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Effects of NeemAzal-T/S on different developmental stages of rose aphid, *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 [*Azadirachta indica* A. Juss (Meliaceae)] product NeemAzal-T/S and its influence on stage-specific mortality, development, and reproduction of rose aphids, *Macrosiphum rosae* L. (Hemiptera: Aphididae), 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 juvenile 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 sublethal concentrations of neem as first instars. 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 to control rose aphids in integrated pest management (IPM) systems. Because this insecticide has no rapid knock-down effect and no effect on adult aphids, combinations with natural enemies of pest insects as well as with other insecticides are possibilities to enhance the efficacy.

KEYWORDS
aphids, rose, neem, azadirachtin, IPM, Hemiptera, Aphididae, *Azadirachta indica*, natural insecticide, efficacy, mode of action, greenhouse trial

INTRODUCTION

Natural insecticides based on extracts from seed kernels of the neem tree, *Azadirachta indica* A. Juss (Meliaceae), have gained importance for pest management in the last decades. Due to the public pressure to eliminate synthetic chemical pesticides, biological products for pest management are considered as a promising alternative. Neem products are especially convenient for home and garden use and for high-value crops such as ornamentals. Their low persistence in the environment and low mammalian toxicity (Sundaram, 1996; Raizada et al., 2001; Boeke et al., 2004; Kleeberg, 2004) are advantageous for use in public areas. One common pest of roses is the rose aphid, *Macrosiphum rosae* L. (Hemiptera: Aphididae). It has an almost global distribution and infests all sorts of *Rosa* species as its primary host plant. In Europe, *M. rosae* can be found on roses from spring 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 8–10 days to mature at temperatures between 20 and 25 °C. During her 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 & Isman, 1993, 1994; Lowery et al., 1993; Fournier & 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 & Cullen, 2008; Quratulain et al., 2015; 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 p.p.m. 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 p.p.m. azadirachtin [e.g., West & 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 & Isman, 1993; Koul, 1999; Mordue (Luntz) & Nisbet, 2000]. Insect growth regulatory effects result from the influence of azadirachtin on ecdysone and juvenile hormone titers in insect hemolymph (Barnby & 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, whereas juvenile hormone controls the developmental stage at the time of molting [Mordue (Luntz) & Blackwell, 1993; Mordue (Luntz) & Nisbet, 2000; Mordue (Luntz) et al., 2010]. Treated aphid nymphs failed to complete the molting process and died (Lowery & Isman, 1994). By contrast, lethal effects of neem on adults of hemipteran insects seemed to be less clear. Nevertheless, juvenile hormone is involved in the production of eggs and mobile offspring in adult insects (Hardie, 1987; Riddiford, 2012). Sterility and reduced fecundity have been reported as effects of neem treatments on adult aphids [Stark & Rangus, 1994; Lowery & Isman, 1994; Fournier & Brodeur, 2000; Mordue (Luntz) & 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 host plant (Dimetry & Schmidt, 1992; Lowery et al., 1993; Lowery & Isman, 1994, 1996; Stark & Rangus, 1994; Koul, 1998; Pavela et al., 2004). In *Aphis fabae* Scopoli, exposure of adults to azadirachtin concentrations as low as 1 p.p.m. reduced the fecundity (Dimetry & Schmidt, 1992), whereas in *Brevicoryne brassicae* L., 50–60 p.p.m. azadirachtin were necessary to be effective (Koul, 1998; Pavela et al., 2004). In *Myzus persicae* Sulzer and *Nasonovia ribisnigri* (Mosley), 40–80 p.p.m. 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* (Cockerell) remained unaffected even at concentrations of 80 p.p.m. azadirachtin (Lowery & Isman, 1996). Older instars and adult aphids are generally less sensitive to lethal effects of azadirachtin (Dimetry & Schmidt, 1992; Pavela et al., 2004). However, lethal concentrations for nymphs also vary with aphid species: LC_{50} (median lethal concentration, causing 50% mortality) of azadirachtin for second instars on leaf disks is only 2.4 p.p.m. for *M. persicae*. Intermediate values were recorded for *Macrosiphum euphorbiae* Thomas, with an LC_{50} of 8 p.p.m. or *Aphis gossypii* Glover, with 90 p.p.m. azadirachtin. The LC_{50} of 635 p.p.m. for *C. fragaefolii* forms a remarkable exception from the common rates (Lowery & Isman, 1994). In addition to lethal effects, sublethal effects and hormesis effects may also occur, especially when the active ingredient degrades due to environmental influences (Caboni et al., 2006; Desneux et al., 2007; Cutler et al., 2009; Ullah et al., 2019).

This summary indicates that it is difficult to predict the effects of neem treatments for a specific aphid species by comparison with results for other species. For *M. rosae*, only limited data are available and partly contradictory. 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 p.p.m. azadirachtin. The survival rate of adults was higher and 70–90 p.p.m. azadirachtin was needed to reach more than 90% mortality. This suggests that currently registered dosages of around 30–50 p.p.m. azadirachtin only result in moderate mortality of adults. Atanasova et al. (2014) observed an insufficient effect 5 days after a single application of NeemAzal-T/S on *M. rosae* populations in field trials at concentrations of 0.3 and 0.5%, equivalent to registered dosages in Europe. In contrast, Quratulain et al. (2015) reached 60–100% mortality of *M. rosae* already 24 h after spraying a self-made neem-solution with unknown concentrations of azadirachtin in the field. Generally, 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 to be a slow-acting insecticide and experimental periods should not be shorter than 1 week; otherwise, relevant effects might be missed (Stark & Rangus, 1994).

