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ORIGINAL PAPER

Local and systemic effect of azadirachtin on host choice and feeding activity of *Macrosiphum rosae* on rose plants

Imke Bartelsmeier[1,2] · Michael Kilian[1] · Cindy J. M. ten Broeke[2] · Daan Mertens[2] · Marcel Dicke[2]

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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 with azadirachtin as the leading active ingredient. 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 are available on the presence of azadirachtin 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 analysis do not show differences in settling or feeding on untreated and NeemAzal-T/S-treated rose plants. Collectively, our data show 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.

Keywords Aphids · Neem · Azadirachtin · Rose · Antifeedant · Mode of action · IPM

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 moulds (Brødsgaard and Albajes 1999; Alford 2012a, b). 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. 2022). 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

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✉ Imke Bartelsmeier
imke.bartelsmeier@gmail.com

[1] Crop Science Division, Bayer AG, Alfred-Nobel-Str. 50, 40789 Monheim am Rhein, Germany

[2] Laboratory of Entomology, Wageningen University & Research, Droevendaalsesteeg 1, Radix building, 6708PB Wageningen, The Netherlands

that developed after the application, and the presence of the active ingredient 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 and Nisbet 2000; Mordue 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 (1996b) and Sundaram et al. (1995) reported, for instance, an impact on spider mites after an upward distribution of azadirachtin in the xylem following 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 repellent or deterrent effects in insects (Mordue and Nisbet 2000; Mordue 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. In the case of aphids, deterrent effects were reported for instance for cereal aphids: *Sitobion avenae* and *Rhopalosiphum padi* clearly preferred to settle on untreated winter barley leaves rather than on azadirachtin-treated leaves (West and Mordue 1992). Another species, the pea aphid *Acyrthosiphum pisum* is 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 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).

Secondary antifeedant effects are caused by internal feedback mechanisms after ingestion of azadirachtin or other neem toxins (Mordue and Blackwell 1993; Mordue and Nisbet 2010). For this effect, toxins need to be present at the feeding site of the insect. The probing activity of cereal aphids 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 1992).

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 one 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 antifeedant effect only lasted up to 24 h (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 secondary 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 deterrent effect by the commercial neem product NeemAzal-T/S is present. Repellence was assessed in terms of host–plant choice and we tested whether the general 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. 2022), 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 vapour lamps (400 W) if sunlight intensity outside fell under 120 W/m^2. 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, fungicides to control mildew were applied, according to current registrations in Germany. Compo Ortiva Universal Pilzfrei (Compo, Germany) or Bayer Garten Rosen-Pilzfrei Baymat (SBM, Germany) was 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 × 78 cm × 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 litre 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 m^2 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 the 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 are 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 h 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 6 and 13 individuals in the first trial and 11 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.

Repellent effects

Repellence of NeemAzal-T/S was tested in five acrylic glass cages with three gauze sides (dimensions: 63 cm × 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 h, 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 analysed with a generalized linear mixed model (GLMM) on count data with Poisson probability distribution and log-link function (package glmmTMB; Brooks et al. 2017). The model included treatment and time as categorical variables and accounted for repeated measures by including cage identity as random intercept (Zuur et al. 2009). Effect size

