural enemies such as the parasitoid *Diaeretiella rapae* or by syrphids like *Episyrphus balteatus* however these natural enemies are not easy to handle and the efficacy is not consistent. A reliable integrated control system could be based both natural enemies and selective biopesticides as a fast “task force”.

These studies therefore were aimed at providing baseline information on efficacy of liquid formulation of NeemAzal without oil and the granular formulation, against economically important pests of vegetables which could be adopted for integrated control systems in organic farming.

CHAPTER 2

EFFICACY AND PERSISTENCE OF SYSTEMIC SLOW - RELEASE NEEM FORMULATIONS IN THE CONTROL OF WHITEFLIES, *ALEYRODES PROLETELLA* AND *TRIALEURODES VAPORARIORUM*

Abstract

The aim of this study was to evaluate the efficacy and persistence (residual effect) of different formulations of NeemAzal for agricultural pest control, comparing foliar with root applications. Currently, most of the registered neem products are formulated for foliar spray applications, with high concentrations of oils and are prone to decomposition under light (photodegradation) and heat. Soil application with uptake of active ingredients by the root systems could preclude such negative effects, hence providing higher levels of efficacy and persistence.

The effects of aqueous solution without surfactants, granules formed from powdered NeemAzal with an inert carrier, and spray applications were compared for the control of *Aleyrodes proletella* L. on cabbage and *Trialeurodes vaporariorum* West on tomato. The treatments were done 0, 3, 5, 7, and 14 days before the plants were exposed to adult whiteflies. All basic experiments were conducted in a greenhouse. The results showed that, while efficacy of foliar treatment decreased significantly over time, soil application treatments proved significantly more persistent, particularly the granular formulation, which provided reliable efficacy for 14 days.

In addition a first field experiment was performed in a cabbage field focusing on the control of *A. proletella* and using liquid formulations of NeemAzal. Significantly lower number of adults and immatures were recorded in treated plants compared to the untreated plants. By

the end of week ten, the number of immatures on untreated plots was approximately four times lower than that of NeemAzal treated plots.

In conclusion results show that soil applications of NeemAzal can efficiently control immature stages of *A. proletella* and *T. vaporariorum* on cabbage and tomato respectively. Fast efficacy, long persistence, no damage by formulation compounds to the sensitive rhizosphere, no risk of direct interference with natural enemies indicates systemic neem application could be a promising IPM tool in the management of both greenhouse and cabbage whiteflies.

Key words: Neem; Azadirachtin efficacy; persistence; *A. proletella*; *T. vaporariorum*

2.1 Introduction

Whiteflies are among the most significant pests worldwide. In central Europe, species like *T. vaporariorum* and *Bemisia tabaci* Gennadius cause severe problems for greenhouse cultivation as well as field crops such as cabbage (Ramsey and Ellis, 1996; Menke and Gerhard, 2010). Whiteflies such as *A. proletella* have become serious pests in recent years (Springate and Colvin, 2012). They cause direct damage by sucking phloem sap, resulting in withdrawal of assimilates, which affects both growth and yield of infested crops. Often more important is the indirect damage caused by honeydew excretion, which provides a substrate for sooty mold fungi (Ramsey and Ellis, 1996). This inhibits photosynthetic efficacy by covering the plant surface with a sticky layer of wax deposition, trapping remains of whiteflies, including exuvia and pupal cases (Van Lenteren et al., 1995; Ramsey and Ellis, 1996)¨ Such impurities reduce the quality of the crop and cause additional costs for cleaning.

Control of whiteflies with synthetic pesticides is efficient only with systemic and more or less persistent pesticides, which creates the risk of selecting resistant populations (Cahill et al., 2009; Springate and Colvin, 2012; Liang et al., 2012). Alternatively, an important option in whitefly management is biocontrol, which satisfies the increasing consumer demand for insecticide residue-free produce (Byrne et al., 1992; Harris and Burress, 2000). In the case of *T. vaporariorum* on several vegetable crops under protected cultivation, biocontrol with parasitoids such as *Encarsia formosa* (Gahan) and predators like *Amblyseius swirskii* (Athias-Henriot) and *Euseius ovalis* (Evans) has been shown to be quite successful (Berndt and Meyhöfer, 2007; Messelink et al., 2008). However, efficacy is not constant and reliable if a broader range of crops is considered. Berndt and Meyhöfer (2007) for instance, reported the ineffectiveness of a curative release of *E. formosa* as the only antagonist to control *T.*

vaporariorum in cut Gerbera. More significant is the situation with *A. proletella*, where the efficacy of, *Encarsia tricolor* (Foerster), is in general still not sufficient to keep the pest population below economical threshold levels (Zhang and Hassan, 2003; Saucke et al., 2011). There is therefore a growing interest for improving biocontrol efficacy in a sustainable and selective manner. Pesticides of natural origin "biopesticides" are an option because of their low human toxicity, low persistence and pronounced selectivity concerning non-target organism if beneficials in integrated control strategies are considered (Sundaram, 1996; Schmutterer, 1997; Biondi et al., 2012a). Moreover, their potential to control pesticide resistant populations is high because of the different and multiple mechanism of action. Bio-pesticides are suitable to be used in biological or ecological production systems (Wen et al., 2009; Menke and Gerhard, 2010). In this regard, neem products containing the biologically active compound azadirachtin, which is derived from the Neem tree *Azadirachta indica* A. Juss (Meliaceae), are promising candidates. Research indicates that azadirachtin, an antifeedant and growth regulator for a wide variety of insects, delays and prevents moulting, reduces growth and development, and can cause significant mortality in whitefly immatures (Hilje et al., 2003; Santos and Costa, 2004; Kumar and Poehling, 2007; Wen et al., 2009). Furthermore, both foliar and systemic applications of azadirachtin have been shown to deter oviposition of whiteflies (Coudriet, et al., 1985; Nisbet et al., 1993; Kumar, et al., 2005; Kumar and Poehling, 2007; Wen, et al. 2009).

Most of the neem products in the market today such as NeemAzal (AZA) T/S® (Trifolio GmbH, Lahnau, Germany) are formulated for foliar application (BBA, 1999). Though effective, they show rapid photodegradation with significant loss in bioactivity when exposed to light, particularly short wavelength light, such as blue and UV. The rapid photodegradation can limit the timespan of bioactivity considerably (Barnby, et al., 1989;

Johnson et al., 2003). Moreover, the foliar treatments may cause residual and topical exposure of natural enemies in the crop canopy and cause toxic effects to sensitive species (Feldhege and Schmutterer, 1993; Arnó and Gabarra, 2011; Biondi et al., 2012b; Gontijo et al., 2014). Application of neem products to the soil and subsequent systemic distribution, as was reported for thrips control by Thoeming et al. (2003), could help to overcome these problems. However, this would require formulations that provide high quantity and long-term supply for uptake of AZA into the root system without detrimental side effects. These negative side effects can be caused by the lipophilic formulation compounds of the products designed for leaf treatments to improve distribution on leaf surfaces and loco-systemic uptake. On the other hand, azadirachtin is a water-soluble compound. Applied to the soil, rapid leaching can cause both the risk of high losses of active ingredient from the rhizosphere and a need for high dosages for effective treatments (Kleeberg, 2001; Daly, 2004).

Formulating azadirachtin as a granular slow-release product could be an option to achieve continuous availability of AZA in the rhizosphere, as well as maintaining low detrimental effects of the formulation ingredients on the sensitive root hairs and low risk of leaching. Hence, this study evaluated the efficacy, persistence and residual effect of "special soil formulations" of NeemAzal. A water-based drenching solution and a solid granular formulation were compared to a foliar spray application for the control of *A. proletella* on Brussels sprouts and *T. vaporariorum* on tomato. Basic comparison with controlled measurement of whitefly performance in different stages of all compounds and both species was performed on potted plants in a greenhouse whereas a first population experiment in the field was restricted to testing the liquid formulations against the cabbage whitefly.

2.2 Materials and Methods

2.2.1 Neem formulations and treatments

Three types of neem products, granules made of hydrophilic carrier material containing 7% azadirachtin (AZA), a water based formulation NeemAzal-T (1% AZA), and the commercial product formulated with oil for foliar treatments NeemAzal-T/S® (1% AZA), all delivered from Trifolio M GmbH, Lahnau, Germany, were used. Water and/or blank formulation served as controls. For application in the greenhouse using potted plants, 150mg/kg (=10.5 mg AZA/kg) granules of NeemAzal were mixed with the growing substrate (Fruhstorfer Erde® Type P). NeemAzal-T was drenched to the plant substrate at a rate of 1ml/kg (=10mg AZA/kg). For the foliar treatment, NeemAzal-T/S® was diluted with water to a treatment concentration of 0.5% (10mg AZA/kg) and sprayed to the plant canopy until run-off.

In the field, two formulations were tested: NeemAzal-T was drenched at a rate of 10ml/m^2. For application in each plot, 60ml NeemAzal-T were diluted with 18liters of water and drenched a few centimeters around the base of the plants. The second neem tretment was NeemAzal-T/S, which was sprayed at the same rate as in the greenhouse. Spraying was done to the crop canopy using a knapsack sprayer until run-off. Water was used as a control.

2.2.2 Plants and insects

In the greenhouse experiments two species of whiteflies were used: the common greenhouse whitefly, *T. vaporariorum*, maintained on tomatoes; and the cabbage whitefly, *A. proletella*., maintained on Brussels sprouts. Both insect species were taken from stock cultures on potted plants kept in insect-proof cages in a greenhouse. For synchronization, female whiteflies were allowed to lay eggs on plants in the cages for 24 hours and then removed using an

aspirator. Plants with same aged eggs were kept separately until synchronized emergence of adults (F1).

Tomato seedlings (*Lycopersicon esculentum*), var. Hildares were pre-germinated for 3 days and Brussels sprouts (*Brassica oleracea*) var. Gemmifera certified seeds were planted in plastic seedling trays (50 × 30 × 6.5cm). Seedlings were grown for 2 weeks under greenhouse conditions of 23 ± 2°C and 65–75% RH with an 18:6 h light:darkness photoperiod (L:D). Thereafter they were transplanted into plastic pots (13 × 7.5 × 8.5 cm) and further kept under greenhouse conditions. Fruhstorfer Erde® Type P; composed of humus, clay, and peat (15, 35, and 50%) served as standard culture substrate. Brussels sprouts were used in the field experiment

2.2.3 Experiments

Separate experiments were conducted for each whitefly species on the respective host plant. Four experiments were conducted in these studies; three in the greenhouse and one in an open field.

Experiment 1: Efficacy of different neem products against *A. proletella* and *T. vaporariorum*.

To evaluate the efficacy of soil-applied and the foliar neem formulations against immature stages of *A. proletella,* 48 (12 plants for each of the treatments A-D, see below) eight weeks old Brussels sprouts plants in plastic pots were used. One well developed middle leaf per plant was chosen for infestation with whiteflies, and five *A. proletella* females were placed on the underside of each of these leaves using clip cages for 24 hours of egg laying.

After removal of adults, the plants (12 each treatment) were (A) treated at the substrate level with NeemAzal-T at 1 ml/kg of soil (10 mg Azadirachtin), (B) treated with NeemAzal granules at 150 mg/kg (10.5 mg Azadirachtin) of soil, by mixing the granules into the upper soil layer (C) treated with NeemAzal-T/S at 0.5% (10 mg Azadirachtin) sprayed to the plant canopy until run-off using a hand-held sprayer and (D) treated with water as control.

Pots treated with different neem formulations were randomly arranged on two adjacent tables in a greenhouse. The number of eggs per each leaf cage (replicate) was recorded, and subsequent development stages were monitored by counting the number of larvae that hatched from the eggs, the number of pupae developing from surviving larvae, and the number of emerging adults for each single plant.

The same setup was repeated for *T. vaporariorum* with again 48 tomato plants (12 replicates each treatment).

Experiment 2: Effect of neem formulations on whitefly oviposition repellence

In this experiment we evaluated the effect of three neem formulations on egg deposition by two species of whiteflies; *A. proletella* and *T. vaporariorum*.

For the set with *A. proletella,* 144 8-week-old Brussels sprouts plants were planted in plastic pots. Randomly selected subsets of 36 plants were treated with NeemAzal-T, NeemAzal granules, NeemAzal-T/S and water as described above.

Eight plants, two plants from each treatment, were arranged in a random manner in insect proof cages placed inside a greenhouse. Approximately 150 whitefly females were released per cage on the same day, three hours after treatment (D0), after three days (D3), or five days after treatment (D5). The adults were left in the cages for 24 hours to lay eggs, and then

removed carefully by means of an aspirator. The total number of eggs deposited was recorded per treatment. Each cage was considered as a replicate; there were 6 cages for each treatment day.

Similar set up was also used for *T. vaporariorum* with 144 tomato plants

Experiment 3: Persistence effect of neem formulations on whiteflies

This experiment aimed to assess the persistence of the different neem formulations over time. 72 Brussels sprouts plants were grown in a greenhouse (see conditions above).

Randomly selected subsets of 24 plants, six per treatment, were treated with (A) NeemAzal-T, (B) NeemAzal granules, (C) NeemAzal-T/S and (D) water as described above.

In a first trial, five same-aged whitefly females were introduced, as in experiment 1, on single leaves on the plants, three hours after treatment at (D0), three days (D3) and five days (D5) after treatment. In a second experiment, the introduction of whiteflies was performed at D0, D7 and D14 after treatment. The adults were allowed to lay eggs for 24 hours and then removed from the plants. Development of whiteflies was monitored until emergence of adults (see experiment 1). For each of the day variants, treated plants were randomly arranged on a greenhouse bench.

Experiment 4: Efficacy of neem formulations in the field

The experiment was conducted on a field of the department of Phytomedicine experimental site (N 52°23, E 9° 42), Hannover, Germany. A plot measuring 13m x 13m was demarcated. The plot was divided into four rows. Each row had three plots each measuring 2m by 3m and plots were surrounded by a 1m walking path. In each plot 35 eight-weeks old Brussels sprouts plants (*Brassica oleracea* var. gemmifera, variety “Genius”), arranged in five rows of

seven plants were planted in summer 2013. Plants were fertilized with NPK "Blaukorn" (12/12/17; dosage 40 g m^{-2}; COMPO GmbH, Münster, Germany). All other agronomic practices like weed control and irrigation were carried out according to practice orientated standards.

In each row, treatments were randomly allocated to the plots. Treatment application was done every two weeks from week 4 onwards, three treatments in total. Ten randomly chosen plants were marked per plot and each week the number of pest insects (adults, and immature stages per plant), was counted for total of 10 weeks. To avoid edge effect, data collection was done only in the inner rows of the plots.

Statistical analysis

Larval and pupal mortalities of *A. proletella* and *T. vaporariorum* were analyzed using binomial generalized linear models (GLMs) with logit link function and overdispersion ("quasi-binomial") (McCullagh and Nelder 1989). Larval mortality was calculated as the proportion of emerging pupae, based on the number of hatched eggs. Similarly, mortality at the pupae stage was calculated as the proportion of emerging adults, based on the number of larvae that pupated.

For experiment 1 separate quasi-binomial GLMs were fitted for the different species, developmental stages, and formulations. The models included formulations and replication as the dependent factors. Statistical significance of factors was assessed with analysis of deviance F-tests. Formulations were significant ($p<0.05$), hence their means were separated using Tukey-type tests controlling the rate of type I errors at 5%.

The number of eggs in experiment 2 was modelled using quasi-Poisson GLM with formulation and days as independent factors, separately for species. Interaction terms of the factors were also included. Analysis of deviance F-tests showed that days were significant ($p<0.05$) in all cases, so subsequent Tukey-type comparisons were carried out at the usual 5% error rate Pairwise comparisons of formulations were done for each day.

Similarly, for experiment 3 quasi-binomial GLMs models were fitted with formulation, days, and replication as independent factors, separately for species and developmental stages. Interaction terms of all factors except replication were also included. Analysis of deviance F-tests showed that days were significant ($p<0.05$) in all cases, so subsequent Tukey-type comparisons were carried out at the usual 5% error rate.

For the field experiment (experiment 4), the total numbers of whitefly immatures and adults was analyzed by GLM with log-link and quasi-Poisson assumption, with models fitted for each monitoring week separately and Tukey-type comparisons between treatments at each week were carried out at 5% error rate

2.3 Results

2.3.1 Efficacy of neem products against whiteflies

The formulation type had a highly significant effect on the efficacy of neem against *A. proletella*, as shown by the analysis of deviance F-tests: $F_{4,44} = 76.1$, $P < 0.001$ for larvae, $F_{3,44} = 70.8$, $P < 0.001$ for pupae (Fig. 1). Similarly, formulation effects on the immature mortality of *T. vaporariorum* were significant: $F_{3,44} = 37.9$, $P < 0.001$ for larvae, $F_{3,44} = 77.1$, $P < 0.001$ for pupae (Fig. 2).

