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Systemic Slow-Release Neem Formulations: The Future of Cabbage Aphids, *Brevicoryne brassicae* Control

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Abstract

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

Subject Areas

Entomology

Keywords

Azadirachtin, *Brevicoryne brassicae*, Efficacy, Dose, Fecundity, Persistence

1. Introduction

The cabbage aphid *Brevicoryne brassicae* L. is native to Europe but has a worldwide distribution. Their short life cycle and high fecundity result in high reproductive rates. Cabbage aphid has been described as a very serious pest and significant losses are associated with its infestation, particularly in many economically important host crops of the Brassicaceae family [1] [2] [3].

Cabbage aphid feeds by sucking sap from plants causing leaves to curl inward and become chlorotic. Moreover, infestations result in a reduction of market value in mature plants by an accumulation of aphid residues such as the exuviae sticking on the plant surface, and contamination by sooty mold fungi growing on the honeydew. Continued feeding by aphids causes yellowing, wilting and stunting of young plants [2] [4]. They also cause indirect damage by vectoring plant viruses [5].

Control of these pests has chiefly relied on the application of synthetic pesticides. These pesticides could potentially be toxic to humans when they are used in vegetable production [2] [6] [7] [8] [9]. Aphids are likely to develop resistance to synthetic pesticides due to overlapping parthenogenetic generations [10]. Apart from biocontrol with natural enemies development of consumer-safe and selective pesticides for the control of this pest is of paramount interest.

In recent years, there is a shift of focus toward naturally occurring pest control agents, and bio-pesticides are suitable to be used in integrated control strategies or ecological production systems. Neem products containing the biologically active compound Azadirachtin, which is derived from the Neem tree *Azadirachta indica* A. Juss (Meliaceae), is an alternative insecticide for organic farming [11] [12] [13] [14]. Besides, these products have low human toxicity, low persistence and pronounced selectivity concerning non-target organism, such as parasitoids, predators, and pollinators [15] [16] and degrade rapidly in the environment [17].

Azadirachtin is an antifeedant and growth regulator for a wide variety of insects (Mordue and Luntz, 1998), inhibits cuticulogenesis [15], delays and prevents molting, and has been shown to prolong development time of aphids [18] [11]. Moreover azadirachtin results in increased adult and nymph mortality, reduced number of nymphal molts and decreased adult fecundity in Brown citrus aphids *Toxoptera citricida* (Kirklady) [19] and cabbage aphid [18].

Most of the Neem products in the market today such as NeemAzal (AZA) T/S®

(Trifolio) are formulated for foliar applications. Despite their high efficacy when in direct contact with the target organism, the oil-based foliar formulations rapidly degrade under high temperatures and UV light [20] [21]. There is, therefore, a need for alternative strategies, which may improve the efficiency of applications. Soil application and uptake of active ingredients by the root systems could avoid these negative effects and hence attain higher levels of pest control sustainably.

Azadirachtin is taken up by the plants through the roots and is systemically translocated acropetal to the feeding sites of insects following soil treatment [10] [22] [23] [24] [25]. The aim of this study, therefore, was to evaluate the efficacy, persistence and residual effects of soil-applied neem formulations in the control of cabbage aphid under greenhouse conditions.

2. Materials and Methods

2.1. Neem Formulations and Treatments

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

2.2. Plants and Insects

Cabbage aphid, *Brevicoryne brassicae*, used in this study was cultured on Brussels sprouts kept in insect-proof cages in a greenhouse (average temperature 20˚C). For synchronization of the culture, young adult aphids were introduced onto plants and allowed to reproduce for 12 hours and then carefully removed using a fine-tip camel hair brush. The same aged offspring was further cultured.

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

2.3. Experiments

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

formulations

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

To assess the effect of azadirachtin on the survival of aphids, monitoring was done daily for 16 days, by counting the number of living and dead aphid larvae. The effect of the azadirachtin on reproduction of aphids was assessed by recording the total number of offspring per day. Fecundity was calculated as the number of offspring (dead and live) per adult per day.

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

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

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

2.4. Statistical Analysis

For Experiment 1, a survival analysis was performed with a Cox proportional hazard model using robust sandwich variances to account for heterogeneity between plants, *i.e.*, considering plants as clusters [26]). Multiple comparisons of hazard rates between dosage groups using a 5% familywise error rate and corresponding 95% simultaneous confidence intervals were calculated [27].

No violation of the proportional hazards assumption was found when inspecting scaled Schoenfeld residuals.