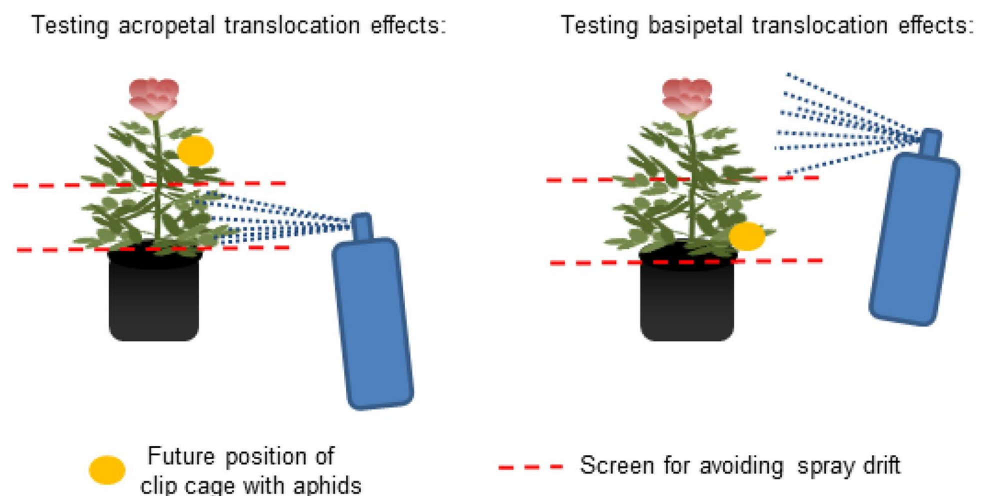

Fig. 1 Application scheme for assessing the effects of acropetal (left) and basipetal (right) translocation of active ingredients of NeemAzal-T/S and the future position of clip cages containing *M. rosae* nymphs (clip cage plus aphids were added after spraying and drying of the plants)

and significance of explanatory variables were determined using likelihood-ratio tests. A Tukey post hoc test was used to analyse the data per time point.

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 h 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 h. Then, new filter papers were placed in the Petri dishes for 24 h and were replaced two times. Thus, the honeydew was collected on the filter papers for 2 h before the application, 2 h and 1, 2 and 3 days after application. Additionally, the 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. The size of the photos was 6000 × 4000 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 were 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 × 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 h. 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 h 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 six 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

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 24 h	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

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 h. Each device was used for a separate plant treatment and recordings of all treatments were randomized 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 2 EPG waveforms that were differentiated and their biological meaning

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

For detailed explanation of the terms, see Van Helden and Tjallingii (2000) and Reese et al. (2000)