Mortality of immature stages of both whitefly species in all neem treatments was highly significant ($P < 0.001$) compared to the control. The mortality in the control was always below 15%, whereas mortality resulting from neem treatment was constantly above 60%. There were, however, no differences in efficacies of the three neem formulations against immature stages of both *A. proletella* and *T. vaporariorum.*

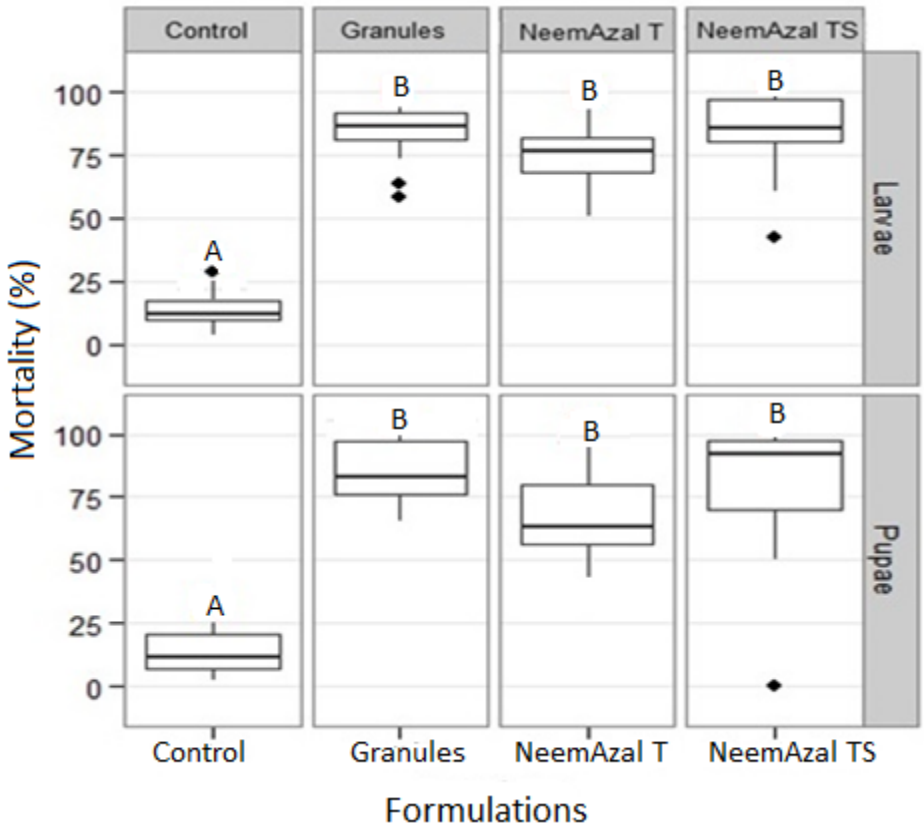

Figure 1: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Aleyrodes proletella* caused by different NeemAzal treatments (granules, NeemAzal-T and NeemAzal T/S - details see text). Different letters indicate significant differences among the treatments at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons). (In a box plot, the thick line shows the median and upper and lower boundaries show upper and lower quartiles, respectively)

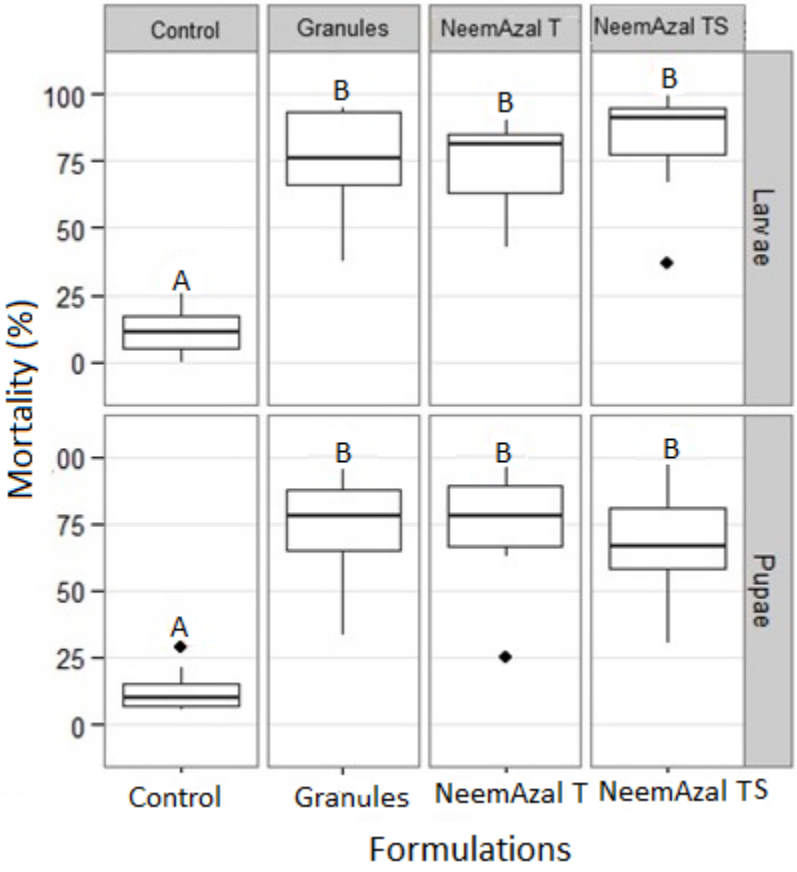

Figure 2: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Trialeurodes vaporariorum* caused by different NeemAzal treatments (granules, NeemAzal-T and NeemAzal T/S - details see text). Different letters indicate significant differences among the treatments at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons).

2.3.2 Effect of neem products on oviposition

There were significant effects of neem treatments on the oviposition of *A. proletella (*$F_{3,66}$ = 11.9, P < 0.001) (Fig. 3) and *T. vaporariorum* ($F_{3,66}$ = 4.8, P < 0.01) (Fig. 4). Overall, neem treatments significantly decreased oviposition by *A. proletella* across the days as compared with the control, in particular NeemAzal T/S when exposure of whiteflies was done same

day, D0 (P = 0.004) or D3 (P = 0.04) and for NeemAzal T (P = 0.001) and NeemAzal granules (P = 0.001) at day 5 after treatment. However, the number of eggs did not differ among the days, except NeemAzal granule at day 5 after treatment. Similarly, effects of treatments on egg deposition by *T. vaporariorum* were observed when plants' substrates were treated five days before being infested with whiteflies (D5) where NeemAzal T and NeemAzal granules had significantly lower number of eggs deposited compared to the control. Furthermore, we could observe for *T. vaporariorum* that oviposition tended to decrease, although not statistically significant (P = 0.07) at D5 in comparison to D0 with NeemAzal-T and granules but not with NeemAzal T/S.

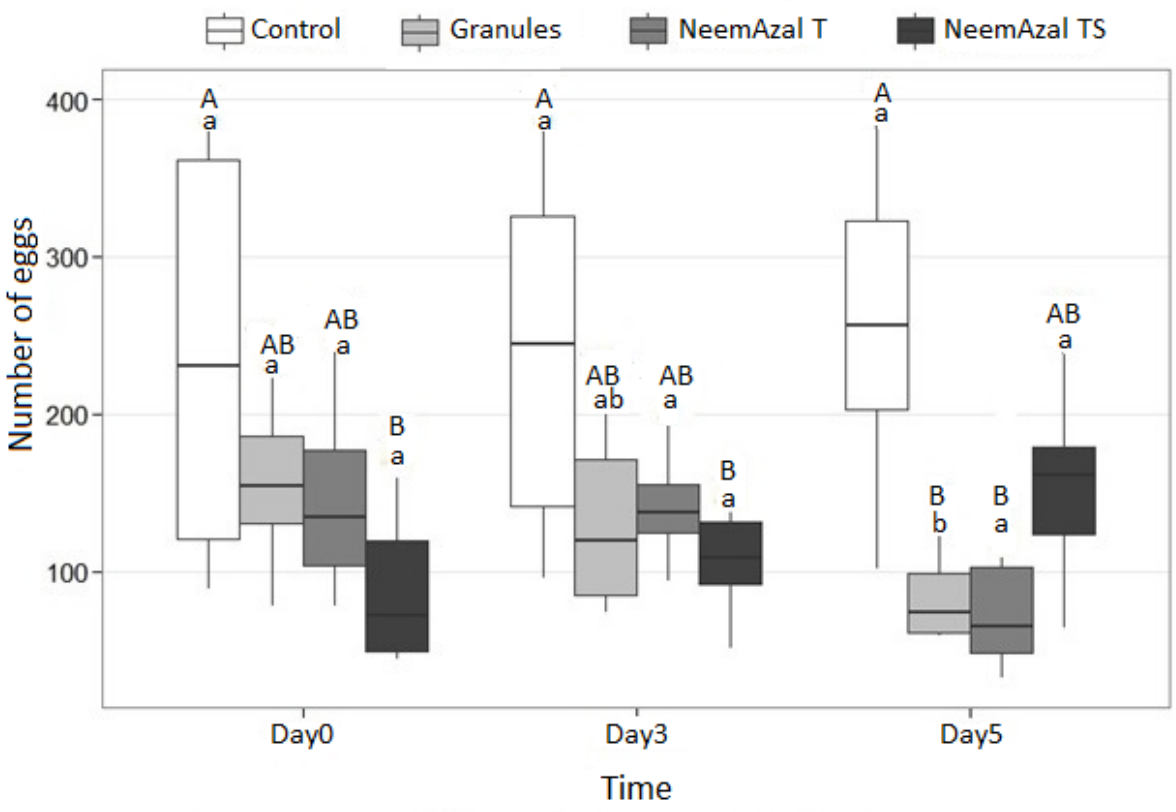

Figure 3: Boxplots of the number of eggs of *A. proletella* recorded on plants treated with different formulations of NeemAzal (NeemAzal granule, NeemAzal-T, NeemAzal T/S and control - details see text). Plants were infested with whiteflies at zero (D0), three (D3) or five

(D5), days after treatment. Different letters within each panel indicate significant differences among treatments (upper case). Significant differences among days are indicated with lower case letters at a multiple type I error level of 5% (quasi-poison GLM, Tukey's pairwise mean comparisons).

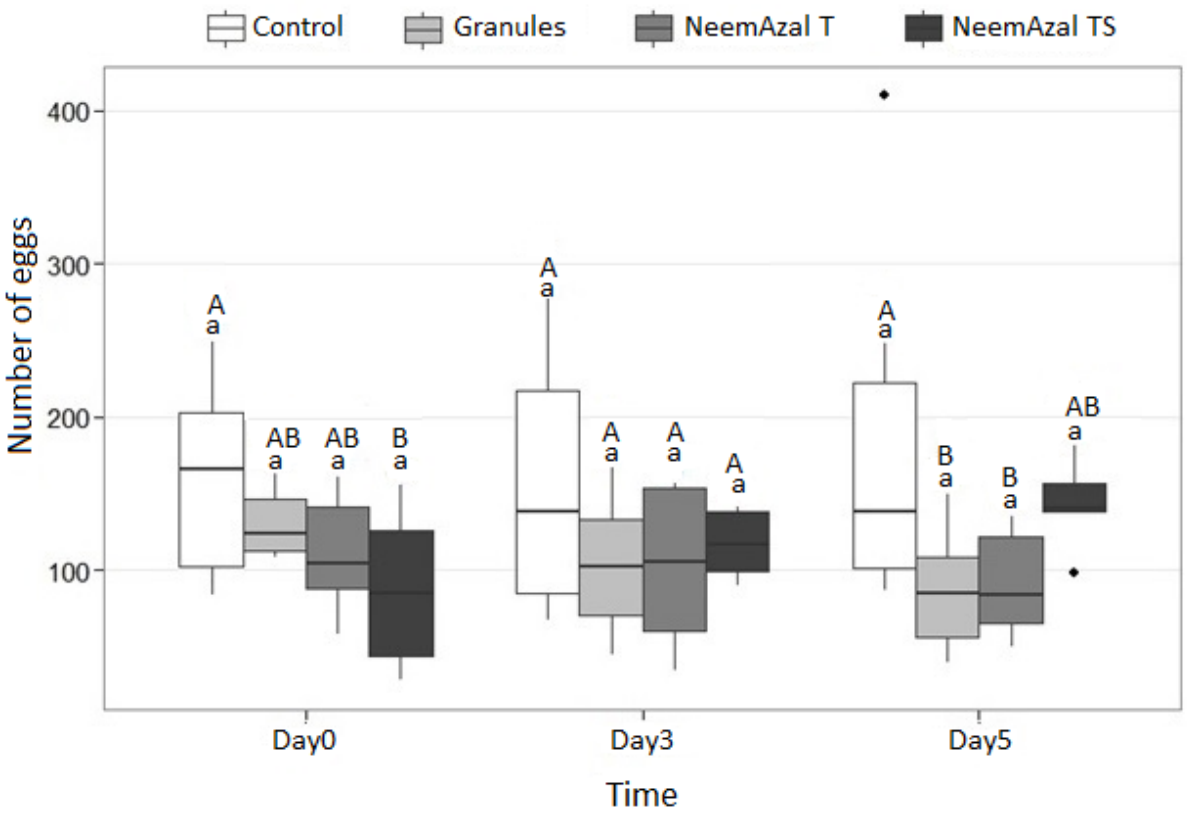

Figure 4: Boxplots of the number of eggs of *T. vaporariorum* recorded on plants treated with NeemAzal treatments (NeemAzal granule, NeemAzal-T, NeemAzal T/S and control - details see text). Plants were infested with whiteflies at zero (D0), three (D3) or five (D5), days after treatment. Different letters within each panel indicate significant differences among treatments (upper case). Significant differences among days are indicated with lower case letters at a multiple type I error level of 5% (quasi-poison GLM, Tukey's pairwise mean comparisons).

2.3.3 Persistence and residual effect of neem formulations on whiteflies

The results of the two trials did not differ, for *A. proletella* (P = 0.34 for larvae and P = 0.49 for pupae) and *T. vaporariorum* (P = 0.79 for larvae and P = 0.85 for pupae), thus the data was pooled and compared. The effects of time (days) were highly significant in the analysis of deviance F-tests (*A. proletella*: $F_{4,136} = 55.9$, $P < 0.001$ for larvae, $F_{4,136} = 25.8$, $P < 0.001$ for pupae; *T. vaporariorum*: $F_{4,136} = 36.5$, $P < 0.001$ for larvae, $F_{4,136} = 21.2$, $P < 0.001$ for pupae).

The efficacy of the two soil-applied neem formulations against *A. proletella* (Fig. 5) and *T. vaporariorum* (Fig.6) remained high and did not significantly change with time when plants were infested with whiteflies either 0 (D0), 3 (D3), 5 (D5) or 7 (D7) after neem application. However there was significant loss of efficacy when plants were infested with whiteflies 14 days (D14) after neem application as compared with D0 and D3.

On the other hand, there was evidently high loss of activity with the foliar formulation, indicated by significantly low immature mortality, when plants were infested with whiteflies 5 days (D5) after being sprayed compared with D0 and D3, for both whitefly species.

Soil treatments were more persistent than foliar treatments, attaining over 50% mortality up to 7 days after application for both whitefly species. As expected, foliar treatment was only effective when whiteflies were exposed to fresh residues.

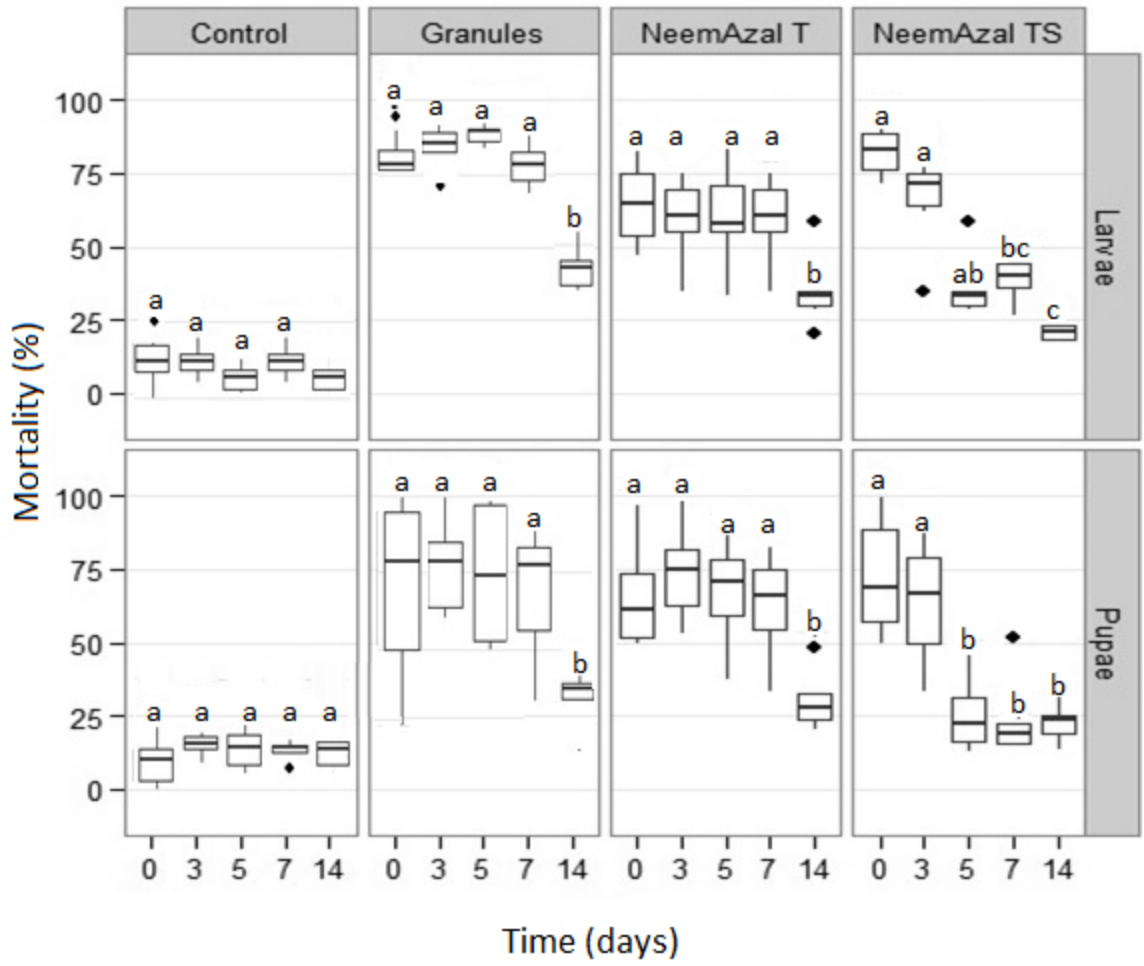

Figure 5: Boxplots of mortality (%) of immature stages (larvae and pupae) of *A. proletella* caused by three NeemAzal formulations at company recommended rates (granules: 10.5mg AZA per kg of soil; NeemAzal-T: 10mg AZA per kg of soil; NeemAzal T/S: 10mg AZA per kg of soil). Plants were infested with whiteflies at zero (D0), three (D3), five (D5), seven (D7) or fourteen (D14) days after treatment. Different letters within each panel indicate significant differences among the days at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons).

