Evaluation of azadirachtin used to protect plants against arthropod pests

Imke Bartelsmeier

Propositions

1. Insecticides based on azadirachtin are valuable for integrated pest management strategies of aphids on high value crops.
   (this thesis)

2. Active ingredients of neem products are translocated systemically in acropetal and basipetal direction after spraying and are present in the phloem of rose plants.
   (this thesis)

3. Sex-specific health issues are underrepresented in medical sciences.

4. Science will become more collaborative and cross-functional due to Covid-19.

5. Microplastics will remain a global human and environmental threat even if we are immediately stopping the production of new plastics.

6. Educating about food production in secondary schools contributes to a more sustainable society.

7. Application of genome editing contributes to the European Union's agricultural strategy.

Propositions belonging to the thesis, entitled:

Evaluation of azadirachtin used to protect plants against arthropod pests

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Evaluation of azadirachtin used to protect plants against arthropod pests

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Thesis

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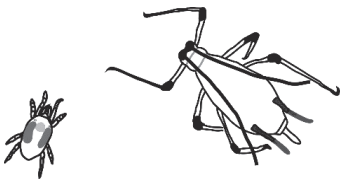
Chapter 1

General introduction

Evaluation of azadirachtin used to protect plants against arthropod pests



The use of synthetic agrochemicals is one of the most discussed topics about European agriculture today. Farmers, consumers, environmental associations and politicians are debating about the need, use, environmental impact and human safety of pesticides. The pressure from governments, supermarket chains and the public on growers is high. However, are there alternatives to plant protection with synthetic pesticides for protecting crops from losses up to 80% (Oerke and Dehne 2004, Oerke 2006)? The human population is growing and the global arable area is limited. Consequently, we cannot afford to accept yield losses – the yield even needs to increase in the future, despite more challenging conditions due to climate change.

In organic crop production, synthetically produced pesticides are banned. The total organic crop production area continues to increase in the European Union (EUROSTAT 2020). The political intention is that in 2035, less than 15 years from now, more than 20 % of the agricultural area in Germany should be farmed organically (BMEL 2020). In 2019, the organically farmed area in Germany was still below 10 % of the total agricultural area (BMEL 2021), thus reaching 20 % is an ambitious goal. The European Union (EU) aims to reach that at least 25 % of the EU's agricultural land are under organic farming by 2030 (EC 2021a). At the same time, also conventional farming and horticulture are developing strategies to reduce synthetic pesticides more and more. Some countries and trade channels are phasing out synthetic pesticides for home and garden use and public areas (e.g EC 2015). Consequently, finding alternative control strategies, agents and products is needed. Integrated Pest Management (IPM) is a systems approach combining prevention by resistant or tolerant crop variety selection, cultivation techniques, physical barriers, biological control and other non-chemical methods to collectively manage pests in crops. Horticultural production of fruits, vegetables and ornamental plants is of huge economic importance for several countries such as the Netherlands, Colombia, Kenya and Ecuador - the largest cut flower export countries (Rabobank/Royal FloraHolland 2016). One element of IPM in these productions of high-value crops can be botanical pesticides. Insecticides based on extracts from the neem tree (*Azadirachta indica*) and their use in horticulture are in focus of this thesis.

Integrated Pest Management

Modern plant protection in general should follow the guiding principle of Integrated Pest Management (IPM). In short, it means to consider and combine all biological control techniques, host plant resistance and cultivation techniques to avoid or reduce the use of synthetic pesticides to a justifiable minimum, while ensuring the balance between economy (yield losses, costs) and ecology (environmental impact, sustainability) (Barzman et al. 2015, EC 2021b) The integration of different approaches is a characteristic of IPM. Monitoring and setting action thresholds are crucial to identify the economic threat and to make decisions on when and what crop protection activities to initiate. Prevention by proper cultivation techniques is another important pillar of IPM: Smart crop rotation, seed bed preparation, tillage, sowing dates and -techniques, under-sowing, irrigation, fertilization, selecting resistant varieties and hygiene measures are included. Beneficial organisms may be promoted, e.g. by keeping habitats next to the field such as flowering strips. Biological control means the use of living organisms to control populations of pest organisms (Eilenberg et al. 2001). Biological control utilizes natural control mechanisms using predatory, parasitoid, pathogenic or herbivory organisms. Beneficial organisms can be released in the field, orchard or protected cultures in greenhouses either by a mass release (inundative biological control) or with the aim that they will multiply (inoculative biological control) and control the pest for a certain time period (Eilenberg et al.

2001). However, not only biological control can be used for organic production and as alternative to synthetic pesticides. The field of biological insecticides of natural origin is large. In the following section, an overview is given on substances that are currently registered in the EU.

Biological insecticides and current status in the EU

In the following, the term "insecticide" might also refer to acaricides and means pesticides against arthropod pests in general. If pesticide application is needed for plant protection, the possibility of choosing within a wide range of different modes of action is crucial for avoiding resistance of pest species. For synthetic insecticides, it seems to be more and more challenging to discover new active molecules, which are meeting with registration requirements (Fuglie et al. 2011). The output of new insecticides has been relatively constant during the last two decades (Sparks et al. 2019). Compared to the number of molecules screened, the outcome of new active ingredients is even decreasing, as requirements for registration are becoming more challenging and expensive (Thacker 2002, Sparks 2013). Major criticism on existing synthetic products is the toxicity for pollinators and other non-target organisms, which leads to a decrease in biodiversity, and the risk of resistance (Geiger et al. 2010). Furthermore, their widespread and persistence in the environment, followed by an impact on soil and water ecosystems is of concern, especially for systemic pesticides (Chagnon et al. 2015, Gross 2014, Chaudhary et al. 2017). Several compounds of the major insecticide class of neonicotinoids have been banned in the European Union for field crops since 2018 (Bass and Field 2018, EC 2021b). Furthermore, the acceptance of pesticides by society is declining.

Biological products have the reputation of being more eco-friendly. Nevertheless, also these are agents that are released in ecosystems, might cause negative side effects and might impact non-target species. Nature provides a broad range of naturally occurring toxic substances and this is a great resource for finding pesticidally active ingredients. Biological or natural insecticides comprise all kinds of substances that have a natural origin and are not synthetically made. These can be based on microorganisms, plant-derived compounds or so-called botanical insecticides, mineral compounds or soaps. Not yet commercially relevant, but in focus of recent EU-promoted research, are biopesticides based on insect peptide hormones. They mimic neuropeptides and can be thus highly selective (CORDIS 2020). For the European Union, the currently most relevant biological insecticides are pyrethrum, rapeseed oil and neem extracts/azadirachtin. In addition to horticultural production, a major area of application for biopesticides is the non-professional use. In view of the new legislation in EU countries like France, banning synthetic pesticides for non-production areas and home and garden use (EC 2015, Yacoub 2019), developing safe biological products for private consumers with good ecotoxicological characteristics is required.

Neem and pyrethrum are the best-known examples of botanical insecticides. Pyrethrum is a powder made of dried flower heads of *Chrysanthemum cinerariaefolium* (Astaeraceae). This powder contains six natural insecticide compounds, the pyrethrins, of which pyrethrins I and II are the most abundant and effective ones. They are acting as contact neurotoxins and are causing a fast knockdown effect. Their susceptibility to photodegradation prevents on the one hand accumulation in the environment, on the other hand it is also lowering the efficacy in outdoor uses (Mueller-Beilschmidt 1990, Khambay and Jewess 2010, El-Wakeil 2013). Synthetic pyrethroids (e.g. deltamethrin, cyhalothrin, permethrin) are worldwide used and cheaper than the natural product, but not registered for organic crop production. These synthetic pyrethroids are more stable, thus more persistent in the

environment (Mueller-Beilschmidt 1990, Gan et al. 2005). A further disadvantage is the development of resistance in target organisms (Khambay and Jewess 2010).

Many of the registered pyrethrum products contain rape seed oil as formulation ingredient as solvent or carrier. Oils have a contact activity by blocking the respiratory system of insects, resulting in asphyxiation, but they can also deter insect feeding or settling, cause cellular and nervous disruptions (Taverner et al. 2001, Cranshaw and Baxendale 2013, Najar-Rodriguez et al. 2008, Yu 2015a). Oil can improve the permeability of the insect's cuticle for insecticidal substances and act as carrier (de Licastro et al. 1983, Mulrooney et al. 1993). Some products are pure rape seed oil formulations. Also oils of other origin (e. g. paraffin oil) are used for pest control with a similar contact effect on insects, especially on larval stages and soft-skinned insects. A disadvantage can be phytotoxicity. Furthermore, even if their solely contact toxicity and low persistence is a safety benefit, such products without an orally active substance need to be well applied, ensuring a good coverage of pest insects to achieve a good control. Many pest species are well hidden on crops, sitting underneath the leaves or in buds. The physical effect can cause a quick knock-down effect, but remaining individuals may recover quickly.

Other groups of insecticides are plant essential oils and microorganisms. Plant essential oils have various effects on insects, both lethal and sublethal (e.g. repellent). Examples are e.g. rosemary, thyme and peppermint oils (Isman 2006). Natural terpene blends of *Chenopodium ambrosioides* were used as basis for commercial insecticides and acaricides (Manker 2012, Musa et al. 2017). Microorganisms can be used to combat insect pests. Bacteria such as *Bacillus thuringiensis* and granuloviruses are registered in Europe (BVL 2020, 2021). Spinosyns (Spinosad) are produced by fermentation of the soil-borne bacterium *Saccharopolyspora spinosa*. Spinosad is widely used as insecticide and acaricide (Thompson et al. 2000).

One of the main barriers for the commercialization of products of natural origin is a varying concentration of the active ingredient in the raw material. Often, a natural mixture of several active compounds is present. Furthermore, compared to synthetic standards, natural products often lack sufficient efficacy. Variable effects, natural active ingredient mixtures, stability and standardization of the product might complicate the registration processes and raw material of natural origin is more expensive than synthesized active ingredients (Schmutterer 1990, Isman 1997, Isman 2004, Isman 2006, Balog et al. 2017). Therefore, such products are economically more convenient for high value horticultural crops (Isman 2004).

Next to pyrethrum and rape seed oil, neem products are commercially the most important and known biological insecticide (Isman 2006, Isman 2020). The history and current status of neem products is reviewed in the following.

Plant protection with neem extracts

What is "neem"?

Since 1959, when the scientist Heinrich Schmutterer observed in the Sudan the phenomenon that locust swarms destroyed all plants with the exception of neem trees (*Azadirachta indica*) (Maramorosch 1995), thousands of publications have dealt with neem and its insecticidal compounds. Although neem was hyped by many researchers as a very promising alternative to synthetic pesticides, it still does not play an essential role in plant protection today (Isman and

Grieneisen 2014, Isman 2020). The following paragraphs will give an overview about ingredients of neem extracts, the mode of action and possibilities and problems relating to an efficient use in practice.

Neem, either as seed extract or neem oil, is nearly worldwide used for plant protection and as antiparasitic agent for medicinal purposes (Schmutterer 1990, Mordue (Luntz) et al. 2010). Self-made neem formulations provide valuable possibilities for small-scale farming in developing countries, where the neem tree naturally occurs (Schmutterer 1988, Isman 2006, 2008 and 2017, Constantine et al. 2020). However, such applications are not within the scope of this study. Also neem oil is only of minor commercial relevance in Europe. Here, I focus on the use of commercially relevant neem products in integrated pest management (IPM) for horticultural crops and the home and garden use.

The neem tree naturally occurs in the tropics and subtropics (Schmutterer 1995). The seed kernels of neem fruits are the most important source of insecticidal ingredients. Neem seed extract contains a variety of structurally related active compounds such as nimbin, salanin and azadirachtin. In addition to azadirachtin A, numerous other azadirachtins (e.g. 3-tigloyl-azadirachtol: "azadirachtin B") are present in neem extracts (Kraus 1995, Mordue (Luntz) et al. 2010). They belong to triterpenoids, more precisely to the group of limonoids (Schmutterer 1990, Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010). Azadirachtin A is normally declared as the leading substance of neem products. The main reasons for this are that it is quantitatively the most common ingredient and secondly, the effect against insects of a neem extract is closely correlated with its content of azadirachtin A (Isman et al. 1990, Kleeberg 2010). In contrast to pyrethrins, of which synthetic mimics, the pyrethroids, are a commercially well-established insecticidal class, synthetic azadirachtin has no relevance for pest control. Even through azadirachtin was synthetically made in 2007 (Jauch 2008, Ley et al. 2008), costs and benefits seem to be not attractive for commercialization, as no products are on the market.

Mode of action of neem products

The mode of action of azadirachtin varies among different insects orders, but also species. The mode of action is officially classified as "unknown" or "uncertain" (Sparks et al. 2020, IRAC 2021). Various effects have been reported: lethal and sublethal effects by feeding inhibition, repellent or deterrent effects and impact on development (insect growth regulating (IGR)) and reproduction. The mode of action is subject of thousands of scientific publications from the last decades, covering pests of Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Orthoptera and Acari (Mordue (Luntz) and Blackwell 1993, Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010).

Antifeedant effects

Lepidoptera are most sensitive to the antifeedant effect of azadirachtin. In this order, an inhibition of feeding is reached at very low doses of < 0.001 – 50 ppm azadirachtin, whereas Coleoptera, Hemiptera and Hymenoptera require higher doses of 100 to 500 ppm azadirachtin (ED_{50}) (Mordue (Luntz) and Nisbet 2000). In Orthoptera, the effective dose for antifeedant effects has a broad range from depending on species (Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 1996, Mordue (Luntz) 2004). Antifeedant effects can be either primary, which means at the sensory level, i.e. insects are repelled prior to ingestion of neem-treated material through their contact chemoreceptors, or secondary, where the insects stop feeding after ingestion of neem compounds. Secondary effects are caused by toxic effects, such as necrosis of cells and an impact on the midgut

peristalsis. The death occurs a few days after these histopathological effects were observed (Nasiruddin and Mordue (Luntz) 1993, Trumm and Dorn 2000). For instance, caterpillars stop feeding, remain inactive for several days or might drop from the leaves before dying, resulting in no minimal damage of the neem-treated crops (Hasan and Ansari 2011). Not only feeding can be deterred, but also oviposition deterrence by neem products was shown in choice tests, e.g. for the important lepidopteran cabbage pest *Pieris brassicae* (Hasan and Ansari 2011). Studies on antifeedant effects are reviewed in further detail in, for instance, Mordue (Luntz) et al. 2010.

Insect-growth-regulating (IGR) effects and impact on reproduction

Physiological effects resulting in inhibition of juvenile development, mortality and reduced reproduction are much more consistent in arthropod pests than the antifeedant effects. These effects can be summarized as neuroendocrine effects of azadirachtin, as they can be explained with an impact of azadirachtin on hormone levels involved in molting and juvenile development (Mordue (Luntz) and Blackwell 1993, Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010). Azadirachtin manipulates ecdysone and juvenile hormone titers in insect hemolymph. The synthesis and release of PTTH (prothoracicotropic hormone) is inhibited by azadirachtin, resulting in a reduction of receptivity of prothoriacic glands. As a consequence, ecdysone and juvenile hormone titers are lower than in untreated insects. In addition, azadirachtin has direct effects on the production of ecdysone 20-monooxygenase, which catalyzes the transformation of ecdyson to its active form 20-hydroxyecdysone (Barnby and Klocke 1990, Mitchell et al. 1997). 20-hydroxyecdysone is needed for the development of a new skin and removal of exuvia. A prolonged molting process was observed after treatment of insects with azadirachtin, or morphological abnormalities if molting was achieved, for instance in lepidopteran larvae (Martinez and van Emden 2001). Generally, juvenile stages are more lethally affected by azadirachtin than adult insects. A reduced survival of juvenile stages and capacity to develop to adult insects was reported for numerous agricultural and horticultural pests, for instance for cabbage caterpillars (Hasan and Ansari 2011), whiteflies (Basedow et al 2002, von Elling et al. 2002), fruit flies (Van Randen and Roitberg 1998), leaf miners (Hossain and Poehling 2008, Hossain et al. 2008, Tomé et al. 2013), thrips (Otieno et al. 2015), Colorado potato beetle (Zabel et al. 2002) and aphids (Lowery and Isman 1994).

In addition, the affected hormones are also involved in the production of eggs and juveniles (Rembold and Sieber 1981, Hardie 1987, Barnby and Klocke 1990, Riddiford 2012). Sterility and reduced fecundity are often observed. For instance, the egg hatchability and survival of *Pieris brassicae* were reduced on neem-treated plants (Hasan and Ansari 2011) and reproduction of the wine weevil *Otiorhynchus sulcatus* was ceased on azadirachtin-treated leaves (Cowles 2004).

The mode of action of azadirachtin is even more complex. In addition to the presented effects, also cytotoxic effects in insect cell lines were observed and effects on cell division and protein synthesis, which are contributing to the biologically observable effects of azadirachtin (reviewed in Mordue (Luntz) 2004, Mordue (Luntz) et al. 2010). The reduction of fecundity and egg hatching, increased larval mortality and delayed developmental times to adults can result in a reduced population growth in the field (e.g. Zaki 2008, Ahmad et al. 2015, Shah et al. 2019).

Advantages and Disadvantages

Beneficial for using neem products for crop protection are their possible broad spectrum of target pests (Schmutterer and Singh 1995). Even more important is a low mammalian toxicity (Raizada et al.

2001, Boeke et al. 2004, Yu 2015b) and a low risk for persistence in the environment (Ruch et al. 1997, Troß et al. 2000, Pussemier 2000). Neem products have furthermore a low risk of resistances of the pest species. Neem extracts which are used for today's commercial products contain a mixture of active ingredients, even when azadirachtin A is considered the leading substance. The naturally occurring mixture of different ingredients in neem seed kernel extracts seems to avoid a desensitization of insects to neem products in comparison to purified azadirachtin, attributed to a diffusion of the selection process by different modes of action and target sites (Feng and Isman 1995, Bomford and Isman 1996).

Another benefit for the practical use of neem products is the systemic action. Azadirachtin and most likely other neem compounds are at least partly systemic. After application, they can be translocated in the plants and so the ingredients can reach plant parts which were not directly treated (Sundaram 1996, Pavela et al. 2004, Thoeming et al. 2003, 2006). Azadirachtin is penetrating through the plant cuticle and is distributed within the plant (Basedow et al. 2002, Schulz et al. 1994) or can be taken up by roots and translocated with plant sap to upper parts of the plant (Thoeming et al. 2006). However, it might depend on the treated plant if only translaminar effects are present or systemic action (Schulz et al. 1994). Systemic activity is an advantage for pest management, because also hidden insects will be affected when they feed on the plant and after the application newly growing plant tissue is protected as well. On the other hand, systemic pesticides in general came under criticism due to high mobility and accumulation in the environment (Chagnon et al. 2015, Gross 2014). However, compared to many synthetic products, azadirachtin is quickly degraded within a few days, depending on UV radiation, temperature, pH value and microbial activity and the risk for bioaccumulation is considered very low (Stark and Walter 1995, Ruch et al. 1997, Troß et al. 2000, Pussemier 2000, Johnson et al. 2003, Caboni et al. 2002, Caboni et al. 2006, Chaudhary et al. 2017).

That azadirachtin acts mainly as a toxin upon feeding would be favorable for integrated pest management systems with beneficial arthropods and for the natural regulation in general (Schmutterer 1990). Neem products, e.g. NeemAzal-T/S, are rated as relatively harmless for beneficials in general and as nontoxic for bees (EFSA 2011, Trifolio-M 2014). Under field conditions, beneficials might be unaffected by the neem product. Hence, the effect of neem products in combination with an intact natural regulation may result in a sufficient control of pest species.

On the other hand, operators might face a high variability in efficacy and a late occurring effect might not promote their adoption (Schmutterer 1990). Due to the IGR effects, nymphal stages are more affected compared to adults and neem is a slow-acting pesticide (Stark and Rangus 1994, Lowery and Isman 1994, Koul 1999). With neem as a stand-alone product, it is unlikely to reach a 90-100% mortality of pest populations with all kind of developmental stages. If reproduction is not significantly affected, timing of application has a significant role to bring the pest populations back under thresholds. Speed of population development is also dependent on external factors such as temperature, but also the performance of neem products might be dependent on environmental factors due to degradation of active substances. The environmental benefit that active ingredients are quickly degraded in the environment (short half-life) has on the other hand the consequence that efficacy is quickly decreasing as well. As neem has no knock-down effect, application timing and intervals and environmental factors might influence the performance and control success. However, when adding neem products as an element to IPM approaches, its effect to slow down pest population growth in combination with other control measures can result in an effective pest

management. The broad-target spectrum of azadirachtin might have negative consequences here, as also beneficial organisms released as biological control agents might be affected, depending on the species (Aggarwal and Brar 2006, Zaki 2008, EFSA 2011).

Another negative aspect for growers is the costs. Commercial neem products are generally more expensive than synthetic pesticides; thus their main area of application are high value crops such as vegetables, fruits and ornamental plants (Isman 2004). Only few studies are available comparing the efficacy of neem products with synthetic standard products including costs. Considering action thresholds to reach a specific marketability in cauliflower infested with mixed pest species resulted for instance in a comparably good performance of NeemAzal-T/S, a commercial neem product, compared to a synthetic standard (Voliam Flexi, active ingredients thiamethoxam and chlorantraniliprole). However, due to higher costs of NeemAzal-T/S, revenue was higher using the synthetic standard. Self-made neem seed extract prepared as an aqueous extract was less expensive, but also less effective than NeemAzal-T/S (Shah et al. 2019). Reviewing these pros and contra arguments underlines the difficulty of replacing synthetic pesticides with natural products such as neem one-on-one. The aim and the discussion should be rather about finding sustainable pest management strategies, with neem products as one possible part, resulting in sufficient control levels, while using synthetic pesticides is not needed.

Neem products in practice

Registered products containing azadirachtin in the European Union are for example NeemAzal-T/S, Oikos, Azatin or BloomAzal (BVL 2021, CTGB 2021). These products are emulsifiable concentrate formulations with azadirachtin A as active substance. Azadirachtin products contain a natural neem tree seed kernel extract, with a standardized amount of Azadirachtin A as leading substance. However, neem extracts have a mixture of several compounds next to azadirachtin A. Such mixtures might complicate the registration process for natural products compared to synthetic products.

Neem oil is also used worldwide as a pesticide, but it is of minor commercial relevance in Europe. Neem products are mainly used as spay applications. Also drenching, for instance against soil dwelling pests such as fungus gnats is registered (BVL 2021). Soil applications using the systemic effect of azadirachtin, with the aim to control pests also on above ground plant parts, are the subject of some publications (e.g. Thoeming et al. 2003, 2006, Hossain et al. 2008, Farah et al. 2010), but are not yet commercially relevant in Europe.

The low toxicity profile regarding humans and the low persistence qualifies neem products also for the home and garden use. As stated above, neem products are quite expensive and, thus, not suitable for large scale crops. However, for high value horticultural crops especially in the house and garden field of application, consumer-safe neem products are promising. The pesticide market for non-professional users is large: 6.8 billion USD in 2018 and it is expected to grow at an annual growth rate of 3.8% from 2019 to 2025 (Grandviewresearch 2019).

Two major, omnipresent arthropod pest groups in home and garden context, but also in professional horticultural production, are aphids and spider mites. Both pests have high reproduction rates and short development times, consequently effective and safe control strategies are a challenge. The next part will review the potential of neem products for controlling aphids and spider mites.

Control of aphids

Aphids are worldwide distributed and especially in protected cultures such as greenhouses their rapid mass development with parthenogenic and viviparous reproduction is critical (Sullivan 2008, Alford 2012a) The impact of neem compounds on aphids in general is diverse. Physiological activity such as the insect growth regulatory (IGR) effect is the most important lethal effect in the case of aphid control with neem. Aphids do not seem to be killed by any neurotoxic effect (Lowery and Isman 1993, Koul 1999, Mordue (Luntz) and Nisbet 2000). Immature aphids show a high mortality a few days after neem treatments especially during molting (Koul 1998, Pavela et al. 2004, Lowery and Isman 1994). Treated larvae were swollen and expected to molt soon, but they failed to complete this process or to remove the exuvia (Lowery and Isman 1994) due to the impact of azadirachtin on insects hormone levels (Barnby and Klocke 1990). Generally, nymphs are more affected than adult aphids. Mortality of immature aphids caused by neem extracts or azadirachtin formulations are reported for several species, such as *Brevicoryne brassicae* (Pavela et al. 2004), *Aphis craccivora* (Dimetry and El-Hawary 1995), *Aphis fabae* (Dimetry and Schmidt 1992), *Aphis glycines* (Kraiss and Cullen 2008), *Nasonovia ribis-nigri*, *Macrosiphum euphorbiae*, *Acyrthosiphum pisum*, *Aphis gossypii*, *Rhopalosiphum padi*, *Fimbriaphis fimbriata* and *Myzus persicae* (all Lowery and Isman 1994). Nymphs that survived had significant prolonged developmental times (e.g. Dimetry and El-Hawary 1995, Kraiss and Cullen 2008).

For lethal effects in older larvae and adults, much higher concentrations are required than for younger nymphs (Dimetry and Schmidt 1992, Stark and Rangus 1994, Lowery and Isman 1994, Dimetry and El-Hawary 1995, Basedow et al. 2002). Even if no mortality was observed for adult aphids, the sterilization effect or a decrease in fecundity was observed after treatments with azadirachtin or neem extracts. The fecundity was significantly lower for aphids which develop on neem-treated plant material or which were exposed as adults, for instance for *A. pisum* (Stark and Rangus 1994), *B. brassicae* (Koul 1998) *A. craccivora* (Dimetry and El-Hawary 1995), *N. ribis-nigri* and *M. persicae* (Lowery and Isman 1994, Nisbet et al. 1994, Lowery and Isman 1996). *M. persiace* nymphs that were still born were malformed (Nisbet et al. 1994). Mordue (Luntz) et al. (1996) as well reported a higher percentage of non-viable nymphs of total nymphs produced for *M. persicae* on azadirachtin-treated diets (Mordue (Luntz) et al. 1996). As also the longevity and reproductive period might be decreased (Dimetry and El-Hawary 1995), these indirect impacts on adult aphids significantly contribute to a control of pest populations in the field. However, previous results are partly contradictory. Fecundity and also longevity of *A. glycines* was for instance not affected after treatment with the product Neemix or neem seed oil (Kraiss and Cullen 2008). Even in the same experiment, species might react differently. In contrast to *N. ribis-nigri* and *M. persicae*, neem oil and azadirachtin did not affect the fecundity of *Chaetosiphon fragaefolii* in the same study (Lowery and Isman 1996).

IGR effects and an impact on reproduction are obviously the main toxic effects of neem formulations on aphids at moderate doses, but also antifeedant effects were reported in the past. Repellency was not observed in *M. persicae* by Azatrol, a commercial neem product, at registered concentrations. However, their honeydew excretion was significantly reduced if the aphids fed on treated leaves, an indicator for the reduced feeding activity of aphids (Shannag et al. 2014). In another study with *M. persicae*, similar effects were observed on azadirachtin-treated diets, but effects do occur not before 26 h after treatment earliest. Furthermore, very high concentrations of 100 ppm azadirachtin were necessary to reach a significant feeding-inhibiting effect (Nisbet et al. 1994). Similar results are

reported for *B. brassicae*: For a significant antifeedant effect, 200 ppm of azadirachtin were necessary (Koul et al. 1997). Today's registered concentrations are equivalent to 30-50 ppm of azadirachtin A. Therefore, published results from laboratory experiments might be not given in field or greenhouse applications with registered doses of formulated products. Effects on aphids are closely correlated to the azadirachtin content, even if neem extracts are used (Koul 1999), but also the kind of formulation is important. Plant oils in the formulation are common in commercial neem products today for improving the stability and can increase the efficacy (Schulz et al. 1994). Therefore, the formulation may have certain effects on aphids itself.

Not only different species among Aphididae are responding differently, even in same trials (Lowery and Isman 1994, 1996, Basedow et al. 2002), but also the sensitivity of the same species is different depending on the host plant as tested for instance for *M. persicae*. Second instar of *M. persicae* was most susceptible on mustard cabbage and lest susceptible on corn, requiring 40 times higher lethal concentration for 50% mortality (LC_{50}) than on mustard cabbage and four times higher LC_{50} than on lettuce (Lowery and Isman 1994). As effects of neem are normally occurring not before three to seven days after exposure (Dimetry and El-Hawary 1995) and no knock-down effect is given, several factors can influence the efficacy. Depending on formulation, experimental set up or route of exposure, concentration, developmental stage, species and host plant results might differ (Lowery and Isman 1994, Dimetry and Schmidt 1992, Basedow et al. 2002). Only few results are available, where these details are addressed and efficacy in the field is hard to predict from laboratory trials. Weather conditions might influence the efficacy as well, as temperature and humidity can influence on the one hand the food intake, but also the speed of population growth (Lowery et al. 1993) and the degradation of active ingredients from neem-extracts. Furthermore, the pest densities at the beginning are important, whether slow-acting neem products are able to significantly decrease aphid populations in the field and to hinder the exponential growth of colonies (Shah et al. 2019). This discussion shows that neem formulations might not be able to control aphids in the field, as mortality rates vary and are influenced by numerous factors.

Control of spider mites

Spider mites, for instance the two-spotted spider mite *Tetranychus urticae*, are arthropods that belong to the Acari. *T. urticae* is polyphagous and uses a lot of different trees, shrubs, home and garden plants as hosts. They usually feed on the lower leaf surface and damage plants by sucking cell contents through their stylets. This leads to cell death, appearing as speckled leaves, and can result in yellowing and wilting (Capinera 2008, Alford 2012b). The small mites, sitting underneath the leaf, are difficult to hit directly with pesticides. For this reason, contact substances such as pyrethrins can fail to have the desired effect. In addition, due to their very fast reproduction, developing resistance to commonly used compounds is problematic and already an issue worldwide (Van Leeuwen et al. 2010, Khajehali et al. 2011). Neem products with their natural mixture of several active ingredients and especially azadirachtin as an at least partly systemic compound would be favorable for controlling spider mites. Beyond that, azadirachtin is assumed to be mainly a feeding rather than a contact poison (Schmutterer 1990).

In contrast to aphids, fewer results have been published for the potential of neem for controlling spider mites, but several sublethal and population decreasing effects are reported. Significant deterrent and antifeedant effects of different neem extract or azadirachtin formulation were proven

in the past (e.g. Dimetry et al. 1993, Sundaram and Sloane 1995, Dabrowski and Seredynska 2007, Bernardi et al. 2013, Marcic and Medo 2015).

Multiple reasons for an impact of azadirachtin on the population growth of *T. urticae* were recorded. For instance, the viability and reproduction of survived mites was reduced on bean leaves treated with the commercial product NeemAzal-T/S. In addition, female longevity was significantly reduced (Marcic and Medo 2015). Population reduction was proven also for the azadirachtin containing product Azamax, however, compared to the synthetic standard abamectin, similar levels of reduction were later reached (Bernardi et al. 2013). Furthermore, reduction in fecundity, oviposition as well as a decrease in egg hatchability was observed (Dimetry et al. 1993, Sundaram and Sloane 1995, Marcic and Medo 2015), which can lead to lower population growth. Basha and Kelarny (2001) found additionally a prolongation of the life cycle of *T. urticae* and neem-treated spider mites stayed longer in immature stages (Basha and Kelarny 2001). In contrast, azadirachtin in sublethal concentrations caused mortality and affected fecundity, even if the resulting offspring was not affected in its development. Nevertheless, the tested concentrations led to declining populations and incorporation of azadirachtin in IPM programs of *T. urticae* is suggested (Martinez-Villar et al. 2005). Specific effects on spider mites might differ as a consequence of formulation or product used, concentration (Sundaram and Sloane 1995) and experimental set up.

Why is further research on neem products needed?

The current state of the art indicates that results are diverse, partly contradictory, but difficult to compare. Self-made neem solutions, pure azadirachtin with various solvents or neem oil were used, with unknown formulation and exact concentration of azadirachtin. Isman and Grieneisen (2014) reviewed the situation on botanical insecticide research and concluded that only one third included any chemical data or characterization of the tested extracts and only a quarter of them included positive controls (Isman and Grieneisen 2014). Positive controls can be a well-known, highly efficient biological product, or a synthetic standard. Thousands of papers are available testing sublethal effects in the laboratory, but results are not transferable to practical application. When repellent effects were recorded in the laboratory, does that have any meaning for the application in gardens or a greenhouse for rose production? Is the oviposition-deterrent effect on spider mites (e.g. Dimetry et al. 1993) or the reduced reproduction of aphids (e.g. Lowery and Isman 1996) significant, when registered doses of commercial neem products are used?

Neem products, compared to the number of publications, lag behind expectations regarding the commercial relevance (Isman 2020). Experiments with neem should not only address whether lethal and sublethal effects are present, but also if these lead to a sufficient control. Furthermore, the reported variable efficacy needs to be explained: Temperature and UV radiation have an impact on the degradation of active ingredients of neem, but it is not clear whether they have this any impact in practice. To use neem as an alternative to synthetic insecticides and an element of IPM strategies, their efficacy needs to be improved to avoid application failures. For achieving a stable efficacy, detailed knowledge is needed about critical factors that affect efficacy.

Objectives of this thesis

The main objective of this thesis was to evaluate the potential and limitations of neem products as a valuable tool in IPM strategies for controlling horticultural pests such as aphids and spider mites. The efficacy and mode of action of registered concentrations of NeemAzal-T/S, a commercial neem-

product, on rose aphids (*M. rosae*) and spider mites (*T. urticae*) was investigated. To close the gap how effective a biological neem product is compared to synthetic insecticides, a synthetic standard was added to the experiments as positive control when possible. To exclude effects of formulation ingredients the blank formulation of the product was included as negative control. Generally, experiments with intact plants in the greenhouse were chosen over leaf disk or and excised leaf laboratory experiments to reflect realistic conditions. Experiments with systemic application were performed to determine whether indirect ways of exposure are effective as well.

Furthermore, the thesis addresses possible reasons for low efficacy of neem products and will test possible environmental influencing factor such as temperature, UV intensities and daytime of application. Results should help to improve the application and efficacy and to discuss the capabilities of neem products for IPM strategies.

Experimental system

Roses are one of the most widely grown ornamentals in professional horticulture and gardens. Relevant, nearly globally distributed pests for floriculture and private customers on roses are the rose aphid *Macrosiphum rosae* and the spider mite *Tetranychus urticae*. *M. rosae* is nearly worldwide distributed, very common in Europe and often occuring on roses in gardens. They infest preferably new shoots and buds. An infestation with *M. rosae* leads to a sticky covering of plants with honeydew, thus promoting the risk of sooty mold contamination. Their sucking activity results in deformed flowers and leaves (Alford 2012c). Only few results are available about the control of *M. rosae* with neem products. As effects among aphids are diverse, further research on effects specifically addressing *M. rosae* and the commercially relevant product NeemAzal-T/S (Trifolio-M GmbH, Lahnau, Germany) are of interest. Spider mites such as *T. urticae* can cause severe damage to ornamental plants (Capinera 2008, Alford 2012d), especially in protected cultures of roses. Resistance of *T. urticae* to common acaricides is already an issue in rose productions, e.g. in greenhouses in the Netherlands (Khajehali et al. 2011). The potential of commercial neem products as NeemAzal-T/S for controlling spider mites such as *T. urticae* needs to be addressed.

Thesis Outline

Chapter 2 focuses on the mode of action of registered doses of a commercial neem formulation, NeemAzal-T/S, on the rose aphid *M. rosae*. In standard efficacy trials in the greenhouse, the effect is compared to that of a synthetic standard product. Additionally, the population development starting with one adult female was compared on plants treated with different concentrations of NeemAzal-T/S. In clip-cage experiments, the mode of action was investigated in more detail: the effect on different developmental stages, nymphal development and mortality and the impact on reproduction was determined. The effects of exposing aphids as juveniles to the neem product or as adult females were compared.

Chapter 3 presents a study of the antifeedant and systemic effects of NeemAzal-T/S. Bi-directional (upwards and downwards) systemic action was assessed to answer the question whether active ingredients will be available in the phloem of rose plants, where aphids are feeding on. On this basis, I tested whether registered concentrations have repellent and antifeedant effects on *M. rosae*. The impact of neem on host-plant choice and honeydew production was investigated and on the probing and feeding behavior. Feeding activity was assessed quantitatively with the amount of honeydew excretion and qualitatively with the electrical penetration graph (EPG) technique. This chapter

complements the findings of chapter 2 and completes the mode of action testing with registered doses of a commercial neem product.

Chapter 4 presents efficacy results of neem products on rose aphids at different temperatures and UV intensities to evaluate possible influencing factors. Furthermore, the effects of time of the day of application and thus conditions at application (direct sunlight and higher temperatures compared to an application in the evening) were investigated in field experiments. Results are discussed with regard to opportunities to improve the application of NeemAzal-T/S.

Chapter 5 presents the effects of neem formulations and blank-formulation ingredients in greenhouse trials on the two-spotted spider mite *T. urticae*. The efficacy is compared to a synthetic standard product. Furthermore, deterrent effects of NeemAzal-T/S and its blank formulation on *T. urticae* were tested in the laboratory. To exclude formulation effects, also soil application was performed in the greenhouse.

In Chapter 6, the results are summarized and placed in the wider context of the overall objective of this thesis. The findings are discussed within the broader framework of integrated pest management in horticulture and home and garden use.

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Chapter 2

Effects of NeemAzal-T/S on different developmental stages of rose aphids (*Macrosiphum rosae*)

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Abstract

Natural insecticides often do not provide a strong knock-down effect and have a lower efficacy than synthetic pesticides. For an effective application of natural insecticides, it is essential to know the product's mode of action in detail. The efficacy of the commercial neem product NeemAzal-T/S and its influence on stage specific mortality, development and reproduction of rose aphids, *Macrosiphum rosae*, were determined in greenhouse trials. NeemAzal-T/S had an efficacy of 40 % against *M. rosae* in standard efficacy trials with initial infestations between 100 and 270 aphids per plant. However, it has a significant impact on the survival of nymphs in the first to third instar as well as on the development. Nymphs treated with NeemAzal-T/S exhibit a significant delay in molting to the second instar and most of them die before the first molt. Furthermore, reproduction of adult *M. rosae* females is reduced when aphids are exposed to sub-lethal concentrations of neem already as first instars. The increase in population growth is inhibited or delayed on rose plants treated with NeemAzal-T/S. The results indicate that applications of NeemAzal-T/S can be used for controlling rose aphids in integrated pest management (IPM). Due to a lack of a rapid knock-down effect and no effects on adult aphids, combinations with natural enemies of pest insects as well as with other insecticides are possibilities to enhance the efficacy.

Introduction

Natural insecticides based on extracts from seed kernels of the neem tree (*Azadirachta indica*) have gained importance for pest management in the last decades. Due to a public pressure for eliminating synthetic chemical pesticides, biological products for pest management are considered as a promising alternative. Especially for home and garden use and for higher value crops such as ornamentals, neem products are convenient. Their low persistence in the environment and a low mammalian toxicity (Sundaram 1996, Raizada et al. 2001, Boeke et al. 2004, Kleeberg 2004) are advantages for their use in public areas. One common pest of roses is the rose aphid *Macrosiphum rosae* (Aphididae). It has an almost global distribution and infests all kinds of *Rosa* species as its primary host plant. In Europe, *M. rosae* can be found on roses from April to autumn. Preferred feeding sites are young leaves and developing buds. Severe infestations cause deformed small flowers and leaves, leading to stunted growth. In addition to such direct damage, the excreted honeydew promotes the growth of sooty mold fungi. *Macrosiphum rosae* can severely reduce the quality of roses (Maelzer 1977, Alford 2012). It needs about eight to ten days to mature at temperatures between 20 and 25 °C. During their lifetime, each rose aphid female is able to produce more than 30 nymphs, depending on the environmental conditions and rose cultivar (Ölmez et al. 2003, Golizadeh et al. 2017). Due to their parthenogenetic and viviparous reproduction, colonies of aphids grow rapidly.