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

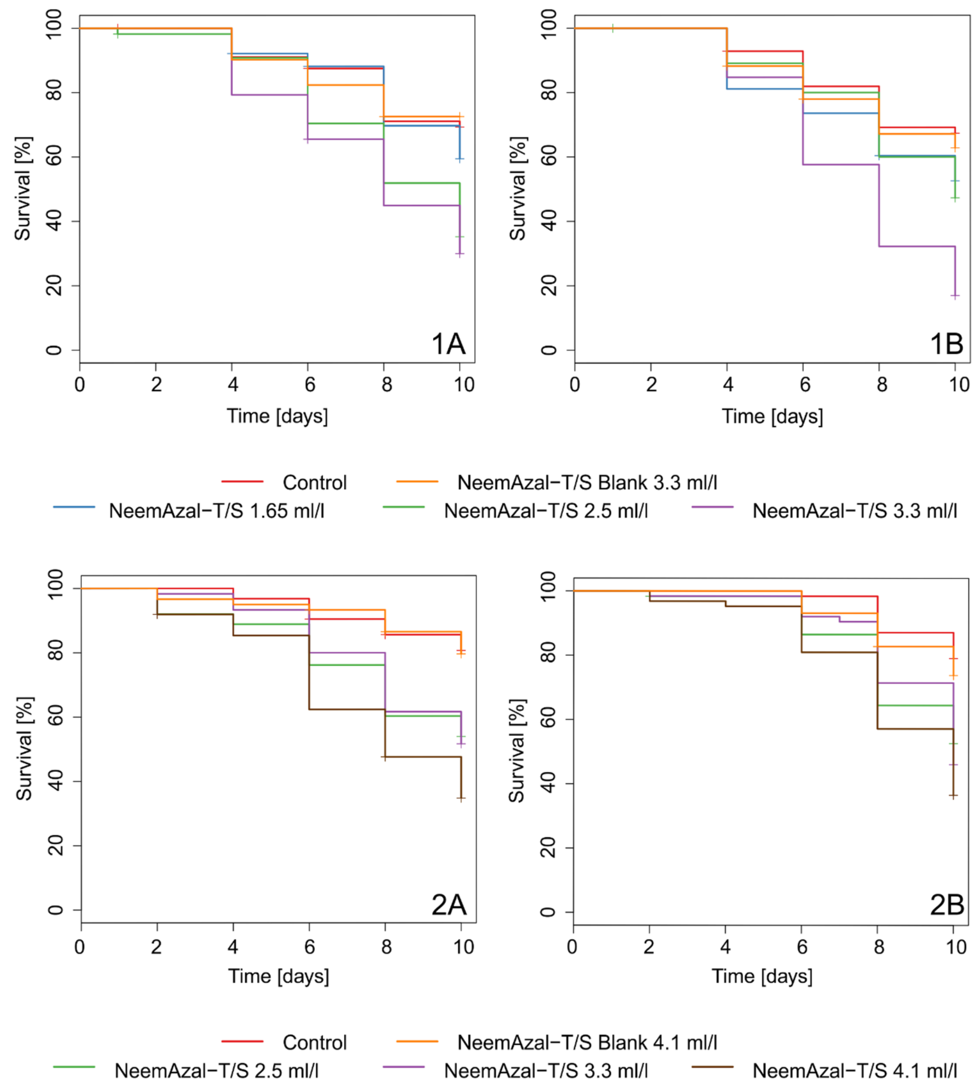

Fig. 2 Systemic effects of NeemAzal-T/S in rose plants in trial 1 (panels 1A and 1B) and trial 2 (panels 2A and 2B). **A** Percentage survival of *M. rosae* nymphs on untreated plant parts higher than the sprayed part (acropetal translocation). **B** Percentage survival of *M. rosae* nymphs on untreated 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

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. The hazard in the control group is set to 1 as the baseline hazard, while the hazard to die is significantly increased for *M. rosae* with the 3.3 ml/l and 4.1 ml/l NeemAzal-T/S treatment (Table 3).

Repellent effects

Nearly all released apterous *M. rosae* settled on the rose plants in the test arenas during the experimental period. Both treatment and time are important in predicting the number of aphids on plants ($\chi^2 = 10.19$, df = 2, $P = 0.006$ and $\chi^2 = 18.91$, df = 5, $P = 0.002$, resp) whereas the change over time is similar for both treatments because there is no significant interaction effect ($\chi^2 = 1.27$, df = 4, $P = 0.866$). We did not find any significant post hoc differences among treatments at any of the time points (Fig. 3). The most informative time point is 72 h, when almost all aphids had settled on one of the plants and at this time point, the numbers did not differ between treatments (Tukey adjusted $P = 0.304$). These data indicate that NeemAzal-T/S does not repel *M. rosae*.

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 numbers of honeydew droplets increase with