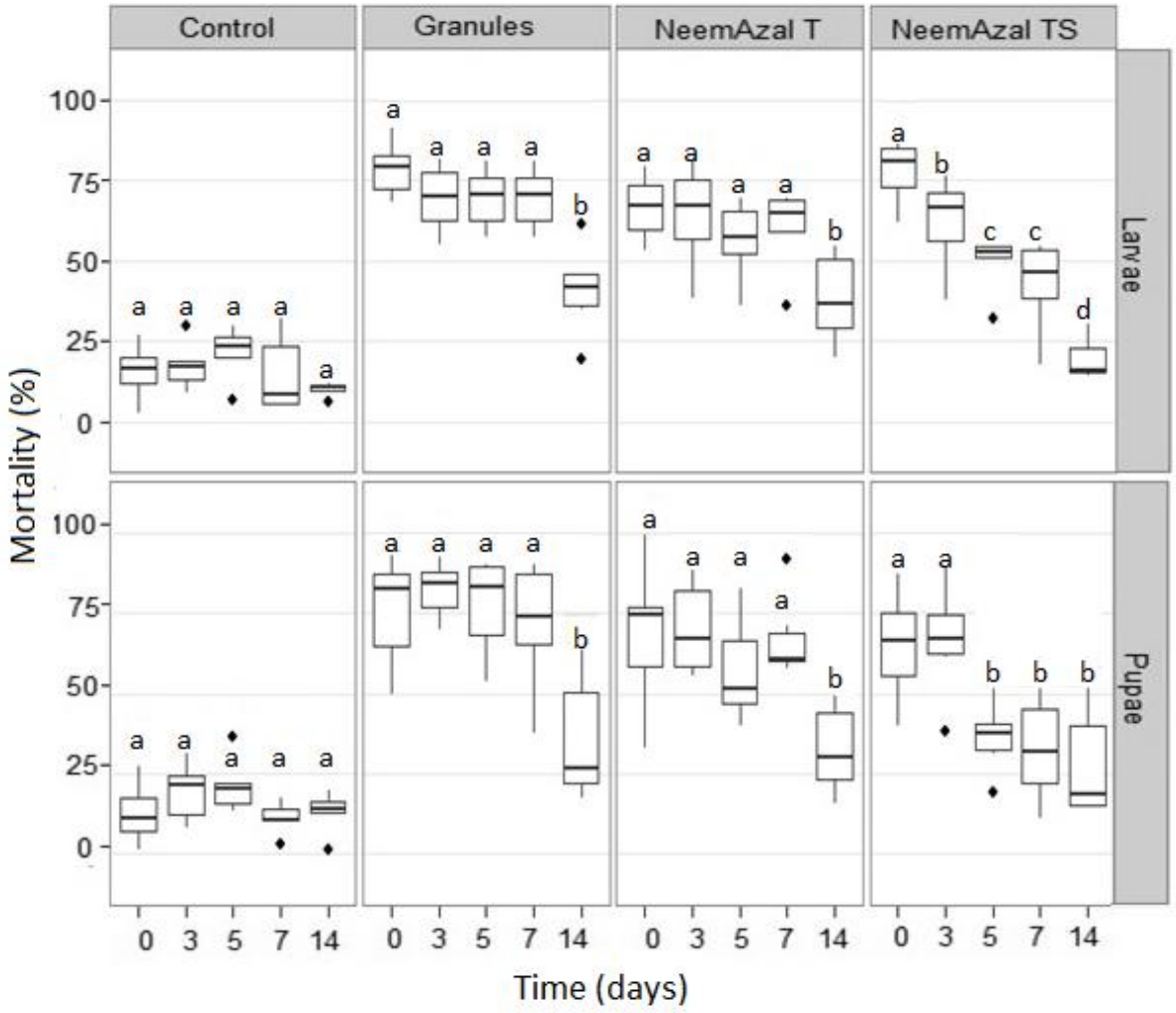

Figure 6: Boxplots of mortality (%) of immature stages (larvae and pupae) of *T. vaporariorum* caused by three NeemAzal formulations at company recommended rates (granules: 10.5mg AZA per kg of soil; NeemAzal-T: 10mg AZA per kg of soil; NeemAzal T/S: 10mg AZA per kg of soil). Plants were infested with whiteflies at zero (D0), three (D3) five (D5), seven (D7) or fourteen (D14) days after treatment. Different letters within each panel indicate significant differences among the days at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons).

Experiment 4: Efficacy of neem formulations in the field

The aim of this experiment was to asses the efficacy of two neemAzal formulations against *A. proletella* and *B. brassicae*, under open field condition. However the numbers of other insects for instance aphids were too low during the experimental period that the numbers in different treatments could not be evaluated and compared. Overall, the effects of neem treatments on both adults (Fig. 7) and immatures (Fig. 8) of *A. proletella* were highly significantly ($P < 0.001$).

The mean number of whiteflies on treated plants was significantly lower on week four, after the first treatment application. There was a steady, though slow increase in the number of whiteflies in the untreated plots, whereas after every treatment, we observed a reduction in population in the treated plots. Furthermore, the two NeemAzal treatments did not differe but both significantly differed from the control. For instance, there was a significantly lower population of adults on week 6, 8 and 10 on treated plots compared to control (Fig. 7).

Similarly, the population of immature stages (both larvae and pupae) was significantly lowered by the tretaments and we observed almost a population crash after treatmant in week four, from 114±23.3 (mean ± SE) immatures per plant to 33.±7.7 in week five under NeemAzal T/S treatment. By the end of week ten, the number of immatures on untreated plots was approximetly four times higher than that of NeemAzal treated plots. In general, the highest number of immatures recorded in treated plots was 165 ± 21.8 in week eight. On the same week, the number of immatures in the control plots was 330.6 ± 19.3. (Fig. 8)

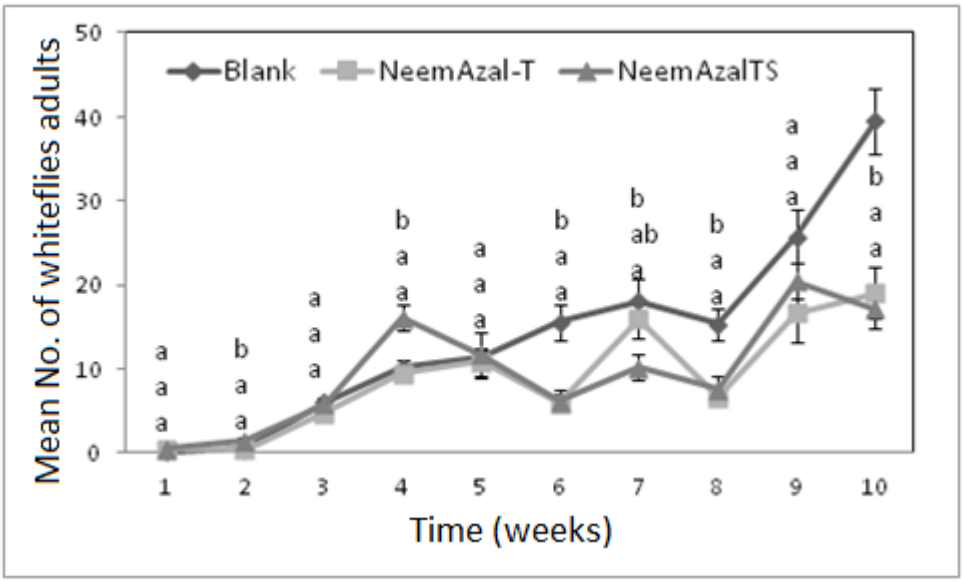

Figure 7: Mean ± SE number of *A. proletella* per plant, on Brussels sprouts planted on an open field during summer 2013 and treated with two neem formulations (NeemAzal T/S and NeemAzal T). Different letters above the line show significant difference between the treatment and control at each monitoring week (quasi-Poisson GLM, Tukey's pairwise mean comparisons).

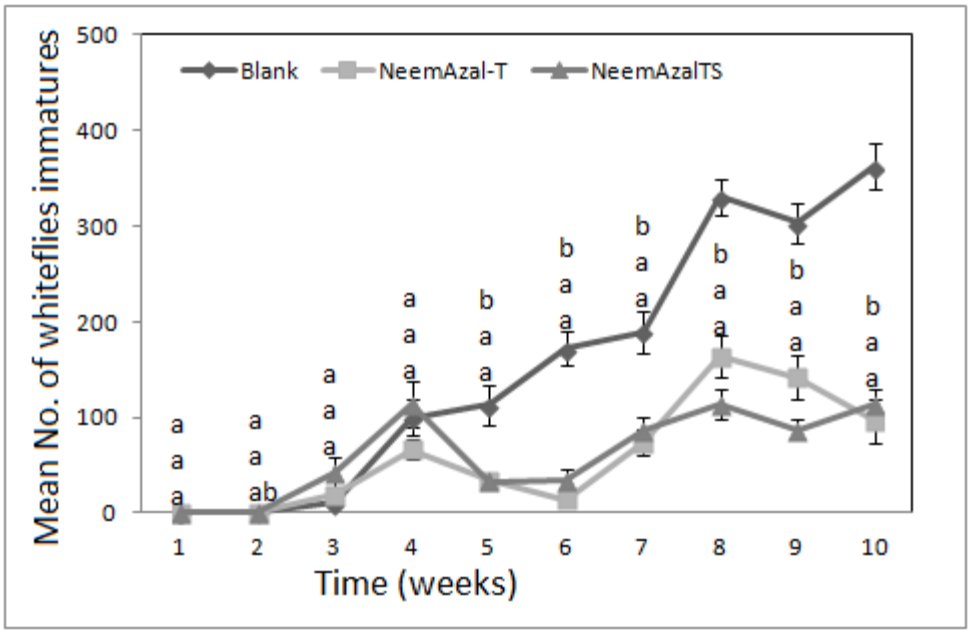

Figure 8: Mean number of *A. proletella* immatures (larvae and pupae) per plants, on Brussels sprouts planted on an open field during summer 2013 and treated with two neem formulations (NeemAzal T/S and NeemAzal T). Different letters above the line show significant difference between the treatment and control at each monitoring week (quasi-Poisson GLM, Tukey's pairwise mean comparisons).

2.4 Discussion

The results of this study indicate that neem, either as foliar or systemically (soil) administered formulation, is very effective against immature stage of both the Greenhouse whitefly, *T. vaporariorum* and the cabbage whitefly *A. proletella*. We recorded a mortality of over 60% of immature stages of both *T. vaporariorum* and *A. proletella,* an indication that azadirachtin, the biologically active compound in all the three formulations, was effective. The high sensitivity of whiteflies to neem products has been documented in previous research (von Elling et al., 2002; Kumar et al., 2005; Kumar and Poehling, 2006; Dehghani et

al., 2012; Dehghani and Ahmadi, 2013). A primary objective of our study was the comparison of foliar treatments and soil application, mainly to ensure better selectivity for an integrated management (see also introduction). We observed a high efficacy after soil treatments comparable to the foliar treatment. Several studies, (Gill and Lewis, 1971; Nisbet et al., 1993; Osman and Port, 1990; Thoeming, et al., 2003; Daly, 2004), have shown that azadirachtin can actually be absorbed through the roots, which ensures that the tetraterpenoids are systemically translocated acropetal in the plant.

Most of the commercial Neem products in the market are formulated for foliar application. Despite evidence of translaminar translocation and high efficacy when in direct contact with the target organism, the duration of active compounds (AZA) in Neem products, hence efficiency, is influenced not only by abiotic factors but also environmental conditions (Kumar and Poehling, 2006). Soil application and uptake of active ingredients by the root systems could avoid these negative effects, resulting in a higher level of pest control (Koul et al., 1990; Schmutterer, 1990; Showler et al., 2004; Kumar and Poehling, 2006). However, the foliar products are formulated with liphophilic ingredients, such as oils, to achieve a complete wetting of the plant or target surface. These oil-formulated ingredients could have detrimental effects when delivered to the plant rhizosphere, although azadirachtin by itself has been shown to have no or synergistic effect on some soil microorganisms (Spyrou et al., 2009; Gopalet al., 2007). In the view of these challenges, special soil treatment formulations have been developed which are hydrophilic formulated. A first test compound was NeemAzal®-U, (17% azadirachtin), which resulted in strong systemic effects against different life stages *Liriomyza sativae* Blanchard on *L. esculentum* (Hossain et al., 2008) and *Bemisia tabaci* (Kumar and Poehling, 2006).

Granular formulation in particular could ensure longer periods of activity (persistence) through slow release of azadirachtin. NeemAzal granules are formulated with hydrophilic carrier material, which enhances continuous release and uptake through the root (Daly, 2004; Farah, 2009). The high efficacy of this product against the whiteflies species could result from these factors.

The results of this study indicated that neem treatments resulted in fewer eggs being deposited by *A. proletella,* compared to the control. NeemAzal T/S reduced egg deposition significantly 0 and 3 days, but not day 5. The reverse was true for the soil - applied formulations where significant effect on egg deposition only occurred when treated plants were infested with whiteflies after 5 days. This difference in the formulations could be due to the application method which results into different amounts the active ingredient on the oviposition sites of the plant canopy. Since NeemAzal T/S was sprayed directly on the plant canopy, the adults were coming into direct contact with the treatment. The residues were effective up to 3 days after application but there was high loss of activity over time by day 5, due to degradation of active ingredients after exposure to high temperature and light in the greenhouse (Johnson et al., 2003). On the other hand, following soil treatments with NeemAzal granules and NeemAzal T, azadirachtin must be translocated from the roots to the upper parts of the plants (Kumar and Poehling, 2006; Thoeming et al., 2006; Farah, 2009) thus the concentration on the leaves and other oviposition sites might not have been enough to affect egg deposition by 3 days after treatment. Similar results have been observed by several authors: Azadirachtin deterred the settling of *Bemisia tabaci* adults on tomato, and hence reduced egg deposition on treated plants (Kumar and Poehling, 2006, 2007). Reduced oviposition in *Sesamia calamistis* (Bruce et al., 2004), and cabbage moth (Jõgar et al., 2009) as a result of neem treatment has also been reported.

Reduced oviposition in our studies could probably be a result of deterrence and/or antifeedant effects of azadirachtin, as earlier reported in several other studies with whiteflies (Hilje, et al., 2003; Kumar et al., 2005; Kumar and Poehling, 2007; Wen et al., 2009). We cannot distiguish oviposition and feeding deterrence since it was not the intention of this study to analyse feeding activity in detail.

Similarly, with *T. vaporariorum* we only observed a significant reduction in the number of eggs after soil treatment with NeemAzal granule and NeemAzal-T, 5 days before exposure to whiteflies. Again, this could have been a result of the relatively slow systemic translocation and accumulation of the active ingredient at the plant canopy. NeemAzal granules are formulated to be a slow release-formulation. Therefore, due to the continuous but slow release of azadirachtin, oviposition deterrent or antifeeding effects were retarded. Decrease or loss of oviposition deterrence with time as a result of degradation of azadirachtin was expected and observed in NeemAzal T/S, due to the fast degradation of the exposed residues (see above). Our findings corroborate those of other authors, (Showler et al., 2004; Kumar and Poehling, 2006) who reported decreased loss of oviposition deterrence on the test species with time after topical application of neem.

Persistence is a major important quality parameter for any plant protection compound, determining duration and reliability of action. In the current study, soil applications were more persistent than foliar treatments, and with soil treatments, there was no decrease in mortality with time. However, in the foliar treatment, efficacy decreased with time, with the lowest mortality at day 5. The initially high but quickly decreasing efficacy of topical application could be due to fast degradation of the active ingredient when exposed to high intensity of sunlight and relatively high temperatures in the greenhouse (Johnson et al., 2003;

Barrek and Paisse, 2004; Kumar and Poehling, 2006). Similar results were also reported in a greenhouse experiment, where 5 days post application of NeemAzal-T/S, the mortality of *B. tabaci* had dropped to the level of the control (Kumar and Poehling, 2007). On the other hand, strong systemic effects of NeemAzal-T/S were reported on western flower thrips at least 6 days after soil application (Thoeming, et al. 2003).

Azadirachtin has been shown to have a large number of oxygen groups (Morgan, 2009) thus it is rapidly biodegradable in soil, with a half-life of a between 1 to 12 days depending on soil type (Daly, 2004, Farah, 2009). Slow release formulations in the form of granules would therefore, offer an alternative that is more stable in the soil, thus protects the plant against pests over extended periods of time through the slow release of the active ingredient. In our study, NeemAzal granule formulation gave the most intensive control with > 60% mortality of WF immature stages even when exposure to whiteflies was done 7 days after treatment, and a significant loss of activity was observed only if exposure of the whiteflies was performed 14 days after treatment. This is in contrast to foliar treatments, where efficacy of NeemAzal T/S significantly decreased over time with a sharp gradient. Similar results were reported by Kumar and Poehling (2007). The authors observed that fresh residuals of foliar neem treatments resulted in up to 100% mortality of immature stages of *B. tabaci*, but only 7% on seven-day-old, whereas with soil treatments, mortality attenuated during the same period only from 88% to 45%. The high stability in efficacy of the neem soil treatments is in agreement with our studies. Comparing the two formulations, persistence of neem effects after soil application is significantly longer, compared to foliar application. This has also been recorded in other studies with soil neem treatments. Soil drenching with NeemAzal-T/S resulted in longer persistence than foliar application with the same product in control effects on *Frankliniella occidentalis* Pergande (Thoeming et al., 2003).