For effective aphid control, treatments are needed which rapidly disrupt the exponential population growth. Insecticides containing neem extracts are generally active against aphid species, but the mode of action and effective concentrations can be variable (Lowery and Isman 1993, 1994, Fournier and Brodeur 2000). Previous studies are usually difficult to compare to current use of neem products. Many studies used self-made neem extract formulations, preparations of pure azadirachtin or neem seed oil without defining the content of the neem toxins (e.g. Kraiss and Cullen 2008, Shah et al. 2017). For pure azadirachtin solutions, it remains often unclear, whether only azadirachtin A or all types of azadirachtin were used. The registered dose in Europe is equivalent to 30-50 ppm

azadirachtin A in the spraying solution for the commercial product NeemAzal-T/S (BVL 2017). However, some studies resulted in effective concentrations higher than 50 ppm azadirachtin (e.g. West and Mordue (Luntz) 1992, Nisbet et al. 1993). Results for these concentrations and varying azadirachtin formulations provide information that is of limited relevance for current application of neem products.

In general, neem products or azadirachtin formulations lead to an increased mortality and inhibition of development of aphid nymphs. This insect growth regulatory (IGR) effect is the most important lethal impact on aphids (Lowery and Isman 1993, Koul 1999, Mordue (Luntz) and Nisbet 2000). IGR effects result from the influence of azadirachtin on ecdysone and juvenile hormone titers in insect hemolymph (Barnby and Klocke 1990, Mitchell et al. 1997). These hormones are crucial for successful molting of insects. Ecdysone is responsible for the development of a new integument and exuvia shedding, while juvenile hormone controls the developmental stage at the time of molting (Mordue (Luntz) and Blackwell 1993, Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010). Treated aphid nymphs fail to complete the molting process and die (Lowery and Isman 1994). By contrast, lethal effects of neem on adults of hemipteran insects seem to be less clear. Nevertheless, juvenile hormone is involved in the production of eggs and living offspring in adult insects (Hardie 1987, Riddiford 2012). Sterility and reduced fecundity have been reported as effects of neem treatments on adult aphids (e. g. Stark and Rangus 1994, Lowery and Isman 1994, Fournier and Brodeur 2000, Mordue (Luntz) and Nisbet 2000, Pavela et al. 2004).

Many studies, especially between 1990 and 2010, have dealt with the impact of azadirachtin on aphids. A comparison of these studies reveals that within the family of Aphididae, the effective dose varies with species, developmental stage, type of exposure (e. g. systemically through roots, topical or via previously sprayed and dried leaves), formulation and also with the host plant (e.g. Dimetry and Schmidt 1992, Stark and Rangus 1994, Lowery et al. 1993, Lowery and Isman 1994, 1996, Koul 1998, Pavela et al. 2004). In *Aphis fabae*, exposure of adults to azadirachtin concentrations as low as 1 ppm reduced the fecundity (Dimetry and Schmidt 1992), while in *Brevicoryne brassicae* 50 to 60 ppm azadirachtin were necessary to be effective *(*Koul 1998, Pavela et al. 2004). In *Myzus persicae* and *Nasonovia ribisnigri* 40 to 80 ppm azadirachtin reduced numbers of offspring significantly, when provided as neem seed oil or as a pure azadirachtin formulation. In contrast, the reproduction of *Chaetosiphon fragaefolii* remained unaffected even at concentrations of 80 ppm azadirachtin (Lowery and Isman 1996). Older nymphal instars and adult aphids are generally less sensitive to lethal effects of azadirachtin (Dimetry and Schmidt 1992, Pavela et al. 2004). However, lethal concentrations for nymphs also vary with aphid species: LC_{50} (lethal concentration causing 50 % mortality) of azadirachtin for second instar nymphs on leaf disks is only 2.4 ppm for *Myzus persicae*. Intermediate values were recorded for *Macrosiphum euphorbiae* with an LC_{50} of 8 ppm or *Aphis gossypii* with 90 ppm azadirachtin. The LC_{50} of 635 ppm for *C. fragaefolii* forms a remarkable exception from the common rates (Lowery and Isman 1994).

This summary indicates that it is difficult to predict effects of neem treatments for a specific aphid species by comparison with results for other species. For *M. rosae*, only limited data are available. Koul (1999), for instance, observed fewer individuals molting and 100 % mortality for second instars of *M. rosae* on leaf disks treated with 30 or 60 ppm azadirachtin. The survival rate of adults was higher and 70 to 90 ppm azadirachtin was needed to reach more than 90 % mortality. This suggests that currently registered dosages of around 30 to 50 ppm azadirachtin only result in moderate

mortality of adults. It is recommended to evaluate neem products on more effects than only mortality. A reduction in fecundity is also an important effect for pest management; especially in cases where mortality of adult insects is not achieved. Neem is further considered as a slow-acting insecticide and experimental periods should not be shorter than one week; otherwise, relevant effects might be missed (Stark and Rangus 1994).

Many studies used leaf disks dipped in azadirachtin or neem seed oil solutions as food for aphids (e.g. Lowery and Isman 1993a, 1994, 1996, Monteiro dos Santos et al. 2004). This might represent a much higher coverage of leaves with the treatment solution than would be the case in common spraying applications just before runoff. In applications with fine droplets provided by high-pressure spraying devices, plant leaves are normally not totally covered by the solution. With the following translocation to untreated plant parts, concentrations of azadirachtin on leaves will be diluted. Dipped leaf disks can have a higher impact on aphids than a realistic spray application on intact plants might have. Additionally, leaf disks can influence the resistance of plants against aphids compared to intact plants (ten Broeke et al. 2016). Here, experiments with clip cages on intact plants were chosen over leaf disk and excised leaf experiments to have realistic conditions.

The objective of this study was to assess in detail how registered doses of a commercial formulation, NeemAzal-T/S, affect *M. rosae*. First, standard efficacy trials were carried out to compare the effect of this neem product to that of a synthetic pesticide. Second, the influence of NeemAzal-T/S on *M. rosae* population increase was investigated. For a better evaluation of effects, clip-cage experiments were carried out, which allow for the exact counting and observing of aphids. Nymphal development and mortality were assessed as well as the mortality of all developmental stages separately. Furthermore, the reproduction of adult rose aphids was compared, when they were exposed to neem as early as juveniles and as adults. The blank formulation of NeemAzal-T/S was included in all experiments to allow a differentiation between effects of the active ingredients and formulation effects, e.g. mortality due to asphyxiation by the contained oils (Cranshaw and Baxendale 2013).

Materials and methods

General materials for all experiments

Insects and plants

The rose aphid *Macrosiphum rosae* was used in all experiments. A red phenotype of *M. rosae*, originated from a naturally occurring female on a cultivated rose in Monheim am Rhein, Germany, was reared on potted miniature roses (family Rosaceae, genus *Rosa*, different Kordana® varieties by W. Kordes' Söhne Rosenschulen GmbH & Co KG) in cages made of acrylic glass with three sides of gauze. Dimensions of the cages were 63 cm x 78 cm and 63 cm high. Aphids were maintained at 22±2 °C, 60±10 % relative humidity and 16:8 light:dark (L:D) photoperiod in a climate chamber.

Miniature rose plants were used as freshly rooted cuttings in pots. For the *M. rosae* rearing and experiments, one or two plants - depending on the experiment - were transferred to 11 or 12 cm diameter pots with Einheitserde Classic, Type ED 73, 155 fine, as substrate. Plants were kept in an air-conditioned greenhouse at 21±3 °C with a relative humidity set at 60 %. A photoperiod of 16:8 L:D was maintained. During this time, additional light was provided by sodium vapor lamps if sunlight intensity outside the greenhouse fell under 120 W/m². A few days after repotting, plants were

drenched with the liquid fertilizer Wuxal Top N (Manna, Germany), an NPK (12-4-6) fertilizer solution with micronutrients, used in a dose of 0.3 %. Fertilization was repeated if required until the start of the experiments. In the case of a rare powdery mildew infestation before the experiments, plants were sprayed with fungicides according to current registrations for fungicides in roses in Germany (Compo Ortiva Universal Pilzfrei, active ingredient azoxystrobin and Bayer Garten Rosen Pilz-frei Baymat, active ingredient tebuconazole). Fungicide treatments were latest done one week before the experiments started to exclude unforeseen side effects by contact. Furthermore, all plants were sprayed to maintain equal conditions between the treatments. In these conditions, plants were maintained until use for experiments for three to four weeks. For experiments, plants were used in a stage where flower buds had already developed, but were still closed (BBCH stage 54-58, Meier et al. 2009). Experimental plants were preselected for similar habitus and condition and equally distributed over treatments and control. All trials were conducted in an air-conditioned greenhouse as described above between 2014 and 2016.

Treatments

NeemAzal-T/S produced by Trifolio-M GmbH, Lahnau, Germany, was used as a common and registered neem-based insecticide in Europe. It is an emulsifiable concentrate (EC) with 10 g of azadirachtin A per liter as its major active ingredient, by a maximal amount of 3-4 % natural neem seed kernel extract (NeemAzal technical, Trifolio-M GmbH 2015). Azadirachtin is a tetranortriterpenoid and belongs to IRAC group “UN: Compounds of unknown or uncertain Mode of Action” (IRAC 2017). In most experiments, different concentrations of NeemAzal-T/S were used. For miniature roses smaller than 50 cm, as used in this study, 0.3 ml of the product per m² is registered as dose rate. With a slurry volume of 900 l/ha, 3.3 ml product/l water are the common dose rate. Plants were sprayed until run off while ensuring a complete coverage of plants. This dose rate is equivalent to 30 g azadirachtin A/ha per application. Up to four applications per culture per year are allowed in Germany (BVL 2017) at an interval of seven to ten days.

To be able to discriminate between active-ingredient and formulation effects, a treatment with the blank formulation of NeemAzal-T/S (referred to ‘NeemAzal-T/S Blank’ below, provided by Trifolio-M GmbH, Lahnau, Germany) was added to most of the studies. This product does not contain the NeemAzal extract, but only tensides and plant oils in the same amount as used as formulation in the original product. In most experiments, NeemAzal-T/S Blank was only used in the highest concentration as NeemAzal-T/S was used to minimize numbers of treatments and to allow more replicates for the remaining treatments. As a positive control, a novel flupyradifurone product was added to the efficacy studies described below. Flupyradifurone is a butenolide and belongs to IRAC MoA group 4D: Nicotinic acetylcholine receptor (nAChR) competitive modulators and has an impact on the nervous system of insects (IRAC 2017). The used formulation is a soluble (liquid) concentrate, containing 50 g flupyradifurone per liter. It was used in a concentration with 1.33 ml/l water. Spraying solutions were either applied with a manual trigger spray bottle or a handheld sprayer, operated by compressed air with 3 bar pressure and a 1.1 mm bore hollow cone nozzle. A detailed description of generally used treatments and application is given in Table 1.

Table 1 Overview of treatments, type of formulation and corresponding dosages and active ingredient (a.i.) contents. Formulation type: EC: Emulsifiable concentrate, SL: Soluble concentrate.

Treatment name	**Formulation type**	**Active ingredient** (a.i.)	**a.i. in product** [g/l]	**Dose per application** [g a.i./ha]	**Application dose spraying solution** [ml product/l water]
Control	(untreated)	-	-	-	-
NeemAzal-T/S Blank	EC	no a. i.	0	-	-
NeemAzal-T/S 1.65 ml/l	EC	Azadirachtin A	10	15	1.65
NeemAzal-T/S 2.5 ml/l	EC	Azadirachtin A	10	22.5	2.5
NeemAzal-T/S 3.3 ml/l	EC	Azadirachtin A	10	30	3.33
Flupyradifurone	SL	Flupyradifurone	50	60	1.33

Efficacy trials

Two efficacy trials with potted miniature rose plants and *M. rosae* were conducted in the greenhouse. Conditions in the greenhouse compartment are as described above. The experimental design was in accordance to EPPO guideline PP 1/023(2) for efficacy evaluation of plant protection products “Aphids on ornamental plants”, but with half the number of plants per plot: Four plots with five rose plants per plot were used per treatment instead of ten plants per plot (EPPO 1997). Each plot represented a replicate. Two miniature rose plants per 11 cm diameter pot were used as experimental plants. Rose plants were maintained as described previously. The plots were arranged in a completely randomized design in the greenhouse after artificial infestation with aphids from the main culture. Approximately 50 aphids of mixed developmental stages and ages were transferred with a fine brush onto experimental plants one week before the start of the experiment. In the second trial, the application was made four days after aphid infestation to treat plants with a lower number of aphids. Plants were watered before and throughout the experiment directly onto the soil and via the irrigation mats underneath the pots. Greenhouse conditions were the same as described above.

The following treatments were used: An untreated control, water control, 3.3 ml/l NeemAzal-T/S Blank, 1.65 ml/l, 2.5 ml/l or 3.3 ml/l NeemAzal-T/S and 1.33 ml/l Flupyradifurone SL 50. Water and products were applied with a handheld sprayer, operated by compressed air with 3 bar pressure and a 1.1 mm bore hollow cone nozzle. Water, NeemAzal-T/S blank and NeemAzal-T/S were applied two times, i.e. at day 0 and day 7. Flupyradifurone SL 50 was only applied at day 0.

Total numbers of aphids per plant were counted as estimated groups of five to ten individuals. The evaluations were made before the first application and 7 and 14 days after the first application (DAA) for trial 1. For trial 2, infestation was also assessed on days 21 and 29. Evaluation at 7 DAA was completed before the second application was implemented.

Efficacy was calculated as follows (Henderson and Tilton 1955):

$$Efficacy_{HT}\ [\%] = \left(1 - \frac{T_a}{T_b} \times \frac{C_b}{C_a}\right) \times 100$$

$$T_b = Infestation\ on\ treated\ plant\ before\ application$$
$$T_a = Infestation\ on\ treated\ plant\ after\ application$$
$$C_b = Infestation\ on\ control\ plants\ before\ application$$
$$C_a = Infestation\ on\ control\ plants\ after\ application$$

Statistical analysis was carried out with the trial management software ARM (Agriculture Research Manager, revision 2017, Gylling Data Management, Inc., United States of America). Numbers of aphids per plant and efficacy, calculated by the method of Henderson-Tilton (Henderson and Tilton 1955, see formula above) were analyzed with Student-Newman-Keuls (SNK) test ($P < 0.05$) separately for each evaluation day after data transformation (square root or log10), if the data was not normally distributed.

Population development

For assessing the population development of *M. rosae* as affected by NeemAzal-T/S, a synchronized aphid rearing was used. For obtaining adult females of the same age, about 50 random adult females from the main culture were separated into five clip cages on untreated miniature rose plants in pots. They were kept in a climate chamber at 22±2 °C, 60±10 % relative humidity and 16:8 L:D photoperiod for 24 hours to produce nymphs. After this period, clip cages and adult females were removed and the nymphs were left on the rose plants without clip cages. The plants were placed in an acrylic glass cage with three gauze sides of 63 cm x 78 cm and 63 cm high for another 11 days. Ten miniature rose plants per treatment were sprayed with a handheld spraying bottle for the following treatments: 3.3 ml/l NeemAzal-T/S Blank, 1.65 ml/l and 3.3 ml/l NeemAzal-T/S. In addition, ten untreated plants for the control were added to the study. After drying of the plants, each plant was placed onto a saucer. Ten water-filled bowls of 40 cm x 59 cm x 6 cm high were placed on a greenhouse table. In the water, additional saucers in an upside-down position served as islands on which the experimental plants on saucers were placed in a randomized manner. The water around the plants prevented escaping of apterous aphids or migration to other plants. An 11-12 day-old (since larviposition) *M. rosae* female from the synchronized rearing was transferred to each plant and the total number of aphids was counted 1, 2, 4, 6, 8, 10, 12 and 16 days later. The experiment was located in an air-conditioned greenhouse compartment as described previously. Modelling of population growth over time was carried out in R (version 3.1.3, The R Foundation for Statistical Computing, 2015) with the function “gamm” for generalized additive mixed models (GAMM). A Poisson distribution with log link was used for the count data and a smoothing spline per treatment was fitted. Required packages were ggplot2, reshape2 and mgcv. Predicted values of GAMM were plotted with function “ggplot”.

Nymphal mortality and developmental time

A clip-cage experiment in the greenhouse was conducted to investigate the mortality and development of *M. rosae* nymphs on untreated and NeemAzal-T/S treated plants. The following

treatments were used: untreated control, 3.3 ml/l NeemAzal-T/S Blank and 1.65 ml/l, 2.5 ml/l or 3.3 ml/l NeemAzal-T/S. Ten miniature rose plants per treatment were sprayed with a handheld sprayer, operated by compressed air with 3 bar pressure and a 1.1 mm bore hollow cone nozzle. After drying of the plants, a clip-cage with five to ten randomly picked adult *M. rosae* from the main culture were introduced onto each rose plant. 24 hours later, adults were removed from the clip cage and their offspring was observed for 14 days. Mortality and development were evaluated once per day. To determine the developmental stages, exuvia were counted daily to identify the nymphal stages. Mortality of aphids was analyzed in R (version 3.1.3, The R Foundation for Statistical Computing, 2015) with the functions "survfit" and "coxph" of the package survival. With the function "coxph", a Cox proportional hazards model was fitted and the Tukey test was used as a post-hoc test for an all-pair comparison of the risk for aphids to die in the different treatments. This was realized via the function "glht" of the package multcomp. Mortality of the control group was used as the baseline hazard. Results were interpreted with regard to the increase in hazard to die for aphids under the different treatments compared to the untreated control group. Interpretation of output of "coxph" was in accordance to Thernau and Grambsch (2000) and Fox and Weisberg (2011). Developmental times were analyzed by comparing the time until the first molting from the first nymphal stage to the second. The probability to develop earlier was also calculated with the Cox proportional hazards model as a time to event (molting). The packages and functions used were the same as described above for mortality. For further analysis of the developmental times, the mortality was too high in some treatments and, therefore, numbers of aphids were too small for meaningful statistics.

Developmental-stage-specific effect

For testing the effect of NeemAzal-T/S separately on each developmental stage of *M. rosae*, synchronized rearing for each stage was arranged before the experiment. Eleven days before the start of the experiment, randomly picked adult females from the main culture were placed in clip cages on non-infested miniature rose plants for 24 hours. After this time, adults were removed and emerged nymphs were maintained without clip cages in a rearing cage for additional ten days until the beginning of the experiment. The same procedure was done again 8, 6, 4 and 1 days before start of the experiment to have five separated *M. rosae* cultures of different ages. Every rearing was consisted of one specific developmental stage and these aphids were used for the clip-cage experiment. A total of 30 experimental plants per treatment were prepared. These 30 potted rose plants remained untreated for the control, 30 plants were sprayed with 3.3 ml/l NeemAzal-T/S Blank and 30 were sprayed with 3.3 ml/l NeemAzal-T/S. After drying, aphids were transferred onto the rose plants in clip cages. Per treatment and developmental stage, six clip cages were used, each with five aphids. A clip cage represents a replicate per stage. Clip cages were checked daily and dead aphids were counted for seven days. Plants were arranged in a greenhouse compartment in completely randomized design. Mortality was analyzed with the Cox proportional hazards model in R (version 3.1.3, The R Foundation for Statistical Computing) for each developmental stage separately. A post-hoc Tukey test was used for an all-pair comparison between the treatments. Applied packages are described in the previous section.

Reproduction

Reproduction of *M. rosae* as affected by NeemAzal-T/S was investigated in two clip-cage experiments in the greenhouse. Different cultures of *M. rosae* were prepared for these experiments: aphids cultured on either untreated, NeemAzal-T/S Blank or NeemAzal-T/S treated plants. First, nymphs of the same age were produced by keeping adult females from the main rearing in clip cages for 24 hours. Their offspring were then transferred with a fine brush to untreated plants and plants which were either sprayed with 2.5 ml/l NeemAzal-T/S Blank, 1.25 ml/l NeemAzal-T/S or 2.5 ml/l NeemAzal-T/S. Each rearing was maintained separately in rearing cages of 63 cm x 78 cm and 63 cm high in a climate chamber at 22±2 °C, 60±10 % relative humidity and 16:8 L:D photoperiod for further nine days. Consequently, aphids were ten days old since larviposition at the start of the experiment and already exposed to sub-lethal concentrations of NeemAzal-T/S during their development. The experimental plants were sprayed either with 3.3 ml/ NeemAzal-T/S Blank or with 1.25 ml/l, 2.5 ml/l or 3.3 ml/l NeemAzal-T/S. In addition, one completely untreated group was added to the study. This resulted in 13 different treatment combinations which are listed in Table 2. Per combination, eight replicates were prepared, each comprising of one plant with one clip cage containing a single adult aphid. Reproduction was assessed daily by counting numbers of nymphs in the clip cages and removing them, during a period of seven days. Mean numbers of offspring per female and day were compared using ANOVA with Tukey HSD post hoc test in SPSS software (version 22, IBM). Generalized linear models were fitted separately for each day with offspring per female as dependent variable. Treatment of plants for aphid rearing and treatment of experimental plants were added as factors. Either the Poisson or negative binomial distribution with log-link function was chosen depending on the model fit. Significance was assessed by pairwise comparisons with Bonferroni adjustment in SPSS (version 22, IBM). Graphs were built in SPSS and adjusted in CorelDraw Graphics Suite 2017 (Corel Corperation).

Table 2 Combinations of treatment of *M. rosae* culture and treatment of experimental plants for reproduction trials.

Combination No.	Treatment of plants for *M. rosae* rearing before the experiment	Treatment of experimental plants
1	Untreated	Untreated
2	Blank 2.5 ml/l	Blank 3.3 ml/l
3	Blank 2.5 ml/l	NeemAzal-T/S 1.25 ml/l
4	Blank 2.5 ml/l	NeemAzal-T/S 2.5 ml/l
5	Blank 2.5 ml/l	NeemAzal-T/S 3.3 ml/l
6	NeemAzal-T/S 1.25 ml/l	Blank 3.3 ml/l
7	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 1.25 ml/l
8	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 2.5 ml/l
9	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 3.3 ml/l
10	NeemAzal-T/S 2.5 ml/l	Blank 3.3 ml/l
11	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 1.25 ml/l
12	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 2.5 ml/l
13	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 3.3 ml/l

Results

Efficacy trials

None of the tested NeemAzal-T/S concentrations led to a reduction in numbers of *M. rosae* per plant compared to the initial infestation in both efficacy trials (Tables 3 and 4). However, the total number of aphids per plant was not as high as in the untreated control and the highest tested NeemAzal-T/S concentration reached around 40 % efficacy in both trials. Numbers of aphids on flupyradifurone treated rose plants (positive control) were significantly lower than in the other treatments on all evaluation dates after application. The efficacy of flupyradifurone was significantly higher compared to all other treatments with the exception of 14 DAA in trial 1, where differences to NeemAzal-T/S 2.5 ml/l and 3.3 ml/l were not significant (Table 3). Application of flupyradifurone resulted in efficacies over 90 % on 3 DAA and 7 DAA in both trials. In contrast, the efficacies of water and NeemAzal-T/S Blank were generally lower than those of 2.5 ml/l and 3.3 ml/l NeemAzal-T/S, but differences were only significant on 7 DAA in trial 1 (Table 3) and 14 DAA in trial 2 (Table 4).

Table 3 Results of efficacy trial 1: Mean (±SE) numbers of *M. rosae* per plant and mean (±SE) efficacy in % (Henderson-Tilton) before application and 3, 7 and 14 days after first application (DAA) of different concentrations of NeemAzal-T/S and flupyradifurone. N = 4 plots per treatment, each plot with 5 plants.DA2: Days after second application (Flupyradifurone only one application). Means within a column marked with different letters are significantly different according to Student-Newman-Keuls test after data transformation (square root or log10, $P < 0.05$).

	Before application		**3 DAA**			**7 DAA**			**14 DAA/7 DA2**		
	Numbers of aphids per plant		Numbers of aphids per plant		Efficacy [%]	Numbers of aphids per plant		Efficacy [%]	Numbers of aphids per plant		Efficacy [%]
Control (untreated)	222 ± 14	a	444 ± 32	a	-	676 ± 24	a	-	821 ± 53	a	-
Water	240 ± 11	a	423 ± 34	a	12 ± 2 b	684 ± 37	a	6 ± 8 c	713 ± 24	a	18 ± 8 b
NeemAzal-T/S Blank 3.3 ml/l	239 ± 14	a	412 ± 17	a	13 ± 5 b	626 ± 32	a	13 ± 7 c	818 ± 77	a	6 ± 10 b
NeemAzal-T/S 1.65 ml/l	229 ± 28	a	358 ± 71	a	25 ± 6 b	503 ± 48	a	27 ± 4 bc	778 ± 69	a	3 ± 16 b
NeemAzal-T/S 2.5 ml/l	246 ± 17	a	285 ± 18	a	39 ± 9 b	490 ± 49	a	34 ± 6 bc	576 ± 47	a	36 ± 5 ab
NeemAzal-T/S 3.3 ml/l	270 ± 11	a	362 ± 33	a	32 ± 7 b	462 ± 55	a	43 ± 9 b	571 ± 47	a	43 ± 5 ab
Flupyradifurone SL 50 1.3 ml/l	255 ± 13	a	29 ± 22	b	94 ± 4 a	35 ± 28	b	95 ± 4 a	269 ± 88	b	72 ± 10 a

Table 4 Results of efficacy trial 2: Mean (±SE) numbers of *M. rosae* per plant and mean (±SE) efficacy in % (Henderson-Tilton) before application and 3, 7 and 14 days after first application (DAA) of different concentrations of NeemAzal-T/S and flupyradifurone. N = 4 plots per treatment, each plot with 5 plants. DA2: Days after second application (Flupyradifurone only one application). Means within a column marked with different letters are significantly different according to Student-Newman-Keuls test after data transformation (square root or log10, $P < 0.05$).

	Before application	**3 DAA**		**7 DAA**		**14 DAA/7 DA2**		**21 DAA/14 DA2**		**29 DAA/21 DA2**	
	Numbers of aphids per plant	Numbers of aphids per plant	Efficacy [%]	Numbers of aphids per plant	Efficacy [%]	Numbers of aphids per plant	Efficacy [%]	Numbers of aphids per plant	Efficacy [%]	Numbers of aphids per plant	Efficacy [%]
Control (untreated)	115 ± 8 a	189 ± 22 a	-	235 ± 23 a	-	346 ± 14 ab	-	531 ± 26 a	-	630 ± 19 a	-
Water	118 ± 7 a	185 ± 13 a	1 ± 12 b	250 ± 17 a	-5 ± 8 b	369 ± 14 a	-4 ± 6 c	494 ± 27 a	9 ± 9 b	596 ± 16 a	7 ± 8 b
NeemAzal-T/S Blank 3.3 ml/l	112 ± 12 a	151 ± 16 a	14 ± 13 b	222 ± 8 a	-2 ± 12 b	254 ± 13 ab	22 ± 8 bc	429 ± 22 a	14 ± 12 b	546 ± 44 a	8 ± 13 b
NeemAzal-T/S 1.65 ml/l	103 ± 5 a	153 ± 17 a	6 ± 15 b	216 ± 30 a	-6 ± 17 b	270 ± 36 ab	14 ± 9 bc	462 ± 44 a	2 ± 11 b	560 ± 51 a	1 ± 10 b
NeemAzal-T/S 2.5 ml/l	117 ± 6 a	141 ± 5 a	25 ± 6 b	186 ± 13 a	21 ± 6 b	229 ± 15 ab	34 ± 8 b	342 ± 20 a	33 ± 12 b	477 ± 43 a	23 ± 12 b
NeemAzal-T/S 3.3 ml/l	105 ± 4 a	142 ±17 a	12 ± 17 b	177 ± 27 a	13 ± 18 b	193 ± 2 b	39 ± 2 b	337 ± 30 a	31 ± 6 b	475 ± 29 a	18 ± 6 b
Flupyradifurone SL 50 1.3 ml/l	131 ± 12 a	9 ± 5 b	96 ± 2 a	21 ± 12 b	93 ± 4 a	63 ± 39 c	86 ± 8 a	136 ± 57 b	77 ± 10 a	154 ± 52 b	79 ± 7 a

Population Development

An application of 1.65 ml/l and 3.3 ml/l NeemAzal-T/S led to a stagnating population growth, compared to the untreated control and a NeemAzal-T/S Blank treatment, when population development had started with one adult *M. rosae* female (Fig. 1). An exponential increase in numbers of aphids per rose plant was assessed after 12 days, while this increase was missing in both NeemAzal-T/S treatments. 3.3 ml/l NeemAzal-T/S had a significant ($P < 0.05$) influence on the numbers of aphids per plant for the observation period of 16 days.

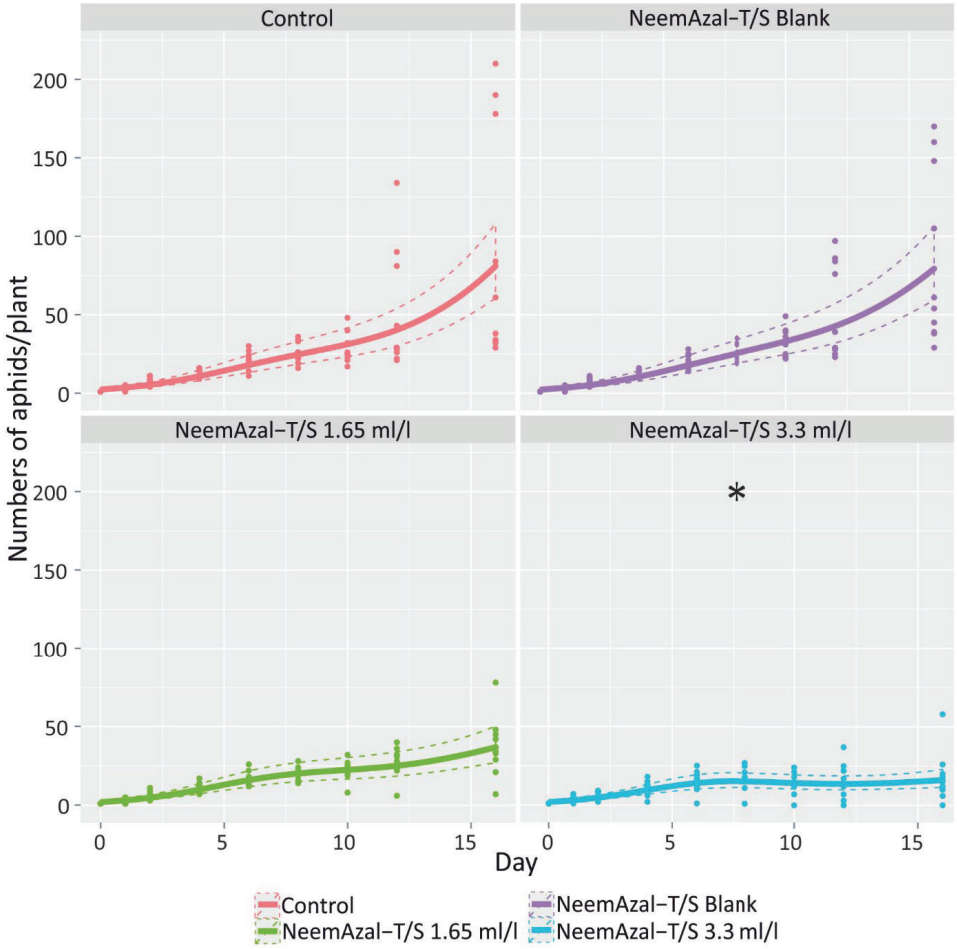

Fig. 1 Population growth of *M. rosae* on untreated, NeemAzal-T/S Blank or NeemAzal-T/S treated plants starting with one 12-day-old (since larviposition) adult female. Results of a generalized additive mixed model (GAMM) with Poisson distribution and log-transformed data for each treatment separately. Thin lines: Absolute numbers of aphids per plant for each replication. Thick line: function for average numbers of *M. rosae* per plant predicted by the GAMM for each treatment. The area within the dashed lines around this function describes an approximation of a 95 % confidence interval for the predicted values. Asterisk means significant ($P < 0.05$) influence of treatment compared to the untreated control on numbers of aphids per plant.

Nymphal mortality and development

Mortality of *M. rosae* nymphs was significantly higher in all tested NeemAzal-T/S treatments compared to the untreated control and NeemAzal-T/S Blank. All observed nymphs in the neem treatments died after 7 (3.3 ml/l NeemAzal-T/S), 9 (1.65 ml/l NeemAzal-T/S) and 11 (2.5 ml/l NeemAzal-T/S) days. Until day 4, more than 50 % of these aphids had already died (Fig. 2). The risk for *M. rosae* nymphs to die on NeemAzal-T/S-treated rose plants is significantly increased and higher with increasing NeemAzal-T/S concentrations. However, differences between the three tested product concentrations were not significant (Table 5). NeemAzal-T/S Blank showed a similarly low mortality as the control (Fig. 2) and did not pose a significantly higher risk for aphids to die (Table 5).

First instar nymphs of *M. rosae* have a significantly increased probability to molt later to the second instar compared to nymphs on NeemAzal-T/S Blank or untreated rose plants (Table 6). The delay in development becomes clear if developmental stages are compared for all treatments. On day 5, the majority of living nymphs had reached the second or third nymphal instar on untreated and NeemAzal-T/S Blank treated plants while in the NeemAzal-T/S treatments only first and second instars were found (Fig. 3). NeemAzal-T/S Blank did not influence the nymphal developmental time to the second instar (Table 6) and showed a similar percentage distribution of the developmental stages as the control on day 5.

Developmental stage specific efficacy

The mortality of *M. rosae* on rose plants treated with 3.3 ml/l NeemAzal-T/S decreased with development to older instars (Fig. 4). First, second and third instar nymphs had a significantly ($P < 0.001$) higher risk to die due to NeemAzal-T/S compared to the untreated control (Table 7). The hazard ratio of dying (HR) is the highest for the first instar, followed by the second and third instar in the NeemAzal-T/S treatment. The HR for the first instar is nearly ten times higher than for the third instar, but differences in HR between instars were not statistically analyzed. The mortality of fourth instars and adult *M. rosae* was not significantly higher on NeemAzal-T/S treated rose plants compared to the control and NeemAzal-T/S Blank. Furthermore, NeemAzal-T/S Blank did not pose a significantly higher hazard to die for all instars compared to the control (Table 7).

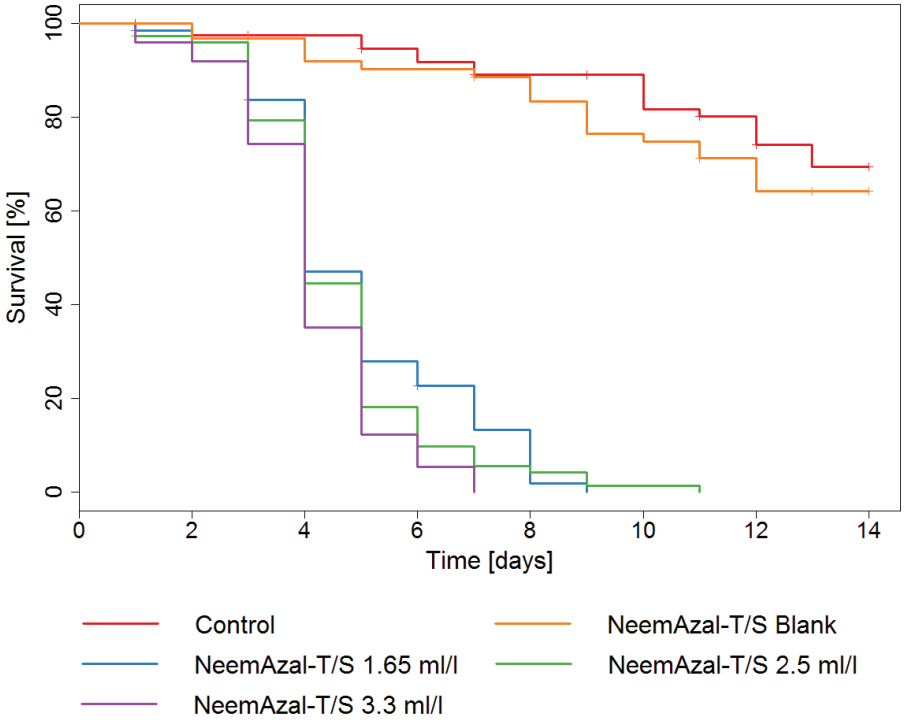

Fig. 2 Survival of *M. rosae* first instar nymphs over 14 days on untreated (control) rose plants and plants treated with NeemAzal-T/S Blank or 1.65 ml/l, 2.5 ml/ and 3.3 ml/l NeemAzal-T/S. Initial total numbers of observed aphids varied between 62 and 78 individuals per treatment.

Table 5 Mortality of *M. rosae* first instar nymphs on untreated, NeemAzal-T/S Blank and NeemAzal-T/S treated rose plants. Results of Cox proportional hazards model with treatment "Control" as the baseline hazard group. Hazard ratios (HR) mean the multiplicative change in risk to die compared to the control. N = 10 replications (clip cages with 4 to 14 aphids) per treatment.

Treatment	Hazard ratio (HR)[1]		95 % CI	p-value
Control	1[2]	b	-	-
NeemAzal-T/S Blank	1.3	b	0.7 - 2.3	0.61
NeemAzal-T/S 1.65 ml/l	22.8	a	12.53 - 41.4	< 0.001
NeemAzal-T/S 2.5 ml/l	26.9	a	15 - 47.9	< 0.001
NeemAzal-T/S 3.3 ml/l	36.3	a	19.9 - 66.2	< 0.001

[1] Different letters indicate significant ($P < 0.05$) differences in hazard ratios according to Tukey post-hoc test.
[2] HR for Control set to 1 as baseline hazard.

Table 6 Mean developmental time (±SE) in days for *M. rosae* nymphs from first to second instar and probability to develop to the second instar later than in the Control treatment. Results of Cox proportional hazards model. Significance codes: *** p < 0.001, n.s. = not significant.

Treatment	Developmental time to second instar [days, mean ±SE]	n[1]	Probability to develop later than in Control [%]	
Control	2.9 ± 0.3	73		
NeemAzal-T/S Blank	2.9 ± 0.2	53	8	n.s.
NeemAzal-T/S 3.3 ml/l	4 ± 0	2	96.9	***
NeemAzal-T/S 2.5 ml/l	3.1 ± 0.4	10	85.8	***
NeemAzal-T/S 1.65 ml/l	3.5 ± 0.4	11	84.2	***

[1] n = numbers of aphids for which molting has been observed, pooled over all replications

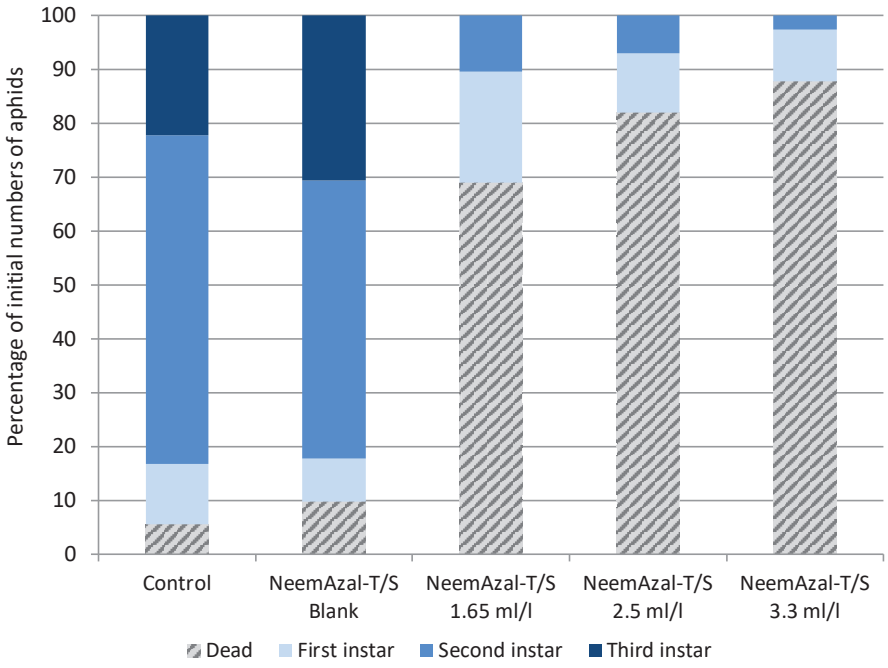

Fig. 3 Comparison of development progress of *M. rosae* nymphs. Percentage of initial numbers of *M. rosae* individuals in the first, second and third instar, as well as percentage of dead aphids after 5 days of exposure to untreated, NeemAzal-T/S Blank or NeemAzal-T/S treated rose plants. Pooled data over all replicates per treatment. Initial numbers of observed aphids varied between 62 and 78 aphids per treatment.