Many studies used leaf disks dipped in azadirachtin or neem seed oil solutions as food for aphids (e.g., Lowery & Isman, 1993, 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 obtain more realistic conditions.

The objective of this study was to assess in detail how registered doses of the 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 possible insufficient efficacies, clip-cage experiments were carried out, which allowed 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. These experiments are meant to unravel the effect of azadirachtin on populations of *M. rosae*. 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 & Baxendale, 2013).

MATERIALS AND METHODS

General materials for all experiments

Insects and plants

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 (*Rosa* spec., Rosaceae, various Kordana varieties by W. Kordes' Söhne Rosenschulen, Klein Offenseth-Sparrieshoop, Germany) in cages (63 × 78 × 63 cm) made of acrylic glass with three sides of gauze. Aphids were maintained in a climate chamber at 22 ± 2 °C, 60 ± 10% r.h., and L16:D8 photoperiod.

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 (Hermann Meyer, Rellingen, Germany), as substrate. Plants were kept in an air-conditioned greenhouse at 21 ± 3 °C, 60% r.h, and L16:D8 photoperiod. During this time, additional light was provided by sodium vapor lamps if sunlight intensity outside the greenhouse fell under 120 W m^{-2}. A few days after repotting, plants were drenched with the liquid fertilizer Wuxal Top N (Manna, Nürnberg, 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 (Münster, Germany), active ingredient azoxystrobin and Bayer Garten Rosen Pilz-frei Baymat (Leverkusen, Germany), active ingredient tebuconazole). Fungicide treatments were done at the latest 1 week before the experiments started, to exclude unforeseen side effects. Furthermore, all plants were sprayed to maintain equal conditions between the treatments. Plants were maintained under these conditions for 3–4 weeks until used for experiments. For experiments, plants were used at a stage in which 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 (Lahnau, Germany) was used as a common and registered neem-based insecticide in Europe. It is an emulsifiable concentrate with 10 g of azadirachtin A per liter as its major active ingredient, with a maximal amount of 3–4% natural neem seed kernel extract (NeemAzal technical; Trifolio-M, 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 m^2 is registered as dose rate. With a slurry volume of 900 l ha^{-1}, 3.3 ml product l^{-1} water is the common dose rate. Plants were sprayed until run-off, ensuring a complete coverage of plants. This dose rate is equivalent to 30 g azadirachtin A ha^{-1} per application. Up to four applications per culture per year are allowed in Germany (BVL, 2017) at an interval of 7–10 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) 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 formulation used is a soluble (liquid) concentrate, containing 50 g flupyradifurone per liter. It was used in a concentration with 1.33 ml l^{-1} 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.

Efficacy trials

Two efficacy trials with potted miniature rose plants and *M. rosae* were conducted in the greenhouse. Conditions in the greenhouse compartment were 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 10 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 1 week before the start of the experiment. In the second trial, the application was made 4 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: untreated control, water control, 3.3 ml l^{-1} NeemAzal-T/S Blank, 1.65, 2.5, or 3.3 ml l^{-1} NeemAzal-T/S, and 1.33 ml l^{-1} 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 2×, i.e., on days 0 and 7. Flupyradifurone SL 50 was only applied on day 0.

Total numbers of aphids per plant were estimated by counting groups of 5–10 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 & Tilton, 1955):

$$\text{Efficacy } (\%) = \left(1 - \frac{T_a}{T_b} \times \frac{C_b}{C_a}\right) \times 100,$$

where T_b and T_a is the infestation on treated plants before and after application, respectively, and C_b and C_a is the infestation on control plants before and after application.

Statistical analysis was carried out with the trial management software ARM (Agriculture Research Manager, revision 2017; Gylling Data Management, Brookings, SD, USA). Numbers of aphids per plant and efficacy, calculated by the formula of Henderson & Tilton (1955; see above) were analyzed with Student-Newman-Keuls (SNK) test ($\alpha = 0.05$) separately for each evaluation day after data transformation [$\sqrt{x}$ or $\log_{10}(x)$], if the data were 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% r.h., and L16:D8 photoperiod for 24 h 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

TABLE 1 Overview of treatments, type of formulation, corresponding dosages, and active ingredient contents

Treatment	Formulation type[1]	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	–	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

[1]EC, emulsifiable concentrate; SL, soluble liquid concentrate.