Similarly, high efficacy of NeemAzal against whiteflies was also attained in the field experiment. From our results NeemAzal T and NeemAzal T/S did not differ in their efficacy against *A. proletella.* After treatment application (week 4 after sowing) clear effect of the treatments were observed with significantly lower number of whiteflies in treated plants. This is an indication that NeemAzal could also be used in open field. However it could be more effective when used before heavy infestations to keep the populations below the economic threshold. After every treatment, lower numbers of adult and hence immatures were recorded on treated plants but then the population started building up again. This would mean that for an effective control of whiteflies in the field, repeated application of Neem would be necessary. Combinations of neem with other control methods in an IPM program might be an alternative. High efficacy of neem extracts have been reported in other studies. Biswas (2013) reported a reduction of mustered aphid 63.16-72.55% in the field as a result of different concentrations of neem leaf extracts and between 73-81% as a result of neem seed extract. Similarly, Flint and Parks (1989) recorded a 60% reduction in the numbers of immatures of *B tabaci* in small field plots after applications of aqueous sprays of Margosan-O, a commercial formulation of azadirachtin. Our results also corroborate findings of Aziz et al. (2013) who reported significant reduction of English grain aphid following application of different neem formulations in the field. Other studies, Roy and Gurusubramanian (2011) also reported significant reduction of pest population density of three sucking pests, tea mosquito bug, thrips and jassids in the field as a result of azadirachtin.

In summary, considering our results and those reported by other researchers, soil applications of NeemAzal can efficiently control immature stages and reduce egg deposition by adults of *A. proletella* and *T. vaporariorum* on cabbage and tomato respectively. Fast efficacy, long persistence, no damage by formulation compounds to the sensitive rhizosphere, and last but

not least, no risk of direct interference with natural enemies indicates this kind of neem application being a most promising IPM tool in the management of both greenhouse and cabbage whiteflies.

CHAPTER 3

EVALUATION OF EFFICACY AND DOSE RESPONSE OF SOIL-APPLIED NEEM FORMULATIONS IN SUBSTRATES WITH DIFFERENT AMOUNTS OF ORGANIC MATTER, IN THE CONTROL OF WHITEFLIES, *ALEYRODES PROLETELLA* AND *TRIALEURODES VAPORARIORUM*

Abstract

Neem products have been used frequently as an alternative to synthetic pesticides, because of their insecticidal, insect anti-feedant, and growth regulating effects. Moreover, new formulations are continually being developed and therefore, they have to be evaluated for their efficacy and persistence. In this regard, two soil-applied products, a liquid based drenching solution NeemAzal-T and NeemAzal granules, were evaluated against two whitefly species, *Aleyrodes proletella* L. and *Trialeurodes vaporariorum* (West) on Brussels sprouts and tomatoes, respectively. The plants were grown in two substrates: a commercial substrate (CS) composed of 15% humus, 35% clay, and 50% peat and a CS/sand mixture in 1:1 ratio. The main objective of the study was to evaluate the efficacy, persistence and dose response of the two soil applied NeemAzal formulations in substrates with different amount of organic matter. The results show that the efficacy of neem formulations was dose-dependent, with the highest doses of NeemAzal granules (300mg/kg =21mg azadirachtin (AZA) /kg of substrate) and NeemAzal T (2ml/kg = 20mg AZA/kg of substrate) achieving up to 100% mortality of immature stages of whiteflies. NeemAzal caused significantly higher mortality of immature stages of both whitefly species with CS+sand mixture than with pure CS. Persistence of the NeemAzal formulations was not influenced by the substrate type but rather by time span between treatment and infestation with significant decrease in efficacy when whiteflies were exposed 10 days after treatments.

Key words: Azadirachtin; organic matter; substrates: whiteflies

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3.1 Introduction

There is an increasing interest in the use of so-called biopesticides, compounds containing active ingredients from natural sources, such as neem products. The main reasons are needs for more ecologically sound approaches to pest control, with environmentally friendly pesticides in general and in particular demands for food free of insecticide residuals, and the necessity for growers to abide by the rules of organic farming. Neem products, derived from the neem tree, *Azadirachta indica*, influence many important pests of different crops and cause feeding deterrent, repellent and growth-regulating effects. Azadirachtin is the main bioactive ingredient with the highest concentration in the seeds (Thoeming et al., 2006; Brunherotto et al., 2010). The extent of these effects would however depend on variables such as concentration, formulation of the active principle, and application methods, among other factors (Stark and Walter, 1995; Daly, 2004; Khattak and Mamoon-ur-Rashid, 2006). The potential for their use in the control of crop pests, and here particularly whiteflies in organic farming systems, needs more investigation to determine optimal formulations and application schemes.

The oil based foliar formulations being registered and available in the market, though effective, are rapidly degraded under high temperature and UV, hence have a short persistence (Barnby et al., 1989; Johnson et al., 2003). Application of Neem products to the soil with uptake of active ingredients by the root systems and subsequent systemic distribution, as was reported for *Bemisia tabaci* (Kumar and Poehling, 2006) and *Liriomyza sativae* Blanchard (Hossain et al., 2008), could help to overcome these problems. These studies have demonstrated the systemic properties of soil-applied Azadirachtin, uptake by the root system and translocation to other parts of the plants. Moreover, Thoeming et al. (2003),

working with western flower thrips, observed significant mortality as a result of soil applied Azadirachtin, which was taken up by beans from the soil and successfully translocated acropetally through the plant to the insect feeding sites. However, the efficacy and persistence of soil-applied neem products can be influenced among other factors by soil organic matter content which affects the mobility and the amount of active ingredients adsorbed to the substrate, hence the availability of active solute components of neem in the rhizosphere (Sundaram and Curry, 1994; Ruch et al., 1997; Pussemeier, 2000; Barrek et al., 2004).

The physico-chemical characteristics of pesticides mainly determine the distribution of the active compound, either fixed to organic matter, or free as solute between the soil particles. Some pesticides, or their degradation products, become bound by organic matter when they enter into the soil (Kerle et al, 2007; Tiryaki and Temur, 2010). Moreover, Spark and Swift (2002), argued that for soils with high organic matter (>5%), the mobility and sorption processes of the pesticides are mostly influenced by the total organic matter content, rather than the nature of the organic matter. Therefore, the nature and properties of the soil and of the pesticide determine the extent of adsorption of the pesticide under any particular substrate type. Nevertheless, since azadirachtin has been shown to be adsorbed by organic matter, hence reducing the efficacy of some NeemAzal products (Pussemeier, 2000; Daly, 2004), we therefore conducted experiments to address the following questions: Does the amount of organic matter in the substrate have an effect on the availability of Azadirachtin from NeemAzal T and NeemAzal granules for root uptake in the control of whiteflies? What is the dose-effect relationship for the substrate-applied formulations in controlling whiteflies? Finally, is the amount of organic matter in the growing substrate influencing the long-term effect of the soil applied NeemAzal formulations?

3.2 Materials and Methods

3.2.1 Neem formulations and treatments (general)

To test the efficacy of substrate-applied Azadirachtin, two types of Neem products were used. They included a granular formulation, constituting of hydrophilic carrier material containing 7% Azadirachtin (AZA) and a water based formulation NeemAzal-T (1% AZA), both produced by Trifolio M GmbH, Lahnau, Germany. Three dosage levels of NeemAzal formulations were tested, as well as a control consisting of a blank formulation of NeemAzal-T. There were two substrate types. The first was a commercial substrate (CS), Fruhstorfer Erde® Type P, composed of humus, clay, and peat in the proportion of 15, 35, and 50% respectively. The second was a substrate mixture of Fruhstorfer Erde® and sand (CS+Sand) in a 1:1 ratio. The two substrates were selected to compare the effect of the Neem products' dose-response and persistence in substrates containing differing amounts of organic matter. For application, granules of NeemAzal were mixed with the culture substrates at 75mg/kg (=5.25 mg AZA/kg), 150mg/kg (=10.5 mg AZA/kg) and 300mg/kg (=21 mg AZA/kg) per kilogram of substrate. NeemAzal-T was drenched to the plant substrate as 1ml/kg (=10mg AZA/kg), 1.5ml/kg (=15mg AZA/kg) and 2ml/kg (=20mg AZA/kg).

3.2.2 Experimental Material

Tomato seedlings (*Lycopersicon esculentum*), var. Hildares were pre-germinated for 3 days, and Brussels sprouts (*Brassica oleracea*) var. gemmifera certified seeds were planted in plastic seedling trays (50 × 30 × 6.5cm). Seedlings were grown for 2 weeks under greenhouse conditions of 23 ± 2°C and 65–75% RH with an 18:6 h light:dark photoperiod, thereafter transplanted into plastic pots (13 × 7.5 × 8.5 cm) and further kept in a greenhouse under conditions of 23 ± 2°C and 50–60% RH with an 18:6 h light: dark photoperiod.

Two species of whiteflies were used; the common greenhouse whitefly, *Trialeurodes vaporariorum* maintained on tomatoes, and the Cabbage whitefly, *Aleyrodes proletella* maintained on Brussels sprouts. Both populations were from stock cultures on potted plants kept in insect-proof cages in a greenhouse (average temperature 20°C). For synchronization, female whiteflies were allowed to lay eggs on plants in the cages for 24 hours and then removed using an aspirator. Plants with same-aged eggs were kept separately until synchronized hatching of adults (F1).

3.2.3 Experiments

Experiment 1: Substrate effect and dose-response of *Aleyrodes proletella* and *Trialeurodes vaporariorum* to various NeemAzal formulations

In this experiment we evaluated the effect of soil-applied neem formulations at different concentrations, applied to the two types of substrate with varying amounts of organic matter, on mortality of immature stages of *A. proletella* and *T. vaporariorum*.

The set up for *A. proletella* consisted of 160 Brussels sprouts plants in plastic pots, 80 of them grown in CS and 80 in CS+sand. One well developed middle leaf per plant was chosen for infestation with whiteflies, and five *A. proletella* females were placed on the underside of each of these leaves using clip cages for 24 hours of egg laying. After removal of adults, the sets of 80 plants were randomly subdivided into portions of ten to which the selected dosages of NeemAzal granules (75 mg/kg, 150 mg/kg, 300 mg/kg, blank) and NeemAzal-T drench (1 ml/kg, 1.5 ml/kg, 2 ml/kg, blank) were applied as described above. A second application was performed after 15days.

Pots with different substrates and neem dosages were randomly arranged on tables in a greenhouse (conditions see above); for practical reasons, however, pots with NeemAzal granules were placed on different tables than pots drenched with NeemAzal-T. The number of eggs per cage was recorded, and subsequent development stages were monitored by counting the number of larvae that hatched from the eggs, the number of pupae developing from surviving larvae, and the number of emerging adults.

The same setup was repeated for *T. vaporariorum* with 160 tomato plants.

Experiment 2: **Persistence effect of neem formulations in different growing substrates**

This experiment aimed to assess the long-term effect of the soil-applied neem formulations in two substrates with varying amounts of organic matter, with respect to mortality of immature stages of *A. proletella* and *T. vaporariorum*.

For the set with *A. proletella*, 54 8-week-old Brussels sprouts plants~~,~~ were planted in plastic pots, in CS and 54 in CS+sand. Randomly selected subsets of 18 pots were treated with (A) a blank control, (B) NeemAzal-T at 1 ml/kg of soil (10mg Azadirachtin), or (C) NeemAzal granules at 150 mg/kg of soil (10.5mg Azadirachtin) by mixing the granules into the upper soil layer.

Five same-aged whitefly females were introduced as in experiment 1 (i.e., in a clip cage attached to one well developed leaf per plant) to the plants. Six plants of both soil variants were infested on the same day (D0), after five days (D5), or ten days after treatment (D10). The adults were allowed to lay eggs for 24 hours and then removed from the plants. The pots were arranged on three tables in the greenhouse, separately for D0, D5, and D10, but on each

table randomized for substrates and neem treatments. The number of eggs laid was recorded, as well as the subsequent development stages until emergence of adults (see experiment 1).

The entire experiment was repeated at a later time with another 108 Brussels sprouts plants and data pooled to achieve a total sample size of 216 (i.e., 12 plants per combination of substrate, neem treatment, and days).

The same experimental setup was used for *T. vaporariorum* with 108 7-week-old tomato plants in a first run and another 108 in a second replication.

Statistical analysis

Larval and pupal mortalities of *A. proletella* and *T. vaporariorum* were analyzed using binomial generalized linear models (GLMs) with logit link function and overdispersion ("quasi-binomial") (McCullagh and Nelder 1989). Larval mortality was calculated as the proportion of emerging pupae, based on the number of hatched eggs. Similarly, mortality at the pupae stage was calculated as the proportion of emerging adults, based on the number of larvae that pupated.

For experiment 1 separate quasi-binomial GLMs were fitted for the different species, developmental stages, and formulations. The models included dose and substrate as independent factors as well as the interaction of the two. Statistical significance of factors was assessed with analysis of deviance F-tests. Doses proved significant in all cases ($p<0.05$), hence their means were separated using Tukey-type tests controlling the rate of type I errors at 5%. Groups with an average mortality of 100% were excluded from the Tukey comparisons because of having zero variance, thus making inference unfeasible. In addition, substrates were compared pairwise for each dose level.

Mortalities in experiment 2 were modelled using quasi-binomial GLMs with substrate, formulation, days, and replication as independent factors, separately for species and developmental stages. Interaction terms of all factors except replication were also included. Analysis of deviance F-tests showed that days were significant ($p<0.05$) in all cases, so subsequent Tukey-type comparisons were carried out at the usual 5% error rate (the effects of days are to be interpreted with caution due to being non-randomized across greenhouse tables). Like for experiment 1, groups with variance zero were excluded. Pairwise comparisons of substrates were done for each day.

All statistical computations were performed in R 3.1.2 (R Core Team 2014), using the add-on packages "multcomp" (Hothorn *et al.* 2008) for multiple comparisons and "ggplot2" (Wickham 2009) for graphics.

3.3 Results

3.3.1 Substrate effect and dose response of NeemAzal formulations against *Aleyrodes proletella*

The efficacy of NeemAzal-T on larval stages of *Aleyrodes proletella* (Fig. 1, top right) was dose-dependent with significantly greater mortality (P = 0.007 with CS only, P = 0.027 with CS+sand) in the highest dose (2ml/kg of soil = 20mg AZA) as compared to the manufacturer's recommended dose (1ml/kg of soil = 10mg AZA). The overall substrate effect was found significant ($F_{1,78}$ = 8.6, P = 0.004); however, of the pairwise substrate comparisons at several dose levels, a significant difference (P = 0.003) could only be detected at 1.5ml/kg of soil.

Considering the efficacy of NeemAzal-T on pupae of *A. proletella* (Fig. 1, bottom right) we could not establish any dose-related dependency (apart from the control being inferior). The overall effect of substrate across all doses was significant ($F_{1,74}$ = 7.3, P = 0.009) in favor of CS+sand. However, due to the high variances, none of the pairwise comparisons turned out to be significant.

Like NeemAzal-T also NeemAzal granules affected the mortality of *A. proletella* in a dose-dependent manner (Fig 1, top left). A substrate effect was clearly recognizable for the granules: in pure substrate the overall mortality of whiteflies was significantly lower compared to substrate + sand across all doses ($F_{1,78}$ = 9.5, P = 0.003), and in particular at dose rates of 75mg/kg (P = 0.020) and 150 mg/kg (P = 0.007). At the highest dose of 300 mg/kg mortality of the larval stage reached 100% in the CS+sand mixture, and no pupation of larvae occurred.

The efficacy of NeemAzal granules on the mortality of *A. proletella* pupae showed a clear dose-related increase (Fig 1, bottom left). At 300mg/kg, 100% mortality was achieved at

pupal stage with both substrate types, and consequently no adults emerged. CS+sand increased pupal mortality in comparison to pure CS across all doses ($F_{1,67} = 5.1$, $P = 0.028$) and especially at 75mg/kg ($P < 0.001$).

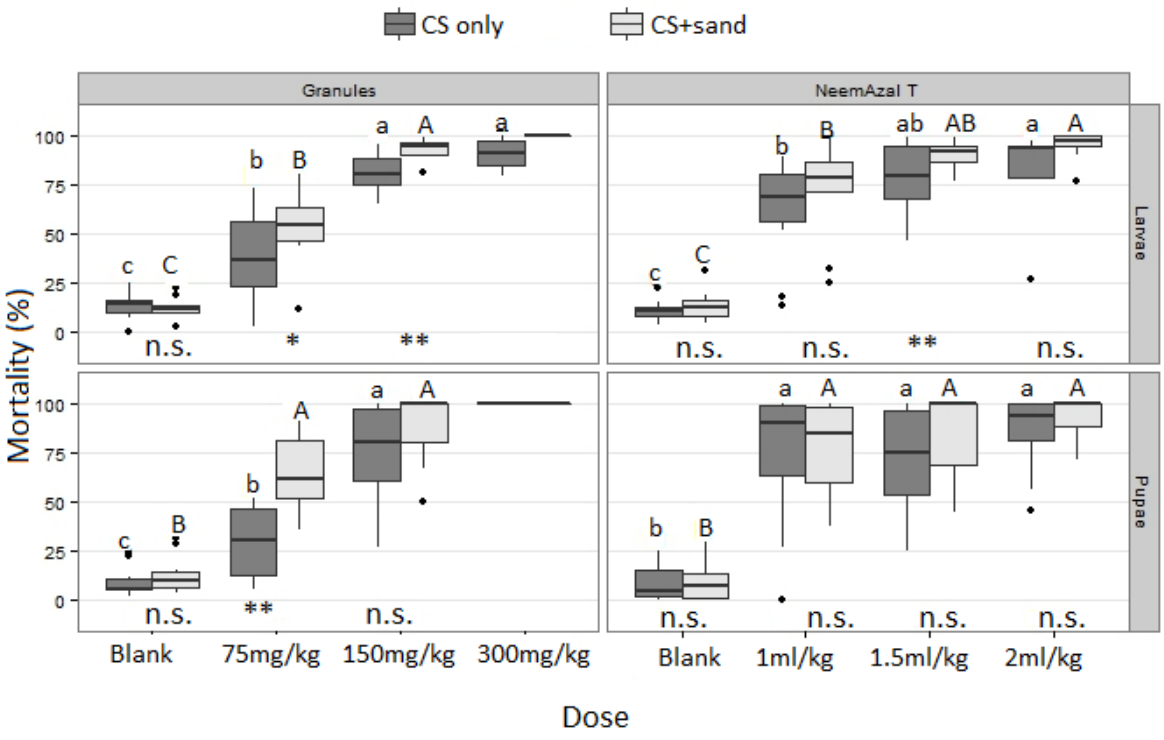

Figure 1: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Aleyrodes proletella* caused by different rates of NeemAzal granules (0, 5.25, 10.5, 21mg AZA per kg of soil) or NeemAzal-T (0, 10, 15, 20mg AZA per kg of soil) in two substrates, the commercial substrate (CS) Fruhstorfer Erde, and a 1:1 CS+sand mixture. Different letters within each panel indicate significant differences among the doses (lower-case for CS, upper-case for CS+sand) at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons). Significant differences between CS and CS+sand are indicated with stars (*** p<0.001, ** p<0.01, * p<0.05, "n.s." not significant).