Table 7 Mortality risk for different instars of *M. rosae* on NeemAzal-T/S treated rose plants. Results are shown as hazard ratio (HR) for mortality with the control as baseline hazard group according to Cox proportional hazards model. Hazard ratios mean the multiplicative change in risk to die compared to the control. N = 6 replications (clip cages, each with 5 aphids) per instar. Within each instar, asterisks are showing a significant influence of the treatment. Significance codes: *** $p < 0.001$, n.s. = not significant.

Treatment	Hazard ratio (HR) (95 % CI)				
	First instar	**Second instar**	**Third instar**	**Fourth instar**	**Adult**
Control[1]	1	1	1	1	1
NeemAzal-T/S Blank	1 n.s. (0.1-13.2)	2.1 n.s. (0.2-18.6)	1.1 n.s. (0.1-14)	1 n.s. (0.4-2.4)	1 n.s. (0.3-3.3)
NeemAzal-T/S 3.3 ml/l	275.5*** (13-5838.1)	63.2*** (9.4-422.6)	30.7*** (4.7-199.1)	1.1 n.s. (0.6-2)	1.3 n.s. (0.4-4.2)

[1] Values for Control as baseline hazard are set to 1.

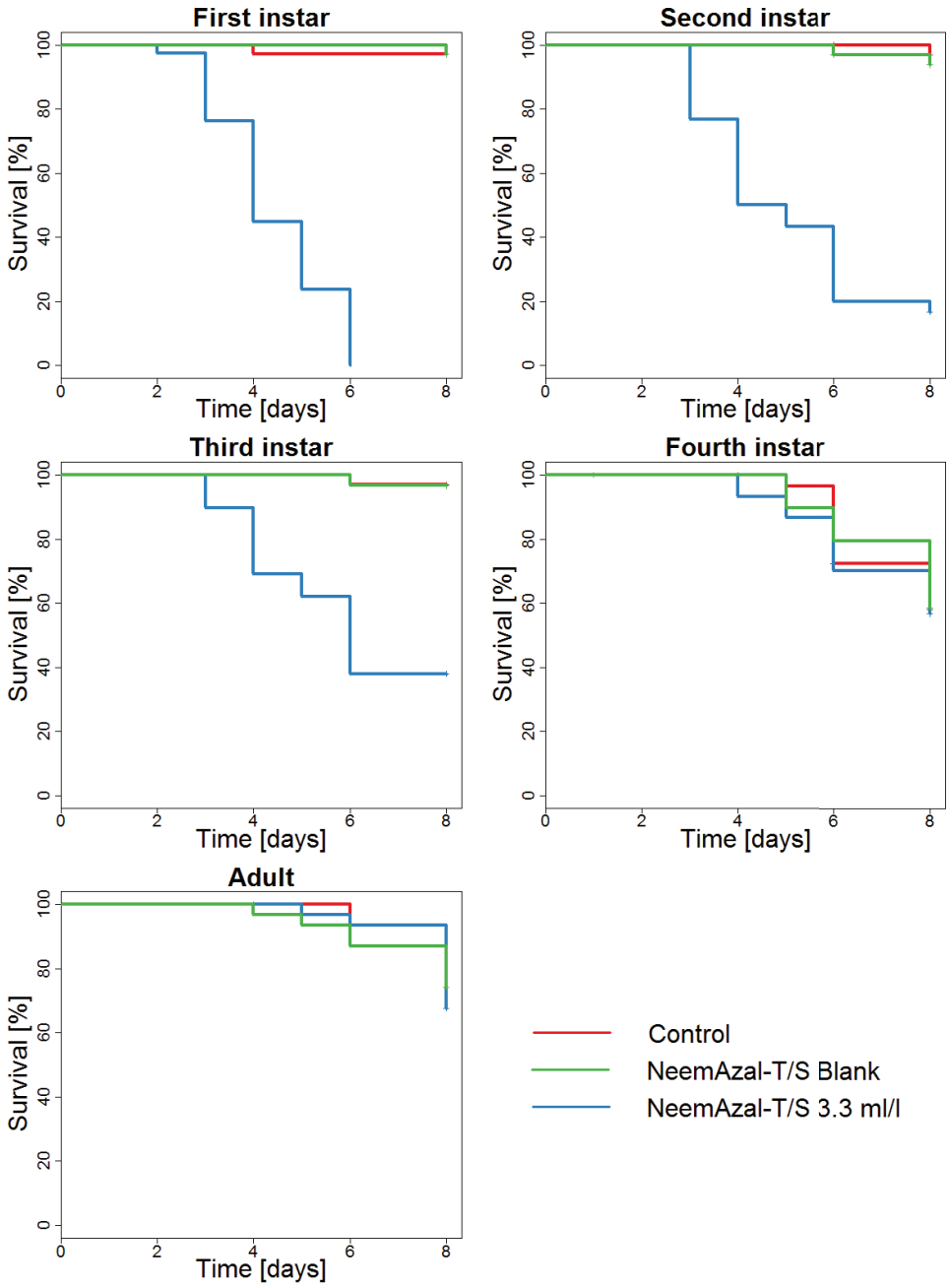

Fig. 4 Survival of *M. rosae* exposed as different developmental stages to untreated (Control) rose plant or plants treated with NeemAzal-T/S Blank or 3.3 ml/l NeemAzal-T/S. N = 30 aphids per instar and treatment.

Reproduction

Reproduction of *M. rosae* was negatively affected by exposure to sublethal concentrations of NeemAzal-T/S during their juvenile phase (Table 8). The mean number of offspring was significantly lower in both trials, when *M. rosae* were exposed to rose plants sprayed with 2.5 ml/l NeemAzal-T/S during their development, followed by an exposure to a plant without azadirachtin (NeemAzal-T/S Blank, treatment combination 10) compared to treatment combination 5, where the adult aphids were transferred from Blank treated rearing plants to experimental plants treated with 2.5 ml/l NeemAzal-T/S (Table 8). In both trials, the treatment during juvenile development was more often the significant factor influencing reproduction, when days were considered separately ($P < 0.05$, Table 9).

Regarding numbers of offspring per female on days 2, 4 and 6, a significant influence of the treatment combination was recorded on days 4 and 6 in experiment 1 and on day 2 in experiment 2 (Table 9). An all-pair comparison (GLMs, Bonferroni adjusted) resulted in statistical differences between treatment combinations 1, 2, 3, and 5 compared to combination 10 (Fig. 5) on day 6 in the first experiment and combination 5 compared to combinations 7, 9, 10 and 13 on day 2 in the second experiment (Fig. 6). No statistically significant differences were recorded on the other days.

Table 8 Reproduction of *M. rosae* as mean numbers (±SE) of offspring per day per female *M. rosae* over seven days in different combinations of pre-experimental rearing treatments and experimental plant treatments with NeemAzal-T/S. Different letters within the same column mean significant ($P < 0.05$) differences in mean numbers of offspring per female and day according to ANOVA with Tukey post hoc test. N = 8 adult female aphids per treatment combination.

	Treatment plants of *M. rosae* rearings before the experiment	**Treatment of experimental plants**	**Mean offspring/day/female ±SE**			
			Trial 1		**Trial 2**	
1	Untreated	Untreated	3.9 ± 0.4	ab	3.6 ± 0.3	ab
2	Blank 2.5 ml/l	Blank 3.3 ml/l	4.0 ± 0.4	a	3.5 ± 0.3	ab
3	Blank 2.5 ml/l	NeemAzal-T/S 1.25 ml/l	4.1 ± 0.4	a	3.0 ± 0.3	abcd
4	Blank 2.5 ml/l	NeemAzal-T/S 2.5 ml/l	4.1 ± 0.4	a	3.3 ± 0.3	abc
5	Blank 2.5 ml/l	NeemAzal-T/S 3.3 ml/l	4.3 ± 0.5	a	4.4 ± 0.3	a
6	NeemAzal-T/S 1.25 ml/l	Blank 3.3 ml/l	2.3 ± 0.6	ab	1.8 ± 0.5	bcde
7	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 1.25 ml/l	2.2 ± 0.4	ab	1.5 ± 0.5	cde
8	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 2.5 ml/l	3.0 ± 0.5	ab	1.9 ± 0.6	bcde
9	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 3.3 ml/l	2.8 ± 0.6	ab	1.1 ± 0.5	de
10	NeemAzal-T/S 2.5 ml/l	Blank 3.3 ml/l	1.4 ± 0.4	b	1.0 ± 0.3	e
11	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 1.25 ml/l	2.4 ± 0.8	ab	2.1 ± 0.5	bcde
12	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 2.5 ml/l	2.3 ± 0.6	ab	2.1 ± 0.4	bcde
13	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 3.3 ml/l	1.8 ± 0.7	ab	1.2 ± 0.4	de

Table 9 Reproduction of *M. rosae* depending on NeemAzal-T/S treatments during the development (rearing treatment) or as adults (experimental plant treatment): Statistical results of model effects of GLMs separately for numbers of offspring per female for every day of both experiments. Significance codes: ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, n.s.: not significant. Due to mortality, n varied with time between 104 observed females on day 1 to 86 (experiment 1) and 65 (experiment 2) females on day 7.

Exp. No.	Day	Model type with log-link	Results of model effects: *P*-value		
			Rearing treatment	Experimental plant treatment	Interaction (Treatment combination)
1	1	neg. binomial	0.067 n.s.	0.729 n.s.	0.933 n.s.
	2	neg. binomial	0.012*	0.528 n.s.	0.920 n.s.
	3	Poisson	< 0.001 ***	0.160 n.s.	0.533 n.s.
	4	Poisson	< 0.001 ***	0.845 n.s.	0.049 *
	5	Poisson	< 0.001 ***	0.717 n.s.	0.209 n.s.
	6	Poisson	< 0.001 ***	0.002 **	0.016 *
	7	Poisson	0.009 **	0.981 n.s.	0.221 n.s.
2	1	Poisson	< 0.001 ***	0.198 n.s.	0.006 **
	2	Poisson	< 0.001 ***	0.082 n.s.	0.003 **
	3	Poisson	< 0.001 ***	0.379 n.s.	0.510 n.s.
	4	Poisson	< 0.001 ***	< 0.001 ***	0.118 n.s.
	5	Poisson	< 0.001 ***	0.932 n.s.	0.039 *
	6	Poisson	0.004 **	0.728 n.s.	0.399 n.s.
	7	Poisson	0.259 n.s.	0.836 n.s.	0.744 n.s.

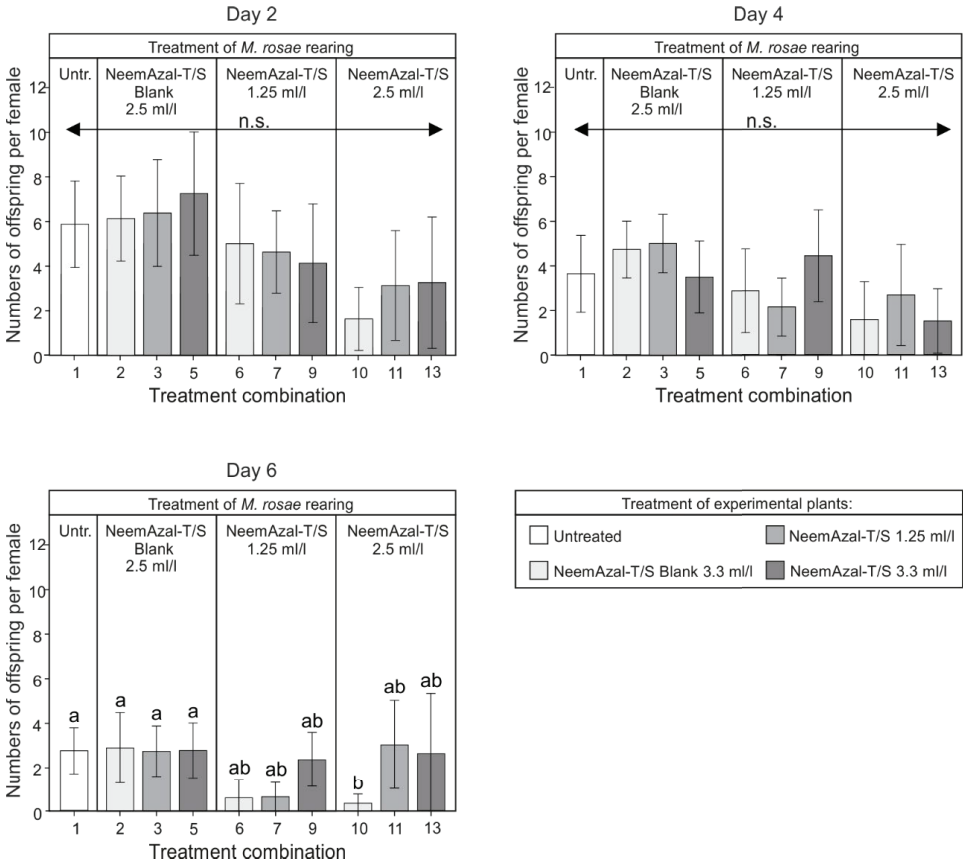

Fig. 5 Mean number of offspring (±SE) per *M. rosae* female for selected treatment combinations (see Table 8) on day 2, 4 and 6 of the first reproduction experiment after start of the experiment. Treatment of plants for *M. rosae* rearing before the experiment is stated on top of the figures, while treatment of experimental plants is colour-coded. Different letters indicate statistically significant (Bonferroni adjusted all-pair test within GLMs for each day, $P < 0.05$) differences in numbers of offspring over all treatment combinations within one day. Due to mortality, numbers of observed females varied with days: n = 8 adult female aphids on day 2, 6-8 on day 4 and 5-8 females on day 6 per treatment combination.

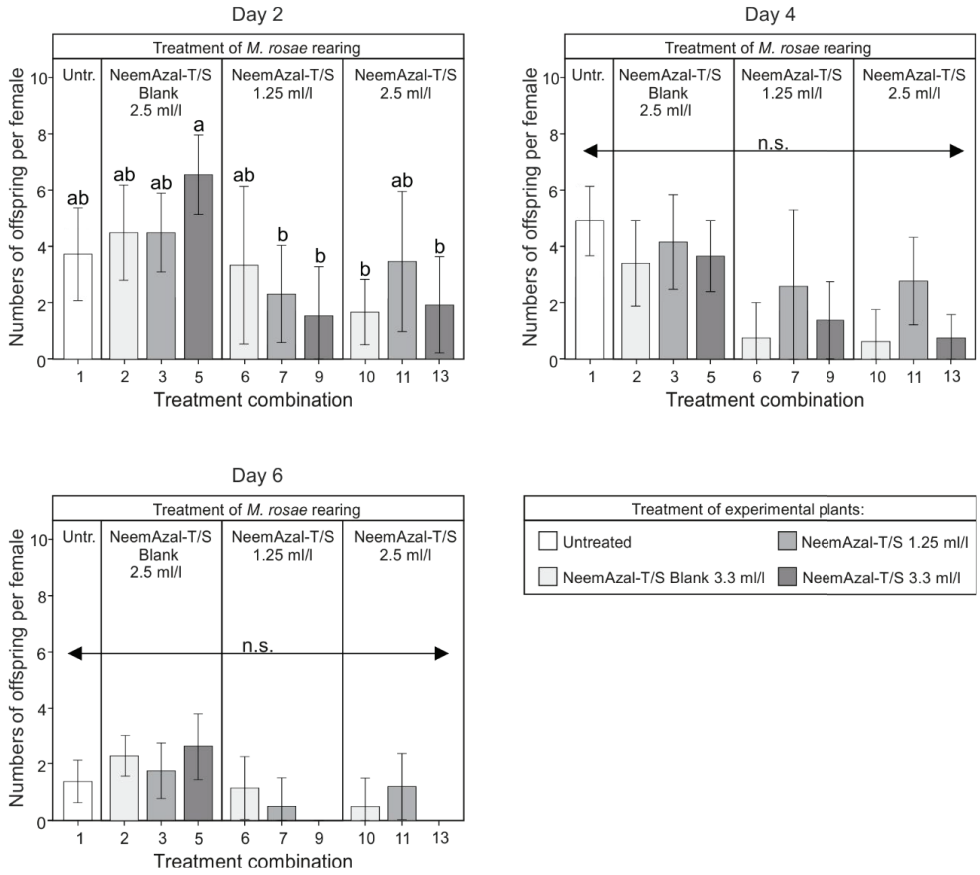

Fig. 6 Mean number of offspring (±SE) per *M. rosae* female for selected treatment combinations (see Table 8) on day 2, 4 and 6 of the second reproduction experiment after start of the experiment. Treatment of plants for *M. rosae* rearing before the experiment is stated on top of the figures, while treatment of experimental plants is colour-coded. Different letters indicate statistically significant (Bonferroni adjusted all-pair test within GLMs for each day, $P < 0.05$) differences in numbers of offspring over all treatment combinations within one day. Due to mortality, numbers of observed females varied with days: n = 8 adult female aphids on day 2, 7-8 on day 4 and 2-8 females on day 6 per treatment combination.

Discussion

Studies on the effects of neem-based products often use non-standardized products or non-formulated azadirachtin. Here, we used a standardized product with specific formulation at different doses. We employed the commercial azadirachtin product NeemAzal-T/S to assess the potential of formulated azadirachtin to control rose aphids through efficacy trials in combination with detailed mode-of-action experiments on different developmental stages of *M. rosae* with the commercial azadirachtin product NeemAzal-T/S. Juvenile stages of *M. rosae* were severely affected by NeemAzal-T/S. Direct effects of the treatment were a delayed development and a high mortality of first, second and third instar nymphs. Furthermore, as an indirect effect, the reproduction of adults that developed from NeemAzal-T/S-treated juveniles was reduced. These results explain why population growth of *M. rosae* on NeemAzal-T/S treated plants in inhibited and reveal that neem products can be effective for controlling rose aphids. In contrast, the mortality of fourth instar nymphs and adult *M. rosae* was not affected and standard efficacy trials with populations of mixed developmental stages only showed a moderate efficacy of currently registered concentrations of NeemAzal-T/S in the greenhouse.

Neem products usually have a low impact on adult aphids, but mortality of juvenile stages is significantly higher due to an insect growth regulating effect (IGR) of azadirachtin. Neem seed oil and a pure azadirachtin formulation, both in concentration equivalent to 40 ppm azadirachtin, resulted in 100 % mortality of second instar nymphs of *Nasonovia ribisnigri* and *Myzus persicae*. Moreover, the treatments significantly reduced the number of molts compared to the control (Lowery and Isman 1994). An increased mortality of aphid nymphs exposed to neem products, neem extracts or pure azadirachtin formulations was reported for several other aphids species, e.g. for *Aphis fabae* (Dimetry and Schmidt 1992, Dimetry and El-Hawary 1995), *Acyrthosiphum pisum* (Stark and Rangus 1994), *Macrosiphum sanbornii* (Koul 1999), *Toxoptera citricida* (Tang et al. 2002), *Brevicoryne brassicae* (Pavela et al. 2004) and *Aphis glycines* (Kraiss and Cullen 2008). Koul (1999) further tested the nymphal mortality of *M. rosae* on azadirachtin and neem seed oil treated leaf disks, rose buds, sepals or twigs. His results of 100 % mortality of second instar nymphs due to azadirachtin concentrations of 30 ppm (Koul 1999) are similar to our results for a spray treatment of complete rose plants with 33 ppm azadirachtin (3.3 ml/l NeemAzal-T/S). The delayed development of *M. rosae* nymphs after treatments with azadirachtin was also reported for *A. craccivora* (Dimetry and El-Hawary 1995) and *B. brassicae* (Pavela et al. 2004).

For lethal effects on adult aphids, previous results were contradictory. For *M. rosae*, concentrations of more than 70 ppm azadirachtin were needed to cause 90 % mortality of adults (Koul 1999). These findings underline that no relevant lethal effects on adult rose aphids can be expected with the registered concentration of 30 to 50 ppm azadirachtin per application, equivalent to a spraying solution of 3-5 ml/l NeemAzal-T/S as we used in this study. For *M. persicae*, *N. ribisnigri* and *C. fragaefolii*, no significant mortality effects on adults were found after treatments with azadirachtin in concentrations up to 80 ppm (Lowery and Isman 1994). However, other aphid species are more sensitive: Adults of the brown citrus aphid *T. citricida*, for instance, had a significantly lower survival on azadirachtin-treated citrus seedlings already at concentrations of 11 ppm azadirachtin after four days. Percentage survival decreased with increasing azadirachtin concentration and 90 % mortality was reached after 7 days with 22 ppm azadirachtin (Tang et al. 2002).

Even if azadirachtin has only minor mortality effects on adult *M. rosae*, the sterilizing action of this compound in adults contributes to aphid control. Azadirachtin seems to affect the reproduction of adult *M. rosae* mainly if the aphids had been exposed to NeemAzal-T/S already during their juvenile development. Exposure of adult *M. rosae* to NeemAzal-T/S treated plants, however, did not significantly reduce the numbers of offspring. Cutler et al. (2009) found similar results for *M. persicae* adults placed on potato leaf disks treated with sublethal concentrations of azadirachtin. They found no reduced numbers of offspring per adult aphid for azadirachtin concentrations up to 1 mg/L. By contrast, on leaf disks dipped in the highest tested azadirachtin concentration, F1 survival was significantly reduced as well as the numbers of F2 progeny produced per F1 aphid (Cutler et al. 2009). These results are consistent with Kraiss and Cullen (2008), who also found no effect on reproduction after adult *A. glycines* were sprayed topically with Neemix or neem seed oil compared to water-sprayed aphids (Kraiss and Cullen 2008). These results indicate that to affect reproduction, rose aphids need to be exposed to azadirachtin already as juveniles. This may be caused by azadirachtin interfering with the vitellogenin synthesis in insects for egg and living offspring production as a consequence of its influence on the titers of juvenile hormone (Rembold and Sieber 1981, Hardie 1987, Barnby and Klocke 1990, Riddiford 2012). The synthesis cascade might not be influenced anymore by azadirachtin if insects have already reached the adult stage. However, effects of azadirachtin regarding aphid reproduction vary: for the brown citrus aphid, by contrast, Tang et al. (2002) found that the brown citrus aphids *T. citricida* reproduce significantly less on citrus seedlings treated with Neemix at azadirachtin concentrations of 11 up to 180 ppm and their longevity was shorter compared to aphids on water treated seedlings (Tang et al. 2002). A direct reduction in number of living offspring was also recorded for *M. persicae* and *N. ribisnigrii* on neem-seed-oil treated leaf disks, but the reduction was higher when aphids were exposed already as fourth instar nymphs (Lowery and Isman 1996).

Although no direct effects on adult aphids were found, our experiment for population growth starting with one adult female shows that NeemAzal-T/S in concentrations of 3.3 ml/l can significantly reduce the numbers of aphids per plant compared to an untreated control. Most remarkable is that an exponential increase in numbers of aphids per plant did not happen on neem-treated rose plants. Reasons for the observed stagnation in population growth may be on the one hand a high mortality and inhibited development of nymphs, as previously discussed, and on the other hand, a sterility of aphids which developed on the treated plants. Moreover, Koul (1999) recorded that the survival of first generation (F1) aphids produced by adult *M. rosae*, which were exposed to neem seed oil or azadirachtin for 48 hours, was significantly lower compared to the offspring of untreated adults (Koul 1999). This effect ensures a long-lasting efficacy of neem products, since the next generation of aphids will also be affected even when the existing azadirachtin concentrations in plants might be already lower a few days after an application. They also tested the efficacy of neem seed oil on *M. rosae* in the field using lower azadirachtin concentrations than used in this study with 14 and 28 ppm azadirachtin, but the efficacy was higher. Aphid numbers were reduced by over 65 % with 14 ppm azadirachtin and 75 % with 28 ppm azadirachtin seven days after the second application and 14 days after the first spraying (Koul 1999).

In conclusion, application of neem products against aphids needs to be done early when infestation levels are still low. Several short application intervals additionally ensure to target sensitive young developmental stages. Thus, neem products are generally active against aphids as presented and

discussed in this study. However, a standard application strategy, when a high infestation pressure is reached and the aphid population consists of mixed developmental stages, neem products might not be able to control aphids sufficiently with the recommended spraying intervals of 7 to 10 days. Neem is a slow-acting insecticide and its efficacy should be evaluated based on reproduction, longevity, behavior and population growth (Stark and Rangus 1992). Neem products are generally compatible with other products and beneficial insects in IPM strategies (Raguraman et al. 2004). The combination of an azadirachtin-based product with a contact knock-down insecticide such as pyrethrum can ensure a higher efficacy as well as a good resistance management. Furthermore, the potential of azadirachtin to reduce pest population growth provides good opportunities for an effective combination with other elements of IPM such as natural enemies or entomopathogenic control agents.

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Chapter 3

Local and systemic effect of NeemAzal-T/S on host choice and feeding activity of *Macrosiphum rosae* on rose plants

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Abstract

Many studies in the last decades addressed the antifeedant and repellent effects of neem (*Azadirachta indica*)-based pesticides on arthropod herbivores. However, results for aphids regarding repellence and feeding were often contradictory and mainly based on laboratory studies with self-made neem extracts in high concentrations. Moreover, no data is available on the presence of azadirachtin, the main active ingredient of neem, in the phloem of treated plants – the tissue that most aphids feed on. We investigated how the application of the commercial neem product NeemAzal-T/S onto only one plant half (upper or lower) affects nymphs of the aphid *Macrosiphum rosae* in clip cages on the untreated plant half. Results for aphid mortality indicate that active ingredients of NeemAzal-T/S are translocated both upwards and downwards in rose plants and that active ingredients are transported in the phloem. Furthermore, we investigated whether NeemAzal-T/S has a repellent or antifeedant effect on *M. rosae*. A choice test in the greenhouse as well as the quantification of honeydew excretion and electrical penetration graph (EPG) analysis do not show differences in settling or feeding on untreated and NeemAzal-T/S-treated rose plants. Collectively, our data shows that the effect of approved concentrations of NeemAzal-T/S on *M. rosae* is based on the toxicity after feeding on treated plants, not on starvation.

Introduction

Aphids can cause severe damage to ornamental plants. Direct damage resulting from their phloem-feeding activities includes, for instance, stunted growth and leaf and flower deformations. However, their indirect damage is even more important: aphids can transmit economically significant plant viruses and their stylet punctures in the plant tissue are possible entrance points for plant pathogens such as bacteria or fungi. Additionally, the honeydew that the aphids secrete causes quality losses of plants, especially by promoting the growth of sooty molds (Brødsgaard and Albajes 1999, Alford 2012a, 2012b). One of the major pests of rose plants is the rose aphid *Macrosiphum rosae*. Its feeding activity on roses, preferably on new shoots and buds, leads to deformed leaves and flowers (Maelzer 1977, Alford 2012b). As an alternative to synthetic pesticides for pest control, biological insecticides from natural sources such as the neem tree (*Azadirachta indica*) are promising. These products, if properly applied, are characterized by relatively low mammalian toxicity (Raizada et al. 2001, Boeke et al. 2004, Morgan 2009), low persistence in the environment (Szeto and Wan 1996, Sundaram 1996a, Sundaram et al. 1997) and reduced risk of resistance development in the pest (Feng and Isman 1995). However, they often do not provide efficacies as high as found for synthetic pesticides (Bartelsmeier et al. 2021). Beyond causing mortality, additional characteristics of insecticides are beneficial for efficient aphid control: a systemic distribution of active ingredients in the plants and a repellent (before contact to treated plant tissue) or deterrent (after contact) effect. A transportation of active ingredients via the vascular system enhances the intake by insects, particularly by phloem feeders. Especially a distribution to plant parts that are not themselves treated, e.g. shoots and buds that developed after the application, and the presence in the phloem is a benefit for targeting phloem-feeding aphids. In addition, feeding reduction can decrease damage and honeydew production and consequently improve plant quality.

These characteristics are reported for neem products (Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010). The basis for most neem products is an extract from the seed kernels of the

neem tree (*Azadirachta indica*) that contain several natural insecticidal compounds such as azadirachtin, nimbin and salannin (Ley et al. 1993, Kraus 1995). However, the main active ingredient is the terpenoid azadirachtin A, normally referred to as azadirachtin. Biological activity of neem formulations against insects is strongly correlated to their azadirachtin content (Isman et al. 1990, Koul 1999). Azadirachtin treatments to roots and soil were effective against different arthropod species: Sundaram (1995, 1996b) reported for instance an impact on spider mites after an upwards distribution of azadirachtin in the xylem after root treatment of spruce and aspen plants (Sundaram et al. 1995, Sundaram 1996b). A soil treatment of bean plants led to a significantly higher mortality of thrips and an upward translocation of azadirachtin A and B (Thoeming et al. 2006). Regarding hemipteran insects, significant impacts have been reported for instance on whiteflies after soil treatments with neem extract or azadirachtin (Kumar et al. 2005, Karanja et al. 2015).

A clear proof for transport of azadirachtin in the phloem has not been reported so far. Although neem toxins move from the leaf surface to the inner tissue of the leaf in chrysanthemum, only limited movement to other leaves was recorded (Larew 1988). If azadirachtin is only or mainly translocated in the xylem, the control of phloem-feeding insects might be less effective. This may explain a low efficacy of neem products in the control of aphids and phloem-feeding hemipteran pests in general (Schmutterer 1988, National Research Council 1992).

Azadirachtin may have antifeedant or repellent effects in insects (Mordue (Luntz) and Nisbet 2000, Mordue (Luntz) et al. 2010). Interference with feeding activity by neem formulations is mainly reported for orthopteran and lepidopteran rather than hemipteran insects. Neem formulations can lead to both primary and secondary antifeedant effects. Primary antifeedant effects, also known as deterrent effects, are caused by contact chemoreception and occur, for instance, in locusts and caterpillars at low concentrations of azadirachtin. These insects often prefer to starve instead of ingesting azadirachtin-treated plant material. Secondary antifeedant effects are caused by internal feedback mechanisms after ingestion of azadirachtin or other neem toxins (Mordue (Luntz) and Blackwell 1993, Mordue (Luntz) and Nisbet 2010). For this deterrent effect, toxins need to be present at the feeding site of the insect. In the case of aphids, repellent effects were reported for cereal aphids: *Sitobion avenae* and *Rhopalosiphum padi* clearly preferred untreated winter barley leaves to azadirachtin-treated leaves. Furthermore, aphid probing activity was significantly reduced two and four days after 250 ppm azadirachtin had been applied topically on barley seedlings and after 500 ppm azadirachtin had been applied systemically through roots (West and Mordue (Luntz) 1992). *Acyrthosiphum pisum* aphids are repelled prior to leaf contact by the azadirachtin-containing product RD-Repelin (Hunter and Ullmann 1992). However, because the product contained also other plant extracts and no blank formulation was tested, this does not prove that repellence was caused by azadirachtin. In contrast, Nisbet et al. (1994) reported that azadirachtin-treated diets in concentrations up to 100 ppm did not affect the settling behaviour of *Myzus persicae*. Nevertheless, after 24 h, honeydew production by *M. persicae* aphids, a proxy for feeding intensity, was significantly reduced already at 25 ppm azadirachtin, but settling behaviour was still not affected (Nisbet et al. 1994). This suggests that only high concentrations of neem extracts clearly affect aphid settling behaviour (Mordue (Luntz) and Blackwell 1993, Nisbet et al. 1994), but that secondary antifeedant effects after ingestion of treated plant material may occur in aphids at lower concentrations than 100 ppm (Nisbet et al. 1994). However, repellent or deterrent effects are different among aphid species. Only three of six tested species were deterred by neem seed oil in a

study by Lowery and Isman (1993). Furthermore, the rapid disruption of feeding in less than 1 hour was not attributed to the presence of azadirachtin according to the authors, but other neem toxins seem to be responsible for this effect. Additionally, the repellence only lasted up to 24 hours (Lowery and Isman 1993). This summary of the literature indicates that aphid species react differently to azadirachtin in terms of settling and feeding and that effective concentrations of azadirachtin are high. Effects caused by 100 to 500 ppm azadirachtin cannot be expected by the currently registered dosage of only 30-50 ppm. The kind of formulation - neem oil, watery formulation or the use of acetone or ethanol – and adjuvants might also affect the results.

For *M. rosae*, no results are available regarding a repellent or antifeedant effect caused by commercial neem products under practical conditions. However, a leaf-disc laboratory test with an aqueous neem extract solution and second instar *M. rosae* nymphs, revealed an effective dose causing 50 % of deterrence by 11 ppm azadirachtin (Koul 1999). This is in the range of currently registered doses of up to 50 ppm azadirachtin, but it is questionable if this effect is also visible in experiments with complete plants under realistic plant growth conditions. For a secondary antifeedant effect, active ingredients of neem extracts need to be available at the feeding site, in the phloem. For this reason, the first objective of this study was to assess whether the commercial product NeemAzal-T/S has systemic effects in rose plants and whether active ingredients may be expected to occur in the phloem. We first tested whether treatment with azadirachtin has effects on *M. rosae* nymphs that feed on untreated plant parts, either above or below the treated tissues of the plant. The second objective was to investigate whether a repellent or antifeedant effect by the commercial neem product NeemAzal-T/S is present. Repellence was assessed in terms of host-plant choice and whether feeding activity of *M. rosae* is affected by commonly registered doses of this product. The feeding was assessed quantitatively by analysing the amount of secreted honeydew as well as qualitatively with the Electrical Penetration Graph (EPG) technique. This technique allows to compare the probing and feeding behaviour of aphids (van Helden and Tjallingii 2000) and can reveal which components of aphid feeding are affected by treating plants with NeemAzal-T/S. The honeydew production of *M. rosae* was assessed for populations of mixed developmental stages to determine the impact for the practical use of this product. Furthermore, because the effect of azadirachtin on mortality of adult aphids is normally low (Lowery and Isman 1994a, Bartelsmeier et al. 2021), repellence and feeding deterrence were investigated for adult aphids. This study thus investigates whether any advantageous effects of NeemAzal-T/S treatment can be expected on adult stages, even when their mortality is not increased.

Materials and methods

General materials for all experiments

Insects and plants

As experimental plants and plants for the aphid culture, miniature rose plants (*Rosa hybrida*, different Kordana® Classic-varieties by W. Kordes' Söhne Rosenschulen GmbH & Co KG) were used. Rose plants were obtained as freshly rooted cuttings with 3-4 plants per pot. These plants were separated and one or two plants were transferred to pots of 11 or 12 cm diameter with the soil Einheitserde Classic, Type ED 73, 155 fine. Plants were kept in an air-conditioned greenhouse at 21±3 °C, 60±10 % RH and a 16:8 L:D photoperiod. Additional light in the greenhouse was provided by

sodium vapor lamps (400 W) if sunlight intensity outside fell under 120 W/m². A few days after transplanting, rose plants were drenched with a liquid fertilizer (Wuxal Top N by Manna, Germany, in a dose of 0.3 %). Fertilizer application was repeated when required. When needed, plants with fungicides to control mildew, according to current registrations in Germany. Compo Ortiva Universal Pilzfrei (Compo, Germany) or Bayer Garten Rosen Pilz-frei Baymat (SBM, Germany) were used. Fungicide treatments were latest done one week before an experiment started to exclude contact effects on aphids. If fungicides were applied, all plants were sprayed to ensure equal conditions. Plants were kept in these conditions for three to four weeks until their use for insect rearing or experiments. At the beginning of an experiment, experimental plants were in a stage where the inflorescence emerged (BBCH stage 51-59, Meier et al. 2009).

As experimental insects, a red phenotype of *M. rosae* was reared, originating from a naturally occurring female on a cultivated rose in Monheim am Rhein, Germany. Rose aphids were maintained on potted miniature roses (Kordana Classic varieties, as described above) in cages made of acrylic glass with three gauze sides (63 cm x 78 cm x 63 cm high). Cages were placed in a climate chamber (22±2 °C, 60±10 % RH and a 16:8 h L:D photoperiod). During three to four weeks before every experiment, aphids were reared on the rose variety that was used for the experiment as well.

Treatments

NeemAzal-T/S (Trifolio-M GmbH, Lahnau, Germany), a common and registered neem-based insecticide in Europe, was used to treat plants. This product is an emulsifiable concentrate with 10 g azadirachtin A per liter as its major active ingredient (NeemAzal technical, Trifolio M-GmbH 2014). For miniature roses smaller than 50 cm, as used in this study, 0.3 ml of the product per m² is registered as dose (German Federal Office of Consumer Protection and Food Safety (BVL) 2017). With a slurry volume of 900 l/ha, 3.3 ml product/l water is the recommended dose. Plants were sprayed till run-off while ensuring a complete coverage of plants. Spraying solutions were either applied with a manual trigger spray bottle or a handheld sprayer, operated by compressed air with 3 bar pressure and a 1.1 mm bore hollow cone nozzle. NeemAzal-T/S was used in different concentrations in most experiments and also its blank formulation was used, referred to as NeemAzal-T/S Blank (provided by Trifolio-M GmbH, Lahnau, Germany). The blank does not contain the NeemAzal-extract, but tensides and plant oils in the same amounts as in the original product NeemAzal-T/S.

Greenhouse conditions and plant care during experiments

Experiments took place in the same air-conditioned greenhouse as described before with the exception of the EPG experiment. A temperature of 21±3 °C, 60±10 % RH and a photoperiod of 16:8 h LD were ensured during the experiments. The EPG experiment was conducted under laboratory conditions with 20±2 °C and continuous artificial light by fluorescent tubes. Plants were watered directly onto the soil as required.

Systemic action of NeemAzal-T/S

To investigate systemic effects of NeemAzal-T/S treatments, two experiments were conducted in which the mortality of *M. rosae* was assessed on untreated plant parts of treated plants. Single rooted cuttings of rose plants (variety Kordana Classic 'Sunstar') were transferred to 11 cm diameter pots. Before the experiment, all but one shoot were cut at soil level to have a single plant shoot per pot. NeemAzal-T/S application was done with a handheld trigger spraying bottle. The soil and the

part of the plant that should stay untreated were covered with a plastic screen before application. The screen was removed directly after spraying and drying of the plants. The effects of acropetal translocation were assessed by attaching a clip cage with *M. rosa*e first instar nymphs on unsprayed leaves above the sprayed plant half. To investigate the effects of basipetal translocation, the clip cage was attached below the sprayed plant half. The scheme of application and position of clip cages is shown in Fig. 1.

In the first trial, mortality of aphids was compared for both translocation directions between an untreated control, 3.3 ml/l NeemAzal-T/S Blank and plants treated with 1.65, 2.5 and 3.3 ml/l NeemAzal-T/S. In the second trial, maximum concentrations were higher and treatments were an untreated control, 4.1 ml/l NeemAzal-T/S Blank and 2.5, 3.3 and 4.1 ml/l NeemAzal-T/S.