acrylic glass cage (63 × 78 × 63 cm) with three gauze sides 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^{-1} NeemAzal-T/S Blank, 1.65 and 3.3 ml l^{-1} NeemAzal-T/S. In addition, 10 untreated plants for the control were added to the study. After the plants were dried, each plant was placed onto a saucer. Ten water-filled bowls of 40 × 59 × 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 the escape of apterous aphids or migration to other plants. An 11- or 12-day-old (time since larviposition) *M. rosae* female from the synchronized rearing was transferred to each plant and all aphids were 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 v.3.1.3 (R Core Team, 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^{-1} NeemAzal-T/S Blank, and 1.65, 2.5, or 3.3 ml l^{-1} 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 the plants were dried, a clip-cage with 5–10 randomly picked adult *M. rosae* from the main culture was introduced onto each rose plant. Then, 24 h 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 v.3.1.3 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 various 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 with Thernau & Grambsch (2000) and Fox & 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 h. After this time, adults were removed and emerged nymphs were maintained without clip cages in a rearing cage for additional 10 days until the beginning of the experiment. The same procedure was done again 8, 6, 4, and 1 days before the start of the experiment to have five distinct *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^{-1} NeemAzal-T/S Blank, and 30 were sprayed with 3.3 ml l^{-1} 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 represented a replicate per stage. Clip cages were checked daily and dead aphids were counted for 7 days. Plants were arranged in a greenhouse compartment in a completely randomized design. Mortality was analyzed with the Cox proportional hazards model in R v.3.1.3 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 h. Their offspring were then transferred with a fine brush to untreated plants and plants which were either sprayed with 2.5 ml l^{-1} NeemAzal-T/S Blank, 1.25 ml l^{-1} NeemAzal-T/S, or 2.5 ml l^{-1} NeemAzal-T/S. Each rearing was maintained separately in rearing cages (63 × 78 × 63 cm) in a

climate chamber at 22 ± 2 °C, 60 ± 10% r.h., and L16:D8 photoperiod for further 9 days. Consequently, aphids were 10 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 l^{-1} NeemAzal-T/S Blank or with 1.25, 2.5, or 3.3 ml l^{-1} NeemAzal-T/S. In addition, one completely untreated group was added to the study. This resulted in the 13 treatment combinations listed in Table 2. Per combination, eight replicates were prepared, each comprising one plant with one clip cage containing a single adult aphid. Reproduction was assessed daily by counting nymphs in the clip cages and removing them, during a period of 7 days. Mean numbers of offspring per female and day were compared using ANOVA with Tukey honestly significant difference (HSD) post hoc test in IBM-SPSS v.22 software (IBM, Armonk, NY, USA). 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 IBM-SPSS v.22. Graphs were built in SPSS and adjusted in CorelDraw Graphics Suite 2017 (Corel Corporation, Ottawa, Canada).

TABLE 2 Combinations of treatment of *Macrosiphum 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 than all other treatments with the exception of 14 DAA in trial 1, where differences to NeemAzal-T/S 2.5 and 3.3 ml l^{-1} were not significant (Table 3). Application of flupyradifurone resulted in efficacies >90% on 3 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 and 3.3 ml l^{-1} 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 Efficacy trial 1. Mean (± SE; n = 4 plots per treatment, each plot with five plants) number of *Macrosiphum rosae* per plant and efficacy (%) before application and 3, 7, and 14 days after first application (DAA) of various concentrations of NeemAzal-T/S and flupyradifurone

	Before application	3 DAA		7 DAA		14 DAA/7 DA2[1]	
Treatment	No. aphids/ plant	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)
Control (untreated)	222 ± 14a	444 ± 32a		676 ± 24a		821 ± 53a	
Water	240 ± 11a	423 ± 34a	12 ± 2b	684 ± 37a	6 ± 8c	713 ± 24a	18 ± 8b
NeemAzal-T/S Blank 3.3 ml/l	239 ± 14a	412 ± 17a	13 ± 5b	626 ± 32a	13 ± 7c	818 ± 77a	6 ± 10b
NeemAzal-T/S 1.65 ml/l	229 ± 28a	358 ± 71a	25 ± 6b	503 ± 48a	27 ± 4bc	778 ± 69a	3 ± 16b
NeemAzal-T/S 2.5 ml/l	246 ± 17a	285 ± 18a	39 ± 9b	490 ± 49a	34 ± 6bc	576 ± 47a	36 ± 5ab
NeemAzal-T/S 3.3 ml/l	270 ± 11a	362 ± 33a	32 ± 7b	462 ± 55a	43 ± 9b	571 ± 47a	43 ± 5ab
Flupyradifurone SL 50 1.3 ml/l	255 ± 13a	29 ± 22b	94 ± 4a	35 ± 28b	95 ± 4a	269 ± 88b	72 ± 10a

Means within a column followed by a different letter are significantly different (SNK test: $P<0.05$).

[1]DA2, days after second application (flupyradifurone only one application).

TABLE 4 Efficacy trial 2. Mean (± SE; n = 4 plots per treatment, each plot with five plants) number of *Macrosiphum rosae* per plant and efficacy (%) before application and 3, 7, 14, and 21 days after first application (DAA) of various concentrations of NeemAzal-T/S and flupyradifurone