3.3.2 Substrate effect and dose-response of *Trialeurodes vaporariorum* to NeemAzal formulations

The results for *Trialeurodes vaporariorum* (Fig. 2) were broadly similar to the ones described above for *A. proletella*. Again the dose-response relationship was more pronounced with neem granules than with liquid NeemAzal-T in both substrate types for larval as well as pupal mortality. The latter did not differ significantly among active doses of NeemAzal-T at all. By contrast, the highest dosage of granules (300mg/kg) led to 100% mortality at pupal stage with both CS and CS+sand.

Overall substrate effects on larval mortality were significant for NeemAzal-T ($F_{1,78} = 5.5$, $P = 0.022$) as well as for the granules ($F_{1,78} = 8.3$, $P = 0.005$), but none of the pairwise comparisons at single dose levels. On the contrary, there was no significant substrate effect on pupal mortality, neither with NeemAzal-T ($F_{1,78} = 0.6$, $P = 0.442$) nor granules ($F_{1,73} = 0.1$, $P = 0.746$).

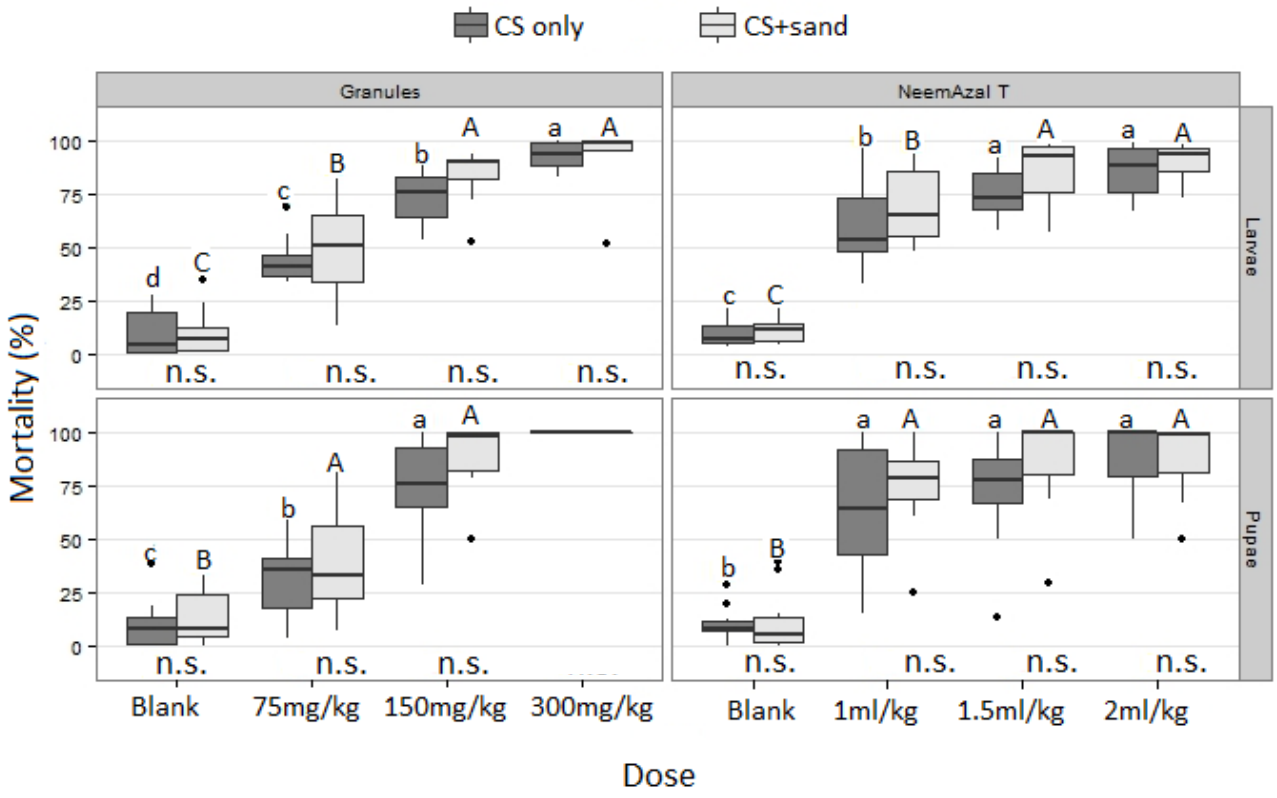

Figure 2: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Trialeurodes vaporariorum* caused by different rates of NeemAzal granules (0, 5.25, 10.5, 21mg AZA per kg of soil) or NeemAzal-T (0, 10, 15, 20mg AZA per kg of soil) in two substrates, the commercial substrate (CS) Fruhstorfer Erde, and a 1:1 CS+sand mixture. Different letters within each panel indicate significant differences among the doses (lower-case for CS, upper-case for CS+sand) at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons). Significant differences between CS and CS+sand are indicated with stars (*** p<0.001, ** p<0.01, * p<0.05, "n.s." not significant).

3.3.3 Persistence effect of neem formulations in different growing substrates

The efficacies of the two neem formulations against *Aleyrodes proletella* (Fig. 3) and *Trialeurodes vaporariorum* (Fig. 4) decreased when plants were infested with whiteflies 10 days after neem application (D10) as compared with D0 and D5; most of these comparisons

proved significant in the Tukey-type tests for both pure CS and CS+sand. In addition, the effects of days were highly significant in the analysis of deviance F-tests (*A. proletella*: $F_{2,210}$ = 38.3, P < 0.001 for larvae, $F_{2,205}$ = 29.0, P < 0.001 for pupae; *T. vaporariorum*: $F_{2,210}$ = 33.5, P < 0.001 for larvae, $F_{2,210}$ = 7.9, P < 0.001 for pupae). Furthermore, we could observe for both whitefly species that larval and pupal mortalities tended to decrease (although not statistically significant) at D5 in comparison to D0 with NeemAzal-T but not with granules.

The comparison of *A. proletella* mortalities between the two s ubstrate types revealed two marginally significant differences (P = 0.012 with larvae and granules; P = 0.014 with pupae and NeemAzal-T) for D10 (Fig. 3). As regards *T. vaporariorum*, significant differences between the substrates could be found only for the granules: CS+sand led to significantly increased larval and pupal mortality (P < 0.001) than pure CS for D0 but then again to significantly lower pupal mortality (P = 0.017) for D10 (Fig. 4).

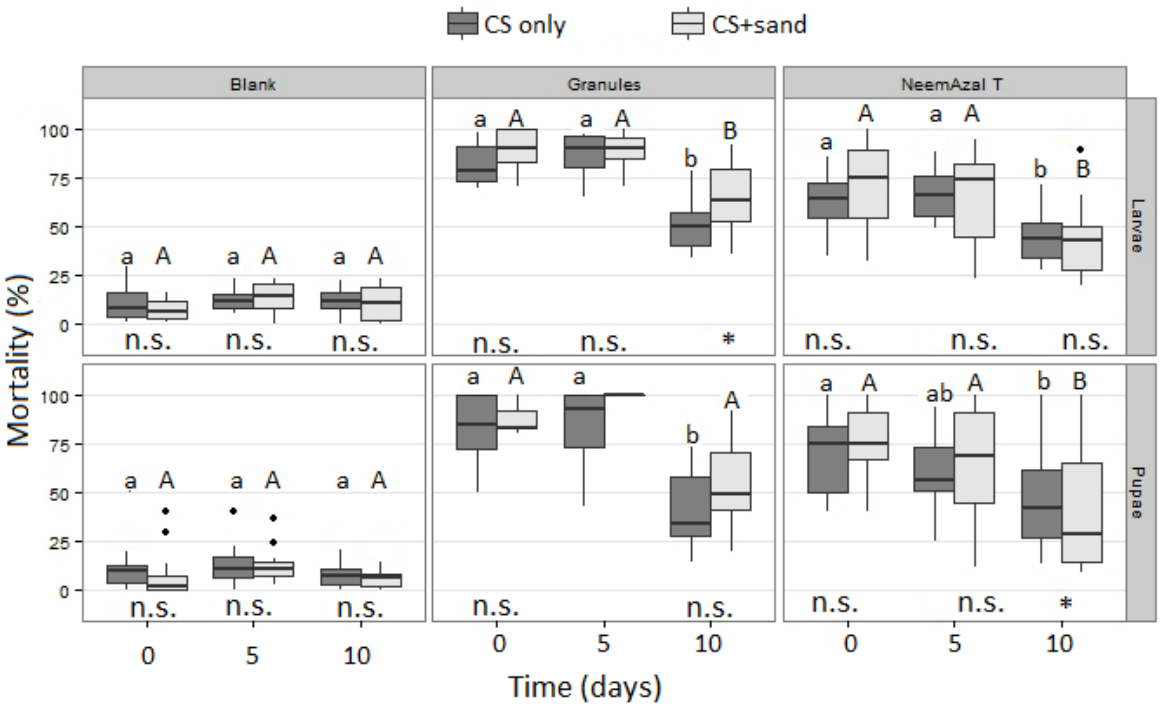

Figure. 3: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Aleyrodes proletella* caused by two soil-applied NeemAzal formulations at their company recommended rates (granules: 10.5mg AZA per kg of soil; NeemAzal-T: 10mg AZA per kg of soil) in two substrates, the commercial substrate (CS) Fruhstorfer Erde, and a 1:1 CS+sand mixture. Plants were infested with whiteflies at zero (D0), five (D5), or ten (D10) days after treatment. Different letters within each panel indicate significant differences among the days (lower-case for CS, upper-case for CS+sand) at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons). Significant differences between CS and CS+sand are indicated with stars (*** p<0.001, ** p<0.01, * p<0.05, "n.s." not significant).

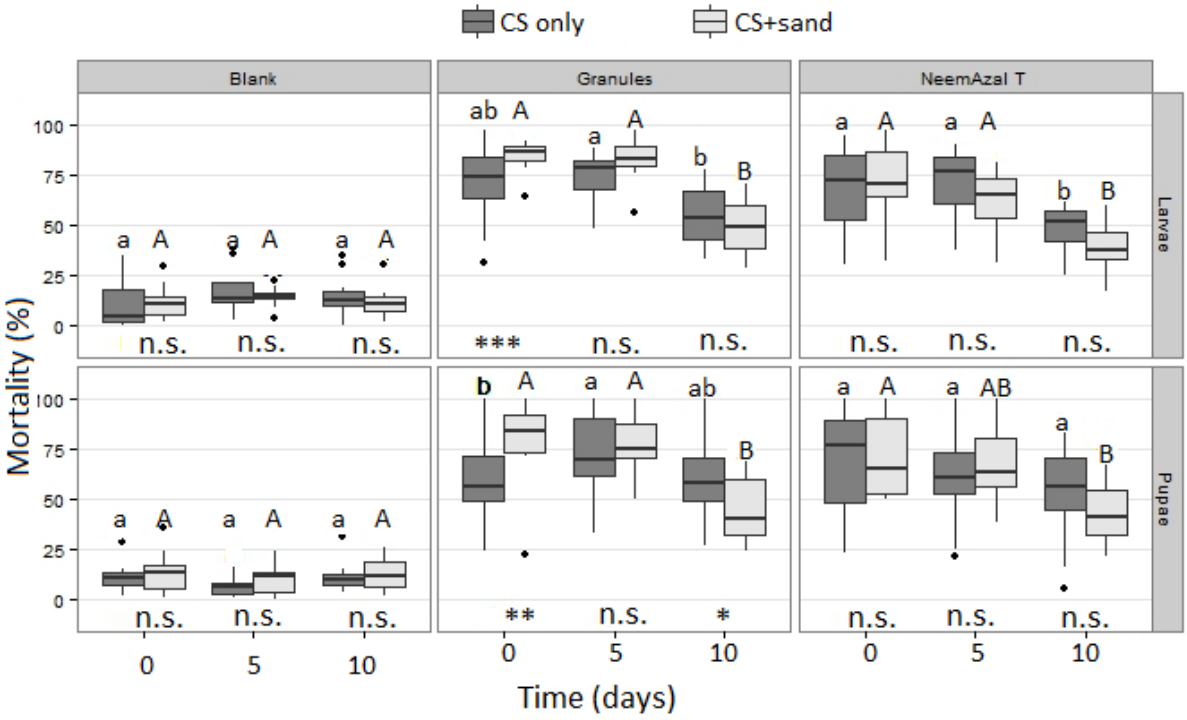

Figure 4: Boxplots of mortality (%) of immature stages (larvae and pupae) of *Trialeurodes vaporariorum* caused by two soil-applied NeemAzal formulations at their company recommended rates (granules: 10.5mg AZA per kg of soil; NeemAzal-T: 10mg AZA per kg of soil) in two substrates, the commercial substrate (CS) Fruhstorfer Erde, and a 1:1 CS+sand mixture. Plants were infested with whiteflies at zero (D0), five (D5), or ten (D10) days after treatment. Different letters within each panel indicate significant differences among the days (lower-case for CS, upper-case for CS+sand) at a multiple type I error level of 5% (quasi-binomial GLM, Tukey's pairwise mean comparisons). Significant differences between CS and CS+sand are indicated with stars (*** $p<0.001$, ** $p<0.01$, * $p<0.05$, "n.s." not significant).

3.4 Discussion

Dose effects

The substrate-applied neem biopesticide proved to be effective in the control of immature stages of both *T. vaporariorum* and *A. proletella* at all three concentrations as compared to control. In addition, mortality of both whitefly species rose with increased dosage rate in both neem formulations. Significant differences of larval mortality were recorded among different neem concentrations from both formulations, with the highest dose of NeemAzal granules (300mg/kg) attaining up to 100% mortality of immature stages of *A. proletella*.

Dose-dependent efficacy of neem-based formulations applied with different methods (topical, foliar, soil) has been reported for several target insects by other authors (Koul et al., 2004; Kumar and Poehling, 2006; Ahmed et al., 2009; Das et al., 2010).The development of larvae of the Rice moth *Corcyra cephalonica* (Stainton) for instance, was inhibited following topical application of azadirachtin, at the highest dose (10µg/larva) tested, with 55% of the insects remaining in the larval stage, compared to 3.3% at the lowest tested dose of 2.5µg/larva (Sharma, 1992). Seljåsen and Meadow (2006) testing different concentrations of NeemAzal–T (5% Azadirachtin) in a leaf dip experiment, reported significant increase in mortality of cabbage moth larvae with increasing concentrations of azadirachtin. Concentrations of 2 $mgml^{-1}$ azadirachtin-A restricted larval development to 2^{nd} instar while concentrations of 8 $mgml^{-1}$ or greater also protected the plants from any observable damage, indicating in addition increasing feeding deterrent effects with rising neem concentrations. Moreover, Ahmed et al. (2007) reported a gradual increase in toxicity of NeemAzal-T/S using foliar spray solutions with elevated concentrations resulting in a marked reduction in the number of nymphs of *Aphis fabae* (Scop). Similar observations were made on dose-response studies with soil applied (drenching) Neem products. For instance, significantly

increasing mortality of L1 larvae of *Ceratothripoides claratris* (Shumsher) (Premachandra et al. 2005) and soil stages of *Frankliniella occidentalis* (Pergande) (Thoeming et al., 2003) was reported with increasing concentrations of NeemAzal-T/S, a neem product foremost formulated for the foliar application. A mortality of 71.8% of the thrips was recorded with highly concentrated drenching solutions (200 mg azadirachtin/L) compared to 48.8 and 49.5% mortality obtained with solutions containing only 50 and 100 mg/L respectively. According to the results of Hossain et al. (2008), NeemAzal®-U (17% Azadirachtin) applied to the substrate of tomato plants resulted in larval mortality ranging from 9.38% (0.75 g $/l^{-1}$) to 100% (2.25 and 3 g $/l^{-1}$) of the leaf miner *Liriomyza sativae* (Blanchard), and most larvae surviving on the treated plants were killed during the pupal stage by the higher dose rates. In these studies, increasing efficacies of the neem products against the pests were attributed to a comparable increase in the amount of Azadirachtin absorbed from the soil and translocated systemically in the plant. Likewise, in our studies, we could conclude that the observed effects were caused by increasing amounts of NeemAzal around the roots (rhizosphere) with rising dosages applied, which resulted in higher concentrations of AZA ultimately in the plants, following dose-dependent intensity of root uptake.

Nevertheless, there is a threshold above which further increasing the dose of a pesticide does not necessarily amount to an increase in efficacy against the pest. From our results, increasing the dose of NeemAzal granules to twice the recommended dose did not significantly increase the mortality of whiteflies, and in case of NeemAzal-T, a dose increase from 1.5 to 2ml/kg did not significantly raise the mortality, although we achieved still significantly higher mortality than with the manufacturer's recommended dose. This indicates a kind of saturation level for the density dependent root uptake of AZA.

Substrate effects

Besides direct effects after topical treatments such as reported by von Elling et al. (2002), a number of studies have been published dealing with systemic properties of Azadirachtin on a number of insects following soil /substrate treatments. When applied to the soil, azadirachtin (AZA) can be efficiently absorbed by the roots from the substrate, transported via the vascular system, and systemically translocated acropetally in the plant to insect feeding sites (Kleeberg et al., 2006; Ahmed et al., 2007, Hossain et al., 2008; Aziz et al., 2013). This has lead to increased attention being given to the systemic properties of neem extracts in insect control as a substitute and/or complement to topical application, which has the drawbacks of rapid degradation(Pavela et al., 2004; Kumar and Poehling, 2006) and stronger side effects to non-target organisms dwelling in the crop canopy. However, the amount of AZA available for translocation is dependent on the concentration of AZA in the solute around the plants' rhizosphere. Hence translocation of neem ingredients from the soil to the foliage can be influenced by the substrate type, since soils with low organic matter content should have lower absorption potential for AZA to organic soil particles and allow comparable higher rates of "free AZA" around roots, but inclined also to higher risks of leaching (Thoeming et al., 2006).