After spraying and drying of the plants, three to five randomly selected adult *M. rosae* from the main culture were placed into each clip cage. 24 hours later, adults were removed and the mortality of the remaining nymphs was assessed 1, 4, 6, 8 and 10 days after application. The initial number of first instar nymphs per clip cage was between 8 and 12 individuals in the first trial and 8 to 14 individuals in the second trial. Five rose plants, each with one clip cage, were used per treatment and translocation direction (acropetal and basipetal). In the untreated control groups, clip cages were placed on leaves in similar positions to have comparable conditions of microclimate and leaf age.

Statistical analysis was conducted with R (version 3.1.3, The R Foundation for Statistical Computing, 2015). A Cox proportional hazards model was fitted with the function “coxph” of the package survival (Thernau 2012). A Tukey post-hoc test (function “glht” of the package “multcomp” (Hothorn et al. 2008)) was used for a comparison between all treatments regarding the mortality risk for *M. rosae* in the specific treatments and translocation directions (acropetal and basipetal). The mortality in each control group was set as the baseline mortality hazard.

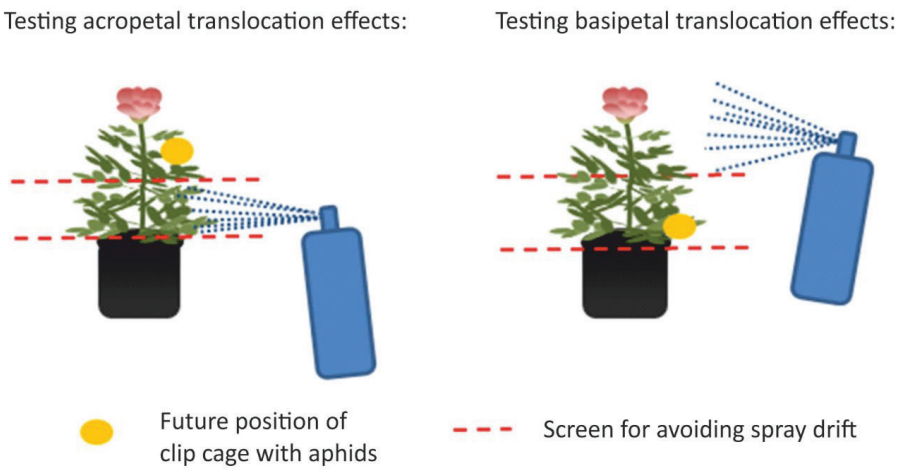

Fig. 1 Application scheme for assessing the effects of acropetal (left) and basipetal (right) translocation of active ingredients of NeemAzal-T/S and the future position of clip cages containing *M. rosae* nymphs (clip cage plus aphids were added after spraying and drying of the plants).

Repellent effects

Repellence of NeemAzal-T/S was tested in five acrylic glass cages with three gauze sides (dimensions: 63 cm x 78 cm and 63 cm high). Ten adult apterous *M. rosae* were released per cage in the middle between one untreated rose plant and one rose plant treated with 3.3 ml/l NeemAzal-T/S of the variety Kordana Classic 'Flirt'. Each cage represents one replication. After 1, 2, 5, 24 and 72 hours, the numbers of adults on each plant were assessed. The numbers of aphids on untreated and NeemAzal-T/S treated rose plants per cage were compared for each time point with a generalized linear model (GLM) with Poisson distribution and log-link function. Statistical analysis was performed in SPSS (version 22, IBM).

Feeding activity: Honeydew secretion by mixed *M. rosae* population or by single adults

To compare honeydew secretion by *M. rosae* on untreated and NeemAzal-T/S treated rose plants, an experimental method with ninhydrin for colouring honeydew droplets was used. Two experiments were conducted: The first one examined the honeydew production of small *M. rosae* populations of mixed developmental stages and the second experiment that of single adult aphids.

For the first trial, single leaflets from the apex of rose plant leaves (Kordana Classic 'Apache') were detached and placed with the petioles into tubes filled with tap water. The experiment was conducted in a climate chamber (22±2 °C, 60±10 % RH, 16:8 L:D). 30 aphids from the *M. rosae* culture were transferred with a fine brush to the lower side of each leaf. Five adult aphids, 10 third-fourth instar nymphs and 15 first-second nymphs were selected from the culture to have similar initial conditions for each population. Stems of the leaves were fixed with parafilm to avoid a turn of the leaf and leakage of water. The tubes were laid down on the seam of a Petri dish (85 mm diameter) underneath the leaf to ensure that the leaf is in horizontally position. The aphids were allowed to settle overnight. Next day, a filter paper (85 mm diameter) was placed in the Petri dish under each leaf. The filter paper was removed after 2 hours and the upper side of the leaf was sprayed with the insecticide with 100 ml handheld trigger bottles. Only the upper side was treated to avoid that aphids fell down due to the application and to minimize contact effects by the oily formulation. The following treatments were compared: an untreated control, 3.3 ml/l NeemAzal-T/S Blank, 1.65, 2.5 and 3.3 ml/l NeemAzal-T/S and 1.67 ml/l Flupyradifurone SL 50 as positive control. Per treatment, five leaves were sprayed. After application, a new filter paper was placed under the leaves for 2 hours. Then, new filter papers were placed in the Petri dishes for 24 hours and were replaced two times. Thus, the honeydew was collected on the filter papers for 2 hours before the application, 2 hours and 1, 2 and 3 days after application. Additionally, numbers of aphids per leaf were counted directly after removing the filter paper. After the experiment, the filter papers were sprayed with a 0.1 % ninhydrin-acetone solution and allowed to dry. Ninhydrin coloured the honeydew droplets purple. After the experiments, all filter papers were photographed individually on a black background with a camera in the same position and settings. Size of the photos was 6000x4000 pixels. The photos were analysed individually for the amount of purple coloured pixels as a measurement of the area covered with honeydew with the image software Gimp, version 2.8.22 (The GIMP Development Team). Data was analysed for each time point as honeydew production per aphid by dividing the coloured area in pixels by the numbers of living aphids for each replication at each time point. The honeydew excretion during two hours before the application was set at 100% and the change in amount of honeydew was analysed. This method allows to take two important aspects into

consideration: First, the natural honeydew production for each population individually before the application and second, the increase or decrease in numbers of aphids with time, e.g. mortality due to treatment effects. Some filter papers were dismissed from the analysis because of unclear colouring (purple water's edges on the filter papers). Due to the small number of replicates (maximum five, for some treatments and time points only four) and a non-normal distribution, no meaningful statistical analysis was possible.

For the second honeydew experiment with single adult aphids, pieces of filter paper (2 x 2 cm) were saturated with a 0.1 % ninhydrin-acetone solution before the experiment and allowed to dry for a day. For each treatment, six rose plants (Kordana Classic 'Flirt') were sprayed using a handheld sprayer. After drying of the plants, a clip cage with one adult rose aphid, randomly selected from the culture, was attached onto each plant. Underneath the clamped leaf with the aphid, one ninhydrin-treated filter paper piece was placed on the bottom of the clip cage and left there for 24 hours. Then, the filter paper was replaced by a new one. This was repeated twice for collecting the honeydew during 0-24, 24-48 and 48-72 hours after application. Purple coloured honeydew spots were counted on each filter paper the next day (time for colour development). Two similar trials were performed with this method. Per treatment and trial, six replications were used. One rose plant with a clip cage containing one adult aphid represents one replication. The following treatments were compared: an untreated control, 4.1 ml/l NeemAzal-T/S Blank and 2.5, 3.3 and 4.1 ml/l NeemAzal-T/S. The experiments took place in the greenhouse at 21±3 °C, 60±10 % RH and a 16:8 L:D photoperiod.

For the statistical analysis, the data of the two experiments with each 6 replications were pooled, resulting in 12 replications per treatment. Cumulative honeydew production over time was assessed by summing of honeydew droplets. Influence of the treatments on the number of honeydew droplets was analysed with a GLM (Poisson distribution, log-link function) for each time period separately. Analysis was performed in SPSS (version 22, IBM).

Feeding activity: Electrical penetration graph

To assess the probing and feeding behaviour of *M. rosae* on untreated and NeemAzal-T/S treated rose plants, an Electrical Penetration Graph (EPG) experiment was performed. Potted miniature rose plants of the variety Kordana Classic 'Sunstar' were used. An overview of the treatments of rose plants for the EPG recordings is given in Table 1. Plants were usually treated in the morning by spraying the solutions with a handheld trigger spraying bottle until a complete coverage was reached. Application was made on the day of recording (treatments 2-6) or one day earlier in the case of the treatments 7 and 8.

For the EPG set-up, a fine golden wire was attached with silver glue to the abdomen of wingless, randomly selected adult *M. rosae* of unknown age. The golden wire was connected with an electrode and was included in an electrical circuit with another electrode in the soil of the rose plant. Directly after wiring, two DC-EPG devices (Giga-8, EPG-Systems, Wageningen, The Netherlands) were used for recording of probing and feeding behaviour for 8 hours. Each device was used for a separate plant treatment and recordings of all treatments were randomised over the experimental period of three weeks. EPG signals of 15 aphids were recorded simultaneously per day. Each wired aphid was placed on a young, nearly completely developed leaf. This leaf was fixed upside down with tape on a plastic

bar to prevent aphids from contact loss due to leaf movement. The software Stylet+d, version 01.28 (EPG Systems, Wageningen, The Netherlands) was used to monitor and record the EPG waveforms.

Analysis of recorded EPG waveforms was done with the software Stylet+a, version 01.30 (EPG Systems, Wageningen, The Netherlands). Recordings with complete hours of no activity/no signals, unclear potential drops or obviously escaped aphids were discarded. Table 2 presents the waveforms that were distinguished in the EPG analysis. For details of the EPG (DC) method and the analysed waveforms see Tjallingii (1978, 1988, 1990, 1994), Van Helden and Tjallingii (2000) and Reese et al. (2000). Statistical analysis of numbers of probes and phases and the time spent in the different phases were compared with a Kruskal-Wallis test and if significant ($P < 0.05$), all pairwise comparisons were made with the Bonferroni-Dunn correction for multiple testing. The software SPSS version 22 (IBM) was used for the statistical analysis.

Table 1 Treatments used for EPG experiment.

No.	Treatment name	Product	Concentration	Application
1	Control	*(untreated)*		
2	Blank 3	Blank formulation of NeemAzal-T/S	3.3 ml/l	Spray application on same day as EPG
3	Blank 10	Blank formulation of NeemAzal-T/S	10 ml/l	
4	NeemAzal-T/S 3	NeemAzal-T/S	3.3 ml/l	
5	NeemAzal-T/S 6	NeemAzal-T/S	6.6 ml/l	
6	NeemAzal-T/S 10	NeemAzal-T/S	10 ml/l	
7	NeemAzal-T/S 24h	NeemAzal-T/S	3.3 ml/l	Spray application 24 h before EPG
8	NeemAzal-T/S Soil	NeemAzal-T/S	3.3 ml/l	Drenching with 50 ml of a 3.3 ml/l NeemAzal-T/S solution per plant

Table 2 EPG waveforms that were differentiated and their biological meaning. For detailed explanation of the terms see Van Helden and Tjallingii (2000) and Reese et al. (2000).

Abbreviation	Biological meaning
NP	Non-penetration period/no activity
pd	Potential drop/cell puncture
C	Stylet pathway including pd
E1	Salivation in phloem
E2	Ingestion of phloem
E	Phloem phase (E1 and E2)
G	Xylem ingestion
F	Penetration difficulties/mistakes

Results

Systemic effect of NeemAzal-T/S

In both trials, systemic effects of a NeemAzal-T/S treatment were recorded on aphids placed on untreated plant parts. Mortality of *M. rosae* that were present on leaves that were not directly treated was higher than on control untreated plants for all tested concentrations of NeemAzal-T/S and for both positions of the clip cages: higher than the treated plant parts (acropetal translocation, Fig. 2 panels 1A and 2A; Table 3) and lower than the sprayed plant half (basipetal translocation, Fig. 2, panels 1B and 2B). Mortality in the NeemAzal-T/S Blank treatments was similar as in the untreated control (Table 3). The hazard of mortality for *M. rosae* increases with increasing concentrations of NeemAzal-T/S in all cases. However, the lowest tested concentration, 1.65 ml/l NeemAzal-T/S, did not lead to a significantly higher mortality risk of *M. rosae* compared to the control (Table 3). With 3.3 ml/l NeemAzal-T/S, a significantly higher mortality was recorded in both trials and for both tested directions of translocation (Table 3).

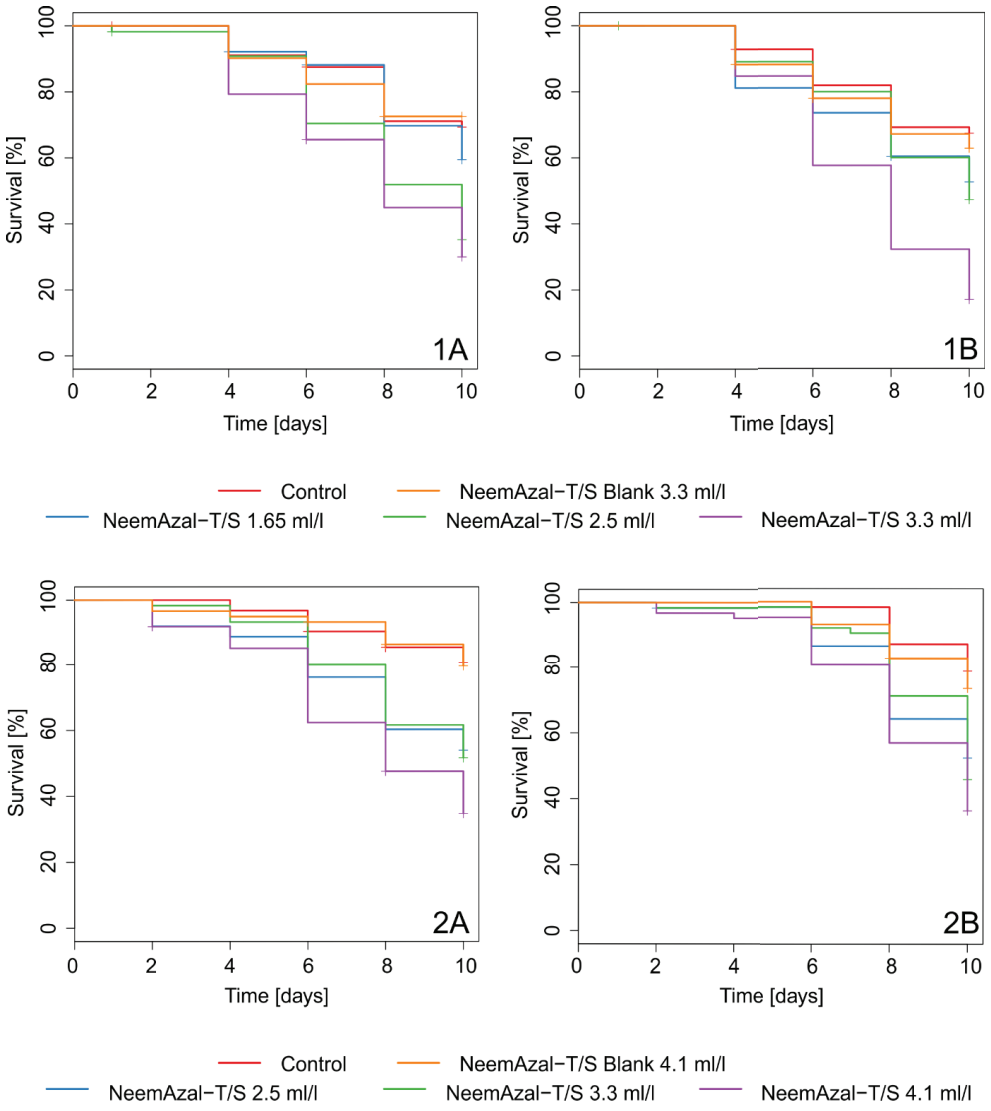

Fig. 2 Systemic effects of NeemAzal-T/S in rose plants in trial 1 (panels 1A and 1B) and trial 2 (panels 2A and 2B). A: Percentage survival of *M. rosae* nymphs on untreated plant parts higher than the sprayed part (acropetal translocation). B: Percentage survival of *M. rosae* nymphs on untreated plant parts below the sprayed part (basipetal translocation). N=5 rose plants per treatment and tested direction of translocation (panels A and B), each with one clip cage containing several aphids. Initial total number of aphids ranged from 51 to 58 aphids per treatment for panel 1A and from 51 to 59 aphids per treatment for panel 1B in trial 1. In trial 2, initial total number of aphids ranged from 60 to 63 aphids per treatment for panel 2A and from 58 to 63 aphids per treatment for panel 2B.

Table 3 Effects of systemic treatments with NeemAzal-T/S on the mortality of *M. rosae*, presented as the mean hazard ratios (HR) to die in the different treatments according to Cox proportional hazards model. Acropetal translocation effects were tested with clip cages attached onto leaves above the treated plant parts. Basipetal translocation effects were tested with clip cages attached on leaves below treated plant parts. Control groups functioned as baseline hazards. N = 5 rose plants, each with one clip cage, per treatment and tested direction (acropetal and basipetal), for each experiment. Every clip cage contained 6 to 14 *M. rosae* nymphs at the start of the experiment. Aphid mortality was assessed for 10 days.

Treatment	Acropetal				Basipetal			
	Trial 1		Trial 2		Trial 1		Trial 2	
	HR[1]	*P*[2]	HR[1]	*P*[2]	HR[1]	*P*[2]	HR[1]	*P*[2]
Control	1[3] b		1[3] c		1[3] b		1[3] bc	
NeemAzal-T/S Blank[4]	1.0 b	0.94	1.0 bc	0.93	1.2 b	0.71	1.3 c	0.53
NeemAzal-T/S 1.65 ml/l	1.5 b	0.37	-	-	1.6 ab	0.27	-	-
NeemAzal-T/S 2.5 ml/l	2.1 ab	0.008*	2.9 abc	0.023*	1.8 ab	0.20	2.9 abc	0.06
NeemAzal-T/S 3.3 ml/l	3.6 a	0.001*	3.0 ab	0.003*	3.8 a	<0.001*	3.1 ab	0.020*
NeemAzal-T/S 4.1 ml/l	-	-	4.9 a	<0.001*	-	-	4.3 a	0.004*

[1] HR: hazard ratio. Different letters within the same column indicate significant ($P < 0.05$) differences in HR according to Tukey post-hoc all-pair comparison in the Cox proportional hazards model.

[2] *P*-value represents the significance of the influence of the treatment on the mortality for *M. rosae* according to Cox proportional hazard model with "Control" as baseline hazard. Significant *P* values are marked with an asterisk.

[3] HR for Control set to 1 as baseline hazard.

[4] NeemAzal-T/S Blank was applied with 3.3 ml/l in experiment 1 and with 4.1 ml/l in experiment 2.

Repellent effects

Nearly all released apterous *M. rosae* settled on the rose plants in the test arenas during the experimental period. Numbers of settled aphids, either on untreated or NeemAzal-T/S treated plants increased with time (Fig. 3). Even if slightly more aphids were found on NeemAzal-T/S plants at all evaluated time points, differences were not significant (GLM, $P > 0.05$). These data indicate that NeemAzal-T/S does not repel *M. rosae*.

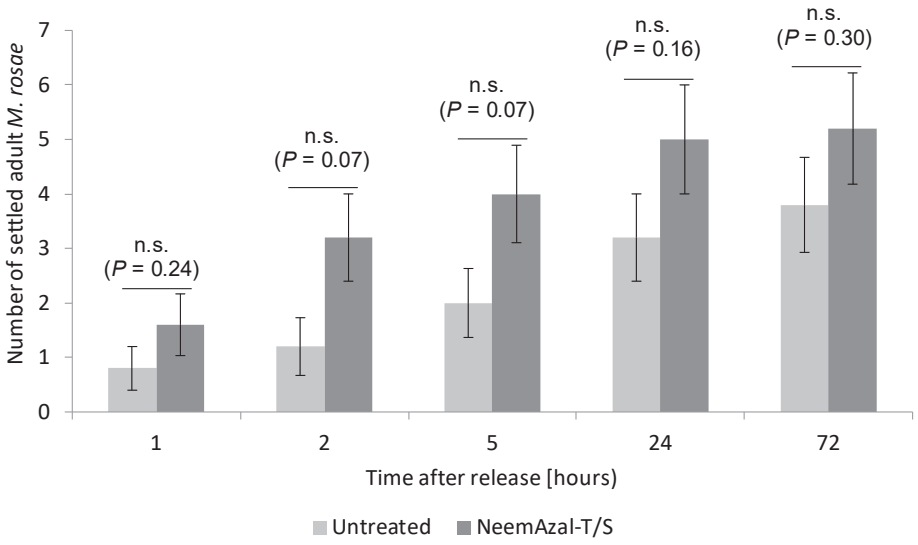

Fig. 3 Host-plant preference of *M. rosae*. Mean number (±SE) of adult *M. rosae* on untreated rose plants and plants treated with 3.3 ml/l NeemAzal-T/S at 1, 2, 5, 24 and 72 hours after release of 10 aphids in each test arena. N = 5 test arenas, each with 10 released aphids. Data for non-settled aphids is not shown. n.s.: non-significant ($P > 0.05$) differences between number of aphids on treatment vs. control groups within one time point based on GLM (Poisson, log-link).

Feeding activity: Honeydew

The honeydew excretion of *M. rosae* populations on untreated and treated rose leaflets during two hours before the application was set at 100%. The honeydew excretion rate per living aphid up to three days is similar in all treatments including the control, with the exception of Flupyradifurone. In this treatment, the numbers of aphids decreased and also the honeydew excretion per living aphid approaches 0% at one day after application (Fig. 4).

For aphids that were individually present on a leaf, the cumulative number of honeydew droplets increases with time. Aphids excreted approximately one droplet per day in all treatments. We found no significant differences in the number of honeydew droplets secreted by adult *M. rosae* on untreated plants and plants treated with different concentrations of NeemAzal-T/S or the blank formulation (Fig. 5).

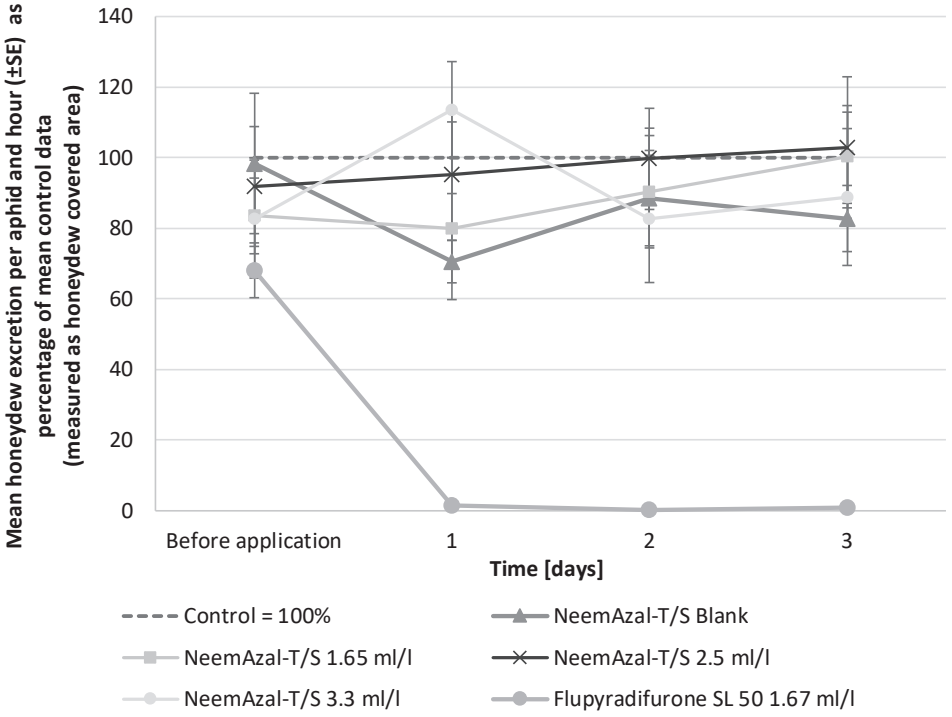

Fig. 4 Mean honeydew excretion (±SE) per hour and per individual living *M. rosae* aphid before and 1, 2 and 3 days after application as percentage of honeydew excretion in the control group (=100 %), assessed as honeydew covered area in pixels of small populations. Total covered area of each replication was divided by the number of living aphids in that replication for each assessment time to exclude mortality effects. N = 4 to 5 observed *M. rosae* populations on single excised leaves per treatment at each time point with varying numbers of aphids of different developmental stages.

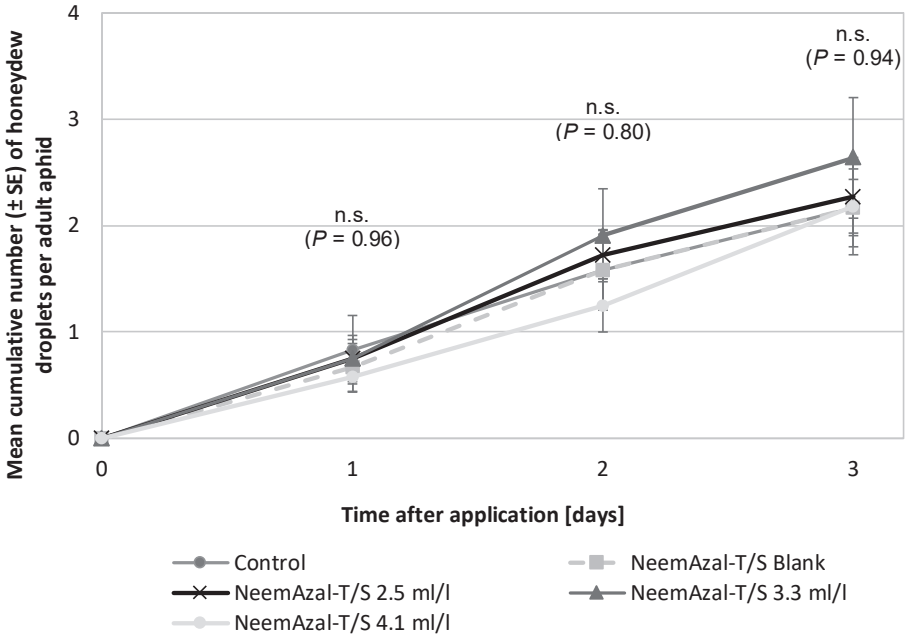

Fig. 5 Honeydew secretion by individual adult *M. rosae* as mean (±SE) cumulative number of droplets per aphid over three days after application of NeemAzal-T/S and the blank formulation. N = 12 aphids per treatment (pooled data of two replicate experiments, each with 6 observed aphids per treatment). n.s. = not significant ($P > 0.05$) influence of treatment on number of honeydew droplets per aphid according to GLM (Poisson, log-link) for each time point.

Feeding activity: Electrical penetration graph (EPG) analysis

EPG recordings of 16 to 22 aphids per treatment were analysed for the complete recording time of 8 hours. From the EPG recordings, parameters regarding no activity (NP), pathway with probing punctures (C/pd) and phloem ingestion (E) were analysed as well as xylem-ingestion (G) and penetration difficulties (F). For most parameters, the number of periods as well as the total duration was compared between treatments.

In the untreated control, aphids had significantly ($P < 0.05$) lower numbers of probes in 8 hours compared to the soil treatment of NeemAzal-T/S (NeemAzal-T/S Soil) (Table 4). No differences were found for numbers and/or duration of no-activity-periods (NP), total time of pathway-phases (C/pd) (Table 4), numbers and duration of xylem-ingestion periods (G) or for periods with penetration difficulties (F). Patterns G and F were only recorded occasionally and not in all recordings (Supplementary Table S1).

For the comparison of phloem salivation and ingestion, the total duration of E1 (phloem salivation) and E2 (phloem ingestion) were included in the analysis, as well as the total duration of phloem phases. No significant differences were found for any of these parameters between the treatments

(Supplementary Table S2). Total duration of phloem-phases includes E1, E2 and unknown phloem waveforms. These unknown waveforms were found in some recordings (Supplementary Table S3 and Supplementary Fig. S1), but number and duration of these patterns did not significantly differ between treatments and have not been analysed in further detail. Additionally to these parameters, the time to the first E2 phase was analysed as well as the time to the first sustained phloem phase and total duration of sustained E2. Sustained phloem phase is inferred from the E2 phase lasting longer than 10 minutes. An E2 phase longer than 10 minutes lasts in most cases longer than 1 hour (Tjallingii 1994) and this time can be used as the threshold time for the acceptance of a sieve element by aphids (Tjallingii 1990, 1994, 2006). No significant differences were found for these parameters. Additionally, no correlation with increasing concentrations of NeemAzal-T/S was found (Supplementary Table S2).

On average, the first sustained E2 phase (phloem ingestion longer than 10 minutes) began after 107 minutes (NeemAzal-T/S 3) up to 230 minutes (NeemAzal-T/S 6). For this reason, the analysis of recordings was conducted separately for hours 1 to 4 and 5 to 8. The aim was to assess possible treatment effects after aphids had obtained the first phloem sap ingestion (sustained E2) in the second time period of 5 to 8 hours. However, no significant differences were found between the treatments in both time periods for number of probes, duration of no activity (NP), pathway period (C/pd) and the phloem phases (E, E1, E2, sustained E2). Furthermore, no pattern is visible with increasing concentrations of NeemAzal-T/S (Supplementary Tables S4, S5 and S6).

Table 4 EPG parameters (mean ± SE) of *M. rosae* regarding probing, non-penetration- (NP) and pathway-periods (C) on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc pairwise comparison.

Treatment	N[1]	No. probes in 8 hours		No. NP[2] periods		Total duration NP[2] (minutes)		Total duration of C[3] including pd (minutes)	
Control	22	130.6 ± 20.9	b	11.5 ± 1.9	a	45.2 ± 11.5	a	125.7 ± 20.4	a
NeemAzal-T/S Blank 3	19	183.8 ± 24.1	ab	16.5 ± 3.1	a	49.6 ± 15.2	a	159.8 ± 19.9	a
NeemAzal-T/S Blank 10	22	193.5 ± 21.7	ab	13.2 ± 3.9	a	37.4 ± 9.2	a	166.0 ± 17.5	a
NeemAzal-T/S 3	21	150.4 ± 20.4	ab	16.5 ± 3.8	a	53.1 ± 15.9	a	121.5 ± 16.0	a
NeemAzal-T/S 6	16	202.8 ± 25.5	ab	18.8 ± 3.0	a	64.8 ± 12.2	a	165.5 ± 18.1	a
NeemAzal-T/S 10	21	158.1 ± 17.3	ab	11.0 ± 3.2	a	38.4 ± 10.8	a	141.2 ± 16.0	a
NeemAzal-T/S 24h	20	175.9 ± 22.3	ab	13.8 ± 2.4	a	41.0 ± 8.3	a	139.6 ± 18.5	a
NeemAzal-T/S Soil	19	227.8 ± 25.3	a	18.9 ± 3.7	a	59.8 ± 17.5	a	193.2 ± 19.8	a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] NP: non-penetration time/no activity

[3] C/pd: Stylet pathway including probes (pd=potential drops)

Discussion

The present study shows that local treatment of rose plants with NeemAzal-T/S has systemic effects on mortality of *M. rosae* nymphs. NeemAzal-T/S did not repel *M. rosae* adults or interfere with their feeding activity, as assessed by quantification of honeydew production and EPG recording. Our results indicate that the effect of NeemAzal-T/S on *M. rosae* is based on toxic effects after ingestion of azadirachtin-containing phloem sap, and not a consequence of lower food intake or starvation.

The first objective of our study was to examine the systemic effect of NeemAzal-T/S. Systemic effects of insecticides are a benefit and usually enhance the potential for an efficient insect control, because hidden individuals will be affected and newly growing shoots will be protected. Our results show that *M. rosae* nymphs exhibited a significantly higher mortality when they fed on untreated plant parts both above and below the plant part which was sprayed with the neem product. These results indicate that active ingredients of NeemAzal-T/S are translocated systemically, acropetally as well as basipetally. An acropetal uptake and translocation of azadirachtin and other toxins from neem was expected and reported before (e.g. Sundaram et al. 1995, Thoeming et al. 2003, 2006, Pavela et al. 2004, Kumar et al. 2005). However, in previous studies the systemic effect usually followed a soil or root treatment. A spread of active ingredients from other aboveground plant parts, e.g. from treated leaves to untreated leaves, has only been reported for chrysanthemum so far. In chrysanthemum plants, spraying the upper or lower half of the plants with a neem extract equivalent to 23 ppm azadirachtin slightly reduced the numbers of pupae and adults of a leaf mining dipteran pest (*Liriomyza trifolii*) on the untreated plant half. However, the translocated amounts of neem extract did not effectively control the leaf miner that feeds on mesophyll cells (Larew 1988). The translocation or presence of azadirachtin or other neem toxins in the phloem is generally regarded as low (Larew 1988, Schmutterer 1988, National Research Council 1992). A basipetal translocation and the spread from treated leaves to higher plant parts indicate that toxins from neem extracts are not only distributed in the xylem of plants, but most likely also in the phloem. In our study, after applying NeemAzal-T/S in common doses the ingredients seem to be sufficiently distributed in rose plants to control *M. rosae* nymphs not only on treated but also on untreated plant parts. Although the concentration of compounds in untreated plant parts was not analysed directly, our results of a bilateral translocation are important for the control of *M. rosae* and other aphids for two reasons. First, the data suggests that the insecticidal compounds are available in the phloem of rose plants after spraying. Second, newly growing shoot tips and buds, the preferred feeding sites of *M. rosae*, that develop after a neem treatment, will be protected as well. In general, the effectiveness of the translocation of azadirachtin and other active substances from neem might be significantly influenced by the plant and pest species (Larew 1988, Lowery et al. 1993, Lowery and Isman 1993, 1994a, 1994b).

The second objective of our study was to investigate whether NeemAzal-T/S has repellent or antifeedant effects on *M. rosae*. Because the neem product does not have a direct effect on adults in terms of mortality or reproduction (Bartelsmeier et al. 2020), a repellent effect against adult stages would be advantageous for the protection of rose plants with neem. However, neither a repellent nor an antifeedant effect has been recorded in our experiments on apterous females of *M. rosae*.

Repellence was also not recorded for *M. persicae* on artificial diet treated with 25-100 ppm azadirachtin (Nisbet et al. 1994). In contrast, 50 ppm and higher concentrations of azadirachtin sprayed on barley leaves repelled *S. avenae* and *R. padi* in the first 25 minutes after the start of

exposure (West and Mordue (Luntz) 1992). Repellent effects only contribute to insect control if the effect is sustained – preferably for a few days at least to prevent aphid settling and population growth. In the case of *M. rosae* and rose plants, the common dose of NeemAzal-T/S, equivalent to 33 ppm azadirachtin A, did not cause any repellent effect in our studies with adult aphids. Thus, aphids most likely did not leave treated plants to settle on untreated plants during the assessed 72 hours period. Other instars might react differently. To deter second instar *M. rosae*, concentrations of a neem seed extract equivalent to only 11 ppm azadirachtin were necessary to cause 50 % deterrence. Initial observations showed that no repellent effect was present. 100% deterrence was achieved with 100 ppm azadirachtin after 6 hours in a leaf disc study (Koul 1999). Experiments to assess the effect of commercial products such as NeemAzal-T/S in terms of repellence or deterrence of *M. rosae* nymphs remain to be done.

Neither of the feeding tests, i.e. a quantitative method assessing the honeydew production and a qualitative method, EPG recording, revealed differences in feeding activity of *M. rosae* with increasing neem concentrations. No effects on honeydew production, a proxy for feeding intensity, were recorded in our experiments. Honeydew excretion was compared for small *M. rosae* populations of mixed developmental stages on detached leaves as well as for single adults. Interestingly, Nisbet et al. (1994) determined that honeydew production of adult *M. persicae* was reduced during the exposure to azadirachtin-treated artificial diets after an initial period of 26 hours. During the first 26 hours, no differences were found, but in the subsequent period of 24 hours, honeydew production was significantly reduced compared to the control (Nisbet et al. 1994). Already 25 ppm azadirachtin had significant effects. The concentrations in our study ranged from 25 to 41 ppm azadirachtin, but no effects on honeydew excretion of adult *M. rosae* were recorded. Different aphid species may respond differently (Lowery and Isman 1993, 1994a, 1994b), but a major difference was also the use of artificial diet versus plants as food source. Plants contain a variety of secondary metabolites that may interact with the effect of azadirachtin. Honeydew production of *M. rosae* populations was compared to aphids on rose leaves sprayed with the synthetic insecticide flupyradifurone. Flupyradifurone is known for its very strong antifeedant effects on aphids and a rapid decrease in honeydew production (Nauen et al. 2015). This effect of flupyradifurone was also remarkable in our study in contrast to the effect of NeemAzal-T/S which had no impact on honeydew production of *M. rosae*.

Studying feeding activity through EPG recording resulted in only one significant difference: mean number of probes was significantly higher in the NeemAzal-T/S soil treatment compared to the control. However, no differences compared to the blank formulation treatments were recorded and there was no correlation with the dose applied. Based on our study, common doses of NeemAzal-T/S do not appear to affect the feeding of adult *M. rosae*. Only one other study has been published that investigated the effect of azadirachtin on phloem-feeding insects with the EPG technique. Nisbet (1992) studied whether probing and feeding by *M. persicae* is affected by root treatments of tobacco seedlings with high azadirachtin doses, i.e. 100 to 1000 ppm. Roots of tobacco seedlings were immersed in azadirachtin solutions 27 hours before the EPG recordings. Apterous *M. persicae* displayed a higher percentage of total recording time expressing non-penetration and pathway periods at very high doses of at least 300 and 500 ppm azadirachtin. Furthermore, the aphids showed more phloem periods, but these were significantly shorter than in the control (Nisbet 1992, Nisbet et al. 1993). Interestingly, when a topical application was compared to a root treatment with 500 ppm

azadirachtin, significantly more time was spent on non-penetrating and pathway time in the root treatment, but not in the topical application (Nisbet 1992). Also in our experiment, soil application with 33 ppm azadirachtin (3.3 ml/l NeemAzal-T/S) led to significantly more probes. However, because no other EPG parameters differed, the impact of azadirachtin on feeding behaviour seems to be limited.

Phloem feeding by the leafhopper *Nephotettix virescens* on neem-oil-treated rice plants was significantly reduced. It is remarkable that the leaf hopper probed more, seemed restless and changed from phloem feeding to xylem feeding on treated rice plants (Saxena and Khan 1985). In our EPG experiment, however, xylem-feeding by *M. rosae* rarely occurred and no differences between the treatments were found for this parameter.

In the EPG experiment, we tested NeemAzal-T/S as foliar applications in doses of 3.3, 6.6 and 10 ml/l, equivalent to 33, 66 and 100 ppm azadirachtin, and therefore exceeded the registered dose (30-50 ppm) already two- to threefold with the highest concentration tested. Nevertheless, no effects of NeemAzal-T/S on feeding behaviour were found. Previous studies showed that very high concentrations of azadirachtin were effective. For absolute antifeedant and repellent effects, concentrations of 100 to 500 ppm azadirachtin are recommended and activity only persists for a few days (Koul et al. 1997, Koul 1999, Nisbet 1992, Nisbet et al. 1993, West and Mordue (Luntz) 1992). Such concentrations are not allowed in today's practical use of neem products. Furthermore, effects differ remarkably between aphid species and ages (Lowery and Isman 1993, Koul 1999). Repellent and antifeedant effects of insecticides in addition to mortality are advantageous, because the excretion of honeydew will be reduced as well as the transmission of aphid-transmitted plant viruses. However, according to our studies and previous work discussed above, repellent and antifeedant effects of azadirachtin or neem formulations do not provide a main contribution to aphid control.