Treatment	Before application	3 DAA		7 DAA		14 DAA/7 DA2[1]		21 DAA/14 DA2		29 DAA/21 DA2	
	No. aphids/ plant	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)	No. aphids/ plant	Efficacy (%)
Control (untreated)	115 ± 8a	189 ± 22a		235 ± 23a		346 ± 14ab		531 ± 26a		630 ± 19a	
Water	118 ± 7a	185 ± 13a	1 ± 12b	250 ± 17a	−5 ± 8b	369 ± 14a	−4 ± 6c	494 ± 27a	9 ± 9b	596 ± 16a	7 ± 8b
NeemAzal-T/S Blank 3.3 ml/l	112 ± 12a	151 ± 16a	14 ± 13b	222 ± 8a	−2 ± 12b	254 ± 13ab	22 ± 8bc	429 ± 22a	14 ± 12b	546 ± 44a	8 ± 13b
NeemAzal-T/S 1.65 ml/l	103 ± 5a	153 ± 17a	6 ± 15b	216 ± 30a	−6 ± 17b	270 ± 36ab	14 ± 9bc	462 ± 44a	2 ± 11b	560 ± 51a	1 ± 10b
NeemAzal-T/S 2.5 ml/l	117 ± 6a	141 ± 5a	25 ± 6b	186 ± 13a	21 ± 6b	229 ± 15ab	34 ± 8b	342 ± 20a	33 ± 12b	477 ± 43a	23 ± 12b
NeemAzal-T/S 3.3 ml/l	105 ± 4a	142 ±17a	12 ± 17b	177 ± 27a	13 ± 18b	193 ± 2b	39 ± 2b	337 ± 30a	31 ± 6b	475 ± 29a	18 ± 6b
Flupyradifurone SL 50 1.3 ml/l	131 ± 12a	9 ± 5b	96 ± 2a	21 ± 12b	93 ± 4a	63 ± 39c	86 ± 8a	136 ± 57b	77 ± 10a	154 ± 52b	79 ± 7a

Means within a column followed by a different letter are significantly different (SNK test: P<0.05).
[1]DA2, days after second application (flupyradifurone only one application).

FIGURE 1 Population growth of *Macrosiphum rosae* on untreated control and NeemAzal-T/S Blank or NeemAzal-T/S treated plants starting with one 12-day-old (since larviposition) adult female. The thick lines represent functions for average numbers of *M. rosae* per plant predicted by the GAMM for each treatment. The shaded area around this function describes an approximation of a 95% confidence interval for the predicted values. The asterisk indicates a significant influence of treatment compared to the untreated control on numbers of aphids per plant (GAMM: P<0.05)

Population development

An application of 1.65 and 3.3 ml l^{-1} 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 (Figure 1). An exponential increase in numbers of aphids per rose plant was assessed after 12 days, whereas this increase was missing in both NeemAzal-T/S treatments. The application of 3.3 ml l^{-1} NeemAzal-T/S had a significant influence on the numbers of aphids per plant for the observation period of 16 days.

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^{-1} NeemAzal-T/S), 9 (1.65 ml l^{-1} NeemAzal-T/S), and 11 (2.5 ml l^{-1} NeemAzal-T/S) days. By day 4, more than 50% of these aphids had already died (Figure 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 untreated control (Figure 2) and did not pose a significantly higher risk for aphids to die (Table 5).

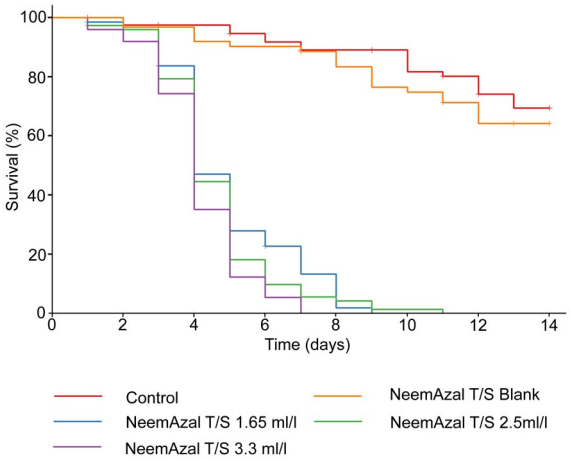

FIGURE 2 Survival of *Macrosiphum rosae* first instars over 14 days on untreated (control) rose plants and plants treated with NeemAzal-T/S Blank or 1.65, 2.5, and 3.3 ml l^{-1} NeemAzal-T/S. Initial total numbers of observed aphids varied between 62 and 78 individuals per treatment

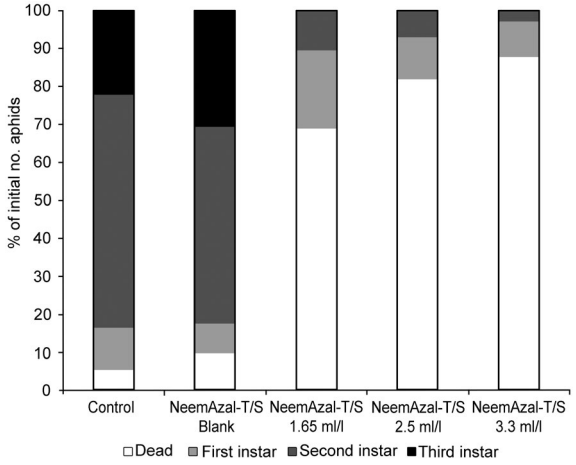

FIGURE 3 Comparison of development progress of *Macrosiphum rosae* nymphs. Frequency (%) of initial numbers of *M. rosae* individuals in the first, second, and third instar, as well as dead aphids (%) after 5 days of exposure to untreated, NeemAzal-T/S Blank, or NeemAzal-T/S-treated rose plants. Pooled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