For Experiment 2, mortality of larvae of *B. brassicae* over time was analyzed using binomial generalized linear models (GLMs) with logit link function and overdispersion ("quasi-binomial") [28]. The models included formulation, days, and trial as independent factors as well as the interaction of formulation and days. Statistical significance of factors was assessed with analysis of deviance F-tests.

All statistical computations were performed in R 3.4.1 [29] using the add-on packages "survival" (version 2.41-3) and "multcomp" (version 1.4-8).

3. Results

3.1. Efficacy and Dose-Response of *B. brassicae* to NeemAzal Formulations

Systemic application of NeemAzal resulted in a marked reduction in the survival of aphid larvae compared to control. Toxicity of azadirachtin to the larvae of both tested formulations increased with dosage, which is reflected in the increasing hazard ratios and highly significant ($P < 0.001$) effects of NeemAzal compared with the control (**Table 1**). The highest doses of NeemAzal granules and NeemAzal-T (300 mg and 2.5 ml/kg of substrate) led to 0% survival rate by 14 days after treatment (**Figure 1** & **Figure 2**). On the other hand, the lowest tested doses of NeemAzal granules and NeemAzal-T (75 mg and 1 ml/kg of substrate) saw 34% and 42% survival, respectively, by day 14 after treatment. Overall, there were no significant differences ($p > 0.05$) between the two highest concentrations NeemAzal granules (225 and 300 mg/kg) of NeemAzal-T (2 and 2.5 ml/kg) did not differ significantly in larval survival rate.

Comparing the efficacies of the two neem formulations at their recommended doses (150 mg/kg NeemAzal granules versus 1 ml/kg NeemAzal-T): the test is highly significant in favor of the granules. The estimated hazard ratio was 2.94 with a 95% confidence interval of (1.74, 4.96), *i.e.* the hazard of aphids dying from feeding on a plant treated with NeemAzal granules at 150 mg/kg substrate was almost 3 times as high as with NeemAzal-T at 1ml/kg of substrate.

Exposing adults of *B. brassicae* to NeemAzal drastically reduced their fecundity (offspring per aphid per day). The effect was on a dose-dependent manner,

Table 1. Cox model for survival analysis: log hazard ratios (hazard ratios), robust sandwich standard errors, and z statistics for each treatment in relation to the blank control. All comparisons are statistically significant with $P < 0.001$.

	log HR (HR)	Robust SE	z
Granules 75 mg	1.054 (2.87)	0.184	5.72
Granules 150 mg	1.915 (6.79)	0.213	9.01
Granules 225 mg	2.167 (8.73)	0.149	14.56
Granules 300 mg	2.633 (13.91)	0.168	15.64
NeemAzal-T 1 ml/kg	0.837 (2.31)	0.190	4.41
NeemAzal-T 1.5 ml/kg	1.595 (4.93)	0.151	10.54
NeemAzal-T 2 ml/kg	1.888 (6.61)	0.139	13.61
NeemAzal-T 2.5 ml/kg	2.286 (9.84)	0.105	21.71

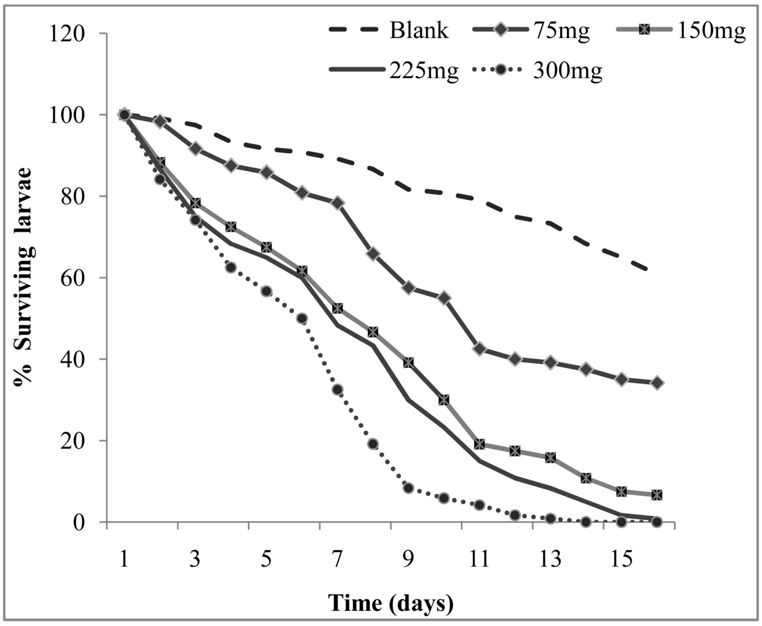

Figure 1. Survival curves for *B. brassicae*: Mean survival rates per day over a time period of 16 days after treatment with NeemAzal granules at 5.25 (75 mg), 10.5 (150 mg), 15.75 (225 mg) and 21 (300 mg) mg AZA per kg of substrate.