Across all doses of the active ingredient, the type of substrate affected the efficacy of NeemAzal T as well as NeemAzal granules against the two species of whiteflies. Mortality of immature stages of the WF was higher using the CS+sand mixture than with the CS only. At the highest dose of granules (21mg AZA/kg) there was no pupation of *A. proletella* with CS+sand mixture at all (i.e., 100% larval mortality), compared to 91% larval mortality with CS alone. These results are in line with other studies which reported higher pest mortalities with substrates containing less organic matter (Basedow 2003; Thoeming et al., 2006). In

their extensive study on distribution of AZA in plants following soil application, Thoeming et al. (2006), observed that there were higher residues of the active ingredients in the foliage than in substrate, roots and stem using CS+sand than with CS. This could explain our results, considering that the foliage is the primary feeding site for the WF, hence the corresponding high pest mortality with the CS+sand mixture. Moreover, Daly (2004) argued that application of azadirachtin to soils of high organic matter content may give rise to contrasting distribution problems. This might in turn increase levels of adsorption, with reduced aqueous availability of azadirachtin at root uptake sites, resulting in decreased concentrations absorbed by the plant.

It has been shown from other studies that soils with low organic matter content result in higher rates of leaching and lower absorption of AZA (Sundaram, 1996; Pussemeier and Kleeber, 1998; Thoeming et al., 2006). Sorption, binding of a chemical to soil particles, is influenced by soil moisture, organic matter content, and texture. Soils high in clay and organic matter have a high potential to adsorb pesticides while sand particles provide less surface area and active or charged binding sites for sorption (Kerle et al., 2007; Tiryaki and Temur, 2010). Azadirachtin is adsorbed principally by organic matter (Daly, 2004). This affinity of azadirachtin towards organic matter may potentially affect its behavior within the soil. The degree of plant uptake of pesticides is determined partially by the pesticide's water solubility. Plant uptake of pesticides prevents runoff or leaching (Kerle et al., 2007). Since AZA is absorbed by the organic matter in a substrate, it could be more tightly bound and accumulated in substrates with higher organic matter (Đurović et al., 2009). This might have resulted in slower release to the rhizosphere, hence limited uptake by the roots in the CS in our experiments.

Persistence of neem in different substrates

Persistence is characterized by the change in efficacy of the neem products over time. We observed that with increasing time span between treatment and exposure of both species of whiteflies, a significant reduction in efficacy occurred, most pronounced after 10 days. Moreover we observed a higher persistence of the granular neem formulation compared to the liquid formulation of NeemAzal T. Similar time courses for loss of activity have been reported in other studies with neem products. Kumar and Poehling (2006) reported a decrease in *Bemisia tabaci* (Gennadius) mortality from 88 to 45%, 7 days after soil treatment with NeemAzal®-U, under greenhouse conditions and from 90 to 64% mortality under laboratory conditions. The relatively long persistence of soil-applied NeemAzal on WF immatures was also demonstrated by findings of Thoeming et al. (2003), who reported strong systemic effects of NeemAzal-T/S on western flower thrips at least 6 days after soil application.

The degradation of AZA is influenced by factors such as light intensity, temperature, pH and physical soil properties, including organic matter content (Pussemeier, 2000; Barrek et al., 2004; Thoeming et al., 2006). From this, factors in the soil system, in particular the absence of intensive radiation and photolytic degradation is the most important reason for the high stability compared to neem when applied to the crop canopy. On the other hand the difference in persistence when comparing both used formulations in the soil should be mainly related to the different adsorption vs. release properties. As discussed above, the type of soil /substrate, in particular the content of organic matter, influences the availability of free AZA in the rhizosphere. However, according to our data, the persistence of the two neem formulations was not dependent on the amount of organic matter in the substrate. We could hypothesize that the amount of AZA in the rhizosphere will decrease much faster when AZA is applied as a solution as compared to granular application. The stronger persistence of the

neem granules is more or less a result of the slow but constant release from the granule matrix, whereas in solution, there is an initial short period of high concentrations available for root uptake, followed by increased leaching, reducing the availability of AZA.

In summary, these results indicate that use of soil-applied NeemAzal is a promising tool in the management of both greenhouse and cabbage whiteflies. It was evident, from our current and previous findings, that formulation type is important in determining both efficacy and persistence of neem products. Moreover, NeemAzal granules proved to be more efficient formulation which can be adopted by growers in bio-production, with little regard to the type of substrate they are using. The Azadirachtin (the biologically active component against insect pests) is slowly released from the granules, taken up by roots and translocated acropetally to the feeding sites of insects, providing fast efficacy and long persistence, which could in turn provide efficient plant protection properties.

CHAPTER 4

EFFICACY AND PERSISTENCE OF SYSTEMIC SLOW-RELEASE NEEM FORMULATIONS IN THE CONTROL OF CABBAGE APHIDS, *BREVICORYNE BRASSICAE*

Abstract

The efficacy and dose-response, residual effect, and effect on fecundity of neem formulations on cabbage aphid *Brevicoryne brassicae* applied systemically through root tissues of Brussel sprouts (*Brassica oleracea*), was studied in the greenhouse. Two formulations were tested; NeemAzal granules containing 7% azadirachtin (AZA), at 75, 150, 225 and 300mg per kilogram of substrate and a water based formulation, NeemAzal-T (1% AZA) at 1, 1.5, 2 and 2.5ml/kg of substrate. The efficacy of the neem formulations was dose-dependent, with the highest doses of NeemAzal granules and NeemAzal T, (300 mg and 2.5 ml/kg of substrate) respectively, having up to 0% survival of aphids by 14 days after treatment. The manufacturer's recommended doses, NeemAzal granules at 150mg and NeemAzal-T at 1ml/kg of substrate, were used to evaluate the persistence and bioresidual effect of the azadirachtin on cabbage aphid over time. After treatments, plants were infested with one day old aphid larvae on the same day (D0), three days (D4) and eight days (D8) after treatment. There was a sharp decrease in persistence with NeemAzal-T when plants were infested 8 days after treatment, and there was no difference in survival of aphids with control plants. However, there were no differences in the survival rate of cabbage aphid larvae if exposed 0, 4 or 8 days after treatment with NeemAzal granules but the survival rate was significantly lower compared to that in the control.

The fecundity of aphids decreased significantly after the application of azadirachtin. In conclusion results show high efficacy of soil applied NeemAzal against cabbage aphid, with NeemAzal granules, which is a slow-release formulation, giving the longest period of bioactivity hence offering longest period of protection.

Key words: Azadirachtin, *Brevicoryne brassicae,* efficacy, dose, fecundity, persistence,

4.1 Introduction

The cabbage aphid *Brevicoryne brassicae* L, is native to Europe but has a worldwide distribution. Their short life cycle and high fecundity result in high reproductive rates. Cabbage aphid has been described as a very serious pests and significant losses are associated with its infestation particularly in many economically important host crops of the Brassicaceae family (Farah, 2009; Opfer and McGrath, 2013; Gill, et al., 2013).

Cabbage aphid feeds by sucking sap from plants causing leaves to curl inward and become chlorotic. Moreover, infestations result in reduction of market value in mature plants by accumulation of aphid residues such as the exuviae sticking on the plant surface, and contamination by sooty mould fungi growing on the honeydew. Continued feeding by aphids causes yellowing, wilting and stunting of young plants (Griffin and Willianson, 2012; Opfer and McGrath, 2013). They also cause indirect damage through vectoring plant viruses (Chivasa et al., 2002).

Control of these pests has chiefly relied on application of synthetic pesticides. These pesticides could potentially be toxic to humans when they are used in vegetable production (Lu, et al., 2008, 2010; Łozowicka et al., 2012; Phoofolo et al., 2013). Aphids are likely to develop resistance to synthetic pesticides due to overlapping parthenogenetic generations, (Ahmed, 2007). Apart from biocontrol with natural enemies development of consumer safe and selective pesticides for control of this pest is of paramount interest.

In the recent years there is a shift of focus to naturally occurring pest control agents, bio-pesticides suitable to be used in integrated control strategies or ecological production

systems. Neem products containing the biologically active compound Azadirachtin, which is derived from the Neem tree *Azadirachta indica* A. Juss (Meliaceae), is an alternative insecticides for organic farming (Kraiss and Cullen, 2008; Wen et al., 2009; Menke and Gerhard, 2010; Lee et al., 2013). Besides, these products have low human toxicity, low persistence and pronounced selectivity concerning non-target organism, such as parasitoids, predators, and pollinators (Lowery and Isman, 1995; Tang, et al., 2002) and degrade rapidly in the environment (Ahmad, 2012).

Azadirachtin, is an antifeedant and growth regulator for a wide variety of insects (Mordue and Luntz, 1998), inhibits cuticulogenesis (Lowery and Isman, 1995), delays and prevents moulting, and has been shown to prolong development time of aphids (Pavela et al., 2004; Kraiss and Cullen, 2008). Moreover azadirachtin resulted in increased adult and nymph mortality, reduced number of nymphal molts and decreased adult fecundity in Brown citrus aphids *Toxoptera citricida* (Kirklady) (Tang et al., 2002) and cabbage aphid (Pavella et al., 2004).

Most of the Neem products in the market today such as NeemAzal (AZA) T/S® (Trifolio) are formulated for foliar applications. Despite their high efficacy when in direct contact with the target organism, the oil based foliar formulations rapidly degraded under high temperatures and UV light (Barnby et al., 1989; Johnson et al., 2003). There is therefore a need for alternative strategies, which may improve the efficiency of applications. Soil application and uptake of active ingredients by the root systems could avoid this negative effects hence attain higher level of pest control sustainably.

Azadirachtin is taken up by the plants through the roots and is systemically translocated acropetal to the feeding site of insects following soil treatment (Kleeberg et al., 2006; Kumar and Poehling, 2006; Ahmed et al., 2007; Hossain et al., 2008; Aziz et al., 2013). The aim of this study, therefore, was to evaluate the efficacy, persistence and residual effects of soil applied neem formulations in the control of cabbage aphid under greenhouse conditions.

4.2 Materials and Methods

4.2.1 Neem formulations and treatments

To test the efficacy, residual effect/persistence and effects on fecundity of substrate applied Azadirachtin on aphids, two types of neem products were used: a granular formulation (NeemAzal granules), constituting of hydrophilic carrier material containing 7% Azadirachtin (AZA) and a water based formulation NeemAzal-T, containing 1 % AZA, both from Trifolio M GmbH, Lahnau, Germany. Four dosage levels of each formulation were tested and water and a blank formulation of NeemAzal-T were used as controls. For application, granules of NeemAzal were mixed with the commercial substrate at 75 mg (= 5.25 mg AZA), 150 mg (= 10.5 mg AZA), 225 mg (= 15.75 mg AZA) and 300 mg (=21 mg AZA) per kilogram of substrate. NeemAzal-T was drenched to the plant substrate as 1 ml (=10 mg AZA), 1.5 ml (15 mg AZA), 2 ml (20 mg AZA) and 2.5 ml (25 mg AZA) per kg of soil.

4.2.2 Plants and Insects

Cabbage aphid, *Brevicoryne brassicae,* used in this study was cultured on Brussels sprouts kept in insect-proof cages in a greenhouse (average temperature 20 $^{\circ}$C). For synchronization of the culture, young adult aphids were introduced on plant and allowed to reproduce for 12

hours and then carefully removed using a fine-tip camel hair brush. The same aged offspring was further cultured.

Brussels sprouts (*Brassica oleracea*) var. Gemmifera certified seeds were planted in plastic seedling trays (50 × 30 × 6.5cm). Seedlings were grown for 2 weeks under greenhouse conditions of 23 ± 2 °C and 65–75% RH with an 18:6 h L:D period. Thereafter they were transplanted into plastic pots (13 × 7.5 × 8.5 cm) and further kept under the same conditions in the green house. Fruhstorfer Erde® Type P; composed of humus, clay, and peat (15, 35, and 50%) served as standard substrate.

4.2.3 Experiments

Experiment 1: Efficacy and dose-response of *B. brassicae* to NeemAzal formulations

To evaluate the efficacy of NeemAzal formulations against cabbage aphids, 100 Brussels sprouts plants in plastic pots were used. One well-developed middle leaf per plant was chosen for infestation of aphids. Six adult aphids from the synchronized culture were placed on the underside of the leaves using clip cages and allowed to reproduce for 12 hours. After removal of adults, two sets of 50 plants were randomly subdivided into portions of ten to which the selected dosages of NeemAzal granules and NeemAzal T (as described above) and the respective control treatments were applied. A blank formulation of NeemAzal T was used as control. The number of larvae was reduced to 12 per cage. Pots with different neem dosages were randomly arranged on tables in a greenhouse.

To assess the effect of azadirachtin on the survival of aphids, monitoring was done daily for 16 days, by counting the number of living and dead aphid larvae. The effect of the azadirachtin on reproduction of aphids was assessed by recording the total number of

offspring per day. Fecundity was calculated as the number of offspring (dead and live) per adult per day.

Experiment 2: Persistence effect of Neem formulations on *Brevicoryne brassicae*

To assess the persistence / residual effect of the azadirachtin on cabbage aphid over time, 108 8-weeks old Brussels sprouts plants were planted in plastic pots in a greenhouse. Randomly selected subsets of 54 plants were (A) treated at the substrate level with NeemAzal-T at 1 ml/kg of soil (10 mg Azadirachtin), and (B) treated with NeemAzal granules at 150 mg/kg (10.5 mg Azadirachtin) of soil, by mixing the granules into the upper soil layer. These are the manufacturer's recommended dosages. A blank formulation of NeemAzal T was used as control

Ten aphid larvae (one-day old) were introduced in a clip cage attached to one well developed leaf per plant. Six plants, from each treatment and control, were infested on the same day (D0), after four days (D4), or eight days after treatment (D8). The trial was repeated twice over time and the data was pooled. Monitoring and data collection followed the aforementioned procedure in experiment 1.

Statistical analysis

For experiment 1, the survival analysis was performed with a Cox proportional hazard model (Cox, 1972). The hazard function or death rate is the instantaneous probability of death for individuals still alive. The Cox model assumes proportionality i. e. the ratio of hazard for any two aphids was assumed to be time dependent. In order to take into account the heterogeneity between plants, plant-specific frailties that follow e.g., a Gaussian or gamma distribution were introduced. The hazard for aphid *i* on plant *j* at time *t* is modeled as:

$$\lambda_{ij}(t|x_{ij}) = \lambda_0(t)\ e^{x_{ij}\beta+\gamma_j}$$
$$= \lambda_0(t)\ \nu_j\ e^{x_{ij}\beta}$$

where $\lambda_0(t)$ is the baseline hazard rate at time t (assumed identical for all aphids), the vector x_{ij}includes the covariates (treatments), and β is the vector of regression coefficients to be estimated. The frailty $v_j = e^{\gamma j}$ is the 'excess risk' of plant *j*; the frailties are modeled as independent and identically Gaussian distributed with mean O and variance ɵ

For experiment 2, Mortality of larvae of *B. brassicae* over time was analyzed using binomial generalized linear models (GLMs) with logit link function and overdispersion ("quasi-binomial"). The models included formulation, days, and trial as independent factors. Interaction terms of all factors except trial were also included in each experiment. Statistical significance of factors was assessed with analysis of deviance F-tests.

All statistical computations were performed in R 3.1.2 (R Core Team 2014)

4.3 Results

4.3.1 Efficacy and dose-response of *B. brassicae* to NeemAzal formulations

Systemic application of NeemAzal resulted in marked reduction in the survival of aphid larvae. Toxicity of azadirachtin to the larvae at all the concentration of both tested formulations increased with increasing exposure time and / or feeding period. This is seen in the highly significant ($P < 0.001$) effects of NeemAzal compared with the control. The

relative hazards within each plant was estimated (but not population-averaged). For instance, the hazard of an aphid on a plant treated with the lowest dose of NeemAzal (granules 75 mg/kg, NeemAzal T1ml/kg,) was estimated to be $e^{1:120}$ = 3:066 and $e^{0:884}$ = 5.04 times respectively, the hazard of an untreated aphid. Furthermore, the effects were dose dependent, with the highest doses of NeemAzal granule and NeemAzal T, (300 mg and 2.5 ml/kg of substrate) respectively, having 0% survival rate by 14 days after treatment (Fig.1 & 2). On the other hand the lowest tested doses of NeemAzal granule and NeemAzal-T, (75 mg and 1 ml/kg of substrate) had 34 and 42% survival rate respectively by day 14 after treatment. Overall, there were no significant differences ($p > 0.05$) between the two highest concentrations NeemAzal granules (225 and 300 mg/kg) of NeemAzal-T (2 and 2.5ml/kg) did not differ significantly in larval survival rate.