TABLE 5 Mortality of *Macrosiphum rosae* first instars on untreated (control), NeemAzal-T/S Blank-, and NeemAzal-T/S-treated rose plants (n = 10 replications, i.e., clip cages with 4–14 aphids per treatment), based on Cox proportional hazards model with 'control' as baseline hazard group. Hazard ratios (HR) indicate the multiplicative change in risk to die compared to the control (HR for control set to 1)

Treatment	HR	95% confidence interval	P
Control	1b	–	–
NeemAzal-T/S Blank	1.3b	0.7–2.3	0.61
NeemAzal-T/S 1.65 ml/l	22.8a	12.53–41.4	<0.001
NeemAzal-T/S 2.5 ml/l	26.9a	15–47.9	<0.001
NeemAzal-T/S 3.3 ml/l	36.3a	19.9–66.2	<0.001

Hazard ratios followed by a different letter are significantly different (Tukey test: P<0.05).

TABLE 6 Mean (± SE) developmental time (days) for *Macrosiphum rosae* from first to second instar after 5 days of exposure to untreated, NeemAzal-T/S Blank, or NeemAzal-T/S-treated rose plants, and probability to develop to the second instar later than in the control, based on Cox proportional hazards model

Treatment	Days to second instar	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 ns
NeemAzal-T/S 3.3 ml/l	4.0 ± 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 = number of aphids for which moulting has been observed, pooled over all replications.

***P<0.001; ns, P>0.05.

First instars of *M. rosae* have a significantly increased probability to molt later to the second instar than 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 live nymphs had reached the second or third instar on untreated and NeemAzal-T/S Blank treated plants, whereas in the NeemAzal-T/S treatments only first and second instars were found (Figure 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^{-1} NeemAzal-T/S decreased with development to older instars (Figure 4). First, second, and third instars had a significantly 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 10× 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).

Reproduction

Reproduction of *M. rosae* was negatively affected by exposure to sublethal concentrations of NeemAzal-T/S

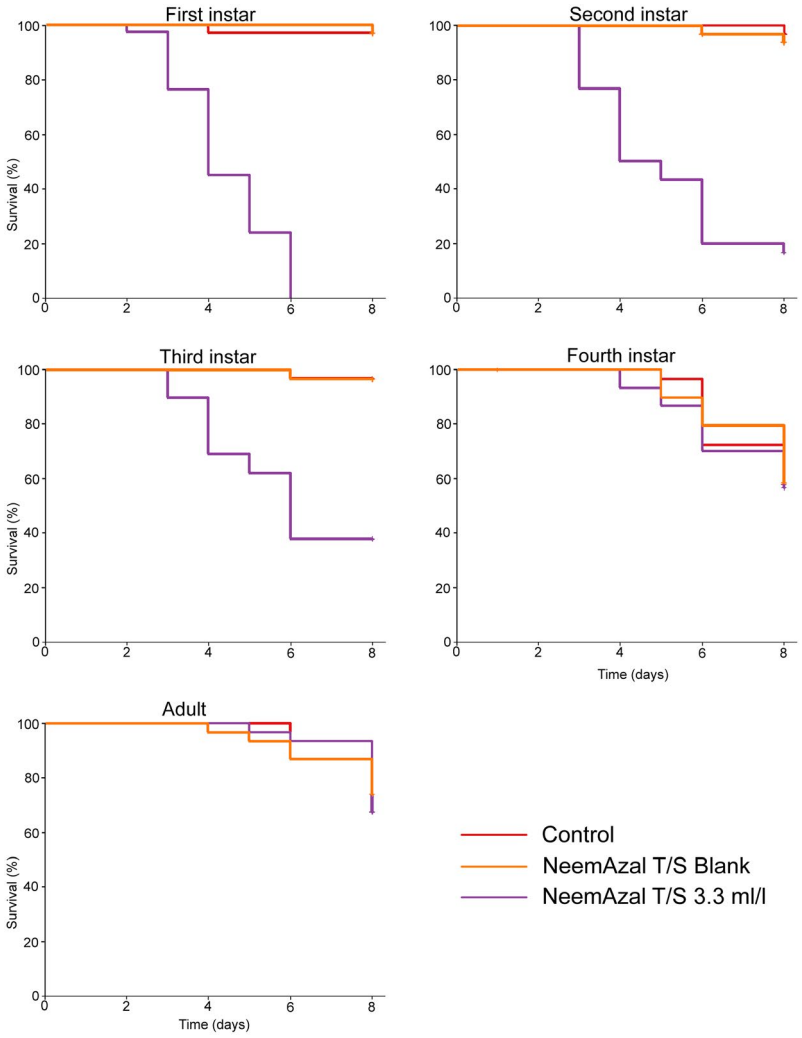

FIGURE 4 Survival of *Macrosiphum rosae* exposed as different developmental stages to untreated (control) rose plant or plants treated with NeemAzal-T/S Blank or 3.3 ml l^{-1} NeemAzal-T/S; n = 30 aphids per instar and treatment

TABLE 7 Mortality risk for four instars and adults of *Macrosiphum rosae* on NeemAzal-T/S-treated rose plants (n = 6 replications per instar, i.e., clip cages, each with five aphids), based on Cox proportional hazards model with 'control' as baseline hazard group. Hazard ratio (HR, with 95% confidence interval in parentheses) indicates the multiplicative change in risk to die compared to the control (HR for control set to 1)

Treatment	First instar	Second instar	Third instar	Fourth instar	Adult
Control	1	1	1	1	1
NeemAzal-T/S Blank	1 ns (0.1–13.2)	2.1 ns (0.2–18.6)	1.1 ns (0.1–14)	1 ns (0.4–2.4)	1 ns (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 ns (0.6–2)	1.3 ns (0.4–4.2)

Within a column, asterisks indicate a significant influence of the treatment (***P<0.001; ns, P>0.05).