In conclusion, the bidirectional systemic effect of NeemAzal-T/S that was reported here benefits control of *M. rosae*, because it enhances aphid mortality. Moreover, the common application of spraying 3.3 ml/l NeemAzal-T/S does not influence the settling behaviour and feeding of *M. rosae*. Thus, for the practical application of NeemAzal-T/S, insect growth regulating effects in immature aphids after feeding on treated plants and a reduced reproduction are the relevant effects for controlling rose aphids (Bartelsmeier et al. 2021).

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Supplemental Data

Table S1 EPG parameters (mean ± SE) of *M. rosae* regarding xylem-ingestion (G) and penetration-difficulties (F) on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc pairwise comparison.

Treatment	N[1]	No. of G events[2]	Total duration G[2] (minutes)	No. of F events[3]	Total duration F[3] (minutes)
Control	22	0.2 ± 0.1 a	16.6 ± 10.6 a	0.4 ± 0.2 a	7.4 ± 4.3 a
Blank 3	19	0.0 ± 0.0 a	0.0 ± 0.0 a	0.9 ± 0.5 a	9.7 ± 4.1 a
Blank 10	22	0.2 ± 0.1 a	7.1 ± 4.5 a	0.3 ± 0.1 a	8.0 ± 4.7 a
NAz[4] 3	21	0.0 ± 0.0 a	0.0 ± 0.0 a	0.3 ± 0.2 a	10.8 ± 8.3 a
NAz[4] 6	16	0.1 ± 0.1 a	8.3 ± 6.1 a	0.4 ± 0.2 a	6.7 ± 3.4 a
NAz[4] 10	21	0.4 ± 0.3 a	13.9 ± 10.2 a	0.6 ± 0.4 a	13.7 ± 7.9 a
NAz[4] 24h	20	0.0 ± 0.0 a	0.0 ± 0.0 a	1.3 ± 0.5 a	36.9 ± 16.0 a
NAz[4] Soil	19	0.0 ± 0.0 a	0.0 ± 0.0 a	1.4 ± 0.7 a	16.7 ± 8.3 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] G: Xylem-ingestion

[3] F: Penetration difficulties/mistakes

[4] NAz: Abbreviation for NeemAzal-T/S

Table S2 EPG parameters (mean ± SE) of *M. rosae* regarding the different phloem phases on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc pairwise comparison.

Treatment	N[1]	Total duration E1[2] (minutes)	Total duration E2[3] (minutes)	Total duration all phloem phases (minutes)[4]	Nr. sustained E2[5] (>10 m)	Total duration sustained E2[5] (>10m) (minutes)	Time to first E2[3] (minutes)	Time to first sustained E2[5] (>10m) (minutes)
Control	22	17.2 ± 3.7 a	249.9 ± 35.0 a	285.1 ± 29.1 a	2.2 ± 0.5 a	237.7 ± 37.5 a	97.7 ± 25.6 a	161.4 ± 38.4 a
Blank 3	19	15.5 ± 2.3 a	198.2 ± 32.0 a	260.7 ± 25.8 a	3.3 ± 0.6 a	173.1 ± 35.9 a	69.7 ± 23.5 a	164.1 ± 39.7 a
Blank 10	22	16.1 ± 3.1 a	192.3 ± 29.1 a	261.5 ± 19.9 a	2.9 ± 0.6 a	167.9 ± 33.6 a	63.5 ± 10.0 a	186.3 ± 40.3 a
NAz[6] 3	21	11.5 ± 3.4 a	267.2 ± 28.9 a	294.6 ± 25.8 a	3.7 ± 0.5 a	255.6 ± 30.7 a	63.8 ± 14.5 a	107.8 ± 27.9 a
NAz[6] 6	16	13.8 ± 2.3 a	182.1 ± 31.3 a	234.7 ± 24.8 a	2.4 ± 0.5 a	159.1 ± 36.5 a	60.6 ± 9.7 a	230.2 ± 49.6 a
NAz[6] 10	21	16.5 ± 2.5 a	204.3 ± 28.8 a	269.7 ± 21.5 a	3.8 ± 0.6 a	187.9 ± 31.4 a	63.0 ± 12.4 a	121.4 ± 30.0 a
NAz[6] 24h	20	11.8 ± 2.5 a	235.8 ± 36.3 a	262.5 ± 31.7 a	2.5 ± 0.4 a	222.8 ± 38.6 a	93.9 ± 21.9 a	172.5 ± 39.3 a
NAz[6] Soil	19	20.3 ± 3.9 a	144.6 ± 27.5 a	210.3 ± 22.9 a	2.8 ± 0.6 a	122.6 ± 30.7 a	67.5 ± 10.2 a	216.3 ± 45.7 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] E1: Phloem salivation

[3] E2: Phloem sap ingestion

[4] Total duration of all phloem phases includes E2, E2 and unknown phloem-phase waveforms (see supplementary Table S3 in Appendix for unknown waveforms)

[5] Sustained E2: E2 phase longer than 10 minutes

[6] NAz: Abbreviation for NeemAzal-T/S

Table S3 EPG parameters (mean ± SE) of *M. rosae* regarding unknown phloem phases on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc pairwise comparison.

Treatment	N[1]	% recordings of total recordings with unknown waveforms	Total duration unknown phloem-phases (minutes)	Nr. unknown phloem phases
Control	22	31.8	18.0 ± 6.2 a	2.7 ± 0.9 a
NeemAzal-T/S Blank 3	19	42.1	46.9 ± 14.2 a	3.8 ± 1.2 a
NeemAzal-T/S Blank 10	22	50.0	53.1 ± 12.2 a	5.1 ± 1.2 a
NeemAzal-T/S 3	21	19.0	15.9 ± 9.6 a	1.5 ± 0.8 a
NeemAzal-T/S 6	16	43.8	38.9 ± 12.8 a	3.5 ± 1.2 a
NeemAzal-T/S 10	21	52.4	48.9 ± 11.1 a	4.0 ± 0.9 a
NeemAzal-T/S 24h	20	40.0	14.8 ± 6.8 a	1.8 ± 0.8 a
NeemAzal-T/S Soil	19	68.4	45.3 ± 9.9 a	4.9 ± 1.0 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

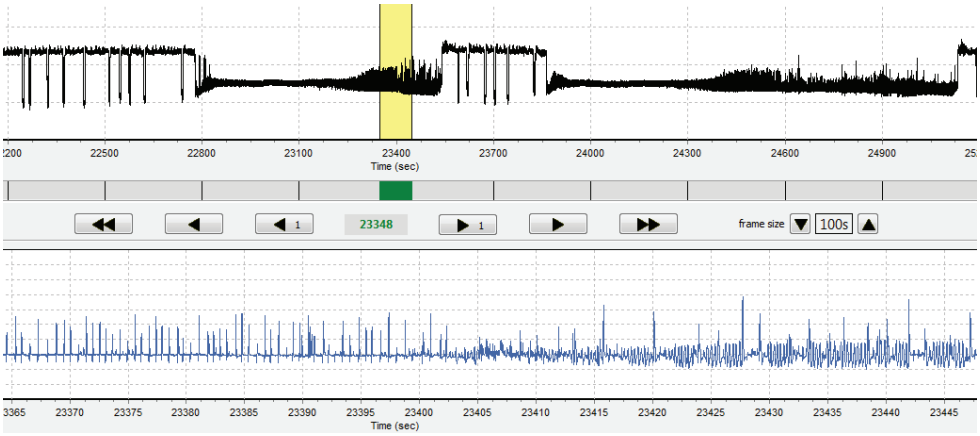

Fig. S1 Example of a recording with unknown waveforms in the phloem-phase after a typical E1 and E2-phase. Waveforms on the lower panel are the magnification of the yellow marked area above. The figure represents a screenshot of data from the software Stylet+a, version 01.30 (EPG Systems, Wageningen, The Netherlands).

Table S4 EPG parameters (mean ± SE) of *M. rosae* regarding probing, non-penetration- (NP) and pathway-periods (C) on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. Results are separated for recording hours 1 to 4 and 5 to 8. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc pairwise comparison.

Treatment	N[1]	No. probes				Total duration NP[2] (minutes)				Total duration of C[3] including pd (minutes)			
		Hours 1-4		Hours 5-8		Hours 1-4		Hours 5-8		Hours 1-4		Hours 5-8	
Control	22	71.6 ±	9.6 a	59.0 ±	13.2 a	34.7 ±	9.7 a	10.4 ±	4.4 a	66.9 ±	9.8 a	58.9 ±	12.5 a
NeemAzal-T/S Blank 3	19	98.1 ±	10.4 a	85.7 ±	14.7 a	25.5 ±	6.8 a	24.1 ±	8.7 a	84.8 ±	8.9 a	75.1 ±	11.8 a
NeemAzal-T/S Blank 10	22	107.4 ±	10.3 a	86.0 ±	15.8 a	27.4 ±	5.7 a	10.0 ±	4.7 a	92.0 ±	7.5 a	74.0 ±	12.0 a
NeemAzal-T/S 3	21	76.2 ±	10.0 a	74.2 ±	14.7 a	25.9 ±	7.7 a	27.2 ±	9.8 a	62.5 ±	7.7 a	59.1 ±	11.1 a
NeemAzal-T/S 6	16	104.6 ±	13.5 a	98.2 ±	14.7 a	40.4 ±	8.5 a	24.4 ±	5.6 a	77.4 ±	10.0 a	88.1 ±	10.6 a
NeemAzal-T/S 10	21	83.6 ±	8.4 a	74.5 ±	11.7 a	26.0 ±	8.0 a	15.6 ±	4.3 a	72.8 ±	7.9 a	68.4 ±	9.9 a
NeemAzal-T/S 24h	20	92.7 ±	12.2 a	83.2 ±	15.5 a	23.8 ±	4.0 a	17.2 ±	5.8 a	75.7 ±	10.1 a	63.9 ±	12.0 a
NeemAzal-T/S Soil	19	118.4 ±	14.7 a	109.4 ±	14.2 a	27.9 ±	5.8 a	32.0 ±	12.5 a	99.5 ±	11.5 a	93.7 ±	11.1 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] NP: non penetration time/no activity

[3] C/pd: Stylet pathway including probes (pd=potential drops)

Table S5 EPG parameters (mean ± SE) of *M. rosae* regarding the different phloem phases (E1: salivation, E2: phloem ingestion) on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. Results are separated for recording hours 1 to 4 and 5 to 8. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc all pair-comparison.

Treatment	N[1]	Total duration E1[2] (minutes)		Total duration E2[3] (minutes)		Total duration all phloem phases[4] (minutes)	
		Hours 1-4	Hours 5-8	Hours 1-4	Hours 5-8	Hours 1-4	Hours 5-8
Control	22	6.8 ± 2.1 a	10.4 ± 2.9 a	110.5 ± 16.4 a	139.3 ± 20.7 a	125.7 ± 14.8 a	159.5 ± 17.3 a
NeemAzal-T/S Blank 3	19	8.9 ± 2.1 a	6.6 ± 1.1 a	91.5 ± 13.6 a	106.8 ± 18.8 a	123.3 ± 11.5 a	137.3 ± 15.2 a
NeemAzal-T/S Blank 10	22	8.6 ± 1.3 a	7.6 ± 2.1 a	81.9 ± 12.0 a	110.4 ± 18.8 a	115.5 ± 9.2 a	146.0 ± 14.3 a
NeemAzal-T/S 3	21	4.9 ± 1.2 a	6.6 ± 2.5 a	128.1 ± 14.7 a	139.1 ± 17.0 a	142.6 ± 13.3 a	152.0 ± 15.5 a
NeemAzal-T/S 6	16	4.8 ± 1.0 a	9.0 ± 1.9 a	90.9 ± 17.9 a	91.1 ± 17.8 a	114.8 ± 15.3 a	119.9 ± 14.2 a
NeemAzal-T/S 10	21	8.6 ± 1.8 a	7.9 ± 1.6 a	90.1 ± 14.1 a	114.2 ± 16.0 a	120.4 ± 12.3 a	149.3 ± 11.7 a
NeemAzal-T/S 24h	20	4.3 ± 0.9 a	7.5 ± 2.3 a	99.7 ± 17.1 a	136.1 ± 20.4 a	110.3 ± 16.5 a	152.2 ± 16.8 a
NeemAzal-T/S Soil	19	11.2 ± 2.6 a	9.1 ± 1.8 a	66.5 ± 12.4 a	78.1 ± 17.7 a	99.0 ± 11.5 a	111.3 ± 15.1 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] E1: Phloem salivation

[3] E2: Phloem sap ingestion

[4] Total duration of all phloem phases includes E2, E2 and unknown phloem-phase waveforms

Table S6 EPG parameters (mean ± SE) of *M. rosae* regarding the sustained (longer than 10 minutes) E2 phases on untreated (Control) rose plants and plants treated with different concentrations of NeemAzal-T/S Blank or NeemAzal-T/S. Results are separated for recording hours 1 to 4 and 5 to 8. See Table 1 for an overview of the different treatments. Different letters within the same column indicate significant ($P < 0.05$) differences in means based on a Kruskal-Wallis Test and a Bonferroni-corrected post-hoc all pair-comparison.

Treatment	N[1]	Nr. sustained E2[2] (> 10 m)		Total duration sustained E2[2] (> 10m) (minutes)	
		Hours 1-4	Hours 5-8	Hours 1-4	Hours 5-8
Control	22	1.2 ± 0.2 a	1.0 ± 0.3 a	105.8 ± 17.4 a	131.9 ± 21.9 a
Blank 3	19	1.9 ± 0.4 a	1.9 ± 0.4 a	77.0 ± 15.7 a	77.0 ± 15.7 a
Blank 10	22	1.6 ± 0.3 a	1.2 ± 0.3 a	69.2 ± 14.2 a	98.7 ± 21.0 a
NAz[3] 3	21	2.0 ± 0.3 a	1.8 ± 0.3 a	122.8 ± 15.6 a	132.8 ± 18.1 a
NAz[3] 6	16	0.8 ± 0.2 a	1.6 ± 0.4 a	78.2 ± 20.4 a	80.9 ± 20.0 a
NAz[3] 10	21	1.9 ± 0.3 a	1.9 ± 0.4 a	82.0 ± 14.9 a	106.0 ± 17.5 a
NAz[3] 24h	20	1.4 ± 0.3 a	1.1 ± 0.2 a	91.3 ± 17.5 a	125.2 ± 21.9 a
NAz[3] Soil	19	1.4 ± 0.3 a	1.4 ± 0.4 a	56.2 ± 13.6 a	66.4 ± 19.4 a

[1] N: total number of replicates (recordings of single aphids analysed for each treatment)

[2] sustained E2: E2 (phloem sap ingestion) phase longer than 10 minutes

[3] NAz: Abbreviation for NeemAzal-T/S

Chapter 4

Factors influencing the efficacy of azadirachtin for aphid control: Temperature, UV-radiation and moment of application

Imke Bartelsmeier, Michael Kilian and Marcel Dicke

Abstract

Botanical insecticides such as neem products are a promising alternative to synthetic pesticides. However, active ingredients of neem extracts rapidly degrade due to environmental factors. High temperatures and high UV intensities can result in varying efficacies of neem products, but practical trials to assess this with formulated neem products are missing so far. The aim of the present study was to assess the impact of different temperatures (18, 22 and 26 °C), UV-A intensities and moments of application on warm, sunny days on the efficacy of the commercial neem-product NeemAzal-T/S to control rose aphids (*Macrosiphum rosae*). The application of different doses of NeemAzal-T/S resulted in high efficacies (> 80 %) at all tested temperatures and UV intensities in the greenhouse. Due to formulation (contact) effects on the aphids, the efficacy of NeemAzal-T/S was even better at the highest tested temperature of 26 °C. Two outdoor trials, comparing the efficacy when the product was applied in the evening or in direct sunlight at noon on warm days, showed a significantly better efficacy when applied in the evening. This effect also seems related to contact effects of the formulation, but not by degradation of active ingredients. We conclude that NeemAzal-T/S can be used to control *M. rosae* in the tested temperature range of 18 to 26 °C and also at higher UV-conditions. However, to take advantage of the contact effect of the formulation, application in the evening is preferred over application in sunlight.

Introduction

For modern plant protection, insecticides with a low persistence in the environment and with different modes of action are needed to prevent the occurrence of resistance. Botanical insecticides are promising alternatives to synthetic pesticides for the control of insect pests on plants. Neem products provide common examples of botanical insecticides. These insecticides are based on extracts of seed kernels from the neem tree *Azadirachta indica*. Advantages of such insecticides are their broad target spectrum (Schmutterer and Singh 1995), low human and mammal toxicity and a relatively low persistence in the environment (Szeto and Wan 1996, Sundaram 1996a, Sundaram et al. 1997, Raizada et al. 2001, Boeke et al. 2004, Morgan 2009). One severe disadvantage, however, is a varying and sometimes low efficacy of neem products in the control of rapidly developing insects such as aphids, especially in comparison to synthetic insecticides (Basedow et al. 2002, Nikolova and Georgieva 2014, Bartelsmeier et al. 2018).

Two characteristics of such botanical insecticides may limit efficacy. First, azadirachtin, the main active ingredient of neem products, is a slow acting and insect-growth regulating substance (Stark and Rangus 1994, Mordue (Luntz) et al. 2010) and mainly active against juvenile stages of aphids. Second, azadirachtin is rapidly degraded which may hamper a stable and long-lasting efficacy of such products. Azadirachtin has a short half-life time of only 4 days in water at pH 7 and 30 °C and the degradation accelerates with increasing pH and temperature (Szeto and Wan 1996, Ruch et al. 1997, Pussemier 2000, Barrek et al. 2004). Another important factor for degradation is sunlight or UV radiation (Dureja and Johnson 2000, Johnson et al. 2003, Barrek et al. 2004). In field trials in Italy, azadirachtin A and other neem-extract compounds that were sprayed on olive and strawberry foliage had a half-life time of less than one day due to photodegradation (Caboni et al. 2002, Caboni et al. 2006). On castor leaves exposed to sunlight, the calculated half-life time of azadirachtin A was 2.5 days (Johnson et al. 2003). Degradation rate of azadirachtin depends on formulation and

environmental conditions. These influencing factors might lead to differences in field-trial efficacy. Professionally formulated azadirachtin-containing products, such as the commercial NeemAzal-T/S, enhance the stability of active compounds, but susceptibility to temperature and/or UV-radiation and a subsequent loss of efficacy has also been reported for formulated products (Ascher et al. 2000, Caboni et al. 2006, Kumar and Poehling 2006, 2007).

Even when azadirachtin and other neem compounds such as nimbin and salannin rapidly degrade under UV exposure, one or more of the photodegradation products of neem extracts remained biologically active against insects in the laboratory (Barnby et al. 1989, Jarvis et al. 1997). However, laboratory experiments with single compounds do not elucidate whether the efficacy of products containing neem-seed extracts is affected under field conditions. Furthermore, the stability of active ingredients is improved in formulated products by surfactants and oily substances. For a successful application of such products, greenhouse or field trials assessing the impact of environmental factors on efficacy are necessary. To the best of our knowledge, no studies have been reported that specifically tested the efficacy of commercial neem products against insects under different environmental conditions.

One major pest of rose plants is the rose aphid *Macrosiphum rosae*. An infestation with this aphid often leads to deformed leaves and flowers because new shoots and buds are the preferred feeding sites (Maelzer 1977, Alford 2012). If botanical insecticides such as neem products are to replace synthetic insecticides, their efficacy needs to be ensured. To achieve a stable efficacy, detailed knowledge is needed about critical factors during application and post-application that may affect efficacy.

The aim of the present study was to investigate the effects of environmental factors on the efficacy of the neem product NeemAzal-T/S for controlling rose aphids (*M. rosae*) under practical conditions under greenhouse and field conditions. Efficacy experiments were conducted in the greenhouse at different temperatures and UV intensities. A clip-cage experiment with first instar nymphs was conducted at the same time as the temperature trial to assess juvenile mortality and developmental times. In field experiments, application in direct sunlight during the day was compared to application in the evening, when no UV radiation was present anymore. Due to the described rapid degradation of azadirachtin depending on temperature and UV radiation, we expected a lower efficacy at high temperatures and high UV intensities. For these reasons, we also expected a better efficacy of NeemAzal-T/S when applied in the evening compared to an application at noon in direct sunshine. This study aims to improve the application and to ensure a good efficacy of NeemAzal-T/S, particularly against aphids.

Materials and methods

General materials and methods for all experiments

Insects and plants

The tested aphid species was a red phenotype of *M. rosae*. The culture originated from a female on a cultivated rose in Monheim am Rhein, Germany. *M. rosae* was maintained in rearing cages with three gauze sides (63 cm x 78 cm x 63 cm high) in a climate chamber (22±2 °C, 60±10 % RH and a 16:8 h L:D photoperiod). As host plants, miniature rose plants (*Rosa* hybrids, different varieties of Kordana-

Classic by W. Kordes' Söhne Rosenschulen GmbH & Co. KG) in pots of 11-13 cm diameter were used. Rose plants were obtained as freshly rooted cuttings with 3-4 plants per pot. The cuttings were separated and one cutting was planted per pot with the soil Einheitserde Classic, Type ED 73, 155 fine. Three to four weeks before every experiment, aphids were reared on the same rose variety that was used in the experiments.

Experimental plants were the same miniature rose plants as described before. Until use for experiments or rearing, rose plants were maintained in an air-conditioned greenhouse at 21±3 °C, 60±10 % RH and a 16:8 L:D photoperiod. Additional light in the greenhouse was provided by 400 W sodium vapor lamps if natural light intensity outside fell below 120 W/m². Plants were watered directly onto the soil as required during cultivation and experiments. A few days after transplanting, the cuttings were drenched with a liquid fertilizer (dose of 0.3 %, Wuxal Top N, Manna, Germany). This was repeated during the subsequent weeks as needed. The plants were sprayed with fungicides to prevent powdery mildew infestation before the start of the experiments, if required. The fungicides Compo Ortiva Universal Pilzfrei (Compo, Germany) or Bayer Garten Rosen Pilz-frei Baymat (SBM Life Science GmbH, Germany) were used in doses according to current registrations in Germany. Fungicides were only applied if necessary and not later than one week before the start of the infestation with aphids to avoid possible side effects on the aphids by the formulation. If applied, all plants were treated to obtain equal conditions among treatments. Experimental plants were in a stage where flower buds were already developed, but still closed (BBCH stage 51-59, Meier et al. 2009).

Treatments

As a neem-based insecticide, NeemAzal-T/S (Trifolio-M GmbH, Lahnau, Germany) containing 10 g/l azadirachtin A was used. This product is registered as a botanical insecticide in Europe for ornamental plants, orchards, vegetables, agriculture and for the home and garden use. See chapter 2 for more details on NeemAzal-T/S. NeemAzal-T/S was sprayed in different concentrations to investigate dose-dependent effects. The blank formulation of NeemAzal-T/S was included in each experiment in the highest formulation dose, as used for the NeemAzal-T/S treatment. The blank contains only oils and tensides, but no neem-seed-kernel extract with active ingredients. Additionally, a mixture of natural pyrethrum and azadirachtin was tested in the temperature experiment. With the exception of the UV experiment, the synthetic insecticide flupyradifurone was included as positive control. Table 1 provides an overview of the insecticides used. Concentrations of NeemAzal-T/S and applications are described separately for each experiment in the following sections. Solutions were sprayed till run-off with a handheld sprayer, operated by compressed air with 3 bar pressure. A 1.1 mm bore hollow cone nozzle was used. As a result, a complete coverage of the leaves was reached.

Table 1 Overview of treatments, type of formulation, corresponding doses and active ingredient (a.i.) contents. Formulation Type: EC: Emulsifiable concentrate, SL: Soluble concentrate.

Treatment name	**Formulation type**	**Active ingredient** (a.i.)	**a.i. in product** [g/l]	**Dose per application** [g a.i./ha]	**Application dose spraying solution** [ml product/l water]
Control	(untreated)	-	-	-	-
NeemAzal-T/S Blank	EC	no a. i.	0	-	-
NeemAzal-T/S 1.65 ml/l	EC	Azadirachtin A	10	15	1.65
NeemAzal-T/S 2.5 ml/l	EC	Azadirachtin A	10	22.5	2.5
NeemAzal-T/S 3.3 ml/l	EC	Azadirachtin A	10	30	3.3
NeemAzal-T/S 4.1 ml/l	EC	Azadirachtin A	10	37	4.1
Pyrethrum-Azadirachtin	EC	Pyrethrum (4.2 g/l) + azadirachtin A (4.1 g/l)	8.3	37.35	5
Flupyradifurone SL 50	SL	Flupyradifurone	50	60	1.3

After spraying the rose plants, they were placed randomly on saucers that were put upside down in plastic trays filled with 1-2 cm of water. The saucers functioned as islands in the water; the plants were not in contact with the water around them. These “islands” prevented apterous aphids from walking from one plant to another. This method allowed for independence of the data obtained from individual plants. Alate females were also present in all experiments, but in very low numbers. Numbers of plants (replications) used per treatment and environmental condition are presented in the next sections for each experiment separately.

Evaluation of infestation and efficacy calculation

In all experiments, rose plants were infested with approximately 50 *M. rosae* individuals of different stages. After a few days, numbers of aphids per plant were assessed before the application of the products. Numbers of aphids per plant were counted as estimated groups of five to ten individuals. These values represent the 0 DAA (days after application) data. Infestation and evaluation times are described in detail for every experiment in the following sections.

Efficacy was calculated as corrected mortality in %, following Henderson and Tilton (1955). The formula used (see below) includes the infestation before application of insecticide or of the blank formulation and the infestation in the reference control group. Calculations were performed for all evaluation days and for all treatments. In different environmental conditions (different temperatures, UV intensities or application times), efficacy values were calculated relative to the corresponding control group.

Corrected mortality by Henderson and Tilton (1955):

$$Efficacy_{HT}\ [\%] = \left(1 - \frac{T_a}{T_b} \times \frac{C_b}{C_a}\right) \times 100$$

$$T_b = Infestation\ on\ treated\ plant\ before\ application$$
$$T_a = Infestation\ on\ treated\ plant\ after\ application$$
$$C_b = Infestation\ on\ control\ plants\ before\ application$$
$$C_a = Infestation\ on\ control\ plants\ after\ application$$

This calculation was additionally done for the highest used concentration of NeemAzal-T/S with the data for NeemAzal-T/S Blank instead of the untreated control data. This method corrects for mortality effects of the blank formulation.

Temperature

To assess the influence of different environmental temperatures on the efficacy of NeemAzal-T/S against *M. rosae*, a greenhouse trial was conducted in three air-conditioned chambers with different set temperatures: 18 °C, 22 °C and 26 °C. Supplementary Table S1 shows the actual conditions during the experimental period of 22 days.

Plants were infested with approximately 50 *M. rosae* individuals from the main culture, three days before start of the experiment. The following treatments were compared at every temperature: untreated control, 3.3 ml/l NeemAzal-T/S Blank, 1.65 ml/l, 2.5 ml/l and 3.3 ml/l NeemAzal-T/S, 5 ml/l Pyrethrum-Azadirachtin and 1.33 ml/l Flupyradifurone SL 50. All treatments were applied on days 0 and 7 with the exception of Flupyradifurone. Flupyradifurone was only applied once, on day 0. Application of all other products on day 7 was carried out after the evaluation of numbers of aphids per plant. Evaluation dates were 0, 2, 7, 9, 11, 14, 16 and 22 days after start of the experiment. For days 2 to 22, efficacy was calculated as explained above. Per treatment and temperature, eight rose plants were used for this. Each plant represented one replication.

Additionally, a clip-cage experiment was conducted in the three greenhouse chambers to compare developmental times on untreated plants and the nymphal mortality on NeemAzal-T/S treated plants. Six rose plants per treatment (untreated control, 1.65 ml/l, 2.5 and 3.3 ml/l NeemAzal-T/S) were sprayed. After drying of the leaves approximately 1-2 hours after spraying, 3-5 apterous adult females from the main *M. rosae* culture were transferred with a fine brush to one clip cage per plant. Females were removed 24 hours later and new-born first instar larvae were checked daily for molting (only untreated control group) and mortality (all treatments) during 12 days.

UV

To investigate the influence of UV-A radiation on the efficacy of NeemAzal-T/S, an experiment was conducted in a greenhouse compartment under regular 400 W sodium vapour lamps or UV lamps (Osram Eversun L100/79 Super, Osram, Germany). Three different UV intensities were used: 2-3 W/m² (no UV lamps, natural UV dose in the greenhouse), 13-17 W/m² and 23-32 W/m² UV-A. These levels are labeled in the following as 0, 50 and 100 % UV, respectively. UV-B radiation was at an

extremely low level of < 1 W/m² in the highest UV-intensity. The UV lamps had an emission spectrum from 310-420 nm. To adapt the experimental plants to the UV conditions and to avoid burns on the leaves, rose plants were cultured under UV lamps for three weeks before the experiment. Furthermore, to adapt the aphids to UV, three separate cultures of *M. rosae* were maintained in the greenhouse chamber three weeks before the experiment started under 0, 50 and 100 % UV in acrylic glass cages with three gauze sides. An untreated control, 4.1 ml/l NeemAzal-T/S Blank and 2.5, 3.3 and 4.1 ml/l NeemAzal-T/S were used as treatments at every UV level. Due to the limited space on the greenhouse tables under the UV lamps, no positive control (synthetic insecticide) was included. Plants were sprayed as described above on days 0 and 7. Per treatment and UV level, six rose plants were used. Each plant represented one replication. Numbers of aphids per plant were assessed on days 0, 5, 7, 9, 12, 14, 16 and 23.

Timing of application

Two outdoor trials were conducted in May/June 2016 to investigate the effects of different times of application on the efficacy of NeemAzal-T/S. Experimental rose plants were placed outside on tables, two weeks before the experiments to allow the plants to acclimate to the outdoor conditions. Each plant was infested with 30-60 aphids from the main culture one week before the experiment started. In the second trial, plants received a second inoculation at three days before the trial started, because numbers of aphids per plant were low.

At the beginning of the experiments, 10 (trial 1) or 12 (trial 2) plants were sprayed per treatment at 12:30 pm in the presence of sunlight and 10 or 12 other plants were treated at 8:30 pm. Supplementary Table S2 shows an overview of temperature and UV conditions for both trials at both times of application. In these experiments, products were only applied once at the start of the experiment (0 DAA). The following treatments were used: untreated control, 3.3 ml/l NeemAzal-T/S Blank, 1.65, 2.5 and 3.3 ml/l NeemAzal-T/S and 1.3 ml/l Flupyradifurone SL 50. After spraying, plants were randomly placed on water-filled trays as described above to avoid migration of apterous aphids from one plant to another. Infestation was recorded as numbers of aphids per plant before the application on day 0 and on day 4 (trial 2) or 5 (trial 2), 8 and 15 days after application. Naturally occurring aphid predator insects such as ladybird or hoverfly eggs and larvae were removed from the plants every second day when recorded. For the efficacy calculation and statistical analysis, data of both trials was pooled, resulting in 22 replications per treatment and application time.

Statistical analysis

For the statistical analysis of efficacy, data distribution was checked for normality with Shapiro-Wilk test for each treatment and day. Homogeneity of variances was examined with Levene's test. If both assumptions for ANOVA were met, an ANOVA with Tukey post-hoc test was performed. If only Levene's test was significant (no equal variances), Welch-ANOVA was used with Games-Howell post-hoc test. In the case of non-normally distributed data, data transformation (e.g. arcsin(x/100)) was applied, followed by testing for compliance with ANOVA assumptions. If no data transformation was successful to obtain normal distribution, the non-parametric Kruskal-Wallis test was used for comparing the efficacies of a treatment in different conditions. If significant, a post-hoc all-pair comparison with Bonferroni-Dunn correction was executed. For the statistical analysis of efficacy

data for comparing application timing, ANOVA was performed or Mann-Whitney U test, if assumptions for ANOVA were not fulfilled as described before. All analyses were performed in SPSS (version 22, IBM).

Nymphal development and mortality were each analyzed using the Cox proportional hazards model in R (version 3.1.3, R Foundation for Statistical Computing, 2015) with functions "survfit" and "coxph" of the package survival (Thernau 2012). Nymphal mortality was analysed for 10 days. See Chapter 2 for more details on the statistical analysis of nymphal mortality or development in R. The control group at every temperature functioned as the baseline mortality hazard for the corresponding NeemAzal-T/S treatments.

Results

Effects of temperature

Efficacy

NeemAzal-T/S reached average efficacies of up to 95 % (3.3 m/l NeemAzal-T/S, 14 DAA, Fig. 1D). Treatment with 1.65 ml/l NeemAzal-T/S had significantly lower efficacies at 18 °C than at 26 °C on days 7, 9 and 11 (Fig. 1B). Similar results were found for 2.5 ml/l NeemAzal-T/S on days 9, 11 and 14 and for 3.3 ml/l on day 7 (Fig. 1C, D). After the second application on day 7, the efficacy of 3.3 ml/l NeemAzal-T/S was higher than 85 % at all three temperatures until the end of the experiment. The efficacy of the blank formulation (NeemAzal-T/S Blank) was also significantly lower at 18 °C than at 26 °C or 22 °C from day 9 onwards (Fig. 1A). Efficacy of the blank was generally lower than of the NeemAzal-T/S treatments. Overall, the efficacy increased with increasing NeemAzal-T/S concentrations at all three temperatures. Treatment with Pyrethrum-Azadirachtin (Fig. 1E) and Flupyradifurone (Fig. 1F) resulted in efficacies higher than 94 % from 2 DAA onwards. In both treatments, the efficacies were not significantly different between the different temperatures. The efficacy of 3.3 ml/l NeemAzal T/S relative to the blank formulation (Fig. 1G) was similar to the efficacy of 3.3 ml/l NeemAzal T/S compared to the untreated control (Fig. 1D).

Effect of temperature on the development of *M. rosae*

Temperature influences aphid development and reproduction rate. To assess the efficacy of NeemAzal-T/S at different temperatures in more detail, development times for each instar and start of reproduction were compared in clip cages during the efficacy trial. *Macrosiphum rosae* nymphs developed significantly faster at higher temperatures. Total developmental time to adult females at 22 °C and 26 °C was 8.7±0.4 and 8.6±0.3 days, respectively. This was significantly shorter than at 18 °C: 10.6±0.3 days (Table 2). First offspring were found on day 11 at 18 °C and on day 10 at 22 °C. Although the mean developmental time to the adult stage was only 8.6 days at 26 °C, reproduction started on day 11 (reproduction data not shown).

Nymphal mortality in clip cages at different temperatures

NeemAzal-T/S treatments significantly enhanced nymphal mortality at all three temperatures (Fig. 2, Table 3). At 22 and 26 °C, all nymphs died within 10 days at all three NeemAzal-T/S concentrations (Fig. 2). At 26 °C, application of 3.3 ml/l NeemAzal-T/S resulted in 50% mortality already at day 3 (LT_{50}: time to 50% mortality). At 18 and 22 °C, LT_{50} was 4 days (Fig. 2). Survival analysis of *M. rosae*

nymphs on plants treated with different concentrations of NeemAzal-T/S shows a highly significant effect of treatment on mortality risk. At all three temperatures and for all tested concentrations of the product, the hazard ratio (HR) to die was significantly increased ($P < 0.001$, Table 3). Hazard ratios increase with increasing concentrations of NeemAzal-T/S as well as with increasing temperatures.

Table 2 Time in days for each developmental stage of *M. rosae* on untreated rose plants at different temperatures. Mean values ± standard deviations. Different letters within an instar represent significant ($P < 0.05$) differences in the time to next molting according to Cox Proportional Hazard Model with Tukey post-hoc test. N represents the total number of observed aphids from five replications (clip cages).

°C	First instar	Second instar	Third instar	Fourth instar	Total time to adult	N
18	3.6 ±0.5 a	2.2 ±0.3 a	2.2 ±0.3 a	2.7 ±0.3 a	10.6 ±0.3 a	27-29
22	3.3 ±0.2 a	1.4 ±0.2 b	1.7 ±0.2 b	2.3 ±0.3 a	8.7 ±0.4 b	41-44
26	2.8 ±0.2 b	1.5 ±0.3 b	1.8 ±0.2 b	2.5 ±0.2 a	8.6 ±0.3 b	46-48

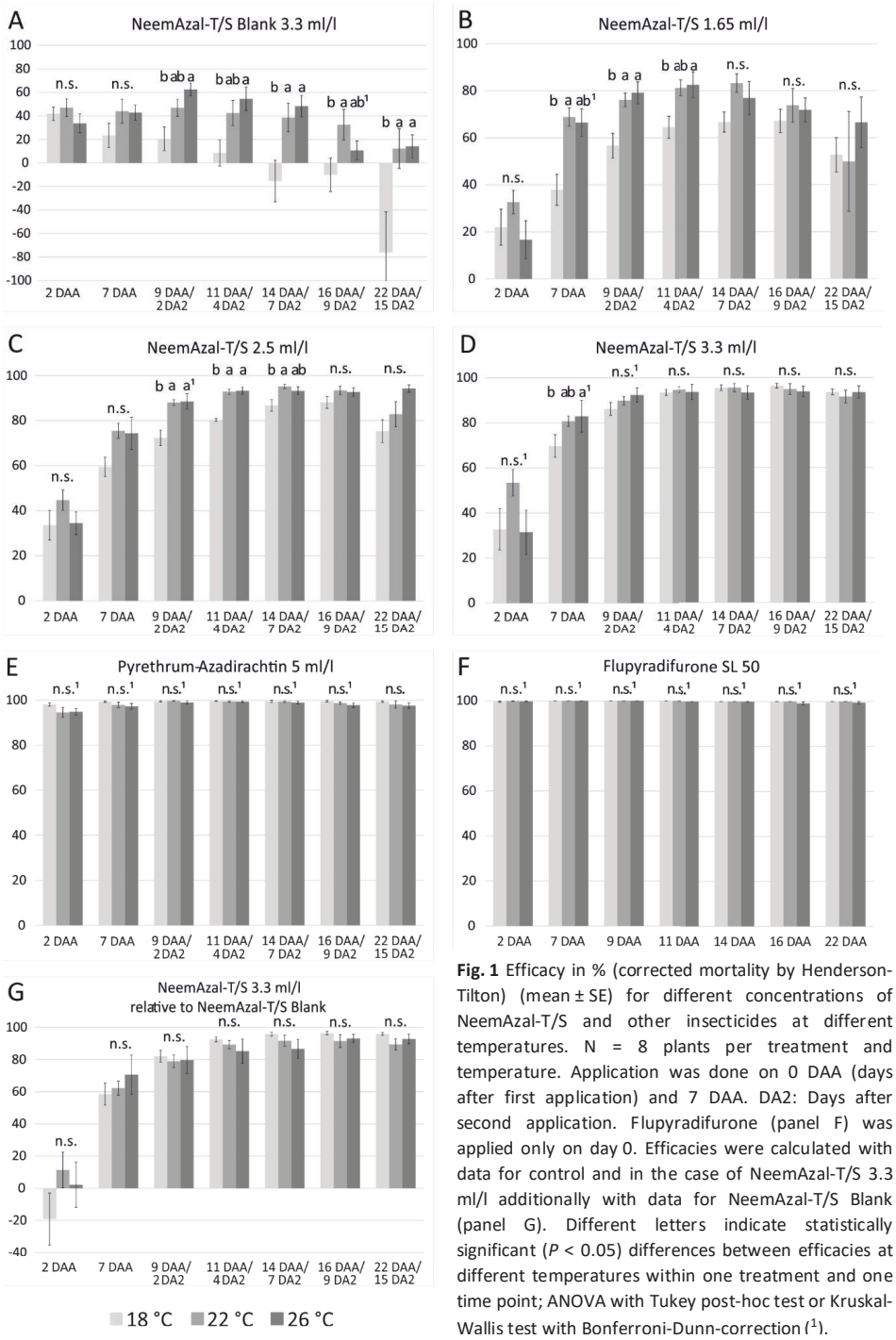

Fig. 1 Efficacy in % (corrected mortality by Henderson-Tilton) (mean ± SE) for different concentrations of NeemAzal-T/S and other insecticides at different temperatures. N = 8 plants per treatment and temperature. Application was done on 0 DAA (days after first application) and 7 DAA. DA2: Days after second application. Flupyradifurone (panel F) was applied only on day 0. Efficacies were calculated with data for control and in the case of NeemAzal-T/S 3.3 ml/l additionally with data for NeemAzal-T/S Blank (panel G). Different letters indicate statistically significant ($P < 0.05$) differences between efficacies at different temperatures within one treatment and one time point; ANOVA with Tukey post-hoc test or Kruskal-Wallis test with Bonferroni-Dunn-correction ([1]).