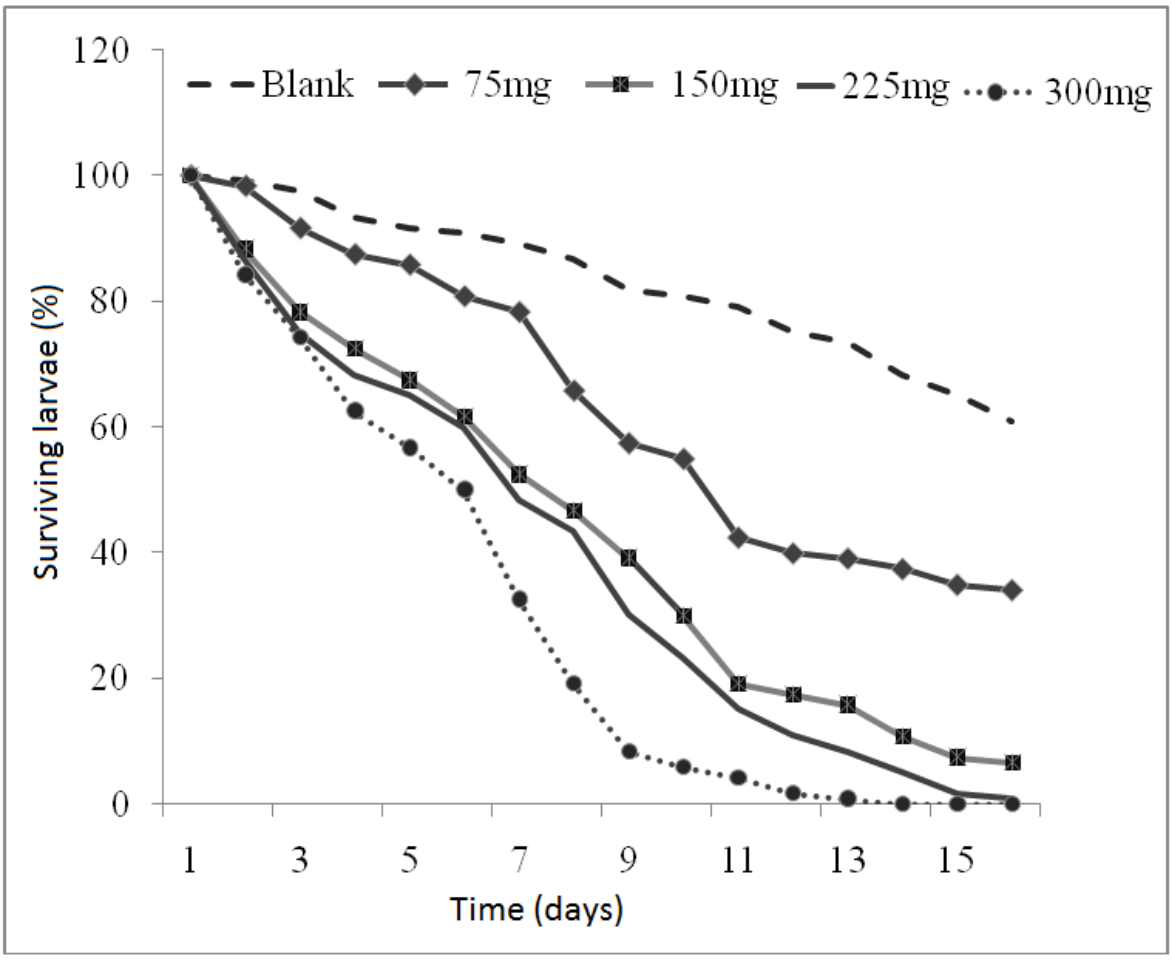

Figure 1. Survival curves for *B. brassicae*: Mean survival rates per day over a time period of 16 days after treatment with NeemAzal granules at 5, 10.5, 15.75 and 21 mg AZA10/kg of substrate.

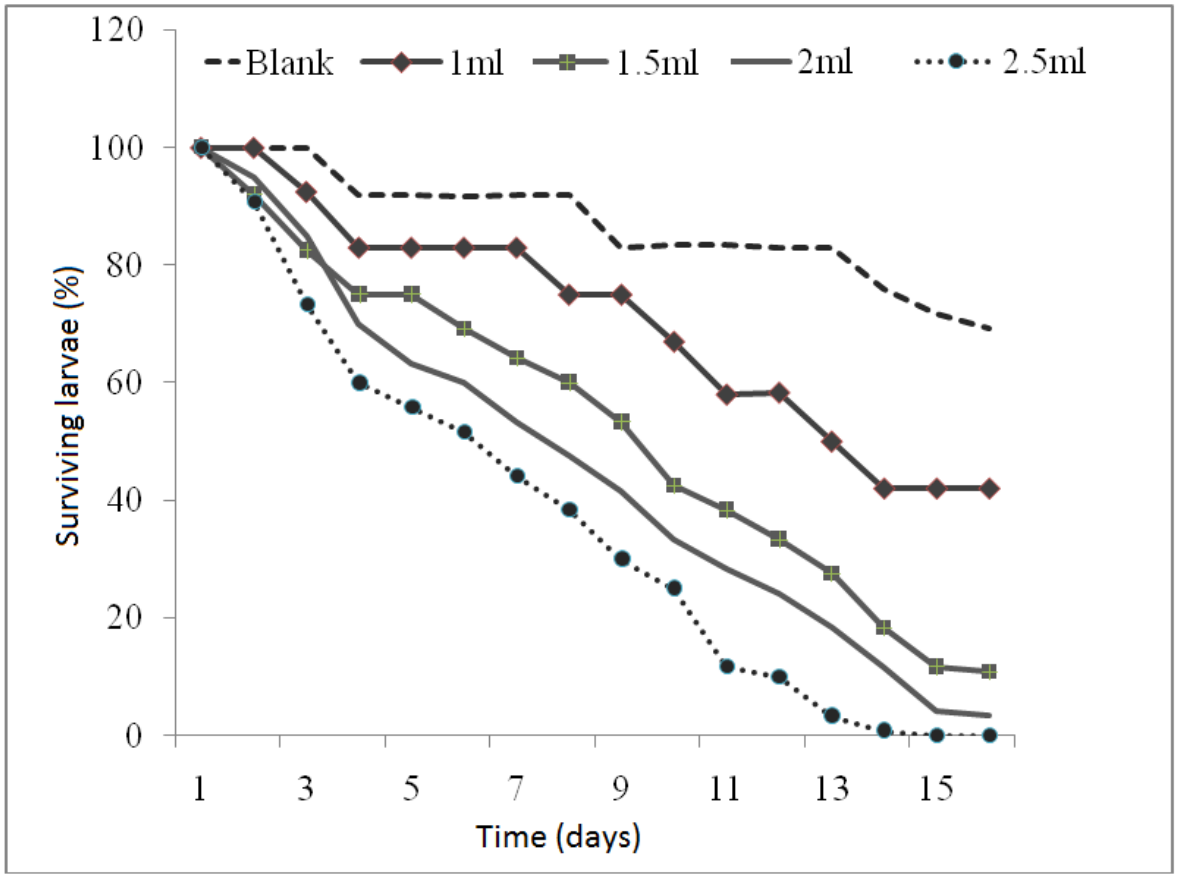

Figure 2: Survival curves for *B. brassicae*: Mean survival rates per day over a time period of 16 days after treatment with NeemAzal-T at, 15, 20 and 25 mg AZA per kg of substrate.

Comparing the efficacies of the two neem formulations at their recommended doses (150 mg/kg NeemAzal granules versus 1 ml/kg NeemAzal-T): the test is highly significant in favor of the granules. The estimated hazard ratio was 3.606 with 95% confidence interval of (2.216, 5.869), i. e. the hazard/risk of aphids dying from feeding on a plant treated with NeemAzal granules at 150 mg/kg substrate was 3.606 times higher as with NeemAzal T 1ml/kg of substrate (Fig. 3).

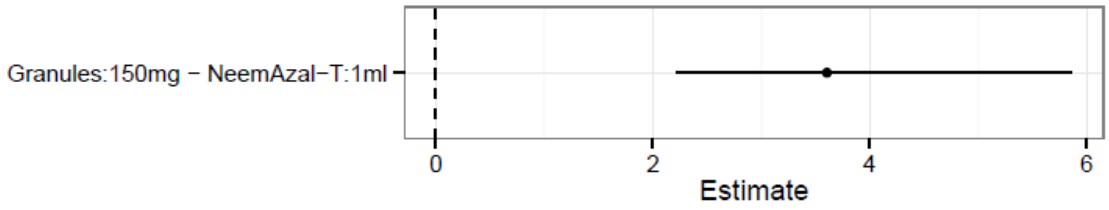

Figure 3. Estimated hazard ratio of NeemAzal granules compared to that of NeemAzal-T, Frailty Cox Model.

Exposing adults of *B. brassicae* to NeemAzal drastically reduced their fecundity (offspring per aphid per day). The effect was on a dose-dependent manner, for instance the average number of offsprings produced on plants treated with NeemAzal T at 1ml/kg was 1.82 ± 0.7 (Mean ± SE) compared to 0.14 ± 0.1 larvae per females per day in plants receiving the same treatment at the dose of 2.5ml/kg of soil. Same scenario was also true for NeemAzal granules, where significantly lower number of larvae were produced on treated plant compared to the control. Although some aphids survived on neem-treated plants, results indicated that their reproduction was greatly affected and at the two higher doses of NeemAzal reproduction was almost completely prevented. Adult fecundity data is summarized in tables 1.

Table 1. Offspring per female per day (from day 8 to 16) **of the mature adult's life following treatment with various doses of NeemAzal-T (ml/kg). mean ± S.E; n = 10.**

	NeemAzal-T				
	Blank	**1 ml**	**1.5 ml**	**2 ml**	**2.5 ml**
Females	8.63 ±0 .3a	6.42 ± 1.1b	3.66 ± 1.0c	2.59 ± 0.7cd	1.43 ± 0.3d
Larvae /female	3.02 ± 0.6a	1.82 ± 0.7b	0.90 ± 0.5c	0.49 ± 0.4cd	0.14 ± 0.1d

* Means followed by the same lowercase letters within column are not significantly different (p = 0.05) Tukey's multiple comparison test.

Table1. Offspring per female per day of the mature adult's life following treatment with various doses of NeemAzal granule (mg/kg). mean ± S.E; n = 10.

	NeemAzal Granules				
	Blank	**75 mg**	**150 mg**	**225 mg**	**300 mg**
Females	8.73 ± 0.3a	6.42 ± 1.1b	2.38 ± 1.0c	1.66 ± 0.6cd	0.48 ± 0.3d
Larvae /female	3.33 ± 1.4a	1.82 ± 0.7b	0.27 ± 0.1c	0.2 5± 0.1c	0.04 ± 0.0c

* Means followed by the same lowercase letters within column are not significantly different (p = 0.05) Tukey's multiple comparison test

4.3.2 Persistence effect of the neem formulations on *Brevicoryne brassicae*

Although the mortality rate of aphids increased with increasing time of exposure, there were no significant differences in percentage mortality of cabbage aphid larvae ($P > 0.05$) when exposed at 0, 4 or 8 days after the substrate was treated with NeemAzal granules. Additionally, at all the three exposure days, the neem treatments attained a significantly higher ($p < 0.05$) mortality compared to the control (Fig 4).

Conversely, the efficacy of NeemAzal T decreased significantly ($P < 0.001$) when plants were infested with aphids 8 days after neem application (D8) as compared with D0 and D4. (Fig 5). At this time no significant difference in mortality rate was observed between larvae in the control and the treatment. However, larval mortality rate was significantly higher for the other treatment (0 and 4 days) compared to the control.

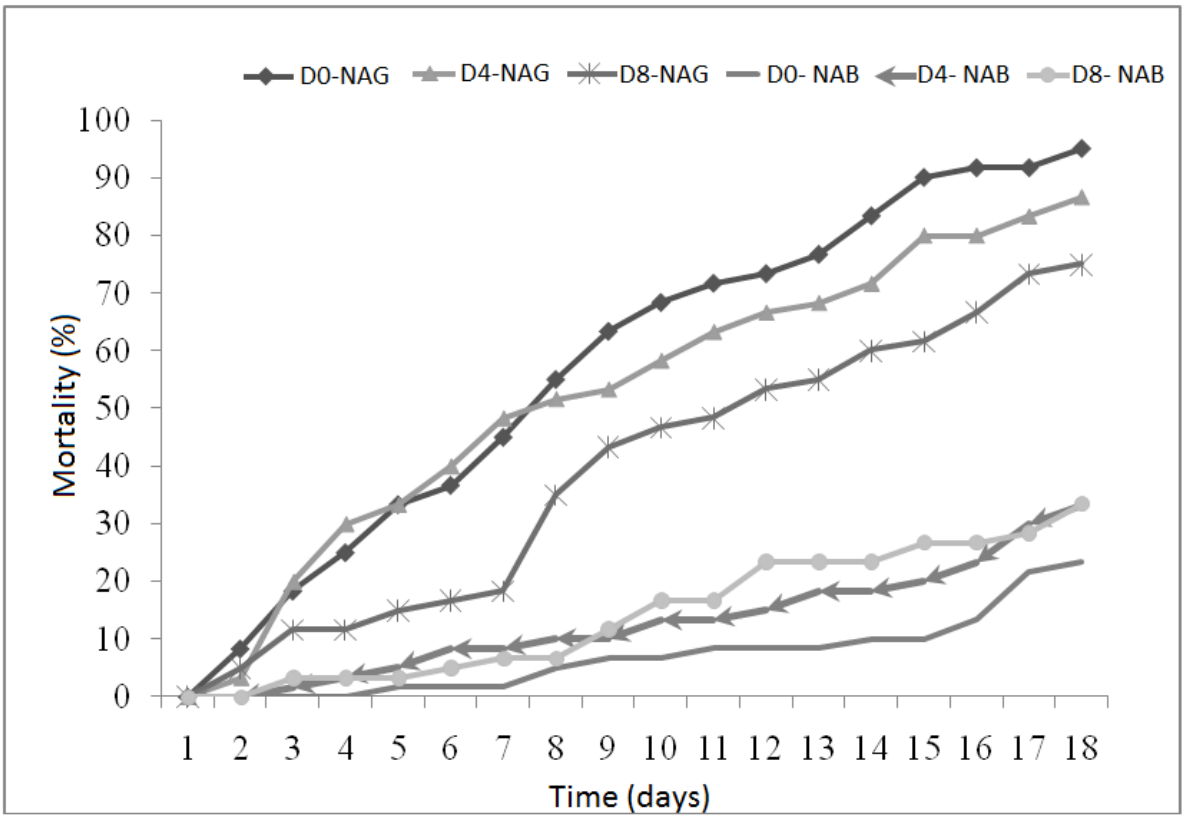

Figure 4: Mortality (%) of *Brevicoryne brassicae* over a time period of 18 days after treatment with NeemAzal granules (NAG) at, 10.5 mg AZA per kg of substrate and a blank formulation of NeemAzal (NAB). Plants were infested with aphids at day zero (D0), four (D4), or eight (D8) days after treatment.

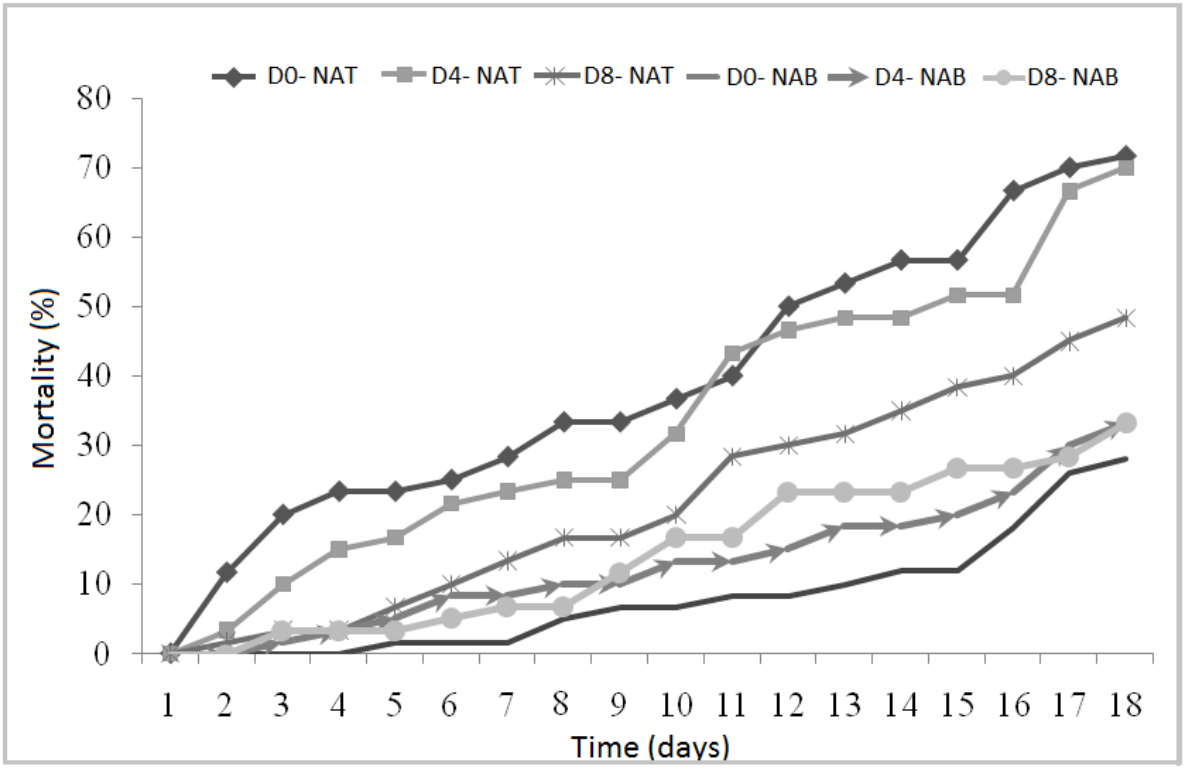

Figure 5: Mortality (%) of *Brevicoryne brassicae* over a time period of 18 days after treatment with NeemAzal-T (NAG) at, 10.5 mg AZA per kg of substrate and a blank formulation of NeemAzal (NAB). Plants were infested with aphids at zero (D0), four (D4), or eight (D8) days after treatment.

4.4 Discussion

Efficacy and dose response

The cabbage aphid is considered a serious insect pest of plants of the brassicae family, not only because it efficiently vectors plant viruses (Tang et al., 2002), but also because of its direct damage by sucking activity and contamination of the produces. The economic losses are huge (Gill et al., 2013). The over reliance on synthetic pesticides for the control of aphids has raised a lot of environmental concerns on the biosafety of these pesticides (Devine and Furlong, 2007). Therefore, biopesticides with low toxicity to humans and fast degrading residues on the produce such as products based on Azadirachtin have been considered safe alternatives for the environment and the health of consumers (Pavela et al., 2004; Pavela and Teixeira da Silva 2006; Pavela et al., 2013). On the other hand the low persistence when applied to the crop canopy and the still existing side effects on non-target organism when directly contaminated demands for improved application strategies such as systemic application via the root system of plants. This could be more desirable in terms of efficiency, persistence and even selectivity (Farah, 2009).