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^{-1} NeemAzal-T/S during their development, followed by 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^{-1} 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 (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 (Figure 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 (Figure 6). No significant differences were recorded on the other days.

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*

TABLE 8 Mean (± SE; n = 8 adult female aphids) number of offspring per female *Macrosiphum rosae* per day over 7 days in various combinations of pre-experimental rearing and experimental plant treatments with NeemAzal-T/S, in two replicate trials

Combination no.	Treatment of plants for *M. rosae* rearing before the experiment	Treatment of experimental plants	Trial 1	Trial 2
1	Untreated	Untreated	3.9 ± 0.4ab	3.6 ± 0.3ab
2	Blank 2.5 ml/l	Blank 3.3 ml/l	4.0 ± 0.4a	3.5 ± 0.3ab
3	Blank 2.5 ml/l	NeemAzal-T/S 1.25 ml/l	4.1 ± 0.4a	3.0 ± 0.3abcd
4	Blank 2.5 ml/l	NeemAzal-T/S 2.5 ml/l	4.1 ± 0.4a	3.3 ± 0.3abc
5	Blank 2.5 ml/l	NeemAzal-T/S 3.3 ml/l	4.3 ± 0.5a	4.4 ± 0.3a
6	NeemAzal-T/S 1.25 ml/l	Blank 3.3 ml/l	2.3 ± 0.6ab	1.8 ± 0.5bcde
7	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 1.25 ml/l	2.2 ± 0.4ab	1.5 ± 0.5cde
8	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 2.5 ml/l	3.0 ± 0.5ab	1.9 ± 0.6bcde
9	NeemAzal-T/S 1.25 ml/l	NeemAzal-T/S 3.3 ml/l	2.8 ± 0.6ab	1.1 ± 0.5de
10	NeemAzal-T/S 2.5 ml/l	Blank 3.3 ml/l	1.4 ± 0.4b	1.0 ± 0.3e
11	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 1.25 ml/l	2.4 ± 0.8ab	2.1 ± 0.5bcde
12	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 2.5 ml/l	2.3 ± 0.6ab	2.1 ± 0.4bcde
13	NeemAzal-T/S 2.5 ml/l	NeemAzal-T/S 3.3 ml/l	1.8 ± 0.7ab	1.2 ± 0.4de

Means within a column followed by different letters are significantly different (Tukey test: P<0.05).

TABLE 9 Reproduction of *Macrosiphum rosae* depending on NeemAzal-T/S treatments during the development (rearing treatment) or as adults (experimental plant treatment): P values for GLMs models separately for numbers of offspring per female for every day of both replicate trials. 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

Trial no.	Day	Model type with log-link	Rearing treatment	Experimental plant treatment	Interaction (treatment combination)
1	1	Neg. binomial	0.067	0.73	0.93
	2	Neg. binomial	0.012	0.53	0.92
	3	Poisson	<0.001	0.16	0.53
	4	Poisson	<0.001	0.85	0.049
	5	Poisson	<0.001	0.72	0.21
	6	Poisson	<0.001	0.002	0.016
	7	Poisson	0.009	0.98	0.22
2	1	Poisson	<0.001	0.20	0.006
	2	Poisson	<0.001	0.082	0.003
	3	Poisson	<0.001	0.38	0.51
	4	Poisson	<0.001	<0.001	0.12
	5	Poisson	<0.001	0.93	0.039
	6	Poisson	0.004	0.73	0.40
	7	Poisson	0.26	0.84	0.74