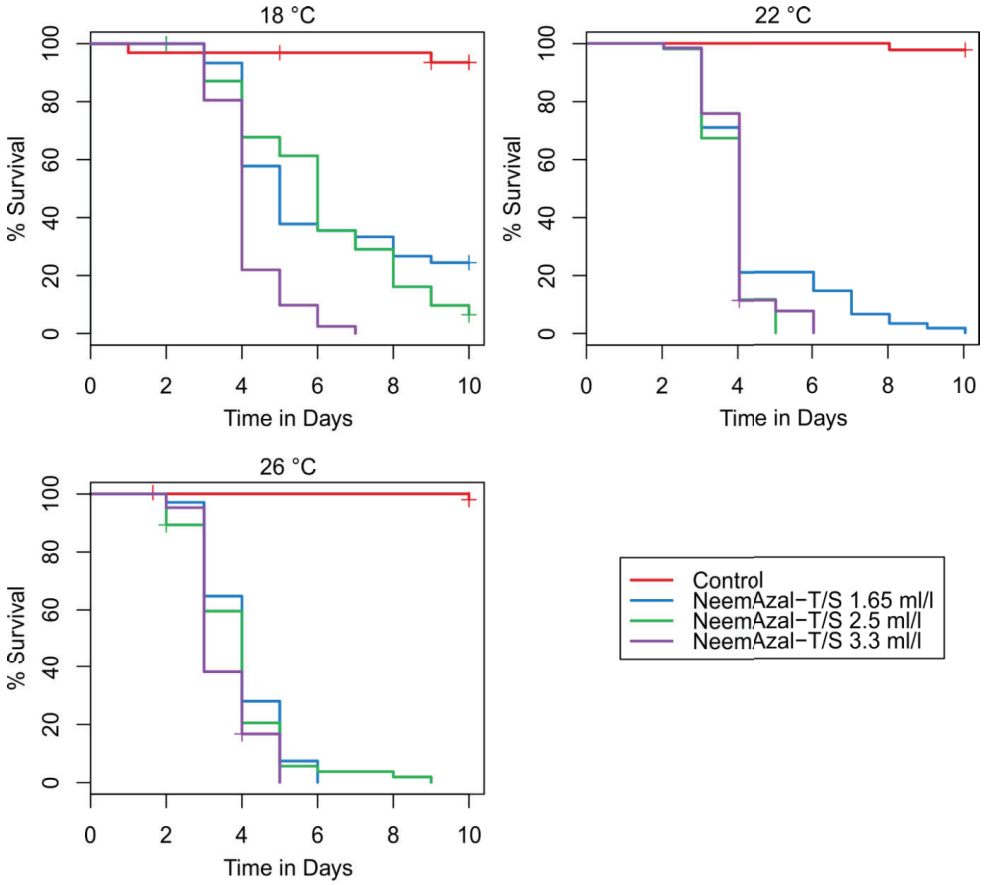

Fig. 2 Survival of *M. rosae* nymphs in clip cages during 10 days at 18 °C, 22 °C and 26 °C on untreated ("Control") and NeemAzal-T/S treated plants. Total numbers of observed aphids per treatment varied between 32 and 46 aphids at 18 °C, 44 - 62 aphids at 22 °C and 42 - 68 aphids at 26 °C.

Table 3 Mortality of *M. rosae* nymphs as hazard ratios (HR) to die (95 % CI) during 10 days after application of different concentrations of NeemAzal-T/S at 18, 22 or 26 °C. Results according to according to Cox Proportional Hazard Model. N = 6 clip cages, each with 4-13 aphids, per treatment and temperature.

	18 °C		**22 °C**		**26 °C**	
	HR	***P***	**HR**	***P***	**HR**	***P***
NeemAzal-T/S 1.65 ml/l	31.8 (4.8 – 212.2)	< 0.001	227.3 (32.1 - 1612)	< 0.001	246.2 (39.1-1550)	< 0.001
NeemAzal-T/S 2.5 ml/l	41.6 (7.2 – 237.4)	< 0.001	371.0 (52.31 – 2659)	< 0.001	267.2 (40.4 – 1768)	< 0.001
NeemAzal-T/S 3.3 ml/l	110.7 (17.7 – 692.7)	< 0.001	328.3 (42.24 - 2331)	< 0.001	382.2 (60.42-2417)	< 0.001

Effects of UV

NeemAzal-T/S reached a high efficacy of 80-90 % at all UV levels and concentrations after the second application (Fig. 3, panels B to E). Efficacy of 3.3 and 4.1 ml/l NeemAzal-T/S was significantly ($P < 0.05$) lower at the intermediate UV level (50 %) on days 12, 14, 16 and 23 (Fig. 3C, D). In the NeemAzal-T/S Blank treatment, mean efficacies were lower, also including negative values and higher standard errors than for NeemAzal-T/S (Fig. 3A).

Efficacies of treatment with 4.1 ml/l NeemAzal-T/S compared to NeemAzal-T/S Blank (Fig. 3E), were similar to efficacies that are calculated relative to untreated control (Fig. 3C). Also here, efficacies were significantly ($P < 0.05$) smaller at the intermediate UV level than at the 0 or 100 % UV levels from days 12 to 23 (Fig. 3E).

Outdoor trials: Effects of timing of application

In the outdoor trials, efficacy of all tested concentrations of NeemAzal-T/S and its blank formulation was consistently higher when applied in the evening until day 8 (Fig. 4). Significant differences between day and evening applications were recorded for NeemAzal-T/S Blank and 2.5 ml/l NeemAzal-T/S on 4/5 and 8 DAA. The highest efficacy (50 %) was recorded on day 8 for the evening application of 3.3 ml/l NeemAzal-T/S, relative to the untreated control (Fig. 4D). In contrast, no significant differences were recorded when efficacy for 3.3 ml/ NeemAzal-T/S is calculated relative to NeemAzal-T/S Blank instead of control (Fig. 4F). Flupyradifurone reached nearly 100 % efficacy at 4-5 DAA and no significant differences were found between midday and evening application (Fig. 4E).

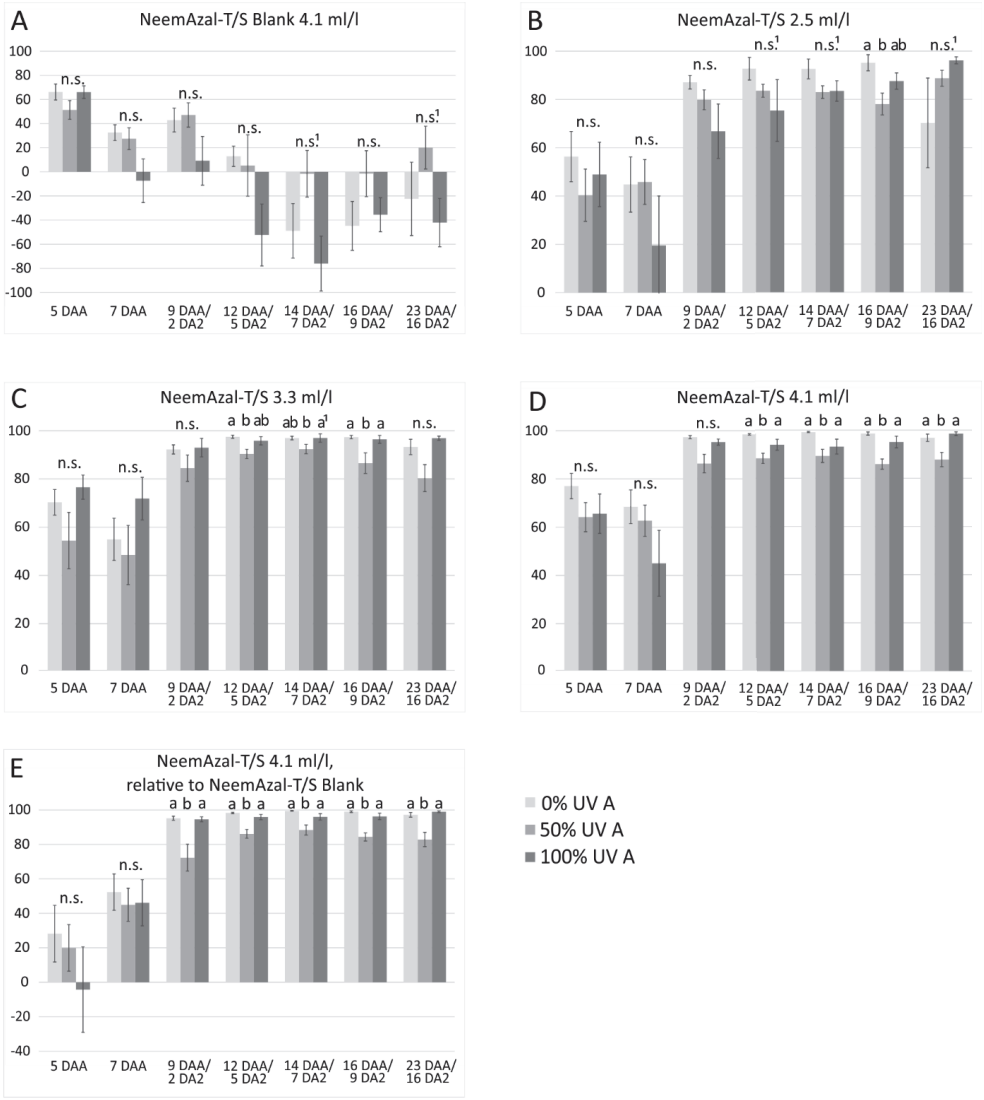

Fig. 3 Efficacy in % mortality (corrected by Henderson-Tilton) (mean ± SE) for different concentrations of NeemAzal-T/S and other insecticides at different UV-A intensities during the experimental period. N = 6 plants per treatment and temperature. Application was done on 0 DAA (days after first application) and 7 DAA. DA2: Days after second application. Efficacies were calculated compared to the untreated control and in the case of NeemAzal-T/S 3.3 ml/l also compared to NeemAzal-T/S Blank (panel E). Different letters indicate statistically significant ($P < 0.05$) differences between efficacies at different UV treatments within one spray treatment and time point; ANOVA (with Tukey or Games-Howell post-hoc test) or Kruskal-Wallis test with Bonferroni-Dunn-correction ([1]).

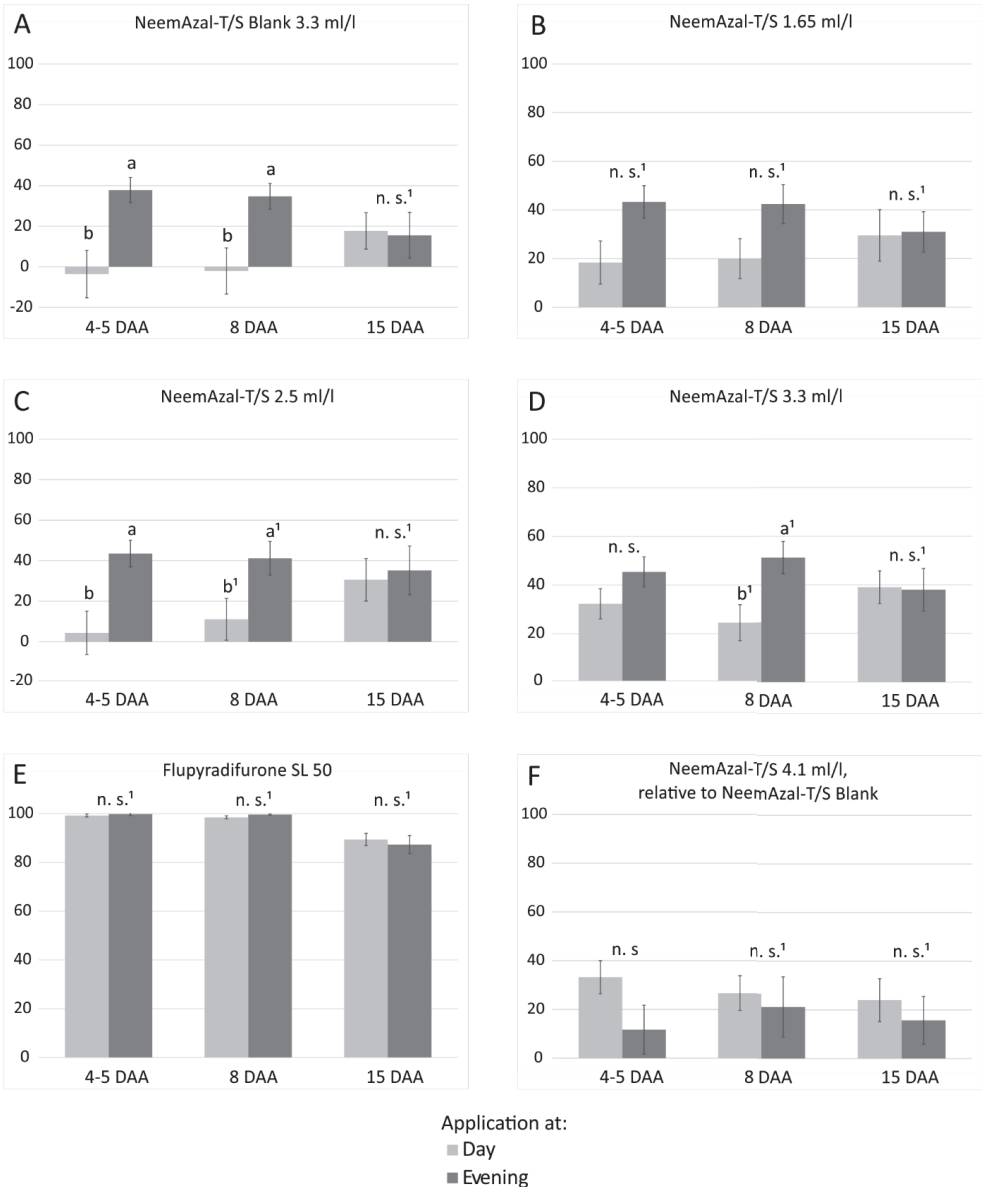

Fig. 4 Efficacy in % mortality (corrected by Henderson-Tilton) (mean ± SE) for different concentrations of NeemAzal-T/S and Flupyradifurone applied in sunlight during midday or in the evening. N = 20-22 plants per treatment and application time (pooled data of two similar trials with 10 and 12 plants per variant). Products were applied once at day 0. Different letters indicate statistically significant ($P < 0.05$) differences between efficacies at different time of application within one treatment and one time point; ANOVA or Mann-Whitney U test([1]).

Discussion

The aim of this study was to assess the effects of environmental factors such as temperature and UV-A radiation on the efficacy of NeemAzal-T/S and to establish recommendations for the practical application of neem products for the control of rose aphids. Our results show that NeemAzal-T/S can reach high efficacies of 90 % and higher at temperatures ranging from 18 to 26 °C and also at high UV-A intensities. Another remarkable result is the higher efficacy when the product is applied in the evening in outdoor trials compared to an application at 12:30 pm on a sunny, warm day.

Our hypothesis was that azadirachtin as the main active ingredient in NeemAzal-T/S would be degraded faster at warm temperatures and at high UV levels, thus resulting in lower efficacies. It was expected that NeemAzal-T/S would reach higher efficacies at 18 °C than at 26 °C due to a faster degradation of azadirachtin with increasing temperatures (Szeto and Wan 1996, Ruch et al. 1997). In contrast, the product tended to have higher efficacies at 26 °C compared to 18 °C, but this effect seems to be a formulation effect as there is no temperature effect when efficacy is assessed relative to the blank formulation. The recorded better efficacy, also of the blank formulation, at 26 °C may result from a better spreading of the emulsifying spray solution on the rose leaves after application. In the temperature range of 18 °C to 26 °C, no negative impact of higher temperatures was apparent.

The clip-cage experiment assessing nymphal mortality also shows a strong lethal effect of NeemAzal-T/S on *M. rosae* nymphs at all tested temperatures, but with a slightly earlier mortality at 26 °C than at the lower temperatures. This effect can be explained by the captured earlier development to later instars at higher temperatures. Azadirachtin typically affects nymphal development because it interferes with the molting hormone cascade (Barnby and Klocke 1990, Mitchell et al. 1997, Mordue (Luntz) et al. 2010). Treated nymphs die at the time when untreated nymphs molt (Lowery and Isman 1994). From the clip-cage experiment it became furthermore clear that reproduction started later at 26 °C, even if development of adult females was completed earlier. These effects, earlier development and nymphal mortality, but later reproduction of surviving aphids, could have supported the slightly better efficacy at 26 °C in the efficacy trial as well. Compared to the pyrethrum-azadirachtin mixture and Flupyradifurone, high efficacy rates are reached later with NeemAzal-T/S. These products have a better knock-down effect. However, after a second application on day 7, the efficacy of 3.3 ml/l NeemAzal-T/S is similar to that of the mixture and the positive control Flupyradifurone at the tested temperature range.

High UV intensities were expected to interfere with efficacy of the neem product. However, efficacies of NeemAzal-T/S on *M. rosae* were high at all tested UV intensities. Still, at the intermediate UV level, the product had slightly, but significantly, smaller effects on *M. rosae*. Hence, impact of UV at the tested concentrations might not be a result of an impact on azadirachtin. Moreover, the temperature was approx. 4-6 °C higher under the UV lamps than under no-UV-emitting regular greenhouse lamps. Consequently, effects cannot be ascribed to the UV intensity alone. The used UV-lamps emitted mainly UV-A (310-420 nm). Severe negative impacts of UV on the degradation of azadirachtin and an activity loss only seem to occur after exposure to shorter wavelengths in the UV-B and UV-C range (e.g. about 250 nm), but not at 300-360 nm (Barnby et al. 1989, Dureja and Johnson 2000). To assess the effects under realistic outdoor conditions, two outdoor trials were conducted to assess the efficacy of NeemAzal-T/S under warm and sunny conditions when the treatment was made at different time of the day.

An application in the evening on warm and sunny days in May resulted in higher efficacies of NeemAzal-T/S compared to an application on the same days at midday at higher temperatures and UV intensities. Formulation effects seem to influence this, because the efficacy of the neem product relative to the blank did not show an effect of time of application. A higher humidity at the evening application resulting in a slower drying of the leaves and a better spread of spray droplets on the leaves, may contribute to this. The formulations contact effect (asphyxiation of soft skinned insects by the oily solution) can be enhanced, if drying is slower and aphids have a higher risk of being in contact with the spraying solution. This may explain the differences of the 12:30 pm versus 8:30 pm application due to formulation effects. It was not measured, but spraying droplets dried indeed very rapidly within minutes during midday and much slower after spraying in the evening. Humidity was higher in the evening and the temperature was decreasing in the evening, most likely leading to the slower drying of spraying droplets. To our knowledge, no other results from previous studies are available that compared different application daytimes of neem-products.

In this trial, efficacy of NeemAzal-T/S was quite low with a maximum of 50 % efficacy in the field trial. Efficacy of flupyradifurone was much better with nearly 100 %. Nevertheless, for biological insecticides, 50 % efficacy with only one application is good enough to include neem products in integrated pest management strategies. Up to four applications are allowed in ornamental plants per growing season (BVL 2017) and will increase the efficacy. Furthermore, combinations with other insecticides, but also biological control agents such as entomopathogenic fungi or bacteria can result in an even more effective aphid control. The lower efficacy compared to the synthetic standard pointed out that especially for such biological products, environmental factors need to be taken into consideration. To enhance at least the formulation's contact effect, an application in the evening compared to an application in direct sunlight is beneficial and a reasonable recommendation. Due to the known degradation of azadirachtin dependent on UV intensity, photostabilizers were tested and discussed in previous laboratory studies to improve the photo-stability of azadirachtin and consequently the efficacy of neem-based products (Sundaram and Curry 1996a, 1996b, Kumar and Parmar 1999, Johnson et al. 2003, Tan and Song 2006). However, experiments about the practical meaning of such improvements for insect control remain to be done.

To our knowledge, no other studies have been published that tested the effects of application time or environmental factors on the efficacy of neem products for controlling aphids. From our results we conclude that NeemAzal-T/S had a good efficacy against *M. rosae* in a broad temperature range up to 26 °C and different UV-A intensities. To ensure a good control of aphids and to take advantage of the blank effect, applications in the evening are preferred.

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Supplemental Data

Table S1 Actual conditions during experiment on effects of temperature on the efficacy of NeemAzal-T/S.

	Set temperature		
	18 °C	**22 °C**	**26 °C**
Temperature (mean ± SD)	17.4 ±1.4 °C	21.3 ±1.2°C	25.1 ±1.3°C
Temperature sum	52937.7	64575.9	76315.5
Temperature range (min. - max.)	12.4–20.2 °C	17.7–24.7 °C	19.7–28.7 °C
Rel. humidity (mean ± SD)	66.5 ±5.1 %	62.2 ±3.5 %	63.9 ±7.4 %
Rel. humidity range (min. - max.)	42–82 %	42-100 %	30-81 %
Temperature at 1st application	19.2 °C	20.0 °C	22.4 °C
Temperature at 2nd application	18.6 °C	22.7 °C	25.9 °C

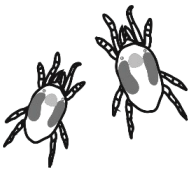
Table S2 Conditions at application times for both outdoor experiments about influence of application daytime on the efficacy of NeemAzal-T/S.

		Trial 1 Start date: 09-05-2016	**Trial 2** Start date: 12-05-2016
Application at 12:30 am	Temperature	24.5 °C	24.6 °C
	UV-A:	22 - 28 W/m²	23 - 29 W/m²
	Humidity:	37 %	30 %
Application at 8:30 pm	Temperature:	21.9 °C	21.4 °C
	UV-A:	0-1 W/m²	0 W/m²
	Humidity:	44 %	48 %

Chapter 5

Efficacy of NeemAzal-T/S and other azadirachtin formulations against the two-spotted spider mite *Tetranychus urticae*

Imke Bartelsmeier, Michael Kilian and Marcel Dicke



Abstract

Efficacy and deterrent effects of different neem (*Azadirachta indica*) formulations containing azadirachtin A as the main active ingredient were tested in laboratory and greenhouse trials against the two-spotted spider mite *Tetranychus urticae* on rose plants. NeemAzal-T/S reached high efficacies of more than 80 % against *T. urticae*. Especially for a short-term effect, adjuvants such as plant oils seem to be critical for strong effects while azadirachtin might be responsible for longer-lasting effects on pest populations. Oil-containing formulations with and without neem extract were furthermore highly deterrent to *T. urticae* females regarding presence, oviposition and feeding. Efficacy of NeemAzal-T/S seems to be limited to sprayed applications. Controlling *T. urticae* with soil applications in rose plants was not successful. Neem products containing surfactants and oils as adjuvants can be used as spraying applications in integrated pest management for controlling *T. urticae*.

Introduction

The two-spotted spider mite *Tetranychus urticae* is a globally distributed severe pest of agricultural and horticultural plants including roses (*Rosa*). Spider mites damage plants by piercing-sucking feeding activity, resulting in extensive chlorosis of leaves and deformed flowers up to complete losses of plants. Their development period from egg to adult lasts less than two weeks at temperatures above 20 °C (Alford 2012). Especially in protected conditions as in greenhouses, effective plant protection products are needed to ensure sufficient control of *T. urtic*ae. One major problem for the control of spider mites is the already existing resistance against many ingredients and the fast development of resistance to new pesticides. In Germany, registered acaricides contain for instance acequinocyl and abamectin as active ingredients. As contact poisons, products based on canola oil, paraffin oil or soap are registered (BVL 2019) which lead to suffocation or mechanically destroy soft-skinned arthropods such as spider mites. However, many publications suggest furthermore neem products or the main active ingredient azadirachtin as effective agents against spider mites (e.g. Sundaram and Sloane 1995, Martinez-Villar et al. 2005, Bernardi et al. 2013). Products based on neem seed kernel extracts are a biological alternative to synthetic pesticides with the benefit of a broad-target spectrum (Schmutterer and Singh 1995), low persistence in the environment (Szeto and Wan 1996, Sundaram 1996, Sundaram et al. 1997), low human toxicity (Raizada et al. 2001, Boeke et al. 2004), low resistance risk (Feng and Isman 1995) and systemic distribution in plants (Thoeming et al. 2003, 2006, Bartelsmeier et al. 2021). Especially the last two characteristics are interesting for controlling *T. urticae*. This spider mite is one of the most resistant arthropods globally and develops resistance due to a high reproduction rate very quickly. Neem-based pesticides are containing a natural seed-kernel extract from the neem tree with azadirachtin A as leading substance, but also other active compounds and thus a broader selection pressure and lower risk of resistance (Feng and Isman 1995). The systemic distribution of azadirachtin in plants is a benefit to reach also spider mites at plant parts that are difficult to reach with contact poisons by normal topical application techniques. In *T. urticae*, neem products can cause mortality, reduce the reproduction by affecting oviposition, hatching success and survival of emerged mites as well as being deterrent (Dimetry et al. 1993, Sundaram and Sloane 1995, Sundaram et al. 1995, Dabrowski

and Seredynska 2007, Bernardi et al. 2013). However, despite these results, nearly no acaricides based on neem are available on the market.

In this study we evaluate the potential of neem products for controlling spider mites. The first aim was to evaluate the efficacy of the commercial neem product NeemAzal-T/S (Trifolio-M GmbH, Germany) relative to its formulation and in comparison to synthetic standard products on populations of *T. urticae* in a standard efficacy trial in the greenhouse. In a second trial, the efficacy of different concentrations of formulation agents such as oil and surfactant were tested with and without the NeemAzal-extract. Additionally, a laboratory test to test repellent effects of NeemAzal-T/S and its blank formulation and a greenhouse trial with soil application were conducted. These approaches should answer the question, whether the neem-seed extract has an effect or the blank formulation.

Materials and methods

Efficacy trial 1

Potted miniature rose plants (family Rosaceae, genus *Rosa*, different Kordana® varieties by W. Kordes' Söhne Rosenschulen GmbH & Co KG) were used as experimental plants. Plants were obtained as freshly rooted cuttings and two cuttings were planted in pots of 13 cm diameter with Einheitserde Classic, Type ED 73, 155 fine, as substrate. Plants were kept at 23±3 °C, 60±20 % relative humidity and 16:8 light:dark (L:D) photoperiod in an air-conditioned greenhouse until the use for experiments. Additional light was provided by sodium vapor lamps if sunlight intensity outside the greenhouse fell under 120 W/m². Rose plants were drenched with the liquid fertilizer Wuxal Top N (Manna, Germany), an NPK (12-4-6) fertilizer solution with micronutrients, used in a dose of 0.3 %, as required until the start of the experiments. At the start of experiments, plants were in a stage where flower buds had already developed, but were still closed (BBCH stage 54-58, Meier et al. 2009).

Two-spotted spider mites (*T. urticae*) were reared on bean plants (*Phaseolus vulgaris*) in a greenhouse chamber with 24±3 °C, 60±10% rel. humidity, and a 16:8 L:D photoperiod. For infesting the experimental plants, two or three small pieces (each piece 1-2 cm diameter) of infested bean leaves were placed on the rose plants in the greenhouse. The trial took place in the same conditions where rose plants were maintained before the experiment.

Two days after infestation, four plots, each with 5 plants, were assessed per treatment. Plots were arranged in a randomized block design on four greenhouse tables (one table represented one block). For assessing the infestation with *T. urticae*, numbers of adults, nymphs and larvae were counted on the visually highest infected leaflet on each plant by using a magnifying glass (10x magnification). Assessment was done before the application (0 DAA, days after application) and 7, 14, 25 and 33 DAA. The treatments used are listed in Table 1. The commercial product NeemAzal-T/S (Trifolio-M GmbH, Germany) and its blank formulation (provided by Trifolio-M GmbH, Germany) were applied twice, on day 0 and 7 after the infestation assessment. The formulation contains 4 % seed kernel extract from the neem tree (*Azadirachta indica*), emulsifiers and plant oils (Trifolio-M GmbH, Germany). As synthetic standard, the registered product “Spinnmilben frei” (Bayer Garten/SBM, France) with acequinocyl as active ingredient, was used. This product is referred to as acequinocyl in the following. This product was only applied once at day 0.

Table 1 Overview of treatments, corresponding doses and active ingredient (a.i.) contents used in the first efficacy trial.

Treatment name	Active ingredient (a.i.)	a.i. in product [g/l]	Application dose spraying solution [ml product/l water]	Application
Control	-	-	-	untreated/no application
NeemAzal-T/S Blank	no a. i.	0	3.3	on day 0 and 7
NeemAzal-T/S 1.65 ml/l	Azadirachtin A	10	1.65	on day 0 and 7
NeemAzal-T/S 2.5 ml/l	Azadirachtin A	10	2.5	on day 0 and 7
NeemAzal-T/S 3.3 ml/l	Azadirachtin A	10	3.3	on day 0 and 7
Acequinocyl	Acequinocyl	150	1.25	on day 0

Efficacy in % was calculated as follows from total numbers of adults, nymphs and larvae on the highest infested leaf per plant (Henderson and Tilton 1955):

$$Efficacy_{HT}\ [\%] = \left(1 - \frac{T_a}{T_b} \times \frac{C_b}{C_a}\right) \times 100$$

$$T_b = Infestation\ on\ treated\ plant\ before\ application$$
$$T_a = Infestation\ on\ treated\ plant\ after\ application$$
$$C_b = Infestation\ on\ control\ plants\ before\ application$$
$$C_a = Infestation\ on\ control\ plants\ after\ application$$

Efficacy in % (Henderson and Tilton 1955, see formula above) was statistically analyzed with a Student-Newman-Keuls (SNK) test ($P < 0.05$) separately for each evaluation day after data transformation (square root or log10), if the data was not normally distributed. Statistical analysis was carried out with the trial management software ARM (Agriculture Research Manager, version 2014, Gylling Data Management, Inc., United States of America).

Efficacy trial 2 with different concentration of blank formulation ingredients

Experimental plants handling and infestation with *T. urticae* were conducted as described above. For this trial, a neem formulation by Bayer AG (Monheim, Germany) containing the NeemAzal-extract (Trifolio M-GmbH, Lahnau, Germany), vegetable oil and a non-ionic surfactant was used. To test the efficacy of different formulations with varying contents of oil and surfactant, an efficacy trial was conducted in the greenhouse with the formulations and doses presented in Table 2.

Plants were infested with *T. urticae* three days before the start of the trial. Before the first application, the number of spider mites (adults, nymphs and larvae) on the highest infested leaf was counted with the help of a magnifying glass. After the first assessment, seven plants per treatment were sprayed. A second application was done after the evaluation of day 7. An acequinocyl product (Bayer Garten Spinnmilben-frei) was added as a positive control and was only sprayed on day 0. The

experiment was located in the greenhouse with roses on water bowls to avoid migration of spider mites to other plants. For all formulations, blank treatments were included, consisting only of water mixed with the surfactant and oil, or with only surfactant.

Product A is a common neem-extract mixture. Product B contains a double amount of the neem extract, to reduce the content of oil and surfactant. Product C contains no oil and Product D contains also no oil, but the double amount of neem extract, to reduce the amount of surfactant. Exact amount of neem extract/azadirachtin A, surfactant and oil are given in Table 2. All products were used in a dose which has the same amount of neem extract, but different amounts of oil and tensides.

From day 3 onward, the percentage of leaf area infested with spider mites was estimated for each plant. This was done by inspecting the underside of leaves and estimating the spider-mite infested leaf area in % of total leaf area. From day 7 onward, the percentage damaged leaf area was evaluated additionally. Percentage of area with visible damage by spider mites (chlorotic spots due to feeding activity) was estimated by inspecting the upper side of the leaves. Influence of the factors azadirachtin, oil and surfactant content was tested with a generalized linear model with Poisson distribution and log-link function. Wald-chi-square statistics for the three factors and their interaction were checked for every evaluation day. Additionally, numbers of spider mites (all stages without eggs) were counted with the help of a magnifying glass 3, 7, 10, 14, 21 and 35 days after the first application. Efficacy in % by Henderson-Tilton (1955) was calculated from this data as described above. Efficacy was analyzed with the non-parametric Kruskal-Wallis test followed by Bonferroni corrected all-pair comparisons. Statistical analysis was performed in SPSS (version 25, IBM).

Table 2 Overview of treatments, corresponding doses and active ingredient (a.i.) contents used in the first efficacy trial.

No.	Name	Dose rate [ml product/l water]	Amount of neem extract (contains 33% azadirachtin A) in dose rate [ml]	Amount of surfactant in dose rate [ml]	Amount of vegetable oil in dose rate [ml]
1	Control		Untreated		
2	Product A	5	0.0634	1.75	3.186
3	Blank A	5	0	1.75	3.186
4	Product B	2.5	0.0634	0.875	1.5615
5	Blank B	2.5	0	0.875	1.5615
6	Product C	1.81	0.0634	1.75	0
7	Blank C	1.81	0	1.75	0
8	Product D	0.91	0.0634	0.85	0
9	Blank D	0.91	0	0.85	0
10	Acequinocyl	1.25	No neem extract (0.1875 g acequinocyl per dose rate)	unknown	unknown

Deterrent effect of NeemAzal-T/S and its blank formulation

For testing the deterrent effect of NeemAzal-T/S and its blank formulation, a Petri dish experiment was conducted in the laboratory. The two areas right and left from the mid rib of a single rose leaflet were treated in different ways by the help of a brush. The leaves were placed upside down on wet cotton wool in open Petri dishes. After drying (approx. one hour later), five adult spider mite females (not older than 2 days) were released on the mid rib. After 24, 48, 72 and 96 hours, the positions of females were recorded and eggs on each side were counted under a stereo microscope. After 24, 48 and 72 hours, eggs were removed after counting. At the end of the experiment (after 96 hours), leaves were placed upside up and feeding damage was estimated in % per side. All damage on both sides was taken as 100 %. Per treatment combination, five leaves were used, each with five adult *T. urticae* females. The treatment combinations used are presented in Table 3.

Position of females (choice of females) was analyzed in R (version 3.4.4) with the package asreml (version 3) and a binomial GLM with log-link function. Each comparison (Untreated vs. Neem, Blank vs. Neem and Untreated vs. Blank) was analyzed separately. Concentration, replication and time point (24, 48, 72 and 96 h after release) were included as factors.

Oviposition preference was analyzed in R (version 3.4.4) with the package asreml (version 3). The ratio of eggs laid on the treated side and the total numbers of eggs was used as the response factor for every time point (24, 48, 72 and 96 h after release, no additive data). Data was analyzed for each treatment combination with GLM with Poisson-distribution and log-link function. Concentration, replication and time point (24, 48, 72 and 96 h after release) were included as factors.

A t-test for paired samples after arcsin-square root-transformation (asin(sqrt(x)) was conducted in SPSS (version 25, IBM) to check for differences in feeding damage between the two treatments.

Table 3 Treatment combinations on both leaf sides for testing repellent effects by NeemAzal-T/ and its blank formulation against *T. urticae* females. Per combination, 5 leaves were treated.

Side A	Side B
Untreated	0.5 ml/l Blank
Untreated	1 ml/l Blank
Untreated	2.5 ml/l Blank
Untreated	5 ml/l Blank
Untreated	7 ml/l Blank
0.5 ml/l Blank	0.5 ml/l NeemAzal-T/S
1 ml/l Blank	1 ml/l NeemAzal-T/S
2.5 ml/l Blank	2.5 ml/l NeemAzal-T/S
5 ml/l Blank	5 ml/l NeemAzal-T/S
7 ml/l Blank	7 ml/l NeemAzal-T/S
Untreated	0.5 ml/l NeemAzal-T/S
Untreated	1 ml/l NeemAzal-T/S
Untreated	2.5 ml/l NeemAzal-T/S
Untreated	5 ml/l NeemAzal-T/S
Untreated	7 ml/l NeemAzal-T/S

Soil application of NeemAzal-T/S

To assess systemic effects and exclude formulation effects an experiment with a soil application was done. The soil of rose plants was treated with a neem solution and because of its systemic action, active ingredients of neem such as azadirachtin A, were translocated to upper plant parts. Spider mites were released on the plants and infestation level was quantified. This method excludes formulation effects and if spider mites are affected by azadirachtin, infestation on neem-treated plants is expected to be lower than in control, water-treated, plants.

Ten miniature roses, each in pots of 11 cm diameter, were used per treatment. To ensure a better uptake of active ingredients from the soil, common substrate was mixed with sand and perlite. This leads to a reduced amount of organic matter. Azadirachtin binds on organic substances in the soil and is then no longer available for plants. Soil applications with azadirachtin are more efficient in sandy substrates (e.g. Thoeming et al. 2006). Roses were planted as small plants in the substrate, after roots had been cleaned from old soil. Substrate was mixed as follows: 40 % commercial substrate (Einheitserde Classic, Type ED 73, 155 fine), 40 % sand and 20 % perlite by volume. Rose plants were drenched with a liquid fertilizer Wuxal Top N (Manna, Germany), an NPK (12-4-6) fertilizer solution with micronutrients, used in a dose of 0.3 %, as required until the start of the experiments.

Oily compounds can damage roots, thus an oil-free neem formulation was used. It contained 3.5 % neem-extract (33 % azadirachtin A in the extract) and a non-ionic surfactant. One ml of the formulation contained 0.01 ml azadirachtin A. For 10 plants, 1 l drenching solution contained either

2, 3.5 or 5 ml of the neem formulation. Each rose was drenched with 100 ml of the corresponding solution three times every 6-7 days.

The following treatments were used:

1) Untreated ("Control")
2) Blank (only tween) 5 ml/l
3) Neem product 2 ml/l
4) Neem product 3.5 ml/l
5) Neem product 5 ml/l

On every evaluation date (6, 12 and 18 days after first application), all well-developed leaves per plant were counted and the number of leaves per plant that were visibly infested with spider mites. The percentage of infested leaves was calculated per plant. Differences in the percentage of infested leaf area were analyzed at 18 days after first application with ANOVA after data transformation (sqrt(x)) in SPSS (version 25, IBM).

For controlling whether azadirachtin was transported upwards in the plants, clip cages with adult rose aphids (*Macrosiphum rosae*) from the stock rearing were placed on the plants after the second drenching. Three clip cages (3 replications) were used per control treatment, neem-product 5 ml/l and neem product 2 ml/l. Each clip cage was on another plant (3 plants per treatment). One day later, adults were removed and newly born nymphs were checked for survival in the clip cages. Per treatment, 39 to 42 nymphs were observed. This test was used as a bioindicator, to assess whether active ingredients from the neem solutions translocated to the upper plant parts. Statistical analysis of aphid mortality was conducted with R (version 3.1.3, The R Foundation for Statistical Computing, 2015). A Cox proportional hazards model was fitted with the function "coxph" of the package survival (Thernau 2012). A Tukey post-hoc test (function "glht" of the package "multcomp" (Hothorn et al. 2008)) was used for a comparison between all treatments regarding the mortality risk for *M. rosae* in the specific treatments and translocation directions (acropetal and basipetal). The mortality in each control group was set as the baseline mortality hazard.