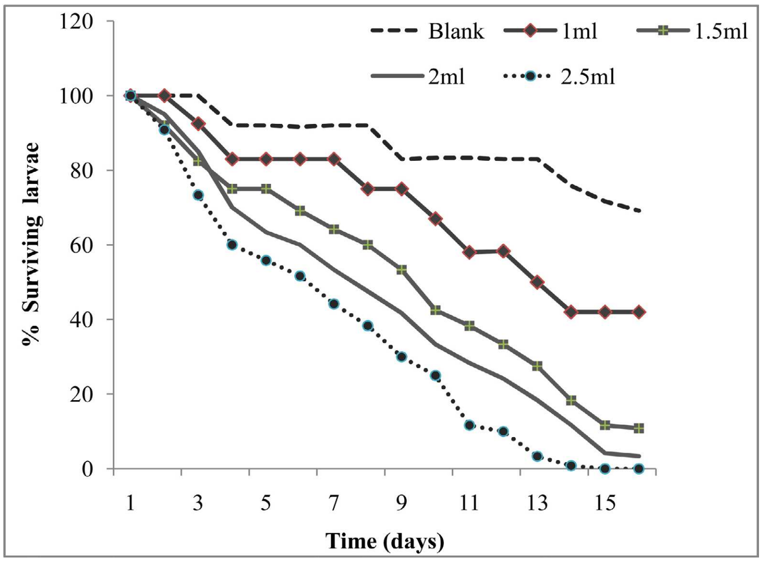

Figure 2. Survival curves for *B. brassicae*: Mean survival rates per day over a time period of 16 days after treatment with NeemAzal-T at 10 (1 ml), 15 (1.5 ml), 20 (2 ml) and 25 (2.5 ml) mg AZA per kg of substrate.

for instance, the average number of offspring produced on plants treated with NeemAzal T at 1ml/kg was 1.82 ± 0.7 (Mean ± SE) compared to 0.14 ± 0.1 larvae per females per day in plants receiving the same treatment at the dose of 2.5 ml/kg of soil. Same scenario was also true for NeemAzal granules, where significantly lower number of larvae were produced on treated plant compared to the control. Although some aphids survived on neem-treated plants, results indicated that their reproduction was greatly affected and at the two higher doses of NeemAzal reproduction was almost completely prevented. Adult fecundity data is summarized in Table 2 and Table 3.

3.2. Persistence Effect of the Neem Formulations on *Brevicoryne brassicae*

Although the mortality rate of aphids increased with increasing time of exposure, there were no significant differences in percentage mortality of cabbage aphid larvae ($p > 0.05$) when exposed at 0, 4 or 8 days after the substrate was treated with NeemAzal granules. Additionally, at all the three exposure days, the neem treatments attained a significantly higher ($p < 0.05$) mortality compared to the control (Figure 3).

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

4. Discussion

4.1. Efficacy and Dose Response

The cabbage aphid is considered a serious insect pest of plants of the brassicae

Table 2. Offspring per female per day (from day 8 to 16) of the mature adult's life following treatment with various doses of NeemAzal-T (ml/kg). Mean ± S.E; n = 10.

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

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

Table 3. Offspring per female per day of the mature adult's life following treatment with various doses of NeemAzal granule (mg/kg). Mean ± S.E; n = 10.