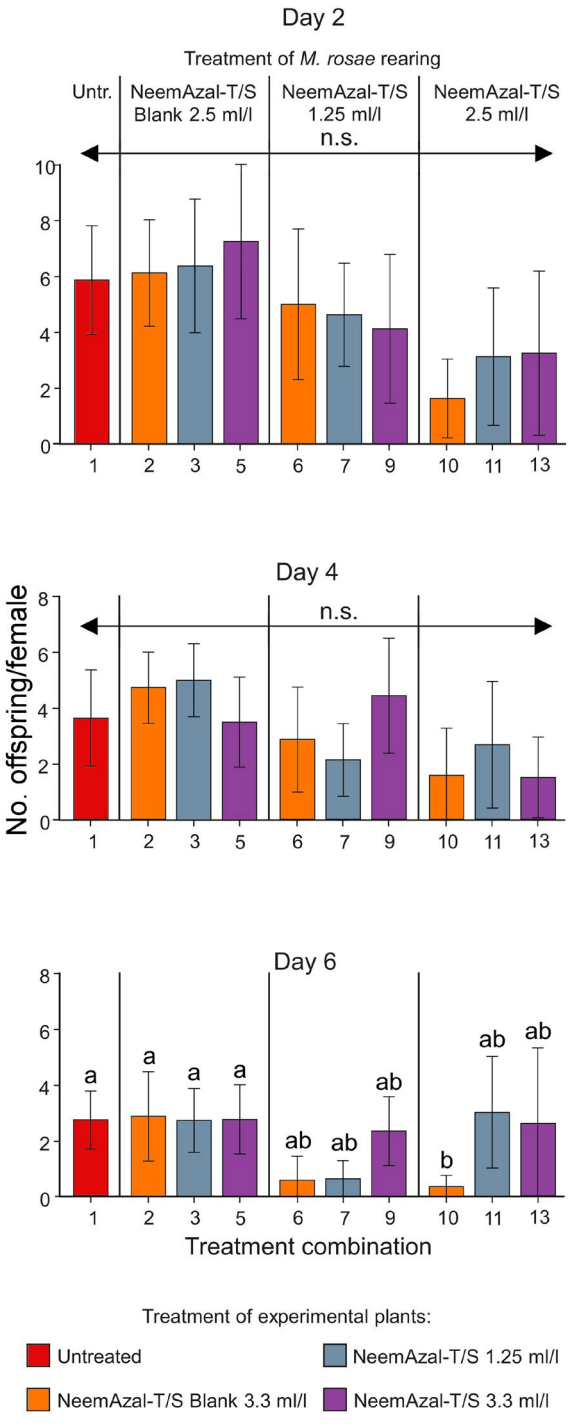

FIGURE 5 Mean (± SE) number of offspring per *Macrosiphum rosae* female for selected treatment combinations (see Table 2 for description) on days 2, 4, and 6 since the start of the first reproduction experiment. Treatment of plants for *M. rosae* rearing before the experiment is stated on top of the 'Day 2' panel, whereas the shading of the bars indicates the treatment of experimental plants. Means capped with different letters are significantly different (Bonferroni adjusted all-pair test within GLMs for each day: $P<0.05$; n.s., $P>0.05$). Due to mortality, numbers of observed adult female aphids per treatment combination varied with days: n = 8 on day 2, 6–8 on day 4, and 5–8 on day 6

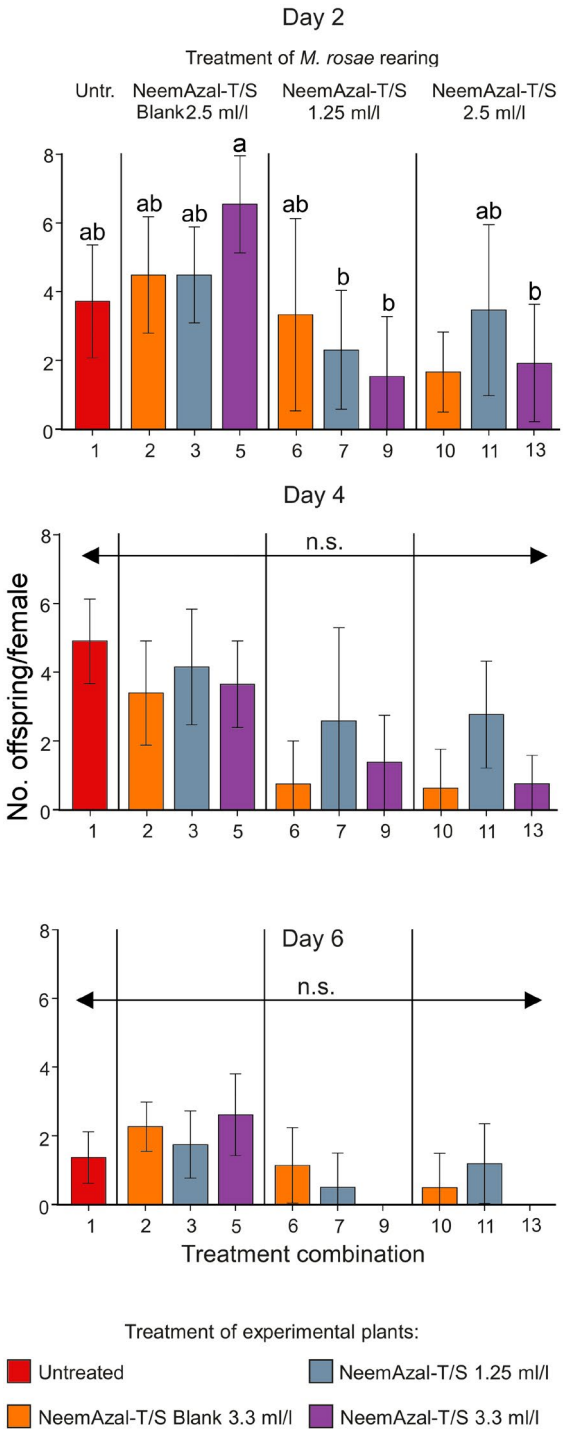

FIGURE 6 Mean (± SE) number of offspring per *Macrosiphum rosae* female for selected treatment combinations (see Table 2 for description) on day 2, 4, and 6 since the start of the second reproduction experiment. Treatment of plants for *M. rosae* rearing before the experiment is stated on top of the 'Day 2' panel, whereas the shading of the bars indicates the treatment of experimental plants. Means capped with different letters are significantly different (Bonferroni adjusted all-pair test within GLMs for each day: $P<0.05$; n.s., $P>0.05$). Due to mortality, numbers of observed adult female aphids per treatment combination varied with days: n = 8 on day 2, 7–8 on day 4, and 2–8 on day 6