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

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

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

[a]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

[b]*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

[c]HR for control set to 1 as baseline hazard

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

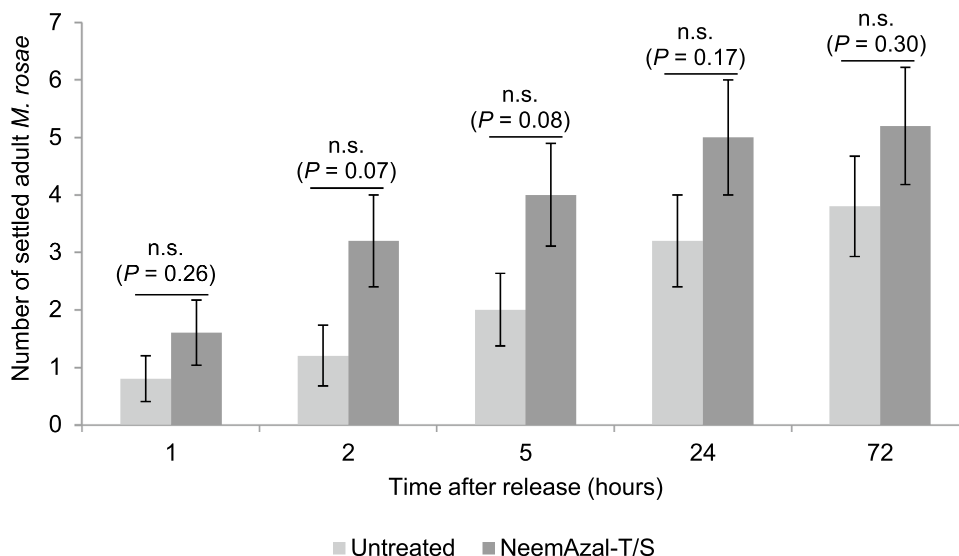

Fig. 3 Host–plant preference of *M. rosae*. Mean number (±SE) of adult *M. rosae* on untreated rose plants and plants treated with 3.3 ml/l NeemAzal-T/S at 1, 2, 5, 24 and 72 h after release of 10 aphids in each test arena. $N=5$ test arenas, each with 10 released aphids. Data for non-settled aphids are not shown. n.s.: non-significant (Tukey adjusted $P>0.05$) differences between the number of aphids on treatment vs. control groups within one time point based on GLMM (Poisson, log-link) with time and treatment as categorical variables and including the cage identity to account for repeated measurements

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).

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 h. 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 h 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

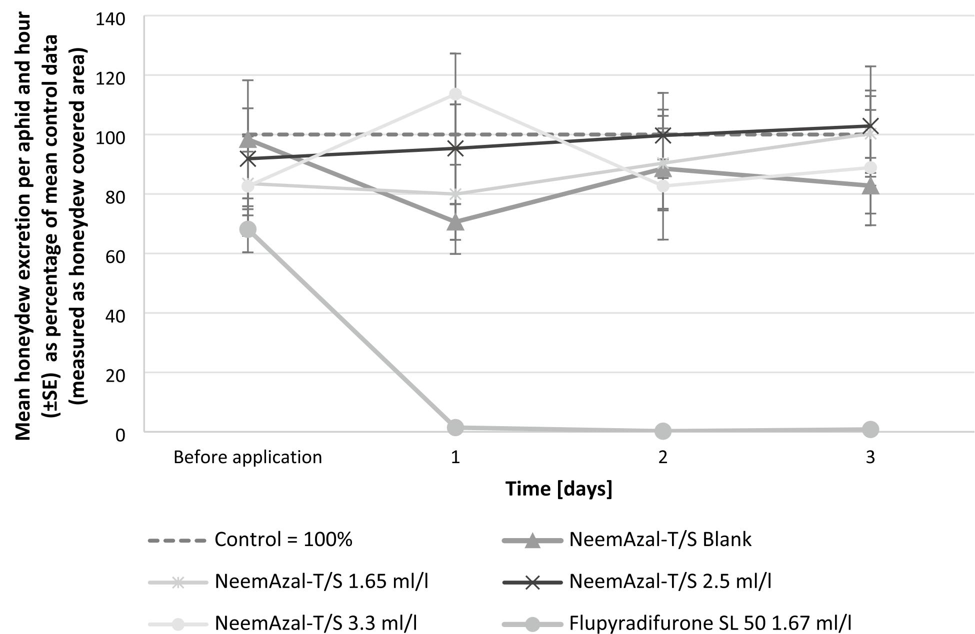

Fig. 4 Mean honeydew excretion (± SE) per hour and per individual living *M. rosae* aphid before and 1, 2 and 3 days after application as percentage of honeydew excretion in the control group (= 100%), assessed as honeydew covered area in pixels of small populations. Total covered area of each replication 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