Results

Efficacy trial 1

All tested NeemAzal-T/S concentrations and the blank formulation reached high efficacies of 90 % (Henderson-Tilton corrected) after 33 days with two applications (Fig. 1). Efficacies of different NeemAzal-T/S concentrations were not significantly different on any evaluation date and similar to the efficacy of the blank formulation. Acequinocyl, the synthetic standard product, was only applied on day 0. Acequinocyl was significantly more effective with more than 60 % efficacy at 7 days after first application (DAA) compared to the other treatments. NeemAzal-T/S reached less than 20 % mortality. From 14 DAA on, no differences between the treatments were present anymore.

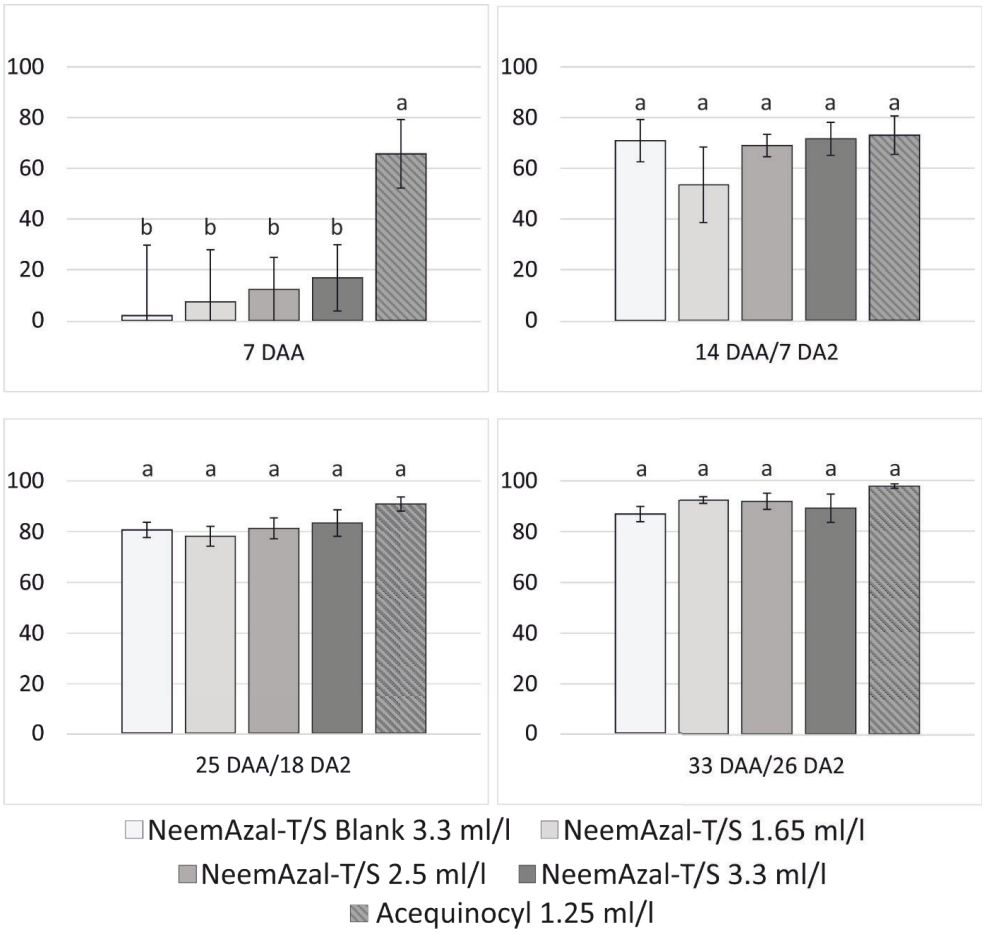
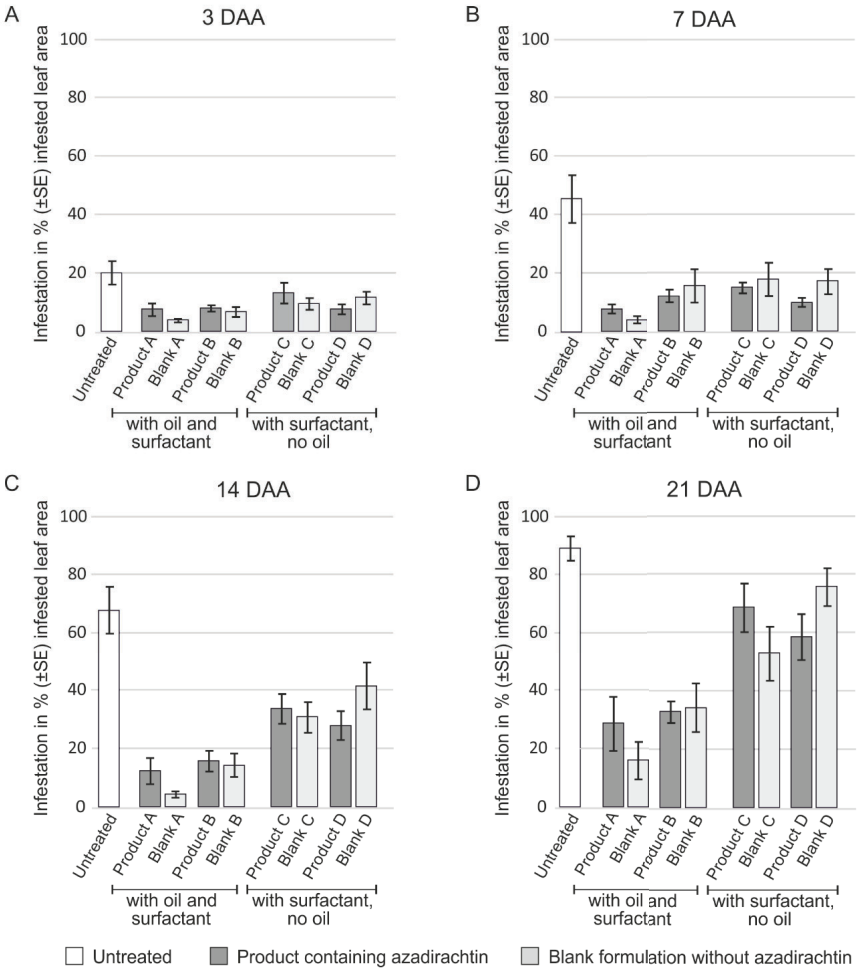


Fig. 1 Efficacy in % (mean ± SE, corrected by Henderson-Tilton) at 7, 14, 25 and 33 days after first application (DAA) of different concentrations of NeemAzal-T/S and an acequinocyl product as synthetic standard. N = 4 plots per treatment, each plot with 5 plants. DA2: Days after second application (Acequinocyl only one application). Second application was done after data was recorded for 7 DAA. Means within a panel marked with different letters are significantly different according to Student-Newman-Keuls test after data transformation (square root or log10, $P < 0.05$).

Efficacy trial 2 with different concentrations of blank-formulation ingredients

The second efficacy trial, comparing different concentrations of blank-formulation ingredients, resulted in generally higher efficacy of the oil-containing products A and B and their blank formulations compared to products C and D without oil in the formulation regarding infestation and damage (Fig. 2 and 3). Infestation by *T. urticae* and the proportion of leaves damaged are lowest on plants treated with Product A or Blank A. Oil and surfactant are highly significant factors throughout the experiment (Fig. 2 and 3, GLM, Poisson). Azadirachtin has a significant influence from day 14 onwards for infestation and on day 35 for damage. From day 7 onwards treatment with Product A and Blank A exhibit a lower infestation with spider mites compared to the untreated control (Fig. 2 and 3). Acequinocyl had the highest effect from the beginning onwards with only slight damage at the beginning of the trial (3 DAA) and no infestation and damage for the remaining time of the trial (Fig. S1 and S2 in supplemental data).

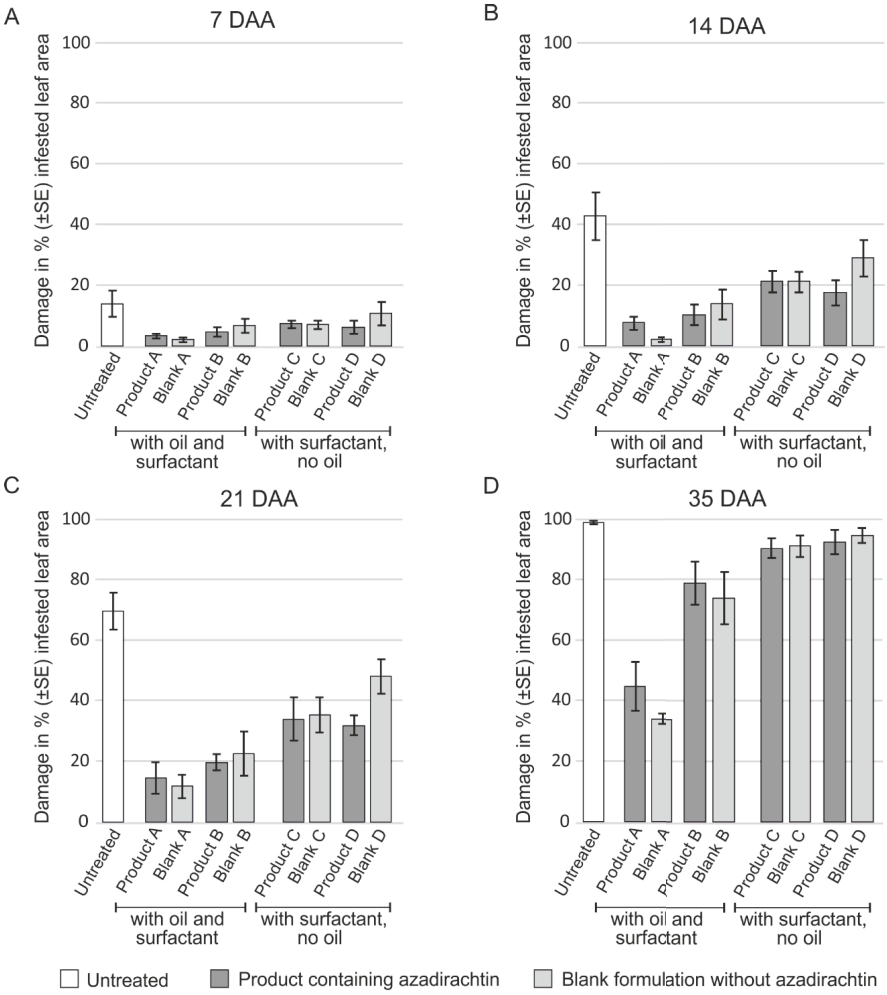
Efficacy calculated by Henderson-Tilton (Table S1 in supplemental data) shows similar results as for infestation and damage. Blank A had a significantly higher efficacy on 14 DAA compared to Product C, Product D and Blank D, while efficacy of Product A differs not significantly from other treatments. Blank A and Product A do not differ in efficacy (Table S1 in supplemental data).



Influence of factors:

Factor:	3 DAA	7 DAA	14 DAA	21 DAA
azadirachtin	n.s. (P = 0.052)	n.s. (P = 0.326)	P = 0.004	P = 0.002
oil	P < 0.001	P < 0.001	P < 0.001	P < 0.001
surfactant	P < 0.001	P < 0.001	P < 0.001	P < 0.001
interaction of factors	P = 0.001	P < 0.001	P < 0.001	P < 0.001

Fig. 2 Infestation with *T. urticae* as infested leaf area (±SE), estimated in % of total leaf area of rose plants on 3, 7, 14 and 21 days after first application (DAA). Products (containing azadirachtin) and their blank formulations were applied on days 0 and 7. Table in the bottom states *P*-values for the influence of the factors azadirachtin, oil and surfactant content on infestation according to GLM (Poisson, log-link function). n = 7 plants per treatment.



Influence of factors:

Factor:	7 DAA	14 DAA	21 DAA	35 DAA
azadirachtin	n.s. (P = 0.415)	n.s. (P = 0.151)	n.s. (P = 0.093)	P = 0.018
oil	P < 0.001	P < 0.001	P < 0.001	P < 0.001
surfactant	P < 0.001	P < 0.001	P < 0.001	P = 0.027
interaction of factors	P = 0.025	P < 0.001	P = 0.001	P = 0.021

Fig. 3 Damage by *T. urticae* on rose plants as damaged leaf area (±SE), estimated in % of total leaf area of rose plants on 7, 14, 21 and 35 days after first application (DAA). Products (containing azadirachtin) and their blank formulations were applied on days 0 and 7. Table in the bottom states *P*-values for the influence of the factors azadirachtin, oil and surfactant content on damage according to GLM (Poisson, log-link function). n = 7 plants per treatment.

Effect of NeemAzal-T/S and its blank formulation on spider mite distribution

Observing adult *T. urticae* females at 24, 48, 72 and 96 h after release on rose plant leaflets with differently treated sides right and left from the mid rib shows that significantly larger numbers of mites are present on untreated sides compared to either Blank or NeemAzal-T/S treated sides. Giving female spider mites the choice between NeemAzal-T/S and the blank formulation, resulted in higher preference for the NeemAzal-T/S treated side (Fig. 4). Dose and time point (24, 48, 72 and 96 h) are significant factors for all three comparisons ($P \leq 0.001$ for both factors in all three combinations according to binomial GLM with log-link function).

Results for distribution of eggs over the two sides is similar. The untreated leaf sides received more eggs than either the NeemAzal-T/S or the blank treated sides, with concentration as a significant factor ($P \leq 0.001$ for both treatment-combinations; GLM with Poisson log-link, Fig. 5). Giving a choice between NeemAzal-T/S and its blank formulation, more oviposition was recorded on the NeemAzal-T/S treated site, but neither concentration, nor the time point have a significant influence ($P = 0.109$ for concentration, $P = 0.749$ for time point, GLM, Poisson, log-link). Preference of the untreated side is decreasing with days for both adults and oviposition, but in the case of oviposition, time point has no significant influence for all three treatment-combination (Time Point: Untreated vs. Blank: $P = 0.248$, Blank vs. NeemAzal-T/S: $P = 0.109$, Untreated vs. NeemAzal-T/S: $P = 0.060$, GLM, Poisson, log-link).

Doses of 1 ml/l NeemAzal-T/S or higher resulted in significant more feeding damage on the leaf sides treated with the Blank formulation compared to the untreated side and on leaf sides treated with NeemAzal-T/S compared to the side treated with the Blank. If the Untreated leaf side was compared to NeemAzal-T/S, feeding damage was significantly higher on the untreated side in all tested doses (Fig. 6, t-test).

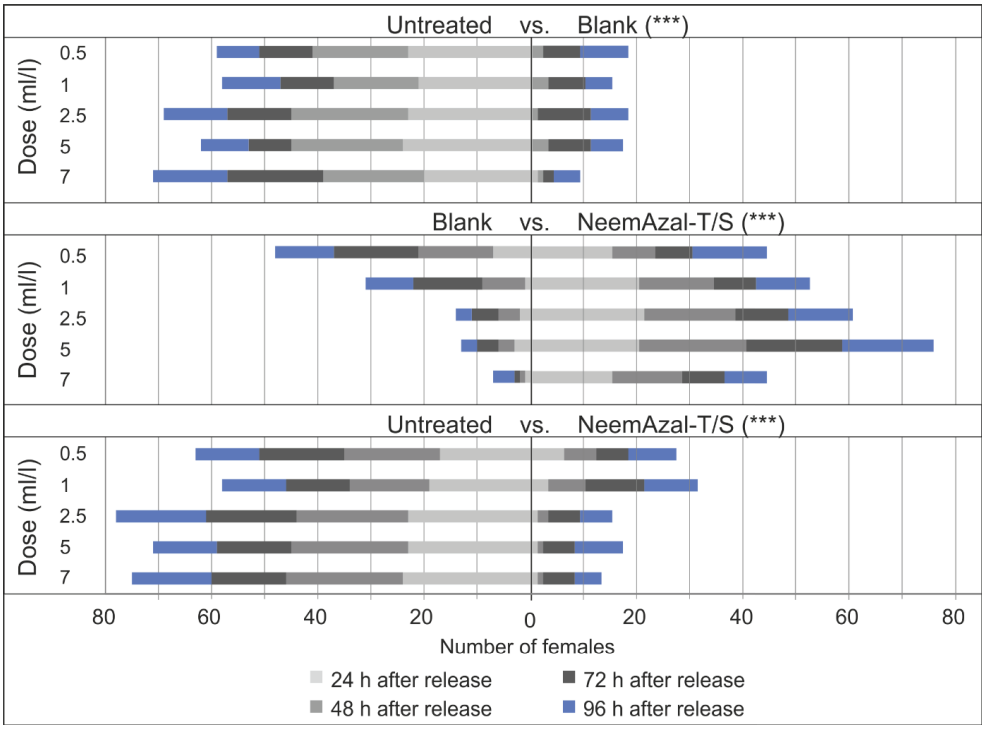


Fig. 4 Distribution of adult *T. urticae* spider mites over differently treated sides of a leaf. Treatments include different doses of NeemAzal-T/S, its blank formulation or no treatment. Number of females 24, 48, 72 and 96 hours after release on both leaf sides, summed over all replications. n = 5 replications per treatment, each with 5 adult females. One replication represents a rose leaflet with differently treated leaf sides placed in a Petri dish. For treatment-comparisons marked with asterisks (***), dose and time point significantly ($P \leq 0.001$) influence the probability whether spider mites chose the treated side or NeemAzal-T/S treated side in case of Blank vs. NeemAzal-T/S according to binomial GLM with log-link function.

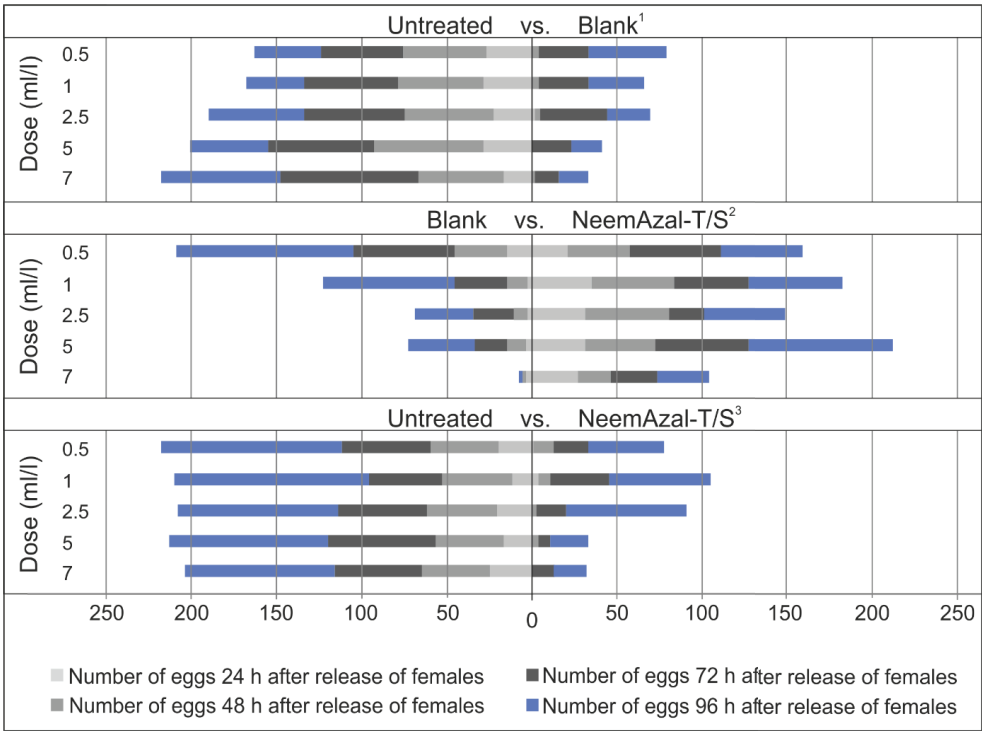


Fig. 5 Distribution of *T. urticae* eggs over differently treated sides of a leaf. Treatments include different doses of NeemAzal-T/S, its blank formulation or nor treatment. Number of eggs 24, 48, 72 and 96 hours after release of 5 females per replications on both leaf sides, summed over all replications. n = 5 replications per treatment. One replication represents a rose leaflet with differently treated leaf sides placed in a Petri dish. Influence of dose and time point according to GLM (Poisson, log-link function): [1]: dose: $P \leq 0.001$, time point: n.s. ($P = 0.248$), [2]: dose: n. s. ($P = 0.109$), time point: n. s. ($P = 0.749$), [3]: dose: $P \leq 0.001$, time point: n.s. ($P = 0.060$).

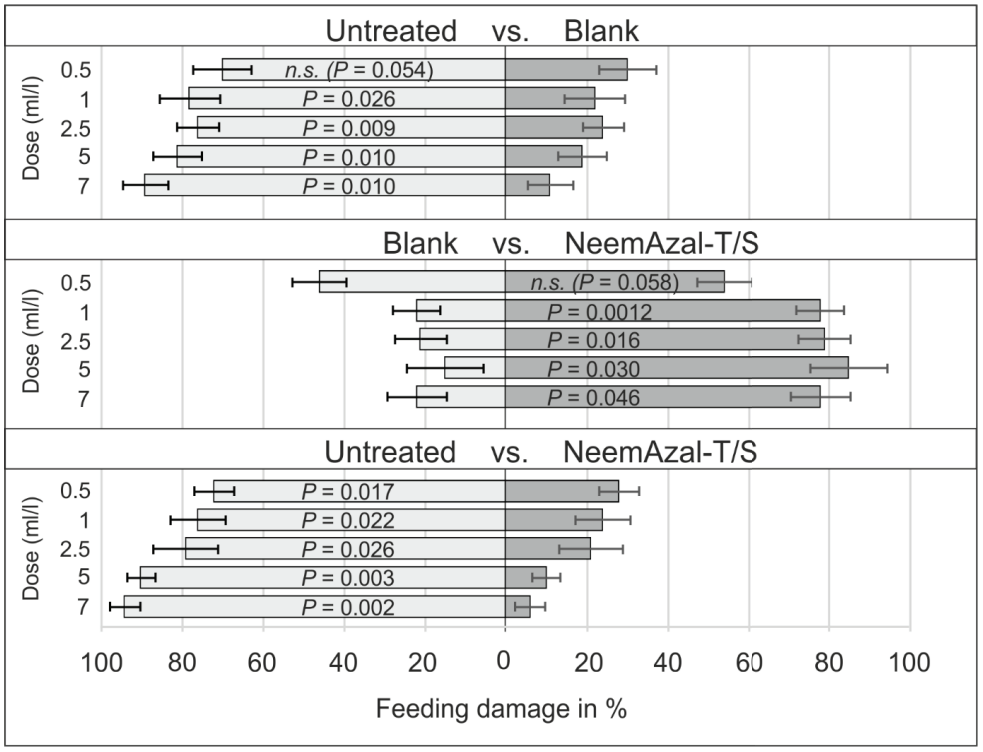


Fig. 6 Distribution of feeding damage by *T. urticae* on control and treatment side of the leaf for NeemAzal-T/S and its Blank formulation. Average % of leaf area with feeding damage (± SE). 100% means the sum of damage of both leaf sites. n = 5 replications per treatment. One replication represents a rose leaflet with differently treated leaf sides placed in a Petri dish. n.s. = not significantly different (t-test for paired samples after arcsin-square root-transformation).

Soil application of NeemAzal-T/S

The percentage of leaves infested with *T. urticae* was assessed after soil applications with different concentrations of neem-extract formulations or a blank formulation. Infestation with *T. urticae* did not differ significantly (P = 0.199) between the treatments, including a blank treatment and an untreated control, after 18 days and three soil applications (Fig. 7). Results for the bioindicator test with *M. rosae* nymphs are presented in Fig. 8. All nymphs died on the neem-treated plants within 5 days, while no mortality was recorded on untreated plants (Fig. 8), indicating that the NeemAzal-T/S application was successful in terms of aphid mortality.

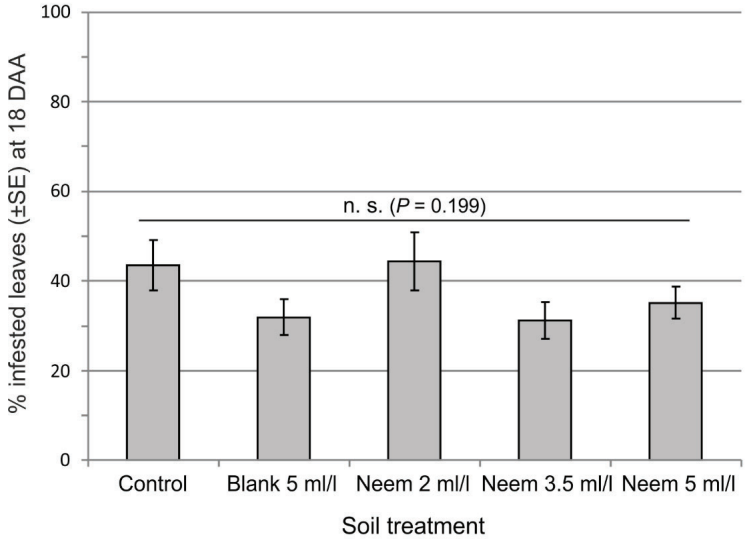


Fig. 7 Percentage of leaves infested (±SE) with *T. urticae* at 18 days after first soil application (DAA) with different concentrations of a neem-extract formulation or the blank formulation. Control: untreated rose plants. All other treatments were applied three times on day 0, 6 and 12 days. n.s.: no significant ($P > 0.05$) differences between the treatments (ANOVA after data transformation (sqrt(x))). n = 10 plants per treatment.

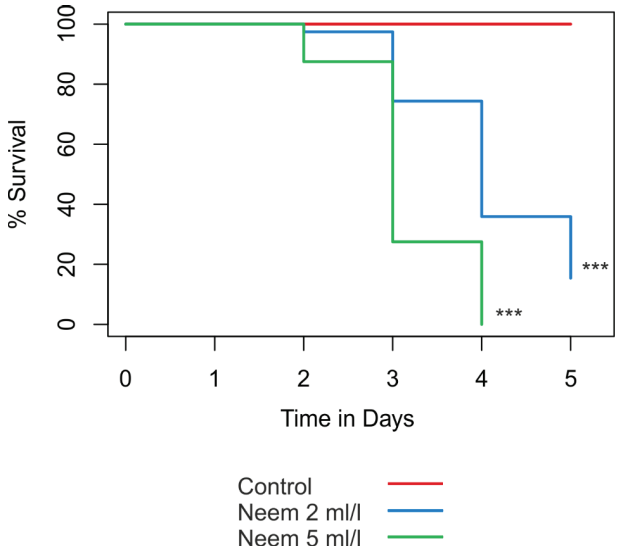


Fig. 8 Survival of *M. rosae* first instar nymphs over 5 days on untreated (control) rose plants and plants in soil drenched with 2 ml/l and 5 ml/l neem formulation. Initial total numbers of observed aphids varied between 39 and 43 individuals per treatment. Aphids were exposed to plants in clip cages after the second soil drenching. Treatments marked with *** have a significant ($P \leq 0.001$) impact on *M. rosae* survival (Cox proportional hazards model with data for treatment "Control" as the baseline hazard).

Discussion

We have tested the efficacy and repellent effects of NeemAzal-T/S and other formulations with the NeemAzal extract on *T. urticae* in greenhouse and laboratory trials. NeemAzal-T/S can reach high levels of efficacy (> 80 %) against the two-spotted spider mite *T. urticae* in trials with rose plants. However, our experiments show that this effect is limited to the directly sprayed product. The formulation, especially the oil content, is a key factor for a high efficiency.

Azadirachtin is quickly degraded in aqueous solutions (Szeto and Wan 1996). Thus, formulated products normally contain oil in addition to surfactants. Oil prevents quick degradation and tensides are improving the emulsification in water for application and, thus, the spread on the leaves. Consequently, when a formulated product is tested against *T. urticae*, the effect of active ingredients cannot be separated from the effect of adjuvants such as oil. Our results indicate that formulated azadirachtin is responsible for a long-term effect as it has a significant influence on infestation and damage from day 14 on. Azadirachtin is known for a population-growth reduction due to its influence on the reproduction and IGR (insect growth regulating) effects in insects (Barnby and Klocke 1990, Lowery and Isman 1994, Koul 1999), while a knock-down effect is often missing (Bartelsmeier et al. 2021).

As we found a strong efficacy of oil and surfactants in the presented efficacy trials, we tested additionally the efficacy of soil applications. Soil applications with neem products are not commercially relevant so far, but this method excludes any effects caused by the formulation and allows to assess only the efficacy of active ingredients from neem products. No effect was found on spider mites after soil applications with neem on rose plants in the greenhouse. This is in contrast to studies from Sundaram et al. who found a decrease in population development of *T. urticae* on aspen plants in azadirachtin-treated soil (Sundaram et al. 1995). A possible reason is that active ingredients were not available for spider mites in upper plant parts. However, we recorded strong mortality in phloem-feeding aphids on the same plants we tested, indicating that the azadirachtin had reached the upper plant parts. Previous studies have also clearly indicated that azadirachtin is translocated upwards in plants and is available for spider mites and other mesophyll-feeding pest species such as thrips (Sundaram et al. 1995, Thoeming et al. 2003, 2006). In insects, a clear influence of azadirachtin on the hormone cascade regulating insect development was found (Barnby and Klocke 1990, Rembold and Sieber 1981, Riddiford 2012). It generates a longer-lasting population reduction, when this effect occurs in following generations (Koul 1999, Bartelsmeier et al. 2021). However, the hormone system and, thus, the response to azadirachtin may be different in Acari, resulting in a lower efficiency compared to results reported for insect control.

Similar to our results, NeemAzal-T/S also significantly repelled *T. urticae* females on bean plants (Marcic and Medo 2015). Azamax, another commercially available azadirachtin-containing product, has shown high efficacies and repellence against *T. urticae* on strawberry leaves in laboratory tests (Bernardi et al. 2013). However, the effect of blank formulations is unknown in both studies, because efficacies were only compared to a water or untreated control, but not to blank formulations. We found a significant deterrent effect for feeding and oviposition also for the blank formulation of NeemAzal-T/S up to four days after application. The preference of the spider mites for NeemAzal-T/S treated leaf sides compared to blank treated leaf sides may be related to an even more soapy and thus deterrent surface in the case of the blank side, because the dirtying of the neem-seed kernel

extract is missing as in the NeemAzal-T/S treatment. Preference and thus the repellent effect is decreasing with time.

In summary, although neem products have been reported as valuable components of integrated pest management for controlling *T. urticae* (Sundaram et al. 1995, Mansour et al. 1997, Bernardi et al. 2013), there is a strong effect of the formulation. To ensure a good efficiency, the contact knock-down effect and deterrent effect of the formulation should be exploited. This may provide a more important tool than the neem component.

Acknowledgements

We would like to thank Dr. Edmund Hummel from Trifolio-M GmbH for providing the blank formulation of the tested product NeemAzal-T/S and Dr. Johan Kijlstra and his team from Bayer AG for providing us the different neem-extract and blank formulations used in our experiments. Furthermore, we would like to thank Lukas Busswinkel from Bayer CropScience Deutschland GmbH for his support with the statistical analysis of the choice experiment.

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Supplemental Data

Table S1: Mean (±SE) efficacy in % (Henderson-Tilton) 7, 10, 14, and 21 days after first application (DAA) of different neem-formulations and their blanks and an acequinocyl product as synthetic standard. N = 7 plants per treatment. DA2: Days after second application (Acequinocyl only one application). Means within a column marked with different letters are significantly different according to Kruskal-Wallis test with Bonferroni corrected all-pair comparison, $P < 0.05$).

		7 DAA			10 DAA/3 DA2			14 DAA/7 DA2			21 DAA/14 DA2		
1	Product A	6.2	± 29.5	abc	55.5	± 13.5	ab	28.6	± 14.1	ab	57.5	± 12.1	ab
2	Blank A	66.7	± 11.9	ab	72.1	± 5.9	ab	83.3	± 4.5	a	76.4	± 7.6	ab
3	Product B	41.0	± 12.6	abc	48.8	± 17.6	ab	24.5	± 15.7	ab	45.0	± 14.1	ab
4	Blank B	-36.1	± 52.9	bc	-3.8	± 35.1	b	28.0	± 18.4	ab	16.1	± 21.4	b
5	Product C	-56.4	± 46.3	bc	-93.0	± 64.7	b	-95.5	± 80.9	b	-34.9	± 43.5	b
6	Blank C	-34.7	± 38.8	bc	34.5	± 14.4	ab	30.9	± 12.1	ab	8.7	± 17.1	b
7	Product D	-5.2	± 27.1	abc	-44.1	± 34.6	b	-57.1	± 47.2	b	-22.6	± 38.2	b
8	Blank D	-106.5	± 24.9	c	-52.3	± 24.0	b	-68.6	± 59.2	b	-20.1	± 39.3	b
9	Acequinocyl	100.0	± 0.0	a	100.0	± 0.0	a	100.0	± 0.0	a	100.0	± 0.0	a

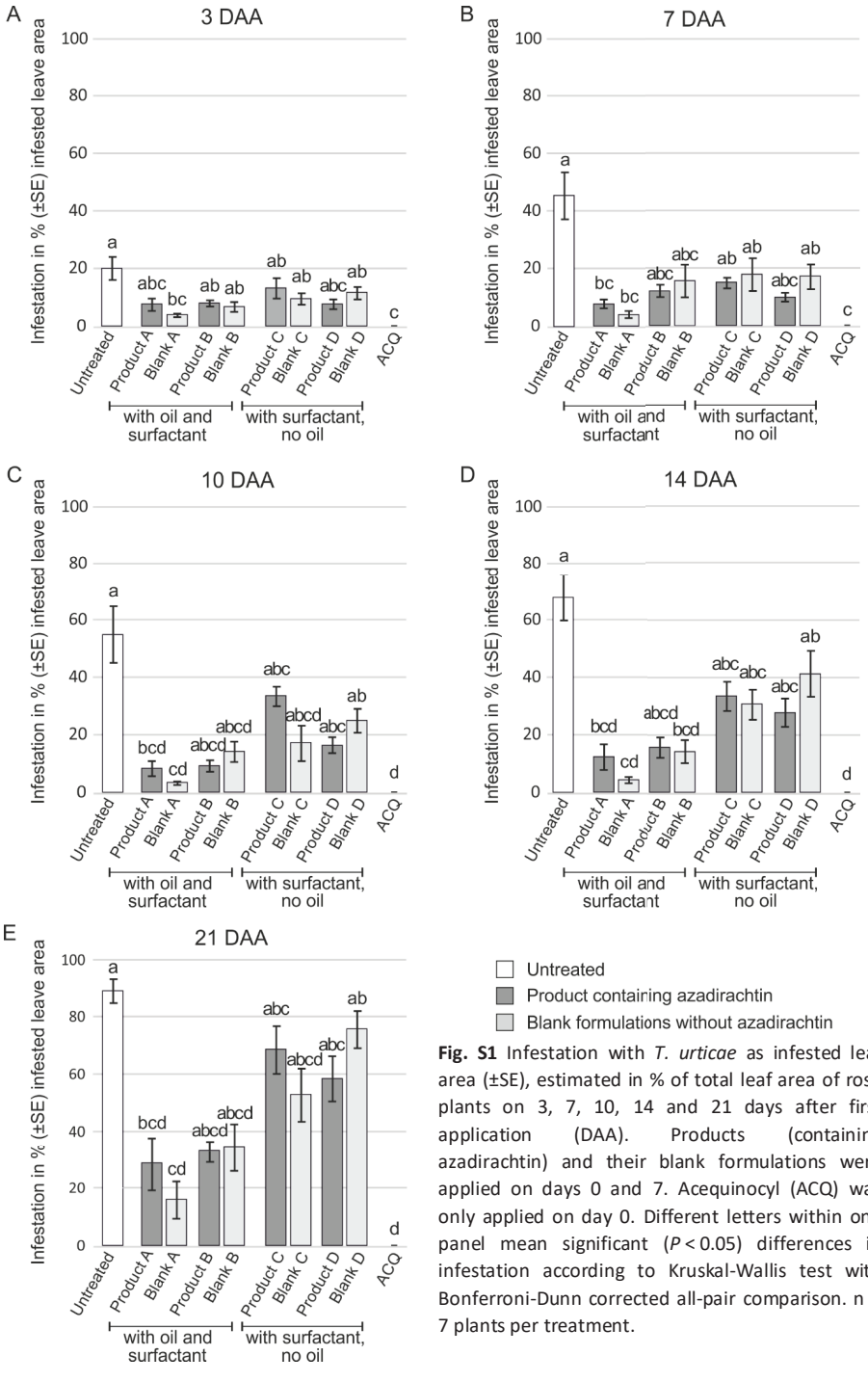

Fig. S1 Infestation with *T. urticae* as infested leaf area (±SE), estimated in % of total leaf area of rose plants on 3, 7, 10, 14 and 21 days after first application (DAA). Products (containing azadirachtin) and their blank formulations were applied on days 0 and 7. Acequinocyl (ACQ) was only applied on day 0. Different letters within one panel mean significant ($P < 0.05$) differences in infestation according to Kruskal-Wallis test with Bonferroni-Dunn corrected all-pair comparison. n = 7 plants per treatment.

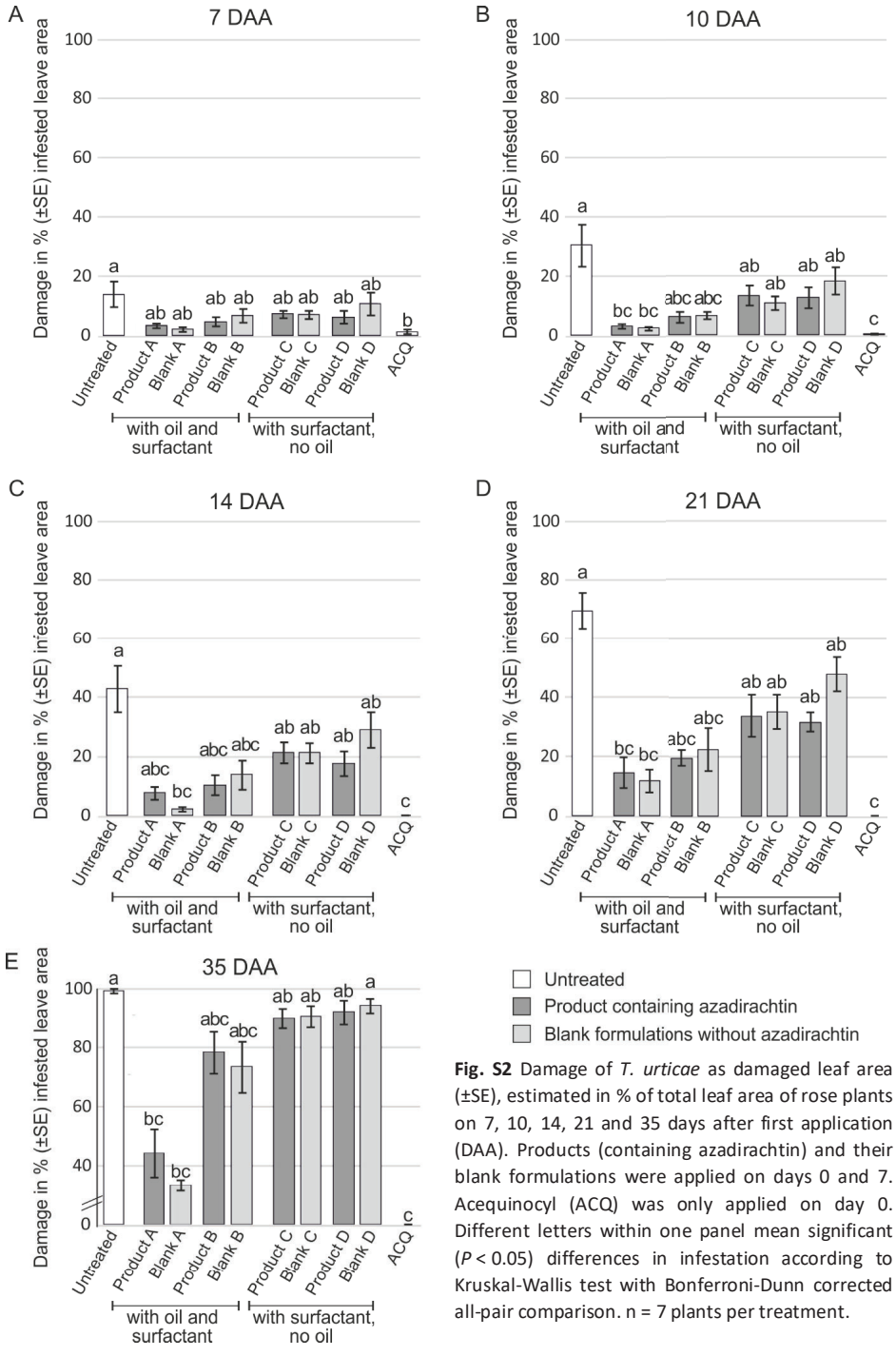

Fig. S2 Damage of *T. urticae* as damaged leaf area (±SE), estimated in % of total leaf area of rose plants on 7, 10, 14, 21 and 35 days after first application (DAA). Products (containing azadirachtin) and their blank formulations were applied on days 0 and 7. Acequinocyl (ACQ) was only applied on day 0. Different letters within one panel mean significant ($P < 0.05$) differences in infestation according to Kruskal-Wallis test with Bonferroni-Dunn corrected all-pair comparison. n = 7 plants per treatment.