Our greenhouse evaluations of the effects of two NeemAzal formulations indicate that Azadirachtin, the active ingredient in the formulations, can significantly reduce survival rates of aphid larvae as well as adult fecundity. Both neem-derived biopesticides (NeemAzal-T and NeemAzal granules) in the present study significantly decreased *B. brassicae* larval survival. Our findings are in agreement with numerous studies. First Koul (1999) concluded that neem based formulations (neem seed extracts) were effective against rose aphid, and Chrysanthemum aphid, both in the laboratory and field. Many other studies reported thereafter high efficacy of neem-based insecticides against different aphid species (Weathersbee and McKenzie, 2005; Sanjeev and Singh, 2008; Melesse and Singh, 2012).

Kraiss and Cullen (2008) for instance working with two neem-derived insecticides Neemix® 4.5 EC and neem seed oil against *Aphis glycines* (Matsumura) reported significantly increased aphid nymphal mortality (80 and 77% respectively) and development time and attributed the effects to neem mode of action as an insect growth regulator.

The efficacy of the NeemAzal formulations in our studies was dose dependent. No aphids survived beyond 14 days of monitoring with the highest doses tested. Similar activities of neem-based biopesticides have been reported by other authors. Hummel and Kleeberg (2002) reported that efficacy of Neem-Azal-PC® (0.5% Azadirachtin) against *Aphis fabae* (Scopoli) was concentration and time dependent. Ahmed et al. (2007) reported that systemic effect of Neem Azal-T/S® and Neemix® on bean aphids gradually increased according to increase of feeding period and concentrations. In their study, Neem Azal-T/S at 2.5µl/100 ml completely prevented the maturation of born nymphs, and caused over 80% mortality compared to concentrations of 1.0µl/100 ml, which resulted in 58.2% maturation rate and < 60% mortality. Our results also corroborate those of Seljåsen and Meadow (2006) who reported a dose-dependent efficacy of NeemAzal-T (5% AZA) against *Mamestra brassicae* L. The authors recounted that concentrations of 2 mg/ml Azadirachtin increased mortality and restricted larval development to 2nd instar, while concentrations of 8 mg/ml or greater also protected the plants from observable damage. Moreover, Hossain et al. (2008) reported that NeemAzal-U® (17% Azadirachtin) at 0.75 g/l drenched to the plant substrate resulted in 9.38% larval mortality compared to 100% mortality resulting for higher doses of 2.25 and 3 g/l in *Liriomyza sativae* (Blanchard). Several other studies have also demonstrated dose-dependent efficacy of neem-based biopesticides on various pest species such as *Trialeurodes vaporariorum* (West) (Pavela and Teixeira da Silva, 2006); *Corythucha ciliata* (Say) (Pavela et al., 2013), *Myzus persicae* (Sulzer) (Déla et al., 2014). Hence we could attribute the

increasing efficacies of the two neem formulations against cabbage aphid to a comparable increase in the amount of Azadirachtin systemically translocated from substrate through the roots and ultimately to the aphid feeding site.

Besides the high efficacy in terms of mortality of the tested neem formulations against cabbage aphid, fecundity, number of offspring per female, was greatly affected, also in a dose-dependent manner. From our results, even if aphids survived on NeemAzal T and NeemAzal Granules treated plants, Azadirachtin significantly reduced their reproduction. Significant reduction of fertility of aphids, even at very low concentrations of azadirachtin has been documented. Pavela et al. (2004) reported a decrease in fecundity of *B. brassicae* after feeding on rape plants, systemically treated with different concentrations of water-based solutions of crystalline Azadirachtin A 97.5%.

Coventry and Allan (2001) showed that exposure of adult *M. persicae* to neem seed oil and azadirachtin not only influenced the survival of offspring produced by treated adults but also adults emerging from neem treated nymphs were undersized with abnormal wings, legs and stylets. While neem products have been shown to reduce the fecundity and fertility of adults, and molting of nymphs of various aphid species (Fournier and Brodeur, 2000; Tang et al., 2002; Pavela et al., 2004) other studies**,** (Kraiss and Cullen, 2008) reported that fecundity of *A. glycines* adults treated with azadirachtin and neem seed oil was not affected. This may be an indication that growth regulation effects of neem depends on host plant, aphid species and / or, treated aphid instar (Shannag et al., 2014). Mode of application, systemically through the root versus direct spray, of the neem based biopesticide and perhaps differences in routes of exposure of the target insect to the test substance (Shannag et al., 2014) could explain the difference in our studies and also the contradicting findings mentioned above. Vimala et al.

(2010) attributed the effect of neem-based biopesticides on the reproductive potential of aphids to blocking the neurosecretory cells responsible for hormone production controlling the aphid maturation process, egg production and embryonic development.

Persistence and residual effect

NeemAzal granules were effective for at least 8 days after soil application in our greenhouse experiments. In contrast only low mortality could be obtained when plants were infested with aphids eight days (D8) after application of NeemAzal-T. NeemAzal granules were more persistent and there was no significant reduction of efficacy. This is an indication that the granules slowly but steadily released azadirachtin ensuring long availability of the active ingredient to the plant.

Basedow. et al. (2002) reported that mortality and fecundity of nymphs of *M. persicae*, and *B. brassicae* were severely affected up to 10 days after soil treatment with a neem seed kernel water extract. Comparable persistence effects with Neem products drenched to the plant substrate were obtained also with other pests. Soil drenching with NeemAzal-T/S® (1% azadirachtin, Trifolio-M) against *F. occidentalis* was recorded up to 3weeks after treatment (Thoeming et al., 2003), and significant systemic effect of NeemAzal-T (5% AZA) against *Mamestra brassicae* were reported up to two weeks after treatment application (Seljåsen and Meadow 2006). Furthermore, NeemAzal-U was found to be effective against larval stages of *Liriomyza sativae* for at least 7 days after soil drenching, in both laboratory and greenhouse (Hossain et al., 2008). The authors recorded a larval mortality of up to 100% with neem concentrations of 3 g l^{-1} water. Azadirachtin is readily mobile in soil and systemic in plants (Daly, 2004; Thoeming et al., 2006; Hossain et al., 2008). Since its likely to be destroyed by light and high temperatures after spraying it on plants(Akhtar et al., 2008), soil application

has become more desirable. On the other hand residual activity of Neem based products sprayed to the crop canopy have low persistence against aphids. For instance, Salam (2009) reported a sharp decline in residual insecticidal activity of NeemAzal T/S on cherry-oat aphid and rose-grain aphid, from 22.2–20.0% on zero time and reached 0.0–7.9% respectively, seven days post application.

In conclusion, our studies and those of other authors show that soil-applied neem-based formulations are effective against cabbage aphids and efficiently inhibit their reproduction. Liquid based formulation such as NeemAzal T, with less oil, which proved to be effective against Cabbage aphid in our experiments would ensure effective crop protection without detrimental effects on the roots. Controlled slow release formulation like NeemAzal granules makes it possible for the active ingredient to be delivered gradually to its target over a period of time thus reducing loss of active compound in the soil, due to run off and leaching hence ensure longer periods of crop protection against aphids and other pests

CHAPTER 5

GENERAL DISCUSSION

Bio-safety and the need for environmentally friendly pesticides have necessitated development of products from natural sources to be used against insect pests. Neem, extracted from the neem tree *Azadirachta indica* (A) Juss (Meliaceae), is one such product and has been shown to be effective against over 400 species of insect pests (Saxena, 1989; Schmutterer and Singh, 1995). Azadirachtin, the biologically active compound in neem, has increasingly been used as a biopesticide, which produces multiple toxic effects in insects. Most commercially available products containing Azadirachtin as active ingredient are formulated as liquid sprays for foliar application. The rapid photodegradation of azadirachtin upon exposure to intensive light in particular of short wavelength can limit the time span of bioactivity considerably (Barnby et al., 1989; Johnson et al., 2003). Alternative approach, which may improve efficiency and persistence include development of formulations that could provide high quantity and long-term supply for uptake of AZA into the root system. In this respect, the aim of this project was to evaluate efficacy of two new neem formulations NeemAzal-T and NeemAzal granule against phloem feeding pests; greenhouse whiteflies, cabbage whiteflies and cabbage aphids.

Therefore, to study the efficacy of neem formulations against *T. vaporariorum* and *A. proletella*, greenhouse experiments were carried out. The two soil-applied formulations were compared at the manufacturer's recommended rate with a registered foliar spray formulation, NeemAzal T/S and blank formulation or water. Experiments were also set up to study the effect of the formulations on whitefly oviposition intensity. Furthermore, persistence or the residue effect of the neem formulations on whiteflies was also assessed.

Our results show that all three neem formulations were very effective against immature stages of *T. vaporariorum* and *A. proletella*. Mortality of the immature stages resulting from neem treatment was over 60% compared to below 15% in the control. Similarly, egg deposition was greatly affected by the treatments and there were significantly fewer eggs deposited on neem treated plants. Moreover soil treatments were more persistent than foliar spray formulations both in efficacy and inhibiting egg deposition. The results of the present study suggest that substrate-applied azadirachtin is effective against foliar-feeding insect pests. This is consistent with the findings of Thoeming et al. (2003), Kumar and Poehling (2006), Hossain et al.(2008) and Aziz et al.(2013) that azadirachtin can actually be absorbed through the roots, and is systemically translocated acropetal in the plant and to the insect feeding sites. In our study egg deposition was affected by neem treatments. The number of eggs deposited by *A. proletella* and *T. vaporariorum* was significantly affected when whiteflies were exposed to plants treated at their substrate level 5 days before exposure to whiteflies. Probably, it was only 5 days after treatment that enough azadirachtin, to deter oviposition, had accumulated at the oviposition and feeding sites. Several studies have demonstrated uptake and systemic translocation of AZA acropetal following soil treatments. However, Thoeming et al. (2006) and Farah (2009) observed that following soil application, maximum AZA concentrations on the foliage and soil were reached between 2 to 5 days after application. This could explain our lack of significant influence of the soil treatment on egg deposition when whiteflies were infested 0 and 3 days after treatments. Fresh foliar residues of NeemAzal T/S, significantly deterred egg deposition but, as would be expected, efficacy decreased with time to the level of the control by 5 days after treatment.

In this study, the persistence of soil-applied formulations was hypothesized to be longer than foliar spray, and indeed we found the two soil-applied formulations to be far more persistent

than foliar spraying. Granular formulation was the most persistent and only showed reduced activity when exposure of whiteflies was done 14 days after treatment. Reduced efficacy in foliar treatments was expected since azadirachtin has been shown to rapidly degrade upon exposure to high temperatures and light (Johnson et al., 2003; Barrek and Paisse, 2004).

Delayed delivery of active ingredient to the soil could be achieved by encapsulation of azadirachtin in pellets or granule. Previous work (Daly, 2004) was able to show that there was controlled - release of AZA from laboratory-made pellets loaded with the radio-active tracer, into an aqueous medium and ultimately to the soil. The author observed that the rate of release was dependent on the nature of the pellets. A follow-up of that study was carried out by Farah (2009) who tested two types of pellets, one with hydrophilic material and one with hydrophobic material. The author reported that concentration of AZA in the soil reached its maximum after 5 days with hydrophilic granule and concluded that the delivery of AZA from the granule into the soil could be delayed by the inclusion of hydrophobic material. This ensures slow and steady release of the a.i. over time, which could explain the long persistence of NeemAzal granules in our experiments. The current results also agree with those of Kumar and Poehling (2006, 2007) and Thoeming et al. (2003) who reported prolonged persistence of drenched NeemAzal T/S compared to its topical application.

An outdoor experiment was also carried out to assess the efficacy of neem formulations against aphids and whiteflies. There was very low aphid population during the study period, hence only results from white flies were analyzed. Significantly high reduction of whitefly population was achieved with NeemAzal T and NeemAzal T/S compared to the control. After every treatment application, lower numbers of adult and hence immatures were recorded on treated plants but then the population gradually built up again. Although

treatments achieved significant level of control compared to the untreated plots, it was difficult to achieve complete control due to probable movement of adults from neighbouring field. Other authors have reported efficacy of neem extract against insects in the field; Flint and Parks, (1989) reported a 60% reduction in the number of immature stages of *B. tabaci* on cotton leaves after applications of aqueous sprays containing 160 ppm of azadirachtin. Roy and Gurusubramanian (2011), also observed 50-75.8% reduction of three tea pests tea mosquito bug, thrips and jassids after week 4 with two rounds of foliar application of azadirachtin in various neem formulations. Our results also corroborate findings of Biswas, (2013) who reported significant reduction in the population of mustard aphids in mustard fields. In the light of our results, soil application of neem in the field could be a alternative to spray application to avoid negative effects of neem on natural enemies foraging on plant canopy (Biondi et al., 2012; Gontijo et al., 2014), when they come into contact with fresh residues. Combinations of neem with other control methods in an IPM program might be an alternative to reduce the necessity for repeated application. Moreover use of formulations like granules might help to prolong the effective presence in the soil (Darvari and Hasirci, 1996; Farah 2009). However, since the concentration of azadirachtin might be too low in bigger plants and upper parts of the plant (Thoeming et al, 2006) effective control may be achieved in small plants or early in the onset of infestation. Due to time and resources constraint, this study did not test the efficacy of granular formulation in the open field. This is an area that could be studied in details in the future, to evaluate its performance in the field where conditions are not controlled. Moreover, the effects of soil-applied azadirachtin on beneficials and soil microflora was not within the scope of the current study but it would be interesting to explore in order to determine how to use this products in an IPM system.

The efficacy and persistence of soil-applied azadirachtin can be influenced, among other factors by organic matter content which affects the mobility of active components of neem (Sundaram and Curry 1994, Ruch et al. 1997, Pussemeier 2000). Further experiments were therefore set up to study the effects of the amount of organic matter in a substrate, on the availability of azadirachtin for root uptake as well as the dose-effect relationship for the substrate-applied formulations in controlling whiteflies. Clearly from our results, the efficacy of the tested formulations significantly increased with increasing dosage rates. These results corroborate those of Koul et al. (2004), Kumar and Poehling (2006), Ahmed et al. (2009) and Das et al. (2010) who also reported dose-dependent efficacy of neem products. The results of this study indicated that efficacy of NeemAzal was significantly affected by the type of substrate present. Higher immature mortalities were recorded using CS+sand mixture than with the CS only. This could imply that the amount of organic matter in the substrate might have affected the amount of AZA available for translocation to the insect feeding site, hence higher mortalities in substrates with less organic matter content. Substrates with low organic matter content have lower absorption potential for of AZA to organic soil particles and allow comparable higher rates of "free AZA" around the roots (Thoeming et al. 2006). Furthermore, some AZA could have been adsorbed by organic matter (Daly 2004; Farah, 2009), hence decreased concentrations were absorbed by the plants in the pure CS. The amount of organic matter, however, in the test substrate did not influence the persistence of the two neem formulations. As seen also in other experiments, granular formulation is more persistent than the liquid formulation. This could be attributed to slow but constant release of AZA from the granule matrix, while in solution, possible leaching over time could have affected the availability of AZA.

It was also the aim of this study to assess the efficacy of the systemic soil applied formulation in the control of *B. brassicae.* Like in the case of whiteflies, azadirachtin was very effective against the cabbage aphid and the efficacy was dose-dependent. The highest doses tested inhibited survival of larvae beyond 14 days after treatment application. The fecundity of aphids was also greatly reduced by azadirachtin. Similarly, the two soil applied formulation were persistent, more so NeemAzal granules which did not show loss of activity even when infestation of aphid was done eight days after soil treatment thus longer duration of crop protection which in turn might reduce the need for repeated application

In conclusion the results of these studies demonstrated that Azadirachtin is very effective against whiteflies and aphids and high concentrations can completely deter population growth. Formulation type is important in determining both efficacy and persistence of neem products. NeemAzal granules proved to be the most efficient formulation which could be an option for growers in bio-production, with little regard to the type of substrate they are using. The Azadirachtin (the biologically active component against insect pest) is slowly released from the granules, taken up by roots and translocated acropetally to the feeding site of insects, providing fast efficacy and long persistence, which could in turn provide efficient plant protection. Although higher doses of the azadirachtin gave the best results in terms of efficacy, there is a threshold above which further increasing the dose of a pesticide does not necessarily amount to a further linear increase in efficacy against the pest. From our results increasing the dose of NeemAzal granules to two folds the recommended dose did not significantly increase the mortality of whiteflies or aphids and in the case of NeemAzal-T, dose increase from 1.5 to 2ml/kg did not significantly raise the mortality. This indicates a kind of saturation level for the density dependent root uptake of AZA. Fast efficacy, long persistence, no risk of direct interference with natural enemies indicates that soil application

of azadirachtin is the most promising IPM tool in the management of both these pests that could be adopted by growers.

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PUBLICATIONS IN REFEREED JOURNALS FROM THIS THESIS

1. **Josephine Karanja** & Hans-Michael Poehling (2015) Efficacy and persistence of systemic slow-release neem formulations in the control of whiteflies, *Aleyrodes proletella* and *Trialeurodes vaporariorum.* (*In Prep*)

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1. Bartelsmeier[1], H-M. Poehling[1], **J. Karanja**[1], E. Hummel[2]. Kontrolle von Blattläusen und Weißen Fliegen an Kohl mit Quassia-MD und neuen Formulierungen von "NeemAzal". Deutsche Pflanzenschutztagung, September 11th-14th, 2012, Braunschweig, Germany.

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DECLARATION

I Josephine Karanja declare that this thesis is my original work, performed by myself and that it has not been presented for a degree in any other university

Josephine Karanja

Hannover, 2015