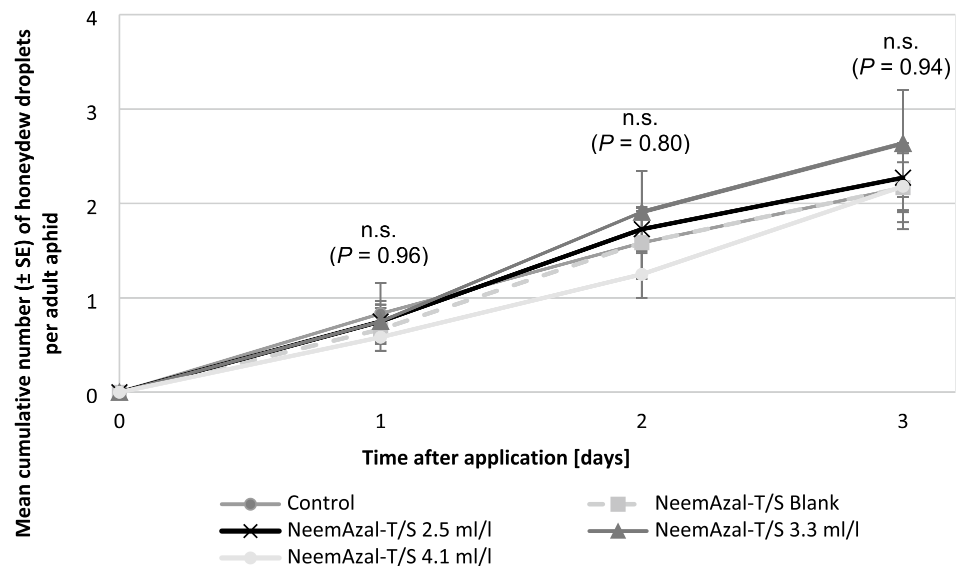

Fig. 5 Honeydew secretion by individual adult *M. rosae* as mean (± SE) cumulative number of droplets per aphid over 3 days after application of NeemAzal-T/S and the blank formulation. $N = 12$ aphids per treatment (pooled data of two replicate experiments, each with 6 observed aphids per treatment). 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

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 (Supplement Table 5).

For the comparison of phloem salivation and ingestion, the total duration of E1 (phloem salivation) and E2 (phloem ingestion) was 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 (Supplement Table 6). Total duration of phloem phases includes E1, E2 and unknown phloem waveforms. These unknown waveforms were found in some recordings (Supplement Table 7; Fig. 6), 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 min. An E2 phase longer than 10 min lasts in most cases longer than 1 h (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 (Supplement Table 6).

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

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

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

N total number of replicates (recordings of single aphids analysed for each treatment)

NP non-penetration time/no activity

C/pd Stylet pathway including probes (pd = potential drops)

On average, the first sustained E2 phase (phloem ingestion longer than 10 min) began after 107 min (NeemAzal-T/S 3) up to 230 min (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 h. 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 (Supplement Tables 8–10).

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.

Systemic action

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 were 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 active 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 suggest 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 developed 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).

Repellent effect

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. 2022), 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 min after the start of exposure (West and Mordue 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-h 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 h 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.

Feeding activity: honeydew production

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 h. During the first 26 h, no differences were found, but in the subsequent period of 24 h, 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*.

Feeding activity: electrical penetration graph analysis

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* are 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 h 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 2- to 3-fold 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 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 deterrent 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 deterrent effects of azadirachtin or neem formulations do not provide a main contribution to aphid control.

Conclusion

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*. A significant nymphal mortality is a typical effect of azadirachtin in aphids (e.g. Lowery and Isman 1994a; Koul 1999) and mainly contributes to an effective control. For the practical application of NeemAzal-T/S, insect growth-regulating (IGR) effects in immature aphids after feeding on treated plants and a reduced reproduction are the relevant effects for controlling rose aphids (Lowery and Isman 1993; Bartelsmeier et al. 2022). These IGR effects might allow a combination of azadirachtin products with biological control agents in integrated pest management (IPM) strategies to achieve good pest control levels. However, possible side effects to beneficial insects needs to be taken into consideration (Desneux et al. 2007), e.g. the active ingredient azadirachtin might be present in nectaries of treated plants or in the honeydew of treated aphids and thus in the energy source of many predators and parasitoids as found for other systemic active ingredients (Calvo-Agudo et al. 2019; Jones et al. 2020). Some studies support the possibility of combining azadirachtin products with beneficial arthropods or other biological control agents in IPM strategies (Medina et al. 2001; Otieno et al. 2017), also in roses (Put et al. 2016). Future research should address such combinations under greenhouse conditions to support growers in implementing smart and sustainable pest control programmes for aphids and other pests.

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Author contributions IB, MK, CTB and MD conceived and designed the research. IB conducted the experiments, analysed the data, with DM, and wrote the manuscript with the input from MK, CTB, DM and MD. All authors approved the manuscript.

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Data availability Data are available on request.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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