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

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

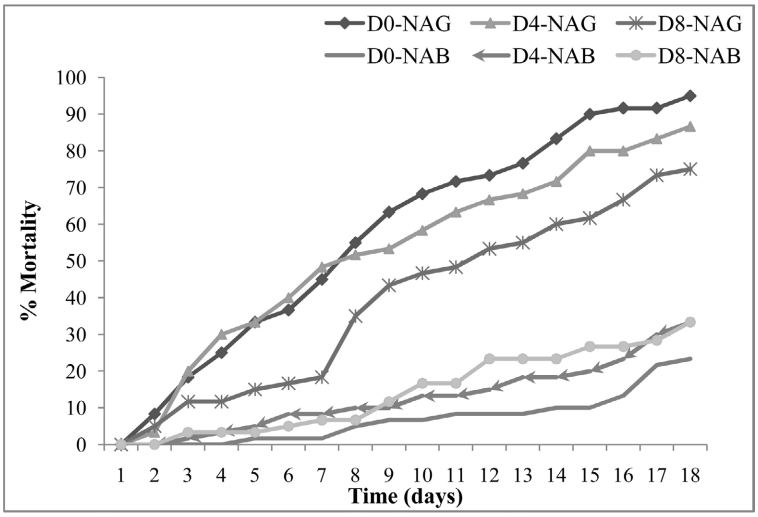

Figure 3. Mortality (%) of *Brevicoryne brassicae* over a time period of 18 days after treatment with NeemAzal granules (NAG) at 10.5 mg AZA per kg of substrate and a blank formulation of NeemAzal (NAB). Plants were infested with aphids at day zero (D0), four (D4), or eight (D8) days after treatment.

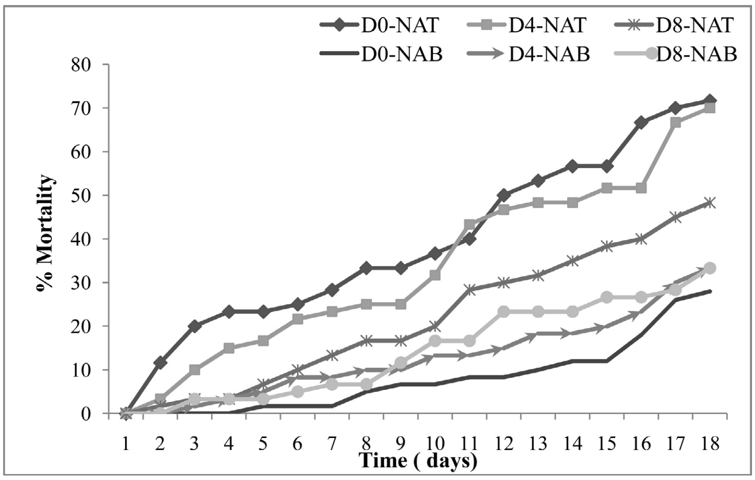

Figure 4. Mortality (%) of *Brevicoryne brassicae* over a time period of 18 days after treatment with NeemAzal-T (NAT) at 10 mg AZA per kg of substrate and a blank formulation of NeemAzal (NAB). Plants were infested with aphids at zero (D0), four (D4), or eight (D8) days after treatment.

family, not only because it efficiently vectors plant viruses [16], but also because of its direct damage by sucking activity and contamination of the produces. The economic losses are huge [3]. The over reliance on synthetic pesticides for the control of aphids has raised a lot of environmental concerns on the biosafety of these pesticides [30] [31] [32]. Therefore, biopesticides with low toxicity to humans and fast degrading residues on the produce such as products based on Azadirachtin have been considered safe alternatives for the environment and the health of consumers [18]. On the other hand, the low persistence when applied to the crop canopy and the still existing side effects on non-target organism when directly contaminated demands for improved application strategies such as systemic application via the root system of plants. This could be more desirable in terms of efficiency, persistence [1].

Our greenhouse evaluations of the effects of two NeemAzal formulations indicate that Azadirachtin, the active ingredient in the formulations, can significantly reduce survival rates of aphid larvae as well as adult fecundity. Both neem-derived biopesticides (NeemAzal-T and NeemAzal granules) in the present study significantly decreased *B. brassicae* larval survival. Our findings are in agreement with numerous studies. First [33] concluded that neem-based formulations (neem seed extracts) were effective against rose aphid, and Chrysanthemum aphid, both in the laboratory and field. Many other studies reported thereafter high efficacy of neem-based insecticides against different aphid species [30] [31] [32]. [11], for instance, working with two neem-derived insecticides Neemix® 4.5 EC and neem seed oil against *Aphis glycines* (Matsumura) reported significantly increased aphid nymphal mortality (80% and 77% respectively) and development time and attributed the effects to neem mode of action as an insect growth regulator.