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 delayed development and high mortality of first, second, and third instars. 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 is inhibited and reveal that neem products can be effective for controlling rose aphids. In contrast, the mortality of fourth instars and adult *M. rosae* was not affected and standard efficacy trials with populations of mixed developmental stages only showed moderate efficacy of currently registered concentrations of NeemAzal-T/S in the greenhouse. Our efficacy results are similar to those of Atanasova et al. (2014), who observed 30–45% efficacy with similar concentrations of NeemAzal-T/S after a single application in field trials with *M. rosae*. Here, we also investigated the underlying mechanisms leading to the efficacy effect.

Neem products usually have a low impact on adult aphids, but mortality of juvenile stages is significantly higher due to an insect growth regulating (IGR) effect of azadirachtin. Neem seed oil and a pure azadirachtin formulation, both in concentration equivalent to 40 p.p.m. azadirachtin, resulted in 100% mortality of second instars of *N. ribisnigri* and *Myzus persicae* (Sulzer). Moreover, the treatments significantly reduced the number of molts compared to the control (Lowery & Isman, 1994). Increased mortality of aphid nymphs exposed to neem products, neem extracts, or pure azadirachtin formulations was reported for several other aphid species, e.g., for *A. fabae* (Dimetry & Schmidt, 1992; Dimetry & El-Hawary, 1995), *Acyrthosiphon pisum* (Harris) (Stark & Rangus, 1994), *Macrosiphoniella sanbornii* (Gillete) (Koul, 1999), *Toxoptera citricida* Kirkaldy (Tang et al., 2002), *Brevicoryne brassicae* L. (Pavela et al., 2004), and *Aphis glycines* Matsumura (Kraiss & 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 instars due to azadirachtin concentrations of 30 p.p.m. (Koul, 1999) are similar to our results for a spray treatment of complete rose plants with 33 p.p.m. azadirachtin (3.3 ml l^{-1} NeemAzal-T/S). The delayed development of *M. rosae* nymphs after treatments with azadirachtin was also reported for *A. craccivora* (Dimetry & 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 p.p.m. 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 30–50 p.p.m. azadirachtin per application, equivalent to a spraying solution of 3–5 ml l^{-1} 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 p.p.m. (Lowery & 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 p.p.m. azadirachtin after 4 days. Survival decreased with increasing azadirachtin concentrations and 90% mortality was reached after 7 days with 22 p.p.m. 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. However, exposure of adult *M. rosae* to NeemAzal-T/S-treated plants 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^{-1}. 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 & 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 & 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 live offspring production as a consequence of its influence on the titers of juvenile hormone (Rembold & Sieber, 1981; Hardie, 1987; Barnby & 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: by contrast, Tang et al. (2002) found that *T. citricida* reproduce significantly less on citrus seedlings treated with Neemix at azadirachtin concentrations of 11–180 p.p.m. and their longevity was shorter than that of aphids on water-treated seedlings (Tang et al., 2002). A direct reduction in number of live offspring was also recorded for *M. persicae* and *N. ribisnigri* on neem seed oil-treated leaf disks, but the reduction was higher when aphids were exposed already as fourth instars (Lowery & 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^{-1} 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, sterility of aphids that 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 h, was significantly lower compared to the offspring of untreated adults (Koul, 1999). This effect ensures a long-lasting efficacy of neem products, as 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 p.p.m., but the efficacy was higher. Aphid numbers were reduced by >65% with 14 p.p.m. azadirachtin and by 75% with 28 p.p.m. azadirachtin 7 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, with a standard application strategy, administered when a high infestation pressure has already been 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 7–10 days. Neem is a slow-acting insecticide and its efficacy should be evaluated based on reproduction, longevity, behavior, and population growth (Stark & Rangus, 1992). Other ways of application which might increase the efficacy, for instance systemically through soil application (Pavela et al., 2004; Karanja et al., 2015) or encapsulation to protect azadirachtin against degradation (Forim et al., 2013; Chaudhary et al., 2017), are not yet registered with the exception of soil application against fungus gnats (BVL, 2021). The efficacy might be increased by combining neem with synergistic products such as karanja oil (Kovarikova & Pavela, 2019) or with a contact knock-down insecticide such as pyrethrum. This 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 (Otieno et al., 2017). Neem products are generally compatible with other products and beneficial insects in IPM strategies (Raguraman et al., 2004), but possible lethal and sublethal effects on non-target organisms and consequences as pest resurgence need to be taken into consideration (Ahmad et al., 2003; Desneux et al., 2007; Turchen et al., 2016; Drobnjakovic et al., 2018).

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AUTHOR CONTRIBUTIONS

Imke Bartelsmeier: Conceptualization (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Project administration (lead); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review & editing (lead). Michael Kilian: Conceptualization (supporting); Funding acquisition (lead); Project administration (supporting); Resources (supporting); Supervision (equal); Visualization (supporting); Writing – original draft (supporting). Marcel Dicke: Conceptualization (supporting); Formal analysis (supporting); Methodology (supporting); Project administration (supporting); Supervision (equal); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal).

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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