Chapter 6

General discussion

Background and scope of the thesis

Research about botanical insecticides based on neem extracts has intensified during the past decades, but regarding commercialisation and market share of such products, they lag expectations (Isman 2020). Main problems for their use are a varying and lower efficacy compared to standard synthetic pesticides. Much is known about biological effects of azadirachtin or self-made neem extracts on insects from laboratory studies (reviewed e.g. in Mordue (Luntz) and Blackwell 1993, Mordue (Luntz) & Nisbet 2000, Mordue (Luntz) et al. 2010). However, the connection to today's practical use and registered concentrations of azadirachtin is often missing (Isman and Grieneisen 2014, Isman 2020). For a better knowledge on how standardized products are working, we addressed the following questions with our experiments: What is the mode of action of a commercially available neem product against aphids and spider mites, how do we need to expose the pest species to the product and what kind of environmental factors are influencing the efficacy? For explaining the mode of action, developmental-stage-specific effects, impact on reproduction, on behaviour and on feeding were considered in the experiments. To explain a possible lag in efficacy, nymphal mortality on dried spraying layers as well as on plant parts that had been treated directly was included in the study in addition to efficacy trials at different temperature- and UV-radiation levels and at different application times during the day. The results should help to understand how insects and spider mites need to be exposed to the product to ensure a good efficacy and what kind of factors lead to significant decreases in efficacy. As neem products are a promising alternative for synthetic pesticides for high value horticultural crops and for the consumer market (home and garden), the rose aphid *Macrosiphum rosae* and the two-spotted spider mite *Tetranychus urticae* were selected as target pests on rose plants. Major limitations of existing studies with neem products are the lack of a comparison to standard synthetic products, the use of products with unknown chemical composition and assessing the impact on target pests only under laboratory conditions (Isman and Grieneisen 2014). We addressed these issues by including standard synthetic insecticides and acaricides and by executing greenhouse trials and two outdoor trials with potted rose plants rather than leaf-disc studies. The findings of this thesis can be used for further product development and for user instructions.

Efficacy of neem products

Impact on population development of aphids

The presented results of standard efficacy trials (Chapter 2, design based on EPPO guidelines) with the standardized product NeemAzal-T/S show a moderate control of *M. rosae* with two applications resulting in 40 % mortality (corrected by Henderson-Tilton 1955). However, the synthetic standard with flupyradifurone as active ingredient reached more than 90 % mortality with only one application. The following detailed mode-of-action experiments should clarify where this lack of efficacy might come from. The results of my research demonstrate a high impact on juvenile stages, as expected for insect growth regulators such as azadirachtin. The mortality caused by NeemAzal-T/S decreases with increasing developmental stage of the *M. rosae* nymphs and was only significant from the first to third stage. The product did not affect survival of adult aphids. This clear stage-specific activity was also reported for other aphid species (Lowery and Isman 1994). Koul (1999) reported that 70 ppm azadirachtin is needed to have significant mortality of adult *M. rosae,* which is much higher than today's (by current EU registrations) allowed concentration, which is equivalent to

30 - 50 ppm azadirachtin. Consequently, after treatment of plants with mixed *M. rosae* populations and even continuous migration, there will always remain aphids on the plants, when no other control agents such as natural enemies are effective. This was also obvious from our following experiments about reproduction and population development.

Regarding reproduction, time of exposure to the product needs to be considered: NeemAzal-T/S affects the reproduction of *M. rosae* females that were exposed to the product as first instar nymphs already, even if they were transferred to untreated plants as an adult. In contrast, if the aphids get in contact with the product as an adult female, no decrease in the reproduction was observed (Chapter 2). Depending on the aphid species and the neem extract or azadirachtin formulation used, the effect on reproduction may vary as discussed in Chapter 2. For *Aphis glycines*, Neemix and neem seed oil did not affect the reproduction of adult aphids (Kraiss and Cullen 2008). In contrast, reproduction of adult citrus aphids (*Toxoptera citricida*) was directly affected upon exposure to different azadirachtin concentrations (Tang et al. 2002). For *Myzus persicae*, results are similar to our findings: the sterilizing effect was stronger when aphids were exposed already as juveniles to neem-seed oil (Lowery and Isman 1996). Generally, the effect of neem formulations on aphid reproduction is a good example for the inconsistency of published results depending on the experimental set-up, test system and neem formulation.

Both effects – the high mortality of juvenile stages and the reduced reproduction of aphids exposed to NeemAzal-T/S during juvenile development - reduce the population growth of *M. rosae* after applications of NeemAzal-T/S. However, in populations of mixed developmental stages, the product is slow-acting compared to standard synthetic pesticides as our results show (Chapter 2). Therefore, early application may be important whereas when pest pressure is high already, the product might fail to protect plants sufficiently. Combinations with insecticides providing the missing knock-down effect are promising to reach satisfying control. Especially for ornamental plants and vegetables where the tolerance threshold of visible insects and quality-reducing by-products such as honeydew is quite low, a reduction in feeding of virus-transmitting and honeydew producing insects would be a benefit.

Ways of exposure: Systemic action

After the developmental-stage-specific effects were clarified, the question how aphids need to be exposed to the product was addressed. According to our results, active ingredients of NeemAzal-T/S seem to be translocated systemically both upwards and downwards in rose plants. This finding is important for two reasons: First, the product will affect aphids at hidden plant parts that cannot be sprayed directly and also buds developing after the applications, a preferred feeding site for rose aphids, are protected. Second, the basipetal translocation suggests that active ingredients from NeemAzal-T/S are available to aphids in the phloem. The basipetal translocation was never tested before to the best of my knowledge. If active ingredients would have been only translocated upwards, active ingredients might not be available in the phloem and this could have explained a lack of efficacy against phloem-feeding insects such as aphids. I conclude from the experiments presented in Chapter 3 that a bidirectional systemic action of NeemAzal-T/S is present in rose plants which supports the efficacy on *M. rosae* if no direct contact to the spraying solution is given.

Impact on feeding activity of aphids

In lepidopteran and orthopteran insects, neem products often reduce feeding activity after ingestion (secondary effect) or are even repellent (sensory, primary effect) (Nasiruddin and Mordue (Luntz) 1993, Mordue (Luntz) & Nisbet 2000, Mordue (Luntz) et al. 2010). For aphids, this effect needs high concentrations of azadirachtin (Nisbet 1992, Nisbet et al. 1994, Mordue (Luntz) et al. 1996, Koul 1999) – probably higher than today's approved ones. From published results, it remains unclear whether we can expect repellent or deterrent effects on aphids from standardized neem products under practical conditions. As no impact on adult *M. rosae* was recorded in Chapter 2 regarding mortality and efficacies (Henderson-Tilton corrected) only reached 40 % in a test with mixed populations, it would be a benefit if the aphids still alive on the plants would stop feeding and thus producing honeydew. For other aphid species, this effect is also relevant regarding plant virus transmission. Thus, we assessed both honeydew production of *M. rosae* populations on excised leaves, of adult females in clip cages on complete plants, and the feeding and probing behaviour of adult *M. rosae* females in an EPG set-up with complete rose plants treated with NeemAzal-T/S (Chapter 3). None of these approaches resulted in significant effects on feeding activity by NeemAzal-T/S. In conclusion, data presented in Chapters 2 and 3 indicate that IGR effects are more relevant for the control of *M. rosae* with this product and an effect on adult aphids is missing.

Factors influencing the efficacy of neem products

To improve the application of neem products, next steps in my investigations were to assess which environmental conditions can decrease the efficacy and should be taken into consideration (Chapter 4). It is known that azadirachtin is degraded quickly depending on temperature and sunlight or UV radiation (Szeto and Wan 1996, Ruch et al. 1997, Pussemier 2000, Dureja and Johnson 2000, Johnson et al. 2003, Barrek et al. 2004). However, to the best of my knowledge, no studies have been published so far which tested the efficacy of standardized neem products under different environmental conditions. To close this gap, I have conducted efficacy trials at different environmental temperatures and UV conditions in a greenhouse with *M. rosae* and rose plants. Furthermore, to get closer to practical conditions, two outdoor trials were performed to investigate whether the time of application has an influence. NeemAzal-T/S was applied at noon in direct sunlight and warm conditions and compared to applications in the evening. Against our expectations, NeemAzal-T/S reached high efficacies of 90 % and higher at the chosen temperature levels of 18 °C to 26 °C and also at different UV levels. We found no decrease in efficacy with increasing environmental temperature or UV intensity. As the UV trial set-up resulted in different temperature conditions and, therefore, results cannot be ascribed to UV alone, outdoor trials should address this topic from a practical point of view. The moment of application is a factor that is easy to consider for the user. Applying NeemAzal-T/S in the evening compared to an application at midday in warm and sunny conditions resulted in remarkably better efficacy when the product was applied in the evening. However, this was also recorded for the blank formulation and, therefore, effects cannot be attributed to azadirachtin in the product. When applied in the evening, spraying-droplets remained wet on the leaves for a longer period, resulting in a better contact of the solution to the aphids. Oil-based formulations of pesticides always have a certain contact effect on soft-skinned insects. To ensure a good efficacy, contact effects of the formulation should be exploited in addition to the active ingredient effects. We conclude from our results that application in the evening is recommended.

Comparing the maximum reached efficacy for NeemAzal-T/S on *M. rosae* from the trials presented in Chapter 4 (≥ 90%) with the results from Chapter 2 (approx. 40 %), the efficacy was much higher in Chapter 4. The efficacy trials in Chapter 2 had higher infestations at the beginning of the trial than in the trials conducted for testing the influencing factors. As discussed, azadirachtin is not providing a strong knock-down effect and has mainly an impact on juvenile stages. Therefore, an application at still low infestations can improve the effect of neem applications to interrupt to exponential population growth of rose aphids.

Lethal and sublethal effects on the two-spotted spider mite

Another important pest, not only on ornamentals, is the two-spotted spider mite *T. urticae*. This pest has a worldwide distribution and it is resistant to many active ingredients. Because neem extracts contain a natural mixture of several compounds, the risk of developing resistance is low (Feng and Isman 1995, Bomford and Isman 1996). To assess the potential of NeemAzal-T/S for controlling spider mites, we assessed the efficacy and repellent effects of this product and other neem-extract formulations in laboratory and greenhouse trials (Chapter 5). In the first efficacy trial, NeemAzal-T/S, but also its blank formulation, reached high efficacies of more than 80 % (corrected by Henderson-Tilton 1955). However, as also the blank formulation provided this good level of control, the impact of active ingredients was questionable. To assess the formulation effects in more detail, several formulations with and without plant oils as adjuvant were tested. It turned out again that oil and also tensides are the significant factors for spider mite control. Azadirachtin only seems to have a long-term effect and does not contribute primarily to the control of *T. urticae*. To check for sublethal effects, the repellent effect on adult females of *T. urticae*, the oviposition preference and feeding activity were assessed in a choice test in the laboratory. Again, NeemAzal-T/S showed strong repellent, oviposition- and feeding-deterrent effects. Nevertheless, this was also recorded for the blank formulation. To exclude any direct formulation effects, an experiment with soil application of a neem extract was conducted, resulting in no significant differences between treatment and control. I conclude that NeemAzal-T/S as a product can be highly effective against spider mites in terms of mortality and repellence, but the formulation ingredients such as plant oil mainly account for these effects (Chapter 5). Effects of azadirachtin on *T. urticae* may vary with formulation type. However, also the azadirachtin concentration significantly influences the effects on mortality, repellence, fecundity, feeding rate and reproduction in other studies (Sundaram and Sloane 1995).

Practical implications

Taken together, the most relevant effects of NeemAzal-T/S on rose aphids are the reduction in insect development and reproduction, leading to a stagnating population growth. Beneficial for a successful application is the bi-directional systemic effect and the stability also at different temperature and UV levels. To make use of formulation contact effects, applications in the evening are recommended. Especially for another tested pest species, *T. urticae*, main efficacy was attributed to formulation adjuvents in our experiments.

Home and garden use

For aphids, the level of control is sufficient for the home and garden use, but one should be aware that complete elimination is probably not reached. For the non-professional use in home gardening, pesticides need to be easy in use, but at the same time safe without risk of overdosing. Private users

are not economically dependent of a high level of pest control compared to professional users and can accept lower efficacies. Gardens are good refuges and can promote natural beneficial insect communities, particularly pollinators. These ecosystems should not be disrupted by inappropriate pesticide use. Generally, neem products for home and garden use are approved as human safety is high and impact on the environment and persistence is low (Sundaram 1996, Raizada et al. 2001, Boeke et al. 2004, Caboni et al. 2002, 2006).

Alternative to synthetic pesticides

Next to home-and-garden use, products such as NeemAzal-T/S might contribute to modern plant protection strategies for horticultural crops. Even if the efficacy of standardized neem products is not as high as that of synthetic pesticides (Chapter 2, Chapter 5) and they might fail as a stand-alone product, they may replace some synthetic insecticides. For instance, using products such as NeemAzal-T/S at the end of spraying sequences instead of a synthetic one, can help to reduce pests below the economic threshold, by further reducing residues of synthetic pesticides in the harvested goods. The pressure on farmers to produce residue-free products has increased during the past decades. In smart spraying sequences with other pesticides and in combination with e.g. contact insecticides on oil- or soap basis, neem products can play an important role in protecting high value crops. Thinking about combinations with other natural active ingredients, pyrethrum-azadirachtin mixtures would provide both a knock-down effect (pyrethrum) and a longer lasting population growth reducing agent (azadirachtin). This combination was promising in one of our trials with *M. rosae* (Chapter 4). Considering such replacements and combinations can decrease the use of synthetic active ingredients.

Integrated Pest Management

Furthermore, not only as part of spraying sequences, but also in combination with other control agents, neem products can be part of smart Integrated Pest Management (IPM) strategies. When the aphid population growth is slowed down due to neem products as discussed before, biological control agents such as entomopathogenic fungi or beneficial insects might have a chance to control pest insects to a level similar to synthetic pesticides. According to the registration studies of NeemAzal-T/S, summarized in the safety data sheet, the product is considered to be generally harmless to beneficials, e.g. predatory mites (*Typlodromus pyri*), ground beetles (*Poecilius cupreus*), seven-spotted ladybirds (*Coccinella septempunctata*) and braconid wasps (e.g. *Aphidius rhopalosiphi*), but harmful to syrphid flies (*Episyrphus balteatus*) (Trifolio-M 2012). As neem products are active against a broad spectrum of species, it is not surprising that certain negative effects on beneficial arthropods have been reported (e.g. Ahmad et al. 2003, Aggarwal and Brar 2006, Zaki et al. 2008, Kraiss and Cullen 2008). If insect-growth-regulating (IGR) effects are dominating, adult individuals of beneficials might be safe, supporting a quick recovery of natural enemy- or beneficial populations even if larvae are affected (e.g. Castagnoli et al. 2002, Kraiss and Cullen 2008, Bernardi et al. 2013). Normally, direct contact toxicity is low, especially to adult arthropods, but offering azadirachtin-treated host insects or artificial diets can result in higher larval mortality, reduced egg hatching, slowed development, inhibited emergence from pupae or mummies compared to the untreated control (e.g. Lowery and Isman 1995, Aggarwal and Brar 2006, Kraiss and Cullen 2008). Consequently, inoculative biological control, where a natural enemy should establish a population after a single release for long-term pest management (van Lenteren 2012), might be affected by azadirachtin products. In contrast, inundative biological control, the release of large numbers of

natural enemies for rapid reduction of a pest population (van Lenteren 2012), is expected to be unaffected by azadirachtin.

Results from laboratory experiments are not representative for effects in greenhouse and field conditions. Although azadirachtin can show slight to moderate negative effects on non-target arthropods, it is considered to be safe due to its generally quick biodegradation (Raguraman and Kannan 2014). Furthermore, not many studies are available where the effects of neem products in combination with beneficial antagonists are compared to the impact of standard synthetic pesticides, e.g. for protecting horticultural crops in greenhouses, but also in the field. Aggarwal and Brar (2006) found, for instance, a clear dose-dependent effect of NeemAzal-T/S on the whitefly antagonists *Encarsia sophia* and *Chrysoperla carnea* on cotton, while even the highest tested NeemAzal-T/S dose was often less toxic to the beneficials in their experiments than the included synthetic products.

Publications on field studies about the potential of neem products for pest control while protecting beneficial communities are rare. Such studies, considering the potential recovery of beneficial populations, are important to evaluate the full impact and ability of neem products as alternative to synthetic insecticides. However, such studies are difficult to conduct and moreover, difficult to interpret as populations of antagonists are highly depending from population size of the pest species. Zaki (2008), for example, recorded a significant reduction of the cabbage pests *Brevicoryne brassicae* and *Bemisia tabaci* in the field after applications of the azadirachtin product NeemAzal-S, but also a decrease in parasitism from *Diaeretiella rapae* and *Eretmocerus mundus.* Additionally, the populations of the predators *Coccinella undecimpuncata* and *Chrysoperla carnea* were significantly reduced (Zaki 2008). The cause of the reduced pest population remains unclear in this case. Other studies concluded that the impact of neem products under field conditions is low or not existent at all- even when laboratory trials had revealed negative direct effects to predators and parasitoids (e.g. Lowery and Isman 1995, Castagnoli et al. 2002). This is in line with the risk assessment from registration for NeemAzal-T/S: Higher tier studies with NeemAzal-T/S suggest that recolonisation of the in-field area is possible within one year for *Coccinella septempunctata* and *Chrysoperla carnea* (EFSA 2011). The ratio between pest (prey) and predator is important: predator populations might decrease after the application of neem products because the neem products reduce the prey population. Considering the effects independent of the relation to the prey (pest) population, field applications of neem-seed oil or neem extracts should not directly affect aphid predator populations and thus are expected to be compatible with biological control. This is often the case, as shown in trials with multiple crops and evaluating several beneficial insects (Lowery and Isman 1995).

Due to the systemic effect of azadirachtin, also soil treatments (e.g. drenching) in combination with predators can be a suitable approach for protecting crops in the greenhouse while reducing negative side effects by spraying and potential residues on leaf surfaces on the released beneficials. As was demonstrated in this thesis, soil application with neem in potted roses can reduce rose aphid populations (Chapter 3). Advantages of soil applications are not only fewer side effects by spray drift and less direct contact to beneficials, but also the efficacy might be stabilized when active ingredients are translocated from the roots. Active ingredients in spray deposits might be more affected by degradation due to environmental factors than active ingredients translocated within plants. On the other hand, beneficials might be still exposed to active ingredients from neem products by feeding on the excreted honeydew of treated aphids or consuming treated aphids. The first scenario was shown for neonicotinoids in mealybug excretion products (Calvo-Agudo et al.

2019), but likely not investigated for azadirachtin. However, the parasitization by beneficial parasitoids was affected when host aphids were treated with azadirachtin. Parasitism rate was lower, mummy weight and the emergence rate were reduced (Lowery and Isman 1995, Ahmad et al. 2003). Based on these data, it is questionable, whether soil applications should be preferred over spraying applications. However, for other pests of rose plants, especially with a soil-dwelling stage as in thrips, a combination of an azadirachtin-soil-treatment with beneficials is promising (Thoeming and Poehling 2006). For instance, the release of predators in combination with azadirachtin as soil treatment combined with entomopathogenic nematodes can result in synergistic or additive effects as shown for thrips (Otieno et al. 2015).

Conclusion and future perspectives

The main part of my research addressed the effects of a commercial neem-based product on *M. rosae* on rose plants. To summarize the presented results and discussion, for *M. rosae*, but also aphids in general, IGR activity is the major mechanism of efficacy at today's registered dosages (Mordue (Luntz) et al. 1996, Chapters 2 and 3). Another part of the thesis addressed the effects of neem formulations on aphid feeding behavior, but none of our approaches revealed significant effects. However, both approaches only considered a short time period after exposure to NeemAzal-T/S: up to three days in the honeydew trial and up to 8 hours in the EPG experiment. Eventually, feeding behavior is changing later, as azadirachtin is normally slowly acting. *Macrosiphum rosae* is not relevant for transmitting economically important plant viruses. Consequently, a slower acting insecticide is not problematic in the specific case of this aphid species. Benefits from a feeding reduction in aphids are mainly for aesthetic reasons: less honeydew and thus less sooty mold on harvested fruits and ornamental plants increases the marketability. Contribution to an economically effective control of rose aphids by feeding reduction is not expected. However, as different aphid species seem to react differently to azadirachtin and also depending from the host plant (Lowery and Isman 1994), it might be worth to check whether neem products can contribute to a decrease in economically relevant virus transmission in other pest-host plant-combinations, e.g. for vegetable production.

From the data presented in this thesis, it remains unclear, whether azadirachtin or other neem compounds impact spider mites. Due to a very high repellent effect of the formulation of NeemAzal-T/S, we were unable to test in single leaf studies sublethal effects in more detail. Deterrent effects of NeemAzal-T/S were also recorded on bean leaves (Marcic and Medo 2015) and for other commercial neem-formulations on raspberry (Dimetry et al. 1993) and strawberry leaves (Bernardi et al. 2013). In all three studies, effects were greater with increasing concentrations of the products, but none of these studies compared to a blank formulation as we did in the experiments presented in Chapter 5. The comparison we investigated resulted in similar degrees of repellency for the formulation without neem seed kernel extract and the product with neem extract. If sublethal effects such as reduced reproduction and delayed development are caused by azadirachtin or other active ingredients from neem, they seem to be smaller than the effects of the formulation considering our results. Similar to the discussion on aphids, effects might be different in other test systems. Some results support the use of neem in IPM strategies against *T. urticae* as juvenile mortality was increased and fecundity and population growth were reduced by azadirachtin (Dimetry et al. 1993, Sundaram and Sloane 1995, Sundaram et al. 1995, Martinez-Villar et al. 2005, Bernardi et al. 2013). However, most of these

studies refer to experiments under laboratory conditions. The usefulness of neem products for spider mite control remain questionable according to our results from greenhouse trials. Meanwhile, the impact of insecticide formulations became clear, which can contribute to a better efficacy of botanical products and a broader range of pest control.

According to Isman and colleagues (Isman and Grieneisen 2014, Isman 2020), studies with neem products should leave the laboratory environment. If synthetic pesticides should be minimized or replaced, more effort should be invested in field studies and in incorporating biological products to spraying sequences and to combine them with other control agents and crop management approaches under practical conditions following modern IPM strategies. The presented thesis is one step towards achieving this, showing the potential, but also the limitations of a commercial neem product as an efficient pest control tool against aphids and spider mites in greenhouse and field conditions for the protection of horticultural crops.

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Summary

One of the most discussed topics in Europe today is the sustainable production of crops. EU countries restrict the use of pesticides more and more. The organically farmed area should increase, but also in conventional horticulture and agriculture, the use of synthetic pesticides should be reduced. Integrated pest management (IPM) aims to combine biological control, resistant variety selection and cultivation techniques, to avoid or reduce the application of synthetic pesticides to a minimum and as a last resort. For these reasons, alternative control strategies to synthetic pesticides are needed. One possibility is the use of botanical insecticides such as neem (*Azadirachta indica*) products, which have the advantage of a low risk of bioaccumulation in the environment and are registered for organic production and the home and garden use. Neem products based on extracts from seed kernels of the neem tree contain azadirachtin as the leading active substance. Aphids and spider mites are among the most difficult pests to control and are severe pests of horticultural crops such as rose plants. Considering previously published results for the control of aphids, effects of neem products can vary between aphid species. Only few results are available for the rose aphid *Macrosiphum rosae* and the two-spotted spider mite *Tetranychus urticae*. Furthermore, due to different and often not well characterized neem or pure azadirachtin formulations used, results are difficult to compare. The effects that were observed in laboratory experiments, sometimes with very high concentrations, might not occur in the same manner under practical conditions in the greenhouse or field with today's registered doses.

The main objective of this PhD thesis was to evaluate the potential of a commercial neem product (NeemAzal-T/S) with a standardized concentration of azadirachtin for controlling *M. rosae* and *T. urticae* on rose plants with registered doses. The effects if the active ingredient as well as the formulation were addressed. The efficacy was investigated in greenhouse trials and the mode of action was assessed. Furthermore, possible environmental factors were evaluated, which can influence the efficacy of the product. Greenhouse and field trials were carried out to complement laboratory trials to have realistic conditions. Synthetic standard products were added as the positive control group to most experiments and the blank formulation of the product as negative control to exclude formulation effects. The formulation consists of plant oils and surfactants and can provide a certain contact effect to soft-skinned arthropods themselves.

In chapter 1, relevant literature was summarized to provide an overview on the numerous effects that azadirachtin or neem products can cause and to address the challenges for an effective control of arthropod pests. Azadirachtin has mainly an impact on juvenile stages of arthropods and is slow-acting. Therefore, reaching an efficacy similar to those of synthetic products is not realistic with neem products. Furthermore, azadirachtin is quickly degraded, depending from environmental factors such as UV radiation and temperature. The low persistence requires a good knowledge about the mode of action to choose a useful application timing.

To evaluate the general potential of azadirachtin to control *M. rosae,* the experimental part started with efficacy trials in the greenhouse, where the product and a synthetic standard product (flupyradifurone as active ingredient) were applied to populations of *M. rosae* of mixed developmental stages. The neem product in the registered dose corresponding to 33 ppm azadirachtin A reached an efficacy of 40 % (corrected mortality) after two applications, while the

synthetic standard reached 90 % with one application. Further experiments addressing the mode of action with clip cages in a greenhouse revealed a significant impact of NeemAzal-T/S on the survival of first to third instar nymphs and prolonged developmental times to the second and third stage of surviving nymphs. These effects were not observable in the treatment with the blank formulation, containing no neem extract. The reproduction of adult *M. rosae* was found to be significantly reduced when they are exposed to azadirachtin-treated plants already as first instar nymphs. Furthermore, the population growth of *M. rosae* starting with one adult female was tested and revealed a significant, dose-dependent impact of NeemAzal-T/S. The moderate efficacy and the population-growth inhibiting effect of the product are discussed as valuable for IPM strategies, as the combination with e. g. biological control might result in a sufficient control of the pest insects, even if neem provides no strong knock-down effect and has no significant impact on adult aphids (Chapter 2).

For further investigations on the potential of azadirachtin to control aphids and to act as an antifeedant or repellent to adult aphids under realistic conditions, experiments on the systemic action and feeding activity of *M. rosae* were conducted (Chapter 3). Spraying only the upper or lower half of rose plants resulted in significant mortality of *M. rosae* nymphs on the untreated plant parts. This indicates a basipetal as well as acropetal systemic translocation of active ingredients from NeemAzal-T/S. Consequently, it was concluded that active ingredients are present in the phloem – the preferred feeding site of most aphids. However, no significant effects were found testing the repellence of adult aphids in choice tests with NeemAzal-T/S-treated and untreated rose plant. For testing the feeding activity, the honeydew production of mixed populations of *M. rosae* was observed before treatment until three days after application. An untreated control, the blank formulation of the product and different concentrations of the neem product were compared with flupyradifurone as positive control. Only flupyradifurone revealed significant negative effects on honeydew excretion by the aphids. In addition to this quantitative approach, an electrical penetration graph (EPG) experiment was conducted as a quantitative measurement. The EPG trial did not show differences in settling or feeding on untreated and neem-treated plants. The effects of NeemAzal-T/S on *M. rosae* was considered to be a result of insect-growth regulating effects after feeding on treated plants, not on starvation.

One advantage of azadirachtin-based insecticides is their low persistence in the environment. Azadirachtin has a short half-life and high UV intensities or high temperatures are leading to an even quicker degradation. As the efficacy of neem products is not as high as those of synthetic pesticides and our results show no knock-down effect, knowledge about the influencing factors and application timing is important to improve the effect. To evaluate the impact of UV radiation and temperature on the efficacy of NeemAzal-T/S, greenhouse trials were conducted with *M. rosae*. The first trial took place in greenhouse chambers at either 18 °C, 22 °C or 26 °C on average. A standard efficacy trial set-up was used in addition to a clip-cage experiment with first-instar nymphs, which allowed the observation of developmental times and the nymphal mortality. The influence of UV radiation was tested with an efficacy trial under lamps emitting UV-A in two different intensities or without UV radiation in the greenhouse. In contrast to our hypothesis that due to the degradation of azadirachtin, high temperatures and UV intensities interfere with the performance of the neem product, registered doses of NeemAzal-T/S reached high efficacies in all conditions tested. The validity of our results for practice is limited as only UV-A was tested. Finally, two outdoor trials were

conducted to investigate the influence of different times of application: in direct sunlight at noon and in the evening. The application in the evening resulted in significantly better control of *M. rosae*, however, this effect seems to be a formulation effect and not dependent on azadirachtin. In the evening, spraying droplets stay longer fluid on the plant and the formulation itself has a contact effect on *M. rosae*. We concluded that NeemAzal-T/S can be well used in the tested temperature range and also at higher UV radiations. To improve the effect of the product and use the formulation effect, an application in the evening is recommended (Chapter 4).

In Chapter 5, the efficacy and deterrent effects of different neem formulations on spider mites (*T. urticae*) were tested in laboratory and greenhouse trials. With NeemAzal-T/S, 90 % efficacy was reached 33 days after the first application (26 days after the second treatment). The synthetic standard, an acequinocyl product, was only applied once and provides a much stronger knock-down effect with 60 % efficacy seven days after the application. Remarkable was that also the blank formulation was as efficient as NeemAzal-T/S, indicating a significant formulation effect. To further investigate the effect of formulation, four different neem-extract formulations and their blank formulations (only surfactant and vegetable oil) were compared. The test products had the same amount of azadirachtin, only the oil and surfactant content were different. Both oil and surfactant were significant factors influencing the efficacy also short-term, while our results indicate that azadirachtin has a significant influence from day 14 on, thus being responsible for a long-lasting effect on spider mites. In a laboratory choice experiment, we found the formulation being highly deterrent to spider mites for the tested period of up to four days, but increasing with time. Therefore, the deterrent effect is only relevant for a short time after application. To exclude any contact effect of the formulation, a greenhouse experiment with soil application on rose plants was performed, revealing no impact on spider mites, while *M. rosae* nymphs died consequently to the soil application with azadirachtin. We concluded that for an efficient spider mite control, the contact knock-down and deterrent effect of the neem formulation should be exploited.

The results presented in chapters 2 - 5 are discussed in the context of practical implications and IPM strategies (Chapter 6). Neem products are good alternatives to synthetic products for the home and garden use, as their human safety is high and their impact on the environment is low. In addition, for professional horticultural production of high value crops, products based on azadirachtin are valuable and can be part of IPM strategies. They slow down the population growth of aphids and in combination with other control agents such as beneficials, control levels similar to those of synthetic standard product might be reached. Studies with neem products should be conducted in the greenhouse or field to be as close as possible to realistic conditions. Further investigations under practical conditions of such IPM conform combinations or of spraying sequences, in which azadirachtin is replacing synthetic products, are needed to show the full potential.

Acknowledgements

First, I would like to thank my promotor Marcel Dicke and my co-promotor from Bayer AG, Michael Kilian, for their support and guidance throughout my PhD project, their patience and constructive feedback. A big thank you that you still supported me, even after the planned four years. I also would like to thank Margit Doth, my supervisor at Bayer during the first two years, for her support. I am glad that my promotor, co-promotor, WUR and Bayer gave me the possibility to do a PhD project within industry in combination with a great university such as WUR and the possibility to join the EPS training and supervision programme. I learned a lot during these years!

I am grateful for the years within Michael Kilians group at Bayer. I would like to thank my colleagues of that time for being welcome with my unusual experiments, for nice lunch breaks and off-topic talks. Ich habe viel von euch gelernt und hatte eine wirklich gute Zeit bei euch im Team: Michael, Margit, Michaela, Cora, Viola, Peter, Klaudia, Manfred, Natalia, Kai, Julia, Tim, Inga, Sebastian, Carla, Joana, Jörn, Sergio, Jan, Beate, Sebastian L., Nadine und Kathrin. Vor allem möchte ich Klaudia, Manfred und Peter danken: ihr habt mir oft mit meinen Versuchen oder den Vorbereitungen geholfen. Vielen Dank für die Unterstützung!

Cindy, I would like to thank you for your help with the EPG experiment that I conducted in the WUR Entomology lab. I also would like to thank Joop van Loon for his support to ensure that the experiment at WUR was possible.

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Curriculum vitae

Imke Bartelsmeier was born on 12 November 1988 in Stadthagen, Germany. After finishing secondary school in 2007, she started to study Horticultural Sciences at the University of Hannover, Germany. Within her Master's programme in Hannover, she intensified the focus on biological plant protection and entomology. Her Master's thesis dealt with different formulations of azadirachtin for soil application for controlling aphids in cabbage. For this thesis, Imke conducted several greenhouse experiments with *Brevicoryne brassicae* over a period of two years next to her courses. Additionally, Imke worked as an assistant in the Entomology group of the Institute of Plant Diseases and Plant Protection.

After her MSc in 2013, she started her PhD project in cooperation with Wageningen University at the Crop Science division of Bayer AG in Monheim, Germany, supervised by Prof. Dr Marcel Dicke (WUR) and Dr Michael Kilian (Bayer AG). At WUR, she was an external PhD student and participated in the training and supervision programme of The Graduate School for Experimental Plant Sciences (EPS). At Bayer, she was part of the department "Environmental Science", where she gained insights in the product development of Bayer's non-agricultural plant protection business. From summer 2018 to 2020, Imke was a testing operations manager in winter oilseed rape breeding. Today, she is a research associate at a consultancy company in the field of human safety and residue analysis of agrochemicals.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Imke Bartelsmeier
Date: 20 October 2021
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Mechanistic evaluation of the plant protection active ingredient Azadirachtin	20 May 2014	1,5
► **Writing or rewriting a project proposal**		
Mechanistic evaluation of the plant protection active ingredient Azadirachtin	03 Jun 2014	6,0
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		7,5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
Get2Gether 2016, Soest	28-29 Jan 2016	0,6
Get2Gether 2017, Soest	9-10 Feb 2017	0,6
► **EPS theme symposia**		
EPS Theme 2 Symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents' 2014, Amsterdam	25 Feb 2014	0,3
EPS Theme 2 Symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents' 2015, Utrecht	20 Feb 2015	0,3
EPS Theme 2 Symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents' 2017, Wageningen	23 Jan 2017	0,3
► **Lunteren Days and other national platforms**		
Experimental Plant Sciences Meeting 2015, Lunteren	13-14 Apr 2015	0,6
Experimental Plant Sciences Meeting 2016, Lunteren	11-12 Apr 2016	0,6
Entomology Day 2013, Ede	13 Dec 2013	0,3
Entomology Day 2014, Ede	19 Dec 2014	0,3
Entomology Day 2015, Ede	18 Dec 2015	0,3
Entomology Day 2016, Ede	16 Dec 2016	0,3
► **Seminars (series), workshops and symposia**		
Symposium: 59. Deutsche Pflanzenschutztagung, Freiburg, Germany	23-26 Sep 2014	1,2
Workshop: Electrical Penetration Graph (EPG) Workshop, Madrid, Spain	08-10 Jun 2017	0,9
► **Seminar plus**		
► **International symposia and congresses**		
15th international symposium on insect-plant relationships (SIP15), Neuchâtel, Switzerland	17-22 Aug 2014	1,2
18th International Plant Protection Congress (IPPC), Berlin, Germany	24-27 Aug 2015	1,2
Hermipteran-Plant-Interactions Symposium (HPIS), Madrid, Spain	05-07 Jun 2017	0,9
► **Presentations**		
Talk: 'News on azadirachtin 1 - MoA findings', European Technical Project Review Meeting 2014, Mettmann, Germany	19-20 Nov 2014	1,0
Talk: 'News on azadirachtin 2 - PhD project', European Technical Project Review Meeting 2015, Dresden, Germany	18-19 Nov 2015	1,0
Poster: 'Up and Down:Systemic Action of a Neem Product', Entomology Day 2015, Ede, The Netherlands	18 Dec 2015	1,0
Poster: 'Up and Down:Systemic Action of a Neem Product', Experimental Plant Sciences Meeting 2016, Lunteren, The Netherlands	11-12 Apr 2016	0,0
Poster: 'Neem: Mode of action on rose aphids (Macrosiphum rosae)', Entomology Day 2016, Ede, The Netherlands	16 Dec 2016	1,0
Poster: 'Neem: Mode of action on rose aphids (Macrosiphum rosae) and consequences for application' (modification of former poster), HPIS 2017, Madrid, Spain	05-07 Jun 2017	0,0
► **IAB interview**		
► **Excursions**		
Subtotal Scientific Exposure		13,9

3) In-Depth Studies		date	cp
►	**Advanced scientific courses & workshops**		
	Basic Statistics	20-27 May 2015	1,5
	Linear Models	08-10 Jun 2015	0,9
	Generalized Linear Models	15-16 Jun 2015	0,6
	Mixed Linear Models	22-23 Jun 2015	0,6
►	**Journal club**		
►	**Individual research training**		
	Subtotal In-Depth Studies		3,6

4) Personal Development		date	cp
►	**General skill training courses**		
	Scientific Publishing	09 Oct 2014	0,3
	Workshop Presentation Skills	10-24 Oct 2014	1,0
	Scientific Writing	05 Feb - 09 Apr 2015	1,8
	PhD Workshop Carousel (4 workshops)	17 Apr 2015	0,3
	PhD Competence Assessment	17 Sep 2015	0,3
	Efficient Writing Strategies	17 Sep - 29 Oct 2015	1,2
	Project and Time Management	10 Feb - 23 Mar 2016	1,5
►	**Organisation of meetings, PhD courses or outreach activities**		
►	**Membership of EPS PhD Council**		
	Subtotal Personal Development		6,4

TOTAL NUMBER OF CREDIT POINTS*	**31,4**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	
** A credit represents a normative study load of 28 hours of study.*	

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