The efficacy of the NeemAzal formulations in our studies was dose dependent. No aphids survived beyond 14 days of monitoring with the highest doses tested. Similar activities of neem-based biopesticides have been reported by other authors [34] [35] [36]. [35] reported that efficacy of Neem-Azal-PC® (0.5% Azadirachtin) against *Aphis fabae* (Scopoli) was concentration and time dependent. [10] reported that systemic effect of Neem Azal-T/S® and Neemix® on bean aphids gradually increased according to the increase in feeding period and concentrations. In their study, Neem Azal-T/S at 2.5 µl/100ml completely prevented the maturation of born nymphs, and caused over 80% mortality compared to concentrations of 1.0 µl/100ml, which resulted in 58.2% maturation rate and <60% mortality. Similar results have been reported by [37]. Our results also corroborate those of [38] who reported a dose-dependent efficacy of NeemAzal-T (5% AZA) against *Mamestra brassicae* L. The authors recounted that concentrations of 2 mg/ml Azadirachtin increased mortality and restricted larval development to 2nd instar, while concentrations of 8 mg/ml or greater also protected the plants from observable damage. Moreover, [24] reported that NeemAzal-U® (17% Azadirachtin) at 0.75 g/l drenched to the plant substrate resulted in 9.38% larval mortality compared to 100% mortality resulting for higher doses of 2.25 and 3 g/l in *Liriomyza sativae* (Blanchard). Several other studies have also demonstrated dose-dependent efficacy of neem-based biopesticides on various pest species such as *Trialeurodes vaporariorum* (West) [27] [36]; *Corythucha ciliata* (Say) [33], *Myzus persicae* (Sulzer) [39]. Hence we could attribute the increasing efficacies of the two neem formulations against cabbage aphid to a comparable increase in the amount of Azadirachtin systemically translocated from substrate through the roots and ultimately to the aphid feeding site.

Besides the high efficacy in terms of mortality of the tested neem formulations against cabbage aphid, fecundity, number of offspring per female, was greatly affected, also in a dose-dependent manner. From our results, even if aphids survived on NeemAzal T and NeemAzal Granules treated plants, Azadirachtin significantly reduced their reproduction. Significant reduction of fertility of aphids, even at very low concentrations of azadirachtin has been documented [40] [41]. [18] reported a decrease in fecundity of *B. brassicae* after feeding on rape plants, systemically treated with different concentrations of water-based solutions of crystalline Azadirachtin A 97.5%.

[41] showed that exposure of adult *M. persicae* to neem seed oil and azadirachtin not only influenced the survival of offspring produced by treated adults but also adults emerging from neem treated nymphs were undersized with abnormal wings, legs and stylets. While neem products have been shown to reduce the fecundity and fertility of adults, and molting of nymphs of various aphid species [16] [24] [38] [42] other studies, [11] reported that fecundity of *A. glycines* adults treated with azadirachtin and neem seed oil was not affected. This may be an indication that growth regulation effects of neem depend on a host plant, aphid species and/or, treated aphid instar [43]. Mode of application, systemically through the root versus direct spray, of the neem-based biopesticide and perhaps differences in routes of exposure of the target insect to the test substance [43] could explain the difference in our studies and also the contradicting findings mentioned above. [44] attributed the effect of neem-based biopesticides on the reproductive potential of aphids to blocking the neurosecretory cells responsible for hormone production controlling the aphid maturation process, egg production and embryonic development.

4.2. Persistence and Residual Effect

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

[45] reported that mortality and fecundity of nymphs of *M. persicae*, and *B. brassicae* were severely affected up to 10 days after soil treatment with a neem seed kernel water extract. [43] also reported a significant reduction in densities of *M. persicae* after spraying neem-based products onto potted sweet pepper plants in the greenhouse seven days post-treatment. Comparable persistence effects with Neem products drenched to the plant substrate were obtained also with other pests. Soil drenching with NeemAzal-T/S® (1% azadirachtin, Trifolio-M) against *F. occidentalis* was recorded up to 3 weeks after treatment [46], and significant systemic effect of NeemAzal-T (5% AZA) against *Mamestra brassicae* was reported up to two weeks after treatment application [38]. Fur-

thermore, NeemAzal-U was found to be effective against larval stages of *Liriomyza sativae* for at least 7 days after soil drenching, in both laboratory and greenhouse [24]. The authors recorded larval mortality of up to 100% with neem concentrations of 3 g·l^{-1} water. Azadirachtin is readily mobile in soil and systemic in plants [24] [47] [48] [49]. Since it's likely to be destroyed by light and high temperatures after spraying it on plants [45], soil application has become more desirable. On the other hand, residual activity of neem-based products sprayed to the crop canopy has low persistence against aphids. For instance, [50] reported a sharp decline in residual insecticidal activity of NeemAzal T/S on cherry-oat aphid and rose-grain aphid, from 22.2% - 20.0% on zero time and reached 0.0 - 7.9% respectively, seven days post application.

5. Conclusion

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

Conflicts of Interest

The authors declare no conflicts of interest.

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